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It's about time

Rapid detection and susceptibility testing of *Pasteurellaceae* causing respiratory disease in cattle by MALDI-TOF MS

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It's about time: rapid detection and susceptibility testing of *Pasteurellaceae* causing respiratory disease in cattle by MALDI-TOF MS

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SUCCESS CONSISTS OF GOING FROM FAILURE TO FAILURE WITHOUT LOSS OF ENTHUSIASM

Winston Churchill

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LIST OF ABBREVIATIONS

AMCRA	Antimicrobial consumption and resistance in animals
AMPP	Analysing mass peak profiles
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BALf	Bronchoalveolar lavage fluid
BCV	Bovine corona virus
BHIB	Brain heart infusion broth
BHV-1	Bovine herpesvirus type 1
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BVDv	Bovine viral diarrhea virus
BW	Body weight
CAMHB	Cation-adjusted mueller hinton broth
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CRP	C-reactive protein
Ct-value	Cycle threshold value
ELISA	Enzyme-linked immunosorbent Assay
DNS	Deep nasopharyngeal swab
EPS	Extracellular polymeric substance
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
FBS	Fetal bovine serum
ICE	Integrative conjugative element
IgBPs	Immunoglobulin binding proteins
LKT	Leukotoxin
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MALDI-TOF MS	Matrix-assisted laser desorption/ionization- time-of-flight mass spectrometry
MBT-ASTRA	MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA)
MBT-RESIST	MALDI Biotyper resistance test

MEA	Measuring enzymatic activity
MIC	Minimal inhibitory concentration
nBAL	Non-endoscopic bronchoalveolar lavage
PCR	Polymerase chain reaction
PCT	Procalcitonin
PI3	Parainfluenza-3 virus
PK-PD	Pharmacokinetic/Pharmacodynamic
PPLO	Pleuropneumoniae like organism
PTI	Post treatment interval
RG	Relative growth
ROC	Receiver operating characteristics
RTI	Respiratory tract infection
RT-qPCR	Real-time quantitative PCR
TAT	Turnaround time
TTA	Transtracheal aspiration
TTW	Transtracheal wash
WGS	Whole genome sequening
YE	Yeast extract

Bovine respiratory disease (BRD) is one of the most important economic diseases in different cattle production systems worldwide. Since bacterial pathogens are mostly the cause of clinical signs and lung lesions, antimicrobial treatment is a cornerstone of BRD control. Also, due to the major impact of BRD, prophylaxis and metaphylaxis is still commonly implemented. This is currently considered unacceptable due to the global threat of antimicrobial resistance, both in human and in veterinary medicine. An alteration from an empiric group treatment to a rational individual treatment, based on microbiological results, is strongly encouraged. However, this alteration and a decreased use of broad-spectrum antimicrobials is not evident for veterinarians, as they fear an ineffective treatment and subsequently an increased mortality. The biggest drawback, for not systematically applying microbiological results by veterinarians, is time.

Until now, in clinical veterinary laboratories, microbiological results are based on cultivation of samples for identification and disk diffusion for susceptibility testing. These diagnostic techniques comprise a minimum of 2 days and mostly longer. This long turnaround time, from sampling to microbiological results, makes it very challenging to even impossible for veterinarians to fulfill current demands of a rational individual treatment. In order to meet this request, rapid and cheap diagnostic techniques are necessary. Therefore, the present doctoral thesis aimed at developing new diagnostic techniques for rapid identification and susceptibility testing of *Pasteurellaceae* causing infectious bronchopneumonia in cattle. The focus lies on obtaining an acceptable turnaround time and a non-expensive method which can be easily applied in clinical veterinary laboratories. Since the sampling method and transport conditions can play an important role in microbiological outcome, these subjects were also incorporated in this doctoral thesis. The obtained results provide answers to the complete diagnostic chain for infectious bronchopneumonia in cattle, from taking samples of the respiratory tract in the field to susceptibility testing in the laboratory.

CHAPTER 1

GENERAL INTRODUCTION

1. BOVINE RESPIRATORY DISEASE: A NEVER ENDING STORY

Bovine respiratory disease (BRD) is one of the most extensively studied diseases in cattle, with research beginning in the late 1800s and still continuing today (Maier et al., 2019). Throughout this period, BRD received multiple names like 'shipping fever', 'transit fever', 'pasteurellosis', 'stockyards pneumonia' and 'enzootic pneumonia' (Hepburn, 1925; Tweed and Edington, 1930; Anderson, 1939; Turner, 1939; Carter and Rowsell, 1958). The etiology of this disease is multifactorial, including complex interactions between the host immune system, viral and bacterial pathogens and the environment (Griffin et al., 2010). For example, stressful situations like transportation and weaning, but also environmental conditions like cold stress and ammonia can lead to an increased susceptibility to BRD (Rice et al., 2007; Aich et al., 2009; Griffin et al., 2010). Viral pathogens associated with BRD are: bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI3), bovine viral diarrhea virus (BVDv), bovine respiratory syncytial virus (BRSV) and bovine corona virus (BCV). Bacterial pathogens most commonly associated with BRD include *Mannheimia haemolytica (M. haemolytica)*, *Pasteurella multocida (P. multocida)*, *Histophilus somni (H. somni)* and *Mycoplasma bovis* (*M. bovis*) (Griffin et al., 2010).

Morbidity and mortality caused by BRD is mostly, with exception of BRSV and BCV (Ellis et al., 1996; Elvander, 1996; Schreiber et al., 2000; Storz et al., 2000a; Storz et al., 2000b; Decaro et al., 2008), not associated with viral infections alone. Instead, these pathogens aid in establishing a favorable environment leading to bacterial infection of the lung (Griffin et al., 2010; Taylor et al., 2010; Woolums, 2015). Consequently these bacterial pathogens are usually the main cause of severe lung lesions and clinical signs in cattle (Yates, 1982). Furthermore, antimicrobial treatment is commonly used to tackle BRD. A correct identification of the causative pathogen of BRD in combination with a successful antimicrobial treatment can therefore lead to full recovery of diseased animals, minimizing economic losses and maximizing animal welfare. Therefore the current thesis focuses on the bacterial component of BRD, with emphasis on the family *Pasteurellaceae*. In this introduction the economic impact of BRD is discussed first. Second, the family *Pasteurellaceae* is briefly addressed. A short review of the current approaches to tackle BRD is provided, ending with antimicrobial therapy and subconsequently antimicrobial resistance.

1.1. Economic impact of BRD

BRD is economically one of the most important diseases in bovine livestock globally (Smith, 1998; NAHMS, 2000; Assie et al., 2009). In the United States' beef industry, the economic impact of BRD has been estimated to exceed €3,6 billion (€5,7 billion inflation-adjusted) annually (Griffin, 2010). In the feedlot industry this was estimated €12,5 per animal, including costs for treatment and production losses (Snowder et al., 2006). Steers that experienced infectious bronchopneumonia returned €51,8 less per animal (Faber et al., 1999). In Europe, a total loss due to BRD and related illnesses is estimated at €576 million (€724 million inflation-adjusted) annualy (Nicholas and Ayling, 2003). Although economic models have been described to estimate economic losses associated with this disease (Hurd et al., 1995; van der Fels-Klerx et al., 2001), truly calculating these losses is not evident as multiple factors need to be taken into account. Additionally, considering the fact that a subclinical infection in animals can also cause suppressed gains, true costs of infectious bronchopneumonia are probably higher than estimated (Wittum et al., 1995).

A first direct cost is mortality, of which infectious bronchopneumonia is the leading cause in the feedlot, dairy and veal industry (Vogel and Parrot, 1994; Pardon et al., 2013; Dubrovsky et al., 2019). In the feedlot industry, infectious bronchopneumonia is responsible for 44-67% of the mortality rate (Vogel and Parrott, 1994; Edwards, 2010). In veal calves, this is 27.7% (Pardon et al., 2012) with already an increased mortality risk (hazard ratio: 5.5) after one episode of infectious bronchopneumonia (Pardon et al., 2013). In the dairy industry, calves are 1.6 to 5.0 times as likely to leave the herd before first calving when suffering from infectious bronchopneumonia (Schaffer et al., 2016). When animals experience 4 or more episodes of BRD, a statistical negative effect is seen on surviving first lactation (Bach, 2011).

A second cost is morbidity, which is of even higher concern as this can cost more due to expenses associated with extra labor, medication and reduced performance. In feedlot cattle, 65 to 80% of the total morbidity rate is caused by infectious bronchopneumonia (Edwards, 2010). A higher morbidity rate will result in a higher antimicrobial use (DeDonder and Apley, 2015a). In veal calves, infectious bronchopneumonia is the main indication for both group and individual antimicrobial use (Pardon et al., 2012). The costs for treatment of respiratory tract infections (RTIs) obviously depend on the

product being used and the period applied. Interestingly, even when accounting for inflation, treatment costs have increased during the years, with an average cost per case of €11,3 in 1999 (Faber et al., 1999), €14,0 in 2000 (NAHMS, 2000) and €19,2 in 2013 (APHIS USDA, 2013). Animals suffering from infectious bronchopneumonia also endure a decrease in performance. Several studies have shown a reduction in average daily gain (ADG) in beef cattle with RTIs compared to healthy animals. A decrease of ADG of 0.06 kg (Bateman et al., 1990; Rezac et al., 2014), 0.08 kg (Wittum et al., 1995), 0.18 kg (Morck et al., 1993), 0.23 kg (Smith, 1998) and 0.95 kg (Snowder et al., 2006) has been described, depending on the age of the calves and the time period measured. However, it seems that infected calves can develop a 'compensatory gain' once effectively treated (Faber et al., 1999), resulting in no difference in ADG at the end of the feeding period.

Additionally, RTIs can change carcass traits such as weight, marbling and subcutaneous fat cover (McNeill et al., 1996; Gardner et al., 1999; Stovall et al., 2000; Pardon et al., 2013). Cattle that were treated 1, 2, 3 or more times had a decrease in carcass value of €20.9, €27.2 and €48.7 compared to untreated animals (Schneider et al., 2009). Also in the dairy industry, RTIs can cause economic damage. When heifers experience 4 or more respiratory infection episodes, a delay of average age of first calving of 3-13 days has been described. Furthermore, these animals have 1.9 ± 0.1 greater odds of not completing first lactation. As the number of infection episodes increase, the accumulated days in lactation and the proportion of productive days throughout life linearly decrease (Bach, 2011). If dairy calves are exposed to RTIs under the age of 120 days, a 233 kg lower milk production is seen (Schaffer et al., 2016). Furthermore, these young calves have an increased occurrence of dystocia at first calving (Warnick et al., 1994).

1.2. *PASTEURELLACEAE*

The family of the *Pasteurellaceae* is characterized by small (0.2-2µm), Gram-negative, non-motile, facultative anaerobic coccobacilli or rods (Quinn et al., 1994). Although they can be ubiquitously present in respiratory, alimentary and reproductive systems of multiple avian, mammalian, reptilian and likely amphibian hosts (Shewen and Rice Conlon, 1993; Rycroft and Garside, 2000; Christensen et al., 2003), they are notorious for their role in respiratory disease in various animals species, including cattle. *P. multocida*, *M. haemolytica* and *H. somni* can be present in the normal microbiota of the upper respiratory tract in cattle. Under predisposing conditions, these bacteria can start multiplicating in the upper respiratory tract, descend along the trachea and infect the lungs (Lillie and Thomson, 1972; Allen et al., 1991; Kehrenberg et al., 2001). The prevalence of each pathogen can vary between region, industry, age and clinical condition of the animal. Overall, *P. multocida* is the most frequently isolated bacterial pathogen from BRD, followed by *M. haemolytica* (Catry et al., 2005; Rérat et al., 2012; Timsit et al., 2017), although the latter is considered the most important bacterial pathogen associated with clinical disease of BRD (Fulton et al., 2009; Griffin et al., 2010). The prevalence of *H. somni* is mostly lower, whereas isolation rates can be high in countries like Australia (Moore et al., 2015) and North America (Orr, 1992). Also in Belgium, *P. multocida* is the most frequently isolated BRD pathogen, followed by *M. haemolytica* and *H. somni* (Figure 1). In the next paragraph a limited overview of each of these three pathogens will be provided.

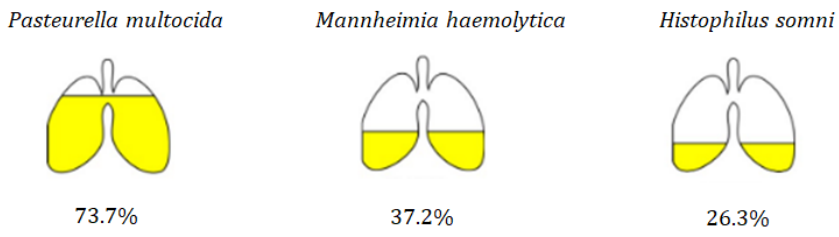

Figure 1: Prevalence of *P. multocida*, *M. haemolytica* and *H. somni* as detected by PCR based on samples of 1742 acute outbreaks of bovine respiratory disease in flanders (December 1, 2016 – August 31, 2019, Griepbarometer Diergengezondheidszorg – Vlaanderen, Animal Health Care – Flanders, https://www.dgz.be/griepbarometer-volgt-de-griepsituatie-op-de-voet)

1.2.1. *Mannheimia haemolytica*

Mannheimia haemolytica, formerly *Pasteurella haemolytica*, is considered the predominant bacterial pathogen associated with infectious bronchopneumonia in cattle (Fulton et al., 2009; Rice et al., 2007; Griffin et al., 2010). Aside from infectious bronchopneumonia, *M. haemolytica* can also be involved in otitis of cattle, pleuropneumonia and septicaemia in small ruminants and cattle, and arthritis and mastitis in sheep (Ehlers et al., 1993; Shewen and Rice Conlon, 1993; Quinn et al., 1994; Mutters et al., 1985a; Mutters et al., 1985b; Duarte and Hamdan, 2004; Mahu et al., 2015; Taunde et al., 2019). *M. haemolytica sensu lato* includes 5 species, namely *M. haemolytica sensu strictu*, *M. varigena*, *M. granulomatis*, *M. glucosida* and *M. ruminalis* (Biberstein et al., 1960; Christensen et al., 2004). Furthermore, *M. haemolytica* comprises 12 capsular serotypes, of which serotype 1, 2 and 6 are most prevalent in cattle and serotype 1 and 6 are most frequently associated with clinical infectious bronchopneumonia (Angen et al., 1999; Highlander, 2001; Griffin et al., 2010; Klima et al., 2014a). Nevertheless, also other serotypes such as A6, A7, A9, A11, and A12 can be isolated from cattle suffering from infectious bronchopneumonia (Grifffin et al., 2010).

M. haemolytica obtains multiple virulence factors. Different virulence factors play a role in the adherence to host cells, including protein adhesins (outer membrane protein A (OmpA), lipoprotein I, N-acetyl-D-glucosamine and fibrinogen-binding proteins) and secreted enzymes (neuraminidase and sialoglycoprotease) (Panciera and Confer, 2010). Furthermore, a polysaccharide capsule is present, which aids in attachment and mediates resisting phagocytosis by neutrophils. Considerable destruction of the respiratory tract is caused by lipopolysaccharide (LPS, endotoxin) and leukotoxin (LKT, exotoxin). LPS interacts with the innate host immune defenses and induces pulmonary inflammation, while LKT is responsible for the lysis of lymphocytes, macrophages, neutrophils and platelets (Czuprynski et al., 2004; Gioia et al., 2006; Rice et al., 2007; Panciera and Confer, 2010)). Additionally, *M. haemolytica* can produce a biofilm (Panciera and Confer, 2010), which consists of microcolonies of bacteria enclosed in extracellular polymeric substance (EPS) (Olson et al., 2002). This biofilm can prevent damage of bacterial cells caused by the host immune system and can reduce susceptibility of bacteria against antimicrobials and detergents (Panciera and Confer, 2010).

1.2.2. *Pasteurella multocida*

P. multocida is associated with disease in many different animal species and is potentially zoonotic (Miyoshi et al., 2012), causing wound infections and septicaemia in humans (Hunt et al., 2000). *P. multocida* contains 5 capsular serogroups (A, B, D, E and F) and 16 somatic serotypes (1-16) (Carter, 1955; Carter 1961; Heddleston et al., 1972; Rimler and Rhoades, 1987; Dabo et al., 2003). Depending on the capsular serogroup, different presentations of a disease can occur. Capsular type A is associated with fowl cholera, although strains have also been isolated from healthy commercial poultry flocks (Muhairwa et al., 2000; Shivachandra et al., 2006), recurrent purulent rhinitis in rabbits and ruminant, porcine, canine and feline respiratory diseases (Mutters et al., 1985a, Quinn et al., 1994). Capsular types B and E are associated with haemorrhagic septicaemia in bovine species in tropical regions (Dutta et al., 1990). Capsular type D has been isolated from atrophic rhinitis of pigs and occasionally pneumonia in other species including ruminants (De Alwis, 1984; Verma, 1988; Quinn et al., 1994; Kalorey et al., 2008) and capsular F from diseased turkeys (Shewen and Rice Conlon, 1993; Quinn et al., 1994) and calves suffering from fatal peritonitis (Catry et al., 2005).

In cattle, *P. multocida* is notorious for its role in infectious bronchopneumonia, although it can also cause mastitis and septicemia (Markey et al., 2013). Isolates from serogroup A can occur as commensal or pathogenic bacteria in the bovine respiratory tract, whereas serotype A3, and to a smaller extent serotype D3, are most commonly isolated from infectious bronchopneumonia (Mutters et al., 1986; Dabo et al., 2003; Harper et al., 2006; Griffin et al., 2010; Sellyei et al., 2015). Although less than *M. haemolytica*, *P. multocida* comprises multiple virulence factors which support adhesion and colonization, evading host defenses and causing tissue damage, therefore responsible for infection. Many of these virulence factors are similar to the above-mentioned of *M. haemolytica*, i.e. a polysaccharide capsule, LPS (endotoxin), the adhesin protein OmpA, the secreted enzyme neuraminidase and a biofilm (Harmon et al., 1991; Panciera and Confer, 2010; Harper et al., 2011). Polymicrobial biofilms with *Histophilus somni* can occur (Elswaifi et al., 2012). Additionally, like other Gram-negative bacteria, outer membrane vesicles can be released into the surrounding medium containing virulence factors such as toxins, enzymes (including beta-lactamases) and adhesions, both by commensals and pathogens (Sellyei et al., 2009; Amano et al., 2010). These outer

membrane vesicles mediate in biofilm formation (Schooling and Beveridge, 2006). Furthermore, adhesins for bacterial adherence to and colonization of cell surfaces are present, including type IV fimbriae and filamentous hemagglutinin (FHA) (Glorioso et al., 1982; Dabo et al., 2003; Ewers et al., 2006; Panciera and Confer, 2010). An exotoxin named *P. multocida* Toxin, has been described, although its role or even existence in strains causing infectious bronchopneumonia remains unclear (Confer, 2009).

1.2.3. *Histophilus somni*

Histophilus somni, formerly *Haemophilus somnus*, has long been recognized to play a role in infectious bronchopneumonia in cattle (Gogolewski et al., 1987). Co-infections of *H. somni* with for example BRSV and *M. haemolytica* have been described (Gershwin et al., 2005; Corbeil, 2007; Murray et al., 2017). *H. somni* is non-encapsulated and does not form exotoxins. However, it produces an endotoxin called lipooligosaccharide (LOS) which can induce apoptosis of endothelial cells (Sylte et al., 2001; Kuckleburg et al., 2008; Panciera and Confer, 2010). Furthermore, *H. somni* can adhere to endothelial cells, inducing thrombus formation by the activation of platelets and can secrete immunoglobulin binding proteins (IgBPs) which mediate dispersion of the bacterium through the alveolar barrier (Bastida-Corcuera et al., 1999; Agnes et al., 2013). The production of histamine by *H. somni* results in vasoconstriction and increased permeability (Ruby et al., 2002) and transferrin-binding proteins permit to obtain iron from host components (Ekins et al., 2004). (Gogolewski et al., 1987; Corbeil 2007). Like other pathogens, *H. somni* is able to form a biofilm (Sandal et al., 2007).

Although *H. somni* is considered a normal inhabitant of the genital tract (preputium and vagina) (Kwiecien and Little, 1992; Griffin et al., 2010), infection with this bacterium can lead to abortion and granular vulvovaginitis (Janzen, 2016). Due to the particular virulence factors, *H. somni* can easily spread within the body resulting in multiple disease outcomes: thrombotic meningo-encephalitis and septicaemia resulting in sudden death (Janzen, 2016), myocarditis (Wessels and Wessels, 2004; Wessels and Wessels, 2005), pericarditis, polyarthritis, polyserositis, laryngitis, mastitis (Janzen, 2016) and otitis media (Duarte and Hamdan, 2004). Besides cattle, *H. somni* is also known to cause pneumonia in sheep and goats (Janzen, 2016).

1.3. Current approaches to tackle BRD

Since BRD is a multifactorial disease, it is obvious to assume that preventive measures can be applied at different levels. There are three hatches which can influence the incidence and clinical outcome of RTIs: immunity of the animal, pathogen virulence, and exposure to environmental risk factors. Despite advances in research on BRD prevention, disease incidence and economic impact have remained unchanged during the last decades (Miles, 2009; Hilton, 2014). Although preventive measurements depend on production type and their importance is age dependent, in the next two paragraphs general aspects on how to tackle BRD will be briefly covered. First, the immunity of the animal will be addressed. Second, environmental risk factors will be shortly discussed.

As for most infectious diseases, prevention of disease includes attaining a good level of both innate and acquired immunity. An innate or aspecific immunity is present in each animal and consists of physical and chemical barriers, for example mucus and surfactant proteins. An acquired or specific immunity is derived by previous exposure of pathogens or vaccination. (Galyean et al., 1999). Furthermore, an acquired immunity already starts at birth, where a proficient volume of good quality colostrum should be provided fast to the neonatal calf in order to acquire maternal immunity. Calves with failure of passive transfer, meaning an inadequate transfer of immunoglobulins from colostrum, are more at risk of developing BRD (Wittum and Perino, 1995; Windeyer et al., 2014; Pardon et al., 2015).

To further enhance acquired immunity, particularly for BRD, several vaccines are commercially available. Besides the numerous vaccines containing viruses like BRSV, PI-3 or BVD, vaccines have been developed containing *P. multocida*, *M. haemolytica* or *H. somni*. A review including 34 studies on vaccination against *P. multocida*, *M. haemolytica* and *H. somni* showed that vaccines containing *P. multocida* or *M. haemolytica* may help in lowering the morbidity incidence, although outcomes of different studies can be contradictory (Larson and Step, 2012). For *H. somni*, vaccination seems to have no added value according to Larson and Step (2012). However, some commercially available vaccines were not evaluated in that study and research to develop more effective *H. somni* vaccines continues (Geertsema et al., 2011; Lo et al., 2012). Noteworthy, vaccination is often combined with other stressful situations such as weaning, castrating, dehorning, commingling and replacement. These stressful situations can

suppress the immune system, decreasing the effectiveness of vaccination and enhancing the risk of severe disease after infection (Lillie, 1974; Irwin et al., 1979; Willard et al., 1996; Callan and Garry, 2002).

Particularly for RTIs, the environmental factors where calves are exposed to in their housing systems are unquestionably important (Louie et al., 2018). This involves on the one hand climate conditions and on the other hand housing infrastructure and associated management practices. Climate conditions that need to be avoid are noticeable changes in temperature, high temperatures with high relative humidity ('heat stress'), low temperatures with draught (wind velocity >0.5m/s ('cold stress'), stable air pollutants (ammonia concentration >10 ppm) dust and over- or underventilation (Lundborg et al., 2005; Lago et al., 2006; Gooch, 2007; Nonnencke et al., 2009; Gorden and Plummer, 2010; Phillips et al., 2010; Louie et al., 2018; Schnyder et al., 2019) . Management and housing factors that can negatively affect the health of animals are commingling older calves with younger ones, high animal density, permitting nose contact between young calves and walking lines from older cattle to younger calves (Sanderson et al., 2008; Gorden and Plummer, 2010; Schnyder et al., 2019). Due to the obligatory group housing of animals from the age of 8 weeks for welfare reasons (CD, 2008), it is challenging to prevent and better control BRD. It is therefore recommended to group animals of equal age, respect walking lines and move animals with clinical signs in quarantine (Callan and Garry, 2002). Other factors that should be taken into account are a hygienic environment, good quality food, fresh drinking water and parasitic control (Duff and Galyean., 2007; Sweiger and Nichols, 2010).

1.4. Antimicrobial therapy and resistance

1.4.1 Antimicrobial therapy

Although management and vaccination strategies are the preferred approach for disease prevention and control, it still seems mandatory to use antimicrobials once animals develop a bacterial infection. Thousands of veterinarians, and in several countries also farmers, take the decision to initiate an antimicrobial therapy. This antimicrobial decision making process contains several steps (Figure 2). After detecting infectious bronchopneumonia in cattle, samples are taken for identification and susceptibility testing of the causative pathogen. These topics will be discussed further in this thesis. While awaiting the microbiological results, an empiric therapy (i.e., therapy based on collective experience and scientific evidence [ESGAP, 2019]), is applied. Current practices of treatment of infectious bronchopneumonia consist of prophylaxis, metaphylaxis or curative therapy, which will be further elaborated in this chapter. Depending on the outcome of the microbiological results, the empiric therapy will be determined inappropriate and therefore adjusted or will be determined appropriate and can therefore be maintained as a definitive therapy. The duration of antimicrobial treatment, period between therapeutic application and evaluation of success or failure of the treatment (post treatment interval) and different antimicrobials, which can be applied for BRD, will be further clarified in this chapter.

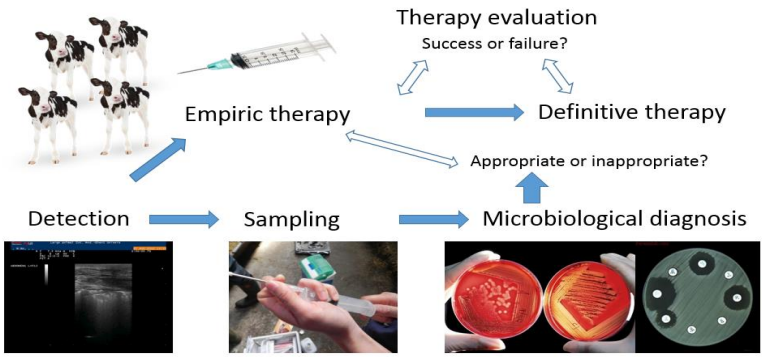

Figure 2: Overview of terminology and steps in the process of antimicrobial decision making in animals (Figure design: B. Pardon)

Whether an antimicrobial is considered to contribute to clinical outcome relies on the concentration of the antimicrobial at the site of infection (pharmacokinetics) related to the effect of the antimicrobial on the pathology of the body of an animal (pharmacodynamics). Antimicrobials can be used in 3 different ways, namely as a prophylactic or metaphylactic group treatment, or as an individual treatment. In the past, antimicrobials were also used as growth promoters. However, this application is banned since 2006 in Europe (EC, 2005) due to mounting evidence about antimicrobial resistance and transfer of resistance genes from animal to human microbiota (Langlois et al., 1984; Aarestrup, 1999; Levy, 2002; Funk et al., 2006; Cogliani et al., 2011; Ajayi et al., 2019; Mascaro et al., 2019). Prophylaxis, applied since 1950, is defined as the administration of an antimicrobial to healthy animals in order to prevent infections based on risk or possible consequences (Johnston, 1998). Metaphylaxis, as such defined since 1980, describes the administration of an antimicrobial to (apparently) healthy animals when the disease of interest is present within the group (Urban-Chmiel and Grooms, 2012). Hence metaphylaxis is indicated for high-risk cases in order to restrict the spread and impact of the disease (Smith et al., 2001; Lees and Shojaee Aliabadi, 2002; Edwards, 2010). This implicates that both prophylaxis and metaphylaxis involve the administration of antimicrobials to 'healthy' or 'apparently healthy' animals to 'prevent' infections, however, in prophylaxis there is a perceived 'risk', whereas in metaphylaxis there is a definable 'hazard'.

Both prophylaxis and metaphylaxis are highly debated terminologies in modern times in face of the need to reduce antimicrobial use. Nevertheless, several studies have shown the economic benefit of implementing these mass medications (Lofgreen, 1983; Van Donkersgoed, 1992; Frank et al., 2002; Step et al., 2007; Catry et al., 2008; Rérat et al., 2012). However, a review summarizing 169 controlled clinical trials over 50 years (1966-2016) demonstrated only a moderate yet highly variable relative risk reduction in morbidity caused by infectious bronchopneumonia when applying prophylaxis and metaphylaxis in cattle (Baptiste and Kyvsgaard, 2017). These reductions were dependent on the antimicrobial classes used, the number of infectious bronchopneumonia episodes and the duration of the controlled clinical trials. The highest impact was noticed when broad-spectrum critically important antimicrobials or a combination of antimicrobials was being used. Furthermore, the observed effects were highest in studies performing short (<40 days) controlled clinical trials, whereas longer

follow-up periods did not evidence any benefit of this strategic antimicrobial group therapy anymore (Baptiste and Kyvsgaard, 2017). This information suggests that switching from group to individual therapy is likely not as unthinkable as currently believed, but is merely based on practical, labor reasons. Baptiste and Kyvsgaard, 2017 also demonstrated in their study that 7 animals should be treated in order to prevent 1 BRD case. Therefore this individual therapy, where only animals showing clinical signs would be treated, would clearly limit the number of animals being exposed to antimicrobials and therefore benefit against antimicrobial resistance. However, due to the tendency of cattle to hide illness, extensive pulmonary damage may already occur prior to therapy, which could be harmful for animal welfare (Fulton, 2009). Additionally, this 'late' treatment could demand higher antimicrobial dosages. Lhermie et al, 2016 demonstrated a more effective treatment outcome when calves were treated in an early stage (2 h after inclusion), when the inoculum concentration of *M. haemolytica* was still low, at a low concentration of marbofloxacin compared to a higher concentration of marbofloxacin at a later stage (35-39 h after inclusion). However, current diagnostic tools, where results are only available after days, hamper such an early treatment strategy. An early detection of infectious bronchopneumonia, including identification of the causative pathogen in combination with susceptibility testing, and an appropriate dosage of antimicrobials could therefore lead to a reduction of antimicrobial use.

Within the context of sustainable use of antimicrobials, the duration of treatment should be as short as possible, but as long as necessary (Kouyos et al., 2014; Laxminarayan, 2014; Toutain et al., 2016). Unfortunately, no data are available to make a definitive conclusion on the duration of treatment of infectious bronchopneumonia in cattle that will optimize efficacy while minimizing the selection of resistant bacteria. In human medicine, several studies have been conducted trying to answer this research question. These studies state that the optimal therapy length is dependent on many factors, including type of pneumonia, condition of the patient, the causative pathogen and antimicrobials being used. However, multiple studies have demonstrated that a treatment duration of 5 days is equally effective compared with longer treatments (Apley, 2015). In cattle, mostly a treatment duration of 7 days or longer is recommended, although this is not scientifically proven. It is plausible that also in veterinary medicine shorter length treatments might lead to good clinical results. In association with the duration of treatment, the post treatment interval (PTI), or period

between therapeutic application and evaluation of success or failure of the treatment, needs to be considered. This interval can be variable depending on the severity of infection and the condition of the animal. In mild or very acute infections, the clinical condition of the animal may immediately reflect a treatment success. In more severe infections, or when the animal is immunosuppressed, evaluating success of treatment may not be evident until the clinical condition of the animal normalizes. Generally for BRD, a PTI of 24 hours after the assumed clinical effect of a daily antimicrobial or antimicrobials that are developed to obtain a clinical effect for two or three days is being used (Apley et al., 2015). In addition, it has been shown that clinical cures of infectious bronchopneumonia in cattle can occur without the continued presence of antimicrobials (DeDonder and Apley, 2015b). Whether clinical cure is sufficient to assure complete healing of the lesion as can be detected by ultrasonography or radiography is unknown in cattle.

A considerable number of antimicrobials in cattle is currently registered for the treatment of infectious bronchopneumonia, namely beta-lactam antibiotics such as aminopenicillins (+clavulanic acid) and extended spectrum cephalosporins (ceftiofur), tetracyclines (oxytetracycline, doxycycline), potentiated sulfonamides (trimethoprim + sulfonamides), macrolides (tylosin, tilmicosin, tulathromycin and gamithromycin), aminoglycosides (gentamicin), aminocyclitols (spectinomycin), lincosamides (lincomycin), phenicols (florfenicol), and fluoroquinolones (marbofloxacin, danofloxacin, enrofloxacin). In Belgium, guidelines have been compiled by the organization 'antimicrobial consumption and resistance in animals' (AMCRA), aiding in the decision making process of using antimicrobials for metaphylaxis or as a treatment of BRD (Table 1). First choice products can be used without diagnostic measurement, although the latter is always recommended. Second choice products should only be obtained when the diagnosis of BRD is confirmed by diagnostic tests. According to legislation (RD 21/07/2016) in Belgium for food producing animals, third choice antimicrobials, namely fluoroquinolones and 3rd – 4th generation cephalosporines, can only be used under specific conditions.

Table 1: Formularium according to the antimicrobial consumption and resistance in animals (AMCRA) for metaphylaxis or treatment of animals suffering from bovine respiratory disease with the exclusion of *Mycoplasma spp.* (https://www.formularium.amcra.be/I/14). The color code is according to the WHO list of critically important antimicrobials for human medicine (WHO CIA list, 2019)

First choice antimicrobials		
Florfenicol	Penicillin	Trimethoprim-sulfonamides
Second choice antimicrobials		
Amoxicillin/Ampicillin	Doxycycline	Lincomycin + Spectinomycine
Amoxicillin-clavulanic acid	Oxytetracycline	Gamithromycin
Penicillin + Streptomycin	Tildipirosin	Tilmicosin
Penicillin + Neomycin	Tulathromycin	Tylosin
Third choice antimicrobials		
Cefquinome/ Ceftiofur	Danofloxacin	Enrofloxacin
Marbofloxacin	Difloxacin	Flumequin

An investigation of the animal in combination with appropriate sampling is necessary. Identification of the clinical pathogen in association with antimicrobial susceptibility testing is performed in an accredited laboratory. In addition, this susceptibility test should prove that first and second choice antimicrobials are no longer effective against this specific disease and only susceptibility is seen against one or more third choice antimicrobials. These antimicrobials can be applied by the veterinarian or by the farmer on prescription. However, there are three exceptions on this legislation. First, if no result by the laboratory was obtained or if sampling was not possible, the veterinarian can apply third choice antimicrobials based on scientific proof. Second, when similar microbiological results are available for the same group (batch) of animals for the same pathology. These results should be no older then 6 months for veal calves, intensively reared poultry and pigs and no older then 12 months for cattle and other species like small ruminants, rabbits, aquaculture and poultry besides chickens. A third exception is in case of emergency to save a (single) animals life. This treatment can only be performed by the veterinarian. Still, clinical investigation in combination with sampling

and analysis remains mandatory. As soon as microbiological results are available, the definitive treatment should be adjusted. This classification is based upon the best available evidence. The color code (yellow, orange and red) is based on the importance of an antimicrobial to human medicine according to the Word Health Organization (WHO), the classification according to the 'Office International des Epizooties (OIE) or World organization for animal health and the reports of the European Medicines Agency (EMA). Code red indicates the 'Highest Priority Critically Important Antimicrobials', including quinolones and 3rd and 4th generation of cephalosporins (WHO, 2019).

In the feedlot industry, 16% of the animals display signs of infectious bronchopneumonia, of which 87.5% is treated. Treatment of clinical cases always consists of injectable antimicrobials and 3% of the treated cattle received oral antimicrobials. Up to 31% received prophylactic antimicrobials on arrival (APHIS, USDA, 2013). Results of the dairy industry show that 18.1% of the preweaned calves are treated for infectious bronchopneumonia (Dargatz and Lombard, 2014). The main indication for treatment in the veal industry is respiratory disease (Pardon et al., 2013; Lava et al., 2016). In a study on Swiss veal calves, the majority of this treatment (84,6%) is performed as group treatment with oral powder fed through an automatic milk feeding system (Lava et al., 2016). A Belgian study shows that of these group treatments, 13% is used as prophylaxis and 87% as metaphylaxis or as a treatment (Pardon et al., 2012). Group treatment in Swiss veal calves mainly consists of combination products like chlortetracycline-tylosin-sulfadimidine or chlortetracycline-spiramycin for 54.9% or amoxicillin for 43.7%. In Belgian veal calves, group treatment consists of oxytetracycline (23.9%), doxycycline (23.9%), or a combination of products like tylosin with oxytetracycline (13.4%) and tylosin with doxycycline (13.4%). As in feedlot cattle, individual treatment of veal calves is mainly applied with injectable antimicrobials (88.5%) using fluoroquinolones (38.3%), penicillins (amoxicillin or benzyl penicillin, 25.6%), macrolides (13.1%), tetracyclines (12%), 3th and 4th generation cephalosporins (4.7%) and florfenicol (3.9%) (Lava et al., 2016). Also in North America, macrolides are often used as metaphylaxis and fluoroquinolones as treatment (APHIS, USDA, 2013). Taking these overall results of a high antimicrobial use for BRD into account, it is not surprising that antimicrobial resistance in bacterial pathogens involved in BRD is common.

1.4.2. Antimicrobial resistance

Both in veterinary and in human medicine, there is an evident link between antimicrobial resistance and the (ab)use of antimicrobials (Chantziaras et al., 2014; Tang et al., 2017). In order to prevent a further spread and increase of antimicrobial resistance in pathogens, global action plans have been initiated emphasizing the importance of prudent use of antimicrobials, especially the critically important antimicrobials (WHO, 2015). The first step in this progress is the collection and reporting of data on the use of antimicrobial agents. For food-producing animals in Europe, this is performed by the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC). The latest report of ESVAC demonstrates that Belgium is the 8th highest consumer of antimicrobials (EMA, ESVAC, 2018). Nevertheless, Belgium is making progress in reducing the amount of antimicrobials used, with a decrease of 35.4% from 2011 until 2018, with a reduction of 12.8% from 2017 to 2018. Furthermore, since 2011, a reduction of 79.1% of the critically important antimicrobials and 69.8% of medicated feed has been attained (AMCRA, 2019).

Notwithstanding the current efforts, resistant isolates of *Pasteurellaceae* are being reported, with a various prevalence depending on the country of origin and corresponding antimicrobials used. An overview of resistant isolation rates of *Pasteurellaceae* from different countries during recent monitoring programs is presented in Table 2. Overall for *Pasteurellaceae*, mainly resistance against tetracycline and tilmicosin is noticed, with varying resistance levels against penicillin, florfenicol, spectinomycin, tulathromycin and enrofloxacin. Interestingly, no resistance of *Pasteurellaceae* was noticed against amoxicillin-clavulanic acid, ceftiofur and trimethoprim-sulphonamide during these different monitoring programs (Table 2). When comparing the Pfizer monitoring program in America-Canada (Portis et al., 2012) with the VethPath study monitoring program in Europe by the European animal health study centre (CEESA, de Jong et al., 2014), a divergence in prevalence of resistance is noticed with overall higher resistance rates in America-Canada. In both monitoring programs, isolates were obtained from diseased animals. However, no information on recent antimicrobial treatment was available in the Pfizer monitoring program of the United States and Canada, whereas in the European VethPath program cases were selected when no prior antimicrobial treatment was present. For the Animal Health Care

program in Belgium (DGZ, 2019), isolates from animals that were already treated with antimicrobials were included in the study. This can potentially explain the higher resistance rates in Belgium compared to other European Countries in the VethPath program, since it is generally accepted that bacterial strains isolated after treatment contain higher resistance rates due to selection pressure (Noyes et al., 2015; Crosby et al., 2018).

Furthermore, a difference in resistance rate can occur between different herd types. A previous study shows resistance rates of *Pasteurellaceae* from dairy, beef and veal calves of 17.6%, 21.9% and 71.9%, respectively (Catry et al., 2005). A higher frequency of resistance in the veal calf industry was seen for ampicillin, tetracyclines, sulfonamides-trimethoprim, tilmicosin, gentamicin and enrofloxacin. Also, multi-resistance (resistance to three or more antimicrobial categories, Magiorakos et al., 2012) was noticed in the veal calf industry, probably because of the routinely administration of oral prophylactic antimicrobial therapy. Extensively drug-resistant bacteria (susceptible to only one or two categories, Magjorakos et al., 2012) or pandrug-resistant bacteria (resistance to all agents in all antimicrobial categories, Magjorakos et al., 2012) were not present in the previous study. In the veal calf production system, a large within-herd variability of susceptibility profiles was seen, likely due to commingling of calves from different origin (Catry et al., 2005). All these discrepancies can influence the outcome of resistance rates. In order to truly compare resistance profiles over time and between different regions, equality in case selection, antimicrobial susceptibility tests and susceptibility test interpretation criteria is mandatory.

During this doctoral thesis, both deep nasopharyngeal swabs (DNS) and non-endoscopic bronchoalveolar lavages (nBAL) were collected from animals of different (veal, dairy and beef) problem herds (herds experiencing economic losses due to respiratory disease which cannot be controlled) presented at the clinic of Large Animal Internal Medicine (Ghent University). In total, 132 isolates of *P. multocida*, 66 isolates of *M. haemolytica* and 8 isolates of *H. somni* were obtained (Van Driessche et al., unpublished data). Overall, resistance rates for all *Pasteurellaceae* isolated from problem herds are worrisomely high (Table 2). The most concerning factor is the increased resistance rate against critically important antimicrobials, namely enrofloxacin, ceftiofur and tulathromycin of *Pasteurellaceae* isolated from these problem herds compared to

isolates obtained from a monitoring program in Belgium in 2018 (DGZ, 2019). Also, at these problem herds isolates of *M. haemolytica* were present who showed resistance against ≥4 antimicrobial classes, including all critically important antimicrobials (Van Driessche et al., 2018). Animals originating from problem herds were possibly already treated multiple times with different classes of antimicrobials. Bringing these animals to the clinic or bringing the clinic to these animals was a last resort due to therapy failure. Since antimicrobial use is directly linked to antimicrobial resistance (Chantziaras et al., 2014; Tang et al., 2017), it can therefore be expected that isolates from problem herds would obtain higher resistance rates compared to isolates from a monitoring program in Belgium. However the presentation of animals from problem herds suffering from unresponsive infectious bronchopneumonia at the clinic of Large Animal Internal Medicine has increased in the last years, addressing the importance of diagnostic samples to prevent and control these herds and to change the way antimicrobials are used in this sector.

Although at first sight resistance rates of *Pasteurellaceae* do not look worrisomely high, the incidence of resistant pathogens is continuously increasing to antimicrobials which are commonly used to tackle BRD (Welsh et al., 2004; Lubbers and Hanzlicek, 2013; DeDonder and Apley, 2015a). Nowadays, the presence of multi-to extensively drug resistant *Pasteurellaceae*, harboring multiple antimicrobial resistance genes, is increasing (Woolums et al., 2018). Furthermore, Integrative conjugative elements (ICE), which are chromosome-borne mobile genetic elements that can carry clusters of resistance genes, have been described (Wozniak and Waldor, 2010). These ICEs can possibly lead to pan-resistant pathogens where antimicrobial treatment is useless. ICE's have been described in *P. multocida*, *M. haemolytica* and *H. somni* (Michael et al., 2012; Eidam et al., 2015). In addition, transmission of these elements by conjugation has been described between *Pasteurellaceae* and from *P. multocida* to *Escherichia coli* (Klima et al., 2014b). Also the transfer of resistance genes through plasmids from other Gram-negative bacteria to *P. multocida* is seen (Kadlec et al., 2011). The fact that resistance genes can be exchanged between different bacterial species and resistant strains are able to clonally expand (Katsuda et al., 2009), is very worrisome and will lead to an exponential increase of therapy failure both in humans and animals when no action is taken.

Table 2: resistant isolation rates of *pasteurellaceae* from different countries during recent monitoring programs. resistance was determined by available clinical breakpoints according to CLSI. when latter was not available, breakpoints of other *pasteurellaceae* were used (for example clinical breakpoint of *M. haemolytica* was used for *P. multocida* for tilmicosin) or resistance was determined according to the guidelines of the manufacturer

Bacteria	Origin	Year(s) of isolation	No. of isolates	Percentage of resistance										Reference
				PEN	AMC	CEF	FFC	ENR	SPE	TET	TIL	TUL	TMP/S	
P.	USA + Canada	2000-2009	328	3.3	/	0.0	11.6	2.1	/	40.8	23.8	4.6	/	Portis et al., 2012
multocida	Germany: calf	2012-2013	48	2.1	/	0.0	0.0	0.0		10.4	2.1	0.0	/	BVL, 2016
	Germany: adult cattle	2002-2006	50	/	0.0	0.0	2.0	0.0	10.0	0.0	/	/	0.0	de Jong et al., 2014
	Czech Republic	2002-2006	18	/	0.0	0.0	0.0	0.0	5.6	22.2	/	/	0.0	de Jong et al., 2014
	France	2002-2006	41	/	0.0	0.0	0.0	0.0	2.4	0.0	/	/	0.0	de Jong et al., 2014
	Ireland	2002-2006	36	/	0.0	0.0	0.0	0.0	0.0	0.0	/	/	0.0	de Jong et al., 2014
	Italy	2002-2006	51	/	0.0	0.0	0.0	0.0	2.0	11.1	/	/	0.0	de Jong et al., 2014
	United Kingdom	2002-2006	28	/	0.0	0.0	0.0	0.0	0.0	0.0	/	/	0.0	de Jong et al., 2014
	Belgium	2018	176	18.2	0.0	0.0	0.0	1.1	19.3	27.8	18.2	8.5	2.3	DGZ, 2019
	Belgium: problem herds	2015-2019	132	4.5	/	2.2	2.3	9.8	/	37.1	/	15.9	37.1	Van Driessche et al., unpublished data*

Bacteria	Origin	Year(s) of isolation	No. of isolates	Percentage of resistance									Reference
				PEN	AMC	CEF	FFC	ENR	SPE	TET	TIL	TUL	TMP/S	
M. haemolytica	USA + Canada	2000-2009	304	27.3	/	0.0	8.6	6.6	/	43.7	27.3	8.9	/	Portis et al., 2012
	Germany: calf	2012-2013	63	11.1	/	0.0	1.6	0.0	/	22.2	1.6	0.0	/	BVL, 2016
	Germany: adult cattle	2012-2013	35	8.8	/	0.0	0.0	0.0	/	0.0	2.9	2.9	/	BVL, 2016
	Germany	2002-2006	27	/	0.0	0.0	0.0	0.0	0.0	14.3	0.0	/	0.0	De Jong et al., 2014
	France	2002-2006	35	/	0.0	0.0	0.0	0.0	0.0	9.5	0.0	/	0.0	De Jong et al., 2014
	Ireland	2002-2006	20	/	0.0	0.0	0.0	0.0	0.0	0.0	0.0	/	0.0	De Jong et al., 2014
	Italy	2002-2006	31	/	0.0	0.0	0.0	0.0	0.0	5.3	6.5	/	0.0	De Jong et al., 2014
	Belgium	2018	145	28.0	1.4	0.7	5.0	7.7	1.4	32.2	/	12.6	15.4	DGZ, 2019
	Belgium: problem herds	2015-2019	66	31.8	/	4.5	9.1	33.3	/	30.3	/	71.2	42.4	Van Driessche et al., unpublished data*
H. somni	USA + Canada	2000-2009	174	4.5	/	0.0	1.7	7.4	/	42.5	18.4	10.9	/	Portis et al., 2012
	Belgium: problem herds	2015-2019	8	12.5	/	0.0	12.5	12.5	/	25	/	25	37.5	Van Driessche et al., unpublished data*

Abbreviations: PEN, penicillin; AMC: amoxycillin-clavulanic acid; CEF: ceftiofur; FFC: florfenicol, ENR: enrofloxacin; SPE: spectinomycin, TET: tetracycline; TIL: tilmicosin; TUL: tulathromycin; TMP/S: trimethoprim-sulfonamides. *The broth dilution technique was used for the combination of *M. haemolytica* and the antimicrobials penicillin, tulathromycin, tetracycline and florfenicol. Standard quality control strains were included, i.e. *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Streptococcus pneumoniae* ATCC 49619. Additionally, *M. haemolytica* ATCC 33396 was used for tulathromycin resistance detection. The antimicrobial gradient diffusion technique was used for tetracycline resistance detection in *P. multocida*, with the inclusion of *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 as quality control stains. All other antimicrobials tested for resistance for *M. haemolytica* and *P. multocida*, and all antimicrobials tested resistance for *H. somni* were performed by disk diffusion without quality control strains.

2. Current diagnostic techniques in BRD

An accurate diagnosis of BRD with the identification of the causative pathogen(s) is mandatory for optimal prevention strategies and a more rational and effective treatment. Pathogens can be isolated from the lower respiratory tract both in healthy, 'apparently' healthy (subclinically affected) and diseased animals, making it challenging to define the causative pathogen of BRD (Angen et al., 2009; Nicola et al., 2017; Timsit et al., 2018). However, studies have shown that isolation rates, both from nasal and pulmonary samples, are higher in diseased animals compared to 'apparently' healthy animals (Autio et al., 2007; Angen et al., 2009; Timsit et al., 2017). Therefore, a proper investigation of the clinical condition of the animal should be executed for an accurate diagnosis of BRD resulting in a fast empiric treatment. In order to correctly identify the causative agent(s) of BRD, sampling is necessary which will result in a correct definitive therapy.

2.1. In the field

2.1.1. Detection techniques to diagnose BRD

2.1.1.1. Clinical investigation

A rapid and accurate diagnosis of BRD is key to prevent morbidity and mortality in cattle. As for any disease, diagnosis starts with a proper clinical investigation. Visual observations that are included are depression, anorexia and respiratory signs like nasal discharge, coughing and increased breathing rate (Perino and Apley, 1998; Buhman et al., 2000; Duff et al., 2007). However, these observations can be rather subjective. Rectal temperature can be monitored as an objective method, although the cutoff value is not consistent (Booker et al., 1999; Pardon et al., 2015; Yves and Richeson, 2015). In order to have a more objective approach, several scoring systems for BRD have been developed (McGuirk, 2008; Love et al., 2014; Pardon et al., 2015). Despite the fact that these scoring systems are easy to use and can be immediately applied into the field, diagnostic accuracy for BRD is not high. This is not surprising considering the fact that clinical signs are often not specific for BRD (Griffin et al., 2010) resulting in a low

sensitivity. A low specificity can be explained by the fact that cattle are prey animals (Weary et al., 2009) and therefore mask weakness and disease, especially in the presence of humans. In order to bypass this human presence, technology has been developed which can remotely monitor cattle in behavior, activity and feeding changes, resulting in a better diagnostic accuracy (White et al., 2016). Several studies have established the diagnostic accuracy of clinical examination based on pulmonary lesions present at slaughter. These studies show that 60% or more of animals, that presented pulmonary lesions at slaughter, were never identified as ill by clinical observations (Wittum et al., 1996; Thompson et al., 2006; Tennant et al., 2014), resulting in an estimated sensitivity and specificity of 61.8% and 62.8%, respectively (White and Renter, 2009). Another review conducting 7 studies in a meta-analysis demonstrated a sensitivity and specificity of 27% and 92%, respectively for clinical illness (Timsit et al., 2016). When diagnostic accuracy of scoring systems was based on thoracic ultrasound and auscultation, higher diagnostic sensitivity (+/- 72%) and specificity (+/-89) results were obtained, depending on which scoring system was used (Love et al., 2016). These results suggest that clinical observation alone is not sufficient for diagnosing BRD.

2.1.1.2. Thoracic auscultation and ultrasonography

To further strengthen the diagnosis of BRD, thoracic auscultation can be performed (Wilkins et al., 2009). When RTIs are present, increased bronchial sounds, crackles, wheezes or absence of respiratory sounds (Buczinski et al., 2014) can be noticed on the ventral and middle part of the thorax since mostly infections are located at these regions (Bryant et al., 1999; Thompson et al., 2006). As this technique can be easily performed in the field, thoracic auscultation is commonly used to diagnose BRD. However, thoracic auscultation alone yielded a very low sensitivity (5.9%) in detecting lung consolidation in a two-observer study (Buczinski et al., 2014). Another study who compared lung auscultation with ultrasonography findings in a much larger group of 41 Dutch veterinarians showed a sensitivity and specificity of 63% and 46%, respectively (Pardon et al., 2019). In combination with clinical observation, sensitivity of auscultation reached 71.4% (Buczinski et al., 2014) Furthermore, a very poor reliability between different practitioners is present (Pardon et al., 2019). These results shows that for most practitioners thoracic auscultation alone has no added value to the diagnosis of BRD. In

order to improve diagnostic accuracy of thoracic auscultation, computer-aided lung auscultation systems were developed, which automatically categorizes acoustic patterns in lung scores, called Whisper stethoscope. This automated system can reach sensitivity and specificity of 92.9% and 89.6%, respectively (Mang et al., 2015), resulting in an added value for BRD diagnostics. However, this technique is not routinely applied, probably due to the high purchase cost (€500).

Since visual observations and thoracic auscultation are not representative for lung lesions, thoracic ultrasonography can be regarded as an additional tool. When animals suffer from pneumonia, lung consolidation will be visible on ultrasonography due to the displacement of air from the lung tissue (Caswell and Williams, 2007). For investigating lungs of calves, identical equipment as used for reproductive examination of cows can be used, making this technique also applicable in the field. For adult cattle, only the surface of the lungs can be visualized with latter equipment, and therefore more specialized material is necessary. Another disadvantage that hinders practitioners to commonly use this technique is the acquirement of specific training. Therefore, a quick scan method only comprising ample minutes and an algorithm of ultrasonographic images for rapid classification of lung lesions was developed at Ghent University (Pardon, 2019). A study where the UGhent quick-scan method and algorithm was demonstrated to two novice observers, demonstrated a fast learning curve and a mean scanning time of approximately 2 minutes, although more exercise is mandatory for better diagnostic accuracy (De Cremer et al., 2018).

Thoracic ultrasonography comprises multiple advantages. First of all the diagnostic accuracy of lung lesions is excellent (Rabeling et al., 1998; Reinhold et al., 2002; Buczinski et al., 2015; Ollivett et al., 2015). A study, using a Bayesian comparison and a cut-off value of ≥ 1cm in consolidation depth, showed a sensitivity and specificity of 79.4% and 93.9%, respectively (Buczinski et al., 2015). When using a cut-off of ≥ 3cm of lung consolidation caudal from the heart, even a sensitivity and specificity of 89.0% and 95.0% could be reached (Berman et al., 2019). Second, in contrast with thoracic auscultation and clinical investigation, little variation in outcome of diagnosis of BRD is observed between practitioners (Buczinski et al., 2013; Buczinski et al., 2018). Third, a differentiation between upper RTIs, subclinical and clinical bronchopneumonia can be made in combination with visual observations. Fourth, the severity of infection and

therefore prognosis can be estimated as this technique can detect consolidation of the caudal lung lobes, lung abscesses and necrosis (Ollivett and Buczinski., 2016). This tool aids in the decision making process, letting the practitioner decide in an objective way whether treatment is still economically justifiable (Rademacher et al., 2014). In addition, if treatment is performed, this method can also monitor the healing process of the lung and therefore aid in the PTI to evaluate efficacy of treatment (De Cremer et al., 2019).

Considering that visual observations in combination with ultrasonography can differentiate between clinical outcomes and that ultrasonography has an incremental value over auscultation (Buczinski et al., 2016), it is reasonable to assume that the combination of clinical observation and ultrasonography will result in the highest probability of diagnosing an animal with BRD. However, these techniques do not allow differentiation between causative pathogens. Therefore, sampling of the respiratory tract is mandatory to identify the causative agents in order to justify a definitive treatment .

2.1.1.3. Other detection techniques to diagnose BRD

Blood samples are described as a sampling technique to determine different parameters indicative of BRD. Total white blood cell count and differential cell count can be used to verify inflammation (Jones and Allison, 2007). However, in case of BRD, poor diagnostic accuracy is obtained compared to clinical signs and lung lesions at necropsy, concluding that this technique cannot be used to predict BRD (Hanzlicek et al., 2010). Acute-phase proteins, including haptoglobin, serum amyloid A and fibrinogen have been investigated in their potential to diagnose BRD. Although several studies showed promising results measuring haptoglobin (Carter et al., 2002; Humblet et al., 2004; Angen et al., 2009), high levels are also obtained when animals suffer from other infections then BRD and is therefore not specific (Svensson et al., 2007). Furthermore, no definitive outcome on the added value of this acute phase protein can be made since these studies contain variable definitions and a high risk of bias (Abdallah et al., 2016). In human medicine, acute phase proteins like C-reactive protein (CRP) and procalcitonin (PCT) can be used as biomarkers for bacterial infections of the lower respiratory tract (Çolak et al., 2017). In cattle, CRP seems of limited value for diagnosing BRD (Petersen et al., 2004) and PCT has only been described as biomarker of sepsis in calves (Ercan et al., 2016; Bonelli et

al., 2018). Also, a stress-related hormone like cortisol is not only increased by infection and therefore shows low specificity considering BRD (Grandin, T., 1997; Schaefer et al., 2012). Although lactate measurement can be used to predict mortality in calves with severe lung lesions (Coghe et al., 2000), it is not helpful in identifying BRD in animals with mild infection (Hanzlicek et al., 2010). Equal results apply for blood gas analysis (Hanzlicek et al., 2010).

Breath analysis studies show some promising results, however the clinical applicability can be questioned (Reinhold et al., 2000; Burciaga-Robles et al., 2009). Histology of lung tissues and cytology of fluid obtained by bronchoalveolar lavage (BAL) or transtracheal aspiration (TTA) are both valuable in demonstrating inflammation in the lung and determining the presence however not the differentiation of bacteria (Schiefer et al., 1978; Allen et al., 1991; van Leenen et al., 2019). Other techniques like radiography and computed tomography have been described for diagnosing BRD (Lubbers et al., 2007; Masseau et al., 2008). However, these are not applicable in practice because these are highly expensive and very labor intensive, which is mostly not economically viable.

2.1.1.4. Postmortem examination

Postmortem examination can aid in the diagnosis of BRD, with a sensitivity and specificity of 91% and 67%, respectively for lung lesions present at slaughter (Timsit et al., 2016b). Although histopathology can be useful in differentiating bacterial etiologies involved in BRD (Zhang et al., 2019), identification of the causative pathogen(s) of BRD is often not possible, and classification of lesions to identify pathogens is therefore rather informative than conclusive. Generally, interstitial pneumonia is caused by viruses, whereas caseonecrotic bronchopneumonia is caused by *M. bovis*. For *Pasteurellaceae*, typical lesions are suppurative bronchopneumonia and fibrinous pneumonia or pleuropneumonia. Suppurative bronchopneumonia and fibrinous pneumonia or pleuropneumonia are both characterized by bilateral, cranioventrally distributed consolidation with a divergent colour in the affected lobes. In suppurative bronchopneumonia, purulent to mucopurulent exudate is present within the bronchial tubes and is mostly, but not exclusively, associated with *P. multocida* (Panciera and Confer, 2010) or *H. somni* (Zhang et al., 2019). In fibrinous pneumonia, a wide distention of the interlobular septa with oedema or fibrin is noticed, resulting in a marbled

appearance of the lobes. These lesions are mostly related to *M. haemolytica* (Zhang et al., 2019) but can also be seen in infections with other bacteria, such as *H. somni* (Panciera and Confer, 2010). In fibrinous pleuropneumonia, fibrinous pleuritis is noticed with fibrin strands and fluid within the pleural cavity (Panciera and Confer, 2010). Considering the pathology of *H. somni*, other lesions like myocarditis, myocardial infarction and fibrinous synovitis can be present at postmortem examination, resulting in more conclusive evidence of *H. somni* infection.

Besides typical lesions, there has been a correlation between the causative pathogen and the acuteness of disease and subsequently mortality. *M. haemolytica* is most frequently isolated from feedlot animals that died peracute, whereas *M. bovis* is more obtained from chronic cases (Booker et al., 2008). However, in more than 60% multiple clinically relevant pathogenic bacteria can be isolated from the lungs at necropsy in cattle, complicating the ability of identifying the truly causative pathogen(s) (Gagea et al., 2006; Booker et al., 2008; Fulton et al., 2009). Other obstacles with postmortem examination are that animals that died may not be representative of the herd problem because of the presence of another illness or an underlying chronic disease. Furthermore, animals presented for postmortem examination are often treated with antimicrobials, inhibiting reliable culture results. Thus lesions at postmortem examination are not conclusive for the identification of the causative pathogen of BRD nor provide susceptibility testing results. To obtain these microbiological results, optimal sampling techniques are necessary which maximize the chance of isolating the causative pathogen and minimize the chance of contamination.

2.1.2. Sampling techniques to identify the causative pathogen of BRD

Currently three methods for sampling the respiratory tract in cattle are practically possible for field application, namely deep nasopharyngeal swab (DNS), bronchoalveolar lavage (BAL) and transtracheal wash (TTW). Which method is preferred in a given country, has become rather a cultural decision than what is based on hard evidence. An overview of the different sampling site, advantages and disadvantages of each technique is given at the end of this chapter (Table 3).

2.1.2.1 Deep nasopharyngeal swab

A deep nasopharyngeal swab samples the nasopharynx and tonsils. Before inserting the swab, the nostrils are disinfected with alcohol in order to prevent contamination in this region. After disinfection, the swab is gently advanced in the medioventral conchae until a depth that is equal to the distance to the medial canthus of the eye, which should result in sampling the tonsils (Figure 3). This technique can be performed with or without a protective sleeve to minimize contamination from the proximal nasal cavity. Advantages of this technique are the ease and speed which makes it possible to sample multiple animals in a short time frame. However, since a DNS samples the upper respiratory tract, many questions arise if these samples are representative for infections of the lower respiratory tract. Regarding the pathology of infectious bronchopneumonia, encountering that under predisposing factors opportunistic bacteria can multiply in the nasopharynx and subsequently descent into the lungs to cause infection, make it plausible that DNS samples are able to collect these causative pathogens.

Indeed, it has been demonstrated that isolates obtained from DNS can be identical to isolates obtained from the lung in 70% (DeRosa et al., 2000), 77% (Timsit et al., 2013) to even 100% (Godinho et al., 2007) of the cases. To better understand this pathology, several studies have been conducted analyzing the microbiota of the upper and lower respiratory tract. These results show that the microbiota of the upper respiratory tract can drastically change over time (Timsit et al., 2016) and although different from the microbiota of the lower respiratory tract, share several operational taxonomic units (Nicola et al., 2017), suggesting a correlation between both microbiota. Studies have

compared the agreement of DNS with samples retrieved from the lower respiratory tract (TTW, BAL). For isolating respiratory viruses, a DNS can be used (O'Neill et al., 2014) although studies describe that applying samples retrieved from the lower respiratory tract are more suitable (Kimman et al., 1986; Heckert, et al., 1997; Caldow, 2001; Doyle et al., 2017). When guarded DNS are compared with lung lavages postmortem, the positive predictive value is 100% both for *M. haemolytica* and *M. bovis*. The negative predictive value was 67% for *M. haemolytica* and 33% for *M. bovis* (Godinho et al., 2007). These results are in agreement with Thomas et al., 2002, who demonstrated a low sensitivity (21.4%) but high specificity (94.4%) for *M. bovis* from unguarded DNS compared to BAL, suggesting that BAL is the best method for isolating *M. bovis* in cattle with respiratory disease (Thomas et al., 2002).

Considering the *Pasteurellaceae*, positive and negative predictive values of DNS samples compared to BAL samples on individual calf level are 67% and 100% respectively for *M. haemolytica*, 75% and 100% respectively for *P. multocida* and 100% and 96% respectively for *H. somni* (Capik et al., 2017). A very good agreement between DNS and BAL/TTW is noticed for *Pasteurellaceae* (Capik et al., 2017, Doyle et al., 2017). In contrast, other studies suggest only a moderate agreement between DNS and BAL samples (Allen et al., 1991) and DNS and TTW samples on individual calf level Timsit et al., 2013). These results show that DNS sampling can be an added value for BRD diagnostics, provided that a correct sampling technique is performed on acutely ill animals (DeRosa et al., 2000) and multiple animals within a group are sampled (Allen et al., 1991).

Figure 3: A deep nasopharyngeal swab

2.1.5.2. Bronchoalveolar lavage

A bronchoalveolar lavage (BAL) samples a part of the lung, resulting in a more clear image which pathogens are actually present at the site of infection. After disinfecting the nostril, a small long sterile catheter can be inserted ventromedial, passing to the conchae and reaching the larynx. When entering the trachea, the animal will display a cough and the tip of the tongue will curl up. Ticking to the cartilage rings of the trachea can be felt while further advancing through the trachea into the bronchi until wedge position is reached (Figure 4). An average of ±100 mL, ranging from 50 mL to 250 mL (Pringle et al., 1988; Allen et al., 1991; Caldow, 2001; Thomas et al., 2002; Capik et al., 2017; Doyle et al., 2017) of sterile physiological saline is inserted into the lung and immediately aspirated, leaving a foam layer in the sample due to contact with surfactants.

This technique can be performed with (Allen et al., 1992) or without an endoscope (Fogarty et al., 1983). Advantages of applying an endoscope are the protection of the catheter from contamination and the option to sample a particular or multiple lung lobes. An endoscope-guided BAL is therefore mostly performed in human medicine (Reynolds, 2011; Bello et al., 2016), cattle (Allen et al., 1991; Allen et al., 1992) companion animals (Johnson and Vernau, 2011), and even exotic animals (Hermes et al., 2018), although contamination of the nasopharyngeal flora by passage of the endoscope cannot be completely avoided (Reynolds, 2011). However in veterinary medicine, and in particular food-producing animals, using an endoscope is very time consuming since proper disinfection is mandatory between sampling different animals. Furthermore, it can be quite risky to use expensive material when working with animals, unless these animals are sedated which again is time-consuming.

A more rapid technique which is more applicable in practice is a non-endoscopic bronchoalveolar lavage (nBAL). Although this technique is becoming more commonly used in practice, evidence of the bacteriological merit of an nBAL is lacking. Before this doctoral thesis, this technique had been described in cattle (Caldow, 2001; Thomas et al., 2002), horses (McKane and Rose, 1993) and humans (Kollef et al., 1995; Tasbakan et al., 2011). However, a lot of concern arises if contamination can occur by passage of the catheter through the nose when performing a BAL. Therefore, in practice it is recommended to perform a non-endoscopic BAL with a protective sleeve or inner- and outer tube (Caldow, 2001). Different studies have been analyzing the diagnostic

accuracy of BAL samples compared to other techniques. Although TTW is generally considered most suitable for microbiological results, no difference is seen between BAL and TTW for the isolation of *M. haemolytica*, *P. multocida* and *M. bovis* in dairy calves with clinical BRD (Doyle et al., 2017). However, BAL samples have greater value for cytological investigation than TTW samples (Hoffman, 2008), which can also be used for the diagnosis of Dictyocaulus viviparus infection (Boon et al., 1987). Additionally, BAL is considered the most appropriate method for postmortem identification compared to swab samples or tissues of the lung (Godinho et al., 2007).

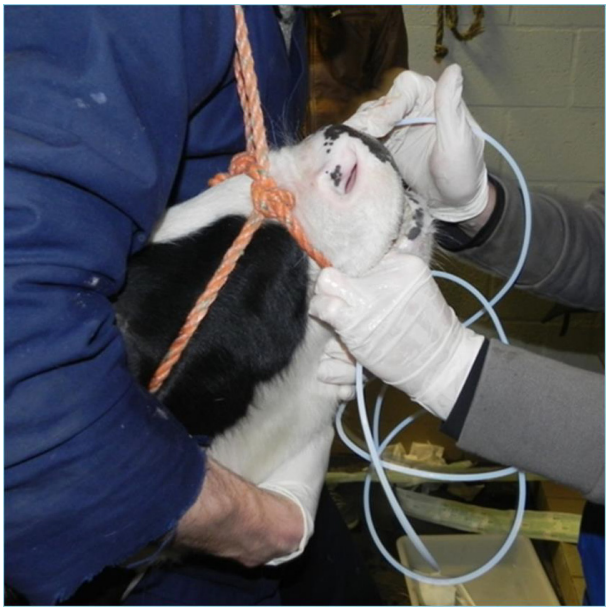

Figure 4: A non-endoscopic bronchoalveolar lavage (nBAL)

2.1.5.3. Transtracheal wash and other transtracheal techniques

Transtracheal techniques has been developed to bypass nasal contamination (Espinasse et al., 1991). Besides a transtracheal wash (TTW) which is more commonly used, a transtracheal bronchoalveolar lavage and a transtracheal swab can be performed. A transtracheal bronchoalveolar lavage combines the advantage of bypassing the nasopharyngeal flora with an equal sampling site as a nasal-induced bronchoalveolar lavage. A transtracheal swab has been described (Heckert et al., 1997; Rohn et al., 1998; DeRosa et al., 2000), but is not commonly used in practice. As for nasopharyngeal swabs, transtracheal swabs need to be performed on multiple acutely ill animals (Heckert et al., 1997; Rohn et al., 1998).

Although in literature a TTW is sometimes referred to as 'transtracheal aspiration' (TTA), this terminology is not correct since in these studies fluid is inserted into the respiratory tract and immediately aspirated resulting in a wash rather than an aspiration (Timsit et al., 2013; Nicola et al., 2017). When performing a TTW, the animal is restrained and the ventral region in the neck, with easy access to the trachea, is shaved, disinfected and locally anesthetised. A small incision is made and a thick needle is perforated through the trachea. Through this needle, a small catheter is further advanced into the trachea till the bifurcation (Figure 5). A volume of physiological fluid, with an average amount of ± 40 mL ranging 30 mL - 50 mL (Timsit et al., 2013; Doyle et al., 2017; Nicola et al., 2017) is inserted into the lungs and immediately aspirated.

An advantage of this technique is that different bronchi and therefore different lung lobes are being sampled in contrast to BAL. However, it needs to be addressed that, when sampling at the bifurcation of the lung, only bronchi, and not bronchioli or alveoli will be flushed. To overcome this hurdle, a transtracheal bronchoalveolar lavage can be performed with a volume ranging from 10 mL to 40 mL (Heckert et al., 1997; Sheehan et al., 2005; Angen et al., 2009). Also, although the bifurcation and deeper parts of the lung are being sampled, TTW can as easily detect a descending infection like tracheitis as it can detect pneumonia due to a netto influx of commensals and pathogens from the nasopharynx to the trachea. This is evidenced by multiple studies stating that the respiratory tract is not sterile, demonstrating that also in healthy calves (Nicola et al., 2017; Timsit et al., 2018) opportunistic bacteria can be found in lower respiratory tract specimens. Therefore, excluding 100% of the nasopharyngeal flora seems impossible.

However it has been demonstrated that transtracheal samples often contain pure cultures (Angen et al., 2009). Furthermore, it has been shown that isolation rates improve with transtracheal bronchoalveolar lavage compared to nasal and tracheal swabs in chronically ill animals (Rohn et al., 1998). Therefore TTW is commonly used in cattle, specifically in Canada, France and Germany. However, this technique also holds disadvantages. A transtracheal technique is more labor intensive and therefore more time consuming compared to other techniques. Also, this technique requires more material than other sampling methods, making transtracheal samples more expensive (Rohn et al., 1998).

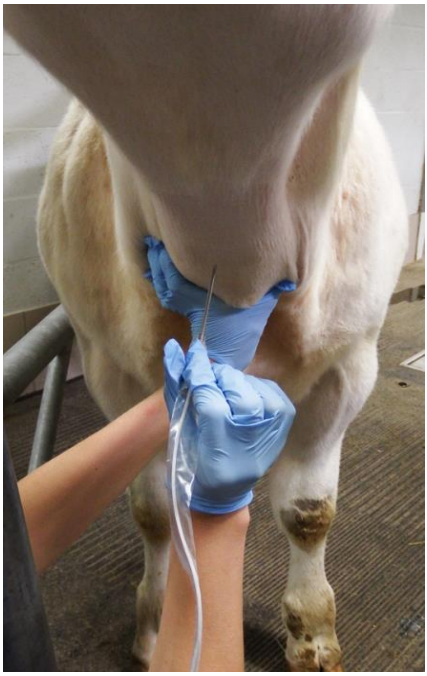

Figure 5: A transtracheal wash

Table 3: Characteristics of three sampling techniques (deep nasopharyngeal swab, bronchoalveolar lavage and transtracheal wash) commonly used to sample the respiratory tract of cattle (Heckert et al., 1997; Rohn et al., 1998; Capik et al., 2017; Doyle et al., 2017)

Characteristics	**Deep nasopharyngeal swab**	**Broncho-alveolar lavage**	**Transtracheal wash**
Sampling site	Nasopharyngeal mucosae	Lung lobe	Tracheal bifurcation
Sampled surface	< 0,5 cm^2	>10 cm^2	5-10 cm^2
Easiness to perform	+++	++	+
Cost for sampling procedure	1,5 euro	5 euro (re-usable catheter)	15 euro (no reusable catheter)
Representative for deep airways	Less likely	More likely (nasal contamination)	More likely (aspiration possible)
Possible contamination through nasal passage	+++	+	-
Animal welfare	+++	++	+

2.2. In the laboratory

2.2.1. Identification of causative bacterial pathogens

2.2.1.1 Cultivation

Before identification of the causative bacterial pathogens can occur, a cultivation step is mostly necessary (Figure 6). For samples derived from the respiratory tract, cultivation of *Pasteurellaceae* can be obtained on Columbia blood agar enriched with 5% sheep blood. An incubation time of 20 to 24 hours at a temperature of 35°C is required for visible growth. For *H. somni*, addition of 5% to 10% carbon dioxide (CO_2) to the atmosphere is necessary for growth on an agar plate. Culture result can have different outcomes. A negative culture result, meaning no bacterial colonies are present on the agar plate, can occur when no bacterial colonization or infection is present, or when antimicrobials are used before sampling, possibly inhibiting growth of bacteria. Culture results can be polymicrobial, meaning the presence of several types of microorganisms. This can result from contamination of nasopharyngeal flora in the sample, or it is just a representation of a normal polymicrobial respiratory microbiome or it can be clinically relevant in for example aspiration pneumonia.

Valuable culture result for identifying the causative bacterial pathogen(s) of infectious bronchopneumonia, thus strongly infer a cause and effect relationship with infection, are results were clinically relevant pathogens are abundantly present on the agar plate, with or without ample contamination. Overall, a good clinical interpretation of a culture result is crucial, though this depends on the clinical skills of the technician. Advantages of cultivation are the quantitative measurement that can be performed, where high concentrations of bacteria are more indicative for infection associated with disease then low concentrations (Autio et al., 2007; Angen et al., 2009; Timsit et al., 2017). Also, when cultivation is performed on non-selective agars, it has the ability of finding 'new bacteria' as possible causative pathogen in a certain disease. This is in contrast with molecular techniques where identification of particular bacteria, which are determined in advance, is performed.

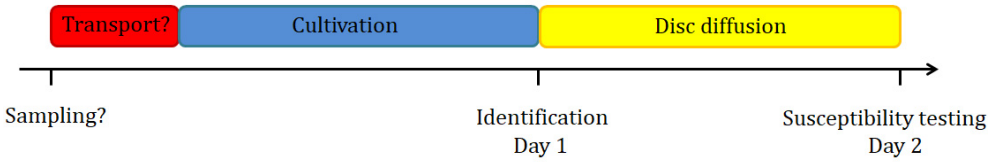

Figure 6: Schematic overview of the different conventional steps acquired for identification and susceptibility testing of the causative bacterial pathogen of infectious bronchopneumonia in cattle over time

2.2.1.2. Phenotypic characteristics

The conventional method for identifying *Pasteurellaceae* is based on the phenotypic characteristics of these bacteria. After incubation, colonial morphology can be inspected. *Pasteurellaceae* are moderate in size, round and greyish. *P. multocida* is characterized by relatively large, mucoid colonies after overnight incubation, is non-haemolytic and does not grow on MacConkey agar. *M. haemolytica* forms smaller colonies after overnight incubation, are beta-haemolytic and can be seen as pinpoint red colonies on MacConkey agar. *H. somni* is characterized by small colonies after 24 to 48 hours of incubation which may appear yellowish and may show a frank clearing around these colonies (Quinn et al., 1994).

Also other *Pasteurellaceae*, like for example non-hemolytic *M. haemolytica*, *Moraxella spp.* and *Gallibacterium anatis* can be involved in infectious bronchopneumonia (Catry et al., 2007a; Mahu et al., 2005; Nicola et al., 2017; Bavananthasivam et al., 2018; Van Driessche et al., 2019). Macroscopically distinguishing *Pasteurellaceae* from other bacterial families and within the family to genus and species level can be challenging. Therefore, biochemical test have been developed, even making it possible to further categorize bacteria into subspecies level (Schiefer et al., 1978; Quinn et al., 1994; Angen et al., 2003; Dousse et al., 2008). However, these biochemical tests are very time-consuming (20 hours) and labor intensive. With the development of new faster and accurate diagnostic techniques, phenotypic identification seems to become outdated in routine diagnostics.

2.2.1.3. DNA-based techniques

Several DNA-based techniques have been described for identifying *Pasteurellaceae*. In contrast with phenotypic identification and MALDI-TOF MS, the cultivation process can be omitted, speeding up the time for identification. Nevertheless, it often takes days before identification results are available due to the laboratory workflow. DNA-based techniques are labor intensive and more expensive. In most clinical laboratories these techniques are not performed daily and multiple samples are collected before performing this technique to increase laboratory workflow. Since no 'viable' bacteria are necessary for molecular techniques, but merely DNA, identification of the causative pathogen can still occur after antimicrobial treatment. However, since no isolates are obtained, susceptibility testing cannot be performed. Furthermore, these techniques are designed for identifying particular pathogens, inhibiting the possibility of detecting less known bacterial pathogens who could also cause a certain disease. Molecular techniques can detect bacteria even present in small numbers and besides quantitative real team PCR do not provide an concentration of bacteria, leaving no room for clinical interpretation with possible over-diagnosis when ample amounts of commensal bacteria are found. In contrast with MALDI-TOF MS, molecular techniques are able to further identify bacteria until subspecies level as well as genotype, providing more epidemiological information during an acute outbreak (Gunawardana et al., 2000).

A commonly used technique in veterinary diagnostics is polymerase chain reaction (PCR). By the use of primers and polymerase, a segment of nucleic acid of a well-known genetic region is amplified during cycli of different temperatures which induce denaturation, annealing and elongation. When a sufficient amount of DNA is present and after gel electrophoresis, this can be identified by using known positive controls (Klima et al., 2010). Newer techniques have made it possible to directly identify the bacterium during the PCR process, omitting gel electrophoresis, namely real-time quantitative PCR (RT-qPCR). A fluorescent signal is measured (expressed as relative fluorescence unit) that will increase by each amplification. When a large amount of DNA is present in the initial sample, an earlier detection of the signal will occur. The cycle of the DNA process where this signal is above the detection limit is determined as Cycle threshold (Ct)-value. Thus the lower the Ct-value, the higher the amount of DNA that was present in the initial sample. Therefore quantitative analysis can be obtained (Guenther et al., 2008). An application of PCR or RT-qPCR is the multiplex PCR, which is capable of identifying

multiple viruses and bacteria by a single test and is therefore more cost-effective although this technique can therefore also be less sensitive. For BRD, different commercial kits using PCR are available: identifying 7 clinically important pathogens, namely PI-3, BRSV, BCV, *M. haemolytica*, *P. multocida*, *H. somni* and *M. bovis* (DGZ, 2015), identifying BHV-1, BVDV and PI-3 (Horwood and Mahony, 2011), identifying the capsular serotype of *M. haemolytica* (Klima et al., 2017) and identifying different *Mannheimia species* (Alexander et al., 2008).

More seldom, in-situ hybridization can be used for identification. This technique uses a labeled complementary DNA probe to localize specific DNA in tissue or within a specific lesion. Finding an infectious agent in a specific lesion gives strong evidence for relatedness to disease (Kureljušić et al., 2016). Microarrays can be used to identify causative pathogens, as well as determine virulence, gene functions, genotyping and investigate host-pathogen interactions (Oiha and Kostrzynska, 2008). Whole genome sequencing can also be performed for identification, genotyping, subtyping, determination of resistance genes and revealing the whole microbiome of the respiratory tract (Edwards and Holt., 2013; Rai et al., 2015; Woolums et al., 2018). However, these techniques are only applied in a research setting and are not used in common clinical diagnostics.

2.2.1.4. MALDI-TOF MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized veterinary clinical microbiology. The technique is based on mass spectrometry of abundant proteins which are mostly ribosomal proteins. After cultivation, a colony of interest is placed on a target plate and covered with a matrix, which assists in the desorption and ionization of bacterial proteins and protects them from fragmentation. After desorption and ionization of bacterial proteins by a laser, these proteins are accelerated in a vacuum tube, which can differ based on their mass-to-charge ratio. An ion detector is situated at the end of the tube, which measures the 'time-of-flight', with smaller proteins reaching the detector earlier than larger proteins. Based on this information, a mass spectrum is generated, comparable to a protein fingerprint of a bacterium (Figure 7). The mass spectrum can be, with the correct software, automatically compared with a database of reference spectra for relatedness

and after few minutes identification until species level is performed. Since the database of reference spectra is continuously expanding, most clinically relevant bacteria and a large number of different yeast can be identified, including *Pasteurellaceae* (Kuhnert et al., 2012).

This technique is nowadays commonly used both in human and veterinary medicine (Bizzini and Greub, 2010). Advantages are the speed, accuracy and usability. Whereas phenotypic identification with biochemical tests demands 20 hours, MALDI-TOF MS identification only requires few minutes (Zangenah et al., 2013). Due to this fast identification the economic impact, like shorter treatment and hospital stay, is substantial (Mok et al., 2016) and the impact on public health is priceless (Rodríguez-Sánchez et al., 2019). One study comparing the accuracy of MALDI-TOF MS with other diagnostic techniques demonstrated excellent accuracy for *Pasteurellaceae* (Puchalski et al., 2016). Also, since the application is user friendly, there is no need for highly specialized personnel. And although the purchase of such a device is costly, this technique has a high return on investment. A bench-by-bench study, assessing the impact of MALDI-TOF MS on the cost-effectiveness compared to standard identification systems, demonstrated that by the implementation of MALDI-TOF MS, a reduction of the reagent and labor costs of identification of 57% is possible within 12 months (Tan et al., 2012).

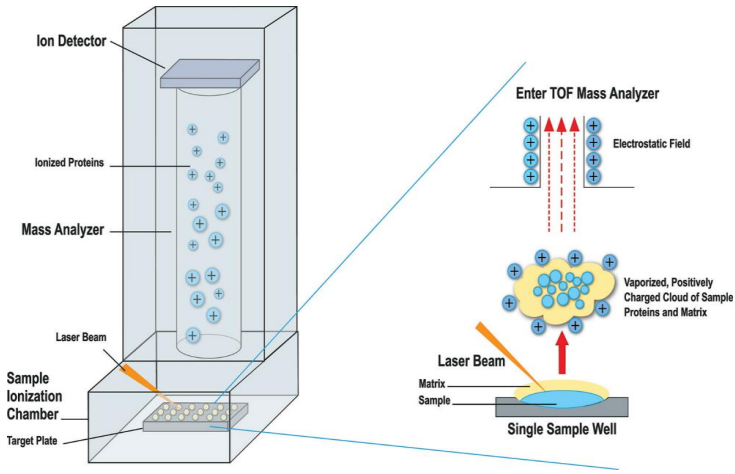

Figure 7: Basic principle of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, Patel, 2014)

2.2.1.5. Serology

Serologic tests are used to identify animals that have recently been exposed to pathogens and have raised a humoral immunological response (antibodies). However, this does not indicate that the animal was clinically ill due to the pathogen, or was shedding the pathogen at that time. Seroconversion, meaning the alteration of an animal from a seronegative to a seropositive condition and therefore antibodies can be detected in the blood, required 3 weeks. This leads to a considerable delay of results and testing of multiple animals can be expensive. Also, no differentiation between antibodies derived from vaccines or from infections can be made (Friend et al., 1977). Antibody detection for bacteria in plasma can be performed using Enzyme-Linked Immunosorbent Assay (ELISA), quantitative fluorometric assay (Confer et al., 1983) and agglutination tests (Wilkie and Markham, 1979; Fodor et al., 1996). An example is the antibody detection to leukotoxin of *M. haemolytica*. Newer technologies demonstrate the use of a direct ELISA with purified exopolysaccharide to differentiate between diseased animals infected with *H. somni* and healthy animals with commensal *H.* somni at a high diagnostic accuracy (Pan et al., 2014).

An overview of the strengths and limitations of the different diagnostic methods for identifying *Pasteurellaceae* is provided in Table 4.

Table 4: Strengths and limitations of different diagnostic techniques used to identify *Pasteurellaceae*

	Strengths	Limitations
Phenotypic characterization	-Inexpensive -Identification possible until subspecies level -Susceptibility testing possible -Ability of finding 'new' bacteria as possible causative pathogen -Quantitative measurement possible	-Culture is required + biochemical tests Overall TAT: 2 days -Labor intensive -Pathogen must be viable. Therefore storage and handling of the sample and previous antimicrobial treatment is important -Animal must be shedding the organisms at the time of sampling
MALDI-TOF MS	-Cost-effective -User friendly -Very accurate -After cultivation, identification only takes couple of minutes. Overall TAT: 1 day -Susceptibility testing possible -Ability of finding 'new' bacteria as possible causative pathogen -Quantitative measurement possible	-Identification possible until species level -Pathogen must be viable. Therefore storage and handling of the sample and previous antimicrobial treatment is important -Animal must be shedding the organisms at the time of sampling
DNA-based techniques	-No culture required -Organism does not have to be viable as it targets the DNA of the organism -Identification possible until genotype -WGS: Detection of resistance mechanisms and subtyping possible	-Although the TAT only takes several hours, in practice this is mostly days due to laboratory workflow -Labor intensive -Higher cost -Risk of over-diagnosis -No identification of 'new' bacteria -Animal must be shedding the organisms at the time of sampling -No susceptibility testing possible -Besides qPCR no quantitative measurement possible
Serology	-No culture required -Animal does not have to be shedding the organisms at the time of sampling -Longevity of antibody expression is possible several months	-Seroconversion takes 3 weeks before antibodies can be detected -No susceptibility testing possible -Higher cost -Risk of over-diagnosis -No identification of 'new' bacteria -Identification until species level -No direct cause-effect relatedness -No differentiation between antibodies derived from infection or vaccination (with exceptions)

Abbreviation: TAT: turnaround time

2.2.2. Antimicrobial susceptibility testing

2.2.2.1. General terms and definitions

Antimicrobial susceptibility testing supports the decision making process of rational antimicrobial use and consequently therapy success. With antimicrobial susceptibility tests, a categorization between susceptible, intermediate and resistant can be made. Antimicrobial resistance is defined as the ability of a microorganism (in this case bacteria, but this can also include viruses and parasites) to stop an antimicrobial from working against it (WHO, 2015). From a clinical perspective, a 'susceptible' outcome indicates that there is a good chance that the animal will respond to therapy with the investigated antimicrobial when using the correct dosage indicated for the specific species and disease. When the result is 'resistant', applying an antimicrobial at the correct dosage will probably not lead to a successful therapy. An 'intermediate' result approaches a susceptible result if infection is at an anatomical location where the drug concentrates (CLSI, 2018a). For example, tildipirosin rapidly and extensively concentrates in the lower respiratory tract (Menge et al., 2012). An intermediate result provides a buffer zone for inherent variability in antimicrobial susceptibility testing, which can be related to dose adjustments for other sites of infections, a chronical disease state or a difference in metabolism and age. These isolates can be interpreted as susceptible or resistant, and therefore these results should always be proceeded with caution (CLSI, 2018a).

To categorize isolates as susceptible, intermediate or resistant, the minimal inhibitory concentration (MIC) value, defined as the lowest concentration of the antimicrobial that inhibits visual growth of the bacterium (Quinn et al., 1994; CLSI, 2008), is compared with specific veterinary clinical breakpoints, established by the Clinical and Laboratory Standards Institute (CLSI). These clinical breakpoints are based on two pillars. First of all, pharmacokinetic/pharmacodynamic (PK-PD) data are evaluated, observing the absorption, distribution, metabolism and elimination of an antimicrobial in vivo. These data merit specific information, as they are derived from the host animal species, which is mostly performed on healthy animals, and encounter a specific dose, route, and duration of therapy. Second, the clinical outcome of in vivo treatment with a specific antimicrobial on the animal, suffering from disease caused by the specific bacterial

pathogen, is observed. Although these results may be non-conclusive, latter is used as a validation of PK/PD cutoffs.

For clinical breakpoints, it is noteworthy to address that these values are host - disease - pathogen - antimicrobial specific, and therefore not direct interchangeable with other bacterial pathogens or hosts. Unfortunately, veterinary clinical breakpoints are not available for every combination. In case of BRD, available clinical breakpoints for *P. multocida*, *M. haemolytica* and *H. somni* are presented in Table 5, according to the most recent guidelines of CLSI (CLSI, 2018b). Recently, the Veterinary Subcommittee on Antimicrobial Susceptibility Testing (VetCAST) has set up new clinical breakpoints for *P. multocida*, which are also presented in Table 5 (VetCAST, 2019). However, these breakpoints are not host –and disease specific.

Susceptibility tests require an acceptable degree of accuracy in order to be used in laboratories. This diagnostic accuracy is indicated by very major, major and minor errors. A very major error is defined as the percentage of false-susceptible results, meaning that a resistant isolate is classified as susceptible and therefore can lead to therapy failure. Since this error can have substantial consequences like increased morbidity and mortality, this rate may not exceed 1.5%. A major error indicates false-resistant results. Although an antimicrobial could lead to therapy success, this is not used as this is incorrectly classified as resistant. This misclassification will not lead to therapy failure and therefore the acceptable rate is limited to 3%. A minor error is defined as misclassification of an intermediate outcome, which mostly has no consequences considering the choice of an appropriate antimicrobial for therapy (CLSI, 2013).

Table 5: Zone diameter and MIC breakpoints for *M. haemolytica*, *P. multocida* and *H. somni* according to CLSI, 2018b and VetCAST, 2019

Bacterium	Antimicrobial agent	Disk content	Interpretive Categories and Zone Diameter Breakpoints, nearest whole mm			Interpretive Categories and MIC Breakpoints, µg/mL			Reference
			S	I	R	S	I	R	
M. haemolytica, P. multocida and H. somni	Spectinomycin	100 µg	≥14	11-13	≤10	≤32	64	≥128	CLSI, 2018b
	Ampicillin	-	-	-	-	≤0.03	0.06-0.12	≥0.25	CLSI, 2018b
	Penicillin G	-	-	-	-	≤0.25	0.5	≥1.0	CLSI, 2018b
	Ceftiofur	30 µg	≥21	18-20	≤17	≤2	4	≥8	CLSI, 2018b
	Enrofloxacin	5 µg	≥21	17-20	≤16	≤0.25	0.5-1	≥2	CLSI, 2018b
	Gamithromycin	15 µg	≥15	12-14	≤11	≤4	8	≥16	CLSI, 2018b
	Tilmicosin	15 µg	≥14	11-13	≤10	≤8	16	≥32	CLSI, 2018b
	Tulathromycin	30 µg	≥18	15-17	≤14	≤16	32	≥64	CLSI, 2018b
	Florfenicol	30 µg	≥19	15-18	≤14	≤2	4	≥8	CLSI, 2018b
	Tetracycline	-	-	-	-	≤2	4	≥8	CLSI, 2018b
P. multocida	Tildipirosin	60 µg	≥20	17-19	≤16	≤4	8	≥16	CLSI, 2018b
M. haemolytica	Tildipirosin	60 µg	≥21	18-20	≤17	≤8	16	≥32	CLSI, 2018b
H. somni	Tildipirosin	60 µg	≥17	14-16	≤13	≤8	16	≥32	CLSI, 2018b
P. multocida and M. haemolytica	Danofloxacin	5 µg	≥22	18-21	≤17	≤0.25	0.5	≥1	CLSI, 2018b
P. multocida	Benzylpenicillin	1 µg	≥17	-	<17	≤0.5	-	>17	VetCAST, 2019
	Ampicillin	-	-	-	-	≤1	-	>1	VetCAST, 2019
	Amoxicillin	-	-	-	-	≤1	-	>1	VetCAST, 2019
	Amoxicillin – clavulanic acid	2-1 µg	≥15	-	<15	≤1	-	>1	VetCAST, 2019
	Cefotaxime	5 µg	≥26	-	<26	≤0.03	-	>0.03	VetCAST, 2019
	Ciprofloxacin	5 µg	≥27	-	<27	≤0.06	-	>0.06	VetCAST, 2019
	Doxycycline	-	-	-	-	≤1	-	>1	VetCAST, 2019
	Tetracycline	30 µg	≥24	-	<24	-	-	-	VetCAST, 2019
	Trimethoprim-sulfonamides	1.25-23.75 µg	≥23	-	<23	≤0.25	-	>0.25	VetCAST, 2019

2.2.2.2. Broth dilution

The broth dilution test is considered the reference test for antimicrobial susceptibility testing. Two-fold dilutions of an antimicrobial are placed in a liquid medium with addition of a bacterial concentration of 1-5 x 10^5 colony forming units (CFU)/mL. After an overnight incubation, a visual inspection of bacterial growth takes place (Figure 8). Although the broth dilution method is considered the reference test, it is quite time-consuming due to the multiple steps that need to be performed, increasing the possibility of errors. In order to make the method more user friendly and reproducible, commercially available kits have been developed obtaining different concentrations of multiple antimicrobials. However, these commercial kits limit the flexibility of using only a couple of particular antimicrobials and are expensive (€5 - €10 per antimicrobial). Also, commercial systems like Sensititre and the Phoenix Automated Microbiology System (BD Biosciences, USA) have been developed to automatically categorize isolates as susceptible, intermediate or resistant (Doern et al., 1985; Horstkotte et al., 2004).

Figure 8: Broth dilution test

2.2.2.3. Disk diffusion

The disk diffusion method, also known as the Kirby-Bauer method, is the most commonly used technique for susceptibility testing of clinical isolates in veterinary laboratories (Figure 9). After placing a concentration of 1-2 x 10^8 CFU/ml of a bacterium on an agar plate, paper disks containing a certain concentration of antimicrobials are transferred on this plate. This is incubated, leaving the bacterium to grow to a certain distance to a disk, which has diffused the antimicrobial through the agar medium (Figure 10). This distance or zone of inhibition depends on how resistant/susceptible the bacterium is against that particular antimicrobial and is measured by the distance from the disk till the growth region of the bacterium (Bauer et al., 1966; Quinn et al., 1994). Depending on this zone of inhibition, the bacterium can be categorized as susceptible, intermediate or resistant, using the clinical breakpoints of disk diffusion according to CLSI (CLSI, 2015). Advantages of this technique are the ease of performance, cost price (€0,07 per antimicrobial) and the adaptability of using the antimicrobials of choice. Also, systems have been developed, like Sirscan, Osiris and BIOMIC, to automatically determine the zone of inhibition which increases standardization and decreases labor (Hubert et al., 1998; Nijs et al., 2003; Hombach et al., 2013). However, with some exceptions (Callens et al., 2016), no MIC-values can be derived with this method, leading to a qualitative rather than quantitative result. Furthermore, when comparing disk diffusion results with broth dilution outcomes for bovine *Pasteurellaceae*, a moderate sensitivity (meaning absence of false susceptible results) is obtained, leading to misclassifications in populations with a high prevalence of resistance (Catry et al., 2007b). Also, disk diffusion does not work very well for large molecules which have difficulties in diffusing, like colistin (Maalej et al., 2011). Additionally, Credille, B., 2019 demonstrated that different outcomes between susceptibility testing methods can be present.

2.2.2.4. Antimicrobial gradient diffusion

The antimicrobial gradient diffusion method, also known as E-test, is used to derive MIC-values with a method comparable with disk diffusion. Instead of using disks, test strips impregnated with an increasing concentration of an antimicrobial are placed on the agar (Figure 10). The MIC value is determined by the lowest point of the ellipse shaped zone where growth of the bacterium is suppressed (Quinn et al., 1994). As with disk diffusion, gradient diffusion allows for the flexibility of using only a couple of antimicrobials, and the antimicrobials of choice. However, these test strips are not cheap (€2 - €3 per antimicrobial) and therefore not recommended when performing an antibiogram of multiple antimicrobials. In general, the antimicrobial gradient diffusion method have demonstrated a good diagnostic accuracy compared to the agar dilution method (Baker et al., 1991; Huang et al., 1992), although no results are available specifically for bovine *Pasteurellaceae*.

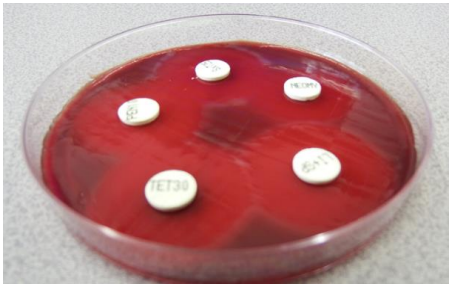

Figure 9: Disk diffusion test

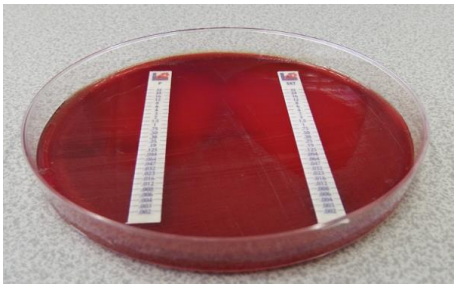

Figure 10: Antimicrobial gradient diffusion

2.2.2.5. Other antimicrobial susceptibility testing methods

Other antimicrobial susceptibility methods have been described like agar dilution (Mirajkar and Gebhart, 2016) and molecular genetic methods. However, these techniques are not commonly used in routine veterinary laboratories. That is because the agar dilution method is very time-consuming and expensive. For molecular genetic methods, the clinical utilization is very limited due to the poor genetic-phylogenetic correlation. For example resistance genes may not yet been discovered and therefore not found. Also, resistance genes may not be expressed and therefore not result in clinically relevant resistance.

Overall, the above discussed antimicrobial susceptibility methods still require an overnight incubation time. Also for identification of the causative pathogen, a minimum time of 20 hours is necessary due to cultivation (phenotypic characterization or MALDI-TOF MS), laboratory workflow (DNA-based techniques) or characteristic of the technique (serology). Nowadays in a conventional clinical laboratory, identification is performed after cultivation with the recently introduced MALDI-TOF MS technique. Susceptibility testing is commonly performed by the disk diffusion method in this clinical setting. This means that the turnaround time (TAT) from sampling to susceptibility results requires a minimum of 2 days (Figure 6). When taking the duration of transport into mind, and the commonly required subculturing of pathogens due to contamination, this TAT often takes 4 days or longer. Given the economic importance of BRD and considering animal welfare, awaiting susceptibility results before treating these animals is unthinkable. However, deciding to initiate an empiric group treatment is nowadays also criticized within the framework of a responsible antimicrobial use in animals and humans. The only solution to meet both requirements is to develop more rapid and accurate diagnostic techniques which are applicable in clinical veterinary laboratories.

2.2.4. New MALDI-TOF MS developments in human medicine

2.2.4.1. Direct detection

Different automated systems have been developed to identify bacterial pathogens and aid in antimicrobial susceptibility testing. Examples are the Phoenix Automated Microbiology System (BD Bioscienes, USA) (Stefaniuk et al. 2003), Vitek 1 and 2 (bioMérieux)(Leverstein-van Hall et al., 2002) and MicroScan WalkAway system (Dade Behring, West Sacramento, CA) (Patteet et al., 2012). These systems increase laboratory workflow and can decrease the turnaround time to a minimum of 10 hours for susceptibility testing (Horstkotte et al., 2004; Mittman et al., 2009). However, cultivation is still necessary. Therefore, direct inoculation of these automated systems from positive blood cultures have been investigated (Chung et al., 2009; Lupetti, et al., 2010; Beuving et al., 2011) shortening TAT with 12-24 hours. These automated systems show merit to diagnostic laboratories.

However, it was the new technique called MALDI-TOF MS who has absolutely revolutionized these laboratories. Since the introduction of this technique, multiple applications of MALDI-TOF MS have been made, focusing on rapid and accurate identification and susceptibility testing. As with the automated systems, MALDI-TOF MS still requires a culture. Thus new research was conducted for direct detection of pathogens in clinical samples by MALDI-TOF MS. The main focus of these publications in human medicine was sepsis, a well-known life threatening condition where rapid identification and consequently effective treatment is crucial (Harbarth et al., 2003; Kumar et al., 2006). Most recent results show that correct identification of positive blood samples by MALDI-TOF MS is possible after 15 minutes in 90% of the cases (Azrad et al., 2019). Commercial kits are available for direct detection of positive blood samples, namely the Sepsityper™ kit (Bruker Daltonics, Bremen, Germany) and Biofire filmarray® Blood culture Identification (BCID) Panel test (bioMérieux clinical diagnostics, Chemin de l'Orme, France), demonstrating the applicability in clinical laboratories.

Besides positive blood cultures, MALDI-TOF MS has also been used for rapid identification of bacteria in other samples like urine, cerebrospinal fluid and other liquids like peritoneal fluid and surgical wound fluid. A brief overview of the major studies investigating rapid detection by MALDI-TOF MS is provided in Table 6. It needs

to be addressed that, besides different techniques applied in each study, technical differences for identification by MALDI-TOF MS were present. For example, Azrad et al., 2019 used the Microflex, Biotyper 3.3 software, while Sakarikou et al., 2018 used the Autoflex, Biotyper 3.1 software and Lin et al., 2018 used the Microflex, Biotyper 3.0 software. These differences could have an influence on the results.

Studies conducting rapid identification by MALDI-TOF can also be of interest in veterinary medicine, although sepsis is mostly caused by Gram-negative bacteria in cattle (76.5% Gram-negative, 2.35% Gram-positive, Aldridge et al., 1993; Fecteau et al., 1997), while in human medicine this ratio is more balanced to 50%-50% (Hariharan et al., 2003). Interestingly, although RTIs are considered of major importance in humans (WHO, 2017b), no literature is available on rapid identification of bacteria in respiratory tract samples by MALDI-TOF MS. A possible explanation for this can be the often polymicrobial character of respiratory tract samples.

Table 6: Major studies investigating rapid detection of bacterial pathogens from positive blood cultures, urine, cerebrospinal fluid and other liquids by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (chronologically ordered per sample type)

Time to identification	Correct ID[1,2] (%)	Number of samples[3]	Sample	Technique	Reference
15 min.	90.0%	186	Positive blood culture	Serum activator tube + centrifugation steps	Azrad et al., 2019
40 min.*	100.0%	102	Positive blood culture	Home-made lysis buffer+ protein extraction	Sakarikou et al., 2018
9 min.	81.8%	324	Positive blood culture	2-stage centr., lysis buffer	Lin et al., 2018
15 min.*	87.0%	101	Positive blood culture	Washing/centrifugation steps	Maelegheer and Nulens, 2017
15 min.	77.8%	266	Positive blood culture	Lysis buffer, washing/centrifugation steps	Randazzo et al., 2016
5 hours	78.6%	925	Positive blood culture	5 hour subculture	Verroken et al., 2015
25 min.	89.3%	152	Positive blood culture	In-house saponin method+ formic acid	Jakovljev and Bergh, 2015
20-30 min.	81.4%	1000	Positive blood culture	2-stage centr.(+protein extraction)	Rodríguez-Sánchez et al., 2014
7.1 hours (4.7-10.2)	91.7%	109	Positive blood culture	In-house method with tween 80	Leli et al., 2013
40 min.*	66.4%	113	Positive blood culture	In-house saponin method+ protein extraction	Meex et al., 2012
20 min.	73.7%	59	Positive blood culture	In-house saponin method	Martiny et al., 2012
40 min.*	85.5%	164	Positive blood culture	Sepsityper kit	Buchan et al., 2012
40 min.*	59.4%	507	Positive blood culture	Sepsityper kit	Kok et al., 2011
<30 min.	39.6%	318	Positive blood culture	2-stage centr., wash + protein extraction	Ferreira et al., 2011a
10 min.	76.0%	68	Positive blood culture	2-stage centr., wash	Ferreira et al., 2011b
1 hour	76.1%	212	Positive blood culture	Serum separator tube, washing/centrifugation steps, lysis buffer+ protein extraction	Stevenson et al., 2010

Time to identification	Correct ID[1,2] (%)	Number of samples[3]	Sample	Technique	Reference
<80 min.	89.7%	503	Positive blood culture	Centrifugation steps + protein extraction	Moussaoui et al., 2010
100 min. (75-140)	95.0%	277	Positive blood culture	Centrifugation/washing steps + protein extraction	Christner et al., 2010
20 min.	91.0%	373	Positive blood culture	In-house saponin method	Ferroni et al., 2010
30-45 min.	57.0%	122	Positive blood culture	Centrifugation/washing steps+ home-made ammonium chloride lysing solution (+protein extraction)	Prod'hom et al., 2010
2 hours	76.0%	240	Positive blood culture	Centrifugation/washing steps+ protein extraction (trifluoroacetic acid or formic acid)	La Scola and Raoult., 2009
2-3 hours	67%	100	Urine samples	Different centr. Steps, diafiltration, wash, desalting (+protein extraction)	DeMarco and Burnham, 2014
<30 min.	94.8%	1456	Urine samples	Flow cytometry, 2-stage centr., wash, protein extraction	Wang et al., 2013
<30 min.	91.8%	260	Urine samples	Flow cytometry, 2-stage centr., wash (+protein extraction)	Ferreira et al., 2010
<30 min.	38.6%	44	Cerebrospinal fluid	2-stage centr., wash, protein extraction	Bishop et al., 2018
<30 min.	100%	1	Cerebrospinal fluid	Centr., wash, protein extraction	Segawa et al., 2014
15 min.	98%	300	Other liquids (after enrichement)	Centr., wash,sonification, centr. Wash, centr.	Oviaño et al., 2018

*estimated time based on centrifugation steps and with or without protein extraction (based on Martiny et al., 2012)

[1]Correct identification of the direct method by MALDI-TOF MS to species level

[2]When different methods for direct detection by MALDI-TOF MS were used, results of the best method or the method compared to the 'conventional direct method' (i.e. Sepsityper) are shown

[3]Number of clinical blood culture samples which was detected as positive and where grown on conventional culture was seen. Positive blood culture broths that contained two or more different species were not included in the results except for Sakarikou et al., 2018

2.2.4.2. Resistance determination

There are three main approaches for antimicrobial susceptibility testing by MALDI-TOF MS that are suitable for use in clinical laboratories. First the detection of antibiotic modifications due to the enzymatic activity of the bacterium, second the analysis of peak patterns of bacteria or mass peak profiles and third the semi-quantitative measurement of bacterial growth with or without an antimicrobial (Oviaño and Bou, 2019). An overview of the first two techniques can be found in Table 7. These studies have mainly been focusing on multiresistant bacteria, which cause the biggest global threat (CDC, 2013).

Measuring of the enzymatic activity of the bacterium is based on the modification of an antimicrobial structure by bacterial enzymes from a resistant bacterium. This modification of an antimicrobial structure can be visible by an alteration in mass spectrum. Advantages of this technique are the usability, speed (±30 minutes) and it can be directly applied from the clinical sample. Additionally, all types of carbapenemase can be detected, including rare ones and those who are not available in commercial molecular tests (Oviaño and Bou, 2019). This is important since nearly 20% of carbapenemase-producing isolates can be missed with the current clinical breakpoints defined by EUCAST or CLSI (Huang et al., 2014). A disadvantage of this technique is that it can only be applied on known resistance mechanisms and it does not provide information regarding the MIC. Nevertheless, this technique has shown great progress during the years by decreasing the incubation time (Lasserre et al., 2015; Oviaño et al., 2016; Oviaño et al., 2017a), applying more antimicrobials (Oviaño et al., 2017c) and automate the analysis of spectra and categorization of susceptible or resistant (Oviaño et al., 2016).

Although analysis of peak patterns or mass peak profiles of bacteria have been described (Wybo et al., 2011; Josten et l., 2014; Lau et al., 2014; Youn et al., 2016), this technique still needs to be validated. Susceptible and resistant microorganisms of the same species can differentiate in spectrum due to the expression of a specific protein involved in the resistance phenotype (=biomarker). Therefore this technique is very simple to apply, easy to automate and requests no extra expenses. However, it cannot be used for all bacteria nor all antimicrobials and again the resistance mechanism should be well known. Protein expression may depend on specific clones or growth terms, and

the data cannot be extrapolated to other antimicrobials from the same class. Therefore, this technique should be interpreted with caution.

Unfortunately, all previous methods using known resistance mechanisms include antimicrobials which are not used in veterinary medicine (Table 6). To develop a method for rapid susceptibility testing in veterinary medicine, new techniques need to be explored. One promising technique is the effect of the antimicrobial on bacterial growth, which overcomes the problem of resistance mechanisms. First, the MALDI Biotyper resistance test with stable isotope-labeled amino acids (MBT-RESIST) was developed (Sparbier et al., 2013; Jung et al., 2014). This technique uses the incorporation of heavy amino acids which increase molecular weight and are therefore visible in the spectrum. And although having potential, specific media are required, making incorporation in clinical laboratories challenging. Another technique, where specific media are unnecessary, is the MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay (MBT-ASTRA). With this technique, the growth of a bacterium is analysed with and without the antimicrobial. After incubation, the growth derived from an increased protein signal is calculated by measuring the area under the curve (AUC) of the obtained spectrum. When the bacterium is susceptible, the AUC of the bacterium with the antimicrobial should be significantly reduced in comparison with the sample of the bacterium without antimicrobial. When the bacterium is resistant, no significant difference in AUC should be noticed. The relative growth (RG) ratio is the ratio of the AUC derived from spectra with and without antimicrobial and is used to distinguish susceptible from resistant bacteria according to an empirically defined cut-off value (Figure 11).

At the start of this doctoral thesis, the MBT-ASTRA had been used for the fast growing *Klebsiella pneumoniae* and meropenem (Lange et al., 2014). The biggest advantage of this technique is that it can be applied on each bacterium and each antimicrobial, as long as favourable conditions for growth of the bacterium are provided. Furthermore, excellent results with this principle of modifications in the mass peak profile, with and without the presence of antifungal agents, have also been demonstrated in fungi (Marinach et al., 2009; De Carolis et al., 2012; Vella et al., 2013). Since growth of the bacterium is necessary, this technique has a longer TAT compared to the other techniques described. For some fast growing bacteria involved in sepsis, 1-4 hours are

needed for accurate susceptibility testing (Lange et al., 2014; Sparbier et al., 2016; Jung et al., 2016; Maxson et al, 2017). Also for slow growing bacteria like mycobacteria this method seems viable (Ceyssens et al., 2017). Since MBT-ASTRA is based on growth, it has an excellent correlation with phenotypic resistance (Lange et al., 2014). Recent developments show that MBT-ASTRA can be performed by microdroplet incubation directly on the MALDI-TOF MS target plate (Idelevich et al., 2018), which only requires 6µL droplets, simplifying the procedure and decreasing preparation costs. To date, the MBT-ASTRA technique has not yet been applied in veterinary medicine.

Table 7: Overview of major studies involving fast susceptibility testing by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Tech-nique	Bacteria	Anti-microbials	TAT	Sensi-tivity	Speci-ficity	Num-ber of sam-ples	Reference
MEA	*Escherichia coli*	ceftriaxone,	30 min	100%	100%	100[a]	Oviaño et al. 2017a
MEA	*Enterobacteriaceae*	imipenem	90 min	100%	100%	3041[b]	Oviaño et al. 2017b
MEA[c]	*Escherichia coli*	Ciprofloxacin, norfloxacin, levofloxacin	30 min	100%	100%	117	Oviaño et al., 2017c
MEA[c]	*Enterobacteriaceae*	Ciprofloxacin, norfloxacin	<1 hour	100%	100%	122[b]	Oviaño et al., 2017d
MEA	*Enterobacteriaceae, Pseudomonas sp.., Acinetobacter spp.*	imipenem	30 min	98%	100%	119[a]	Oviaño et al., 2016
MEA[c]	*Enterobacteriaceae*	Norfloxacin[c]	4 h	98%	100%	113	Pardo et al., 2016
MEA	*Enterobacteriacea and Pseudomons aeruginosa*	meropenem	2.5h	98%	100%	161[a]	Papagiannitsis et al., 2015
MEA	*Enterobacteriacea*	imipenem	<30 min	100%	100%	223[a]	Lasserre et al., 2015
MEA	*Enterobacteriacea*	Cefotaxime, ceftazidime, clavulanic acid	90 min (max 150 min.)	99%	100%	141[a]	Oviaño et al. 2014

Tech-nique	Bacteria	Anti-microbials	TAT	Sensi-tivity	Speci-ficity	Num-ber of sam-ples	Reference
MEA	*Enterobacteriacea*	Cefotaxime, ampicillin	2.5 h	100%	91.5%	85[a]	Jung et al., 2014
MEA	*Enterobacteriacea, Pseudomonas aeruginosa*	meropenem	4h	96.67%	97.87%	124[a]	Hrabák et al., 2011
AMPP	*Escherichia coli*	polymyxin	15 min	100%	100%	87	Dortet et al., 2018
AMPP	*Enterobacteriaceae*	carbapenem	10 min	96%	99%	140	Youn et al., 2016
AMPP	*Staphylococcus aureus*	methicillin	10 min	95%	100%	220	Josten et al., 2014
AMPP	*Enterobacteriaceae*	carbapenem	10-30 min	100%	100%	62	Lau et al., 2014
AMPP	*Bacteroides fragilis*	carbapenem	10 min	100%	100%	248	Wybo et al., 2011
AMPP[d]	*Klebsiella pneumoniae, Escherichia coli*	carbapenem	30 min	100%[e]	100%	18	Hu et al., 2015
AMPPs[d]	*Klebsiella pneumoniae*	carbapenem	30 min	100%	100%	12	Cai et al., 2012

Abbreviations: MEA (measuring enzymatic activity), AMPP (analysing mass peak profiles)
[a] blood cultures
[b] urine samples
[c] detection of the AAC (6')-Ib-cr Enzyme (plasmid-mediated quinolone resistance mechanism). Although this mechanism provides low MIC values by itself, it can facilitate the selection of higher-level resistance during treatment
[d] based on the detection of porins
[e] better sensitivity then reference technique, i.e. SDS-PAGE

Concluding remarks

It is clear that the long turnaround time of classic bacterial culture is a crucial hurdle for veterinarians to enhance the use of bacteriology to support their decision making for antimicrobial treatment of bacterial respiratory tract infections. Adequate detection of animals with bacterial infections of the respiratory tract and a rapid microbiological diagnosis are the essential components to truly rationalize antimicrobial use in food animals.

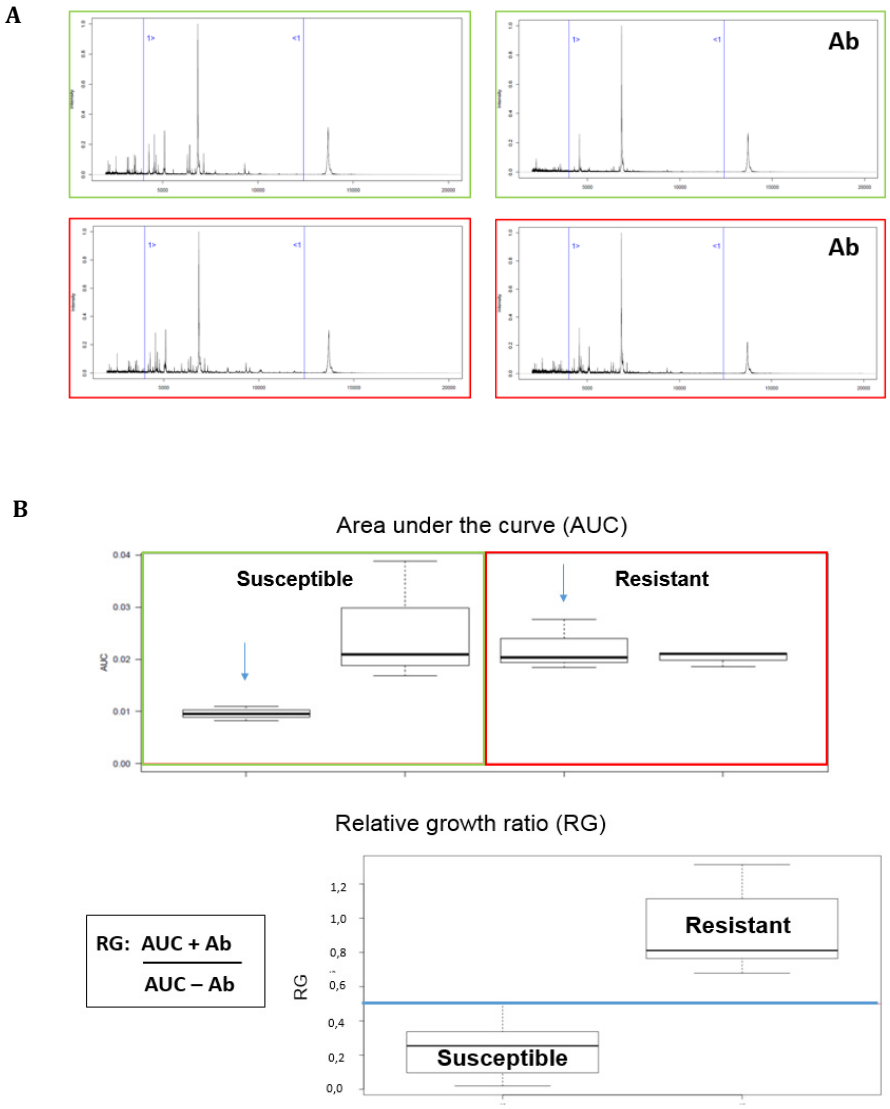

Figure 11: Principle of MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay (MBT-ASTRA). **(A)** Spectrum of a susceptible strain (green) and resistant strain (red) after incubation without (left) and with (right) antimicrobial. **(B)** Calculation of the area under the curve (AUC) of each spectrum. The blue arrow indicates the addition of an antimicrobial. The susceptible bacterium shows a reduction in AUC when an antimicrobial is added, while a resistant bacterium demonstrates an equal AUC. When the relative growth (RG) ratio is calculated according to the above-mentioned formula, the susceptible bacterium will be situated beneath an empirical determined cut-off value and the resistant bacterium above this cut-off value

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CHAPTER 2
SCIENTIFIC AIMS

Despite global action plans for reducing and rationalizing antimicrobial use, prophylaxis and metaphylaxis is still commonly used in veterinary medicine, especially to tackle respiratory tract infections. However, both veterinarians and farmers, consider awaiting microbiological results, which have a minimum turnaround time of 2 days, unacceptable for economic and animal welfare reasons. Providing rapid and reliable diagnostic techniques is therefore a crucial element to evolve towards a reduced and more rational use.

MALDI-TOF MS offers an economically achievable alternative for routine identification and susceptibility testing of respiratory pathogens in cattle. This technique is currently widely used for bacterial indentification, is cheap, and provides accurate results in a limited time frame. MBT-ASTRA is a MALDI-TOF MS based approach which can provide susceptibility testing results in only ample hours, and has the advantage of an unlimited application considering bacterial species and antimicrobial. MALDI-TOF techniques offer great possibilities to reduce the turnaround time from sampling to microbiological results for samples of the respiratory tract in cattle, meeting the demands of veterinarians and farmers to proceed to a more restricted and rational use.

Therefore, the overall objective of this thesis was to develop a diagnostic chain from sampling to susceptibility testing result, which is economically and practically achievable and has a minimal turnaround time.

The specific objectives of the present doctoral thesis were:

(1) To compare commensal overgrowth and bacterial culture results between deep nasopharyngeal swab (DNS) and non-endoscopic bronchoalveolar lavage (nBAL) samples (Chapter 3)

(2) To determine if sedation of the animal has an influence on the sampling site of an nBAL (Chapter 4)

(3) To evaluate the effect of different storage conditions of nBALs on the isolation rate and concentration of *Pasteurellaceae* and contamination (Chapter 5)

(4) To develop a rapid identification technique for *Pasteurellaceae* in nBAL samples by MALDI-TOF MS (Chapter 6)

(5) To develop rapid susceptibility testing of *P. multocida* and tetracycline by MBT-ASTRA (Chapter 7)

CHAPTER 3

A DEEP NASOPHARYNGEAL SWAB VERSUS NONENDOSCOPIC BRONCHOALVEOLAR LAVAGE FOR ISOLATION OF BACTERIAL PATHOGENS FROM PREWEANED CALVES WITH RESPIRATORY DISEASE

A DEEP NASOPHARYNGEAL SWAB VERSUS NONENDOSCOPIC BRONCHOALVEOLAR LAVAGE FOR ISOLATION OF BACTERIAL PATHOGENS FROM PREWEANED CALVES WITH RESPIRATORY DISEASE

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ABSTRACT

BACKGROUND: Nonendoscopic bronchoalveolar lavage (BAL) is a practical alternative for a deep nasopharyngeal swab (DNS) to sample the airways of a large number of calves in a short period of time. The extent of commensal overgrowth and agreement of BAL with DNS culture results in preweaned calves are unknown.

OBJECTIVES: To compare commensal overgrowth and bacterial culture results between DNS and BAL samples.

ANIMALS: A total of 183 preweaned calves (144 with bovine respiratory disease and 39 healthy animals).

METHODS: Cross-sectional study. Deep nasopharyngeal swab and BAL samples were taken from each calf and cultured to detect *Pasteurellaceae* and *Mycoplasma bovis.* Agreement and associations between culture results of DNS and BAL samples were determined by kappa statistics and logistic regression.

RESULTS: Bronchoalveolar lavage samples were less often polymicrobial, more frequently negative and yielded more pure cultures compared to DNS, leading to a clinically interpretable culture result in 79.2% of the cases compared to only in 31.2% of the DNS samples. Isolation rates were lower in healthy animals, but not different between DNS and BAL samples. Only *Histophilus somni* was more likely to be isolated from BAL samples. In clinical cases, a polymicrobial DNS culture result did not increase the probability of a polymicrobial BAL result by ≥ 30%, nor did it influence the probability of a negative culture. A significant herd effect was noted for all observed relationships.

CONCLUSIONS AND CLINICAL RELEVANCE: Nonendoscopic BAL samples are far less overgrown by bacteria compared to DNS samples under the conditions of this study, facilitating clinical interpretation and resulting in a higher return on investment in bacteriologic culturing.

Key words

Bacteria, Bovine Respiratory Disease, Sampling, Comparison

INTRODUCTION

Bovine respiratory disease (BRD) has major economic impact in all cattle production systems worldwide (Snowder et al., 2006). It is the main indication for antimicrobial use in calves and therefore receives considerable attention in countries in which veterinary use of antimicrobials is in question (Pardon et al., 2012). To rationalize antimicrobial use, veterinary formularies have been established in several European countries such as Belgium, The Netherlands and Denmark. These formularies recommend sampling of the respiratory tract, bacterial isolation, and susceptibility testing before certain antimicrobial classes, critical for human medicine, can be used (Mevius et al., 2012). Recently, a change in Belgian law has been made, requiring an antibiogram before fluoroquinolones or cephalosporins can be used (RD July 21., 2016). However, to date, there is no consensus on how the respiratory tract should be sampled to isolate causative pathogens.

In practice, deep nasopharyngeal swabs (DNS) (Catry et al., 2005; Godinho et al., 2007; Pardon et al., 2011), transtracheal aspiration (TTA) (Timsit et al., 2013) and bronchoalveolar lavage (BAL) (Pringle et al., 1988; Thomas et al., 2002) have been used for sampling the respiratory tract. Deep nasopharyngeal swab is the easiest, fastest and cheapest technique and therefore most suitable for sampling large groups of animals (Godinho et al., 2007). One major disadvantage is that DNS does not sample the site of interest (pneumonic lung). Previous work in a single feedlot showed moderate agreement between DNS and BAL culture results in calves for *Pasteurellaceae (Pasteurella multocida, Mannheimia haemolytica sensu lato* and *Histophilus somni)* and Mycoplasmata (Allen et al., 1991). Transtracheal aspiration samples the bronchial bifurcation, but has the disadvantage of being more time-consuming, expensive and invasive, while at the same time holding a certain risk (e.g. haemorrhage, emphysema, infection) for the animal (Rohn et al., 1998). Agreement between DNS and TTA culture results was reported in fattening bulls to be moderate for *M. haemolytica s.l.* (Timsit et al., 2013). A BAL often is performed with an endoscope, which requires costly equipment and carries high risk of contamination when sampling multiple animals successively (Allen et al., 1991). Alternatively, BAL can be performed with a reusable sterilised BAL catheter without endoscopic guidance (Van Driessche et al., 2016). This makes it easier for large numbers of animals to be sampled at the lung level in a short

time frame and with a low cost per calf. However, an important point of criticism is the nasal passage of the BAL catheter, which may inoculate the BAL sample with either respiratory pathogens of the nasal cavity or commensal microflora (Rohn et al., 1998). Despite the high prevalence of BRD in preweaned calves (Pardon et al., 2011; Assie et al., 2004), information on the performance of nonendoscopic BAL and the agreement of DNS and BAL culture results in preweaned calves currently is not available. Results in preweaned calves might substantially differ from those in feedlot cattle, because preweaned calves are more likely to suffer from their first BRD episode, whereas the older feedlot cattle might relapse, and residual pathogenic flora in the lung might differ from the dominant nasopharyngeal flora.

Therefore, the objectives of our study were (1) to determine the outcome of bacterial culture results, isolation rates, and agreement for samples taken with DNS and nonendoscopic BAL with respect to *Pasteurellaceae* and *Mycoplasma bovis* infections in preweaned calves; (2) to determine the polymicrobial nature of DNS and BAL samples; and (3) to determine whether a polymicrobial DNS culture result, caused by the nasopharyngeal flora or unhygienic sampling, influences BAL culture results.

MATERIALS AND METHODS

All sampling techniques and the study protocol were revised by the local ethical committee and permitted under experimental license number EC2014-164.

Sample Size Calculation, Study Design And Animals

Sample size was calculated to detect a 30% difference in culture results (i.e. prevalence of pure cultures) between DNS and BAL samples in calves with BRD (cases) and controls with 95% confidence and 80% power. Required sample size for a 2-sided test was 37 observations per group (Winepiscope 2.0, University of Zaragoza, Spain). The sample size for the cases was increased 3.5 times to increase the probability that all major BRD pathogens would be present in the data set.

A cross-sectional study was performed on 14 commercial herds (4 veal, 10 beef) between September 2014 and May 2015. The study was divided into 2 parts. In 11 herds, animals with clinical BRD (cases) were sampled, and in 3 (2 veal and 1 beef) herds, only healthy animals were sampled (controls).

Veal calves were group-housed (4-8) on a slatted floor and fed milk replacer, concentrates, and roughage according to European legislation (EC2008-119). Beef calves also were group-housed (8-12 calves per group) on straw and received milk replacer, concentrates, and roughage. The herds with clinical BRD were reported by local veterinarians and subsequently visited by the research staff. Calves to be sampled (cases) were selected based on previously described inclusion criteria (Pardon et al., 2015). Briefly, the following clinical signs were scored on a 4-point scale (score 0-3): lethargy (from standing to recumbency and position of the ears), cough (from absent to spontaneous), rectal temperature (from <39°C to >39.5°C) and nasal discharge (from absent to bilateral purulent). An animal with a score ≥5 was considered a case, independent on how many clinical signs were abnormal. Additionally, thoracic ultrasound examination was performed with a 7.5 MHz linear probe (Tringa Linear Vet, Esaote, The Netherlands) as previously described (Buczinski et al., 2013). The definition for a case was the presence of a consolidated zone in the lung of ≥1 cm. In the affected herds, all animals that met the inclusion criterion were sampled. To avoid subclinical infection or inflammation (bronchitis-pneumonia) because of exposure to BRD risk

factors, controls were selected from herds that had not experienced a BRD outbreak in the last month. Controls had to show a normal clinical investigation (0 on the 4-point scale) and absence of any ultrasonographic abnormalities. Animals that were vaccinated against BRD or treated with antimicrobials 14 days prior to sampling were excluded from the study.

Sampling

From each calf, an unguarded DNS and then a BAL sample was taken as previously described (Van Driessche et al., 2016). Before inserting a DNS, the animal was restrained while standing and the nostrils were disinfected with 90% alcohol. A 16-cm sterile transport swab (Transystem™, Copan, Brescia, Italy) was used. The swab was sufficiently long to cover the distance from the nostril to the medial canthus of the eye, hereby sampling nasopharyngeal tissue. The swab was introduced medioventrally in the nasal cavity until the nasopharyngeal tissue was reached. After rotating several times, the swab was taken out and placed in Amies transport medium without charcoal formulas.

Bronchoalveolar lavage fluid was collected by a reusable home-made polytetrafluorethylene catheter (1.5 m length; inner- and outer diameter, 2 and 4 mm, respectively, VWR, Leuven, Belgium) adjusted with a 12-G catheter stylet (Van Driessche et al., 2016). The procedure was performed in standing animals without sedation as previously described (Van Driessche et al., 2016). Briefly, after rinsing the nostril with 90% alcohol, the catheter was inserted medioventrally in the nasal cavity, passed through larynx and trachea, and gently advanced into the bronchi until the wedge position was reached. Next, 20 mL of sterile 0.9% NaCl was injected into the lungs and immediately aspirated (recovery of 30-50% of the fluid (Van Driessche et al., 2016)). If no fluid was recovered, a second 20 mL injection was attempted. Sample validity was checked by inspecting for the presence of the characteristic foam layer, indicating contact with surfactant. Samples were transported at ambient temperature and cultured within 12 hours after sampling. For each calf, a new sterilised catheter was used. Sampling was performed by different veterinarians (3-5 different samplers per herd, 17 different samplers in total).

Bacteriology

Deep nasopharyngeal swab and BAL samples (0.2 mL) were inoculated on Columbia blood agar (Oxoïd, Hampshire, UK) enriched with 5% sheep blood and on pleuropneumoniae-like organism (PPLO) agar (10.6 g D-glucose and 40 g PPLO (Difco, BD Diagnostic Systems, Sparks, MD, USA) in 800 mL of distilled water [pH= 7.8-7.9]) for isolation of *Pasteurellacea*e and *M. bovis*, respectively. Blood agars were incubated overnight and PPLO agars for 5 days, both at 35°C and 5% CO_2. Bacteria were selected based on phenotypic characteristics and subsequently further identified by biochemical tests according to as previously described (Quinn et al., 1994). Identification of *M. bovis* was made by culturing on PPLO agar enriched with polysorbate 80. *Mycoplasma bovis* colonies showed the typical "fried-egg" morphology on microscopic examination. If no growth was observed after this period, incubation was continued for 48 h for *Pasteurellaceae* and 7 days for *M. bovis*. All bacteriological analyses were performed at the department of bacteriology at the Faculty of Veterinary medicine, Gent University, Belgium.

Data Management And Statistical Analysis

Culture results were interpreted as follows. A negative culture result was defined as the absence of growth of the target bacteria or the presence of < 2 colonies of contaminants after 48 h of incubation for *Pasteurellaceae*. A polymicrobial result was defined as the growth of multiple bacterial colonies with different morphologies on the agar of which no target bacteria could be subjected to subculture for further identification. A pure culture result was defined as the presence of 1 bacterial species on the agar (> 2 colonies). The presence of several (< 5) bacterial species on the agar with dominant growth of 1 species was defined as a dominant culture. Isolation rates of the studied bacteria were calculated by dividing the sum of pure and dominant cultures (i.e., positive cultures) by the total number of samples. All results, except for polymicrobial results, were considered clinically interpretable.

The experimental unit was the individual calf. To compare isolation rates between DNS and BAL samples, a multivariable linear mixed model was constructed (PROC GLIMMIX) with the respective bacteriological result (e.g., *P. multocida* or pure culture) as the outcome variable and swab/BAL as a binary variable factor. A binomial distribution and

logit link function with Wald's statistics for type 3 contrasts was used. Herd was added as a random factor to account for clustering. No agreement was investigated among the different veterinarians involved.

Agreement between DNS and BAL for the isolation of *P. multocida*, *M. haemolytica s.l.*, *H. somni* and *M. bovis* was determined by means of the Kappa statistic (Cohen, 1960). Strength of agreement for the Kappa coefficient was interpreted as previously described (Landis and Koch, 1977) (≤0= poor; 0.10-0.20= slight; 0.21-0.40= fair; 0.41-0.60= moderate; 0.61-0.80= substantial and 0.81-1.0= almost perfect).

The association between isolation of a bacterial species from the DNS sample and its isolation from the BAL sample was determined by means of a multivariable linear mixed model (PROC GLIMMIX). Eight different models were constructed, separate for cases and controls, with the respective pure culture (*M. haemolytica s.l.*, *P. multocida*, *M. bovis*, and all pure cultures), a polymicrobial culture, dominant culture or negative result as the outcome variables. A binomial distribution and logit link function with Wald's statistics for type 3 contrasts was used. Herd was added as a random factor to account for clustering.

To determine the effect of a polymicrobial DNS culture result on the probability of a pure culture in the BAL sample in calves with BRD, 5 different general linear mixed models were constructed with *M. haemolytica*, *P. multocida*, *M. bovis* and a negative culture result as outcome variables. The same procedure as described above was followed. Model validity was evaluated by the Hosmer-Lemeshow goodness-of-fit test for logistic models. Significance was set at $P<0.05$. All analyses were performed in SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Details on herd types, number of animals sampled, and sampling results at herd level are provided in Table 1. *M. haemolytica s.l.*, *P. multocida*, and *H. somni* were found in 27.3% (3 of 11), 63.6% (7 of 11) and 18.2% (2 of 11) of the BRD outbreak herds, respectively. *Mycoplasma bovis* was only found in both veal farms with BRD outbreaks (18.2%; 2 of 11). Very few targeted respiratory pathogens (n=7) could be retrieved from the 3 control herds. In 2 herds (herds 13 and 14), 2 *P. multocida* isolates were retrieved, whereas in herd 14, 2 *H. somni* isolates also were retrieved. In herd 13, *M. bovis* was isolated from a single calf. In herd 12, no respiratory pathogens could be isolated (Table 1).

Table 1. Overview of isolated pathogens and polymicrobial culture results in the 11 case and 3 control herds

Herd	Case/ Control	Ty-pe	Age (w)	Calves (n)	Number (Percentage) of Positive Cultures For				% DNS Poly-microbial	% BAL Poly-microbial
					M. haemo-lytica s.l.	*P. multocida*	*H. somni*	*M. bovis*		
1	Case	Beef	5	3	0 (0)	0 (0)	0 (0)	0 (0)	3 (100)	0 (0)
2	Case	Beef	17	7	0 (0)	1 (14.3)	0 (0)	0 (0)	6 (85.7)	0 (0)
3	Case	Beef	9	10	0 (0)	2 (20)	0 (0)	0 (0)	8 (80)	1 (10)
4	Case	Beef	8	10	0 (0)	1 (10)	0 (0)	0 (0)	8 (80)	1 (10)
5	Case	Beef	8	10	3 (30)	0 (0)	0 (0)	0 (0)	5 (50)	2 (20)
6	Case	Beef	8	10	0 (0)	0 (0)	9 (90)	0 (0)	9 (90)	0 (0)
7	Case	Beef	10	15	0 (0)	3 (20)	0 (0)	0 (0)	13 (86.7)	6 (40)
8	Case	Beef	8-12	5	0 (0)	1 (20)	0 (0)	0 (0)	3 (60)	2 (40)
9	Case	Beef	9-13	7	0 (0)	0 (0)	0 (0)	0 (0)	7 (100)	5 (71.4)
10	Case	Veal	6	35	13 (37.1)	25 (71.4)	1 (2.9)	22 (62.9)	10 (28.6)	3 (8.6)
11	Case	Veal	7	32	2 (6.3)	7 (21.9)	0 (0)	4 (12.5)	27 (84.4)	10 (31.3)
12	Control	Beef	3-8	9	0 (0)	0 (0)	0 (0)	0 (0)	5 (55.5)	2 (22.2)
13	Control	Veal	2	18	0 (0)	3 (16.7)	0 (0)	1 (5.5)	15 (82.3)	7 (38.9)
14	Control	Veal	8-28	12	0 (0)	2 (16.7)	2 (16.7)	0 (0)	12 (100)	4 (33.3)

DNS, deep nasopharyngeal swab; BAL, bronchoalveolar lavage

Isolation rates of the targeted pathogens (*M. haemolytica s.l.*, *P. multocida*, *H. somni*, and *M. bovis*) were higher in cases compared to controls both in DNS (43.7% [63 of 144] versus 5.1% [2 of 39]; *P*<0.01) and in BAL (53.5% [77 of 144) versus. 17.9% [7 of 39]; *P*<0.01; Table 2). With DNS and BAL, both in cases and controls, *P. multocida* (n= 67) was isolated most frequently, followed by *M. bovis* (n= 39), *M. haemolytica s.l.* (n=30) and *H. somni* (n=13). In case calves, the isolation rates were not significantly different between DNS and BAL for all studied bacteria, except for *H. somni* which was less frequently isolated from DNS (*P*<0.01; Table 2). Mixed infections (i.e., isolation of ≥2 respiratory target bacteria from the same DNS or BAL sample) were only seen in cases from the veal farms (Table 3). In cases, agreement between DNS and BAL culture results was moderate for all bacteria (κ= 0.41-0.60), with the exception of *H. somni*, for which it was slight (κ=0.16; Table 4). A positive DNS culture result in cases significantly increased the odds of a positive BAL for *M. haemolytica s.l.*, *P. multocida* and *M. bovis* (Table 4). This relationship was significantly affected by the herd effect (*P*<0.001).

Table 2. Differences in isolation rates of bacterial respiratory pathogens and negative, pure culture, or polymicrobial culture results between DNS and BAL samples in 183 preweaned calves

	Cases			Controls		
Bacterial culture result	DNS[a] (n=144) (%)	BAL[a] (n=144) (%)	*P*-value[b]	DNS[a] (n=39) (%)	BAL[a] (n=39) (%)	*P*-value[b]
M. haemolytica s.l.	12 (8.3%)	18 (12.5%)	0.21	0	0	-
P. multocida	31 (21.5%)	30 (20.8%)	0.87	2 (5.1%)	4 (10.3%)	0.40
H. somni	2 (1.4%)	9 (6.3%)	0.01	0 (0%)	2 (5.1%)	0.15
M. bovis	18 (12.5%)	20 (13.9%)	0.68	0 (0%)	1 (2.6%)	0.31
Pure culture	12 (8.3%)	42 (29.2%)	<0.001	0 (0%)	5 (12.8%)	0.02
Dominant culture	12 (8.3%)	14 (9.7%)	0.68	2 (5.1%)	2 (5.1%)	1.0
Polymicrobial culture	99 (68.8%)	30 (20.8%)	<0.001	32 (82.1%)	15 (38.5%)	<0.001
Negative culture	21 (14.6%)	58 (40.3%)	<0.001	5 (12.8%)	17 (43.6%)	<0.01

DNS= deep nasopharyngeal swab; BAL= bronchoalveolar lavage
[a] results are shown as numbers with percentages between brackets
[b] *P*-value referring to the difference between DNS and BAL

Table 3. Mixed infections as diagnosed by bacterial culture on DNS or BAL samples in 144 preweaned calves with respiratory disease

	DNS	BAL
P. multocida + M. haemolytica s.l. + M. bovis	6.7% (1/15)	25.0% (4/16)
P. multocida + M. haemolytica s.l.	20.0% (3/15)	18.8% (3/16)
P. multocida + M. bovis	66.6% (10/15)	37.4% (6/16)
M. haemolytica s.l. + M. bovis	0.0% (0/15)	18.8% (3/16)
M. haemolytica s.l. + H. somni	6.7% (1/15)	0.0% (0/16)

The majority of DNS cultures were polymicrobial (68.8% [99 of 144] in cases, 82.1% [32 of 39] in controls), meaning that no *Pasteurellaceae* or *M. bovis* could be phenotypically identified from the plate. Compared to DNS, BAL samples were significantly less polymicrobial ($P<0.001$ for cases and controls), more often negative ($P< 0.001$ for cases, $P<0.01$ for controls), and more often returned pure cultures of *Pasteurellaceae* or *M. bovis* ($P<0.001$ for cases, $P<0.02$ for controls; Table 2). In summary, BAL samples returned an interpretable result (either negative, pure, or dominant culture result) in 79.2% of the cases and in 61.5% of the controls, compared to 31.2% and 17.9% for DNS in cases and controls, respectively ($P< 0.01$ for both comparisons; Table 2). The polymicrobial nature of a sample result was strongly affected by the herd-effect ($P<0.001$). A polymicrobial DNS and BAL culture result in at least 1 animal was present in almost all herds (11 of 14 herds, the other 3 herds had no polymicrobial BAL culture result), but there was very large variation in the percentage of polymicrobial results among the herds sampled (Table 1). In the cases, a polymicrobial DNS culture result did not increase the probability of a polymicrobial BAL result by ≥ 30% ($P=0.09$), nor did it influence the probability of a negative culture ($P=0.52$). However, the probability of retrieving *M. haemolytica s.l*. and *P. multocida* from the BAL sample still decreased when the DNS was polymicrobial. In contrast, there was no effect of a polymicrobial DNS result on the probability of isolation of *M. bovis* from the BAL sample (Table 5).

Table 4. Associations and agreement between DNS and BAL culture results in 183 preweaned calves

	Cases							Controls					
	Percentage (number) of positive BAL cultures		Association of DNS with BAL			Agreement		Percentage of positive BALs (number)		Association of DNS with BAL			Agreement		
Species	DNS culture result[a] Negative	Positive	OR	95% CI	*P*-value	Kappa	95% CI	DNS culture result[a] Negative	Positive	OR	95% CI	*P*-value	Kappa	95% CI
M. haemolytica s.l.	7.6% (10/132)	66.7% (8/12)	18.9	3.3-111.1	<0.01	0.52	0.36-0.69	0% (0/39)	0% (0/0)			ND		
P. multocida	10.5% (12/114)	63.3% (19/30)	13.3	3.5-50.0	<0.001	0.48	0.25-0.71	8.1% (3/37)(3/37)	50.0% (1/2)(1/2)	11.4	0.5-250	0.12	0.28	0.03-0.54
H. somni	5.6% (8/142)	50% (1/2)	ND			0.16	0-0.46	0% (0/0)(0/0)	5.1% (2/39)(2/39)			ND		
M. bovis	4.8% (6/124)	60.0% (12/20)	8.9	2.0-38.5	<0.01	0.58	0.38-0.78	2.6% (1/39)(1/39)	0% (0/0)(0/0)			ND		
Pure culture	24.2% (32/132)	83.3% (10/12)	7.8	1.4-45.5	0.02	0.28	0.12-0.43	12.8% (5/39)(5/39)	0% (0/0)(0/0)			ND		
Dominant culture	9.8% (13/132)	8.3% (1/12)	ND			ND		5.4% (2/37)(2/37)	0% (0/2)(0/2)			ND		
Polymicrobial culture	8.9% (4/45)	26.3% (26/99)	2.9	0.8-10.2	0.09	0.12	0.03-0.21	14.3% (1/7)(1/7)	43.8% (14/32)(14/32)	0.18	0.46-47.6	0.15	0.15	0-0.33
Negative culture	39.0% (48/123)	47.6% (10/21)	3.5	1.0-11.6	0.05	0.05	0-0.18	38.2% (13/34)(13/34)	80.0% (4/5)(4/5)	6.5	0.60-71.4	0.12	0.21	0.08-0.34

DNS, deep nasopharyngeal swab; BAL, bronchoalveolar lavage; ND, no statistical analysis possible, because a too small number of observations in one of the groups; OR, odds ratio; CI, confidence interval

Strength of agreement for the Kappa coefficient was interpreted according to Landis and Koch: ≤0= poor; 0.10-0.20= slight; 0.21-0.40= fair; 0.41-0.60= moderate; 0.61-0.80= substantial and 0.81-1.0= almost perfect

[a]DNS culture result refers to isolation of the same bacteria as in the BAL. Herd effect was significant for all studied outcomes, except *P. multocida*.

Table 5. Results of univariable logistic regression models on the effect of a polymicrobial DNS on recovery of respiratory bacteria from bal samples in 183 preweaned calves

Species	Percentage (number) of positive BAL cultures		OR	95% CI	*P*-value
	Polymicrobial DNS				
	No	Yes			
Cases (n=144)					
M. haemolytica s.l.	31.1% (14/45)	4.0% (4/99)	0.23	0.08-0.64	<0.01
P. multocida	44.4% (20/45)	11.1% (11/99)	0.20	0.05-0.83	0.03
H. somni	2.2% (1/45)	88.9% (8/99)	ND		
M. bovis	24.4% (11/45)	7.1% (7/99)	1.34	0.33-5.62	0.67
Negative culture	22.4% (13/45)	45.5% (45/99)	1.36	0.53-3.5	0.52
Controls (n=39)					
M. haemolytica s.l.	0% (0/7)	0% (0/32)	ND		
P. multocida	14.3% (1/7)	9.4% (3/32)	0.62	0.05-7.69	0.70
H. somni	0% (0/7)	6.3% (2/32)	ND		
M. bovis	0% (0/7)	3.1% (1/32)	ND		
Negative culture	71.4% (5/7)	37.5% (12/32)	0.24	0.04-1.53	0.12

DNS, deep nasopharyngeal swab; BAL, bronchoalveolar lavage; ND, no statistical analysis possible, because of a too small number of observations in one of the groups; OR, odds ratio; CI, confidence interval
The random herd effect was significant in all models.

DISCUSSION

To determine how the respiratory tract should be sampled to isolate the causative pathogens, a cross-sectional study was performed to compare bacterial culture results and commensal overgrowth between DNS and BAL samples. Sampling procedures returning high isolation rates of the major respiratory pathogens and with a straightforward interpretation of the culture results have the highest return on investment and are therefore most suitable for practice.

In our study, all isolates were identified by biochemical tests and morphology instead of the polymerase chain reaction. This approach might limit the results with respect to bacterial species identification. Biochemical identification was selected because it is the routine identification method used in private laboratories in Belgium and neighboring countries, for reasons of speed and cost of analysis. The objective of our study was to gain insights into the sampling and culture methods currently used in the field. Also, no selective media to increase *Pasteurellaceae* isolation rates were used, because doing so currently is not the standard procedure used in private laboratories. Selective media would likely decrease contamination, whereas 1 of the main objectives was to study differences in contamination between DNS and BAL. A final limitation of this study was that, for practical reasons, the returned lavage fluid volume was not determined. Quantification of the target bacteria was not an objective of the study, but differences in the returned volume might potentially have influenced culture results.

One of the main findings in the study on preweaned calves is that isolation rates of respiratory bacterial pathogens in both DNS and BAL samples were lower in controls compared to cases. The most likely explanation is that the control group consisted of animals originating from other farms than the case farms, whereas in previous work, “apparently healthy” in-contact animals were used as controls (Allen et al., 1991). These apparently healthy animals are likely exposed to the same risk factors as the cases and might be subclinically infected. Therefore, in our study, controls were deliberately chosen from farms without recent BRD exposure, and ultrasound examination was used as an additional tool to aid in selecting truly healthy animals. A disadvantage of this approach is the environmental differences (e.g., bedding, herd size, air quality…) that exist among herds. To definitively determine whether isolation rates differ between

diseased and truly healthy animals in 1 herd, a longitudinal study design would be needed.

Agreement between DNS and BAL samples was moderate for *M. haemolytica s.l.*, *P. multocida* and *M. bovis*, similar to what was observed for *M. haemolytica s.l*. in fattening bulls (Timsit et al., 2013). Agreement was much lower for *H. somni*, which can be explained by the fact that *H. somni* is easily overgrown by other bacteria (Quinn et al., 1994). Given their polymicrobial nature, DNS samples are likely to be falsely negative for *H. somni*, when no selective media are used. Current understanding of the pathogenesis of bacterial pneumonia in calves suggests overgrowth of *Pasteurellaceae* in the nasopharynx and tonsils with subsequent colonization of the trachea and lungs (Grey et al., 1974). Even when applying a transtracheal sampling procedure, in diseased animals, one is probably as likely to isolate bacteria that have descended from the nasopharynx as those originating from the lung. Possible reasons why DNS and BAL samples do not agree are false-negative results caused by polymicrobial overgrowth (sampling technique or presence of resident flora), sampling of a nonaffected lung lobe with the nonendoscopic BAL technique, or the absence of deep bronchitis or alveolitis in case calves. The latter reason was excluded as much as possible by the use of ultrasound examination in this study. Previous work showed that this nonendoscopic BAL approach samples a random lung lobe in nonsedated animals, and not necessarily the most frequently affected cranial lobes (Van Driessche et al., 2016). This might in part explain why some cultures of cases were negative. However, we doubt this is true, and our hympthesis is that passage through trachea and deep bronchi transfers bacteria deeper into the lung.

Interestingly, in the same animal, the DNS could be polymicrobial, whereas the BAL yielded a pure culture, dominant culture or even a completely negative result. Additionally, the polymicrobial nature of the DNS did not affect the presence of a negative or pure culture result in the BAL. Also, *H. somni* could be isolated in pure culture from the lungs of diseased calves, whereas it was overgrown or absent on the nasopharyngeal culture. These observations strongly suggest that, under the conditions of our study, nasopharyngeal contamination of a BAL sample is less common than previously assumed. To what extent a possible cleansing effect of the DNS contributes to a pure culture result in the BAL is unclear. On the other hand, a DNS polymicrobial result

did reduce the probability of isolating *M. haemolytica s.l.* or *P. multocida* from the BAL, whereas this effect was not observed for *M. bovis* for which selective media were used. Again, this observation could be explained by BAL placement in a healthy lung lobe in a case calf or because respiratory bacteria are not necessarily involved in every case. Several viruses (e.g., bovine respiratory syncytial virus, bovine corona virus) are capable of inducing pneumonia and marked disease without bacterial superinfection. Unfortunately, in our study viral analysis in each case was not possible for financial reasons. However, in our opinion, the polymicrobial nature of DNA and BAL is strongly influenced by the sampling (technique and hygiene), given that such a strong herd effect on the sampling results was observed. To overcome the issue of possible nasopharyngeal overgrowth in DNS and BAL samples due to nasopharyngeal passage, both the use of selective media for isolation of *Pasteurellaceae* (e.g., addition of bacitracine (Catry et al., 2005; Pardon et al., 2011)) and a more quantitative approach on BAL results (Rennard et al., 1986) might be suitable. Our study focused on culture results obtained when applying DNS and BAL as in practice. To definitively determine the extent and diagnostic importance of possible overgrowth as a consequence of nasopharyngeal passage, experimental work with intensive strain typing and necropsy to confirm the infective status of the lung will be needed.

As mentioned above, a significant herd effect was noted on many of the outcomes studied. Deep nasopharyngeal swab and BAL were performed only after cleansing the outer nares and without a protective sleeve used in previous work (Allen et al., 1991; Godinho et al., 2007). This could have increased the risk of contamination by bacteria residing in the nostril. In Belgium, DNS for practical reasons is routinely performed without a protective sleeve, again increasing external validity in this study. Multiple samplers participated in the study, and although all of them received at least 1 training from the same trainer before the start of the study, variation in the extent of experience in taking DNS or BAL samples and in the hygienic procedures accompanying these techniques might have influenced the results. Deep nasopharyngeal swab samples might be polymicrobial due to the presence of a highly variable nasopharyngeal microflora (Allen et al., 1992) or due to environmental contamination (e.g., touching the muzzle or other objects during sampling). Other reasons might be environmental or aerosolized dust, endotoxin, bedding conditions, and issues with stable ventilation. Likely, the risk of catheter contamination increases when repeated attempts to enter the trachea are

needed or when the esophagus is accidently entered. Adequate training is likely the only solution, other than considering other procedures such as protective sleeves, agar plugs, or visualization of the larynx trough a low-cost laryngoscope.

CONCLUSION

A nonendoscopic BAL results in less contaminated (and therefore more easily interpretable samples) compared to DNS under the conditions of this study. It returns an interpretable result in 79.2% of the cases, compared to 31.2% in DNS, and has better isolation rates for *H. somni,* offering a better return on investment for bacteriological sampling. It can be performed rapidly in a representative number of animals at low cost and likely has less impact on animal welfare then more invasive techniques.

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CHAPTER 4

EFFECT OF SEDATION ON THE INTRAPULMONARY POSITION OF A BRONCHOALVEOLAR LAVAGE CATHETER IN CALVES

EFFECT OF SEDATION ON THE INTRAPULMONARY POSITION OF A BRONCHOALVEOLAR LAVAGE CATHETER IN CALVES

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SHORT COMMUNICATION

Bovine respiratory disease (BRD) has a major economic impact in different cattle production systems worldwide (Griffin, 1997; Pardon et al., 2013; Lehenbauer, 2014) and is the main reason of antimicrobial use in calves and youngstock (Pardon et al., 2012). Public concern to reduce and rationalize veterinary antimicrobial use has markedly increased in the last decade (BelVetSac, 2015; MARAN 2015). Veterinary formularies have been constructed which advise sampling, bacterial isolation and antimicrobial susceptibility testing before certain antimicrobial classes can be used (Haneveld, 2014; AMCRA, 2015,).

In human beings and different animal species bronchoalveolar lavage (BAL) is considered a safe and reliable sampling technique to identify causal organisms of pneumonia, because it directly samples the lung lobes (Wilkie and Markham, 1979; Pringle et al., 1988; Thomas et al., 2002). Bronchial fluid can be obtained using an endoscope, enabling selection of the lung lobes to be sampled (Pringle et al., 1988; Allen et al., 1991). Disadvantages of the endoscopic guided BAL are the need for disinfecting the scope between two patients, the high purchase costs of the equipment and the risk for damage when using endoscopes on farm in cattle. For these reasons endoscopic guided BAL is not routinely applied to sample large groups of calves. Alternatively, with adequate training, BAL can be performed without endoscope using a BAL catheter. Advantages are that BAL catheters are cheap and can be sterilised and reused, making sampling of a representative number of animals in a limited time frame possible. However, there may be some drawbacks. It is unknown whether blind sampling with a BAL catheter does sample the ventrocranial lung parts, where the vast majority of bacterial pneumonias in calves are situated (Allan et al., 1985; Allen et al., 1991; Dagleish et al., 2010). Also, for animal welfare reasons and to facilitate the sampling procedure, sedation of calves during the BAL procedure might be warranted. Whether sedation affects the intrapulmonary position of the catheter is unknown. Therefore, the objective of this study was to determine the most frequently sampled lung site by BAL and whether the intrapulmonary position of the BAL catheter is affected by sedation with xylazine.

All procedures were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC: 2014/164). The sample size required to determine a 70% difference between the different lung sites with 80% power and 95% confidence was 7 animals in each group (Winepiscope 2.0, University of Zaragoza, Spain). A randomised clinical trial was performed in December 2014 on 18 healthy male Holstein Friesian calves (mean age=7 months +- standard deviation (SD)=3 months, mean weight 193kg +- standard deviation (SD)= 64 kg, no significant differences between both test groups). Animals were individually housed on full concrete floors with deep straw bedding. Diet consisted of commercial concentrates and hay. Animals had been treated for BRD 2 months earlier with florfenicol (20 mg/kg body weight, BW). They were randomised (RAND function, Excel) over the two treatment groups, of which 11 were sedated with a single dose of 0.1 mg/kg BW xylazine intramuscularly (Xyl-M 2%, VMD, Arendonk, Belgium) and 7 served as control, receiving a placebo (the same volume of NaCl 0.9% as xylazine, i. e. 0.01mL/kg BW).

In each calf a BAL was performed as follows. The animal was restrained while standing. After cleaning the nostrils with alcohol 90%, a custom made-catheter (teflon, 1.5 m length, inner and outer diameter of 2 and 4 mm, respectively (VWR, Louvain, Belgium), fitted with a 12 G catheter stylet) was inserted in the ventral meatus, passed through larynx and trachea and gently advanced into the bronchi until the wedge position was reached. Subsequently, 20 ml of sterile NaCl 0.9% was injected and aspirated (average recuperation of 30-50% of the fluid). BAL samples were considered valid if foam was present, indicating presence of surfactant. When no fluid could be aspirated, a second injection of 20 ml was done. The intrapulmonary position of the BAL catheter was determined by endoscopy after sampling and contrast-radiography was performed with a barium sulphate stained catheter. In the control group positioning of the catheter was validated by a post-mortem examination with the catheter in place. No clinical adverse effect was noticed in any of the groups. The operator was not blinded for the treatment group, but the statistician was. Figure 1 shows the different lung lobes in a calf. For statistical analysis the intrapulmonary position of the catheter was characterised with binary values (cranial vs. caudal; left vs. right and dorsal vs. ventral) and logistic regression was used (SPSS V. 22.0, IBM, USA).

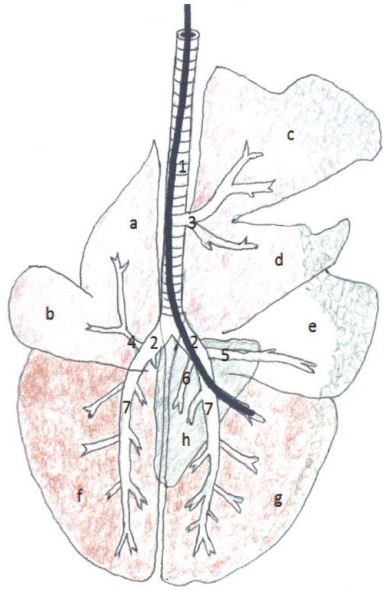

Figure 1: Different lung parts and bronchial pattern of calves. (a) lobus cranialis sinister pars cranialis; (b) lobus cranialis sinister pars caudalis; (c) lobus cranialis dexter pars cranialis; (d) lobus cranialis dexter pars caudalis; (e) lobus medius; (f) lobus caudalis sinister; (g) lobus caudalis dexter; (h) lobus accessorius; (1) trachea; (2) bronchus prinicipalis; (3) bronchus trachealis; (4) bronchus lobaris cranialis; (5) bronchus lobaris medius; (6) bronchus accessorius; (7) bronchus lobaris caudalis

In 72.2% (13/18) of the calves the catheter was positioned in the dorsocaudal part of the lungs. In figure 2 the effect of sedation on BAL catheter positioning is shown. Whereas the probability of sampling the different lung parts is equally distributed in the control animals (CON), in sedated animals (SED) systematically the dorsocaudal lung part was sampled (100% (11/11) vs. 28.6% (2/7)). Sedation had a significant effect on the dorsal vs. ventral (100% (11/11) dorsal positioning for SED vs. 42.9% (3/7) for CON; $P<0.05$) and cranial vs. caudal positioning (100% (11/11) caudal positioning for SED vs 57.1% (4/7) for CON; $P<0.05$), but not on left vs. right positioning (36.4% (4/11) left for SED vs 57,1% (4/7) for CON; $P= 0.49$).

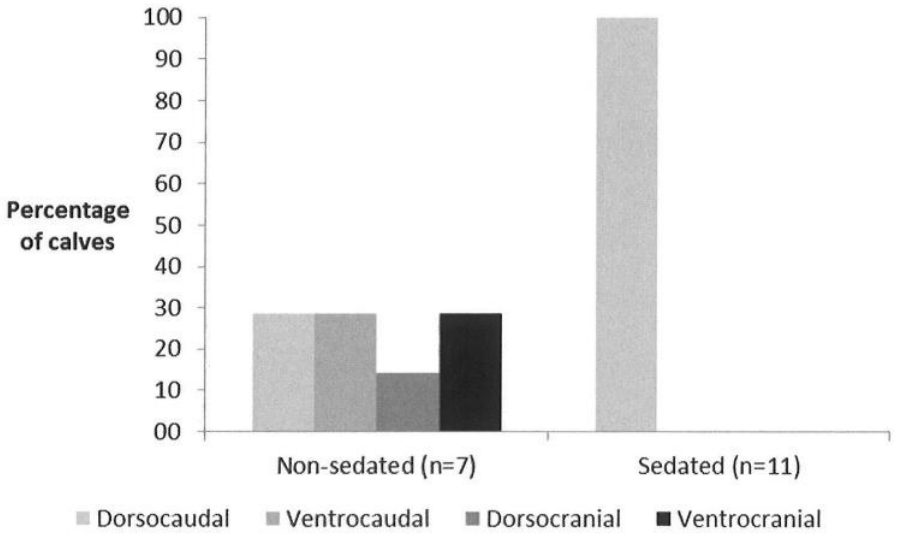

Figure 2: Effect of sedation with xylazine on the intrapulmonary position of a bronchoalveolar lavage catheter in dairy calves

The present study provided two interesting insights relevant for clinicians. First, when performing BAL without endoscopic guidance in unsedated animals, the different parts of the lung have an equal probability to be sampled, including the caudal parts which are much less frequently involved in pneumonia. This might reduce the likelihood to isolate the causative agents from these animals, although no studies on this subject are available. An alternative method for sampling the lower respiratory tract of cattle is a trans-tracheal aspiration (TTA), which samples the bronchial bifurcation and therefore is considered by certain authors more sensitive than BAL to detect focal lesions such as bacterial pneumonia (Sweeney et al., 1991). On the other hand, this technique has the disadvantages of being more time-consuming, expensive and invasive, possibly negatively affecting animal welfare. Further work on the bacteriological outcome of both techniques and on the potential of contamination risk by nasal passage of the BAL catheter is needed.

Secondly, despite that sedation might be beneficial for animal and sampler, it systematically causes sampling of the less affected dorsocaudal parts of the lung. Also in sedated horses a blindly placed BAL tube wedged in a bronchus of the dorsocaudal region of the lung in every case (McKane et al., 1993). Referring to the anatomy of the lung of the horse, the angle between the cranial lobar bronchus and the principal bronchus may make intubation of the cranial lung lobe practically impossible. A similar situation exists for the bronchus trachealis (Figure 1) in calves. However, in unsedated calves, in 28.6% (2/7) the catheter entered this bronchus. Given that there were no significant differences in BW between the test and control groups, it is unlikely that the observed sedation effect is associated with BW. With an increasing BW the needed intromission length of the catheter to reach the wedge position increases (De Schutter et al., 2012), but it is unknown whether the intromised length of the catheter influences pulmonary localization in calves. A first possible explanation for this effect of sedation on intrapulmonary localization of the BAL catheter might be positioning of the head, with a lower head carriage in sedated animals. Secondly, alpha-2-adrenergic agonists are known to induce a general muscle relaxation (Greene and Thurmon, 1988). Laryngopharyngeal muscles are likely also paretic, which might influence orientation and subsequently intrapulmonary positioning of the catheter. Given the use of the wide-spread Holstein breed and the most frequently used sedation procedure in cattle, external validity in this study is estimated to be good. However, differences in test results due to the type of BAL catheter used, cannot be excluded.

In conclusion, a BAL catheter, introduced without endoscopic guidance, samples a random lung lobe and not systematically the most frequently affected cranial lobes. When sedation is applied, the dorsocaudal lung lobes, which are less frequently affected, are systematically sampled. To what extent this difference in intrapulmonary position of the BAL catheter affects the likelihood to isolate the causative bacteria remains to be determined.

AKNOWLEDGEMENTS

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CHAPTER 5

STORAGE TIME AND TEMPERATURE AFFECT THE ISOLATION RATE OF *MANNHEIMIA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* FROM BOVINE BRONCHOALVEOLAR LAVAGE SAMPLES

STORAGE TIME AND TEMPERATURE AFFECT THE ISOLATION RATE OF *MANNHEIMIA HAEMOLYTICA* AND *PASTEURELLA MULTOCIDA* FROM BOVINE BRONCHOALVEOLAR LAVAGE SAMPLES

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ABSTRACT

BACKGROUND: A microbiological diagnosis is essential to better target antimicrobial treatment, control and prevention of respiratory infections in cattle. Under field conditions, non-endoscopic broncho-alveolar lavage (nBAL) samples are increasingly collected. To what extent the highly variable turnaround time and storage temperatures between sampling and cultivation affect the isolation rate of bacterial pathogens is unknown. Therefore, the objective of this experimental study was to determine the effect of different storage temperatures (0°C, 8°C, 23°C and 36°C) and times (0,2,4,6,8,24,48 hours) on the isolation rate and concentration of *Pasteurellaceae* in nBAL samples from clinically affected animals.

RESULTS: Storage at 36°C resulted in a reduced isolation rate already 2 hours after sampling for *Mannheimia haemolytica* and 24 hours after sampling for *Pasteurella multocida*. When samples were stored at 23°C, a decrease in *M. haemolytica* and *P. multocida* isolation rate was noticed, starting at 24 and 48 hours after sampling, respectively. The presence of microbial contamination negatively affected the isolation of *P. multocida* in clinical nBAL samples. An increase in concentration of contaminating bacteria was noticed after 24 hours of sampling at a temperature of 36°C and after 48 hours of sampling at a temperature of 23°C.

CONCLUSION: Optimal *M. haemolytica* and *P. multocida* isolation rates from clinical nBAL samples are obtained after storage at 0°C or 8°C, provided that the sample is cultivated within 24 hours after sampling. The maximum period a sample can be stored without an effect on the *M. haemolytica* and *P. multocida* isolation success varies and is dependent on the storage temperature and the degree of microbial contamination.

Keywords

Pasteurellaceae, transport conditions, bronchoalveolar lavage, cattle, bovine respiratory disease

INTRODUCTION

Respiratory tract infections (bovine respiratory disease (BRD)) have a major impact on farm economics and animal welfare (Snowder et al., 2006). Furthermore, they are the main indication for antimicrobial use in calves (Pardon et al., 2012). In order to rationalize antimicrobial use, diagnostic techniques need to be optimized. Non-endoscopic bronchoalveolar lavage (nBAL) is a practical and economical technique, increasingly used in Western European countries to sample the lower airways of cattle (Van Driessche et al., 2017). Although this technique obtains more pure cultures compared to a deep nasopharyngeal swab, microbial sample contamination can occur, partly depending on the experience of the veterinarian (Van Driessche et al., 2017). In order to minimize microbial contamination and bacterial overgrowth, cultivation of samples needs to be performed as soon as possible after sampling. However, due to the centralization of veterinary laboratories and the limited operating hours of these laboratories (not 24/7 as in some human clinics), the turnaround time between sampling and cultivating of the samples can take 24 hours to even several days.

Optimal storage conditions, supporting survival of causal pathogens and limiting growth of contaminants, are needed to obtain relevant bacterial analysis results (Smismans et al., 2009). False negative or irrelevant results may lead to therapy failure, resulting in increased antimicrobial use, antimicrobial resistance and mortality. Although storage conditions of clinical samples in the field are important, only few studies addressed this subject. Two studies are available comprising the effects of long term survival of *Pasteurellaceae*, namely in swabs from bears (Hansen et al., 2013) or ovine and bovine tracheobronchial washings (Rowe et al., 2001). In the latter experiments, however, sterile lung fluids were spiked, and therefore possible contaminant effects were not taken into account. To what extent nBAL field samples can be stored until analysis, without influencing the isolation rate of clinically import pathogens, is currently unknown. Therefore, the objective of the present study was to determine the effect of storage temperature and duration on the isolation rate of *Pasteurellaceae* from bovine nBAL field samples.

METHODS

The sample size required to determine a 60% difference in isolation rate (80% vs 20%) with 80% power and 95% confidence for a 2-sided test was 8 positive nBAL samples per test group (Winepiscope 2.0, Zaragoza, Spain). Each test group comprises the presence of a clinically relevant bacterial pathogen, namely Pasteurella multocida, Mannheimia haemolytica or Histophilus somni. Therefore samples were taken until a minimum number of 8 positive culture results per test group was reached. An experimental study design was performed on 4 unrelated farms (3 beef, 1 dairy) between March and April 2018. Farms suffering from an acute outbreak of BRD were reported by local veterinarians and subsequently visited by the research staff. Animals to be sampled were selected based on previously described inclusion criteria (Pardon et al., 2015). Additionally, thoracic ultrasound examination was performed with a 7.5-MHz linear probe (Tringa Linear Vet, Esaote, the Netherlands) as previously described (Ollivett et al., 2015). The definition for a case was the presence of a consolidated zone in the lung of ≥1 cm depth (Buczinski et al., 2013). Animals that were treated with antimicrobials within 14 days prior to sampling were excluded from the study.

Cattle that met the inclusion criteria were sampled with the nBAL procedure as previously described (Van Driessche et al., 2016). Briefly, after disinfecting the nostril with 70% alcohol, a reusable home-made polytetrafluorethylene catheter adjusted with a 12-G catheter stylet was inserted in the nasal cavity and gently advanced, through larynx and trachea, into the bronchi. Next, 60 mL of sterile 0.9% NaCl was injected into the lungs and immediately aspirated (recovery of 30-50% of the fluid). Samples were transported at ambient temperature and further processed within 30 minutes after sampling.

Twenty mL of each nBAL sample was used for further analysis and was divided equally over four different 50mL Falcon tubes after vortexing for 1 minute (5mL each). Each Falcon tube was incubated at a different temperature, all monitored with a thermometer, i.e. 0°C +/-1°C (ice), 8°C +/- 1°C (refrigerator), 23°C +/- 1°C (room temperature) and 36°C +/- 1°C (incubator) for 0, 2, 4, 6, 8, 24 and 48 hours. After each incubation period, the sample was vortexed for 30 seconds and 100 µL was transferred to an Eppendorf tube, already containing 900µL phosphate buffered saline (PBS). Ten-fold dilutions were made of each sample for quantitative analysis as previously

described (Van Hecke et al., 2017). From each dilution, 100µl was inoculated on Columbia agar with 5% sheep blood (blood agar; Oxoid, Hampshire, UK) and incubated overnight at 35°C +/-2°C in a 5% CO2 atmosphere. All macroscopically different colonies were counted and identified with MALDI-TOF MS as previously described (Kuhnert et al., 2012). A positive culture result was defined as the macroscopically visible presence of one or more clinically relevant *Pasteurellaceae* (*P. multocida*, *M. haemolytica* and *H. somni*) colonies in pure, dominant or mixed cultures as previously described (Van Driessche et al., 2017). Only samples with a positive culture at time point 0 hours (T0) were included in the experiment. When no clinically relevant pathogen could be isolated at time points 2, 4, 6, 8, 24 or 48 hours, the concentration of the pathogen isolated at T0 in this sample was set at 100 Colony forming units (CFU)/mL (being the detection limit of this plating procedure).

The association between the different conditions for the isolation of *Pasteurellaceae* was determined by means of a multivariable logistic regression model with repeated measures (PROC GLIMMIX). Bonferroni corrections were used to compare between 4 groups. To determine the association between the presence of contaminants and isolation of *M. haemolytica* or *P. multocida* logistic regression was used (PROC LOGISTIC). Model validity was evaluated by the Hosmer-Lemeshow goodness-of-fit test for logistic models. Significance was set at $P<0.05$. All data were collected in Microsoft Excel and statistical analysis was performed in SAS 9.4 (SAS Institute Inc., Cary, NY).

RESULTS

Animals that met the inclusion criteria aged 1 week to 7 months. In total, 13 nBAL samples were collected, of which the initial culture results at T0 showed 6 dominant cultures with 1 clinically relevant pathogen (4 *M. haemolytica*, 2 *P. multocida*) and 7 mixed cultures with both *M. haemolytica* and *P. multocida* (3 pure cultures containing only *M. haemolytica* and *P. multocida* and 4 dominant cultures with also some contaminants present). In total, *M. haemolytica* was isolated from 11 samples (84.6%) with an average log concentration of 4.0 at T0, and *P. multocida* from 9 samples (69.2%) with an average log concentration of 3.7 at T0. *Trueperella pyogenes* was isolated from one sample and *Moraxella bovis* from two samples at T0, both in mixed cultures. *H. somni* was not isolated. Contaminants present in the dominant cultures were *Streptococcus*

spp., *Staphylococcus* spp., *Bacillus* spp., *Escherichia coli* and *Rothia nasimurium* with an average log concentration of 2.8 at T0.

The effect of the various temperatures and storage periods after sampling on the number of positive samples for *M. haemolytica* is presented in Figure 1. In general, the number of samples from which *M. haemolytica* could be isolated, decreased gradually over time. The higher the storage temperature, the earlier the number of positive samples started to decrease, i.e. at 2 hours of storage at 36°C, at 24 hours of storage at 23°C and at 48 hours of storage at 0°C and 8°C. During storage at 0°C and 8°C, the number of positive samples remained stable up to 24 hours after sampling, varying between 10/11 and 11/11 in this period, whereas at 23°C, only 7/11 samples remained positive after 24 hours of storage. When samples were stored at 36°C for 48 hours, *M. haemolytica* could be isolated from only 1 sample, while this was 4/11 for 23°C and 8/11 for both 0°C and 8°C (Figure 1).

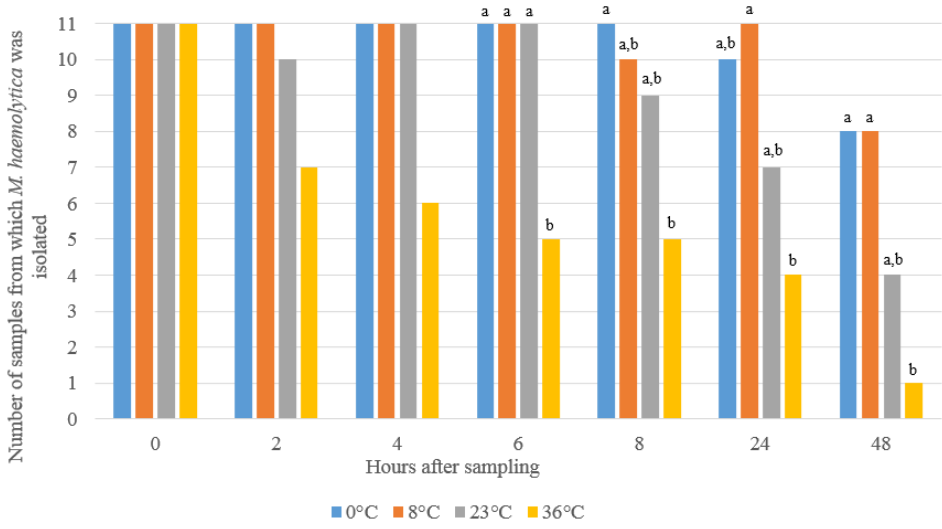

Figure 1: Effect of storage conditions (temperature and time) on the number of bovine nBAL samples from which *M. haemolytica* could be isolated. Different letters (a-b) indicate a significant difference (P<0.05) in temperature within one time after sampling

An overall slight decrease in *M. haemolytica* concentration occurred over time (Figure 2). At a storage temperature of 36°C, the average log concentration of *M. haemolytica* decreased after 2 hours of storage from 4 to 2.9 and remained stable until 48 hours after sampling. No difference in concentration of *M. haemolytica* was noticed between each sampling time between a temperature of 0°C, 8°C and 23°C. Starting from 8 hours after sampling, the different temperatures did not affect the concentration of *M. haemolytica*. (Figure 2). No statistical significant difference was seen between the different temperatures and the time after sampling.

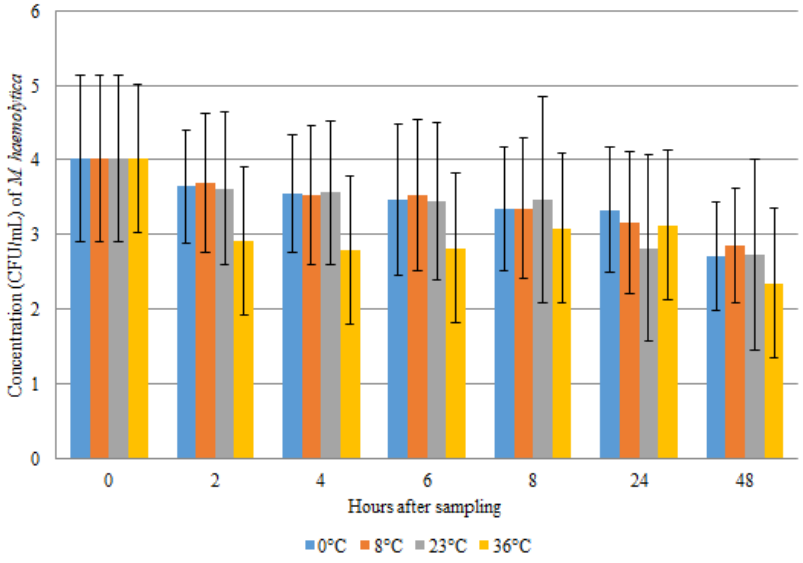

Figure 2: Effect of storage conditions (temperature and time) on the concentration of *M. haemolytica* in 11 bovine nBAL samples. No statistically significant difference was seen between the different temperatures and the hours after sampling

The effect of storage temperature and time on the number of samples from which *P. multocida* could be isolated is presented in Figure 3. A decline in the number of positive samples from which *P. multocida* was isolated was observed throughout the experiment for storage at 36°C, where only 4/9 positive samples were retrieved at 24 hours of storage and no positive samples could be retrieved at 48 hours (Figure 3). This decline in the number of positive samples was due to both contaminant overgrowth and a decreased viability. This decreased viability of *P. multocida* was noticed at a storage temperature of 36°C starting from 24 hours after sampling and at a storage temperature of 23°C starting from 48 hours after sampling. When samples were maintained for 48 hours at 23°C, only 3/9 samples were found positive. At a storage temperature of 0°C or 8°C, the number of positive samples remained stable until 24 hours after sampling, with an isolation rate of 8/9 to 9/9. At 48 hours of storage at a temperature of 0°C, 1 initially *P. multocida* positive sample was negative due to decreased viability. When stored at 8°C for 48 hours, 2 initially *P. multocida* positive samples were negative, one due to decreased viability and one due to contaminant overgrowth.

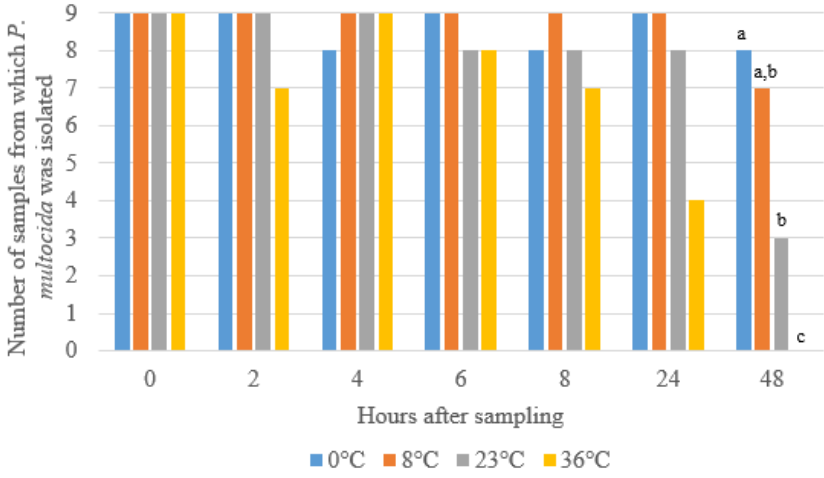

Figure 3: Effect of storage conditions (temperature and time) on the number of bovine nBAL samples from wich *P. multocida* could be isolated. Different letters (a-c) indicate a significant difference (P<0.05) in temperature within one time after sampling

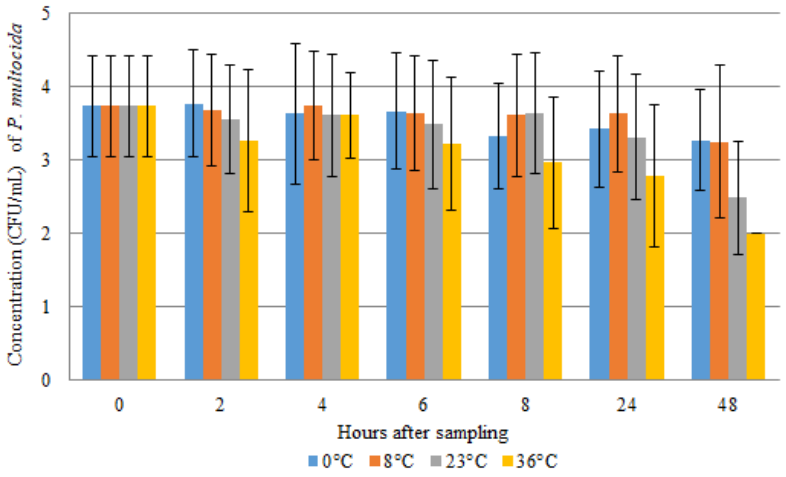

Figure 4: Effect of storage conditions (temperature and time) on the concentration of *P. multocida* in 9 bovine nBAL samples. No statistically significant difference was seen between the different temperatures and the hours after sampling

Compared with *M. haemolytica*, the average concentration of *P. multocida* remained more stable until 24 hours of storage at a temperature of 0°C, 8°C and 23°C (Figure 4). At a storage temperature of 36°C, the *P. multocida* concentration slightly decreased starting from 6 hours after sampling, with a concentration below detection limit at 48 hours after sampling. Accordingly, no positive samples of *P. multocida* were retrieved after storage at 36°C for 48 hours, even in the absence of contamination overgrowth. When samples were stored at 0°C or 8°C, only a slight decrease in log concentration was noticed, from 3.7 at TO to 3.2-3.3 after 48 hours. At 48 hours after sampling, the higher the storage temperature, the lower the concentration of *P. multocida* (Figure 4).

From the 13 samples collected, 10 samples contained microbial contamination at T0. Of the 3 initial samples that were not contaminated at T0, 1 sample showed microbial contamination starting from 2 hours after sampling. The other two initially negative samples showed sporadically microbial contamination with a concentration closely to the detection limit. Results of the influence of bacterial contamination on the isolation rate of *M. haemolytica* and *P. multocida* in the clinical nBAL samples are presented in Table 1. A statistically significant negative association was seen between the presence of

contaminants and the presence of *P. multocida*. This negative association was also noticed for *M. haemolytica*, although not statistically significant. An odds ratio of 0.32 was obtained for *P. multocida* (*P*=0.04), meaning that the presence of contaminants reduced the odds of isolating *P. multocida*. For *M. haemolytica* this odds ratio was 0.7 (*P*=0.651), resulting in a reduced odd of isolating this pathogen when contaminants were present, although this was not statistically significant. An average initial contaminants log concentration of 2.8 CFU/mL was observed (Figure 5). This concentration remained stable during the first 8 hours of storage, regardless of storage temperature. However, after 24 hours of storage at 36°C, the average contaminants log concentration increased to 4.4. After 48 hours of storage, an average contaminants log concentration of 4.7 and 5.1 was reached for storage at 23°C and 36°C, respectively (Figure 5). No statistically significant difference was seen between the different temperatures and the hours after sampling.

Table 1: The effect of the presence of microbial contamination on the isolation of *M. haemolytica* and *P. multocida*

		Mannheimia haemolytica		*Pasteurella multocida*	
		Negative	Positive	Negative	Positive
Contaminants	Negative	37.8% (41/109)	45.9% (117/255)	21.1% (30/142)	45.6% (166/364)
	Positive	62.2% (68/109)	54.1% (138/255)	78.9% (112/142)	54.4% (198/364)
Odds ratio		0.70		0.32	
Confidence Interval		0.58-1.41		0.37-0.83	
P-value		0.651		0.04	

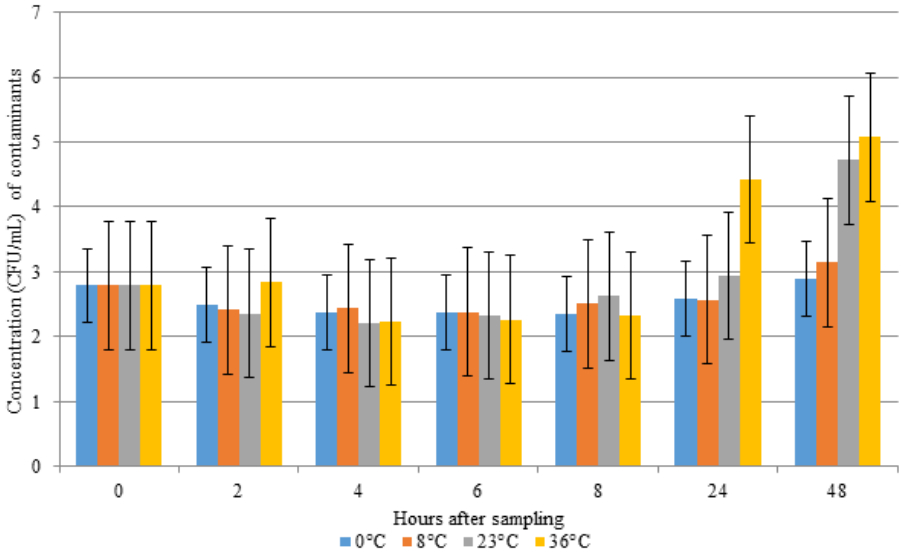

Figure 5 : Effect of storage conditions (temperature and time) on the concentration of bacterial contaminants in 13 bovine nBAL samples. No statistically significant difference was seen between the different temperatures and the hours after sampling

DISCUSSION

This study describes the effects of different storage temperatures and times on the isolation rate and concentration of *Pasteurellaceae* from nBAL samples. When nBAL samples were stored at a temperature of 0°C or 8°C, high isolation rates of *P. multocida* and *M. haemolytica* were obtained until 24 hours of storage. Since storage at 0°C has low feasibility in routine practice, storage of clinical nBAL samples in a refrigerator is a readily available alternative for most practitioners. Also in previous studies low temperatures are recommended for storage (Tefera and Smola, 2002; van Rensburg et al., 2004).

Even though there was no statistically significant decrease in the isolation rate after 24 hours of storage at room temperature (23°C) for both *P. multocida* and *M. haemolytica*, a probably relevant decrease in *M. haemolytica* isolation rate (from 11/11 to 7/11) was observed. The reason for this decreased isolation rate was both due to a decreased viability (2/4 samples) and to microbial contamination (2/4 samples). According to Tano et al. (2011), clinically important bacteria can stay viable for 24 hours at room

temperature, but not in polymicrobial samples. However, these samples were spiked with high pathogen concentrations (10^6 CFU/mL). When lower concentrations, comparable with concentrations obtained in the present study, were used (10^4 CFU/mL and 10^5 CFU/mL), results in viability varied. In another study the viability of *M. haemolytica* remained for a long period of up to 156 days (Rowe et al., 2001). However samples were spiked with high concentration (10^6 – 10^7 CFU/mL) and no bacterial contamination was present. These results stress the importance of a high initial pathogen concentration and avoiding microbial contamination during and after the sampling procedure.

When samples were stored at 36°C, the isolation rate started to decline already 2 and 24 hours after sampling for *M. haemolytica* and *P. multocida*, respectively. When samples were maintained for 48 hours at these temperatures, isolating clinically relevant pathogens was rare or no longer possible. This was mainly, but not exclusively, due to the increased concentration of contaminants, leading to uninterpretable samples when a concentration of ≥1x10^6 CFU/mL of contaminants was reached. These results stress the importance of not leaving samples above room temperature, for example in a closed car or in a tropical environment.

The concentration of *M. haemolytica* slightly decreased over time independent of the storage temperature, though not statistically significant, while for *P. multocida*, this concentration remained more stable. Currently, no information is available on the survival rate of latter 2 bacteria in nBAL samples. One recent study describes the survival rate and density range of *Pasteurellaceae* in the nasopharyngeal microbiota in healthy calves (Thomas et al., 2019). This study showed a longer duration of carriage in the nose and higher concentration for *P. multocida* compared to *H. somni*, however rates of *M. haemolytica* were too low for meaningful survival modelling. Retaining high concentrations of relevant bacteria in clinical samples can be an added value to diagnostics, both for clinical interpretation as for direct detection methods using for example matrix-assisted laser desorption/ionization-time-of- flight mass spectrometry (MALDI-TOF MS) (Van Driessche et al., 2019). A higher negative association between the presence of contaminants and the isolation rate of *P. multocida* was found compared to *M. haemolytica*. This might be partially due to the fact that the average initial load of *M. haemolytica* in the samples was higher compared with the initial *P. multocida* load.

Contaminant overgrowth might therefore negatively impact *P. multocida* isolation rate more quickly than the *M. haemolytica* isolation rate. A previous study showed that the growth of *M. haemolytica* can be inhibited by contaminants like *Escherichia coli* rather than *Staphylococcus* spp. or *Streptococcus* spp. (Kugadas et al., 2014). To what extent different bacterial contaminants had an inhibitory effect on *P. multocida* and/or *M. haemolytica* in this study is unclear, considering the limited number of samples and since different bacterial contaminants were often combined at different concentrations within one clinical sample.

A limitation of current study is the limited sample size. When using 8 positive samples per test group, only 60% difference in isolation rate could be detected. One of the reasons for the limited number of used samples are the strict inclusion and exclusion criteria applied. Indeed, only samples obtained from untreated calves, well-characterized as clinically affected at the level of the lower respiratory tract were included. In addition, only samples from which clinically relevant bacteria could be isolated at T0 were included in the experiment, resulting in the exclusion of various samples. Nevertheless, we feel that the current experimental set-up with a limited number of well-chosen samples and in-depth analysis of the obtained results allows drawing conclusions that are relevant for the practitioner. Another limitation of this study is that, considering the cultivation conditions used in this study, other relevant bacterial pathogens such as *Histophilus somni* and *Mycoplasma bovis* could not be isolated from the current clinical nBAL samples. However, *H. somni* is only rarely isolated because of poor viability and the fact that it is easily overgrown by other bacteria, either clinically relevant or not. Although different studies are available describing the effect of storage conditions on the recovery of *M. bovis*, this was only investigated at low temperatures in milk samples (Al-Farha et al., 2018; Boonyayatra et al., 2010) or colostrum samples (Gille et al., 2017). Therefore, further research into the effect of storage conditions on the recovery of *M. bovis* from nBAL samples is encouraged. Currently, the gold standard technique for identifying these fastidious bacteria is polymerase chain reaction (PCR). Since viability is not mandatory with this technique, it can be expected that the effect of storage conditions for identifying these pathogens will be less important in most veterinary labs using PCR to identify the latter pathogens. Moreover, maximizing the chance of isolating *P. multocida* and *M. haemolytica* is more critical since performing antimicrobial susceptibility testing in

these species can be of major importance for appropriate antimicrobial treatment, while antimicrobial resistance is less prevalent in *H. somni* (DeDonder et al., 2015), or even not routinely tested for in *M. bovis*.

CONCLUSION

This study demonstrates that optimal *M. haemolytica* and *P. multocida* isolation rates from clinical nBAL samples are obtained after storage at 0°C or 8°C, provided that the sample is cultivated within 24 hours after sampling. The maximum period a sample can be stored without an effect on the *M. haemolytica* and *P. multocida* isolation success varies and is dependent on the storage temperature and the degree of microbial contamination.

DECLARATIONS

Ethics approval and consent to participate

All procedures were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2016/20). Verbal informed consent was obtained from all participants by telephone (veterinary officers and farmers), which was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2016/20).

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CHAPTER 6

RAPID IDENTIFICATION OF RESPIRATORY BACTERIAL PATHOGENS FROM CULTURE-ENRICHED BRONCHOALVEOLAR LAVAGE FLUID IN CATTLE BY MALDI-TOF MS

RAPID IDENTIFICATION OF RESPIRATORY BACTERIAL PATHOGENS FROM BRONCHOALVEOLAR LAVAGE FLUID IN CATTLE BY MALDI-TOF MS

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ABSTRACT

Respiratory tract infections are a major health problem and indication for antimicrobial use in cattle and in humans. Currently, most antimicrobial treatments are initiated without microbiological results, holding the risk of inappropriate first intention treatment. The main reason for this empirical treatment is the long turnaround time between sampling and availability of identification and susceptibility results. Therefore the objective of the present study was to develop a rapid identification procedure for pathogenic respiratory bacteria in bronchoalveolar lavage fluid (BALf) samples from cattle by MALDI-TOF MS, omitting the cultivation step on agar plates to reduce the turnaround time between sampling and identification of pathogens. The effects of two different liquid growth media and various concentrations of bacitracin were determined to allow optimal growth of *Pasteurellaceae* and minimise contamination. The best procedure was validated on 100 clinical BALf samples from cattle with conventional bacterial culture as reference test. A correct identification was obtained in 73% of the samples, with 59.1% sensitivity (Se) (47.2-71.0%) and 100% specificity (Sp) (100%-100%) after only 6 hours of incubation. For pure and dominant culture samples, the procedure was able to correctly identify 79.2% of the pathogens, with a sensitivity (Se) of 60.5% (45.0%-76.1%) and specificity (Sp) of 100% (100%-100%). In mixed culture samples, containing ≥2 clinically relevant pathogens, one pathogen could be correctly identified in 57% of the samples with 57.1% Se (38.8%-75.5%) and 100% Sp (100%-100%). In conclusion, MALDI-TOF MS is a promising tool for rapid pathogen identification in BALf. This new technique drastically reduces turnaround time and may be a valuable decision support tool to rationalize antimicrobial use.

INTRODUCTION

Respiratory tract infections are a leading health issue worldwide, both in humans and animals (Radostis, 1984; Dear, 2013; WHO, 2016). Also in cattle, respiratory tract infections have a major impact on farm economics and animal welfare (Snowder et al., 2006). Their role as main indication for antimicrobial use in this species is especially important from a One Health perspective (Pardon et al., 2012). In several food animal industries, antimicrobial resistance is widespread in commensal, pathogenic, and zoonotic bacteria (Hordijk et al., 2013; Catry et al., 2016). To rationalize antimicrobial use for treatment of respiratory tract infections, rapid availability of microbiological results and antimicrobial susceptibility data are equally important in animals and humans. Respiratory tract samples are recommended to guide antimicrobial use (Xin et al., 2017). Different techniques are available, of which bronchoscopic bronchoalveolar lavage is among the most frequently used one (Meyer, 2007). In cattle, a non-endoscopic bronchoalveolar lavage (nBAL), using custom-made low cost catheters, has been developed to meet the demands of veterinary farming practices for a reliable, simple, inexpensive and safe diagnostic technique (Van Driessche et al., 2017).

Most initiated antimicrobial therapies for respiratory tract infections are empirical, meaning that antimicrobials, based on collective experience, are provided before microbiological results are available. This is due to the long turnaround time between sampling and availability of culture and susceptibility testing results, which takes at least 48 hours. A reduction in turnaround time is crucial to avoid inappropriate antimicrobial treatment and has been associated with faster adjustment of this treatment and a shorter intensive care unit stay of patients (Mok et al., 2016).

In recent years, Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) has revolutionized microbiology routine practice by reducing the turnaround time at different levels. First, identification of clinically relevant bacteria after standard culture on agar plates is performed much faster (De Carolis et al., 2014; Nomura, 2015). Second, with the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) method, which compares growth of a bacterium with and without an antimicrobial in order to detect resistance, a susceptibility test result can be reached in only few hours (Sparbier et al., 2016; Van Driessche et al., 2018). To further reduce turnaround time, and achieve the ultimate goal of availability of microbiological identification within one working day, fast identification of the organism from the

sample is essential. Protocols for rapid detection of bacteria in clinical samples by MALDI-TOF MS, skipping cultivation on agar plates, are currently available for blood (Chen et al., 2013), urine (Kitagawa et al., 2018) and other body liquids like peritoneal, synovial and cerebrospinal fluid (Oviaño et al., 2018). To date, no such technique has been developed for broncho-alveolar lavage fluid (BALf) in humans or animals, likely because of the presumed more polymicrobial nature (more contamination expected) of these samples and higher prevalence of mixed infections (Van Driessche et al., 2017). Given the important role respiratory tract infections play in worldwide antimicrobial use, the objective of the present study was to develop and evaluate a MALDI-TOF MS technique for rapid identification of respiratory pathogenic bacteria in BALf samples from cattle.

METHODS

1. PROTOCOL DEVELOPMENT AND OPTIMIZATION

Considering the average concentration of target pathogens in bovine BALf samples varies around $1x10^4$ CFU/mL (van Leenen et al., 2019), whereas MALDI-TOF MS requires a high concentration for reliable identification of pathogens (Mörtelmaier et al., 2019) (minimum $1x10^7$ – $1x10^8$ CFU/mL for *Pasteurellaceae*, data not shown), and considering a doubling time of approximately 30 minutes, a selective enrichment step of maximum 6 hours was deemed necessary to obtain reliable MALDI-TOF identification and still practically feasible for a single-day protocol.

Selection of the optimal bacitracin concentration to minimize contaminant growth

Bacitracin was chosen as selective agent, given its previous successful use to minimise contamination for respiratory samples from cattle on agar plates (Catry et al., 2006). Isolates used in this study were retrieved from a database consisting of clinical isolates of cattle retrieved by non-endoscopic bronchoalveolar lavage. Isolates were stored at -80°C. Starting from a fresh overnight culture, *P. multocida* (Pm 187), *M. haemolytica* (Mh 171) and *H. somni* (Hs 12) were each inoculated in 10 mL of Brain heart infusion broth (BHIB, Difco, BD Diagnostic Systems, Sparks, Md.) supplemented with 0, 8, 16 and 32 µg/mL bacitracin at a final concentration of $1x10^4$ CFU/mL. Immediately after

inoculation and after 6 hours of incubation at a temperature of 35°C +/-2°C and an atmosphere enriched with 5% CO_2, 1 mL sample of each tube was transferred to an eppendorf tube, and ten-fold dilutions were made of each sample for quantitative analysis as previously described (Van Hecke et al., 2017). This experiment was performed 2 times independently.

Selection of the optimal growth medium for Pasteurellaceae

Two different growth media and the incubation time were examined. BHIB and BHIB supplemented with 10% fetal bovine serum (FBS, Hyclone™ Fetal Bovine Serum, GE Healthcare life sciences, UK, Ltd.) and 0.5% yeast extract (YE, Bacto™ Yeast Extract Technical, BD Diagnostic Systems, Sparks, Md.) were used as growth media. Two strains of *P. multocida* (Pm 180, Pm 182), *M. haemolytica* (Mh 171, Mh 178) and *H. somni* (Hs 12, Hs 15) were inoculated in both media at a starting concentration of $1x10^4$ CFU/mL. No antibiotics were added. All tubes were placed in a shaking incubator for 6 hours (35°C +/- 2°C, 5% CO_2). One mL of each tube was transferred to eppendorf tubes 0, 2, 4 and 6 hours after inoculation for quantitative analysis as previously described (Van Hecke et al., 2017). This experiment was repeated twice.

2.VALIDATION ON 100 CLINICAL BALF SAMPLES

Sample collection

Samples originated from cattle (100 different animals, 10 days to 4 years old) from 10 farms with a history of respiratory tract infections. Animals that were treated during the 14-day period prior to sampling were excluded from the study. One hundred nBAL samples were taken from cattle as previously described (Van Driessche et al., 2016). Briefly, the nostril was disinfected with 90% alcohol, and a home-made catheter was inserted medioventrally in the nasal cavity. The catheter was further advanced through larynx and trachea into the bronchi until the wedge position was reached. A volume of approximately 0.6 mL/kg body weight of sterile 0.9% NaCl was injected into the lungs and immediately aspirated. Samples were transported at ambient temperature and processed within 14 hours after sampling. The sampling method was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2019-1). All methods were performed in accordance with the relevant guidelines and regulations.

Sample processing

All samples were simultaneously analysed using conventional bacteriology procedures on the one hand and the new MALDI-TOF MS rapid detection protocol, as described below, on the other hand.

Conventional bacterial culture

All nBAL samples were vortexed for 30 seconds and 1 mL was transferred to an Eppendorf tube. Ten-fold dilutions were made of each sample for quantitative analysis as previously described (Van Hecke et al., 2017). From each dilution, 100 µl was inoculated on Columbia agar supplemented with 5% sheep blood (blood agar; Oxoïd, Hampshire, UK) and incubated for 24-48 hours at 35°C +/- 2 °C in a 5% CO_2 atmosphere. All macroscopically different colonies were counted and identified with MALDI-TOF MS by direct transfer of the colony on the target plate with a toothpick (Kuhnert et al., 2012). Culture results were classified as (1) negative result (≤ $1x10^1$ CFU/mL BALf), (2) pure culture (presence of only 1 bacterial pathogen with a concentration ≥ $2x10^1$ CFU/mL BALf), (3) polymicrobial result (growth of mainly contaminants and no dominant presence of clinically relevant bacteria) (4) dominant culture (abundant presence of 1 clinically relevant pathogen, amongst contaminants, which could be easily subjected to subculture for further identification) (5) Mixed cultures (two or more clinically relevant pathogens, possibly amongst contaminants, which could be easily subjected to subculture for further identification). Some examples of agar plates representing the different classifications are shown in the supplementary data, Figure 1.

Direct detection protocol

The collected nBAL samples were vortexed for 30 seconds and 5 mL of each sample was transferred to a 15 mL falcon tube. After centrifugation (5152 x g for 10 minutes), the supernatant was carefully aspirated, leaving +/- 1 mL of cell pellet in the falcon tube. The cell pellet was vortexed and placed in a 50 mL falcon tube containing 10 mL BHIB supplemented with 10% FBS, 0.5% YE and 32 µg/mL bacitracin. All tubes were placed in a shaking incubator (35 °C +/- 2 °C, 5% CO_2) for 6 hours. After incubation, samples were centrifuged at 5152 x g for 10 minutes and the supernatant was aspirated, again leaving +/- 1 mL of cell pellet in the falcon tube. For each sample, protein extraction and MALDI-TOF MS analysis was performed as previously described (Van Driessche et al., 2018).

Briefly, the cell pellet in the falcon tube was transferred to an eppendorf tube and was centrifuged at 21130 x g for 5 minutes at room temperature. After centrifugation, the supernatant was carefully aspirated and 700 µL of 70% ethanol in high performance liquid chromatography (HPLC) graded water was added to the cell pellet and vortexed. A second centrifugation step with aspiration of the supernatant was performed as described above. After air drying for approximately 10 minutes, 20 µL 70% formic acid (in HPLC graded water) was added to the cell pellet and mixed carefully. Samples were incubated for five minutes at room temperature. In a last step, 20 µL of acetonitrile was added and vortexed. A third centrifugation step (21130 x g for 2 minutes) was performed to clarify the lysates. Noteworthy, the volume of formic acid and acetonitrile was adjusted to the size of the cell pellet (10 µL, 20 µL or 30 µL for a small, medium or large pellet, respectively, according to manufacturer's guidelines).

One µL of the protein extraction was spotted in duplicate on a target plate (MSP 96 target polished steel BC). After air drying, one µL of matrix (10 mg/mL of alpha-cyano-4-hydroxy-cinnamic acid (alpha-HCCA) in 50% acetonitrile - 47.5% water - 2.5% trifluoroacetic acid; Bruker Daltonik GmbH, Bremen, Germany) was placed on each spot. External calibration was included using a bacterial test standard (BTS, Bruker Daltonik GmbH, Bremen, Germany). Analysis was performed with an Autoflex speed MALDI-TOF/TOF MS instrument (Bruker Daltonik GmbH, Bremen, Germany) with commercial software (flexControl 1.4, version 3.4., Bruker Daltonik GmbH, Bremen, Germany), recording the mass range between 2,000-20,000 Dalton using standard settings. The spectra were analysed using MBT Compass version 4.1 (Bruker Daltonik GmbH, Bremen, Germany) that included a reference database of 7926 different bacterial entries, using standard settings. Log score values <1.7 represent no organism identification possible, a (log) score value between 1.7 and 2.0 represents identification at species level at low confidence, and a (log) score value⩾2.0 represents identification at species level at high confidence. The threshold for correct identification was determined at a (log) score value of ≥1.7, as previously described for rapid detection protocols in positive blood cultures (Tanner et al., 2017; Simon et al., 2019).

Diagnostic accuracy (sensitivity (Se) and specificity (Sp)) was determined with bacterial culture as reference test (Winepiscope 2.0 (Zaragoza, Spain)). Pure, dominant and mixed culture results were considered a positive outcome. Negative and polymicrobial test results were considered negative outcomes. Correct identification for 1 pathogen by

MALDI-TOF MS in mixed cultures was considered a positive outcome. 'No peaks found', 'no organism identification possible' or identification of a contaminant by MALDI-TOF MS for negative and polymicrobial test results was considered a positive outcome.

RESULTS

1. PROTOCOL DEVELOPMENT AND OPTIMIZATION

Detection of bacteria depends on the presence of contaminants. Therefore, development consisted of adding an appropriate antimicrobial to minimise bacterial contamination. Since bacitracin has been described to minimise contaminating organisms in respiratory samples in cattle (Catry et al., 2006), this antimicrobial was also used in this study. The optimal concentration of bacitracin, both minimising bacterial contamination of the sample and allowing *Pasteurellaceae* growth, was determined. After 6 hours of incubation, no difference in growth (expressed by CFU/mL) was seen between the various concentrations of bacitracin for *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*. In comparison with the starting concentration of $1x10^4$ CFU/mL, growth increased with 2-4 logs after 6 hours of incubation (mean log concentration: 7.83; standard deviation: 0.22). A concentration of 32 µg/mL was used for further validation.

Since the concentration of pathogens in bovine nBAL samples is generally considered low (average of $1x10^4$ CFU/mL, van Leenen et al., 2019), and the MALDI-TOF MS technique requires high bacterial counts to provide reliable results (Mörtelmaier et al., 2019) (a minimum of $1x10^7$ - $1x10^8$ CFU/mL for *Pasteurellaceae*, data not shown), an incubation step in growth medium is mandatory. Therefore, 2 different growth media were examined. For *P. multocida*, better growth was observed in the supplemented Brain heart infusion broth (BHIB) compared to BHIB (Figure 1). For *M. haemolytica* and *H. somni* no difference in growth was observed between BHIB and supplemented BHIB. Considering the better results for *P. multocida*, the supplemented BHIB was selected for the validation study. In addition, an incubation period of 6 hours was selected for the validation study, given the fact that *P. multocida* and *M. haemolytica* concentrations did not reach the detection limit of MALDI-TOF MS (i.e. $1x10^7$ - $1x10^8$ CFU/mL) after 4 hours of incubation. Longer incubation periods might not have a clear added value over

standard cultivation on plate, since this will probably not lead to identification on the same day of sample inoculation.

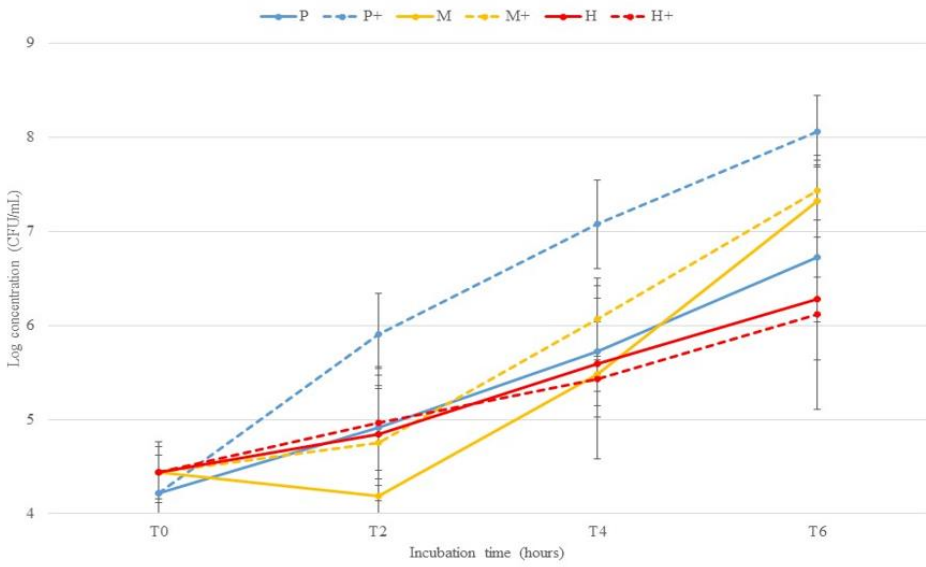

Figure 1: Comparison of BHIB (P, M, H) and BHIB supplemented with 10% fetal bovine serum and 0.5% yeast extract (P+, M+, H+) for *P. multocida* (P), *M. haemolytica* (M) and *H. somni* (H) at different incubation times (0-2-4-6 hours) to optimise bacterial growth (CFU/mL) allowing rapid detection with MALDI-TOF MS. Presented values are the mean of the two strains and the repetitions made including standard deviations

2. VALIDATION ON 100 CLINICAL BALF SAMPLES

Out of the 100 BALf samples collected, 45 different bacterial species were isolated. Seven were considered as clinically relevant pathogens in cattle (*M. haemolytica*, *P. multocida*, *Bibersteinia trehalosi*, *Moraxella ovis*, *Mannheimia varigena*, *H. somni* and *Gallibacterium anatis*). A pure culture, meaning the presence of only 1 bacterial pathogen with a concentration of ≥2 x 10^1 CFU/mL BALf, was obtained in 14% of the samples. A dominant culture, where 1 clinically relevant pathogen was abundantly present, amongst contaminants, which could be easily subjected to subculture for further identification, was seen in 24% of the samples. The majority of the samples obtained was defined as a mixed culture (28%), meaning the presence of ≥ 2 clinically relevant pathogens, possibly amongst contaminants, which could be easily subjected to

subculture for further identification. In 18% of the samples, the culture was polymicrobial, meaning the growth of mainly contaminants and no dominant presence of clinically relevant bacteria and in 16% of the samples the culture result was negative (≤1 x 10^1 CFU/mL BALf). Correct identification rates of the clinically relevant pathogens by the rapid MALDI-TOF MS procedure are listed in Table 1.

Table 1. Identification of clinically relevant pathogens from BALf samples by conventional culture and rapid detection with MALDI-TOF MS

Microorganism	Total number isolated in conventional bacteriological culture	Correct MALDI ID compared to all cultures	Correct MALDI ID for pure and dominant cultures	Correct MALDI ID for mixed cultures
		Number (Percentage)	Number (percentage)	Number (Percentage)
Mannheimia haemolytica	27	14/27 (51.9%)	7/9 (77%)	7/18 (38.8%)
Pasteurella multocida	18	11/18 (61.1%)	7/7 (100%)	4/11 (36.4%)
Bibersteinia trehalosi	9	7/9 (77.8%)	3/3 (100%)	4/6 (66.7%)
Moraxella ovis	22	0/22 (0%)	0/8 (0%)	0/14 (0%)
Mannheimia varigena	8	1/8 (12.5%)	1/3 (33.3%)	0/5 (0%)
Histophilus somni	12	1/12 (8.3%)	0/3 (0%)	1/9 (11.1%)
Gallibacterium anatis	2	2/2 (100%)	2/2 (100%)	ND

ND: not detected

Detailed information on the identification results of the conventional culture and the rapid MALDI-TOF MS method can be found in the supplementary data, Table 1. Considering pure culture samples only, correct rapid identification with MALDI-TOF MS was possible in 71% of the samples, with a Se of 71.4% (47.8%-91.1%) and Sp of 100% (100%-100%). When a dominant culture was obtained, correct identification occurred in 58% of the samples, with a Se of 54.2% (34.2%-74.1%) and Sp of 100% (100%-100%). In pure and dominant cultures, with consideration of the negative culture results correctly identified, the proportion of observed agreement was 79.2%, with a Se of 60.5% (45.0%-76.1%) and a Sp of 100% (100%-100%) (Table 2).

Table 2. 2x2 contingency table for rapid MALDI-TOF MS identification as index test compared to bacterial culture as reference test for identification of respiratory pathogens in pure and dominant BALf culture samples of cattle

Pure and dominant cultures only			
	Bacterial culture +	Bacterial culture -[a]	Total
Rapid MALDI-TOF MS +	23	0	23
Rapid MALDI-TOF MS -	15	34	49
Total	38	34	72

[a] All polymicrobial and negative cultures. When MALDI-TOF MS identified a contaminant, the result was considered rapid MALDI-TOF MS-.

Twenty-eight percent of the clinical samples contained a mixed culture with 2, 3 or 4 different clinically important pathogens in 22, 5 and 1 mixed culture sample, respectively. In mixed cultures, the correct identification rate per clinically relevant pathogen was considerably lower, since MALDI-TOF MS only identified one pathogen (Table 1). Correct identification of one clinically important pathogen occurred in 57% of the cases, and Se and Sp were 57.1% (38.8%-75.5%) and 100% (100%-100%), respectively. All polymicrobial and negative samples were correctly classified. *M. ovis*, *H. somni* and *M. varigena* could rarely to not be identified with the rapid MALDI-TOF MS method (Table 1). Taking all clinical samples into account, the proportion of observed agreement between culture and the direct MALDI-TOF MS method was 73%, with a Se of

59.1 % (47.2%-71%) and a Sp of 100% (100%-100%) (Table 3). In Figure 2, identification percentages stratified on pathogen concentration in BALf are presented. For a bacterial pathogen concentration of $1x10^5$ CFU/mL or more in the original sample, correct identification occurred in all cases. For mixed cultures, a positive association was found between the concentration of the pathogen and the rate of correct identification.

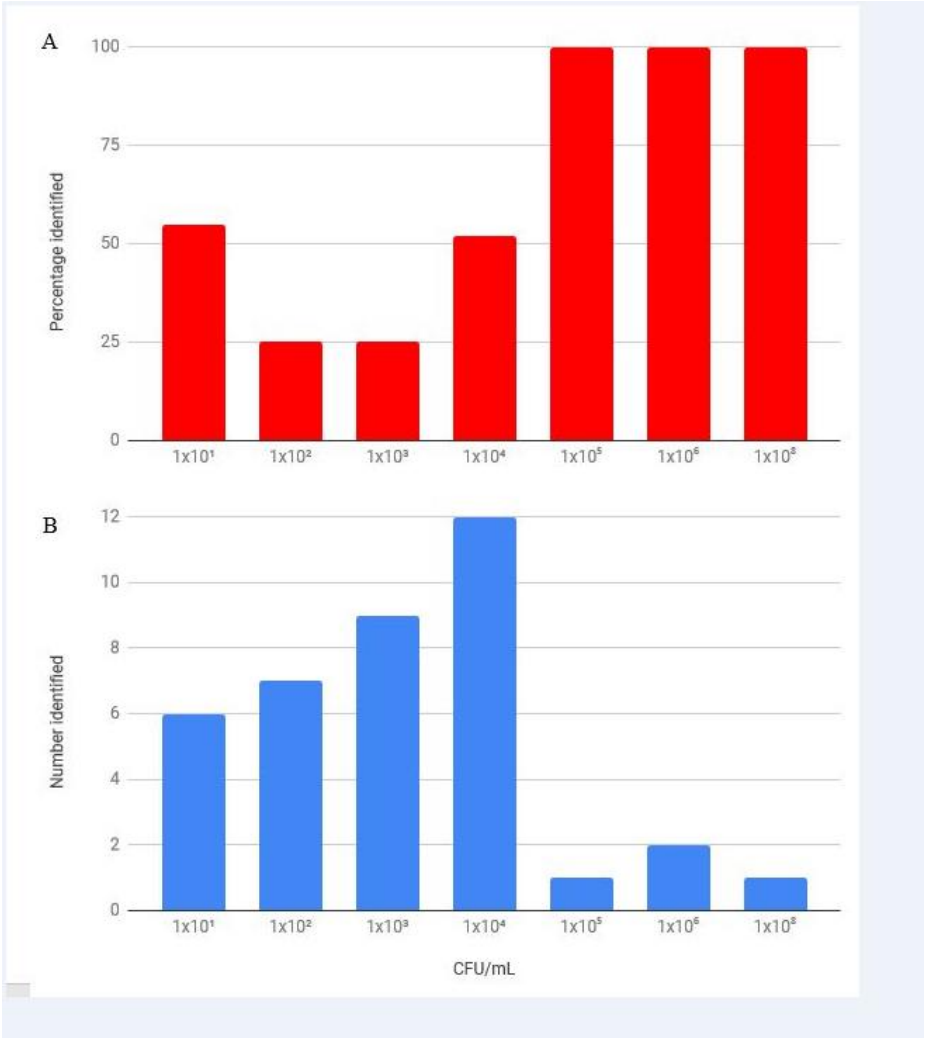

Figure 2: Effect of the initial pathogen concentration (CFU/mL) in dominant and pure cultures in BALf on the percentage of correct identification rate (A) and number of correct identification rate (B) with the rapid MALDI-TOF MS procedure

Table 3. 2x2 contingency table for direct MALDI-TOF MS identification as index test compared to bacterial culture as reference test for identification of respiratory pathogens in 100 clinical BALFf samples of cattle

	Bacterial culture +[a]	Bacterial culture -[b]	Total
Rapid MALDI-TOF MS +	39	0	39
Rapid MALDI-TOF MS -	27	34	61
Total	66	34	100

[a] All samples with isolation of a clinically relevant pathogen, meaning pure culture, dominant culture and mixed culture. For mixed cultures, when one pathogen was correctly identified by MALDI-TOF MS, the result was considered direct MALDI-TOF MS+

[b] All polymicrobial and negative cultures. When MALDI-TOF MS identified a contaminant, the result was considered direct MALDI-TOF MS-.

DISCUSSION

This study showed that next to blood and urine samples (Ferreira et al., 2010; Martiny et al., 2012; Randazzo et al., 2016), rapid identification of bacterial pathogens by MALDI-TOF MS, skipping the cultivation step on agar, is possible for BALf samples. Taking all samples into account, 73% was correctly identified after 6 hours of incubation, which would be a clinically desirable turnaround time for treatment initiation, since this could result in identification on the same day the sample was inoculated. False positive results did not occur, and 3/5 infections were correctly identified. These results were obtained using a cutoff value of ≥1.7. Although a (log) score value between 1.7 and 2.0 should be interpreted as species identification with low confidence, this cutoff value or even lower is commonly used for identification procedures of positive blood cultures by MALDI-TOF MS (Tanner et al., 2017; Simon et al., 2019) and was therefore also applied in the present study.

A limitation of the study was the relatively small sample size, which reduced the number of isolates available for some target pathogens. Whereas Se was above 75% to even 100% for common pathogens like *M. haemolytica* and *P. multocida*, it was below 10% for other bacteria, such as *M. ovis* and *H. somni*, resulting in a lower Se of the technique when considering all samples with pure or dominant cultures. A likely reason why

identification rates for *H. somni* and *M. ovis* were poor, is the slow growth rate of these bacteria. Indeed, *H. somni* was shown to reach a concentration of $1x10^6$ CFU/mL after 6 hours of incubation, starting from a $1x10^4$ CFU/mL inoculum, thereby not reaching the $1x10^7$-$1x10^8$ CFU/mL detection limit (Figure 1). Therefore, successful detection of *H. somni* by the rapid method depended on the initial concentration in BALf and the presence of other clinically relevant pathogens in the sample. Another reason why identification rates for *H. somni*, *M. varigena* and *M. ovis* were low could be due to the small number of spectra present in the Biotyper database, namely 1 entry for *M. ovis* and *M. varigena* and 2 entries for *H. somni*. Therefore possible solutions for increasing the identification rate of these pathogens could be on the one hand increasing the number of entries of each pathogen involved in the database, and on the other hand expanding the incubation time and perhaps the use of other optimised growth media. Another solution to increase identification rates could be a short incubation on agar plates, as previously described for positive blood culture bottles (Zabbe et al., 2015). However, *Pasteurellaceae* are considered fastidiously growing pathogens, in contrast with the fast growing pathogens like *Enterobacteriaceae,* enterococci and staphylococci that were mainly observed in the previous study (Zabbe et al., 2015). Indeed, preliminary tests in our lab suggest that a similar procedure for *Pasteurellaceae* would probably take too long to have an added value over an overnight culture, even though these observations need confirmation. Additionally, differentiating between multiple pathogens among these young subcultures would be difficult, making this method challenging in samples with contaminants or mixed cultures.

Surprisingly, in six samples a correct MALDI-TOF MS identification was obtained from a starting concentration of $1x10^1$ CFU/mL for *M. haemolytica*, *M. varigena*, *P. multocida* and *B. trehalosi*. Given the expected growth rate of the involved species, the most likely explanation for this observation would be that the quantification of $1x10^1$ CFU/mL did not represent the true concentration in the BALf, for example due to clumping of bacteria in the sample. This fenomenon could also have occured in higher dilutions.

An important limitation of the rapid MALDI-TOF technique is that diagnostic accuracy was substantially lower in mixed culture samples. Similar results were previously observed for blood and urine samples (Ferreira et al., 2010; Buchan et al., 2012; Martiny et al., 2012), but its impact on Se and Sp was not shown in these studies. Even though this can vary between studies, often a single bacterial pathogen is present in the lower

respiratory tract of cattle. Mixed infections can occur, but do not make up the majority, which is demonstrated in a study were nBAL samples were taken from 144 preweaned calves with respiratory disease, where 16 samples (=11.1%) obtained a mixed culture (Van Driessche et al., 2017). Since mixed cultures can occur in BALf, it is advised to run the classic bacterial culture in parallel with the rapid detection method. However, as correct identification of one species was still possible in 57% of the samples, depending on the species and initial concentration of the respiratory pathogen, the technique can have an added value for mixed cultures as well. Additionally, new techniques are currently being developed addressing the problem of identifying multiple pathogens within one sample (Yang et al., 2018), leading to promising results of higher diagnostic accuracy by MALDI-TOF MS in the future, also for mixed samples.

Although the current technique only provides identification of respiratory pathogens, it might provide a basis for rapid antimicrobial susceptibility testing such as the MBT-ASTRA method, especially for pure cultures. Because enriched cultures containing a pure culture will only be detected as such by MALDI-TOF MS when a sufficiently high concentration is obtained (10^7-10^8 CFU/mL), the MBT-ASTRA method might be performed subsequently on the same culture, since similar concentrations have been described as inoculum for the MBT-ASTRA method for *Pasteurella multocida* (Van Driessche et al., 2018).

In contrast with human medicine, a different classification of samples was applied in the current study. In human medicine, polymicrobial samples are defined as the presence of >1 pathogen in a sample. Since blood cultures and urine samples are normally sterile, and considering the aseptical method of taking these samples, contamination is only rarely encountered. Therefore, when infection is present, mostly 1 pathogen is derived from the sample. In respiratory tract samples of cattle, the presence of >1 clinically relevant pathogen can occur, and is therefore defined as a mixed culture. A polymicrobial culture is seen as the presence of different micro-organisms who are not clinically relevant. Also, a dominant culture can be present, meaning that a clinically relevant pathogen can be isolated, although still some bacterial contaminants can be present in the sample, due to the sampling procedure.

With the nBAL field sampling technique for cattle, approximately 20% of the samples return a polymicrobial test result (Van Driessche et al., 2017). However, the nBAL technique was deliberately chosen in the present study since it has been shown to give

more pure culture and less polymicrobial culture results compared to other techniques frequently used in cattle such as the deep nasopharyngeal swab (Van Driessche et al., 2017). Possible solutions for reducing polymicrobial samples during sampling could be a more hygienic handling or the implementation of endoscopic-guided bronchoalveolar lavages, although the latter method can also not exclude contamination (Rasmussen et al., 2001). Additionally, bacitracin was added to the samples during incubation in order to limit contamination, which indeed reduced the presence of most Gram-positive contaminants present in the initial samples. After 6 hours of incubation of the samples, *Escherichia coli* and *Bacillus spp*. were the most common contaminants present (data not shown). Finding a method to minimise the growth of latter bacteria without influencing the growth of *Pasteurellaceae*, would indeed increase the diagnostic accuracy of this new rapid technique. This seems, however, very challenging, since *Pasteurellaceae* are commonly more sensitive to various antimicrobial substances than *Enterobacteriaceae*. Nevertheless, the rapid detection protocol by MALDI-TOF MS obtained no false positive result for all polymicrobial samples. Likely, in humans, the issue of sample contamination would be less prominent than in the currently used farm setting.

This study shows that MALDI-TOF MS considerably improves turnaround time, from 24-48 hours to 6.5 hours in total, bringing identification of causal bacteria of lower respiratory tract infections into a clinically desirable timeframe. Furthermore, with respect to the implementation in the laboratory, the most time-consuming steps (6 hours of incubation, 15 minutes centrifugation) require no hands-on time, making this technique easily applicable in current clinical laboratory workflows. In total 3/5 infections were correctly identified by the rapid MALDI-TOF MS technique. In order to support the decision making process of initiating antimicrobial treatment, the classic bacterial culture can be run in parallel with the rapid detection method. However, no false positive results did occur, leading to a specificity of 100%, and antimicrobial treatment should be initiated when a bacterial compound is present in BALf samples. When an animal with a positive result by the rapid MALDI-TOF MS method is treated the same day, this will probably result in less animal suffering and a more effective treatment. Further research including clinical data on the positive outcome of this reduced turnaround time in veterinary medicine, i.e. reduced time of disease and reduced inappropriate empirical treatment in combination with an increased therapy

success, is encouraged. Additionally, the currently described protocol can be used in future studies aiming at different bacteria and host species, including humans.

CONCLUSION

In conclusion, MALDI-TOF MS is a promising technique for rapid detection of respiratory pathogens in BALf, as demonstrated in cattle. This offers the possibility to practitioners and clinicians to better target their initial antimicrobial treatment.

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AUTHOR CONTRIBUTIONS

L.V.D designed and conducted all experiments and sample collection, collected literature data, analysed data, prepared figures and prepared the paper. J.B. conducted the sample collection in the validation study. P.D. and F.H. supervised the work. F.B. and B.P. designed the study, analysed data and supervised the work. All authors read and reviewed the final manuscript.

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SUPPLEMENTARY DATA

Table 1. Identification of clinically relevant pathogens from BALf samples by conventional culture and rapid detection with MALDI-TOF MS

Sample number	Classification conventional culture	Pathogens (concentration, CFU/mL)	Contaminants (concentration, CFU/mL)	Rapid MALDI-TOF MS identification	(Log) score value
1	Mixed: *M. haemolytica*, *B. trehalosi*	*M. haemolytica*: $5x10^3$, *B. trehalosi*: $4x10^2$	*Aerom.veronii*: $2x10^1$, *Bacillus spp.*: $2x10^1$, *Staph. sciuri*: $2x10^1$	*M. haemolytica*	2.49
2	Dominant: *M. haemolytica*	*M. haemolytica*: $5x10^2$	*Staph. cohnii*: $2x10^2$, *Bacillus spp.*: $2x10^1$, *Coryn. amycolatum*: $6x10^1$, *Strept. suis:* $2x10^2$	*M. haemolytica*	2.32
3	Mixed: *M. haemolytica*, *M. ovis*	*M. haemolytica*: $6x10^4$, *M. ovis*: $3x10^3$	/	*M. haemolytica*	2.35
4	Dominant: *M. haemolytica*	*M. haemolytica*: $2x10^1$	*Staph. sciuri*: $8x10^1$, *Staph. lentus*: $2x10^2$, *Neisseria perflava*: $4x10^2$	*M. haemolytica*	2.40
5	Negative	/	/	No organism identification possible	1.47
6	Dominant: *M. haemolytica*	*M. haemolytica*: $4x10^2$	*Bacillus spp.*: $1x10^3$	*M. haemolytica*	2.03
7	Pure: *M. haemolytica*	*M. haemolytica*: $2x10^3$	/	*M. haemolytica*	2.39
8	Polymicrobial	/	*Staph. rostri*: $1x10^3$, *E. coli*: $2x10^3$	*E. coli*	2.26
9	Mixed: *B. trehalosi*, *M. haemolytica*	*B. trehalosi*: $4x10^3$, *M. haemolytica*: $1x10^2$	*Strept. hyovaginalis*: $1x10^2$	*B. trehalosi*	2.29
10	Mixed: *M. haemolytica*, *B. trehalosi*, *M. varigena*, *H. somni*	*M. haemolytica*: $1x10^3$, *B. trehalosi*: $1x10^3$, *H. somni*: $5x\ 10^4$, *M. varigena*: $7x10^3$	*E. coli*: $1x10^2$	*B. trehalosi*	1.91
11	Mixed: *M. haemolytica*, *H. somni*, *M. ovis*	*M. haemolytica*: $2x10^3$, *H. somni*: $1x10^4$, *M. ovis*: $3x10^4$	/	No organism identification possible	1.23
12	Mixed: *M. haemolytica*, *B. trehalosi*	*M. haemolytica*: $5x10^4$, *B. trehalosi*: $2x10^4$	*Bacillus spp.*: $2x10^3$	*M. haemolytica*	2.15
13	Pure: *B. trehalosi*	*B. trehalosi*: $1x10^6$	/	*B. trehalosi*	2.05
14	Pure: *H. somni*	*H. somni*: $4x10^4$	/	No organism identification possible	1.69

15	Pure: *H. somni*	*H. somni*: $1x10^3$	/	No organism identification possible	1.23
16	Mixed: *B. trehalosi*, *H. somni*, *M. haemolytica*	*B. trehalosi*: $5x10^2$, *H. somni*: $2x10^3$, *M. haemolytica*: $1x10^2$	/	*B. trehalosi*	2.12
17	Mixed: *H. somni*, *M. haemolytica*	*H. somni*: $1x10^3$, *M. haemolytica:* $3x10^1$	*Staph. equorum*: $2x10^1$	No organism identification possible	1.44
18	Mixed: *H. somni*, *M. ovis*, *M. varigena*	*H. somni*: $4x10^4$, *M. varigena*: $8x10^3$, *M.ovis*: $1x10^3$	/	*H. somni*	1.71
19	Mixed: *M. haemolytica*, *P. multocida*	*M. haemolytica*: $4x10^4$, *P. multocida*: $3x10^3$	/	*M. haemolytica*	2.14
20	Mixed: *M. haemolytica*, *B. trehalosi*, *M. varigena*	*M. haemolytica*: $1x10^2$, *B. trehalosi*: $2x10^2$, *M. varigena*: $5x10^1$	*Strept. hyovaginalis*: $1x10^3$, *Neisseria subflava*: $2x10^3$, *Staph. xylosus*: $3x10^2$	*B. trehalosi*	2.24
21	Dominant: *G. anatis*	*G. anatis*: $7x10^3$	*Bacillus spp.*: $6x10^2$, *Lactobacillus salivarius*: $2x10^3$, *Pelistega europaea*: $2x10^4$	*G. anatis*	2.23
22	Mixed: *M. haemolytica*, *H. somni*	*M. haemolytica*: $8x10^2$, *H. somni*: $3x10^2$	*Staph. equorum*: $8x10^2$	*Staph. equorum*	1.73
23	Dominant: *M. haemolytica*	*M. haemolytica*: $6x10^4$	*Staph. aureus*: $1x10^2$	*M. haemolytica*	2.21
24	Negative	/	/	No organism identification possible	1.59
25	Negative	/	/	No peaks found	0
26	Polymicrobial	/	*Strept. suis*: $1x10^3$, *Bacillus spp.:* $1x10^3$	No organism identification possible	1.5
27	Dominant: *M. ovis*	*M. ovis*: $2x10^2$	*Staph. haemolyticus*: $1x10^1$	No organism identification possible	1.46
28	Negative	/	*Staph. haemolyticus*: $1x10^1$	No organism identification possible	1.69
29	Negative	/	/	*Staph. haemolyticus*	1.88
30	Dominant: *M. ovis*	*M. ovis*: $2x10^3$	*Staph. chromogenes*: $1x10^2$	No organism identification possible	1.56
31	Polymicrobial	/	*Staph. xylosus*: $4x10^1$, *Neisseria flavescens*: $2x10^2$, *Strept. suis:* $4x10^2$	No organism identification possible	1.68
32	Mixed: *M. ovis*, *P. multocida*	*M. ovis*: $4x10^4$, *P. multocida*: $1x10^3$	/	*Staph. lentis*	1.77
33	Mixed : *M. ovis*, *P. multocida*	*P. multocida*: $9x10^3$, *M. ovis*: $1x10^3$	/	*Staph. haemolyticus*	1.74

34	Dominant: *M. varigena*	*M. varigena*: $3x10^1$	*E. coli*: $1x10^2$, *Neisseria subflava*: $1x10^3$, *Strept. suis*: $3x10^3$	No organism identification possible	1.62
35	Polymicrobial	/	*Acinetobacter towneri*: $3x10^1$, *Serratia liquefaciens*: $4x10^2$, *Aerococcus viridans*: $9x10^1$	No organism identification possible	1.61
36	Dominant: *M. ovis*	*M. ovis*: $3x10^2$	*Staph. sciuri*: $8x10^1$	No organism identification possible	1.26
37	Mixed: *P. multocida*, *M. haemolytica*, *M. ovis*	*P. multocida*: $2x10^3$, *M. ovis*: $4x10^3$, *M. haemolytica:* $5x10^3$	*Strept. suis*: $3x10^2$	No organism identification possible	1.56
38	Pure: *P.multocida*	*P. multocida*: $7x10^4$	/	*P. multocida*	2.01
39	Dominant: *G. anatis*	*G. anatis*: $1x10^4$	*Strept. suis*: $5x10^2$	*G. anatis*	2.28
40	Pure: *Staph. sciuri*	/	*Staph. sciuri*: $6x10^1$	*Staph. equorum*	1.73
41	Polymicrobial	/	*Morganella morganii*: $5x10^1$, *Lysinibacillus fusiformis*: $1x10^1$	No peaks found	0
42	Pure: *H. somni*	*H. somni*: $5x10^2$	/	No organism identification possible	1.5
43	Pure: *M. haemolytica*	*M. haemolytica*: $1x10^4$	/	*M. haemolytica*	2.33
44	Dominant: *P. multocida*	*P. multocida*: $2x10^3$	*Bacillus spp*: $2x10^3$, *Strept. dysgalactiae*: $2x10^2$	*P. multocida*	2.37
45	Polymicrobial	/	*Pantoea agglomerans*: $1x10^1$, *Staph. fleurettii*: $1x10^3$, *Bacillus spp*: $1x10^1$	No peaks found	0
46	Negative	/	/	No organism identification possible	1.6
47	Mixed: *H. somni*, *M. haemolytica*	*H. somni*: $1x10^4$, *M. haemolytica*: $2x10^3$	*Strept. suis*: $7x10^2$	*Strept. suis*	2.23
48	Negative	/	/	No peaks found	0
49	Dominant: *M. haemolytica*	*M. haemolytica*: $1x10^4$	*E. coli*: $2x10^4$, *Strept. suis*: $4x10^3$	*E. coli*	2.33
50	Negative	/	*E. coli*: $1x10^1$	No peaks found	0
51	Polymicrobial	/	*Lysinibacillus fusiformis*: $5x10^1$, *Providencia rettgeri*: $5x10^2$	*E. coli*	2.18
52	Dominant: *M. haemolytica*	*M. haemolytica*: $1x10^5$	*Strept. suis*: 1x10^4	*M. haemolytica*	2.09
53	Dominant: *B. trehalosi*	*B. trehalosi*: $3x10^1$	*Bacillus spp*: $1x10^1$	*B. trehalosi*	1.99
54	Negative	/	/	No peaks found	0
55	Polymicrobial	/	*Bacillus spp*: $2x10^3$, *Strept. pluranimalium*: $5x10^1$, *E. coli*: $4x10^1$	No organism identification possible	1.27

56	Polymicrobial	/	*Bacillus spp*: $6x10^1$, *Strept. pluranimalium* $3x10^2$, *Staph. chromogenes*: $1x10^1$	No organism identification possible	1.46
57	Dominant: *M. varigena*	*M. varigena*: $2x10^3$	*Bacillus spp.*: $3x10^1$, *Strept. pluranimalium*: $7x10^1$	No organism identification possible	1.31
58	Negative	/	/	No organism identification possible	1.38
59	Mixed : *P. multocida*, *M. ovis*	*P. multocida*: 10^6, *M. ovis*: $4x10^3$	*Strept. suis*: $7x10^2$	*P. multocida*	2.02
60	Mixed: *M. ovis*, *P. multocida*	*M. ovis*: $4x10^3$, *P. multocida*: $8x10^2$	*Strept. pluranimalium*: $3x10^2$	No organism identification possible	1.44
61	Dominant: *M. ovis*	*M. ovis*: $6x10^2$	*Neisseria meningitidis*: $5x10^2$	No organism identification possible	1.17
62	Dominant: *M. ovis*	*M. ovis*: $6x10^3$	*Strept. suis*: $2x10^1$	No organism identification possible	1.61
63	Negative	/	/	No organism identification possible	1.17
64	Mixed : *M. ovis*, *P. multocida*	*P. multocida*: $1x10^8$, *M. ovis*: $4x10^2$	/	*P. multocida*	1.71
65	Dominant: *M. ovis*	*M. ovis*: $1x10^4$	*Strept. pluranimalium*: $1x10^2$	No organism identification possible	1.2
66	Negative	/	*Acinetobacter lwoffii*: $1x10^1$	No organism identification possible	1.25
67	Pure: *M. ovis*	*M. ovis*: $4x10^3$	/	No organism identification possible	1.23
68	Negative	/	*Strept. equorum*: $1x10^1$	No organism identification possible	1.48
69	Pure: *P. multocida*	*P. multocida*: $1x10^4$	/	*P. multocida*	2.24
70	Negative	/	/	*Staph. lentus*	1.73
71	Dominant: *B. trehalosi*	*B. trehalosi*: $4x10^2$	*Staph. equorum*: $3x10^1$, *Neisseria flavescens*: $8x10^1$, *Strept. suis*:$2x10^1$	*B. trehalosi*	1.88
72	Polymicrobial	/	*Pantoea anatis*: $2x10^2$, *Bacillus spp.*: $1x10^1$, *Strept. suis* $2x10^1$, *Staph. chromogenes*: $3x10^1$	No organism identification possible	1.65
73	Dominant: *P. multocida*	*P. multocida*: $3x10^3$	*Staph. chromogenes*: $1x10^3$	*P. multocida*	1.78
74	Mixed: *M. haemolytica*, *M. ovis*	*M. haemolytica*: $4x10^2$, *M. ovis*: $2x10^2$	*Strept. suis*: $1x10^1$	No organism identification possible	1.42
75	Dominant: *P. multocida*	*P. multocida*: $3x10^3$	*Staph. chromogenes*: $3x10^2$, *Strept. pluranimalium*: $1x10^2$	*P. multocida*	1.72

76	Dominant: *P. multocida*	*P. multocida*: $2x10^3$	*Staph. chromogenes*: $1x10^3$, *Strept. suis*: $1x10^3$, *Neisseria flavescens*: $5x10^2$	*P. multocida*	1.74
77	Pure: *M. varigena*	*M. varigena*: $5x10^1$	/	*M. varigena*	2.00
78	Mixed: *M. haemolytica*, *M. ovis*	*M. haemolytica*: $7x10^1$, *M. ovis*: $3x10^2$	*Strept. suis*: $1x10^2$	*M. haemolytica*	2.02
79	Mixed: *P. multocida*, *M. ovis*	*P. multocida*: $1x10^4$, *M. ovis*: $8x10^3$	*Staph. xylosus*: $1x10^2$, *Strept. suis*: $1x10^3$	*P. multocida*	2.33
80	Pure: *E. coli*	*E. coli*: $4x10^2$	/	*E. coli*	2.13
81	Mixed: *M. ovis*, *M. varigena*	*M. ovis*: $4x10^3$, *M. varigena*: $4x10^3$	*Staph. chromogenes*: $3x10^3$	No organism identification possible	1.35
82	Mixed: *M. haemolytica*, *P. multocida*	*M. haemolytica*: $3x10^1$, *P. multocida*: $8x10^1$	*Bacillus spp.*: $1x10^1$, *Strept. suis*: $2x10^1$, *Staph. Aureus* : $6x10^1$	*M. haemolytica*	1.74
83	Polymicrobial	/	*Strept. suis*: $2x10^1$, *E. coli*: $6x10^1$	No organism identification possible	1.30
84	Mixed: *M. haemolytica*, *P. multocida*	*M. haemolytica*: $3x10^1$, *P. multocida*: $4x10^1$	*Neisseria spp.*: $2x10^2$, *Strept. suis*: $2x10^1$	*M. haemolytica*	2.03
85	Negative	/	/	No organism identification possible	1.34
86	Polymicrobial	/	*E. coli*: $1x10^2$, *Bacillus spp.*: $1x10^1$, *Staph. xylosus* : $8x10^1$, *Strept. suis* : $1x10^2$	*E. coli*	2.08
87	Polymicrobial	/	*E. coli*: $3x10^2$, *Bacillus spp.*: $2x10^1$, *Staph. haemolyticus*: $1x10^2$,	*E. coli*	2.23
88	Polymicrobial	/	*Staph. chromogenes*: $7x10^2$, *Bacillus spp.:* $1x10^1$, *Strept. suis*: $1x10^2$, *Kluyvera intermedia*: $1x10^2$	No organism identification possible	1.35
89	Dominant: *M. haemolytica*	*M. haemolytica*: $3x10^2$	*E. coli*: $2x10^3$	No organism identification possible	1.3
90	Mixed: *P. multocida*, *M. haemolytica*	*P. multocida*: $1x10^4$, *M. haemolytica*: $6x10^2$	*Strept. suis*: $4x10^3$	*P. multocida*	2.17
91	Polymicrobial	/	*Strept. pluranimalium*: $2x10^1$, *Staph. aureus*: $8x10^1$	No organism identification possible	1.45
92	Pure: *P. multocida*	*P. multocida*: $2x10^4$	/	*P. multocida*	2.32
93	Polymicrobial	/	*Lactobacillus spp.*: $4x10^2$, *Staph. xylosus*: $9x10^1$	No organism identification possible	1.40
94	Polymicrobial	/	*E. coli*: $6x10^1$, *Strept. suis*: $1x10^2$, *Neisseria spp.*: $2x10^2$	No organism identification possible	1.29

95	Polymicrobial	/	*Lysinibacillus fusiformis*: $1x10^2$, *Staph. chromogenes*: $2x10^2$	No organism identification possible	1.52
96	Mixed: *M. varigena*, *H. somni*	*M. varigena*: $8x10^1$, *H. somni*: $1x10^2$	/	*Staph. haemolyticus*	1.7
97	Mixed: *M. ovis*, *H. somni*	*M. ovis*: $7x10^2$, *H. somni*: $1x10^3$	/	*E. coli*	1.81
98	Negative	/	/	no peaks found	0
99	Dominant: *M. ovis*	*M. ovis*: $3x10^4$	*E. coli*: $2x10^2$	*E. coli*	1.77
100	Pure: *Pseudomonas aeruginosa*	/	*Pseudomonas aeruginosa*: $5x10^1$	*Pseudomonas aeruginosa*	1.92

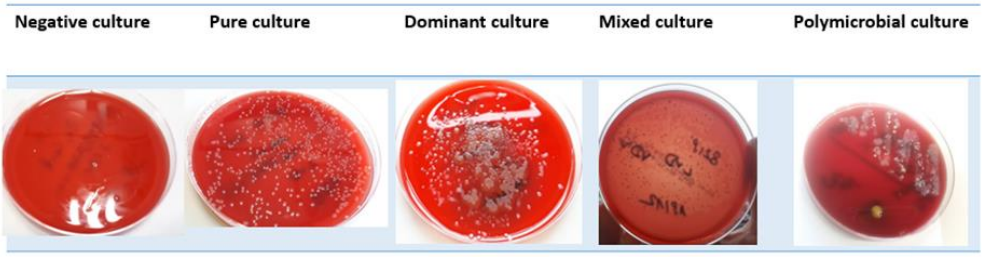

Figure 1: Visualisation of the classification of clinical nBAL samples from cattle obtained by conventional culture

CHAPTER 7

RAPID DETECTION OF TETRACYCLINE RESISTANCE IN BOVINE *PASTEURELLA MULTOCIDA* ISOLATES BY MALDI BIOTYPER ANTIBIOTIC SUSCEPTIBILITY TEST RAPID ASSAY (MBT-ASTRA)

RAPID DETECTION OF TETRACYCLINE RESISTANCE IN BOVINE *PASTEURELLA MULTOCIDA* ISOLATES BY MALDI BIOTYPER ANTIBIOTIC SUSCEPTIBILITY TEST RAPID ASSAY (MBT-ASTRA)

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ABSTRACT

Pasteurella multocida is notorious for its role as an opportunistic pathogen in infectious bronchopneumonia, the economically most important disease facing cattle industry and leading indication for antimicrobial therapy. To rationalize antimicrobial use, avoiding imprudent use of highly and critically important antimicrobials for human medicine, availability of a rapid antimicrobial susceptibility test is crucial. The objective of the present study was to design a MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) procedure for tetracycline resistance detection in *P. multocida*. This procedure was validated on 100 clinical isolates with MIC-gradient strip test, and a comparison with disk diffusion was made. Sensitivity and specificity of the MBT-ASTRA procedure were 95.7% (95% confidence interval (CI) = 89.8-101.5) and 100% (95% CI = 100-100), respectively, classifying 98% of the isolates correctly after only three hours of incubation. Sensitivity and specificity of disk diffusion were 93.5% (95% CI = 86.3-100.6) and 96.3% (95% CI = 91.3-101.3) respectively, classifying 95% of the isolates correctly. In conclusion, this MBT-ASTRA procedure has all the potential to fulfil the need for a rapid and highly accurate tetracycline susceptibility testing in *P. multocida* to rationalize antimicrobial use in outbreaks of bronchopneumonia in cattle or other clinical presentations across species.

INTRODUCTION

Pasteurella multocida, a Gram-negative coccobacillus, causes many important diseases in a wide range of hosts (Quinn et al., 1994). In different ruminant species and pigs it is one of the most frequently isolated pathogens in infectious bronchopneumonia (Boyce et al., 2012; Woolums, 2015), a disease which is the leading cause of morbidity and antimicrobial use in these species (Pardon et al., 2012). In more tropical regions of the world, especially Africa and Asia, *P. multocida* can also cause highly fatal hemorrhagic septicemia in cattle and buffaloes (Carter et al., 1989). In other animal species, this bacterium has been associated with presentations like sepsis (Malhi et al., 2016), but also otitis (Jensen et al., 1983) and peritonitis (Catry et al., 2005a). In humans, *P. multocida* can cause several life-threatening conditions, like sepsis (Konda et al., 2016) and endocarditis (Yuji et al., 2015).

Today, to control bronchopneumonia mass medication is still one of the preferred therapies in different food-producing animal species (Timmerman et al., 2006; Persoons et al., 2011; Pardon et al., 2012). Given the issue of high level antimicrobial resistance in these industries and the one health initiatives founded to combat this (Schantziaras et al., 2013Mukerji et al., 2017; Schrijver et al., 2018), preventive antimicrobial use is no longer considered appropriate and antimicrobial treatment should be targeted (WHO, 2017). In several European countries like Belgium, Germany or the Netherlands, guidelines suggest and legislation requires sampling and susceptibility testing of animal pathogens before specific antimicrobial agents can be used (FDA, 2000; WHO, 2001; MEP, 2012; Fairles, 2013; Chantziaras et al., 2014; RD, 2016; JPIAMR; 2017). In addition, formularies have been initiated in several countries to guide veterinarians to select appropriate antimicrobials mainly based on their clinical efficacy and importance for human medicine (AMCRA, 2015; de Greeff et al., 2017). Especially in more intensive cattle rearing systems which rely on mass medication, antimicrobial multiresistance in *P. multocida* is common, hampering empiric antimicrobial therapy (Catry et al., 2005b; Catry et al., 2006; Hendriksen et al., 2008; Persoons et al., 2011). Multiresistant isolates, that compromise 12 antimicrobial resistance genes, are threatening the future of these production systems (Michael et al., 2012).

To date, mainly disk diffusion susceptibility tests are used in veterinary practice to guide antimicrobial therapy. These take a minimum of 2 days after sampling before results are

available, but in most cases, due to different practical reasons, a waiting period of 3-4 days is average. In most outbreaks of infectious bronchopneumonia postponing an appropriate therapy, until susceptibility testing results are available, is not acceptable for economic and animal welfare reasons. On the other hand initiation of an inappropriate antibiotic treatment will increase antimicrobial resistance selection pressure (Marshall and Levy, 2011). Disk diffusion susceptibility tests have shown a sensitivity of only 85.7% for the detection of tetracycline resistance in *P. multocida* compared to the agar dilution technique as the gold standard in the past (Catry et al., 2007) , which might result to therapeutic failure in practice. The agar dilution technique is however not commonly used in routine veterinary diagnostics, being a more laborious and expensive technique compared with disk diffusion. Although the antimicrobial gradient strip method is not regarded as the gold standard like the broth dilution technique currently, results achieved by both tests have a good correlation (Baker et al., 1991; Huang et al., 1992; Rennie et al., 2012). Other advantages of the antimicrobial gradient strip test are the ease of use in combination with reliability and interpretability, resulting in a simple, near gold standard test. Since previous studies regarding new diagnostic techniques use this method for diagnostic accuracy, this was also performed as reference test in the current study.

Guidance of antimicrobial therapy in food animals is urgently needed, especially given the dense populations and potentially high disease incidences in the current production systems. Latter results in exposure of many animals, their bacteria and environment to antimicrobial selection pressure. To support veterinarians in their decision making process for antimicrobial therapy, not only highly accurate, but especially rapid diagnostic procedures are crucial. This to avoid unnecessary production loss, animal suffering or antimicrobial selection pressure.

The MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA) method, a MALDI-TOF MS-based approach for susceptibility testing, characterized by a semi-quantitative measurement of bacterial proteins, has shown promising results in obtaining rapid antimicrobial susceptibility results for human pathogens (Lange et al., 2014). For some fast growing bacteria involved in sepsis in humans only 1-4 hours are needed for susceptibility testing (Lange et al., 2014; Jung et al., 2016; Sparbier et al., 2016; Maxson et al., 2017). Also for slow growers like mycobacteria this method seems

viable (Ceyssens et al., 2017). Furthermore, this technique shows a high sensitivity and specificity compared to minimum inhibitory concentration (MIC)-gradient strip test (Lange et al., 2014; Jung et al., 2016; Sparbier et al., 2016; Maxson et al., 2017). The potential of MBT-ASTRA for veterinary indications, especially those associated with mass medication, has not been explored. Furthermore applying this method on fastidious growers like *P. multocida* or any other *Pasteurellaceae* has not yet been described. Therefore, the primary objective of the present study was to design and validate an MBT-ASTRA procedure for tetracycline resistance detection in *P. multocida* from bovine origin. The secondary objective was to determine diagnostic accuracy of this MBT-ASTRA procedure and classic disk diffusion compared to MIC-gradient strip testing.

METHODS

1. DETERMINATION OF THE STANDARD CONDITIONS FOR MBT-ASTRA

Selection of the optimal growth medium and starting concentration of P. multocida

BHIB (Difco, BD Diagnostic Systems, Sparks, Md.) and CAMHB (Difco, BD Diagnostic Systems, Sparks, Md.) were used as growth media in this study. One susceptible strain of *P. multocida* (P114, MIC 0.19 µg/mL) was inoculated in 10 mL of BHIB or 10 mL of CAMHB at different starting concentrations. Starting concentrations tested in both media were $1.5x10^6$ CFU/mL, $1.5x10^7$ CFU/mL and $1.5x10^8$ CFU/mL. No antibiotics were added to the medium. All tubes were placed in a shaking incubator for 0, 1, 2, 3, 4 and 6 hours at a temperature of 37°C and an atmosphere enriched with 5% CO_2. After each incubation period, 1 mL samples of each tube were transferred to Eppendorf tubes.

Selection of the optimal tetracycline concentration and incubation time

To determine the optimal concentration of tetracycline and the shortest incubation time necessary to differentiate between susceptible and resistant isolates, three susceptible isolates (P114; MIC 0.19 µg/mL, P103; MIC 0.25 µg/mL, P113; MIC 0.5 µg/mL) and three resistant isolates (P47; MIC 16 µg/mL, P162; MIC 24 µg/mL and P98; MIC 48 µg/mL)

were used. Tetracycline concentrations of 0, 2, 4 and 8 µg/mL were tested (Sigma-Aldrich, Germany) at incubation periods of 0-3-4-5 and 6 hours.

MBT-ASTRA sample preparation

Eppendorf tubes, containing 1 mL samples grown for the indicated incubation time, were centrifuged at 21130 x g for 5 minutes at room temperature. After centrifugation, the supernatant was carefully aspirated and 700 µL of 70% ethanol in high performance liquid chromatography (HPLC) graded water was added to the cell pellet and vortexed. A second centrifugation and aspiration of the supernatant was performed as mentioned above. After air drying for a minimum of 10 minutes, the cell pellets were stored at -20°C for up to a maximum of 3 days. After storing, 20 µL 70% formic acid (in HPLC graded water) was added to the cell pellet and mixed carefully. Samples were incubated for five minutes at room temperature. In a last step, 20 µL of acetonitrile was added and vortexed. Before adding acetonitrile to the cell pellet, an internal standard (Lange et al., 2014, [Bruker Daltonik GmbH, Bremen, Germany, suspended in 25 µL HPLC water]) was added to acetonitrile (ratio of 1/100, using 0.2 µL of internal standard in 20 µL of acetonitrile per sample) for spectra acquisition and facilitating the semi-quantitative analysis of the acquired spectra to quantify the difference in biomass corresponding to the growth between different setups. A third centrifugation step (21130 x g for 2 minutes) was performed to clarify the lysates.

MALDI-TOF MS analysis

One µL of the protein extraction was spotted in triplicate on the target plate (MSP 96 target polished steel BC). After air drying of the spots, 1 µl of matrix (10 mg/mL of α-cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile - 47.5% water - 2.5% trifluoroacetic acid; Bruker Daltonik GmbH, Bremen, Germany) was placed on each spot. External calibration was included in each measurement using a bacterial test standard (BTS, Bruker Daltonik GmbH, Bremen, Germany). Analysis was performed with an Autoflex III smartbeam MALDI-TOF MS instrument (Bruker Daltonik GmbH, Bremen, Germany), recording the mass range between 2.000-20.000 Da using standard settings. Automated data analysis was performed with the MBT-ASTRA software prototype

written in the software package R (Lange et al., 2014). A mass range of 2.500-13.500 was used for analysis. AUC and relative growth rate were calculated automatically for each setup. The duration of MALDI-TOF analysis is around 10-15 minutes for one sample. This duration is not cumulative when analysing multiple samples. The obtained optimal test conditions were subsequently used during assay validation, analysing 100 recent clinical *P. multocida isolates* to determine the cut-off value of the relative growth ratio that allows a differentiation between susceptible and resistant isolates.

2. DETERMINATION OF DIAGNOSTIC ACCURACY OF THE MBT-ASTRA PROCEDURE AND COMPARISON WITH THE DISK DIFFUSION METHOD

Bacterial isolates and cultivation

In this experiment, 100 isolates of *P. multocida* from calves with bronchopneumonia collected in Belgium between 2014 and 2017 were analysed. Sampling of these calves was performed using a deep nasopharyngeal swab or a broncho-alveolar lavage as previously described (Van Driessche et al., 2016). All methods were carried out in accordance with relevant guidelines and regulations. All experimental protocols were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2014/164, EC 2016/20). Identification of *P. multocida* isolates was first achieved by standard biochemical tests (Quinn et al., 1994) and subsequently confirmed with MALDI-TOF MS using the direct transfer protocol as previously described (Kuhnert et al., 2012). Bacteria were cultivated overnight at 37°C and an atmosphere enriched with 5% CO_2 on Columbia agar (Oxoïd, UK) supplemented with 5% sheep blood.

Three susceptibility testing methods were performed on all isolates, namely MIC-gradient strip test, which is comparable to the standard dilution test (Baker et al., 1991; Huang et al., 1992; Rennie et al., 2012), the MBT-ASTRA procedure as described above and disk diffusion as the current standard test in practice.

MIC-gradient strip test

The MIC values of tetracycline were determined using MIC-gradient strips. Briefly, MIC test strips (Liofilchem, Italy) were placed on Mueller Hinton agar plates enriched with 5% defibrinated sheep blood (BD Diagnostics Systems), shortly after the plates had been uniformly inoculated via polyester swabs with 0.5 McFarland suspensions of the pure isolates. Incubation was performed for 18-24 hours under aerobic conditions at 35°C. *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as quality control reference strains. MIC values were determined according to the manufacturer's instructions. Determination of susceptibility was performed according to the current CLSI standards for bovine *P. multocida* (i.e. susceptible ≤ 2 µg/mL, intermediate 4 µg/mL, resistant ≥ 8 µg/mL, [CLSI, 2015]). Used media, incubation conditions, quality control strains and used interpretive criteria were applied according to CLSI standards (CLSI, 2015). Inoculation method was performed according to manufacturer's guidelines.

MBT-ASTRA procedure

All isolates were incubated for 3 hours with and without a tetracycline concentration of 4 µg/mL in CAMHB with a starting concentration of $1.5x10^7$CFU/mL. MBT-ASTRA sample preparation and MALDI-TOF MS analysis were performed as mentioned above.

Disk diffusion test

Susceptibility testing of *P. multocida* and tetracycline was performed in accordance with CLSI standards (CLSI, 2015). Briefly, Mueller Hinton agar (Oxoïd, UK) plates, supplemented with 5% sheep blood, were uniformly inoculated via polyester swabs with 0.5 McFarland suspensions of the pure isolates. Then, tetracycline disks (30 µg/mL, Rosco, Neosensitabs, Taarstrup, Denmark) were placed on Mueller Hinton agar (Oxoïd, UK) supplemented with 5% sheep blood, shortly after the plates had been uniformly inoculated via polyester swabs with 0.5 McFarland suspensions of the pure isolates. Incubation was performed for 18-24 hours at 35°C in ambient air. Analysis of inhibition zones (in mm) was performed and interpretative criteria were according to

manufacturer's guidelines. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as quality control reference strains.

Statistical analysis

Receiver operating characteristics (ROC) curve analysis was used to determine the optimal cut-off of the relative growth ratio to distinguish resistant from susceptible isolates in the MBT-ASTRA procedure (SPSS statistics vs. 24 (IBM, New York, United States)). Performance of the MBT-ASTRA and disk diffusion method compared to the MIC-gradient strip test as the gold standard was determined. Results were presented by distinguishing very major (resistant strain by the MIC-gradient strip test method misinterpreted as susceptible by the disk diffusion/MBT-ASTRA method), major (susceptible strain by the MIC-gradient strip test method misinterpreted as resistant by the disk diffusion/MBT-ASTRA method) and minor errors (intermediate result was obtained by only one method) (Catry et al., 2007; Breteler et al., 2011; Rhodes et al., 2014). Diagnostic accuracy, sensitivity, specificity, RPV and SPV were derived from 2x2 contingency tables using Winepiscope 2.0 (Thrusfield et al., 2001).

RESULTS

1. DETERMINATION OF STANDARD TESTING CONDITIONS FOR MBT-ASTRA

To determine the optimal growth medium and starting concentration of the bacterial suspension, one susceptible isolate (P114: MIC: 0.19 µg/mL) was incubated in two different media using three different starting concentrations for incubation times varying between 0 and 6 hours (Figure 1). This experiment was repeated two times. No difference in growth, expressed by the area under the curve (AUC), was seen between Cation-adjusted Mueller Hinton broth (CAMHB) and Brain Heart Infusion broth (BHIB) (Figure 1). Starting concentration clearly affected growth, reaching sufficient bacterial growth for MALDI-TOF identification after 6, 2-4 and 0 h with $1.5x10^6$ CFU/mL, $1.5x10^7$ CFU/mL and $1.5x10^8$ CFU/mL, respectively (Figure 1).

Second, the antibiotic concentration allowing a clear separation between resistant and susceptible isolates was determined. To obtain this value, three susceptible (P114; MIC 0.19 µg/mL, P103; MIC 0.25 µg/mL and P113; MIC 0.5 µg/mL) and three resistant (P47; MIC 16 µg/mL, P162; MIC 24 µg/mL and P98; MIC 48 µg/mL) isolates of *P. multocida* were tested with tetracycline concentrations of 0, 2, 4 and 8 µg/mL and were incubated for 0, 3, 4, 5 and 6 hours. This experiment was performed 3 times independently, each using 1 resistant and 1 susceptible strain. After incubation, the growth derived from an increased protein signal of these isolates was calculated by measuring the AUC of the obtained spectrum. In the susceptible isolates, the AUC of the sample with antibiotic should be significantly reduced in comparison with the sample without antibiotic. For resistant isolates, no significant difference in AUC should be noticed. The relative growth (RG) ratio is the ratio of the AUC derived from spectra with and without antibiotic and is used to distinguish susceptible from resistant isolates according to an empirical cut-off value. A clear visual difference between susceptible and resistant isolates was achieved at a tetracycline concentration of 4 µg/mL and a RG cut-off value of 0.5 (Figure 2). In this preliminary test, the three resistant isolates showed a RG > 0.5, while the susceptibile isolates showed a RG < 0.5. A correct classification of all strains was already possible after 3 hours of incubation with 4 µg/mL tetracycline (Figure 2).

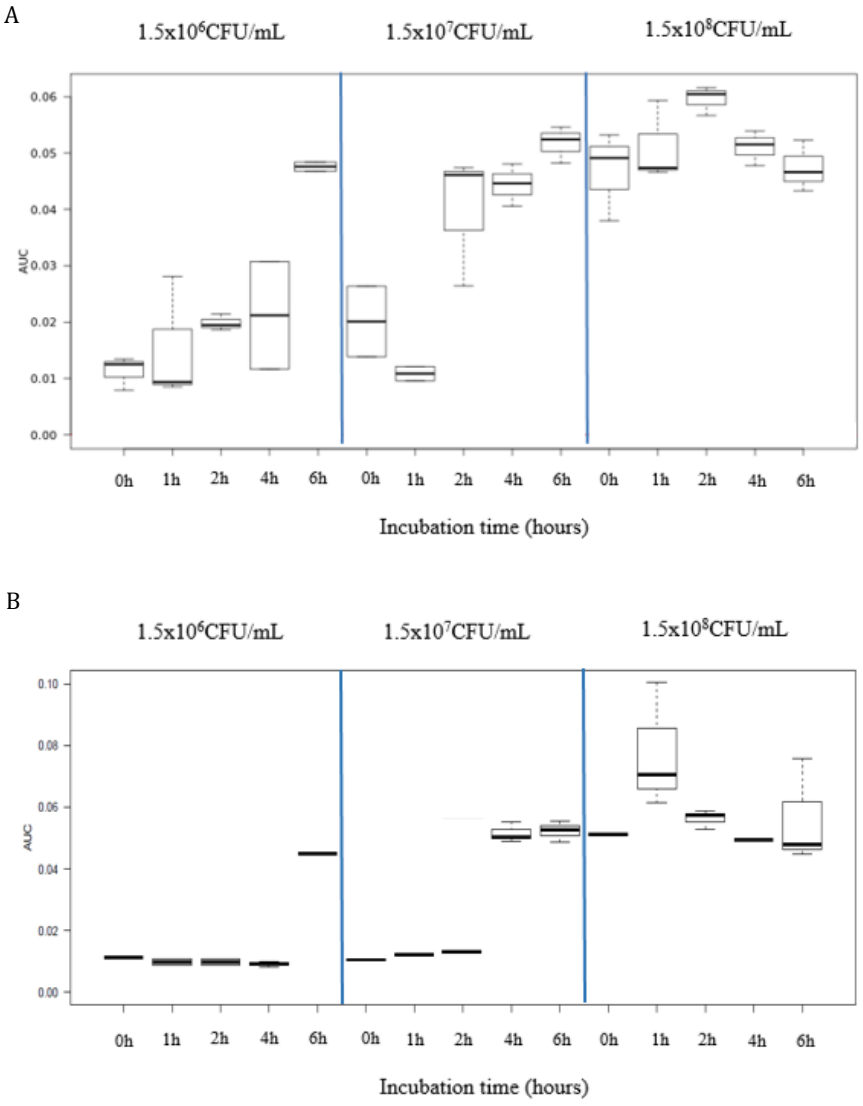

Figure 1: Comparison of CAMHB (a) and BHIB (b) medium and different *P. multocida* starting concentrations to optimize bacterial growth to allow identification in an MBT-ASTRA procedure. Presented spectra are representative for the repetitions made using a single *P. multocida* isolate (P114, MIC: 0.19 µg/mL)

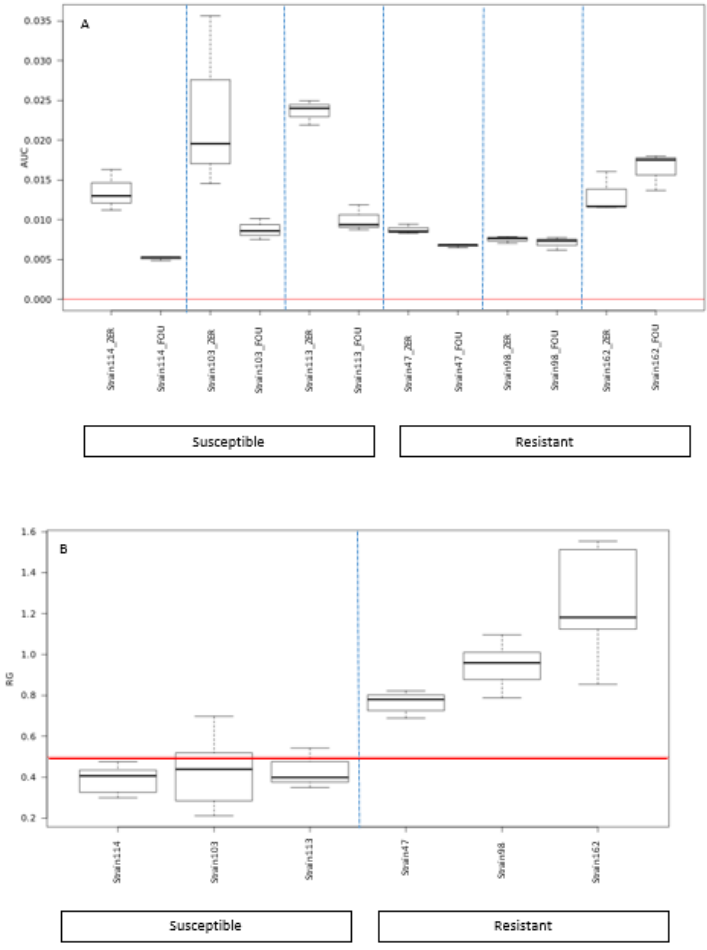

Figure 2: Area under the curve (AUC) (A) and relative growth (RG) (B) box plots of 3 susceptible and 3 resistant *P. multocida* isolates after 3 hours of incubation without antibiotic (=ZER) or with 4 µg/ml of tetracycline (=FOU). For resistant strains, no clear difference between both AUCs is noticed and RGs are high, whereas susceptible strains obtain a lower RG. The horizontal red line represents a RG cut-off value of 0.5

2) DETERMINATION OF THE DIAGNOSTIC ACCURACY OF THE MBT-ASTRA PROCEDURE AND COMPARISON WITH THE DISK DIFFUSION METHOD

MIC distribution of the study population

A total of 100 clinical *P. multocida* isolates were used to validate the MBT-ASTRA with the MIC-gradient strip test as reference test used in the current study. Of the isolates tested, 54 and 46 were classified as susceptible or resistant by MIC-gradient strip test, respectively. This experiment was conducted once. Tetracycline MIC-values for the quality control reference strains were tested twice and were in the acceptable range according to CLSI standards (CLSI, 2015), i.e. *Staphylococcus aureus* ATCC 29213 MIC-value 0.38 µg/mL and 0.5 µg/mL (range 0.12-1 µg/mL), *Escherichia coli* ATCC 25922 MIC-value 0.5 µg/mL and 1.5 µg/mL (range 0.5-2 µg/mL) and *Enterococcus faecalis* ATCC 29212 MIC-value 16 µg/mL (range 8-32 µg/mL). The tested isolates showed a bimodal MIC distribution, ranging between 0.094 and 48 µg/mL (Figure 3). According to veterinary CLSI standards (CLSI, 2015), *P. multocida* isolates are considered responsive to treatment (=susceptible) with tetracycline in cattle when they show an MIC value ≤ 2µg/mL.

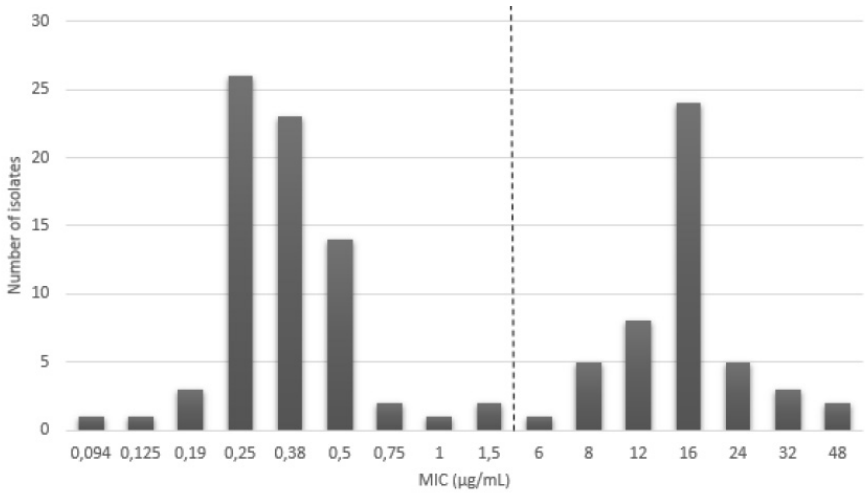

Figure 3: MIC values for tetracycline of 100 recent clinical isolates of *P. multocida* used to determine diagnostic accuracy of an MBT-ASTRA procedure for this antimicrobial-bacterium combination. MIC values were determined by the MIC-gradient strip test. The vertical line represents the CLSI clinical breakpoint for susceptibility (≤ 2 µg/ml, CLSI, 2015)

Diagnostic accuracy of MBT-ASTRA and disk diffusion

The MBT-ASTRA procedure was conducted for 100 clinical isolates with an incubation time of 3 hours and a starting concentration of 1.5 x 10^7 CFU/mL, with and without a tetracycline concentration of 4 µg/mL in CAMHB. This test was performed once. Relative growth values were determined for all tested isolates. Receiver operating characteristics (ROC) curve analysis showed a relative growth value of 0.5 to be the optimal cut-off to differentiate resistant from susceptible isolates for this MBT-ASTRA method (Figure 4). For the disk diffusion tests, quality control reference strains were included and inhibition zones (in mm) were in the acceptable range according to CLSI standards (CLSI, 2015), i.e. *Staphylococcus aureus* ATCC 25923 inhibition zone 24mm (range 24-30mm) and *Escherichia coli* ATCC 25922 inhibition zone 22mm (range 18-25mm). The disk diffusion method with quality control reference strains included was performed once.

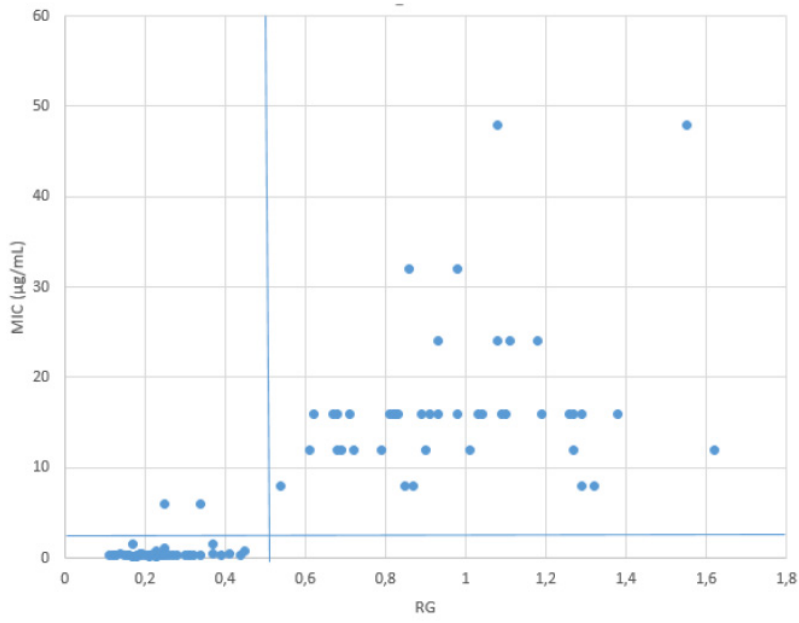

Figure 4: Scatter plot of minimum inhibitory concentrations (MICs) obtained with the MIC-gradient strip test with MBT-ASTRA relative growth (RG) values of 100 recent bovine clinical strains of *P. multocida*. MBT-ASTRA testing conditions were 3 hours of incubation with a concentration of tetracycline of 4 µg/ml. The horizontal and vertical line represent the clinical breakpoint of ≤ 2µg/ml and the RG cut-off value of 0.5, respectively. Two isolates with an MIC value of 6 µg/ml are considered susceptible with MBT-ASTRA, causing 2 false susceptible results

Table 1 shows classification of the test results of both MBT-ASTRA and disk diffusion compared to the MIC-gradient strip test. At a RG cut-off value of 0.5, a correct classification of all 54 susceptible strains and 95.7% (44/46) of resistant strains was achieved. All susceptible isolates showed RG values smaller than 0.5. All resistant isolates had RG values above 0.5, except two isolates (P33, P106) with an MIC value of 6 µg/mL that showed a RG value of 0.34 and 0.25 respectively, causing 2 false susceptible results (Figure 4).

Diagnostic accuracy of the disk diffusion and MBT-ASTRA method was calculated using the MIC-gradient strip test as reference (Table 2). Compared to MIC-gradient strip testing, the MBT-ASTRA method achieved a sensitivity and specificity of 95.7% and 100%, respectively. Sensitivity and specificity of disk diffusion were 93.5% and 96.3%, respectively. The essential agreement of MBT-ASTRA with the MIC-gradient strip test is 98%, due to 2% very major errors (Table 2). In contrast, the disk diffusion method obtained an essential agreement of 95%, including 3% very major errors and 2% major errors (Table 2).

Table 1: 2x2 contingency table showing tetracycline resistance testing results of MBT-ASTRA and disk diffusion compared to MIC-gradient strip test for 100 recent clinical *P. multocida* isolates derived from cattle

Test		Reference test (MIC-gradient strip test)	
		Resistant	Susceptible
MBT-ASTRA	Resistant	95.7% (44/46)	0% (0/54)
	Susceptible	4.3% (2/46)	100% (54/54)
	Total	100% (46/46)	100% (54/54)
Disk diffusion	Resistant	93.5% (43/46)	3.7% (2/54)
	Susceptible	6.5% (3/46)	96.2% (52/54)
	Total	100% (46/46)	100% (54/54)

Table 2: Diagnostic accuracy of disk diffusion and MBT-ASTRA for tetracycline susceptibility testing in 100 clinical *P. multocida* isolates from cattle compared to the MIC-gradient strip test

	Disk diffusion	MBT-ASTRA
Essential agreement	95%	98%
Very major error	3%	2%
Major error	2%	0%
Minor error	0%	0%
Sensitivity	93.5% (86.3%, 100.6%)	95.7% (89.8%, 101.5%)
Specificity	96.3% (91.3%, 101.3%)	100% (100.0%, 100.0%)
RPV	95.6% (89.5%, 101.6%)	100% (100.0%, 100.0%)
SPV	94.5% (88.5%, 100.5%)	96.4% (91.6%, 101.3%)

Values between brackets represent the 95% confidence interval of the estimate. Definitions describing the diagnostic accuracy (Catry et al., 2007; Breteler et al., 2011; Rhodes et al., 2014):
Essential agreement: results of both techniques identical; very major: resistant strain by the MIC-gradient strip test method misinterpreted as susceptible by disk diffusion/MBT-ASTRA; major: susceptible strain by the MIC-gradient strip test method misinterpreted as resistant by disk diffusion/MBT-ASTRA; minor error: intermediate result was obtained by only one method; sensitivity is number of resistant strains by disk diffusion or MBT-ASTRA/number of resistant strains by MIC-gradient strip test; specificity is number of susceptible strains by disk diffusion or MBT-ASTRA/number of susceptible strains by MIC-gradient strip test. RPV, resistant (positive) predictive value; the probability that a strain is truly resistant if the disk diffusion method or MBT-ASTRA categorizes a strain as resistant; SPV, susceptible (negative) predictive value is defined as the probability that a strain is truly susceptible if the disk diffusion method or MBT-ASTRA categorizes a strain as susceptible.

DISCUSSION

This study aimed at developing a MALDI-TOF MS-based approach for rapid tetracycline susceptibility testing in *P. multocida* and to compare this technique and the current standard test in practice (disk diffusion) with the MIC-gradient strip test. The most widely accepted gold standard for susceptibility testing in bacteria is the broth dilution technique (Huber et al., 1998; Jorgensen and Ferraro, 2009). In order to compare the obtained data with previous studies, which commonly used the MIC-gradient strip test, the authors also opted for MIC-gradient strip testing (Lange et al., 2014; Jung et al., 2016; Sparbier et al., 2016; Maxson et al., 2017). MIC-gradient strip test results have a good correlation with MIC values achieved by broth dilution technique, and is often considered as a near gold standard test (Baker et al., 1991; Huang et al., 1992; Rennie et al., 2012). Therefore, the authors believe that current results based on the MIC-gradient strip test will hardly differ from broth dilution results. Further research can include the latter technique as gold standard for determining the accuracy of the MBT-ASTRA method.

Another limitation was that no intermediate results were obtained from neither techniques. In practice, to avoid therapeutic failure, intermediate results are often regarded as resistant, and another antimicrobial is selected. As the MBT-ASTRA method is currently unable to correctly classify intermediate susceptibility isolates for a given antibiotic, including isolates with intermediate results on the reference test would have been an added value. Unfortunately, no isolates classified as intermediate by MIC-gradient strip test were obtained in this study. Also in previous work, this has not been accounted for (Jung et al., 2016; Sparbier et al., 2016). Modifications to this method that would allow classification of intermediate include correlations between the relative growth and MIC value and by using a serial dilution of the given antibiotic to define an MBT-ASTRA minimal breakpoint (Jung et al., 2016). However, classification as intermediate is contra-productive for clinical decision making and may lead to more inappropriate treatments.

The main conclusion of the present study is that the MBT-ASTRA method allows for a tetracycline susceptibility testing result for *P. multocida* in only three hours of incubation with high accuracy. This incubation time is in the same range compared to the MBT-ASTRA method for fast growing bacteria involved in sepsis in humans (Lange

et al., 2014; Jung et al., 2016; Sparbier et al., 2016; Maxson et al., 2017). Although *P. multocida* is considered a fastidiously growing microorganism by CLSI (CLSI, 2015) and tetracycline is considered bacteriostatic, which might cause a longer incubation time to obtain a clear difference in AUC with or without antibiotic. Due to this short incubation period, the MBT-ASTRA method makes it possible to provide the results of the susceptibility test on the same day of identification, avoiding unnecessary production loss or animal suffering. Sensitivity (95.7%) and specificity (100%) of the presented MBT-ASTRA procedure were excessive, in the range of and even higher than compared to previous findings applying MBT-ASTRA for other species under different conditions (Lange et al., 2014; Jung et al., 2016; Sparbier et al., 2016; Maxson et al., 2017). In contrast, the disk diffusion method evaluated in this study had lower sensitivity (93.5%) and specificity (96.3%). A previous study on *P. multocida* isolates showed lower sensitivity (85.7%) and higher specificity (99.1%) of disk diffusion for tetracycline resistance detection (Catry et al., 2007). A possible explanation for this difference could be that at that time disks with higher tetracycline contents (80µg) were used and clinical breakpoints differed with those obtained in the current study. Classification of the 100 isolates with disk diffusion was performed using the interpretative criteria of the manufacturer's guidelines. Currently, no *P. multocida*-specific clinical breakpoints for tetracycline in cattle are available for disk diffusion, while they are described for MIC testing (CLSI, 2015). Zone diameters (mm) were measured and analysed. A clear difference between susceptible (zone diameter ranging from 24-32mm) and resistant (zone diameter ranging from 8-10mm) isolates was noticed. Due to these considerable differences in zone diameter, modifying the clinical breakpoints would have little to no effect on the results.

Compared to the disk diffusion method as used under the current circumstances, MBT-ASTRA shows less discrepancies to the MIC-gradient strip test method. However, in 2% of the isolates tested a very major error was present, potentially resulting in treatment of the animal with tetracycline when the isolate is resistant. Both of these isolates had an MIC value of 6 µg/mL. According to CLSI standards, an MIC value of 4 µg/mL is classified as intermediate and an MIC value ≥ 8 µg/mL is classified as resistant. Considering these clinical breakpoints, both strains with an MIC value of 6 µg/mL can be classified as (borderline) resistant, resulting in 2% very major errors and a sensitivity of 95.7% with

the MBT-ASTRA method. However, the clinical outcome of treatment with tetracycline of *P. multocida* isolates with an MIC value of 6 µg/mL might be difficult to distinctly predict.

Given its rapid results and high diagnostic accuracy, this MBT-ASTRA technique has high potential to be used in the field as a decision aid to direct antimicrobial use in food animals. In order to fulfil this potential, the possibilities of obtaining a full susceptibility test (as many antimicrobials tested at once as in disk diffusion) need to be explored, and the practical issue of availability of a MALDI-TOF MS in the regional laboratories needs to be overcome. However, in recent years the use of MALDI-TOF MS has been advanced to a standard method in clinical microbiology laboratories, due to its rapid and low-cost characteristics (Seng et al., 2009). Considering the simple setup and short incubation time, the MBT-ASTRA method implements an early intervention of adequate antibiotic therapy, resulting in a cost-and –labour efficient tool like the disk diffusion method. Whether the current MBT-ASTRA settings are also applicable to other *Pasteurellaceae* of clinical importance in veterinary medicine, like *Mannheimia haemolytica* or *Histophilus somni*, needs to be determined.

CONCLUSION

The MBT-ASTRA method developed in the present study, has all the potential to fulfil the need for a rapid and highly accurate tetracycline susceptibility testing in *P. multocida*. This is essential to rationalize antimicrobial use in outbreaks of bronchopneumonia in cattle or other clinical presentations across species.

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CHAPTER 8
GENERAL DISCUSSION

Current diagnostic techniques in veterinary medicine fail to provide microbiological results within a limited time frame. Howbeit, this short turnaround time is crucial to convince veterinarians to take samples and achieve a more rational antimicrobial use. This doctoral thesis provided major contributions in reducing this turnaround time for respiratory tract samples from cattle. In this general discussion an overview is given, addressing the different aspects of the complete diagnostic chain for infectious bronchopneumonia in cattle. First, the recommended sampling technique and transport of this sample is discussed. Second, MALDI-TOF MS based techniques for fast identification and susceptibility testing of *Pasteurellaceae* present in respiratory tract samples are explained. Additionally, future prospects are suggested and imperative measurements for routine application in the field and in the laboratory are demonstrated.

NBAL: a practical technique to sample the respiratory tract in cattle

An nBAL has been described as a practical sampling technique for the respiratory tract in cattle (Caldow, 2001). Although this technique is not new, it is not yet commonly used in the field due to a demerit in the amount of scientific work, evidencing the benefits and shortcomings. However, a well-known advantage of a BAL is that it directly samples the lung lobes, making this technique more appropriate for identifying the causative pathogens of infectious bronchopneumonia (Pringle et al., 1988; Thomas et al., 2002). Therefore, this technique has the ability to satisfy current demands for sampling the respiratory tract for identification and susceptibility testing, in order to use adequate antimicrobials. However, there are some drawbacks. Since the catheter is passed through the nose before entering the trachea and lung lobe, criticism arises that these nBAL samples are contaminated by nasopharyngeal flora. This could interfere with microbiological outcomes since on the one hand *Pasteurellaceae* are normal inhabitants of the nasopharyngeal flora and therefore an over-diagnosis of infectious bronchopneumonia can occur. On the other hand, this contamination could also result in polymicrobial culture results, making it challenging to even impossible to identify the causative pathogen of infectious bronchopneumonia. Our work has provided several

insights into this matter (Chapter 3). We showed that nBAL samples are less often polymicrobial, more frequently negative and yield more pure cultures compared to deep nasopharyngeal swab (DNS) samples. The presence of 1 clinically important pathogen (pure culture result) and negative culture results are more easy to interpret in the laboratory. Also, minimizing contamination of a sample was of particular interest in this thesis since these interpretable culture results are more likely to give a correct identification with the rapid detection method by MALDI-TOF MS (Chapter 6). Therefore, disinfection of the nostrils prior to sampling, both for DNS and nBAL, was performed to minimize polymicrobial contamination. Furthermore, several cases in our study demonstrated a polymicrobial culture result in a DNS, while a pure culture result was derived from an nBAL sample within the same animal. When nBAL samples would be systematically contaminated by nasopharyngeal flora, it would be expected that these nBAL samples would also obtain a polymicrobial culture result. Therefore, our results suggest that nBAL samples are less contaminated by nasopharyngeal flora than commonly assumed.

Another point of criticism is animal welfare since, as in this procedure, a lung lobe of an animal is being flushed without sedation. This is in contrast with human medicine, where broncho-alveolar lavage is always performed with sedation (Pugin et al., 1991; Humphreys et al., 1996; Flanagan et al., 2000). Not sedating the animal before performing an nBAL has the advantage of being less time-consuming and consequently less expensive, which makes this technique more applicable in the field considering the current time-economic context. Also, blindly inserting a BAL catheter is very challenging when in particular a young animal (2-3 weeks) is sedated and therefore not able to actively move the larynx. Additionally, our work has shown that, when animals are sedated, the dorsocaudal lung lobes are systematically sampled, whereas without sedation a random lung lobe is being sampled (Chapter 4). The vast majority of bacterial pneumonias in calves is situated in the ventrocranial lung lobes (Allan et al., 1985; Allan et al., 1991; Dagleish et al., 2010). To what extent this difference in intrapulmonary position of the BAL catheter affects the likelihood of isolating the causative bacteria is currently unknown. First steps towards assessing the effects of the sampling method on stress in calves are taken. In a current student thesis, no significant clinical difference between a control group, a group where DNS samples were taken and a group where nBAL samples were taken, was noticed. Only a slight decrease in amount of time that

animals were walking was seen in the two sampled groups compared to the control group (Roelants, 2019).

It is generally accepted that, just like urine (Hilt et al., 2014), also the respiratory tract is not sterile (Timsit et al., 2018). Also in clinically healthy individuals, opportunistic bacteria can be found in lower respiratory tract specimens. This finding was demonstrated in cattle (Allan, 1978; Tanskanen, 1984; Angen et al., 2009; Nicola et al., 2017; Timsit et al., 2018) and horses (Bond et al., 2017). In a human study, only bacteria categorized as normal respiratory flora and not potential pathogenic bacteria were isolated from samples of the lower respiratory tract in healthy individuals (Rasmussen et al., 2001). In the latter study it is suggested that the isolation of normal respiratory flora in the lower respiratory tract is due to contamination of the lower airways by secretions from upper airways transported by the bronchoscope (Rasmussen et al., 2001). While in another study where bronchoscopic BAL samples from clinically normal calves are collected it is suggested that these isolates are microbial inhabitants of the lower respiratory tract (Pringle et al., 1988). Also other studies, where a transtracheal technique was used instead of a bronchoscope and therefore leaving contamination of samples by a bronchoscope or other material out of discussion, demonstrate the presence of opportunistic bacteria in the lower respiratory tract (Angen et al., 2009; Nicola et al., 2017; Timsit et al., 2018; Bond et al., 2017). Also in human medicine, studies are available analyzing the presence of airway microbiota and its homeostatis of diversity or balance, supporting this statement (Dickson et al., 2014; Dickson et al., 2015a; Dickson et al., 2015b). When this balance is disrupted, disease can occur, like for example cystic fibrosis (Lamoureux et al., 2019).

The fact that the respiratory tract is not sterile makes diagnosis of infectious bronchopneumonia more complicated. However, it has been shown that lower isolation rates of clinically important pathogens and different microbial compositions were present in samples of healthy calves compared to animals with clinical signs of pneumonia (Angen et al., 2009; Timsit et al., 2018). Also in this doctoral thesis, isolation rates of clinically important pathogens were lower in healthy animals (Chapter 3). Additionally, isolation of *P. multocida* by PCR or culture was significantly associated with the disease state, whereas for *H. somni* this was only seen for cultivation and not PCR

(Angen et al., 2009). These results stress the importance of only sampling acutely ill animals with confirmation of infectious bronchopneumonia by ultrasound.

It has to be addressed that in this doctoral thesis, when performing an nBAL, a volume of 20-30mL of saline was used in calves in order to obtain a faster and less invasive technique (Chapter 3). In literature an average volume of ±100 mL physiological fluid, ranging from 50 mL to 250 mL is inserted into the lungs (Pringle et al., 1988; Allen et al., 1991; Caldow, 2001; Thomas et al., 2002, Capik et al., 2017; Doyle et al., 2017). The determination of this volume in these studies seemed more empirical rather than evidence-based and was in this doctoral thesis merely decided to limit discomfort to the animals as much as possible. Neither in this doctoral thesis nor in literature a comparison of different volumes on the bacteriological outcome in cattle has been made. Nevertheless, there are several reasons to assume that the impact of reducing the volume on the bacterial outcome can be neglected. One reason is that, in human medicine, performing a BAL with a low volume (20mL) of physiological fluid, also known as the 'minibronchoalveolar lavage', has been described as a safe, less invasive and simpler alternative to a traditional BAL (Tasbakan et al., 2011). One study, comparing the diagnostic value of a BAL and a mini-BAL in the evaluation of pneumonia in immunocompromised patients with respiratory failure, demonstrated a strong correlation between the isolation rates of bacteria and fungi in BAL and mini-BAL samples (Tasbakan et al., 2011). Another study, comparing quantitative cultures between a protected specimen brush and a mini-BAL, showed a good agreement (kappa statistics, 0.63) for quantitative cultures between the brush and mini-BAL (Kollef et al., 1995). A conventional BAL in human medicine comprises of larger volumes (120-150 mL [Tasbakan et al., 2011] or 3 mL/ kg body weight [Grigg et al., 1993]) compared to veterinary medicine. When comparing the volume of the lung of a human and a calf with a body weight of for example 50 kg, a longvolume of 4.5 L and 1.3 L is obtained for humans and cattle, respectively (Reinhold et al., 2015). Nevertheless, a bigger reduction in volume is executed when using a miniBAL, which only makes it plausible that similar results, as described above, could also be obtained in cattle.

Another reason for assuming that lowering the volume of physiological fluid will not negatively affect bacteriological results, is that transtracheal washes in cattle are also performed with a small volume of fluid, namely on average ±40 mL, ranging from 30 mL

to 50 mL (Timsit et al., 2013; Doyle et al., 2017; Nicola et al., 2017). Also transtracheal bronchoalveolar lavage are performed with a small volume of physiological fluid ranging from 10 mL to 40 mL in cattle (Heckert et al., 1997; Angen et al., 2009) and 30 mL in sheep (Sheehan et al., 2005). As these techniques have shown good diagnostic accuracy for identifying the causative pathogen of infectious bronchopneumonia in the lower respiratory tract with low volumes (Heckert et al., 1997; Angen et al., 2009; Timsit et al., 2013; Doyle et al., 2017; Nicola et al., 2017), similar results can be expected for low volumes used in BAL samples.

Overall, a consideration must be made between animal welfare and the economical aspect. In order to be routinely applied in the field, a sampling technique for the respiratory tract of cattle should be cheap, fast, minimally invasive and obtain microbiological results with high diagnostic accuracy. For the laboratory personnel, samples should preferably obtain straightforward clinically interpretable culture results. Also, when MALDI-TOF MS was applied for rapid identification of respiratory pathogens, minimizing contamination of the samples resulted in a higher sensitivity and specificity of correct identification (Chapter 6). Although the nBAL technique currently seems to meet these demands, some points of improvement can be considered. For example the use of an IvetScope (Dairymac Southampton, United Kingdom) to more easily gain access to the larynx. Disadvantages of this technique are that it is more expensive, contamination of the oral flora can more likely occur, and disinfection of the scope between two animals is necessary. However, it can be easily performed in sedated animals and perhaps obtain less contaminated samples since the larynx is visually and therefore more easily reached.

Another suggestion could be the implementation of an endoscope-guided bronchoalveolar lavage, although the latter method can also not exclude contamination (Rasmussen et al., 2001). In human medicine, discarding the first amount of BALf is commonly performed to reduce contamination. Perhaps latter method can be used in veterinary medicine as well. Additionally, the use of a guarded swab or guarded BAL catheter could provide even less polymicrobial results. Unfortunately guarded swabs are more expensive and protected sleeves are not commercially available for BAL catheters. A transtracheal wash (TTW) is shown to provide pure cultures results (Angen et al., 2009). Since this technique perforates the trachea to bypass nasopharyngeal

contamination, it can be considered invasive (Rohn et al., 1998), also questioning animal welfare. Additionally this method is more labor-intensive and more expensive due to the materials used. Generally, it seems that the use of sampling techniques is more cultural based, where a TTW is commonly used in France, Germany and Canada, whereas in the Netherlands and Belgium a BAL or DNS is seemed more appropriate.

Transport conditions

The turnaround time and storage temperatures between sampling and further processing of the sample can be highly variable. In practice, samples are not always kept in the refrigerator and are sometimes stored in a warm car for a long period of time. However, in Belgium the collection of samples is well organized, and therefore the time of sampling can be scheduled. When putting all the effort in taking nBAL samples, why not optimize transport conditions in order to obtain relevant bacterial analysis results and achieve the highest return on investment? It can be expected that different conditions could influence the microbiological outcome of nBAL samples, however this has not yet been investigated. It is also not known which transport conditions are still acceptable for a good microbiological outcome, but also still achievable in practice. Therefore, the effect of these variable transport conditions on the isolation rate of *P. multocida* and *M. haemolytica*, in combination with the evolution of the pathogen and contaminant concentrations were evaluated (Chapter 5).

It is clear that high temperatures (≥36°C) have a detrimental effect on the isolation rate and concentration of clinically relevant pathogens, especially for *M. haemolytica* (Chapter 5). When samples are stored at room temperature (24°C) for 24 hours, no statistically significant difference was seen with lower temperatures concerning the isolation rate and concentration of clinically relevant pathogens or the concentration of contaminants (Chapter 5). These results suggest that storing samples at room temperature for 1 day can still be 'acceptable'. However, during this study only 7/11 samples remained positive for *M. haemolytica* under the latter conditions (Chapter 5), demonstrating that storage at room temperature is not ideal. The concentration of contaminants starts to increase at a temperature of ≥36°C starting from a storage time of 24 hours, and a temperature of 24°C starting from a storage time of 48 hours (Chapter

5). When samples are stored at a temperature of ≤8°C for 24 hours, the isolation rate and concentration of clinically relevant pathogens and the concentration of contaminants is not affected (Chapter 5). Since a minimum concentration of bacteria is necessary for correct identification by MALDI-TOF MS (Chapter 6), and contamination of these samples should be kept to a minimum, these results stress the importance of keeping the storage time within 24 hour and keeping samples cooled (at ≤8°C) during that time (Figure 1). A high initial pathogen concentration and a low contaminant concentration in the sample at arrival in the clinical laboratory could result in a faster identification. Therefore, a possible point of improvement could be to revise transport conditions by directly incubating the nBAL fluid in a custom-made growth medium, thereby using transportation time as incubation time. This might allow to shorten the incubation step at the laboratory considerably, or even allow to directly process the samples for MALDI-TOF MS detection of pathogens at the moment of arrival in the laboratory.

Direct identification of respiratory pathogens by MALDI-TOF MS

During this doctoral thesis, applications for direct detection by MALDI-TOF MS in veterinary medicine have been investigated, for example in feline urine samples (Maeda et al., 2018) and under experimental conditions in milk (Barreiro et al., 2017; Barreiro et al., 2018). Rapid identification of bacterial pathogens in respiratory tract samples by MALDI-TOF MS has not yet been described, both in human as in veterinary medicine. In this doctoral thesis, we successfully managed to develop a rapid identification procedure for pathogenic respiratory bacteria in nBAL samples (Chapter 6). After only 6 hours of incubation in an enrichment medium, a correct identification was obtained in 73% of the clinical samples (Chapter 6). This new technique results in a reduction in time from 24 hours, which is needed with conventional methods, to only 6 hours (Figure 1). This turnaround time might seem longer compared to other studies using positive blood cultures or urine samples for rapid identification by MALDI-TOF MS. However several aspects need to be clarified to put things into perspective.

It has to be addressed that the turnaround time to identification, mentioned in previous studies, starts from a positive blood culture. Blood cultures automatically signal positive in a specific incubator when a detectable concentration of microorganisms is present, based on monitoring the carbon dioxide (CO_2) produced by these growing organisms. This detectable concentration of bacteria is generally at least 10^7 CFU/mL (Croxatto et al., 2012; DeMarco et al., 2013). Therefore, depending on the starting concentration of bacteria and the growth speed of the specific bacterial species, these incubation times can be highly variable (1 hour to 5 days). Since the detectable concentration of a positive blood culture is for most bacteria sufficient for direct identification by MALDI-TOF MS (Croxatto et al., 2012; DeMarco et al., 2013), a TAT for identification of only a couple of minutes to 1 hour starting from these positive blood cultures seems very plausible. Applying such an automated detection system, which indicates when a concentration of bacteria reaches the detection limit of MALDI-TOF MS, yields high efficiency. Since the detection limit of MALDI-TOF MS for *Pasteurellaceae* is 10^7-10^8 CFU/mL (Chapter 6), expectations arises that this automatic system could possibly be used for samples of the respiratory tract. Therefore, we attempted this system for nBAL samples (Bactec Peds Plus pediatric blood culture vial, Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.). However, this was not successful. Experimental studies demonstrated long incubation times and positive signals were obtained at concentrations highly exceeding the detection limit of MALDI-TOF MS.

In previous studies concerning direct detection of pathogens in urine samples by MALDI-TOF MS, high rates of correct identification occurred when flow cytometry was applied (Ferreira et al., 2010; Wang et al., 2013). Urine samples containing pathogens with a concentration >10^5 CFU/mL are automatically detected by the flow cytometer. This concentration is sufficient for detecting pathogens from urine by MALDI-TOF-MS (Ferreira et al., 2010; Wang et al., 2013; DeMarco and Burnham, 2014; Maeda et al., 2018). Similar to positive blood cultures, when urine samples already contain a concentration of pathogens detectible by MALDI-TOF MS, a short turnaround time and high diagnostic accuracy can be expected. Perhaps the use of flow cytometry on nBAL samples could benefit for rapid identification by MALDI-TOF MS. However, the cut-off value of the flow cytometer (10^5 CFU/mL) is below the detection limit of respiratory pathogens by MALDI-TOF MS (10^7-10^8 CFU/mL). Also, the cut-off value of the initial concentration of pathogens in respiratory tract samples used, in order to be considered

clinically relevant, varies between different studies. In human medicine, different cut-off values during quantitative culture studies of BAL samples are practiced: 10^3 CFU/mL (Kollef et al., 1995), 10^4 CFU/mL in general (Flanagan et al., 2000; Rasmussen et al., 2001; Tasbakan et al., 2011) or in monomicrobial samples (Escribano Montaner et al., 2018) and 10^5 CFU/mL when more than one organism is present (Escribano Montaner et al., 2018) or when a miniBAL is used (Tasbakan et al., 2011). In dogs, a concentration of 1.7x 10^3 CFU/mL has been used (Peeters et al., 2000) and for cattle a cut-off value of 10^6 CFU/mL has been described (McGuirk, 2008). In cattle, it has also recently been shown that the initial concentration of opportunistic bacteria in nBAL samples can be highly variable (Chapter 6), and independent of the clinical status of the animal (van Leenen et al., submitted). This is also supported by the fact that a random lung lobe is sampled when performing an nBAL without sedation (Chapter 4). The bacterial pathogen concentration can be lower when sampling the caudodorsal part of the lung compared to the concentration when cranioventral lung lobes are sampled, while the clinical relevance is equal. With our new rapid detection method, 100% of the clinical nBAL samples could be correctly identified by MALDI-TOF MS in pure and dominant cultures when a starting concentration of $\geq 10^5$ CFU/mL was present (Chapter 6). When an initial concentration of 10^4 CFU/mL was present, correct identification only occurred in ±50% of the samples (Chapter 6). Gaining more insight in the association between clinical relevance and the concentration of the respiratory opportunistic pathogen, would help in the interpretation of bacteriological results obtained by the direct detection MALDI-TOF MS procedure as described in this thesis (Chapter 6).

In contrast with blood samples, which are considered sterile, nBAL samples are far more complex. First of all, samples of the lower respiratory tract can be more easily contaminated compared to blood. In chapter 3, 20.8% of nBAL samples obtained a polymicrobial culture result, and 9.7% of nBAL samples obtained a dominant culture. In chapter 6, polymicrobial and dominant culture results were obtained in 18% and 24% of the cases, respectively. Even with addition of the antimicrobial bacitracin, contaminant overgrowth of nBAL samples during incubation could still occur with the direct detection MALDI-TOF MS procedure (Chapter 6). This can explain the higher correct identification rate for pure cultures (71%) compared to dominant cultures (58%) (Chapter 6). Parallel with the current study, research was conducted at our department for rapid identification of *M. bovis* in BALf by MALDI-TOF MS (Bokma et al., 2019), since

M. bovis is also an important pathogen in the BRD-complex. *M. bovis* is intrinsically resistant to different antimicrobial classes, resulting in an easy elimination of possible contaminants and a quite straightforward identification. Results show that correct identification can occur after 2 days of incubation.

Second, in contrast with blood samples, nBAL samples can contain mucus. During microscopic examination of nBAL samples, clumping of bacteria, sometimes embedded in mucus, was noticed. As a result, performing slow and fast centrifugation techniques to separate mucus and cells from bacteria as described in previous studies for urine samples (Iñigo et al., 2016; Zboromyrska et al., 2016; Li et al., 2019), may potentially not be successful in BAL fluid. In order to dissolve this mucus, experimental designs have been tested during this doctoral thesis with acetylcysteine (lysomucil®, Zambon, Brussels, Belgium). However, results of these preliminary experiments showed that a sufficient concentration of acetylcysteine to dissolve mucus would also work bactericidal against *Pasteurellaceae*. Perhaps treatment of infectious bronchopneumonia in cattle by nebulizing acetylcysteine could be an alternative option. Third, samples of the respiratory tract commonly contain mixed cultures (Chapter 3, Chapter 6). With this new described detection method by MALDI-TOF, it was not possible to identify multiple pathogens in a mixed culture (Chapter 6). Nevertheless, still one pathogen could be correctly identified in 57% of the samples. The rate of correct identification was positively associated with the concentration of the pathogen (Chapter 6), again stressing the importance of adequate storage of samples (Chapter 4). This limitation has also occurred in other studies (Ferreira et al., 2010; Moussaoui et al., 2010; Martiny et al., 2012; Buchan et al., 2012). However, new developments show that it is possible to correctly identify up to 6 different pathogens in mixed cultures by MALDI-TOF MS (Yang et al., 2018). Also the creation of a specific database could, besides increasing the rate and speed of pathogen identification (Pinault et al., 2019), aid in the detection of multiple pathogens in mixed samples.

With the rapid MALDI-TOF MS technique, a considerable reduction in TAT is possible. Regarding the epidemiological situation of BRD (the time between the first clinical case and an outbreak), this TAT of 6 hours of incubation brings identification of causal bacteria of lower RTIs into a clinically desirable timeframe. In human medicine, different studies have described the positive impact of rapid identification of pathogens from

blood cultures by MALDI-TOF MS (Clerc et al., 2013; Huang et al., 2013: Osthofff et al., 2017). Also the added value of identification of pathogens from respiratory tract samples, after cultivation, by MALDI-TOF MS have been described, leading to an adjustment of antimicrobial therapy and a shorter intensive care unit (ICU) stay (Mok et al., 2016). Since the TAT is only more reduced with this new technique, it can be assumed that all these benefits would also apply on critically ill animals suffering from infectious bronchopneumonia. However, no research conducting the clinical relevance, and whether this new diagnostic procedure will clearly change or reduce antimicrobial therapy in cattle, is available yet. If no bacterial pathogen can be isolated from BALf by the rapid detection method, the causative pathogen is probably viral and no antimicrobial therapy should be administered. Since 30% of the respiratory samples collected in Belgium obtaines only a viral component, and 100% of BRD cases are treated with antimicrobials (DGZ, Griepbarometer 2019), fast identification of the causative pathogen could reduce antimicrobial use. However, the sensitivity of the rapid MALDI-TOF MS technique is currently 59.1%, which is not yet sufficient enough to replace current classical bacteriological identification. In order to do so, diagnostic accuracy should be improved as discussed in the future prospects. As with most newly developed techniques, there is room for improvement. Nevertheless, this research demonstrates for the first time that it is possible to identify causative bacterial pathogens from nBAL samples by MALDI-TOF MS within ample hours, giving veterinarians the opportunity to await microbiological results before starting an antimicrobial treatment.

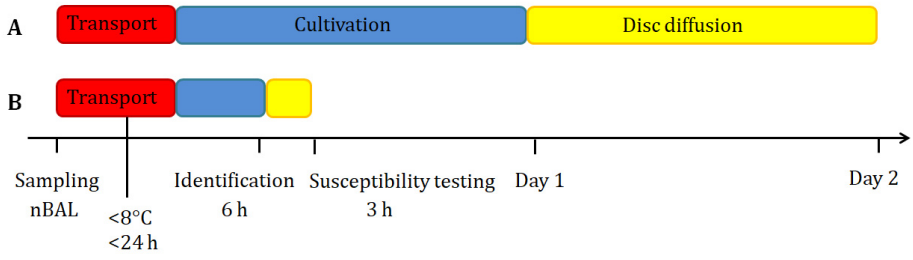

Figure 1: Schematic overview of the conventional method (A) and the achievements of this doctoral thesis (B) concerning identification and susceptibility testing of *Pasteurellaceae* causing infectious bronchopneumonia in cattle

MBT-ASTRA: a new technique for rapid resistance detection

For veterinarians, rapid susceptibility testing results are even more important than rapid identification of the causative pathogen. This is because susceptibility testing results directly lead to therapy success. Also, susceptibility tests are necessary in order to use highly and critically important antimicrobials for human medicine as a second choice or last resort, respectively. In this doctoral thesis it was possible to obtain tetracycline resistance detection in *P. multocida* by MBT-ASTRA after only 3 hours of incubation (Chapter 7). This achieved TAT is comparable with previous studies using MBT-ASTRA for rapid resistance detection (Table 1). This was the first time that the MBT-ASTRA method was applied on an antimicrobial which is commonly used in veterinary medicine. Also, in contrast with the fast-growing microorganisms that were used in previous studies, *P. multocida* is more considered as a fastidious growing microorganism.

Tetracycline was first applied for the MBT-ASTRA method in this PhD thesis due to its economical relevance. As described in the introduction, this antimicrobial is commonly used to treat BRD in calves (Pardon et al., 2012; Lava et al., 2016), especially as a group treatment. As a result, the highest resistant isolation rates of *Pasteurellaceae* derived from different countries are noticed for tetracycline (Kehrenberg et al., 2001; Schwarz and Chaslus-Dancla, 2001; Portis et al., 2012; de Jong et al., 2014; BVL, 2016; DGZ, 2019). To what extent this resistance causes therapy failure for infectious bronchopneumonia in cattle is currently unknown. One dated study demonstrates an increase in mortality of 10% for bovine *Pasteurellaceae* when the causative pathogen is resistant to the administered antimicrobial (Grimshaw et al., 1987). Currently in Belgium, conventional farms commonly use long-acting macrolides for dairy cattle and florfenicol for beef cattle (DGZ, Griepbarometer 2019). Therefore, expanding the MBT-ASTRA method with more antimicrobials would be beneficial. My opinion would be to first expand with long-acting antimicrobials since these are considered highest priority antimicrobials in human medicine according to the World Health Organization (WHO, 2019) and an increasing trend in resistant isolation rates against these 'young' antimicrobials is noticed (Portis et al., 2012; DGZ, 2019). To obtain a full antibiogram

with the MBT-ASTRA method, the concentration and incubation time of each antimicrobial, and the medium and starting concentration of each bacterial species needs to be determined. Also, a sufficient amount of resistant and susceptible isolates needs to be available for validating this new technique. Although it takes some effort to obtain a protocol for a full antibiogram with the MBT-ASTRA method, this can be afterwards automated in the laboratory and can give veterinarians the opportunity to immediately treat with a sufficient antimicrobial. To what extent this new technique would actually change antimicrobial therapy among veterinarians is currently unknown. For this, a cohort study comparing the choice of antimicrobials for the MBT-ASTRA method with the traditional bacteriological susceptibility testing needs to be performed.

One drawback of the MBT-ASTRA method is that no MIC-values can be obtained, giving a qualitative result rather then a quantitative one, wich is comparable with the disk diffusion method. Research, comparing relative growth values with MIC-values obtained by the MIC-gradient strip test, has demonstrated no direct correlation (Sparbier et al., 2016). Also in this doctoral thesis, no direct correlation between both values could be obtained (Chapter 7). This is not surprising, since the relative growth ratio is dependend on the the growth capacity of the individual strain with and without a particular antimicrobial. Nevertheless, an MBT-ASTRA MIC-value can be derived for each individual strain by different titration experiments of the antimicrobial. With latter technique, species –and antimicrobial specific breakpoint concentrations can be obtained, i.e. the minimal antibiotic concentration required for correct classification of the isolates. When comparing these MBT-ASTRA MIC-values with MIC-values obtained by the MIC-gradient strip test, a linear correlation could be noticed between both values (Sparbier et al., 2016). Although it seems devious that experimental breakpoints of MBT-ASTRA are not equal to the current clinical breakpoints, it needs to be addressed that, once each bacterium-antimicrobial combination technique is optimized, this technique has the protential to be fully automated in the laboratory. To validate the MBT-ASTRA method, comparison with other susceptibility testing techniques is necessary like MIC-gradient strip test or broth dilution. For this, clinical breakpoints of the specific pathogen-antimicrobial combination need to be available in order to determine the diagnostic accuracy of the new technique. Unfortunately for *M. bovis*, no clinical breakpoints are available for any antimicrobial. Therefore, this technique cannot be used for this pathogen.

The disk diffusion method is mainly used in clinical veterinary laboratories to guide antimicrobial therapy. However, this technique only shows a sensitivity of 85.7% for the detection of tetracycline resistance in *P. multocida* compared to the agar dilution technique as the gold standard (Catry et al., 2007). In this doctoral thesis, a sensitivity for disk diffusion of 93.5% was obtained (Chapter 7), which could result in therapy failure. Nevertheless this technique is used for its feasibility, although a TAT of 1 day is necessary. With the MBT-ASTRA method, it was not only possible to substantially reduce the TAT from 24 hours to only 3 hours (Figure 1), it was also possible to achieve higher diagnostic accuracy compared to disk diffusion. Since MBT-ASTRA has the potential of being fully automated, this technique meets all requirements to be implemented in clinical veterinary laboratories. Combined with the direct detection method, microbiological results could become available within one working day. This new prospect can motivate veterinarians to take samples, and allows them to await microbiological results before antimicrobial treatment, resulting in a more rational antimicrobial use.

Table 1: Overview of publications using MBT-ASTRA for rapid resistance detection in bacteria

Bacteria	Antimicrobial	TAT	Se	Sp	Number of samples	Reference
Klebsiella pneumoniae	meropenem	1h	93.5%	100%	108	Lange et al., 2014
Enterobacteriaceae	cefotaxime piperacillin-tazobactam gentamicin ciprofloxacin	4h	93.5%	100%	129	Jung et al., 2016
Staphylococcus aureus	ciprofloxacin oxacillin cefepime vancomycin	2h	95%	100%	35	Maxson et al., 2017
Mycobacterium tuberculosis and nontuberculous mycobacteria	rifampin isoniazid linezolid ethambutol clarithromycin rifabutin	6d	99.5%	100%	222	Ceyssens et al., 2017
Klebsiella pneumoniae and Pseudomonas aeruginosa	meropenem	4-5 h	100%	100%	48	Idelevich et al., 2018
Pasteurella multocida	tetracycline	3h	95.7%	100%	100	Van Driessche et al., 2018

Abbreviations: Se, Sensitivity; Sp, Specificity

Future prospects

Despite the fact that this doctoral thesis provides a major contribution in reducing the TAT from sampling to microbiological results for bacterial lower RTIs in cattle, additional research to refine this new diagnostic technique is desirable. In this chain of diagnosing respiratory disease, future research should be conducted in minimizing contamination of samples of the lower respiratory tract even more. Furthermore, possibilities in revising transport conditions and using transport time as incubation time would absolutely further shorten the TAT. For identification purposes, correct identification of mixed bacterial cultures should be investigated, which now seems plausible with current new developments (Yang et al., 2018). The merit of creating a specific database of respiratory pathogens could also be examined (Pinault et al., 2019). Not only bacterial respiratory pathogens, but also respiratory viruses could be implemented with this fast identification. Identification of respiratory viruses in human medicine by MALDI-TOF MS has been described (Calderaro et al., 2016). Providing a full package for fast and reliable identification of respiratory viruses and bacteria in cattle by MALDI-TOF MS could potentially replace PCR, as susceptibility testing afterwards is still possible.

In this doctoral thesis, identification was possible after 6 hours of incubation, and susceptibility testing was possible after 3 hours of incubation. However, performing fast susceptibility testing directly after direct identification of one sample still needs to be developed. Since identification of bacteria is achievable at a concentration of 10^7-10^8 CFU/mL for nBAL samples, and the MBT-ASTRA method requires a starting concentration of *P. multocida* of 10^7 CFU/mL, it seems plausible that identification and susceptibility testing results could be available after ≤ 9 hours of incubation. Additionally, only resistance detection of tetracycline by MBT-ASTRA was demonstrated in this doctoral thesis. Expanding this method to a full antibiogram (containing 12 different antimicrobials) would be necessary in order to be applied in the field.

Translation to the field

Science is one thing, translating new knowledge to the field another. Since it was clear during this doctoral thesis that nBAL samples provide far more clinically interpretable culture results compared to DNS, the use of the nBAL technique was encouraged among veterinarians. However, it was also clear that training and experience was necessary in order to quickly and correctly perform this technique, which would result in a minimum of contamination of the sample and minimized stress or pain to the animal. Therefore, during the course of this Ph.D thesis multiple training sessions in Belgium and neighbouring countries were executed by the department of Large Animal Internal Medicine in collaboration with Animal Health Care-Flanders (DGZ Vlaanderen) and different pharmaceutical companies. The training sessions were preceded by lectures demonstrating veterinarians the possibilities of thoracic ultrasound to improve the diagnosis of animals with pneumonia. Sampling pneumonic animals would likely result in the highest return on investment on microbiological culture results.

Also, alongside this Ph.D project, the department, in cooperation with DGZ, initiated a monitoring initiative for respiratory tract infections in epidemic outbreaks of respiratory disease: 'Griepbarometer'. This project aimed at situating respiratory outbreaks of cattle in Belgium, and monitors the causative pathogens in combination with susceptibility profiles. In the diagnostic laboratory of DGZ, a PCR for 4 bacteria (*P. multocida*, *M. haemolytica*, *H. somni* and *M. bovis*) and 3 viruses (PI3, BRSV and BCV) is offered. By doing so, Griepbarometer convinces veterinarians to collect samples of the respiratory tract and send it to their laboratory. Since viruses are more easily obtained from BAL samples than DNS samples (Kimman et al., 1986; Heckert, et al., 1997; Caldow, 2001; Doyle et al., 2017), training sessions focusing on the nBAL technique were given. As a result, currently 75% of the samples of the respiratory tract send to the lab (DGZ, Griepbarometer 2019) for identification are nBAL samples, which can only be further encouraged. Additionally, the amount of respiratory tract samples of cattle send to the laboratory for PCR sevenfolded the last years, going from approximately 70 samples in 2015 to approximately 520 samples in 2018 (DGZ, Griepbarometer 2019). Despite this immense increase of samples being taken by the veterinarian, no increase in antimicrobial use for respiratory disease in cattle is demonstrated. On the other hand, it should be addressed that more accurate and rapid diagnostic techniques do not

automatically result in a decrease of antimicrobial use. Caution should be taken not to over-diagnose. Therefore, sampling should always be preceded by a proper clinical investigation and thoracic ultrasonography. Also, laboratory results should be interpreted with caution. Where polymerase chain reaction (PCR) is usefull for the diagnosis of viruses and *M. bovis*, *Pasteurellaceae* should be interpreted on culture (both agar or broth), showing clinical relevance when a large amount is present in pure, dominant or mixed culture.

To what extent an increased sampling and detection of respiratory tract infections would result in over-treatment of infections which would otherwise perhaps could heal naturally, is currently unknown. Subclinical infections of BRD are quite common (Thompson et al., 2006; van Leenen et al., 2019), leading to a reduced average daily gain (ADG) of 91 g when not treated (Thompson et al., 2006). When animals with a clinical infection of the lower respiratory tract were not treated, an increased risk of dying within 15 days after diagnosis occurred. This risk was associated with the depth and area of lung consolidation measured with thoracic ultrasonography (Rademacher et al., 2014). In another study, thoracic ultrasonography was used to quantify the effects on relapse rate and ADG of lung lesions in calves first diagnosed with bronchopneumonia with treatment of florfenicol (Timsit et al., 2019). This study showed a positive association between the maximal depth of lung consolidation and a higher risk of relapse and lower ADG. With this information, a more scientific-based decision can be made if treatment of an animal would be necessary and beneficial.

Thus, what are the expectations to truly apply the obtained knowledge and technical advances from this thesis in the field? Besides the aspects described in future prospects, one major contribution to rapid implementation of this technique in clinical veterinary laboratories would be the implementation of automation. In human medicine, clinical laboratories are working 24/7. And this system is the only solution for reducing the TAT as much as possible. Regarding the 'One Health' policy, it seems rather peculiar that this system is not yet operating in veterinary medicine. Since this new MALDI-TOF MS approach in veterinary medicine has the possibility to be fully automatized, this would lead to faster results and higher quality standards whilst also being more economical (Buchan and Ledeboer, 2014; Dauwalder et al., 2016).

General conclusion

This thesis provides major contributions in substantially reducing the TAT, from sampling to susceptibility testing results, of *Pasteurellaceae* causing infectious bronchopneumonia in cattle by MALDI-TOF MS. This new technique will finally make it possible for veterinarians to await microbiological results before initiating antimicrobial treatment. Consequently leading to a more rational use of antimicrobials in the veterinary profession and therefore a reduction in antimicrobial resistance. It's about time.

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SUMMARY

Bovine respiratory disease has a major economic impact on all cattle production systems worldwide. Antimicrobial group treatment is still commonly used to tackle this disease, although this is considered unacceptable in modern times facing antimicrobial resistance. Nowadays, sampling of the respiratory tract for identification of the causative pathogen in combination with susceptibility testing is demanded for a rational and individual treatment. Current diagnostic techniques in veterinary medicine use cultivation for identification and disk diffusion for suscueptibility testing. However, these techniques acquire a turnaround time (TAT), meaning the time from sampling to susuceptibility testing results, of minimum 2 days, and mostly longer. While awaiting these microbiological results, an empiric antimicrobial treatment (i.e., therapy based on collective experience and scientific evidence), for economic reasons and to safeguard animal welfare. Decreasing this TAT would give veterinarians the opportunity to immediately provide an adequate rational antimicrobial treatment. In human medicine, MALDI-TOF MS has been suggested as a reliable diagnostic tool for rapid identification and susceptibility testing. Therefore, in the present doctoral thesis new applications of MALDI-TOF MS in veterinary medicine were explored in order to substantially reduce the TAT into a clinically desirable time frame, of 1 day or less.

As a general introduction (Chapter 1), an overview of bovine respiratory disease was provided, with emphasis on *Pasteurellaceae* since bacterial pathogens are mostly the cause of lung lesions. It is clarified why this disease is 'a never ending story'. Although extensively studied, infectious bronchopneumonia still causes enormous economic damage. Preventive measurements do not seem to be effective enough, and antimicrobial treatment appears necessary in a substantial number of cases in all production systems worldwide. However, this in itself can create a new problem, namely the global threat of antimicrobial resistance. A review of the current diagnostic techniques, both in the field and in the laboratory, is giving focus on their strengths and limitations. From this literature review it became clear that there is no consensus on which sampling technique should be used for the respiratory tract in cattle. Also, current diagnostic techniques conventionally used in clinical veterinary laboratories are time-consuming. Therefore, new developments of MALDI-TOF MS as a rapid practical diagnostic technique in human medicine are briefly discussed.

The overall objective of this doctoral thesis was to obtain rapid identification and susceptibility testing of *Pasteurellaceae* causing infectious bronchopneumonia in cattle by MALDI-TOF MS. Since the sampling method and transport conditions can affect the microbiological outcome of this new technique, these subjects were also incorporated in this doctoral thesis (Chapter 2). Therefore, this doctoral thesis provides answers to the complete diagnostic chain of infectious bronchopneumonia in cattle, from taking samples of the respiratory tract in the field to susceptibility testing in the laboratory.

In chapter 3, bacterial culture results and contaminant overgrowth were compared between DNS and nBAL samples from calves. A cross-sectional study was performed sampling 183 preweaned calves, of which 144 were suffering from respiratory disease and 39 calves who were clinically healthy. nBAL samples were less often polymicrobial, more frequently negative and yielded more pure cultures compared to DNS. Clinically interpretable culture results were obtained in 79.2% of the cases for nBAL samples compared to only 31.2% for DNS samples. Isolation rates were lower in healthy animals, but not different between DNS and nBAL samples, except for *H. somni*, which was more likely to be isolated from nBAL samples. In clinical cases, a polymicrobial DNS culture result did not increase the probability of a polymicrobial nBAL result by ≥30%, nor did it influence the probability of a negative culture. A significant herd effect was noted for all observed relationships. The main conclusion was that nBAL samples are far less overgrown by contaminants compared to DNS samples, facilitating clinical interpretation and resulting in a higher return on investment in bacteriologic culturing.

The effect of sedation on the intrapulmonary position of an nBAL catheter in calves was described in chapter 4. BAL sampling was performed during a randomised clinical trial using 18 healthy Holstein-Friesian calves. Prior to sampling, sedation with xylazine was executed on 11 animals and 7 animals served as control. The intrapulmonary position of the BAL catheter was determined in the sedated group by endoscopy after sampling and contrast-radiography. In the control group the position was validated by post-mortem examination with the catheter in place. In 72.2% of the calves, the catheter was positioned in the dorsocaudal part of the lungs. In the control animals, the probability of sampling the different lung parts was equally distributed, while in the sedated animals the dorsocaudal lung lobes were systematically sampled. However, to what extent this

difference affects the likelihood to isolate the causative bacteria, which mostly affect the cranial lung lobs, remains to be determined.

Chapter 5 describes the effect of storage time and temperature on the isolation rate and concentration of *M. haemolytica* and *P. multocida* in nBAL samples. Thirteen nBAL samples from clinically affected animals were subjected to 4 different temperatures (0°C, 8°C, 23°C and 36°C) for 0, 2, 4,6, 8, 24 and 48 hours. Storage at 36°C resulted in a reduced isolation rate and concentration already 2 hours after sampling for *M. haemolytica* and 8 hours after sampling for *P. multocida*. When samples were stored at 23°C for 24 hours, a decrease in *M. haemolytica* isolation rate and concentration was noticed. When samples were stored at a temperature of 0°C or 8°C for 24 hours, the isolation rate was not affected although the concentration of *M. haemolytica* slightly decreased. The presence of contaminants reduced the odds of isolating *P. multocida* and *M. haemolytica*. An increase in concentration of contamination was noticed after 24 hours of sampling at a temperature of 36°C, and after 48 hours of sampling at a temperature of 23°C. In conclusion, optimal *M. haemolytica* and *P. multocida* isolation rates from clinical nBAL samples are obtained when samples are stored at a temperature of ≤8°C and further processed within 24 hours after sampling.

The next goal was to develop and validate a rapid identification procedure for pathogenic respiratory bacteria in nBAL samples from cattle by MALDI-TOF MS (Chapter 6). For the development phase, results showed that brain heart infusion broth supplemented with fetal bovine serum and yeast extract and 32µg/mL bacitracin resulted in optimal *Pasteurellaceae* growth with the highest expected effect on bacterial contamination. This procedure was validated using 100 clinical nBAL samples from cattle with conventional bacterial culture as reference test. For pure and dominant culture samples, this new procedure was able to correctly identify 79.1% of the pathogens, with a sensitivity and specificity of 60.5% and 100%, respectively. In mixed culture samples, one pathogen could be correctly identified in 57% of the samples with a sensitivity of 57.1% and a specificity of 100%. In these samples, the initial concentration of the pathogen was positively associated with the rate of correct identification. *Moraxella ovis*, *H. somni* and *M. varigena* could rarely to not be identified with this rapid MALDI-TOF MS method. Overall, correct identification was obtained in 73% of the samples, with 59.1% sensitivity and 100% specificity after only 6 hours of incubation.

These results are promising and show a new application of MALDI-TOF MS for direct pathogen identification in culture-enriched BAL fluid. This new technique drastically reduces TAT and can therefore be a valuable decision support tool to rationalize antimicrobial use, both in veterinary and human medicine.

In chapter 7, rapid susceptibility testing was achieved by designing an MBT-ASTRA procedure for tetracycline resistance detection in *P. multocida*. Standard testing conditions were determined and were set on a starting concentration of $1.5x10^7$ CFU/mL for *P. multocida* in Cation-adjusted Mueller Hinton broth (CAMHB) for an incubation period of 3 hours and a concentration of 4µg/mL for tetracycline. This procedure was validated on 100 clinical isolates with MIC-gradient strip test as gold standard, and a comparison with disk diffusion as conventional method was made. The MBT-ASTRA procedure was able to correctly classify 98% of the isolates after only 3 hours of incubation, with a sensitivity of 95.7% and a specificity of 100%. For the disk diffusion method, 95% of the isolates were correctly classified with a sensitivity of 93.5% and specificity of 96.3%. This study showed that this new MBT-ASTRA procedure has all the potential to fulfill the need for a rapid and highly accurate tetracycline susceptibility test in *P. multocida*. A reduction in TAT to only 3 hours makes it possible to rationalize antimicrobial use in outbreaks of bronchopneumonia in cattle or other clinical presentations across species.

In the general discussion, first the practical and microbiological value of the nBAL technique and suggested transport conditions are described. Second, the TAT and diagnostic accuracy of the newly developed techniques by MALDI-TOF MS are compared with techniques described in human medicine, pointing out their strengths and limitations and demonstrating their novelty and associated difficulties. Last, future prospects are suggested and a translation to the field, emphasizing automation, is made.

The developments of these new techniques by MALDI-TOF MS, preceded by the suggested sampling technique and transport conditions, make it very realistic to obtain reliable microbiological results within one working day in the very near future. This gives veterinarians an essential tool for a more rational antimicrobial use in cattle suffering from infectious bronchopneumonia.

SAMENVATTING

Bovine respiratory disease (BRD of ademhalingsstoornissen) hebben wereldwijd een enorme economische impact op alle rundvee productiesystemen. Antimicrobiële groepsbehandeling wordt nog altijd vaak gebruikt om deze ziekte aan te pakken, alhoewel dit tegenwoordig niet meer aanvaard wordt door de opkomende problematiek van antibiotica resistentie. Tegenwoordig wordt staalname van het ademhalingsstelsel, voor identificatie van het oorzakelijk pathogeen in combinatie met gevoeligheidsbepaling, gevraagd voor een rationele en individuele behandeling. De huidige diagnostische technieken in de diergeneeskunde gebruiken cultivering voor identificatie en disk diffusie voor gevoeligheidsbepaling. Echter, deze technieken vereisen een minimum doorlooptijd, dit is de tijd van staalname tot beschikbaarheid van de resultaten van de gevoeligheidsbepaling, van 2 dagen, en meestal langer. Wachtend op deze microbiologische resultaten wordt ondertussen een empirische behandeling ingesteld, gebaseerd op de collectieve ervaring en wetenschappelijk bewijs, omwille van economische redenenen en het vrijwaren van dierenwelzijn. Het verlagen van deze doorlooptijd zou dierenartsen de mogelijkheid geven om onmiddellijk een geschikte en rationele antimicrobiële behandeling in te zetten. In de humane geneeskunde wordt MALDI-TOF MS voorgesteld als een betrouwbaar diagnostisch middel voor snelle identificatie en gevoeligheidsbepaling. Daarom wordt in het huidige onderzoek gezocht naar nieuwe toepassingen van MALDI-TOF MS in de diergeneeskunde, om zo de doorlooptijd te verminderen tot een klinisch gewenste tijdspanne, namelijk 1 dag of minder.

Als een algemene inleiding (Hoofdstuk 1) wordt een overzicht gegeven van BRD, met nadruk op de *Pasteurellaceae* aangezien bacteriën meestal de oorzaak zijn van longletsels. Er wordt duidelijk gemaakt waarom deze ziekte kan gezien worden als 'een verhaal dat nooit eindigt'. Alhoewel deze ziekte al uitgebreid bestudeerd is, blijft infectieuze bronchopneumonie enorme economische schade toebrengen Preventieve maatregelen lijken niet te werken, en een antimicrobiële behandeling lijkt noodzakelijk bij een opmerkelijk aantal gevallen in alle productiesystemen wereldwijd. Maar dit op zich brengt weer een nieuw probleem met zich mee, namelijk de globale bedreiging van antibiotica resistentie. Een overzicht wordt voorzien van de huidige diagnostische technieken, zowel in het veld als in het laboratorium, waarbij hun sterktes en zwaktes worden aangehaald. Uit dit literatuuroverzicht wordt duidelijk dat er geen overeenstemming is omtrent welke staalname techniek moet gebruikt worden om het

ademhalingsstelsel van rundvee te bemonsteren. Ook wordt het duidelijk dat de huidige diagnostische methoden die conventioneel gebruikt worden in de klinische diergeneeskunde laboratoria heel veel tijd vragen. Daarom worden ook de nieuwe ontwikkelingen van MALDI-TOF MS als een snelle en praktische diagnostische methode in de humane geneeskunde kort aangehaald.

De algemene doelstelling van dit doctoraat was om snelle identificatie en gevoeligheidsbepaling van *Pasteurellaceae,* die infectieuze bronchopneumonie bij runderen veroorzaken, te bekomen door middel van MALDI-TOF MS. Aangezien de staalname techniek en transportcondities invloed kunnen hebben op de microbiologische uitkomst van deze nieuwe techniek, werden deze ook geïncorporeerd in the doctoraat (Hoofdstuk 2). Hierdoor biedt dit proefschrift antwoorden over de volledige diagnostische keten van infectieuze bronchopneumonie bij rundvee, van het nemen van stalen van het ademhalingsstelsel in het veld tot gevoeligheidsbepaling in het laboratorium.

In hoofdstuk 3 worden bacteriële cultuur resultaten en overgroei van contaminanten vergeleken tussen een diepe nasopharyngeale swab (DNS) en een niet-endoscopische bronchoalveolaire lavage (nBAL) bij kalveren. Een cross-sectionele studie werd uitgevoerd waarbij 183 nog niet gespeende kalveren bemonsterd werden. Van deze 183 kalveren vertoonden er 144 kalveren ademhalingsstoornissen en 39 kalveren waren klinisch gezond. De isolatiegraad van kiemen was lager bij klinisch gezonde dieren, maar niet verschillend tussen DNS en nBAL stalen, behalve voor *H. somni* die meer kans had om geïsoleerd te worden uit nBAL stalen. Bij klinische gevallen verhoogde een polymicrobiële DNS cultuur noch de waarschijnlijkheid op een polymicrobieel nBAL resultaat met ≥30%, noch de waarschijnlijkheid op een negatieve cultuur. Een significant kudde effect was aanwezig voor alle geobserveerde vergelijkingen. De hoofdconclusie was dat nBAL stalen veel minder gecontamineerd zijn dan DNS stalen, wat de klinische interpretatie eenvoudig maakt en zo een hoger rendement bekomen wordt voor de bacteriologische cultivering.

De invloed van sedatie op de intrapulmonale positie van een nBAL katheter bij kalveren werd beschreven in hoofdstuk 4. Tijdens een gerandomiseerde klinische studie werden nBAL stalen genomen bij 18 gezonde Holstein-Friesian kalveren. Voor staalname werden 11 dieren gesedeerd met xylazine en 7 dieren dienden als controle. De

intrapulmonale positie van de BAL katheter werd in de gesedeerde groep bepaald door endoscopie na staalname en contrast-radiographie. Bij de controlegroep werd de positie vastgesteld door post-mortem onderzoek, waarbij de katheter ter plaatse bleef. Bij 72.2% van de kalveren was de katheter in de dorsocaudale longkwab geplaatst. Bij de controlegroep was de waarschijnlijkheid om de verschillende longkwabben te bemonsteren evenredig verdeeld, terwijl bij de gesedeerde dieren systematisch de dorsocaudale longkwab bemonsterd werd. In welke mate dit verschil in staalnameplaats de waarschijnlijkheid op isolatie van de oorzakelijke kiemen, die meestal de craniale longkwabben infecteren, beïnvloedt is echter nog niet duidelijk.

Hoofdstuk 5 beschrijft de invloed van bewaartijd en temperatuur op de isolatiegraad en concentratie van *M. haemolytica* en *P. multocida* in nBAL stalen. Dertien nBAL stalen van klinisch aangetaste dieren werden blootgesteld aan 4 verschillende temperaturen (0°C, 8°C, 23°C en 36°C) gedurende 0, 2, 4, 6, 8, 24 en 48 uur. Het bewaren van de stalen aan een temperatuur van 36°C resulteerde in een verminderde isolatiegraad en concentratie na amper 2 uur na staalname voor *M. haemolytica* en 8 uur na staalname voor *P. multocida*. Wanneer stalen bewaard werden aan een temperatuur van 23°C gedurende 24 uur, werd een afname van de isolatiegraad en concentratie van *M. haemolytica* waargenomen. De isolatiegraad verandert niet wanneer stalen bewaard worden aan een temperatuur van 0°C of 8°C gedurende 24 uur, alhoewel de concentratie van *M. haemolytica* wel in een kleine mate afneemt. De aanwezigheid van contaminanten verminderde de kans om *P. multocida* en *M. haemolytica* te isoleren. Een vermeerdering van de concentratie van contaminanten werd waargenomen na 24 uur aan een temperatuur van 36°C, en na 48 uur aan een temperatuur van 23°C. Samenvattend, de optimale isolatiegraad van *M. haemolytica* en *P. multocida* uit klinische nBAL stalen wordt bekomen wanneer stalen bewaard worden aan een temperatuur van ≤8°C en verder verwerkt worden binnen 24 uur na staalname.

De volgende doelstelling was om een snelle identificatie procedure te ontwikkelen en valideren voor pathogene ademhalingskiemen in nBAL stalen van rundvee door middel van MALDI-TOF MS (Hoofdstuk 6). In de ontwikkelingsfase vertoonde het gebruik van brain heart infusion broth (BHIB) gesupplementeerd met foetaal kalverserum en gistextraxt en bacitracine aan een concentratie van 32µg/mL een optimale groei van *Pasteurellaceae* met verwachtingen op de hoogste reductie van contaminatie. Deze

procedure werd gevalideerd op 100 klinische nBAL stalen van rundvee met de conventionele bacteriologische cultuur als referentie test. Voor reinculturen en dominante culturen was deze nieuwe techniek in staat om 79.1% van de pathogenen correct te identificeren, met een sensitiviteit en specificiteit van 60.5% en 100%, respectievelijk. Voor mengculturen kon maar 1 pathogeen correct geïdentificeerd worden in 57% van de stalen met een sensitiviteit van 57.1% en specificiteit van 100%. Bij deze stalen was de oorspronkelijke concentratie van het pathogeen positief geassocieerd met de graad van correcte identificatie. *Moraxella ovis*, *H. somni* en *M. varigena* werden nauwelijks tot niet geïdentificeerd met de snelle MALDI-TOF MS methode. Algemeen werd een correcte identificatie bekomen in 73% van de stalen, met een sensitiviteit van 59.1% en specificiteit van 100% na enkel 6 uur incuberen. Deze resultaten zijn beloftevol en tonen een nieuwe toepassing van MALDI-TOF MS, namelijk snelle identificatie van pathogenen in cultuur verrijkende BAL vloeistof. Deze nieuwe techniek vermindert drastisch de doorlooptijd en kan daardoor een waardevolle beslissingstool zijn om antibioticumgebruik te rationaliseren, zowel in de diergeneeskunde als in de humane geneeskunde.

In hoofdstuk 7 werd een snelle gevoelsbepaling bereikt door een MBT-ASTRA procedure te ontwikkelen voor de detectie van tetracycline resistentie bij *P. multocida*. Standaard condities werden bepaald waarbij een startconcentratie van *P. multocida* van $1.5x10^7$ CFU/mL in Cation-adjusted Mueller Hinton broth (CAMHB) gedurende 3 uur incubatie en een tetracycline concentratie van 4µg/mL geschikt leken. Deze procedure werd gevalideerd op 100 klinische isolaten met de MIC-gradiënt strip test als gouden standaard, en een vergelijking met disk diffusie als conventionele methode werd gemaakt. De MBT-ASTRA methode was in staat om 98% van de isolaten correct te classificeren als gevoelig of resistent na enkel 3 uur incubatie, met een sensitiviteit van 95.7% en specificiteit van 100%. Bij de disk diffusie methode werd 95% van de stalen correct geïdentificeerd met een sensitiviteit van 93.5% en specificiteit van 96.3%. Deze studie toont aan dat de nieuwe MBT-ASTRA methode alle potentieel heeft om de nood van een snelle een zeer accurate tetracycline gevoeligheidsbepaling bij *P. multocida* te vervullen. Een reductie in doorlooptijd tot maar 3 uur maakt het mogelijk om antibioticumgebruik te rationaliseren bij uitbraken van bronchopneumonie bij rundvee of andere klinische presentatie bij andere soorten.

Als een algemene discussie werd eerst de praktische en microbiologische waarde van de nBAL staalname techniek en de voorgestelde transport condities besproken. Hierna werd de doorlooptijd en diagnostische accuraatheid van de nieuwe ontwikkelde technieken door middel van MALDI-TOF MS vergeleken met technieken uit de humane geneeskunde, waarbij de sterktes en zwaktes werden benadrukt en de innovatie maar ook moeilijkheden van deze nieuwe technieken werden besproken. Als laatste werden mogelijke toekomstperspectieven voorgesteld en een vertaling naar het veld gemaakt, waarbij een nadruk op automatisatie werd gelegd.

De ontwikkelingen van deze nieuwe technieken door MALDI-TOF MS in de diergeneeskunde, voorafgegaan door de voorgestelde staalname techniek en transport condities, maakt het zeer realistisch om in de nabij toekomst microbiologische resultaten te bekomen binnen 1 werkdag. Dit geeft dierenartsen de essentiele tool voor een meer rationeel antibioticumgebruik bij rundvee die lijden aan infectieuze bronchopneumonie.

CURRICULUM VITAE

Laura Van Driessche werd geboren op 21 januari 1990 te Kortrijk. Na het beëindigen van het secundair onderwijs aan het Sint-Bernardus College in Oudenaarde, richting Wetenschappen-Wiskunde, startte ze in 2008 met de studies Diergeneeskunde aan de Universiteit Gent. Hierbij was ze van 2012-2014 ook actief als jobstudent op de vakgroep Virology, Parasitology en Immunology. In 2014 behaalde ze het diploma van dierenarts (optie Herkauwers) met onderscheiding.

Onmiddellijk na afstuderen trad ze in dienst bij de vakgroep Inwendige Ziekten van de Grote Huisdieren op de Faculteit Diergeneeskunde in Merelbeke als doctoraatstudent, onder begeleiding van Prof. Dr. P. Deprez en Dr. B. Pardon en in samenwerking met de vakgroep Pathologie, Bacteriologie en Pluimveeziekten onder begeleiding van Dr. F. Boyen. In januari 2016 kreeg Laura Van Driessche een beurs van het Fonds voor Wetenschappelijk Onderzoek (FWO, 1S52616N). Zij legde zich toe op de inwendige ziekten en gezondheidszorg van voornamelijk herkauwers en stond, naast dienstverlening voor de kliniek, mee in voor het klinische onderwijs aan en begeleiden van schrijfopdrachten van master studenten. Zij nam eveneens deel aan de nacht- en weekenddiensten van de vakgroep. Daarnaast was zij betrokken bij diverse projecten inzake de diagnostiek van BRD in België en buurlanden.

Laura Van Driessche is auteur en medeauteur van meerdere publicaties in nationale en internationale wetenschappelijke tijdschriften. Verder was ze ook reviewer voor een internationaal tijdschrift en chairman op een internationaal congres. Ze was ook meermaals spreker op (inter)nationale congressen en kreeg prijzen voor de beste presentatie/pitch.

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