Antimicrobial resistance and virulence of bacteria in cattle with bovine respiratory disease syndrome over a period of five years in Bavaria

von Alexander Erich Melchner

Inaugural-Dissertation zur Erlangung der Doktorwürde

der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität

München

Antimicrobial resistance and virulence of bacteria in cattle with bovine respiratory disease syndrome over a period of five years in Bavaria

von Alexander Erich Melchner

aus Weiden in der Oberpfalz

München 2022

Aus dem Veterinärmedizinischen Department

der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Lehrstuhl für Bakteriologie und Mykologie

Arbeit angefertigt unter der Leitung von:

Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.

Angefertigt am Bayerischen Landesamt für Gesundheit und Lebensmittelsicherheit in Oberschleißheim

Mentorin: Priv.-Doz. Dr. Julia M. Riehm

Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan: Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.

Berichterstatter: Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.

Korreferent: Priv.-Doz. Dr. Bianka Schulz

Tag der Promotion: 30. Juli 2022

Meiner Familie

TABLE OF CONTENTS

ABBREVIATIONS

%	percentage
µl	microliter
AMR	antimicrobial resistance
BCoV	bovine corona virus
BHV1	bovine herpes virus type 1
BMEL	Federal Ministry of Food and Agriculture (Bundesministerium für Ernährung und Landwirtschaft)
BMG	Federal Ministry of Health (Bundesgesundheitsministerium)
BMJ	Federal Ministry of Justice (Bundesministerium der Justiz)
bp	base pairs
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
BTK	Federal Veterinary Surgeons'Association (Bundestierärztekammer e.V.)
BVDV	bovine viral diarrhea virus
BVL	Federal Office of Consumer Protection and Food Safety (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit)
C	degrees celsius
CLSI	Clinical and Laboratory Standards Institute
DARTS	German antibiotic resistance strategy (Deutsche Antibiotika Resistenzstrategie)
DNA	deoxyribonucleic acid
FAO	Food and Agriculture Organization
g	relative centrifugal force or *g* force
HGT	horizontal gene transfer
HS	haemorrhagic septicaemia (Hämorrhagische Septikämie)
ICE	integrated conjugative element
IgG	immunoglobulin G
IL	interleukin

INF	interferon
LPS	lipopolysaccharides
MDR	multidrug-resistance
MIC	minimum inhibitory concentration
OIE	World Organisation for Animal Health
OMP	outer membrane protein
PCR	polymerase chain reaction
PI-3	bovine parainfluenza virus type 3
QRDRS	quinolone resistance determining regions
StIKo Vet	Standing Committee on Veterinarian Vaccination (Ständige Impfkommission Veterinärmedizin)
TNF	tumor necrosis factor
WHO	World Health Organisation

REGISTER OF ILLUSTRATIONS & LIST OF TABLES

I. INTRODUCTION

Bovine respiratory disease (BRD) is one of the most important health problems in bovine medicine worldwide and leads to economic losses in both beef production and dairy farms (SNOWDER et al., 2006; HILTON, 2014; DUBROVSKY et al., 2019). The etiology of the BRD complex is described as multifactorial, as both non-infectious factors and infectious agents are involved in the development of the disease (SANDERSON et al., 2008; GRISSETT et al., 2015). Viral pathogens impair the immune system and favour a secondary bacterial infection (LOPEZ et al., 1976; GERSHWIN et al., 2008). Bacterial pathogens from the Pasteurellaceae family such as *Pasteurella multocida* and *Mannheimia haemolytica* are of particular importance, because they have been detected most frequently in cattle with BRD (ANHOLT et al., 2017; HOLSCHBACH et al., 2020). Several virulence factors enable these bacteria to evade the immune system and trigger various forms of pneumonia, especially fibrinous pleuropneumonia, and suppurative bronchopneumonia (CONFER, 2009; PANCIERA & CONFER, 2010). For the therapy and control of those bacterial infections, the administration of antibiotics therefore plays a decisive role in dairy and fattening farms (EDWARDS, 2010). However, studies from around the world show that there is a trend towards increased antimicrobial resistance (AMR) and multidrug-resistance (MDR) to certain antimicrobial agents in bacterial BRD causing pathogens (EL GARCH et al., 2016; KLIMA et al., 2020). To ensure that infectious diseases can continuously and effectively be treated with antibiotics in the future, surveillance programmes as the German Resistance Monitoring "GERM-Vet" are essential (WHO, 2015; BVL, 2020b).

The aim of this work was to complement existing resistance studies by recording current trends in the development of AMR in bacterial pathogens of BRD in Bavaria over the five-year period between July 2015 and June 2020 and to investigate the influence of animal- and farm-specific epidemiological parameters on the resistance pattern. In addition, virulence was investigated from a subset of *Pasteurella multocida* isolates by determining the capsular type by polymerase chain reaction (PCR).

II. LITERATURE OVERWIEW

1. Etiology of bovine respiratory disease (BRD)

1.1. Predisposing factors

Insufficient colostrum intake after birth is one risk factor for the development of BRD. Calves with low postcolostral serum immunoglobulin G (IgG) levels have a two-times higher risk of developing pneumonia than calves with higher levels (VIRTALA et al., 1999)(Figure 1). Holstein calves should consume at least 153 grams IgG in the first two hours after birth. This corresponds to an intake of at least three litres of colostrum (CHIGERWE et al., 2008). In addition, colostrum from cows that have signs of mastitis at the first milking should not be used (VIRTALA et al., 1999). Inadequate hygienic conditions in the keeping of calves also favour BRD. Therefore, young calves should have enough bedding material to nestle in. Ideally, the animal nests so deeply that its legs are no longer visible. This protects against drafts and chilling at temperatures below the thermoneutral zone (LAGO et al., 2006). Also, there should be good drainage under the bedding. It ensures that urine, spilled milk, and water are drained out, so the straw does not get soaked but stays dry (NORDLUND & HALBACH, 2019). Additionally associated with BRD are an increasing number of animals kept within a group. A study from Sweden showed that in groups with 12 to 18 calves the incidence of BRD was higher than in groups with six to nine calves. The recommendation is therefore to keep calves in groups with ten or less animals (SVENSSON & LIBERG, 2006). Increasing herd size is also associated with an increasing risk of BRD in older cattle (MURRAY et al., 2016). Another critical phase is the transport of calves from the farm of origin to the fattening farm. Long-distance transports are associated with temporary dehydration and stress (CERNICCHIARO et al., 2012)(Figure 1). Studies have shown that the stress mediator plasma cortisol is elevated during transport (ISHIZAKI & KARIYA, 2010). Long-distance transports are often associated with the commingling of cattle from different farms, which also increases the risk of developing BRD (SANDERSON et al., 2008). Another predisposing factor is insufficient ventilation of the barns. Adequate air exchange improves air quality

and reduces the number of bacteria in the air, resulting in a lower prevalence of BRD (LAGO et al., 2006). For housing cattle in barns a combined system of natural ventilation based on an eaves ridge system and a positive pressure tube system is recommended (NORDLUND & HALBACH, 2019). Finally, it should be mentioned that the season and climate conditions also had an influence on the incidence of BRD. In the summer months June to August, BRD occurs less frequently than in months with lower average temperatures (SELBITZ et al., 2015; MURRAY et al., 2016)(Figure 1).

1.2. Viral pathogens

The viral pathogens of the BRD complex include bovine coronavirus (BCoV), bovine viral diarrhea virus (BVDV), bovine herpes virus type 1 (BHV-1), bovine respiratory syncytial virus (BRSV), and bovine parainfluenza virus type 3 (PI-3) (GRISSETT et al., 2015; HEADLEY et al., 2018)(Figure 1). Since both BVDV and BHV-1 are notifiable animal diseases in Germany and are thus controlled by the authorities, these two pathogens are not expected to play a role in the etiology of BRD in Germany (BMEL, 2021). PI-3 and BRSV belong to the Paramyxoviridae family and are single-stranded enveloped RNA viruses. The viruses are widespread in German cattle herds with a seroprevalence of 60-80 %. Transmission occurs via droplet infection by virus-contaminated nasal secretions and aerosols. These viruses are very contagious and spread very quickly in the cattle population (SELBITZ et al., 2015). BRSV attaches to respiratory epithelium, replicates in ciliated epithelial cells and type two pneumocytes. The virus induces proinflammatory chemokines and cytokines that cause neutrophilic granylocytes, macrophages, and lymphocytes to migrate into the airways, leading to respiratory disease (VALARCHER & TAYLOR, 2007). Damage to the ciliated epithelium due to PI-3 and BRSV infection limits the mucociliary clearance (LOPEZ et al., 1976; GERSHWIN et al., 2008; SELBITZ et al., 2015). BRSV and PI-3 are therefore classified as primary infectious agents and, in combination with the predisposing factors of BRD, lead to stress and immunosuppression in cattle (GRISSETT et al., 2015; MEHINAGIC et al., 2019). Therefore, certain bacteria that colonise the nasopharynx as commensals, proliferate and cause secondary bacterial inflammation of the lungs (CONFER, 2009). Because predisposing factors as well as viral and bacterial pathogens are

involved in the etiology of BRD, it is referred to as a multifactorial disease (GRISSETT et al., 2015)(Figure 1).

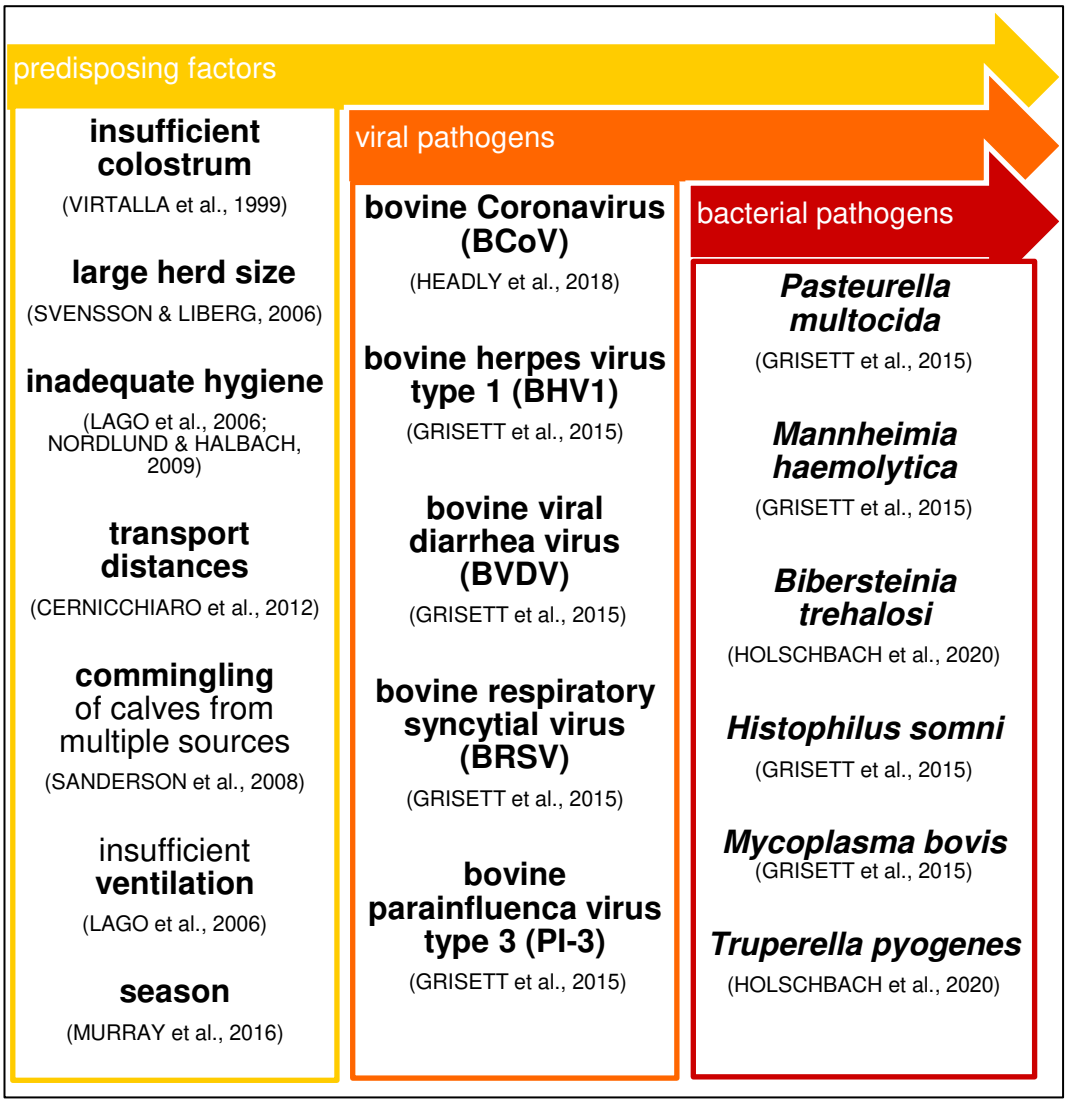

Figure 1. Overview of the multifactorial etiology (predisposing factors, viral pathogens, bacterial pathogens) of BRD.

1.3. Bacterial pathogens

1.3.1. Pasteurellaceae

The most important bacterial pathogens of BRD are members of the Pasteurellaceae family, namely *Pasteurella multocida*, *Mannheimia haemolytica*, *Bibersteinia trehalosi,* and *Histophilus somni* (GRISSETT et al., 2015; HOLSCHBACH et al., 2020)(Figure 2). These are non-sporulating bacteria; they are oxidase- and

catalase-positive (*Histophilus* spp. only oxidase-positive), immotile, predominantly encapsulated, facultative anaerobic rods (SELBITZ et al., 2015). They live as commensals in the mucous membranes and infect the lungs of calves and cattle after exposure to various stressors and viral pathogens (CONFER, 2009)(Figure 1). In *Mannheimia haemolytica*, exposure to those factors leads to a shift in serotypes. While serotype A:2 is predominantly isolated from the respiratory mucous membranes in healthy animals, the two serotypes A:1 and A:6 dominate in cases of disease (KLIMA et al., 2014). In the case of *Pasteurella multocida* isolates, serotype A:3 is the most important serotype (DABO et al., 2007).

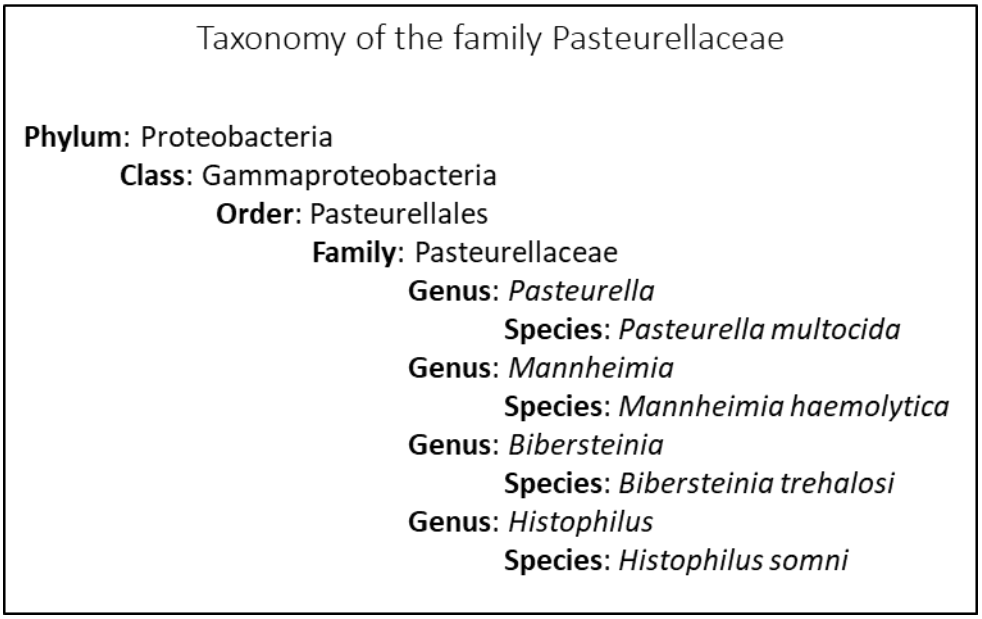

Figure 2. Taxonomy of the family Pasteurellaceae including the bacterial species investigated in the present study. Own illustration based on (SCHOCH et al., 2020).

The pathogens of the Pasteurellaceae family mainly cause fibrinous pleuropneumonia and suppurative bronchopneumonia with acute, subacute, and chronic courses (ANDREWS & KENNEDY, 1997; PANCIERA & CONFER, 2010; PRAVEENA et al., 2014). Suppurative pleuropneumonia is often diagnosed in young calves on dairy farms, while fibrinous pleuropneumonia is often an acute pneumonia in stressed beef cattle (PANCIERA & CONFER, 2010).

1.3.2. Mycoplasma bovis

Mycoplasma bovis is another bacterial pathogen involved in the BRD complex

(GRISSETT et al., 2015). The bacterium belongs to the class of Mollicutes that implies a lack of a true cell wall and pleomorphic phenotype (SELBITZ et al., 2015). This bacterium colonises the nasopharynx as a commensal, is therefore also detected in healthy animals and may infect the lungs after various stressors (GAGEA et al., 2006; CONFER, 2009)(Figure 1). In *Mycoplasma bovis* infections of calves – in case of *Mycoplasma bovis* often caseonecrotic bronchopneumonia – arthritis is diagnosed in some cases at the same time as pneumonia (GAGEA et al., 2006). This pathogen is also involved in ear infections (DUDEK et al., 2020). A special feature of this bacterium is the limited effectiveness of ß-lactam antibiotics, with resistance rates of over 98 % for penicillin and ceftiofur due to the lack of a cell wall (BTK, 2015; ANHOLT et al., 2017).

1.3.3. Truperella pyogenes

Bacteria of the species *Truperella pyogenes* belong to the Actinomycetaceae family and are facultative anaerobic gram-positive pleomorphic rods (SCHOTT et al., 2014; SELBITZ et al., 2015). They are ubiquitous pyogenic pathogens that colonise the surfaces of mucous membranes in the nasopharynx. As a pathogen within the BRD complex, they develop their virulent effect when pneumonia has already been caused by other pathogens (CONFER, 2009). *Truperella pyogenes* is therefore associated with chronic abscessing pneumonia (PANCIERA & CONFER, 2010; RISSETI et al., 2017).

2. Virulence factors of *Pasteurella multocida*

Because the bacterial pathogens of the BRD complex live as commensals on the mucous membranes of the respiratory tract but also have the potential to cause pneumonia, it is crucial to understand the virulence factors that give the bacteria their pathogenicity (CONFER, 2009). Of particular importance are those of *Pasteurella multocida*, because this pathogen is the most frequently isolated from animals suffering from BRD (PORTIS et al., 2012; HOLSCHBACH et al., 2020).

2.1. Polysaccharide capsule

2.1.1. Impact of capsule type on the clinical picture

As an infectious agent, *Pasteurella multocida* causes different clinical pictures depending on the serotype and capsule type. According to the Carter-Heddlestone scheme, *Pasteurella multocida* isolates can be classified into 16 somatic serotypes (serotype 1 to 16) and five capsular serogroups A, B, D, E and F (TOWNSEND et al., 2001; SELBITZ et al., 2015). The main components of the capsule are glycosaminoglycans and polysaccharides, which consist of repeating disaccharide units. The disaccharide units contain an amino sugar. The most important capsule material of type A is hyaluronan (hyaluron acid), of type D unmodified heparin (N-acetylheparosan), and of type F unmodified chondroitin (DEANGELIS et al., 2002). A monosaccharide analysis of a serogroup B strain showed a composition of arabinose, mannose, and galactose in a ratio of 0.5 : 2.0 : 0.8, which indicates that the capsule is formed by a polymer containing these monosaccharides (MUNIANDY, 1992). In BRD, *Pasteurella multocida* isolates of capsule type A dominate, the most important serotype being serotype A:3. However, isolates with capsule types B, D, and F were also detected in cattle with BRD in various publications (EWERS et al., 2006; DABO et al., 2007; ARUMUGAM et al., 2011). Besides BRD, haemorrhagic septicaemia (HS) is another important disease in cattle, buffalo, and wild ruminants. HS is a highly fatal and acute septicaemia with high morbidity and mortality. Geographically, it occurs in some areas of Africa, Asia, the Middle East and southern Europe. However, this disease is not caused by type A strains, but by serotypes B:2 (Asian type) and E:2 (African type) (DE ALWIS, 1992; OIE, 2018).

2.1.2. Protection from the immune system

Components of the capsule, such as hyaluronic acid in type A, also occur in the host in epithelial and neuronal tissue. This protects against a strong antibody reaction against capsular polysaccharides (PETRUZZI et al., 2017)(Figure 3). Furthermore, the presence of a capsule with its polysaccharides, such as hyaluronic acid in type A, impairs the phagocytosis capability of macrophages (PRUIMBOOM et al., 1996; BOYCE & ADLER, 2000). In experiments with

Pasteurella multocida strains of serogroup A, it was seen that depolymerisation of the capsule component hyaluronic acid by a hyaluronidase increases the phagocytosis of bacteria by macrophages (PRUIMBOOM et al., 1996). Further studies have shown that capsule-deficient mutants of Pasteurella multocida type B strains were more readily taken up by macrophages than encapsulated wild-type strains (BOYCE & ADLER, 2000). Encapsulation also appears to provide protection against the bactericidal activity of the complement system (Figure 3). It is hypothesised that the capsule does not prevent the formation of the membrane attack complex, but rather shields the outer membrane (HANSEN & HIRSH, 1989). The extent to which the capsule mediates increased adherence to host tissue does not appear to be clear. While one study demonstrated that components of the capsule, particularly hyaluronic acid, mediate increased adherence to alveolar macrophages, other studies have found no association between the presence of a capsule and increased binding to cells of the respiratory tract and mucus (JACQUES et al., 1993; PRUIMBOOM et al., 1996; BOYCE et al., 2000).

2.2. Biofilm formation

It is known that there is a correlation between the encapsulation of *Pasteurella multocida* A strains and the formation of a biofilm. Biofilm development is significantly stronger in *Pasteurella multocida* isolates that are less encapsulated with polysaccharides. At the same time, biofilm formation increases when capsular polysaccharides are degraded by the addition of hyaluronidase (PETRUZZI et al., 2017). When biofilm is exposed to the stress-related substances noradrenaline and epinephrine, it has been seen in *in vitro*-models that the biofilm disperses with bacteria within it. The virulence factor biofilm could thus play a crucial role in the spread of bacteria that colonise the nasopharynx as commensals and cause inflammation as pathogens in the lower respiratory tract (PILLAI et al., 2018). In addition, biofilm offers protection against the action of antimicrobial substances. The biofilm matrix, which is mainly composed of glycogenic exopolysaccharides in *Pasteurella multocida* Serogroup A, physically shields the bacterial cells from antimicrobial substances (BOUKAHIL & CZUPRYNSKI, 2016; PETRUZZI et al., 2017). In an *in vitro* experiment with *Mannheimia haemolytica* isolates, the minimum inhibitory concentrations (MICs) in biofilms on bovine

bronchial epithelial cells were twice as high for the antibiotic agent tulathromycin, four times as high for the antibiotic agent gentamicin and eight times as high for the antibiotic agent chlortetracycline than in biofilms on polystyrene (BOUKAHIL & CZUPRYNSKI, 2016).

2.3. Lipopolysaccharides

Together with the capsular polysaccharides, the lipopolysaccharides (LPS) form the main portion of the bacterial cell surface (HARPER et al., 2012) (Figure 3). As outlined above, *Pasteurella multocida* strains can be grouped into 16 LPS serovars according to the Carter-Heddlestone scheme. The LPS consist of a highly conserved internal structure and a variable external structure (HARPER et al., 2015). The effect of LPS on bovine leukocytes is summarised in Figure 3. *Pasteurella multocida* LPS induces cell proliferation of bovine leukocytes and the expression of cytokine genes TNF-α, IL-1β, IL-6, IL-8, IL-12 and INF-γ (PERIASAMY et al., 2018). IL-8 has a strong chemotactic effect on neutrophil granulocytes and mediates the transmigration of neutrophils through alveolar endothelial cells. Neutrophils are important mediators of tissue damage. Other cytokines such as TNF-α activate the epithelial cells, increase the transition of further immune cells into the airways and thus also have a proinflammatory effect (CASWELL et al., 1998; GALDIERO et al., 2000; SNYDER & CREDILLE, 2020). Pneumonia with congestion of pulmonary blood vessels, haemorrhages in alveolar spaces, thickened alveolar septa, and oedematous changes in the alveolar and bronchial lumen are the result (PRAVEENA et al., 2014; SNYDER & CREDILLE, 2020). At higher concentrations, *Pasteurella multocida* LPS induce apoptotic cell death of bovine leukocytes mediated by caspases and mitochondrial dysfunction (Figure 3). Apoptotic changes in leukocytes such as membrane blebs, condensed/fragmented nuclei, cellular fragmentation, and mitochondrial swelling may be observed (PERIASAMY et al., 2018).

2.4. Adherence factors

Pasteurella multocida has different adhesins for attachment and colonisation of the respiratory epithelium. These include filamentous hemagglutinin A, type IV fimbriae and outer membrane proteins (Omp), such as OmpA and OmpB. OmpA

is thought to mediate attachment to host cells by bridging with heparin and fibronectin (RUFFOLO et al., 1997; DABO et al., 2003; EWERS et al., 2006).

2.5. Extracellular enzymes

Important representative of *Pasteurella multocida* secreted enzyme are, on the one hand, neuraminidases, which have a nutritional function and participate in the colonisation of the respiratory epithelium (WHITE et al., 1995; MIZAN et al., 2000). On the other hand, the bacterium has the ability to produce proteases that can degrade IgG antibodies(NEGRETE-ABASCAL et al., 1999).

2.6. Mechanisms for iron uptake

Iron is an essential element for the survival and establishment of an infection. Iron plays an important role as a biocatalyst and electron carrier. Because freely available iron is limited in the host organism and is bound to carrier proteins such as haemoglobin or transferrin, *Pasteurella multocida* has numerous proteins for iron uptake. Proteins involved in iron uptake include hemoglobin receptors, hemoglobin-binding proteins A and B, and transferrin binding protein A (PAYNE, 1993; BOSCH et al., 2002; ANDREWS et al., 2003; EWERS et al., 2006; JATUPONWIPHAT et al., 2019).

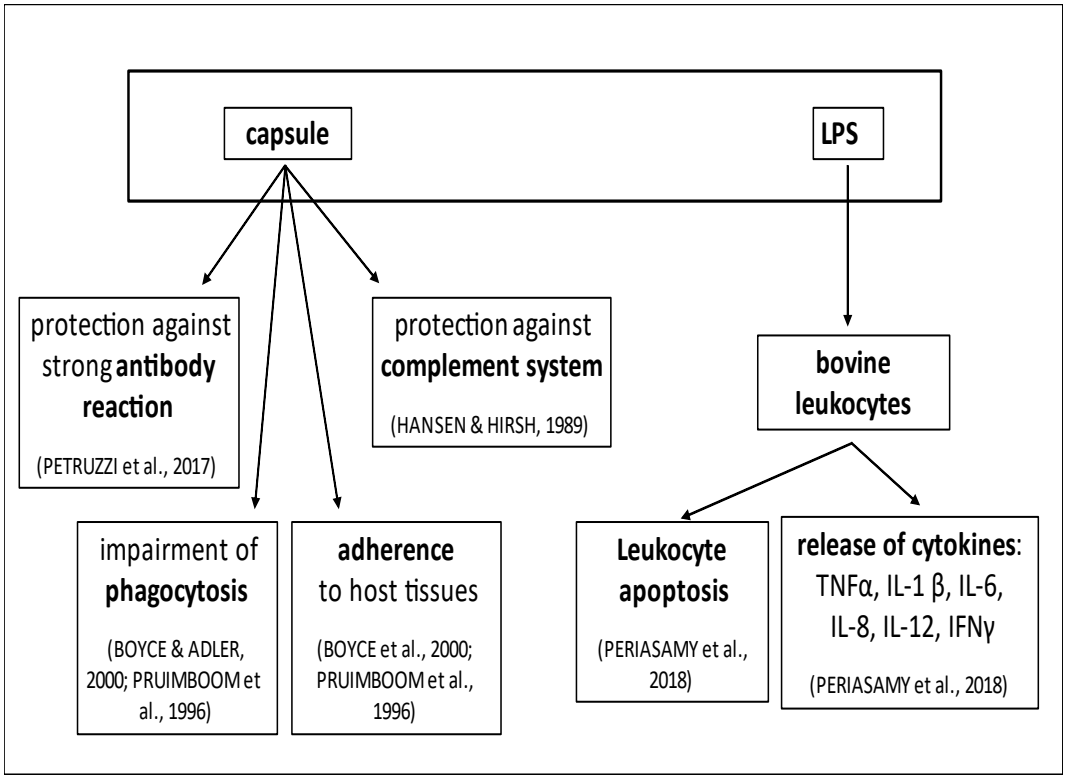

Figure 3. Simplified illustration of virulence originating from the cell surface of *Pasteurella multocida*.

3. Antimicrobial resistance

3.1. Control and therapy of BRD in context of increasing AMR

Vaccines and antibiotics are still the main components for controlling and treating BRD (EDWARDS, 2010). Vaccination against bacterial and viral pathogens can improve animal health and thus also economic losses caused by BRD (SCHUNICHT et al., 2003; WILDMAN et al., 2008). In Germany, 13 vaccine preparations are currently approved by the Paul Ehrlich Institute (PAUL EHRLICH INSTITUTE, 2021). The Standing Committee on Veterinarian Vaccination (StIKo Vet) at the Friedrich-Loeffler Institute suggests vaccination for both beef cattle farms and dairy farms. For dairy farms, it recommends vaccination, if the pathogen in herd is enzootic and endemic in region; for beef cattle farms, the StIKo Vet recommends vaccination regardless of age and regardless of the farm situation (STIKO VET, 2018). Nevertheless, it must be mentioned that systematic reviews and meta-

analyses have shown that there is an inconsistency in the effectiveness of vaccines for BRD prophylaxis. Vaccinations are thus only one part of the control and therapy (EDWARDS, 2010; LARSON & STEP, 2012; THEURER et al., 2015). For the treatment of bacterial pneumonia, antibiotics of eight antibiotic classes (ß-lactams, fluoroquinolones, phenicols, tetracyclines, sulphonamides, aminoglycosides, lincosamides and macrolides) are approved in Germany and available as corresponding preparations (UNIVERSITÄT LEIPZIG, 2020). When selecting an antibiotic for therapy of BRD, bactericidal agents should be preferred to bacteriostatic agents that only inhibit bacterial growth and replication. It could be shown that initial treatments with bactericidal antibiotics were associated with lower odds of further treatments than initial treatment with a bacteriostatic agent (COETZEE et al., 2020). Furthermore, it is important to make a well-founded diagnosis, which include a clinical examination and pathogen determination with a constant evaluation of the resistance situation (BTK, 2015). Studies from the late 1980s and 1990s already saw that the susceptibility of certain antibiotics to bacteria of the BRD complex decreases and changes (WATTS et al., 1994; WELSH et al., 2004). Of particular concern is the increase in MDR isolates from bacteria of the BRD complex. In recent publications from North America, the proportions of MDR *Pasteurella multocida* isolates in feedlot cattles were over 90 % and those of MDR *Mannheimia haemolytica* isolates over 80 % in the study period 2015-2016(LUBBERS & HANZLICEK, 2013; KLIMA et al., 2020).

3.2. Definition of antimicrobial resistance

The term antibiotic resistance is limited to the resistance of bacteria, while antimicrobial resistance (AMR) describes the resistanc of bacteria, viruses, parasites, and fungi to antimicrobials (ROBERT KOCH INSTITUTE, 2020).

3.3. Definition of clinical resistance

Clinical resistance is based on clinically derived breakpoints set by the Clinical and Laboratory Standards Institute (CLSI). Breakpoints are defined MIC values used to categorise a bacterial isolate as susceptible (S), intermediate (I), or resistant (R). The MIC values determined in the susceptibility test with the microbroth dilution method can therefore be interpreted on the basis of the defined breakpoints (CLSI,

2020) (Table 1).

Table 1. Overview of MIC breakpoints and the interpretive criteria susceptible, intermediate, and resistant of the two antibiotic agents florfenicol and enrofloxacin for *Mannheimia haemolytica* isolates in case of respiratory disease in cattle (CLSI, 2020)**.**

antimicrobial agent	**antimicrobial class**	**interpretive categories and MIC breakpoints µg/ml**		
		susceptible	intermediate	resistant
florfenicol	phenicols	≤2	4	≥8
enrofloxacin	fluoroquinolones	≤0.25	0.5-1	≥2

An isolate categorised as susceptible with an MIC value at or below the susceptible breakpoint is expected to be inhibited by the antimicrobial agent when the dosage recommended to treat the infection is used. For an isolate categorised as resistant with an MIC value at or above the resistant breakpoint, it can be expected that it will not be inhibited by the concentration of the antibiotic agent achieved at the normal dosage. Treatment of infections with antibiotic agents tested as susceptible implies a higher probability of therapeutic success compared to antibiotic agent tested as resistant. However, it needs to be mentioned that the MICs determined *in vitro* are objective laboratory values. The antibacterial activity of the antibiotic agent could also be affected by the immune status of the diseased animal or altered pharmacokinetic in the inflamed tissue, so that the result of a susceptibility test does not always correspond to the clinical outcome (CLSI, 2018, 2020; SNYDER & CREDILLE, 2020).

3.4. Spread of antibiotic resistance through horizontal gene transfer

In addition to vertical gene transfer, a transfer of genetic material to the offspring, horizontal gene transfer (HGT) is associated with the spread and acquisition of antibiotic resistance between bacteria. In HGT, genetic material is not exchanged

via the clonal lineage but between strains and also across species and genera (LERMINIAUX & CAMERON, 2019). Transformation, in which extracellular DNA is taken up by bacteria and incorporated into the genome, is one way of HGT. Some members of the Pasteurellaceae family are known to be competent for transformation (WANG et al., 2002; LERMINIAUX & CAMERON, 2019). The second possibility of HGT is transduction, in which bacteriophages – viruses that infects bacteria – transfer antibiotic resistance genes to recipient bacterial strains (BLAHOVÁ et al., 2000; LERMINIAUX & CAMERON, 2019). Conjugation is the third possibility of HGT. In this case, resistance genes are exchanged via mobile genetic elements such as conjugative plasmids or integrated conjugative elements (ICEs), which also have the relevant genes for their own transfer. In contrast to transformation and transduction, cell-to-cell contact is necessary. Plasmids and ICEs establish the connection by using a pilus to transfer themselves to the recipient cell (FROST et al., 2005; LERMINIAUX & CAMERON, 2019). In the spread of resistance genes between bacteria of the BRD complex, conjugation by plasmids and ICEs seems to play a crucial role (KEHRENBERG et al., 2003; KLIMA et al., 2020; STANFORD et al., 2020). It is important to note that in presence of antibiotic agents, all three pathways of HGT are induced. As a result of this, selection pressure for the transfer of antibiotic resistance between bacterial species is increased (PRUDHOMME et al., 2006; MODI et al., 2013; ZHANG et al., 2013).

3.5. Resistance mechanisms and resistance genes in Pasteurellaceae

3.5.1. Resistance against antibiotics of the ß-lactam group

The target site of ß-lactam antibiotics is the cell wall and the mechanism of action is that they bind to penicillin-binding proteins, which are key enzymes in the biosynthesis of the cell wall component peptidoglycan, thus inhibiting cell wall synthesis (MIYACHIRO et al., 2019). The resistance genes *blaoxa-2*, *blaROB-1* and *blaROB-2* are responsible for resistance of ß-lactam antibiotics. While *blaoxa2* encodes a narrow-spectrum ß-lactamase, the product of *blaROB-2* is an extended spectrum ß-lactamase, which hydrolyse in addition to penicillins also third and fourth generation cephalosporins (MICHAEL et al., 2012b, 2012a; KADLEC et al., 2019)(Table 2).

3.5.2. Resistance against antibiotics of the phenicol group

The resistance gene *floR* encodes an efflux pump that is specific for cloramphenicol, florfenicol, and thiamphenicol. The efflux pump actively transports these substances out of the bacterial cell. Active efflux is driven by the proton motive force (BRAIBANT et al., 2005; KEHRENBERG & SCHWARZ, 2005; KEHRENBERG et al., 2008)(Table 2).

3.5.3. Resistance against antibiotics of the sulphonamide group

Unlike mammalian cells, which have uptake systems for folic acid, most prokaryotes are dependent on folic acid synthesis. The point of attack of sulphonamides is the inhibition of synthesis of folic acid compounds by competing as a substrate analogue with p-aminobenzoic acid for dihydropteroate synthase. The *sul2* resistance gene encodes a dihydropteroade synthase which binds to p-aminobenzoic acid despite its structural similarity to the sulfonamides (BROWN, 1962; SKÖLD, 2000; MICHAEL et al., 2012a; EIDAM et al., 2015) (Table 2).

3.5.4. Resistance against antibiotics of the fluorochinolon group

Little is known about resistance of fluoroquinolones in Pasteurellaceae (MICHAEL et al., 2012a). The main mechanisms of action of fluoroquinolones are the inhibition of DNA gyrase and topoisomerase IV. Both enzymes are involved in chromosomal supercoiling, which is important for DNA synthesis, transcription, and cell division. One possibility for the development of fluoroquinolone resistance are mutations in the quinolone resistance determining regions (QRDRS). Mutations in the *gyrA* and *parC* genes for topoisomerase IV, lead to the substitution of amino acids. As a result, target proteins of antibiotic drugs are altered and they can no longer bind (CORREIA et al., 2017). In studies with bovine *Mannheimia haemolytica* and *Pasteurella multocida* strains, it was found that mutations in the QRDRS causes resistant phenotypes with increased MICs (KATSUDA et al., 2009; KONG et al., 2014). The expression of efflux pump genes was also detected in *Pasteurella multocida* (KONG et al., 2014; CORREIA et al., 2017) .

3.5.5. Resistance against antibiotics of the macrolide group

Macrolides bind to the exit tunnel of the newly formed peptide on the ribosomes and as a result of this the protein biosynthesis was blocked (VÁZQUEZ-LASLOP & MANKIN, 2018). One resistance mechanism is that nucleotide A2058 on the 23S rRNA is methylated, preventing macrolide binding to ribosomes. Antibiotic resistance gene *erm(42)* encodes such a rRNA monomethyltransferase, which attaches a methyl group to nucleotide A2058 of the 23S rRNA mediating resistance to macrolides and lincosamides (DESMOLAIZE et al., 2011a). Resistance gene *mph(E)* encodes a phosphotransferase that inactivates macrolides and resistance gene *msr(E)* encode a macrolide efflux pump (DESMOLAIZE et al., 2011b) (Table 2).

3.5.6. Resistance against antibiotics of the tetracycline group

In bovine *Pasteurella multocida* and *Mannheimia haemolytica* strains, the *tetB*, *tetG*, *tetL*, and *tetH* resistance genes are known (KEHRENBERG et al., 2001; KEHRENBERG et al., 2005). They encode an efflux pump that transports tetracycline out of cell (HANSEN et al., 1993; KEHRENBERG et al., 2005). Moreover, in addition to *tetH*, the supressor gene *tetR* has also been shown to regulate efflux activity in response to tetracycline concentration (HANSEN et al., 1993) (Table 2).

3.5.7. Resistance against antibiotics of the aminoglycoside group

Aminoglycosides bind to bacterial ribosomes and interfere with protein synthesis (STERN et al., 2018). Aminoglycoside-resistant strains possess genes of enzymes, which modify the antimicrobial drug so that it is inactive. Genes *strA*, *strB* and *apH1* encode aminoglycoside phosphotransferases that phosphorylate the antibiotics. Gene products of *aadB* and *aadA25* modify aminoglycosides through adenyltransferases (DAVIES & WRIGHT, 1997; WHITE & RAWLINSON, 2001; MICHAEL et al., 2012a) (Table 2).

Table 2. Overview of resistance mechanisms and resistance genes in Pasteurellaceae

antimicrobial class	resistance genes	gene product	resistance mechanism	References
ß-lactams	*blaoxa-2, blaROB-1, blaROB-2*	ß-lactamase	hydrolysis of the ß-lactams	(MICHAEL et al., 2012a, 2012b; KADLEC et al., 2019; MIYACHIRO et al., 2019)
phenicols	*floR*	efflux pump	active transport of phenicols out of cell	(BRAIBANT et al., 2005; KEHRENBERG & SCHWARZ, 2005; KEHRENBERG et al., 2008)
sulphonamides	*sul2*	dihydropteroate synthase	synthesis of dihydropteroate	(BROWN, 1962; SKÖLD, 2000; MICHAEL et al., 2012b; EIDAM et al., 2015)
macrolides	*erm(42)*	rRNA-monomethyltrans-ferase	methylation of 23S rRNA	(DESMOLAIZE et al., 2011a; DESMOLAIZE et al., 2011b; VÁZQUEZ-LASLOP & MANKIN, 2018)
	mph(E)	phosphotransferase	phosphorylation of macrolides	
	msr(E)	efflux pump	active transport of macrolides out of cell	
tetracyclines	*tet(H), tet(R), tet(B), tet(G), tet(L)*	efflux pump	active transport of tetracyclines out of cell	(HANSEN et al., 1993; KEHRENBERG et al., 2001; KEHRENBERG et al., 2005)
aminoglycosides	*strA, strB, apH1*	phosphotransferase	phosphorylation of aminoglycosides	(DAVIES & WRIGHT, 1997; WHITE & RAWLINSON, 2001; MICHAEL et al., 2012a; STERN et al., 2018)
	aadB, aadA25	adenylyltransferase	adenylation of aminoglycosides	

3.6. Antimicrobial resistance as a global health problem

The decreasing susceptibility of antibiotics to certain pathogens and the associated treatment failure is not only a problem in the treatment of BRD (LUBBERS & HANZLICEK, 2013; ANHOLT et al., 2017; KLIMA et al., 2020). In all parts of the world, the prevalence of resistant microorganisms is increasing in both human and veterinary medicine. At the same time, there are currently few replacement products under development. Increasing AMR is attributed to the greater use, overuse, and misuse of antibiotics in recent decades, which has exerted selection pressure on susceptible bacteria with possible survival of resistant bacteria (BELL et al., 2014; WHO, 2015). AMR can spread between

bacterial strains through HGT. In addition, drug-resistant bacteria can circulate between humans and animals via direct human-animal contact, contaminated food or wastewater that pollutes the environment (BERENDONK et al., 2015; WHO, 2015). Also due to the fact that in human and veterinary medicine the same infectious agents are partly treated with the same antibiotic substances, the World Health Organisation (WHO) is calling for a global cross-sector strategy to prevent this impending worldwide health crisis. In cooperation with other organisations such as FAO or OIE, the WHO adopted the “Global action plan on antimicrobial resistance” in 2015. The primary goal is to ensure that infectious diseases can effectively treated and cured with safe and effective drugs in the future. All member states committed to implementing this global plan with national measures (WHO, 2015, 2019).

3.7. Main measures of the German antibiotic resistance strategy

The German authorities implemented the requirements of the WHO's "global action plan on antimicrobial resistance" with the German antibiotic resistance strategy (DARTS), which has been in place since 2008 and has since been adapted and expanded. A central component was the establishment and continuous improvement of surveillance programmes (Figure 4). In human medicine, the monitoring system “Antibiotika-Resistenz-Surveillance (ARS)” for central collection and evaluation of resistance data from the outpatient and inpatient areas was established. In veterinary medicine, two surveillance systems have been established that collect pathogens for resistance testing in a representative manner according to coordinated sampling plans (BMG, 2015). The first is zoonosis monitoring, in which the antibiotic resistance of zoonotic pathogens and animal commensals, for example *Salmonella* spp., *Campylobacter* spp. or Shiga toxin-producing *E. coli*, are determined by sampling from farm, slaughterhouse and retail (BVL, 2020a). In the second monitoring programme "German Resistance Monitoring" (GERM-Vet), the resistance situation of clinically important animal pathogenic bacteria is monitored. This includes samples from both food-producing and non-food-producing animals (BVL, 2020b). Other measures taken by DARTS were mainly changes in legislation (Figure 4). In the course of the 16th amendment to the German Medicines Act, all farmers of fattening farms with more than

20 beef cattle are obliged to report any use of antibiotics to the authorities since July 2014. From the number of animals treated, the treatment days and the average of the total number of animals kept, a farm therapy frequency is calculated for each half year. This frequency of treatment on the farm is compared with nationwide benchmarks. If these benchmarks are exceeded by the respective farm, the farmer is obliged to implement measures to reduce the use of antibiotics (BVL, 2021). Another measure is the documentation of antibiotic consumption levels. Pharmaceutical companies and wholesalers have had to report the quantities of antibiotics dispensed to veterinarians to the Federal Institute for Drugs and Medical Devices since 2011 (BMJ, 2021). In addition, since 2018, the amendment to the "Tierärztlichen Hausapothekenverordnung" requires veterinarians to determine pathogens with antibiotic resistance testing when fluoroquinolones and third and fourth generation cephalosporins are used. Furthermore, resistance testing must be carried out if the antibiotic is changed during treatment or if the antibiotic is used more frequently than once in a certain production and age period (BMJ, 2018).

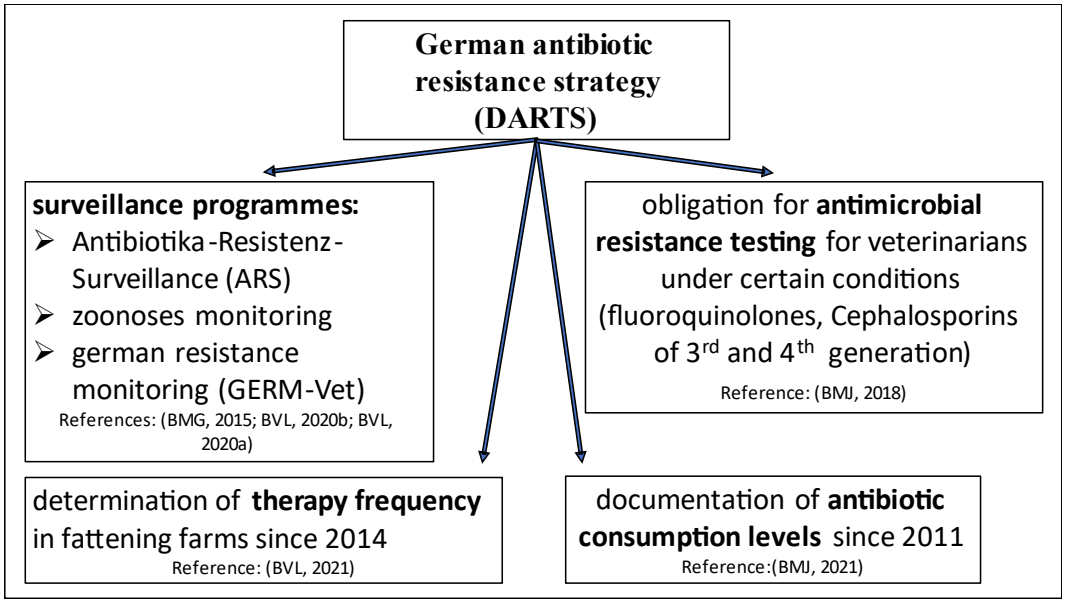

Figure 4. Summary of important measures taken by DARTS to reduce antibiotic use and AMR

III. PUBLICATION

Antimicrobial Resistance in Isolates from Cattle with Bovine Respiratory Disease in Bavaria, Germany

Alexander Melchner, Sarah van de Berg, Nelly Scuda, Andrea Feuerstein, Matthias Hanczaruk, Magdalena Schumacher, Reinhard K Straubinger, Durdica Marosevic, Julia M Riehm

MDPI Antibiotics (Basel) 2021 Dec 15;10(12):1538.

doi: 10.3390/antibiotics10121538.

Supplementary files, „antibiotics-1494441-supplementary.xlsx“:

https://www.mdpi.com/2079-6382/10/12/1538

antibiotics

MDPI

Article

Antimicrobial Resistance in Isolates from Cattle with Bovine Respiratory Disease in Bavaria, Germany

Alexander Melchner [1], Sarah van de Berg [1], Nelly Scuda [1], Andrea Feuerstein [1], Matthias Hanczaruk [1], Magdalena Schumacher [1], Reinhard K. Straubinger [2], Durdica Marosevic [1] and Julia M. Riehm [1,*]

[1] Bavarian Health and Food Safety Authority, 85764 Oberschleissheim, Germany; alexander.melchner@t-online.de (A.M.); Sarah.vandeBerg@lgl.bayern.de (S.v.d.B.); Nelly.Scuda@lgl.bayern.de (N.S.); heubeck.a95@gmail.com (A.F.); Matthias.Hanczaruk@lgl.bayern.de (M.H.); Magdalena.Schumacher@lgl.bayern.de (M.S.); Durdica.Marosevic@lgl.bayern.de (D.M.)

[2] Institute of Infectious Diseases and Zoonoses, Department of Veterinary Sciences, Faculty of Veterinary Medicine, Ludwig-Maximilians-University, 80539 Munich, Germany; Reinhard.Straubinger@micro.vetmed.uni-muenchen.de

* Correspondence: Julia.Riehm@lgl.bayern.de

Abstract: Patterns of antimicrobial resistance (AMR) regarding *Pasteurella multocida* (n = 345), *Mannheimia haemolytica* (n = 273), *Trueperella pyogenes* (n = 119), and *Bibersteinia trehalosi* (n = 17) isolated from calves, cattle and dairy cows with putative bovine respiratory disease syndrome were determined. The aim of this study was to investigate temporal trends in AMR and the influence of epidemiological parameters for the geographic origin in Bavaria, Germany, between July 2015 and June 2020. Spectinomycin was the only antimicrobial agent with a significant decrease regarding not susceptible isolates within the study period (*P. multocida* 88.89% to 67.82%, *M. haemolytica* 90.24% to 68.00%). Regarding *P. multocida*, significant increasing rates of not susceptible isolates were found for the antimicrobials tulathromycin (5.56% to 26.44%) and tetracycline (18.52% to 57.47%). The proportions of multidrug-resistant (MDR) *P. multocida* isolates (n = 48) increased significantly from 3.70% to 22.90%. The proportions of MDR *M. haemolytica* and *P. multocida* isolates (n = 62) were significantly higher in fattening farms (14.92%) compared to dairy farms (3.29%) and also significantly higher on farms with more than 300 animals (19.49%) compared to farms with 100 animals or less (6.92%). The data underline the importance of the epidemiological farm characteristics, here farm type and herd size regarding the investigation of AMR.

Keywords: bovine respiratory disease; antimicrobial resistance; multidrug-resistance; *Pasteurella multocida*; *Mannheimia haemolytica*; *Trueperella pyogenes*; dairy farm

check for updates

Citation: Melchner, A.; van de Berg, S.; Scuda, N.; Feuerstein, A.; Hanczaruk, M.; Schumacher, M.; Straubinger, R.K.; Marosevic, D.; Riehm, J.M. Antimicrobial Resistance in Isolates from Cattle with Bovine Respiratory Disease in Bavaria, Germany. *Antibiotics* **2021**, *10*, 1538. https://doi.org/10.3390/antibiotics10121538

Academic Editor: Clair L. Firth

Received: 19 November 2021
Accepted: 14 December 2021
Published: 15 December 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Bovine respiratory disease (BRD) is one of the most significant health problems in bovine medicine worldwide [1]. The syndrome causes significant economic losses in both beef and dairy production farms [2,3]. Regarding its impact on US feedlots, BRD is the most important disease, with an annual incidence of up to 44%, resulting in economic losses of 13.90 USD per animal regarding treatment costs and lower weight gains [3]. Preweaned calves are most affected by BRD in dairy farms [2]. Furthermore, the pregnancy rates, milk yield, and longevity of dairy cows are also negatively influenced by this syndrome [4–6]. The etiology of BRD is multifactorial, as it is caused by infectious and non-infectious factors [7,8]. Stressful conditions are involved in the development of BRD, such as commingling of calves from different sources or transports over long distances [7,9]. Further, viral agents, such as bovine parainfluenza virus type 3 (PI-3), bovine respiratory syncytial virus (BRSV), bovine herpes virus type 1 (BHV-1), bovine viral diarrhea virus (BVDV) and bovine coronavirus (BCoV) are associated with BRD and may promote secondary bacterial infections by impairing the animals' immune system [8,10–12].

Lastly, bacterial pathogens, such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Bibersteinia trehalosi*, *Histophilus somni*, *Mycoplasma bovis* and *Truperella pyogenes* contribute to the clinical picture [8,10,13,14]. These may cause various forms of pneumonia with an acute, subacute, or chronic course. The different forms of disease representation include mainly fibrinous pleuropneumonia, which is the most common form of acute pneumonia in weaned, stressed beef cattle, and suppurative bronchopneumonia often seen in young dairy calves [15,16].

Suitable preventive measures start from the management of young calves and comprise an adequate colostrum supply [17]. Optimized housing conditions with appropriate ventilation that provide adequate air exchange also show preventive effects on BRD [18–20]. Vaccination against both bacterial and viral pathogens is a valuable prevention measure and leads to improved animal health and fewer economic losses [21–23]. However, antibiotic treatment is indicated for controlling acute bacterial infection and the emerged BRD syndrome [23]. In Germany, the approved classes of antibiotic agents aiming at the treatment of respiratory diseases with bacterial origin include ß-lactam antibiotics, fluoroquinolones, phenicols, tetracyclines, trimethoprim-sulphonamides, aminoglycosides, lincosamides, and macrolides, respectively [24]. Besides the treatment of individual diseased animals, metaphylactic medication of all animals within one epidemiological flock is important in this context [25–27]. Metaphylaxis may include antibiotic treatment of clinically healthy animals, if they had close contact with already infected animals, as these are likely to be infected [28].

Worldwide studies indicate that there is a trend towards increasing bacterial resistance towards certain antimicrobial agents, especially multidrug-resistance (MDR) when pathogens of BRD are investigated [29–37]. In the context of the BRD complex, *P. multocida* and *M. haemolytica* isolates are categorized as MDR if they are not susceptible (resistant or intermediate) to at least one agent in at least three antimicrobial classes [38]. Exposure, overuse, or even misuse of antimicrobial substances do provide evolutionary advantages and may result in resistant bacteria [39–41]. Resistance genes spread between pathogens from the bovine respiratory tract. Two forms of horizontal gene transfer appear to play a key role, namely plasmids and so-called integrative and conjugative elements (ICEs) [30,33,42]. The latter contains an entire collection of resistance genes that may transfer horizontally within one single event between strains, species, and even different bacterial genera [43–45].

The alarming increase of antimicrobial resistance (AMR) in both, human and veterinary medicine, as well as the fact that antimicrobial resistant strains do circulate between humans and animals, set the impulse for the World Health Organization (WHO) to adopt a global action plan against increasing antimicrobial resistance in 2015 [46,47]. The primary goal was to ensure that the treatment and therapy of infectious diseases in both human and veterinary medicine will remain effective in the future. Therefore, WHO statements plead for responsible and prudent use of antimicrobial substances [46]. In Germany, this global action plan of the WHO was implemented in the so-called German Antibiotic Resistance Strategy (DARTS) in 2008, and thoroughly followed since then. Important elements of this strategy were the establishment of monitoring systems for the detection of AMR as well as new legal regulations, such as the documentation of antibiotic consumption levels, the determination of therapy frequency in fattening farms, as well as the obligation of antimicrobial resistance testing under certain conditions for veterinarians [48,49].

The aim of this study was to complement the already existing resistance monitoring programs, to record current trends in the development of AMR and MDR with regard to bacterial pathogens of BRD in Bavaria over the last 5 years and finally to derive treatment recommendations from this. Furthermore, the influence of epidemiological parameters, such as farm type and farm size on the resistance pattern were investigated.

2. Results

2.1. Bacterial Isolates

Between July 2015 and June 2020, a total of 754 isolates were collected from 662 animals with suspected BRD syndrome, origination from 519 farms were included in the present

study. *P. multocida* was the most frequently isolated pathogen with 345 (45.76%), followed by 273 *M. haemolytica* (36.21%), 119 *T. pyogenes* (15.78%), and 17 *B. trehalosi* (2.25%) isolates (Table 1 and Figure 1).

Table 1. Species, absolute and (relative) number of isolates investigated in the present study over the five-year period 2015–2020 in Bavaria, Germany.

	2015/2016 n (%)	2016/2017 n (%)	2017/2018 n (%)	2018/2019 n (%)	2019/2020 n (%)	Total
P. multocida	54 (49.09)	43 (34.96)	70 (46.36)	91 (46.19)	87 (50.29)	345 (45.76)
M. haemolytica	41 (37.27)	52 (42.28)	57 (37.75)	73 (37.06)	50 (28.90)	273 (36.21)
T. pyogenes	14 (12.73)	28 (22.76)	21 (13.90)	27 (13.70)	29 (16.76)	119 (15.78)
B. trehalosi	1 (0.91)	0 (0.00)	3 (1.99)	6 (3.05)	7 (4.05)	17 (2.25)
Total isolates	110 (100)	123 (100)	151 (100)	197 (100)	173 (100)	754 (100)

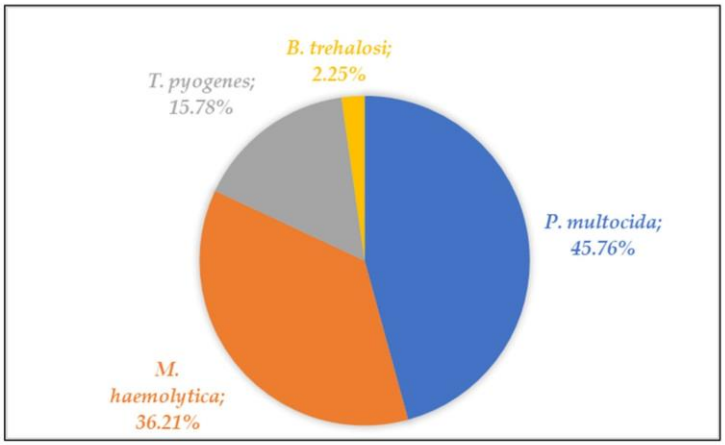

Figure 1. Overall proportion (%) of pathogens detected among the total number of analyzed samples.

2.2. Five-Year Antimicrobial Susceptibility

Low resistance rates with a proportion of not susceptible isolates of less than five percent were found for *P. multocida* isolates (n = 345) in the case of cephalosporin class (ceftiofur), penicillin class (penicillin G), phenicol class (florfenicol), and fluoroquinolone class (enrofloxacin) (Table 2 and Supplementary Table S1). The fraction of not susceptible isolates was higher for the macrolide antibiotic tulathromycin (15.65%) (Tables 2 and S1). The highest proportion of not susceptible *P. multocida* isolates was found for tetracycline (39.42%), and spectinomycin (78.84%) (Tables 2 and S1).

The proportion of not-susceptible *M. haemolytica* isolates (n = 273) collected over the five-year range was below five percent for ceftiofur, for penicillin G, for enrofloxacin, for florfenicol, and for tulathromycin. It was slightly higher for the macrolide compound tilmicosin with 6.59% (Table 2 and Supplementary Table S2). The highest not susceptibility rates were found when isolates were tested with tetracycline (21.25%), and the aminocyclitol class compound spectinomycin, 80.95% (Tables 2 and S2).

For *T. pyogenes* isolates (n = 119) and *B. trehalosi* isolates (n = 17) no defined species-specific minimum inhibitory concentration (MIC) breakpoints according to CLSI VET guidelines are available to categorize these into susceptible and not susceptible (intermediate and resistant) [50–52]. The distribution of MIC values of these two pathogens is shown in Tables S3 and S4.

Table 2. Five-year not susceptible rates of bacterial pathogens with defined species-specific breakpoints according to CLSI VET guidelines.

Antimicrobial Class	Antimicrobial Agent	*P. multocida* % (n)	*M. haemolytica* % (n)	Recommendation for Therapy [1]
cephalosporin	ceftiofur	0.87 (3/345)	0.00 (0/273)	(+/−)
penicillin	penicillin_G	3.48 (12/345)	4.76 (13/273)	(+)
phenicol	florfenicol	4.06 (14/345)	1.10 (3/273)	(+)
fluorochinolone	enrofloxacin	0.29 (1/345)	2.93 (8/273)	(+/−)
macrolide	tilmicosin	no breakpoint [2]	6.59 (18/273)	(+/−)
	tulathromycin	15.65 (54/345)	2.93 (8/273)	(+/−)
tetracycline	tetracycline	39.42 (136/345)	21.25 (58/273)	(−)
aminocyclitol	spectinomycin	78.84 (272/345)	80.95 (221/273)	(−)

[1] recommendation for therapy: (+): suitable for therapy, (+/−): partly suitable for therapy, (−): not suitable for therapy; [2] no breakpoint according to CLSI VET guidelines.

2.3. Trends in Not Susceptibility

The trend analysis of the annual not susceptibility rates pertaining to the species *P. multocida* and *M. haemolytica* revealed a decreasing tendency only for the aminocyclitol agent spectinomycin (Tables 3 and S5, Figure 2a,b). The proportion of not susceptible isolates regarding *P. multocida* isolates decreased from 88.89% in the first study year (July 2015 to June 2016) to 67.82% in the last study year (July 2019 to June 2020; OR = 0.70; 95% CI: 0.56–0.86; $p < 0.001$). Regarding *M. haemolytica* isolates it decreased from 90.24% in the first study year to 68.00% in the last study year (OR = 0.71; 95% CI: 0.55–0.90; $p = 0.005$; Tables 3 and S5; Figure 2a,b). For the investigated *P. multocida* isolates significantly increasing rates of not susceptible isolates were found within the study period for the antimicrobial agents tulathromycin (5.56% to 26.44%; OR = 1.60; 95% CI: 1.25–2.08; $p < 0.001$) and tetracycline (18.52% to 57.47%; OR = 1.62; 95% CI: 1.36–1.94; $p < 0.001$; Tables 3 and S5; Figure 2a).

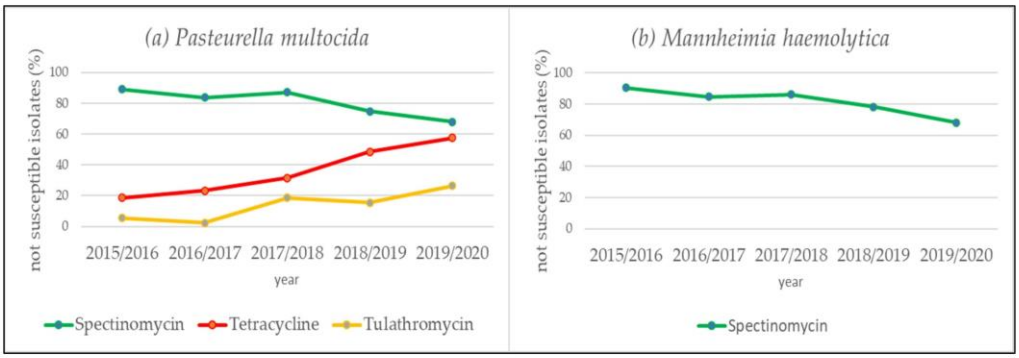

Figure 2. Statistically significant trends regarding the not susceptibility of *P. multocida* (**a**) and *M. haemolytica* (**b**) over the five-year period in Bavaria, Germany. For spectinomycin a significant decrease in not susceptibility could be observed in *P. multocida* (OR = 0.70; 95% CI: 0.56–0.86; $p < 0.001$) (**a**) and in *M. haemolytica* isolates (OR = 0.71; 95% CI: 0.55–0.90; $p = 0.005$) (**b**). For tetracycline (OR = 1.62; 95% CI: 1.36–1.94; $p < 0.001$) and tulathromycin (OR = 1.60; 95% CI: 1.25–2.08; $p < 0.001$) a significant increase in not susceptible *P. multocida* isolates could be observed (**a**).

Table 3. Statistically significant trends, decrease or increase, regarding the not susceptibility of bacterial pathogens investigated in this study over the five-year period 2015–2020 in Bavaria, Germany.

Pathogen	Antimicrobial Class	Antimicrobial Agent	2015/2016 % (n)	2016/2017 % (n)	2017/2018 % (n)	2018/2019 % (n)	2019/2020 % (n)	OR (95% CI)	*p*-Value
P. multocida	Aminocyclitol	Spectinomycin	88.89 (48/54)	83.72 (36/43)	87.14 (61/70)	74.73 (68/91)	67.82 (59/87)	0.70 (0.56–0.86)	<0.001
	Tetracycline	Tetracycline	18.52 (10/54)	23.26 (10/43)	31.43 (22/70)	48.35 (44/91)	57.47 (50/87)	1.62 (1.36–1.94)	<0.001
	Macrolide	Tulathromycin	5.56 (3/54)	2.33 (1/43)	18.57 (13/70)	15.38 (14/91)	26.44 (23/87)	1.60 (1.25–2.08)	<0.001
M. haemolytica	Aminocyclitol	Spectinomycin	90.24 (37/41)	84.62 (44/52)	85.96 (49/57)	78.08 (57/73)	68.00 (34/50)	0.71 (0.55–0.90)	=0.005

2.4. Multidrug-Resistance

In veterinary medicine, *P. multocida* and *M. haemolytica* isolates of BRD are classified as multidrug-resistant (MDR) if they are not susceptible to at least one agent in at least three antimicrobial classes [38]. Following this definition, the prevalence of MDR *P. multocida* and *M. haemolytica* isolates was determined in this study. The eight antibiotic agents penicillin G, ceftiofur, florfenicol, enrofloxacin, tilmicosin (only for *M. haemolytica*), tulathromycin, tetracycline and spectinomycin from the seven antimicrobial classes penicillins, cephalosporins, phenicols, fluoroquinolones, macrolides, tetracyclines, and aminocyclitols were included in this MDR analysis. The highest proportion of MDR-isolates was found for *P. multocida* (13.91%), whereas of the *M. haemolytica* isolates only 5.13% were categorized as MDR (Tables 4 and S6). The analysis of annual MDR rates of the bacterial pathogens showed a significant increase over the five-year period for *P. multocida* from 3.70% (first year) to 22.99% (final year) (OR = 1.61; 95% CI: 1.25–2.14; $p < 0.001$) (Figure 3 and Table S6).

Table 4. Amongst the investigated bacterial species, the absolute and (relative) number of isolates was ranked into the characteristic pan-susceptible, if these were susceptible towards all agents tested. Not susceptible isolates revealed to be resistant against at least two tested antimicrobial classes (shaded in light grey), and multidrug-resistant (MDR) isolates revealed to be resistant against three or more tested antimicrobial classes (shaded in grey).

Pathogen	Number of Isolates	Category/Number of Antimicrobial Classes towards Isolates Were Not Susceptible							
		Pan-Susceptible	Not Susceptible		MDR				
		0	1	2	3	4	5	6	7
P. multocida	345 (100%)	52 (15.07%)	159 (46.09%)	86 (24.93%)	37 (10.72%)	6 (1.74%)	4 (1.16%)	1 (0.29%)	0 (0%)
M. haemolytica	273 (100%)	33 (12.09%)	176 (64.47%)	50 (18.32%)	11 (4.03%)	3 (1.10%)	0 (0%)	0 (0%)	0 (0%)

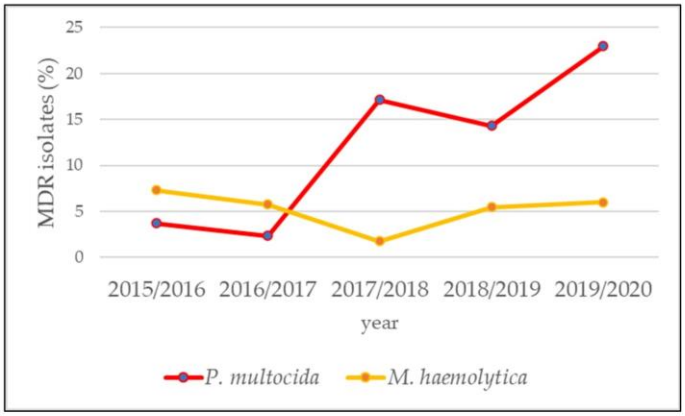

Figure 3. Annual multidrug-resistance (MDR) rates of the bacterial pathogens *P. multocida* and *M. haemolytica* from cattle with bovine respiratory disease (BRD) in Bavaria, Germany. A significant increase of MDR *P. multocida* isolates could be observed over the five-year period 2015–2020 (OR = 1.61; 95% CI: 1.25–2.14; $p < 0.001$).

2.5. Additional Epidemiological Investigations

Further epidemiological investigations were carried out including the 618 MDR *P. multocida* and *M. haemolytica* isolates. Information on the distribution of animal and farm characteristics is displayed in Tables S7 and S8. Most isolates originated from male animals (56.63%), one to two months old (34.95%) and diseased due to BRD (44.98%). PI-3, *My-*

coplasma species and BRSV were detected in 4.05%, 15.37% and 12.94% of the isolates. Most isolates are derived from farms in Upper Bavaria (30.58%), with 101 to 300 animals (58.41%), fattening farms (50.97%) and farms with a therapy frequency of five or less (20.06%).

The results of the univariable and multivariable logistic regression to determine the association of occurrence of MDR *P. multocida* and *M. haemolytica* isolates with certain farm or animal characteristics are shown in Table S7. Regarding the individual animal characteristics, neither sex and age nor the detection of *M. bovis* or PI-3 and BRSV were statistically significantly associated with the occurrence of MDR (Table S7). Additionally, the odds of the occurrence of MDR were not significantly higher among animals, which had died due to the BRD complex as compared to animals that had survived the disease (Table S7). Among the farm characteristics, neither the geographical location in one of the seven administrative districts nor the farm antibiotic therapy frequency was statistically significantly associated with the occurrence of MDR isolates (Table S7). There was a significant association between the occurrence of MDR *P. multocida* and *M. haemolytica* isolates and the size of a farm (Figure 4a). In farms with more than 300 animals, the odds for MDR isolates were significantly higher as compared to farms with a size of 100 animals or less (Adjusted OR = 2.89; 95% CI: 1.26–7.29; $p = 0.017$; Table S7). Our analysis showed that in farms with 100 animals or less, 6.92% of all isolates were MDR, on farms with 101 to 300 animals 8.31% were MDR, while on farms with more than 300 animals 19.49% of all isolates were MDR (Figure 4a).

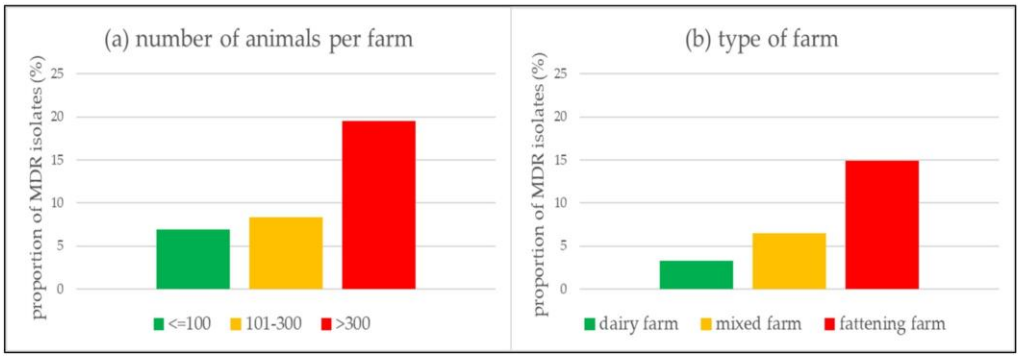

Figure 4. Proportion of multidrug-resistant (MDR) *P. multocida* and *M. haemolytica* isolates depending on farm size (number of animals per farm) (**a**) and type of farm (**b**). In farms with more than 300 animals, the odds for isolating MDR isolates were significantly higher than in farms with 100 or less animals (Adjusted OR = 2.89; 95% CI: 1.26–7.29; $p = 0.017$). In addition, the odds for isolating MDR isolates were significantly lower in dairy (aOR = 0.23; 95% CI: 0.08–0.54; $p = 0.002$) and mixed farms (aOR = 0.46; 95% CI: 0.20–0.93; $p = 0.042$) than in fattening farms.

In addition, the odds for isolating MDR isolates were significantly lower in dairy farms (aOR = 0.23; 95% CI: 0.08–0.54; $p = 0.002$) and mixed farms (aOR = 0.46; 95% CI: 0.20–0.93; $p = 0.042$) as compared to in pure fattening farms (Table S7). Only 3.29% of isolates in dairy farms were MDR, 6.52% were MDR in mixed farms, while in fattening farms 14.92% were MDR (Figure 4b).

3. Discussion

3.1. New Legal Regulations and Increase in Tested Isolates

As a response to the increasing trend in the emergence of resistant pathogens and WHO's global action plan on AMR, the German Antibiotic Resistance Strategy (DARTS) was developed and numerous legal changes have been made [46,48]. Out of these, the amendment to the 2018 "Tierärztliche Hausapothekenverordnung", a national German

law, obliges veterinarians to ensure the efficacy regarding antimicrobial therapy applying prior resistance testing under certain conditions [49]. This legal change is visible, in our study, as the number of all tested bacterial isolates sent to our laboratory has increased since the third investigation year with 151 isolates compared to 197 isolates in the following observation period (Table 1).

3.2. Therapy Guide for the Practitioner

With AMR of bacterial isolates on the rise, precise knowledge of the resistance situation at hand is essential for the targeted treatment of bacterial infections [29,30,32,34]. Since there is only an obligation to determine resistance in certain cases and antibiotic therapy must be started immediately in acute cases of the disease, the practicing veterinarian has to rely on existing data and studies on the local resistance situation [49,53]. Currently, the Germany-wide resistance monitoring program GERM-Vet as well as the Swiss therapy guide for veterinarians advises valuable treatment recommendations considering pharmacological aspects [34,54]. The present study was evaluated on reappraising these guidelines with up-to-date clinical data from Bavaria, Germany (Table 2).

3.3. Antimicrobial Agents with a Favourable Resistance Situation

The Swiss therapy guideline recommends the phenicol agent florfenicol as a first-line antibiotic for the treatment of acutely ill animals with BRD [54]. This compound offers several advantages: firstly, it has a bactericidal effect and thus has advantages over bacteriostatic agents that only inhibit growth and replication and thus depend on good immunocompetence, which may no longer be present in cattle suffering from BRD [53–56]. Secondly, one shot preparations are approved and very practical to use, as a single subcutaneous injection is sufficient [24]. In our analysis, florfenicol showed excellent efficacies for all pathogens (Table 2). However, nine of 14 florfenicol not susceptible *P. multocida* isolates were isolated in the final study year, which might indicate a tendency of upcoming resistance of this bacterial species against florfenicol and needs to be observed in detail in the future (Table S5). Similar increasing trends towards florfenicol-resistant *M. haemolytica* have been reported by the GERM-Vet data in recent years. That underlines the importance of continuous monitoring of resistance trends [34]. We conclude that the benefits of florfenicol outweigh the above-mentioned upcoming risk of resistance development and still recommend florfenicol for therapy in cattle suffering from BRD.

As second-line antibiotics, the Swiss therapy guideline recommends, among others, the use of all compounds of the ß-lactam antimicrobials, with the exception of third-generation cephalosporins, as ceftiofur [54]. In the present study, the proportion of not susceptible isolates for penicillin G regarding the investigated bacterial species was below five percent (Table 2). Consequently, we recommend penicillin G, a member of the ß-lactams, for the therapy of BRD.

At the third-line position, the Swiss therapy guide lists the 3rd and 4th generation cephalosporins, which include the antimicrobial agent ceftiofur and the fluoroquinolone antimicrobial class, including enrofloxacin [54]. It should be noted that these represent important therapeutic reserve antibiotics in human medicine against (MDR) germs, such as methicillin/oxacillin-resistant staphylococci. The WHO classifies these substances, therefore, as "highest priority critically important antimicrobials" and calls on veterinarians to use them prudently, if at all necessary [53,57]. In Germany, too, the cephalosporins of the 3rd and 4th generation as well as the fluoroquinolones are classified as reserve antibiotics and should only be used as antibiotics of last resort if no other effective compounds are available [53]. The legislator, therefore, requires resistance testing or a known resistance situation in the respective farm, which is known from previous antimicrobial susceptibility testing, for every use of these reserve antibiotics [49,53]. Given that ceftiofur is an antimicrobial of last resort, it is quite encouraging that the fraction of not susceptible isolates for *P. multocida* and *M. haemolytica* in our study was less than one percent (Table 2). Data originating from a current North American study regarding feedlot cattle show that the re-

sistance rates for ceftiofur, which are between 3% and 33% for *P. multocida* and between 0% and 4% for *M. haemolytica*, were substantially higher compared to our study from Bavaria (Table 2) [30]. The outcome of the resistance situation regarding the fluoroquinolone agent enrofloxacin is also favorable (Table 2). Given that both ceftiofur and enrofloxacin are important therapeutic reserves, these two antimicrobial agents can only be recommended for therapy to a limited and well-considered extent (Table 2).

3.4. Antimicrobial Agents with Unfavorable Resistance Situation

In the Swiss therapy guidelines, the agent tetracycline is mentioned as a second-line antibiotic for the treatment of acutely ill animals and as an antimicrobial substance for metaphylaxis. However, it is pointed out that its efficacy is limited due to a considerable AMR rate [54]. The latter has been described for BRD pathogens already since the 1990s and was confirmed since then in studies from all over the world [29,30,34,35]. The unfavorable resistance situation was also reflected in the present study. 39.42% of *P. multocida* and 21.25% of *M. haemolytica* isolates were revealed to be not susceptible (Table 2). For *P. multocida*, a significant increase of the portion of not susceptible isolates from 18.52% in the first study year up to 57.47% in the last study year was found (Tables 3 and S5, Figure 2a). Reasons for the decrease in efficacy could be the high use of this compound. In North American feedlots, tetracycline is one of the most frequently used antibiotics for the treatment of BRD, but also for the prevention of liver abscesses [30,31]. In Germany, tetracycline is in terms of volume the most frequently used antibiotic for calves and cattle kept in fattening farms [58]. Due to the demonstrated increase in resistance levels and very poor efficacy, the general use of tetracycline in calf and cattle fattening should be reconsidered and can, therefore, not be recommended for the therapy of BRD (Table 2).

Antibiotics from the macrolide class are also listed as agents for metaphylactic treatment in the Swiss therapy guidelines [54]. We observed a significant increase in not susceptible *P. multocida* isolates from 5.56% in the first study year up to 26.44% in the last study year regarding tulathromycin (Tables 3 and S5, Figure 2a). Within the scope of the Germany-wide resistance monitoring GERM-Vet, an increase of resistant *P. multocida* isolates from 3% in 2016 up to 14% in 2018 was also detected and currently confirms the trend towards a higher resistance rate against tulathromycin [34]. It is of particular concern that although this compound has been authorized in Europe by the European Medicines Agency only since 2003, its resistance situation has increased so rapidly within only few years [59]. This fact is furthermore worrying, as tulathromycin is not only approved and used for therapy but also for metaphylactic treatment [24,30,33]. There is a strong accumulation in inflamed lung tissue and also accomplishes a concentration above the minimum inhibitory concentration (MIC) of over seven days after a single subcutaneous injection [24,60]. Such one-shot preparations, therefore, offer enormous advantages purely from a hands-on point of view, since an animal only needs to be treated once at a time. Nevertheless, it should be emphasized that this antimicrobial class also belongs to the "highest priority critically important antimicrobials" defined by the WHO and represent one of a few available therapeutic options for serious bacterial infections [57]. In this context, the use of tulathromycin in metaphylaxis should also be reconsidered (Table 2).

Spectinomycin from the aminocyclitol class cannot be recommended for the treatment of BRD due to an unfavorable resistance situation (Table 2). With a proportion of 78.84% not susceptible *P. multocida* and 80.95 % not susceptible *M. haemolytica* isolates, spectinomycin represents the antimicrobial agent with the highest proportion of not susceptible isolates in our analysis (Table 2). Moreover, spectinomycin is not among the most frequently used compounds in calf and cattle fattening, and the consumption quantities did not increase in recent years [30,58]. However, a significant decrease of not susceptible isolates against spectinomycin from 88.89% to 67.82% and 90.24% to 68.00% could be seen in *P. multocida* and *M. haemolytica* isolates (Tables 3 and S5, Figure 2a,b).

3.5. Multidrug-Resistance

As described above, eight antibiotic agents from seven antimicrobial classes were included in the MDR analysis because they have species-specific breakpoints according to the CLSI VET guidelines [50]. However, it must be mentioned that there are other antibiotic agents from these seven classes with species-specific minimum inhibitory concentration (MIC) breakpoints for respiratory diseases in cattle. For example, the agent tildipirosin and gamithromycin from the macrolide class or danofloxacin from the fluoroquinolone class were not tested for susceptibility in our laboratory despite the presence of species-specific breakpoints and thus could not be included in the MDR analysis. Ampicillin could also not be included in the analysis because the inhibitory concentrations on the microtiter plate we used were in a higher range than the breakpoints set by CLSI [50]. Since we did not test all antibiotic agents with defined breakpoints for susceptibility, it must be assumed that even more isolates in our data set could be characterized as MDR. A notable finding in our study was the higher rate of MDR *P. multocida* isolates (13.91%) compared to *M. haemolytica* isolates (5.13%) (Tables 4 and S6). This effect was explained in prior publications by different gene transfer and integration rates, or the persistence of ICEs hosting resistance genes and regarding diverse bacterial species [30]. However, the rate of MDR *P. multocida* isolates increased significantly from 3.70% in 2015/2016 to 22.99% in 2019/2020 in the present study (Figure 3, Table S6). This most alarming result was observed also in isolates from North America and illustrates that increasing AMR is a worldwide problem [30,37]. Resistance levels in North America appear high, with proportions of MDR *P. multocida* isolates exceeding 90% and proportions of MDR *M. haemolytica* isolates exceeding 80%, respectively [30]. These numbers exceed those determined in the present study for Bavarian farms (Figure 2, Table S7). It must be mentioned, however, that it is difficult to compare MDR prevalences from different studies, as MIC breakpoints other than those specific to veterinary medicine are often used to divide isolates into susceptible, intermediate and resistant [38].

3.6. Additional Epidemiological Investigations

The investigation of further epidemiological parameters concluded that no animal characteristics were associated with a higher probability of occurrence of MDR *P. multocida* and *M. haemolytica* isolates (Table S7). However, it was seen that the odds for MDR isolates were significantly lower in dairy farms (aOR = 0.23; 95% CI: 0.08–0.54; $p = 0.002$) and mixed farms (aOR = 0.46; 95% CI: 0.20–0.93; $p = 0.042$) compared to fattening farms (Table S7, Figure 4b). The reasons why the resistance problem mainly affects fattening farms can only be speculated and requires further research. However, it is known that the stressful transport from a dairy farm, birthplace, to the fattening farm, as well as the assortment of calves from many individual farms of origin, increases the risk of BRD and thus the need for antimicrobial treatment [7,9,23]. In the U.S., such groups of animals at increased risk for BRD are treated metaphylactically upon arrival in the feedlot to reduce morbidity and mortality rates and achieve better fattening results [25–27,30,31]. In Germany, metaphylactic treatment of an entire group of animals is also permitted. However, it requires diseased animals within this group that show clinical signs and the concern that the healthy animals in the group will also rapidly become ill [28,53]. In prior studies investigating the metaphylactic use of antimicrobial agents in groups of animals, it was shown that the administration of antimicrobial agents favored the shedding of MDR isolates and increased the likelihood of finding such MDR isolates in stablemates after contagious spreading [61,62]. Equally important to mention in this context is the small farm structure of dairy farms within Bavaria with an average herd size of 40 dairy cows per farm [63]. On these farms, calves are often kept individually in calf hutches during the first weeks of life. On the one hand, this is associated with a lower risk of developing BRD and possibly results in a more targeted individual antimicrobial treatment for various diseases compared to the situation in fattening farms [64–66]. There, the beef cattle are kept in groups and subsequently treated possibly as an epidemiologic unit [25–27,30,31]. In

order to limit antimicrobial metaphylaxis and the resulting development of AMR, German law requires laboratory diagnostics including pathogen identification and AMR testing in the case of repeated use of antibiotics in certain age groups and production steps [49].

In addition to the type of farm, the size of the farm is also a critical variable (Figure 4a). In the present study, the odds for MDR isolates were significantly higher on farms with more than 300 animals than on farms with 100 animals or less (aOR = 2.89; 95% CI: 1.26–7.29; $p = 0.017$; Table S7, Figure 4a). At the same time, data from the Federal Ministry of Agriculture and Food show that the frequency of antimicrobial treatment in recent years has been higher for farms with a larger number of animals, and thus more frequently treated with antimicrobials, than on farms with a smaller number of animals [58]. It remains speculative why antimicrobials are used more frequently on farms with a higher number of animals and whether this influenced the higher probability of the presence of MDR isolates. One possible explanation could be that farms with smaller animal numbers have better control of infectious diseases resulting in better individual animal treatment [66]. Other studies have shown that a smaller number of individual animals per group in the animal husbandry departments is advantageous, as the risk of BRD infection increases with the number of animals per group [64,65].

In the present study, there was no statistically significant association between the frequency of therapy and the occurrence of MDR isolates (Table S7). It needs to be mentioned, however, that the values of the treatment frequency only refer to the respective half-year of sampling, but the fattening period lasts more than six months and the values in the preceding or following half-year could differ markedly from the one considered in the analysis. Furthermore, the treatment frequency refers to the entire farm, so it is possible that the animals in our analysis were kept in a barn compartment where fewer antimicrobials were applied. In addition, the treatment frequency refers to all antimicrobials used in the half-year and thus also includes treatments against other diseases. The value of the farm treatment frequency in our study is, therefore, maybe less suitable as an indicator of antimicrobial consumption.

3.7. Limits of the Study

The samples analyzed in the study include samples from the upper respiratory tract, such as nasal swabs but also samples from the lower respiratory tract, such as organ samples from necropsy or bronchoalveolar lavage fluid. However, there is evidence that cultures of nasal swabs from the upper airways are not representative of the pathogen in the lower airways [67]. One study revealed that although samples from both the upper and lower airways were positive for *M. haemolytica*, only 77% showed an identical pulse field gel electrophoresis type [67]. We cannot rule out that the isolates originating from nasal swabs in our study may not be responsible for the clinical picture of BRD.

Another disadvantage of the study is that it is not known whether or how often antimicrobial treatment was applied before sampling. The extent to which immediate antimicrobial treatment before sampling influences the resistance pattern is also controversially discussed in other studies [30,33,61,62]. However, it could be that a previous antimicrobial treatment exerts a selection pressure towards more resistant strains and that the original microbial flora is not represented in these samples.

Another important point to mention is that our study is not an analysis with a clearly defined sampling plan, as is the case, for example, in the national resistance monitoring GERM-Vet, but is a retrospective evaluation of all isolates sent in [34]. Therefore, and following previous publications, only a single individual of each species was included per quarter of a year per farm in our analysis to prevent bias and overrepresentation of clonal isolates [36,68]. Nevertheless, there could also be a potential geographical bias in our dataset, as described in other studies, because our study only includes isolates from Bavaria, a single state of Germany, and even within Bavaria, more samples in our analysis originate from the southern districts than from the northern ones (Supplementary Materials Tables S7 and S8) [30,36,37].

Finally, it should be mentioned that additional molecular screening for AMR genes, as also carried out in recent publications, could provide further insights, especially with regard to the role of ICEs in the spread of MDR isolates and should be part of future endeavors [30,33].

4. Materials and Methods

4.1. Study Design and Origin of Animals

Data included in the present study were collected within the scope of the state veterinary laboratory diagnostics at the Bavarian Health and Food Safety Authority. In the present study, the investigated samples originated from calves, cattle, or dairy cows with putative symptoms of BRD in Bavaria, Germany, from July 2015 to June 2020. In the present study, cows were kept in dairy farms solely for the purpose of milk production, in fattening farms, cattle were kept for meat production, and finally, in mixed farms, both categories of animals were kept. In order to prevent bias and over-representation of clonal isolates, only one isolate of a species per farm per quarter year was included in the data set, following previous publications [36,68].

4.2. Bacterial Isolates

The specimens, here nasal swabs, bronchoalveolar lavage fluid, or lung tissue samples, were analyzed in the ISO 17025 accredited laboratory at the Bavarian Health and Food Safety Authority. Samples were initially inoculated on Columbia sheep blood agar (Oxoid, Wesel, Germany) and incubated at 37 °C for 24 to 48 h under aerobic conditions as well as under a microaerophilic atmosphere, at 10% CO_2. To isolate pure suspicious colonies of *P. multocida*, *M. haemolytica*, *B. trehalosi* or *T. pyogenes*, fresh subcultures were incubated under the above-described conditions. Identification of bacterial species was carried out using MALDI-TOF MS (Bruker, Bremen, Germany).

Regarding the isolation of *Mycoplasma* species, animal samples were inoculated in specific Thermo Scientific™ Mycoplasma/Ureaplasma Broth that inhibits the growth of most gram-negative, gram-positive bacteria, as well as yeasts (Thermo Scientific, Schwerte, Germany), and incubated microaerophilic for 120 h at 37 °C with 10% CO_2.

4.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was carried out according to the protocols published in VET01 5th edition, VET01S 5th edition and VET06 1st edition, by the Clinical and Laboratory Standards Institute (CLSI), Wayne, PA, USA [50–52]. The microbroth dilution method was carried out on 16 different antibiotic substances as commercially available and according to the manufacturer's instructions (Micronaut-S, Grosstiere 4, Merlin, Bruker, Bornheim, Germany). This panel was designed to test on recommended antibiotics for the treatment of farm animals in Germany. The minimum inhibitory concentration (MIC) of each isolate and antimicrobial substance was metered using a photometric plate reader system (Micronaut scan, MCN6 software, Merlin, Bruker, Bornheim, Germany). Subsequently, the MIC value was reconciled with determined species-specific breakpoints to categorize the respective *M. haemolytica* and *P. multocida* isolates into "susceptible", "intermediate" and "resistant" for the tested antimicrobial agents: ceftiofur, penicillin G, florfenicol, enrofloxacin, tilmicosin (only *M. haemolytica*), tulathromycin, tetracyclin and spectinomycin [50]. For the antibiotic agent amoxicillin clavulanic acid, cephalotin, trimethoprim-sulfamethoxazole, colistin, tiamulin, erythromycin and gentamicin, no species-specific breakpoints for bovines with BRD are available. Specific breakpoints for *T. pyogenes* and *B. trehalosi* have not been published by the CLSI for any of the tested antibiotic agents for veterinary medicine either, so that only the distribution of the MIC can be given [50–52].

Regarding the BRD syndrome, *P. multocida* and *M. haemolytica* were termed MDR isolates if they were not susceptible (intermediate and resistant) to at least one antibiotic substance in three or more antimicrobial classes [38]. Following this definition, our

study investigated the prevalence of MDR *P. multocida* and *M. haemolytica* isolates under epidemiological aspects.

4.4. Viral Isolates

Within the scope of the diagnostic services at the Bavarian Health and Food Safety Authority, Germany, results on further viral pathogens were incorporated regarding BRSV and PI-3.

4.5. Epidemiological Data

In addition to the isolated pathogens and respective resistance, epidemiological data on the isolated was collected, including sex of the animal, age of the animal, geographical location of the farm, type of farm, herd size of the farm and antimicrobial therapy frequency of the farm, respectively. Furthermore, it was investigated whether the animal died because of BRD. Data were obtained from the German database "Herkunftssicherungs- und Informationssystem für Tiere" (HIT). The HIT database contains comprehensive data on every single animal, including date of birth, sex, date of death and the status of animal diseases, such as BHV-1. For reasons of animal traceability, the database minutely reveals dates and addresses of trading procedures. Extra data pertaining to farms, such as the geographical location, the age and sex statistics on herds, the number of animals and the corresponding antimicrobial therapy frequency were also be downloaded. All results on animals were connected to the unique ear tag number that is assigned to each animal in the HIT database. It further allowed linking the respective farm characteristics from the HIT database, even beyond the death of an animal. Death due to BRD was defined as death within 14 days after diagnosis, assuming a median recovery time from BRD of 14 days [8]. All data on farms included in the study were determined retrospectively for the initial sampling date. The geographical location of the farm was extracted on administrative district level in Bavaria, here, North Bavaria (Upper, Middle, Lower Franconia and Upper Palatinate), Lower Bavaria, Upper Bavaria, or Swabia. The classification into the type of farm was made by us on the basis of the age and gender statistics in the HIT database. A farm was defined as a dairy farm if it had female animals with calving and male animals only up to the age of four months. If male animals over four months of age were recorded in addition to female animals with calving, we assumed that this farm with cows and female offspring also kept male animals for fattening and, therefore, the farm is categorized as a mixed farm with milk production and beef production. Farms were defined as fattening farms if they kept only male animals or female animals that had not reached first calving age and were, therefore, not used for milk production. Therapy frequency per half-year represents an indicator of the use of antibiotics. It is calculated by multiplying the number of animals treated by the number of treatment days for each active substance used. The sum of all these multiplications per half-year is then divided by the average number of animals kept in the corresponding half-year. In Germany, this parameter is notified officially regarding fattening farms with more than 20 animals since the 16th Amendment to the Medicinal Products Act in 2014 [58,69].

4.6. Statistical Analysis

First, the proportion of isolates containing *M. haemolytica* and *P. multocida*, respectively, per year and for the whole study period was determined. Next, the proportion of not susceptible/MDR isolates was calculated. To investigate whether to proportion of not susceptible/MDR isolates changed over the course of the study period, univariable logistic regression analyses were conducted using the year of sampling as an independent variable. To determine what animal and farm factors are associated with MDR, we conducted multivariable logistic regression analyses. Therefore, the univariable effects of the year the sample was taken, the presence of other pathogens in the isolate (*Mycoplasma* species, BRSV and PI-3), age, sex and disease outcome (diseased vs. deceased) of the animal, as well as region, type (dairy vs. fattening farm), size and therapy frequency of the farm

were assessed. Factors with a p-value ≥ 0.2 were considered for the multivariable model. The most parsimonious model was determined in a stepwise, forward-selection process. All analyses were conducted in R Statistical Software (R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2021).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10121538/s1, Table S1: Distribution of minimum inhibitory concentration (MIC) values over the five-year period 2015–2020 for *Pasteurella multocida* in Bavaria, Germany, Table S2: Distribution of minimum inhibitory concentration (MIC) values over the five-year period 2015–2020 for *Mannhemia haemolytica* in Bavaria, Germany, Table S3: Distribution of minimum inhibitory concentration (MIC) values over the five-year period 2015–2020 for *Trueperella pyogenes* in Bavaria, Germany, Table S4: Distribution of minimum inhibitory concentration (MIC) values over the five-year period 2015–2020 for *Bibersteinia trehalosi* in Bavaria, Germany, Table S5: Annual not susceptibility rates of *Pasteurella multocida* and *Mannheimia haemolytica* over the five-year period 2015–2020 in Bavaria, Germany, Table S6: Annual multidrug-resistance (MDR) rates of *P. multocida* and *M. haemolytica* over the five-year period 2015–2020 in Bavaria, Germany, Table S7: Association between the occurrence of multidrug-resistant (MDR) *Pasteurella multocida* and *Mannheimia haemolytica* isolates and certain animal and farm characteristics over the five-year period 2015–2020 in Bavaria, Germany, Table S8: Overview of *Pasteurella multocida* and *Mannheimia haemolytica* isolates included in the study with corresponding resistance pattern, animal characteristics and farm characteristics.

Author Contributions: Conceptualization, A.M., R.K.S. and J.M.R., methodology, M.H. and M.S.; software, S.v.d.B., M.H., M.S., D.M. and J.M.R.; validation, A.M., S.v.d.B., N.S., A.F., R.K.S., D.M. and J.M.R.; formal analysis, A.M., S.v.d.B., D.M. and J.M.R.; investigation, A.M., S.v.d.B., A.F., D.M. and J.M.R.; resources, M.H., M.S. and J.M.R.; data curation, A.M., M.H., M.S. and J.M.R.; writing—original draft preparation, A.M., A.F. and J.M.R.; writing—review and editing, S.v.d.B., N.S., R.K.S. and D.M.; visualization, A.M. and J.M.R.; supervision, R.K.S. and J.M.R.; project administration, J.M.R.; funding acquisition, M.H. and J.M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors are grateful to the veterinary bacteriology staff members for technical assistance. Furthermore, the authors are grateful to the staff of the StabLab, Ludwig-Maximilians-University Munich, for a basic teaching class in statistical analyses.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Hilton, W.M. BRD in 2014: Where have we been, where are we now, and where do we want to go? *Anim. Health Res. Rev.* **2014**, *15*, 120–122. [CrossRef]
2. Dubrovsky, S.A.; Van Eenennaam, A.L.; Karle, B.M.; Rossitto, P.V.; Lehenbauer, T.W.; Aly, S.S. Bovine respiratory disease (BRD) cause-specific and overall mortality in preweaned calves on California dairies: The BRD 10K study. *J. Dairy Sci.* **2019**, *102*, 7320–7328. [CrossRef]
3. Snowder, G.D.; Van Vleck, L.D.; Cundiff, L.V.; Bennett, G.L. Bovine respiratory disease in feedlot cattle: Environmental, genetic, and economic factors. *J. Anim. Sci.* **2006**, *84*, 1999–2008. [CrossRef]
4. Teixeira, A.G.V.; McArt, J.A.A.; Bicalho, R.C. Thoracic ultrasound assessment of lung consolidation at weaning in Holstein dairy heifers: Reproductive performance and survival. *J. Dairy Sci.* **2017**, *100*, 2985–2991. [CrossRef] [PubMed]
5. Dunn, T.R.; Ollivett, T.L.; Renaud, D.L.; Leslie, K.E.; LeBlanc, S.J.; Duffield, T.F.; Kelton, D.F. The effect of lung consolidation, as determined by ultrasonography, on first-lactation milk production in Holstein dairy calves. *J. Dairy Sci.* **2018**, *101*, 5404–5410. [CrossRef] [PubMed]
6. Bach, A. Associations between several aspects of heifer development and dairy cow survivability to second lactation. *J. Dairy Sci.* **2011**, *94*, 1052–1057. [CrossRef]
7. Sanderson, M.W.; Dargatz, D.A.; Wagner, B.A. Risk factors for initial respiratory disease in United States' feedlots based on producer-collected daily morbidity counts. *Can. Vet. J.* **2008**, *49*, 373–378.

8. Grissett, G.P.; White, B.J.; Larson, R.L. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. *J. Vet. Intern. Med.* **2015**, *29*, 770–780. [CrossRef] [PubMed]
9. Ishizaki, H.; Kariya, Y. Road transportation stress promptly increases bovine peripheral blood absolute NK cell counts and cortisol levels. *J. Vet. Med. Sci.* **2010**, *72*, 747–753. [CrossRef]
10. Headley, S.A.; Okano, W.; Balbo, L.C.; Marcasso, R.A.; Oliveira, T.E.; Alfieri, A.F.; Negri Filho, L.C.; Michelazzo, M.Z.; Rodrigues, S.C.; Baptista, A.L.; et al. Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. *J. Vet. Diagn. Investig.* **2018**, *30*, 249–251. [CrossRef]
11. Lopez, A.; Thomson, R.G.; Savan, M. The pulmonary clearance of Pasteurella hemolytica in calves infected with bovine parainfluenza-3 virus. *Can. J. Comp. Med.* **1976**, *40*, 385–391. [PubMed]
12. Gershwin, L.J.; Gunther, R.A.; Hornof, W.J.; Larson, R.F. Effect of infection with bovine respiratory syncytial virus on pulmonary clearance of an inhaled antigen in calves. *Am. J. Vet. Res.* **2008**, *69*, 416–422. [CrossRef] [PubMed]
13. Kishimoto, M.; Tsuchiaka, S.; Rahpaya, S.S.; Hasebe, A.; Otsu, K.; Sugimura, S.; Kobayashi, S.; Komatsu, N.; Nagai, M.; Omatsu, T.; et al. Development of a one-run real-time PCR detection system for pathogens associated with bovine respiratory disease complex. *J. Vet. Med. Sci.* **2017**, *79*, 517–523. [CrossRef] [PubMed]
14. Anton, B.P.; Harhay, G.P.; Smith, T.P.; Blom, J.; Roberts, R.J. Comparative Methylome Analysis of the Occasional Ruminant Respiratory Pathogen Bibersteinia trehalosi. *PLoS ONE* **2016**, *11*, e0161499. [CrossRef]
15. Panciera, R.J.; Confer, A.W. Pathogenesis and pathology of bovine pneumonia. *Vet. Clin. N. Am. Food Anim. Pract.* **2010**, *26*, 191–214. [CrossRef] [PubMed]
16. Andrews, G.A.; Kennedy, G.A. Respiratory diagnostic pathology. *Vet. Clin. N. Am. Food Anim. Pract.* **1997**, *13*, 515–547. [CrossRef]
17. Virtala, A.M.; Gröhn, Y.T.; Mechor, G.D.; Erb, H.N. The effect of maternally derived immunoglobulin G on the risk of respiratory disease in heifers during the first 3 months of life. *Prev. Vet. Med.* **1999**, *39*, 25–37. [CrossRef]
18. Ollivett, T.L. How Does Housing Influence Bovine Respiratory Disease in Dairy and Veal Calves? *Vet. Clin. N. Am. Food Anim. Pract.* **2020**, *36*, 385–398. [CrossRef] [PubMed]
19. Lago, A.; McGuirk, S.M.; Bennett, T.B.; Cook, N.B.; Nordlund, K.V. Calf respiratory disease and pen microenvironments in naturally ventilated calf barns in winter. *J. Dairy Sci.* **2006**, *89*, 4014–4025. [CrossRef]
20. Nordlund, K.V.; Halbach, C.E. Calf Barn Design to Optimize Health and Ease of Management. *Vet. Clin. N. Am. Food Anim. Pract.* **2019**, *35*, 29–45. [CrossRef]
21. Wildman, B.K.; Perrett, T.; Abutarbush, S.M.; Guichon, P.T.; Pittman, T.J.; Booker, C.W.; Schunicht, O.C.; Fenton, R.K.; Jim, G.K. A comparison of 2 vaccination programs in feedlot calves at ultra-high risk of developing undifferentiated fever/bovine respiratory disease. *Can. Vet. J.* **2008**, *49*, 463–472. [PubMed]
22. Schunicht, O.C.; Booker, C.W.; Jim, G.K.; Guichon, P.T.; Wildman, B.K.; Hill, B.W. Comparison of a multivalent viral vaccine program versus a univalent viral vaccine program on animal health, feedlot performance, and carcass characteristics of feedlot calves. *Can. Vet. J.* **2003**, *44*, 43–50.
23. Edwards, T.A. Control methods for bovine respiratory disease for feedlot cattle. *Vet. Clin. N. Am. Food Anim. Pract.* **2010**, *26*, 273–284. [CrossRef]
24. Universität Leipzig. Veterinärmedizinischer Informationsdienst für Arzneimittelanwendung, Toxikologie und Arzneimittelrecht. Available online: https://vetidata.de/public/quicksearch/do.php (accessed on 14 December 2021).
25. Baptiste, K.E.; Kyvsgaard, N.C. Do antimicrobial mass medications work? A systematic review and meta-analysis of randomised clinical trials investigating antimicrobial prophylaxis or metaphylaxis against naturally occurring bovine respiratory disease. *Pathog. Dis.* **2017**, *75*, ftx083. [CrossRef]
26. Abell, K.M.; Theurer, M.E.; Larson, R.L.; White, B.J.; Apley, M. A mixed treatment comparison meta-analysis of metaphylaxis treatments for bovine respiratory disease in beef cattle. *J. Anim. Sci.* **2017**, *95*, 626–635. [CrossRef] [PubMed]
27. Tennant, T.C.; Ives, S.E.; Harper, L.B.; Renter, D.G.; Lawrence, T.E. Comparison of tulathromycin and tilmicosin on the prevalence and severity of bovine respiratory disease in feedlot cattle in association with feedlot performance, carcass characteristics, and economic factors. *J. Anim. Sci.* **2014**, *92*, 5203–5213. [CrossRef] [PubMed]
28. European Medicines Agency (EMA). Guideline for the Demonstration of Efficacy for Veterinary Medicinal Products Containing Antimicrobial Substances. Available online: https://www.ema.europa.eu/en/documents/scientific-guideline/final-guideline-demonstration-efficacy-veterinary-medicinal-products-containing-antimicrobial_en.pdf (accessed on 7 December 2020).
29. El Garch, F.; de Jong, A.; Simjee, S.; Moyaert, H.; Klein, U.; Ludwig, C.; Marion, H.; Haag-Diergarten, S.; Richard-Mazet, A.; Thomas, V.; et al. Monitoring of antimicrobial susceptibility of respiratory tract pathogens isolated from diseased cattle and pigs across Europe, 2009-2012: VetPath results. *Vet. Microbiol.* **2016**, *194*, 11–22. [CrossRef]
30. Klima, C.L.; Holman, D.B.; Cook, S.R.; Conrad, C.C.; Ralston, B.J.; Allan, N.; Anholt, R.M.; Niu, Y.D.; Stanford, K.; Hannon, S.J.; et al. Multidrug Resistance in Pasteurellaceae Associated With Bovine Respiratory Disease Mortalities in North America From 2011 to 2016. *Front. Microbiol.* **2020**, *11*, 606438. [CrossRef] [PubMed]
31. Anholt, R.M.; Klima, C.; Allan, N.; Matheson-Bird, H.; Schatz, C.; Ajitkumar, P.; Otto, S.J.; Peters, D.; Schmid, K.; Olson, M.; et al. Antimicrobial Susceptibility of Bacteria That Cause Bovine Respiratory Disease Complex in Alberta, Canada. *Front. Vet. Sci.* **2017**, *4*, 207. [CrossRef] [PubMed]

32. Holschbach, C.L.; Aulik, N.; Poulsen, K.; Ollivett, T.L. Prevalence and temporal trends in antimicrobial resistance of bovine respiratory disease pathogen isolates submitted to the Wisconsin Veterinary Diagnostic Laboratory: 2008–2017. *J. Dairy Sci.* **2020**, *103*, 9464–9472. [CrossRef]
33. Stanford, K.; Zaheer, R.; Klima, C.; McAllister, T.; Peters, D.; Niu, Y.D.; Ralston, B. Antimicrobial Resistance in Members of the Bacterial Bovine Respiratory Disease Complex Isolated from Lung Tissue of Cattle Mortalities Managed with or without the Use of Antimicrobials. *Microorganisms* **2020**, *8*, 288. [CrossRef]
34. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL). Resistenzsituation bei Klinisch Wichtigen Tierpathogenen Bakterien. Available online: https://www.bvl.bund.de/SharedDocs/Berichte/07_Resistenzmonitoringstudie/Bericht_Resistenzmonitoring_2018.pdf?__blob=publicationFile&v=4 (accessed on 29 December 2020).
35. Welsh, R.D.; Dye, L.B.; Payton, M.E.; Confer, A.W. Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994–2002. *J. Vet. Diagn. Investig.* **2004**, *16*, 426–431. [CrossRef]
36. Portis, E.; Lindeman, C.; Johansen, L.; Stoltman, G. A ten-year (2000–2009) study of antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex–Mannheimia haemolytica, Pasteurella multocida, and Histophilus somni–in the United States and Canada. *J. Vet. Diagn. Investig.* **2012**, *24*, 932–944. [CrossRef] [PubMed]
37. Lubbers, B.V.; Hanzlicek, G.A. Antimicrobial multidrug resistance and coresistance patterns of Mannheimia haemolytica isolated from bovine respiratory disease cases–a three-year (2009-2011) retrospective analysis. *J. Vet. Diagn. Investig.* **2013**, *25*, 413–417. [CrossRef]
38. Sweeney, M.T.; Lubbers, B.V.; Schwarz, S.; Watts, J.L. Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens. *J. Antimicrob. Chemother.* **2018**, *73*, 1460–1463. [CrossRef] [PubMed]
39. Bell, B.G.; Schellevis, F.; Stobberingh, E.; Goossens, H.; Pringle, M. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. *BMC Infect. Dis.* **2014**, *14*, 13. [CrossRef]
40. World Health Organisation (WHO). WHO Global Strategy for Containment of Antimicrobial Resistance. Available online: https://www.who.int/drugresistance/WHO_Global_Strategy_English.pdf (accessed on 28 August 2021).
41. The World Organisation for Animal Health (OIE). The OIE Strategy on Antimicrobial Resistance and the Prudent Use of Antimicrobials. Available online: https://www.oie.int/fileadmin/Home/eng/Media_Center/docs/pdf/PortailAMR/EN_OIE-AMRstrategy.pdf (accessed on 14 January 2021).
42. Kadlec, K.; Watts, J.L.; Schwarz, S.; Sweeney, M.T. Plasmid-located extended-spectrum β-lactamase gene blaROB-2 in Mannheimia haemolytica. *J. Antimicrob. Chemother.* **2019**, *74*, 851–853. [CrossRef]
43. Michael, G.B.; Kadlec, K.; Sweeney, M.T.; Brzuszkiewicz, E.; Liesegang, H.; Daniel, R.; Murray, R.W.; Watts, J.L.; Schwarz, S. ICEPmu1, an integrative conjugative element (ICE) of Pasteurella multocida: Analysis of the regions that comprise 12 antimicrobial resistance genes. *J. Antimicrob. Chemother.* **2012**, *67*, 84–90. [CrossRef] [PubMed]
44. Michael, G.B.; Kadlec, K.; Sweeney, M.T.; Brzuszkiewicz, E.; Liesegang, H.; Daniel, R.; Murray, R.W.; Watts, J.L.; Schwarz, S. ICEPmu1, an integrative conjugative element (ICE) of Pasteurella multocida: Structure and transfer. *J. Antimicrob. Chemother.* **2012**, *67*, 91–100. [CrossRef]
45. Eidam, C.; Poehlein, A.; Leimbach, A.; Michael, G.B.; Kadlec, K.; Liesegang, H.; Daniel, R.; Sweeney, M.T.; Murray, R.W.; Watts, J.L.; et al. Analysis and comparative genomics of ICEMh1, a novel integrative and conjugative element (ICE) of Mannheimia haemolytica. *J. Antimicrob. Chemother.* **2015**, *70*, 93–97. [CrossRef]
46. World Health Organisation (WHO). Global Action Plan on Antimicrobial Resistance. Available online: https://www.who.int/publications/i/item/9789241509763 (accessed on 28 August 2021).
47. Berendonk, T.U.; Manaia, C.M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Bürgmann, H.; Sørum, H.; Norström, M.; Pons, M.N.; et al. Tackling antibiotic resistance: The environmental framework. *Nat. Rev. Microbiol.* **2015**, *13*, 310–317. [CrossRef]
48. Federal Ministry of Health Federal Ministry of Food and Agriculture Federal Ministry of Education and Research. DART 2020 Fighting Antibiotic Resistance for the Good of Both Humans and Animals. Available online: https://www.bmel.de/SharedDocs/Downloads/EN/Publications/DART2020.pdf;jsessionid=42432EA3DD38EA0D58BFF1DD601CCC04.live842?__blob=publicationFile&v=3 (accessed on 29 December 2020).
49. Bundesministerium der Justiz und für Verbraucherschutz. Verordnung über tierärztliche Hausapotheken (TÄHAV). Available online: https://www.gesetze-im-internet.de/t_hav/BJNR021150975.html (accessed on 15 December 2020).
50. Clinical and Laboratory Standards Institute (CSLI). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals*, 5th ed.; CLSI supplement VET01S; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2020.
51. Clinical and Laboratory Standards Institute (CLSI). *Methods for Antimicrobial Susceptibility Testing of Infrequently Isolated or fastidous Bacteria Isolated From Animals*, 1st ed.; CLSI supplement VET06; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2017.
52. Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disc and Dilution Susceptibility Tests for Bacteria isolated from Animals*, 5th ed.; CLSI standard VET01; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2018.
53. Bundestierärztekammer e.V. Leitlinien für den Sorgsamen Umgang Mit Antibakteriell Wirksamen Tierarzneimitteln. Available online: https://www.bundestieraerztekammer.de/tieraerzte/leitlinien/ (accessed on 14 December 2020).

54. Vetsuisse Fakultät Gesellschaft Schweizer Tierärztinnen und Tierärzte (GST) Bundesamt Lebensmittelsicherheit und Veterinärwesen. Umsichtiger Einsatz von Antibiotika bei Rindern, Schweinen und kleinen Wiederkäuern. Available online: https://www.blv.admin.ch/blv/de/home/tiere/tierarzneimittel/antibiotika/nationale-strategie-antibiotikaresistenzen--star--/sachgemaesser-antibiotikaeinsatz.html (accessed on 12 December 2020).
55. Coetzee, J.F.; Cernicchiaro, N.; Sidhu, P.K.; Kleinhenz, M.D. Association between antimicrobial drug class selection for treatment and retreatment of bovine respiratory disease and health, performance, and carcass quality outcomes in feedlot cattle. *J. Anim. Sci.* **2020**, *98*, skaa109. [CrossRef]
56. Coetzee, J.F.; Magstadt, D.R.; Sidhu, P.K.; Follett, L.; Schuler, A.M.; Krull, A.C.; Cooper, V.L.; Engelken, T.J.; Kleinhenz, M.D.; O'Connor, A.M. Association between antimicrobial drug class for treatment and retreatment of bovine respiratory disease (BRD) and frequency of resistant BRD pathogen isolation from veterinary diagnostic laboratory samples. *PLoS ONE* **2019**, *14*, e0219104. [CrossRef] [PubMed]
57. World Health Organisation (WHO). Critically Important Antimicrobials for Human Medicine. Available online: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf (accessed on 15 December 2020).
58. Federal Ministry of Food and Agriculture. Report of the Federal Ministry of Food and Agriculture on the Evaluation of the Antibiotics Minimisation Concept Introduced with the 16th Act to Amend the Medicinal Products Act (16th AMG Amendment). Available online: https://www.bmel.de/SharedDocs/Downloads/EN/_Animals/16-AMG-Novelle.pdf;jsessionid=8FFAF96307741BA3063F4AE3A8D379A2.live842?__blob=publicationFile&v=3 (accessed on 29 December 2020).
59. European Medicines Agency (EMA). Available online: https://www.ema.europa.eu/en/medicines/veterinary/EPAR/draxxin (accessed on 5 June 2021).
60. Giguère, S.; Huang, R.; Malinski, T.J.; Dorr, P.M.; Tessman, R.K.; Somerville, B.A. Disposition of gamithromycin in plasma, pulmonary epithelial lining fluid, bronchoalveolar cells, and lung tissue in cattle. *Am. J. Vet. Res.* **2011**, *72*, 326–330. [CrossRef]
61. Noyes, N.R.; Benedict, K.M.; Gow, S.P.; Booker, C.W.; Hannon, S.J.; McAllister, T.A.; Morley, P.S. Mannheimia haemolytica in feedlot cattle: Prevalence of recovery and associations with antimicrobial use, resistance, and health outcomes. *J. Vet. Intern. Med.* **2015**, *29*, 705–713. [CrossRef] [PubMed]
62. Woolums, A.R.; Karisch, B.B.; Frye, J.G.; Epperson, W.; Smith, D.R.; Blanton, J., Jr.; Austin, F.; Kaplan, R.; Hiott, L.; Woodley, T.; et al. Multidrug resistant Mannheimia haemolytica isolated from high-risk beef stocker cattle after antimicrobial metaphylaxis and treatment for bovine respiratory disease. *Vet. Microbiol.* **2018**, *221*, 143–152. [CrossRef]
63. Milchreporte Bayern. Available online: https://www.lfl.bayern.de/iba/tier/020223/index.php (accessed on 5 October 2021).
64. Svensson, C.; Liberg, P. The effect of group size on health and growth rate of Swedish dairy calves housed in pens with automatic milk-feeders. *Prev. Vet. Med.* **2006**, *73*, 43–53. [CrossRef] [PubMed]
65. Maier, G.U.; Love, W.J.; Karle, B.M.; Dubrovsky, S.A.; Williams, D.R.; Champagne, J.D.; Anderson, R.J.; Rowe, J.D.; Lehenbauer, T.W.; Van Eenennaam, A.L.; et al. Management factors associated with bovine respiratory disease in preweaned calves on California dairies: The BRD 100 study. *J. Dairy Sci.* **2019**, *102*, 7288–7305. [CrossRef] [PubMed]
66. McEwen, S.A.; Fedorka-Cray, P.J. Antimicrobial use and resistance in animals. *Clin. Infect. Dis.* **2002**, *34* (Suppl. 3), S93–S106. [CrossRef]
67. Timsit, E.; Christensen, H.; Bareille, N.; Seegers, H.; Bisgaard, M.; Assié, S. Transmission dynamics of Mannheimia haemolytica in newly-received beef bulls at fattening operations. *Vet. Microbiol.* **2013**, *161*, 295–304. [CrossRef]
68. Watts, J.L.; Yancey, R.J., Jr.; Salmon, S.A.; Case, C.A. A 4-year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. *J. Clin. Microbiol.* **1994**, *32*, 725–731. [CrossRef] [PubMed]
69. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL). Die Bestimmung der Kennzahlen zu den Therapiehäufigkeiten. Available online: https://www.bvl.bund.de/DE/Arbeitsbereiche/05_Tierarzneimittel/01_Aufgaben/05_AufgAntibiotikaResistenz/02_KennzahlenTherapiehaeufigkeit/KennzahlenTherapiehaeufigkeit_node.html (accessed on 28 August 2021).

IV. ADDITIONAL ANALYSES

1. Material and Methods

1.1. Objective of the examination

A subset of *Pasteurella multocida* isolates was investigated molecularly with regard to their capsule types (A, B, D, E, and F) and the potential presence of the hemorrhagic septicemia (HS)-specific sequence by PCR.

1.2. Bacterial isolates

The bacterial isolates were collected as part of the state veterinary laboratory diagnostic service at the Bavarian Health and Food Safety Authority. The samples, here nasal swabs, bronchoalveolar lavage fluids and lung tissue samples, were obtained from calves, cattle or dairy cows with clinical signs of BRD from Bavaria between July 2015 and June 2020. The incubation conditions of the samples as well as the identification of the bacterial species have already been described in (MELCHNER et al., 2021).

1.3. Extraction of nucleic acid

The extraction of nucleic acid was carried out by thermolysis of a bacterial colony suspended in 100 µl PCR grade water. For this the suspension was heated for 10 minutes at 95 °C and subsequently centrifuged at 15,000 *g* for 10 minutes. The supernatant was used as template in the PCR.

1.4. PCR analysis and gel electrophoresis

Capsule type determination of samples were carried out according to the published OIE protocol (OIE Terrestrial Manual 2018, Chapter 3.4.10) (OIE, 2018). The corresponding primer sequences are summarised in (Table 3). PCR master mix contained the primer mix (Eurofins, Ebersberg, Germany), the Multiplex PCR Kit (Qiagen, Hilden, Germany) and distilled water. Three microliters of template DNA were added to 22 µl master mix, so the total volume was 25 µl in each reaction vessel. The samples were subjected to 35 cycles of amplification in a thermal cycler (Biometra TOne, Analytik Jena, Jena, Germany). The thermal profile of the PCR

consisted of an initial denaturation step at 95 °C for 15 minutes. This was followed by 35 cycles consisting of denaturation (95 °C for 30 seconds), annealing (55 °C for 30 seconds) and elongation (72 °C for 90 seconds). The final elongation lasted 5 minutes at 72 °C. A 2-% agarose e-gel with SYBR Safe DNA Gel stain was used for electrophoresis and jpeg files were generated by a UV gel documentation system (Invitrogen, ThermoFisher SCIENTIFIC, Waltham, USA).

Subsequent detection of potential HS-specific sequence of type B strains was also carried out according to the published OIE protocol (OIE, 2018). Primer sequences for HS-causing serotype B:2 were used as published (Table 3). PCR was carried out as described above.

Table 3. Sequences of primers used in the *Pasteurella multocida* multiplex capsular and HS PCR

sero-group	**gene**	**name**	**sequence (5' to 3')**	**amplimer size (bp)**	**reference**
All	*KMT1*	KMT1T7	ATCCGCTATTTACCCAGTGG	460	(TOWNSEND et al., 1998; OIE, 2018)
		KMT1SP6	GCTGTAAACGAACTCGCCAC		
A	*hyaD -hyaC*	CAPA-FWD	TGCCAAAATCGCAGTCAG	1044	(TOWNSEND et al., 2001; OIE, 2018)
		CAPA-REV	TTGCCATCATTGTCAGTG		
B	*bcbD*	CAPB-FWD	CATTTATCCAAGCTCCACC	760	
		CAPB-REV	GCCCGAGAGTTTCAATCC		
D	*dcbF*	CAPD-FWD	TTACAAAAGAAAGACTAGGAGCCC	657	
		CAPD-REV	CATCTACCCACTCAACCATATCAG		
E	*ecbJ*	CAPE-FWD	TCCGCAGAAAATTATTGACTC	511	
		CAPE-REV	GCTTGCTGCTTGATTTTGTC		
F	*fcbD*	CAPF-FWD	AATCGGAGAACGCAGAAATCAG	851	
		CAPF-REV	TTCCGCCGTCAATTACTCTG		
B:2	HS	KTT72	AGGCTCGTTTGGATTATGAAG	590	(TOWNSEND et al., 1998; OIE, 2018)
		KTSP61	ATCCGCTAACACACTCTC		

2. Results

In total, capsule-type PCR was performed on a subset of 227 *Pasteurella multocida* isolates from calves, cattle, and dairy cows from Bavaria, which were sent to the State Veterinary Diagnostic Laboratory of the Bavarian Health and Food Safety

Authority between July 2015 and June 2020 for pathogen identification and testing for antimicrobial resistance. Of the 227 isolates, 215 (94.7 %) were typed as capsule type A because the multiplex PCR detected the product of *KMT1* (460bp) specific for *Pasteurella multocida* and the product of *hyaD-hyaC* (1,044 bp) specific for capsule type A (Table 4 and Figure 5). In two isolates (0.9 %), the gene product of *hyaD-hyaC* (1,044 bp) was detected, but not the corresponding *KMT1* gene, which is why these were categorised as atypical isolates with capsule type A (Table 4). Five isolates (2.2 %) were identified as capsule type B, as both the gene product of *KMT1* (460 bp) and the *bcbD* product (760 bp) specific for capsule type B were detected (Table 4 and Figure 5). In other five isolates (2.2 %), none of the five specific capsule genes for types A, B, D, E and F, but only the *KMT1* gene specific for all capsule types, were detected (Table 4).

Table 4. Results of the capsular multiplex PCR

capsule Type	type A	atypical isolate of type A	type B	not typeable	total
number of isolates	215	2	5	5	227
%	94.7	0.9	2.2	2.2	100

In a subsequent PCR, it was tested whether the five *Pasteurella multocida* isolates characterised as capsular type B were the HS representing serotype B:2. No gene sequence with the corresponding gene product (590 bp) was detected for the HS representing serotype B:2.

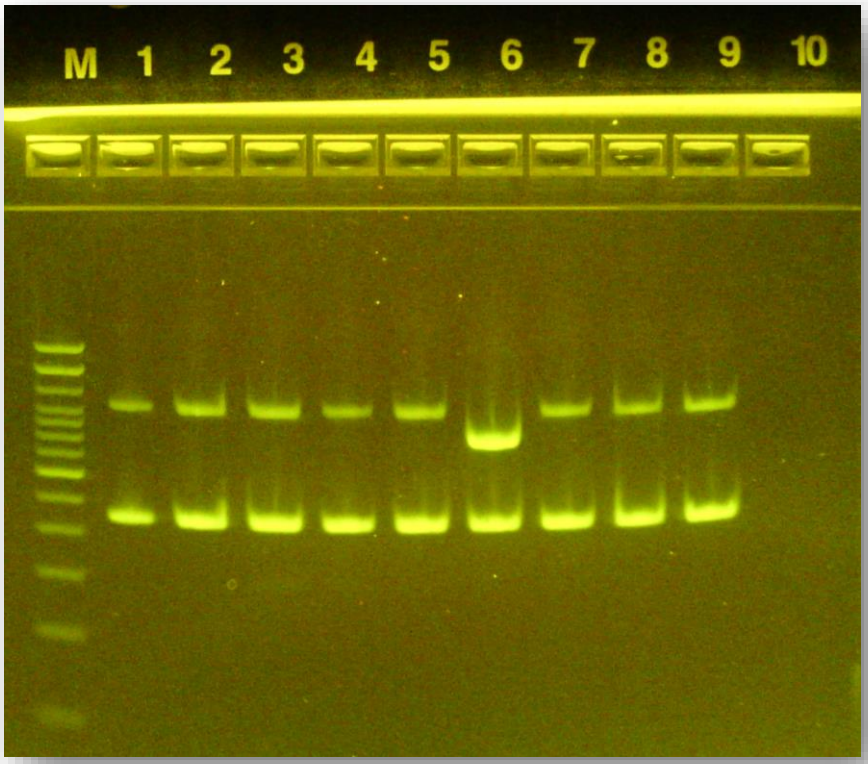

Figure 5. Agarose gel electrophoresis of products from the capsular multiplex PCR. Lane M: 100-bp DNA ladder; Lanes 1, 2, 3, 4, 5, 7, 8 and 9: *Pasteurella multocida*-isolates of capsule type A with the *KMT1* gene product (460 bp) specific for *Pasteurella multocida* and the *hyaD-hyaC* gene product (1,044 bp) specific for capsule type A; Lane 6: *Pasteurella multocida*-isolate of capsule type B with the *KMT1* gene product (460 bp) specific for *Pasteurella multocida* and the *bcbD* gene product (760 bp) specific for capsule typ B; Lane 10: negative control.

V. DISCUSSION

1. Current trends in AMR of pathogens of the BRD complex

In the present study, data on antimicrobial susceptibility of bacterial pathogens of the BRD complex from bovine isolates from the period July 2015 to June 2020 from Bavaria were evaluated retrospectively, taking into account epidemiological parameters. The aim of this study was to complement existing resistance monitoring programmes, such as the German Resistance Monitoring GERM-Vet, and to record current trends and developments in AMR (MELCHNER et al., 2021). In order to discuss current trends in the development of AMR in bacterial pathogens of BRD complex in Germany, our study results from Bavaria were compared with the national resistance monitoring GERM-Vet (BVL, 2020b). It needs to be mentioned that the *Pasteurella multocida*- and *Mannheimia haemolytica*-isolates examined in the GERM-Vet study were collected and evaluated by state and private laboratories according to a defined sampling plan (BVL, 2020b). In our analysis, however, the isolates were not collected according to a defined sampling plan. All isolates obtained from samples sent in by veterinarians were included in the analysis (MELCHNER et al., 2021). However, to prevent overrepresentation and bias due to clonal isolates, only one isolate per species and per farm and per quarter year was included in the analysis following previous publications (WATTS et al., 1994; PORTIS et al., 2012). This also explains why the number of bacterial isolates in our analysis is lower than in the GERM-Vet study. For example, for the study period 2018/2019, 73 *Mannheimia haemolytica*- and 91 *Pasteurella multocida*-isolates were considered in our analysis, while in GERM-Vet there were 82 *Mannheimia haemolytica*- and 149 *Pasteurella multocida*-isolates (BVL, 2020b; MELCHNER et al., 2021). There were differences, but also similarities between our Bavarian study and GERM-Vet in the susceptibilities of antibiotic agents to the pathogens. One major difference is that in the national resistance monitoring in study year 2018/2019, 42.7 % of *Mannheimia haemolytica*-isolates were not susceptible (resistant and intermediate) to penicillin and there was even an increase compared to the same period of the previous year (BVL, 2020b). In our study, only 5.48 % were not

susceptible in 2018/2019 (MELCHNER et al., 2021). Furthermore, in the GERM-Vet study, 15.9 % of *Mannheimia haemolytica*-isolates were not susceptible to enrofloxacin in the study year 2018/2019, while in our study all 73 *Mannheimia haemolytica*-isolates were susceptible to enrofloxacin (BVL, 2020b; MELCHNER et al., 2021). Since our study only contain isolates from Bavaria and GERM-Vet contains isolates from all over Germany, this could possibly be explained by geographical clustering as already described in previous publications (PORTIS et al., 2012; LUBBERS & HANZLICEK, 2013; KLIMA et al., 2020). Nevertheless, the main findings of the GERM-Vet program can also be found in our Bavaria study. The national resistance monitoring reports an increase in florfenicol-resistant *Mannheimia haemolytica*-isolates since 2017 (BVL, 2020b). A trend towards higher numbers of not susceptible *Pasteurella multocida*-isolates to florfenicol is also beginning to emerge as seen in our study, as nine of the total 14 not susceptible isolates were detected from the last study period 2019/2020 (MELCHNER et al., 2021). The same is true for the macrolide antibiotic tulathromycin. In national resistance monitoring, 3 % of *Pasteurella multocida*-isolates were resistant to tulathromycin in 2016/2017, 11 % in 2017/2018 and 14 % in 2018/2019 (BVL, 2020b). In our study, a significant increase in not susceptible *Pasteurella multocida*-isolates to tulathromycin from 5.56 % in 2015/2016 to 26.44 % in 2019/2020 was observed (MELCHNER et al., 2021). It is therefore appropriate to continue monitoring for susceptibility of florfenicol and tulathromycin. In the GERM-Vet program, a 23.5 % proportion of not susceptible *Pasteurella multocida*-isolates to tetracycline was found in 2018/2019 (BVL, 2020b). In our analysis, this was even exceeded with a proportion of 48.35 % in 2018/2019, which is why tetracycline can no longer be recommended for the treatment of BRD (MELCHNER et al., 2021). In both our study and GERM-Vet, the MIC values of the isolates were classified as resistant, intermediate and susceptible according to species-specific veterinary breakpoints published by the Clinical and Laboratory Standards Institute (CLSI, 2020)(Table 1). Unfortunately, species-specific breakpoints for veterinary medicine do not exist for all antibiotic agents approved for the treatment of BRD. For example, amoxicillin-clavulanic acid or sulphonamides could not be included in the analysis, although preparations for both agents are approved for the treatment of BRD in Germany (CLSI, 2020; UNIVERSITÄT LEIPZIG,

2020). Another major problem is that for the bacterial pathogens *Truperella pyogenes* and *Bibersteinia trehalosi* there are no breakpoints at all to classify these isolates into resistant, intermediate and susceptible on the basis of MIC values (CLSI, 2020). Consequently, for almost 20 % of the isolates in our analysis [*Truperella pyogenes* (15.78 %), *Bibersteinia trehalosi* (2.26 %)] only the distribution of the MIC values could be given (MELCHNER et al., 2021). The use of non-species-specific breakpoints, for example breakpoints from human medicine, is not recommended. On the one hand, they do not reflect potential differences in pharmacokinetics, on the other hand, they make it difficult to compare the results with those from other studies. In order to increase the quality of resistance studies, it would be advantageous if there were further breakpoints specific to veterinary medicine (SWEENEY et al., 2018).

2. Importance of epidemiological investigations

In previous studies investigating AMR in pathogens of the BRD complex, further epidemiological investigations have not played a relevant role. They mostly examined only the proportions of resistant/not susceptible isolates to certain antibiotic agents, and later also the proportions of MDR isolates that were resistant/not susceptible to three or more antibiotic classes (WATTS et al., 1994; WELSH et al., 2004; PORTIS et al., 2012; LUBBERS & HANZLICEK, 2013; HOLSCHBACH et al., 2020). In recent studies investigating AMR in feedlots in USA and Canada, associations between MDR profiles and certain epidemiological parameters were determined, which gave us the idea to conduct such investigations as well (ANHOLT et al., 2017; KLIMA et al., 2020; MELCHNER et al., 2021). For example, no associations were found between the number of antibiotic treatments each cattle received and MDR isolates in North American feedlots (KLIMA et al., 2020). Also, in our study from Bavaria, no association was observed between the antibiotic treatment frequency on farm and the occurrence of MDR isolates (MELCHNER et al., 2021). In contrast, there are other studies that show associations between the use of antibiotic drugs and the increased odds of isolating MDR isolates (NOYES et al., 2015; WOOLUMS et al., 2018). For this

reason, the frequency of drug use and the impact of the resistance pattern should therefore be included in future resistance studies. In addition, the epidemiological investigations in the North American studies revealed that there are associations between the MDR profile and the corresponding feedlots from which the tested isolates originated (ANHOLT et al., 2017; KLIMA et al., 2020). This is also reflected in our Bavarian study, because not animal-specific characteristics such as sex, age, disease outcome (deceased vs. diseased) or the detection of further pathogens (PI-3, BRSV, *Mycoplasma* spp.), but farm-specific characteristics were associated with lower/higher odds for the occurrence of MDR isolates in the multivariable regression analysis. The occurrence of MDR isolates was more likely on farms with more than 300 animals than on farms with 100 or less animals, and more likely on fattening farms than on mixed farms or dairy farms in our study (MELCHNER et al., 2021). As already recommended by other authors, it is therefore important to consider epidemiological information, here farm size and farm type, in resistance surveillance in order to develop strategies to reduce the spread of AMR (ANHOLT et al., 2017; MELCHNER et al., 2021).

3. Molecular detection of resistance genes

Regarding the spread of antibiotic resistance genes (ARGs) in bacterial pathogens of the BRD complex, one pathway of HGT, namely conjugation by plasmids and integrated conjugative elements (ICEs), seems to play an important role (KEHRENBERG et al., 2008; KLIMA et al., 2020). It is known that ICEs consist, on the one hand of core genes that encode proteins for their own excision, conjugative transfer, and integration into the genome of the recipient cell. On the other hand, they also contain entire cassettes with more than ten different resistance genes, which can be exchanged between strains, species and furthermore between different bacterial genera within a single HGT event (MICHAEL et al., 2012a, 2012b; EIDAM et al., 2015). As published recently, large numbers of ARGs and core genes were detected in MDR *Pasteurella multocida*- and *Mannheimia haemolytica*-isolates from bovines with BRD. In these studies, ICEs are therefore considered to play a major role in the spread of MDR (KLIMA et al., 2020;

STANFORD et al., 2020). However, it was also found that the presence of ARGs was not completely correlated with the AMR phenotype. This might imply that certain ARGs were inactive, or that the breakpoints used for susceptibility testing need to be reassessed (KLIMA et al., 2020). In conclusion, both approaches, the phenotypic susceptibility testing, as well as molecular ARG screening should be carried out in parallel. This would help to better understand the role of ICEs in the spread of AMR and potentially identify certain genotype-phenotype relationships (CLSI, 2011; SWEENEY et al., 2018).

4. Capsule types of *Pasteurella multocida*-isolates

Capsular multiplex PCR of a subset of 227 *Pasteurella multocida*-isolates collected from Bavaria between July 2015 and June 2020 revealed that capsular type A was the predominant type with 215 isolates (94.7 %) in calves, cattle and dairy cows with putative clinical signs of BRD (Table 4). This result is consistent with a study from Germany in which 92.3 % of all *Pasteurella multocida*-isolates from healthy and diseased bovines were classified as capsular type A (EWERS et al., 2006). Another study from Malaysia also detected capsule type A most frequently in cattle with 53 % (ARUMUGAM et al., 2011). Two isolates (0.9 %) were classified as atypical isolates of capsule type A, because the signal specific for capsule type A with 1,044 bp was visible in the electrophoresis gel, but not the signal specific for all *Pasteurella multocida*-isolates with 460 bp. Instead, a second larger band, only a few bp larger than the 1,044-bp signal specific to capsule type A, was visible in the electrophoresis gel. The previous pathogen identification by MALDI-TOF showed that these isolates were *Pasteurella multocida*-strains. It can therefore be assumed that these two isolates can be also assigned to capsule type A. With five isolates (2.2 %), capsule type B was the second most frequently detected (Table 4). In the Malaysian study, capsule type B was also the second most frequently found in cattle, although with a proportion of 32 %, which is clearly higher than in our study (ARUMUGAM et al., 2011). Not consistent with these results is the German study, which did not detect a single type B isolate from bovines in the capsular multiplex PCR (EWERS et al., 2006). *Pasteurella multocida*-isolates of

capsular type D are mainly isolated from pigs, so it is not surprising that no isolate with capsular type D was detected in our examinations. Type D strains with the dermonecrotic toxin encoding *tox A* gene are mainly associated with progressive rhinitis atrophicans in porcines (DAVIES et al., 2003b; EWERS et al., 2006). Capsule types E and F were not detected in our study and are thus in line with other studies in which they were not detected or only in proportions below 2.5 % from bovine isolates (EWERS et al., 2006; ARUMUGAM et al., 2011). Five isolates (2.2 %) were classified as untypeable, because no specific signals for a capsule type were visible in the electrophoresis gel (Table 4). Such untypeable strains were also seen in the aforementioned comparative studies with proportions of 11.0 % and 2.2 % (EWERS et al., 2006; ARUMUGAM et al., 2011). One may expect that the non-typeable isolates are not encapsulated, because microscopic examinations of those isolates originating from avian hosts using capsule specific stains, India ink, showed that they were not encapsulated (DAVIES et al., 2003a). In summary, *Pasteurella multocida* types A and B dominate in bovines with BRD. It is interesting in this context that isolates with detected gene *tbpa*, which encodes transferrin binding protein A involved in iron acquisition, are significantly associated with disease status in bovines. The *tbpA* gene, in turn, is mainly found in *Pasteurella multocida*-isolates of capsule type A and B (EWERS et al., 2006). This underlines that already with the knowledge of the capsule type, statements can be made about the virulence of *Pasteurella multocida*-isolates. For the determination of capsular types, the multiplex PCR assays performed in our study have major advantages over conventional serological methods, such as the classification of *Pasteurella multocida*-isolates into the five serogroups A, B, D, E and F based on capsular antigens using a passive haemagglutination test according to Carter (CARTER, 1952, 1972; RIMLER & RHOADES, 1987). Typing by PCR is more discriminative because more isolates can be typed by PCR compared to conventional serotyping methods(ARUMUGAM et al., 2011). Besides, colonies from primary isolation plates can be used so that they do not have to be transferred to pure culture. The fact that there are only a few laboratories worldwide that produce and maintain the antisera required for conventional serotyping also speaks clearly in favour of the PCR method (TOWNSEND et al., 2001).

5. Haemorrhagic septicaemia in bovines caused *by Pasteurella multocida*-serotypes B:2 and E:2

In addition to BRD complex, haemorrhagic septicaemia (HS) caused by serotypes B:2 and E:2 is another important disease in bovines (DE ALWIS, 1992; OIE, 2018). The last official case of HS in Germany was reported in 1986 (DE ALWIS, 1999). In 2010, cases of fallow deer, pigs and cattle with HS were detected at the border of Saxony-Anhalt and Brandenburg for the first time since 1986 (SOIKE et al., 2012). In Bavaria, HS was also detected in dead wild boar and wild ruminants (fallow deer, red deer) and shortly afterwards in a neighbouring cattle herd in 2017. Four young cattle were ill showing respiratory clinical signs. A post-mortem examination of one of the two deceased cattle revealed purulent inflammation in the throat area with bleeding and swelling of surrounding cervical and head lymph nodes and a pulpy swelling of the spleen. Detection of *Pasteurella multocida* type B with HS specific gene sequence was obtained after they were cultured after isolation from the phlegmonous area (MÜLLER & LOCHNER, 2017). Among the five *Pasteurella multocida*-isolates classified as capsular type B in our analysis (one isolate from 2016, three from 2018 and one from 2020) the 590-bp gene product specific for HS causing serotype B:2 was not detected in any of them. The results confirm that HS occurs only sporadically in domestic and wild animals in Bavaria and is not an endemic disease as in regions of Africa, Southeast Asia, and the Near and Middle East (MÜLLER & LOCHNER, 2017).

VI. ZUSAMMENFASSUNG

Zwischen Juli 2015 und Juni 2020 wurden insgesamt 662 Tiere aus 519 Betrieben mit vermeintlichem bovinem respiratorischem Syndrom (BRD), kurz Rindergrippekomplex, in Bayern, Deutschland, untersucht. Die 754 gewonnenen Bakterienisolate wurden auf antimikrobielle Resistenz mittels der Mikrodilutionsmethode analysiert. *Pasteurella multocida* war das am häufigsten isolierte pathogene Bakterium mit 345 (45.76 %) Isolaten. Weiterhin wurden 273 *Mannheimia-haemolytica*- (36.20 %), 119 *Truperella-pyogenes*- (15.78 %) sowie 17 *Bibersteinia-trehalosi*-Isolate (2.26 %) gewonnen. Der Anteil an *Pasteurella-multocida*-Isolaten, der in der Resistenztestung über den fünf-Jahreszeitraum als nicht-sensibel (resistent und intermediär) ermittelt wurde, ergab für Ceftiofur 0,87 %, für Penicillin G 3,84 %, für Florfenicol 4,06 %, für Enrofloxacin 0,29 %, für Tulathromycin 15,65 %, für Tetrazyklin 39,42 % und für Spectinomycin 78,84 %. Für *Mannheimia haemolytica* war die Rate an nicht-sensiblen Isolaten für Ceftiofur 0 %, für Penicillin G 4,76 %, für Florfenicol 1,10 %, für Enrofloxacin 2,93 %, für Tilmicosin 6,59 %, für Tulathromycin 2,93 %, für Tetrazyklin 21,25 % und für Spectinomycin 80,95 %. Als Folgerung unserer Ergebnisse können die beiden antibiotischen Medikamente Tetrazyklin und Spectinomycin aufgrund der ungünstigen Resistenzsituation nicht weiter für die Therapie des BRD-Komplexes empfohlen werden. Spectinomycin war der einzige antibiotische Wirkstoff mit einer signifikanten Abnahme in Bezug auf nicht sensible Isolate innerhalb des Studienzeitraums 2015 bis 2020. Diese verliefen für *Pasteurella multocida* von 88,89 % auf 67,82 % und für *Mannheimia haemolytica* von 90,24 % auf 68,00 % rückläufig. Ein signifikanter Anstieg der nicht-sensiblen Isolate wurde für die antibiotischen Wirkstoffe Tulathromycin (5,56 % auf 26,44 %) und Tetrazyklin (18,52 % auf 57,47 %) bei *Pasteurella-multocida*-Isolaten gefunden. Der Anteil an MDR-Isolaten, die nicht sensibel gegenüber mindestens einem Wirkstoff in mindestens drei antimikrobiellen Klassen waren, betrug für *Pasteurella multocida* 13,91 % und für *Mannheimia haemolytica* 5,13 %. Der Anteil von MDR *Pasteurella-multocida*-Isolaten stieg signifikant von 3,70 % (erstes Studienjahr) auf 22,90 % (letztes Studienjahr). Die epidemiologischen

Untersuchungen ergaben, dass in Betrieben mit mehr als 300 Tieren die Wahrscheinlichkeiten für das Isolieren von MDR-Isolaten signifikant höher war als in Betrieben mit 100 oder weniger Tieren. Zusätzlich waren die Wahrscheinlichkeiten für das Isolieren von MDR-Isolaten in Milchviehbetrieben und Gemischtbetrieben signifikant niedriger als in Mastbetrieben. Die besondere Bedeutung von epidemiologischen Parametern wie Betriebsgröße und Betriebstyp, wie sie in unserer Studie gezeigt wurden, sollten deshalb auch in zukünftigen Resistenzstudien berücksichtigt werden.

In einem zweiten Teil der hier vorgestellten Arbeit wurde für 227 der insgesamt 345 *Pasteurella-multocida*-Isolate der Kapseltyp mittels PCR bestimmt. Die Ergebnisse zeigten, dass Kapseltyp A mit 215 Isolaten (92,14 %) der dominierende Typ war. Weiterhin wurden fünf Typ B (2,26 %) und fünf nicht typisierbare (2,26 %), vermutlich nicht gekapselte Isolate, detektiert. Zwei weitere Isolate (0,88 %) konnten nicht eindeutig dem Typ A zugeordnet werden. In der Nukleinsäure von fünf *Pasteurella-multocida-Isolaten* des Typ B konnte keine spezifische Gensequenz der Hämorrhagischen Septikämie (HS) nachgewiesen werden. Dieses Ergebnis bekräftigt, dass die HS nur sporadisch bei Haus- und Wildtieren in Bayern auftritt.

VII. SUMMARY

In a 5-year study, from July 2015 to June 2020, a total of 754 isolates originating from 662 animals with putative BRD syndrome, stemming from 519 farms in Bavaria, Germany, were analysed for antimicrobial resistance via microbroth dilution method. *Pasteurella multocida* was the most frequently isolated pathogen with 345 isolates (45.76 %), followed by 273 *Mannheimia haemolytica* isolates (36.20 %), 119 *Truperella pyogenes isolates* (15.78 %), and 17 *Bibersteinia trehalosi* isolates (2.26 %). The five-year not susceptibility rates of *Pasteurella multocida* isolates were 0.87 % for ceftiofur, 3.48 % for penicillin G, 4.06 % for florfenicol, 0.29 % for enrofloxacin, 15.65 % for tulathromycin, 39.42 % for tetracycline and 78.84 % for spectinomycin. For *Mannheimia haemolytica* isolates, the five-year not susceptibility rates for ceftiofur were 0 %, for penicillin G 4.76 %, for florfenicol 1.10 %, for enrofloxacin 2.93 %, for tilmicosin 6.59 %, for tulathromycin 2.93 %, for tetracycline 21.25 % and for spectinomycin 80.95 %. Thus, due to the unfavourable resistance situation, the two antimicrobials tetracycline and spectinomycin cannot be recommended for the therapy of BRD. Spectinomycin was the only antibiotic agent with a significant decrease regarding not susceptible isolates within the study period from 2015 to 2020 (*Pasteurella. multocida* 88.89 % to 67.82 %, *Mannheimia haemolytica* 90.24 % to 68.00 %). Significant rate of increase of not susceptible isolates were found for the antibiotic agents tulathromycin (5.56 % to 26.44 %) and tetracycline (18.52 % to 57.47 %) in *Pasteurella multocida* isolates. The proportion of MDR isolates that were not susceptible to at least one agent in at least three antimicrobial classes was 13.91 % for *Pasteurella multocida* and 5.13 % for *Mannheimia haemolytica*. The proportion of MDR *Pasteurella multocida* isolates increased significantly from 3.70 % (first study year) to 22.90 % (last study year). The epidemiological investigations revealed that on farms with more than 300 animals, the odds for isolating MDR isolates were significantly higher than in farms with 100 or less animals. In addition, the odds for isolating MDR isolates were significantly lower in dairy and mixed farms compared to fattening farms. The particular importance of epidemiological parameters such as farm size and farm type, as outlined in this study, should therefore also be taken

into account in future resistance studies.

In a second part of the dissertation work, the capsule type of *Pasteurella multocida* was determined by PCR for a subset of 227 isolates. The results showed that capsule type A was the predominant type with 215 isolates (94.17 %). Five type B (2.26 %) and five untypeable (2.26 %), presumably non-encapsulated isolates were detected. Two further isolates (0.9 %) could not be clearly classified as type A. In the five *Pasteurella multocida* isolates of type B, a hemorrhagic septicemia- (HS) specific gene was not detected. This result confirms that HS occurs only sporadically in domestic and wild animals in Bavaria.

VIII. REFERENCES

Andrews GA, Kennedy GA. Respiratory diagnostic pathology. Vet Clin North Am Food Anim Pract 1997; 13: 515-47.

Andrews SC, Robinson AK, Rodríguez-Quiñones F. Bacterial iron homeostasis. FEMS Microbiol Rev 2003; 27: 215-37.

Anholt RM, Klima C, Allan N, Matheson-Bird H, Schatz C, Ajitkumar P, Otto SJ, Peters D, Schmid K, Olson M, McAllister T, Ralston B. Antimicrobial Susceptibility of Bacteria That Cause Bovine Respiratory Disease Complex in Alberta, Canada. Front Vet Sci 2017; 4: 207.

Arumugam ND, Ajam N, Blackall PJ, Asiah NM, Ramlan M, Maria J, Yuslan S, Thong KL. Capsular serotyping of *Pasteurella multocida* from various animal hosts - a comparison of phenotypic and genotypic methods. Trop Biomed 2011; 28: 55-63.

Bell BG, Schellevis F, Stobberingh E, Goossens H, Pringle M. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC Infect Dis 2014; 14: 13.

Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F, Bürgmann H, Sørum H, Norström M, Pons MN, Kreuzinger N, Huovinen P, Stefani S, Schwartz T, Kisand V, Baquero F, Martinez JL. Tackling antibiotic resistance: the environmental framework. Nat Rev Microbiol 2015; 13: 310-7.

Blahová J, Králiková K, Krcméry V, Jezek P. Low-Frequency transduction of imipenem resistance and high-frequency transduction of ceftazidime and aztreonam resistance by the bacteriophage AP-151 isolated from a *Pseudomonas aeruginosa* strain. J Chemother 2000; 12: 482-6.

BMEL. Anzeigepflichtige Tierseuchen. 2021: https://www.bmel.de/DE/themen/tiere/tiergesundheit/tierseuchen/anzeigepflichtige-tierseuchen.html. 20 November 2021.

BMG. DART 2020 Fighting antibiotic resistance for the good of both humans and animals. 2015: https://www.bmel.de/SharedDocs/Downloads/EN/Publications/DART2020.pdf;jsessionid=42432EA3DD38EA0D58BFF1DD601CCC04.live842?__blob=publicationFile&v=3. 9 January 2022.

BMJ. Verordnung über tierärztliche Hausapotheken (TÄHAV). 2018: https://www.gesetze-im-internet.de/t_hav/BJNR021150975.html. 9 January 2022.

BMJ. Verordnung über das datenbankgestützte Informationssystem über Arzneimittel des Bundesinstituts für Arzneimittel und Medizinprodukte*) (BfArM-Arzneimitteldatenverordnung). 2021: https://www.gesetze-im-internet.de/dimdiamv/BJNR014000010.html. 9 January 2022.

Bosch M, Garrido ME, Llagostera M, Pérez De Rozas AM, Badiola I, Barbé J. Characterization of the *Pasteurella multocida* hgbA gene encoding a hemoglobin-binding protein. Infect Immun 2002; 70: 5955-64.

Boukahil I, Czuprynski CJ. *Mannheimia haemolytica* biofilm formation on bovine respiratory epithelial cells. Vet Microbiol 2016; 197: 129-36.

Boyce JD, Adler B. The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B:2). Infect Immun 2000; 68: 3463-8.

Boyce JD, Chung JY, Adler B. *Pasteurella multocida* capsule: composition, function and genetics. J Biotechnol 2000; 83: 153-60.

Braibant M, Chevalier J, Chaslus-Dancla E, Pagès JM, Cloeckaert A. Structural and functional study of the phenicol-specific efflux pump FloR belonging to the major facilitator superfamily. Antimicrob Agents Chemother 2005; 49: 2965-71.

Brown GM. The biosynthesis of folic acid. II. Inhibition by sulfonamides. J Biol Chem 1962; 237: 536-40.

BTK. Guidelines for the prudent use of veterinary antimicrobial drugs 2015: https://www.bundestieraerztekammer.de/btk/downloads/antibiotika/AB_Leitlinien2015_EN.pdf. 9 January 2022.

BVL. Zoonosen-Monitoring 2019. 2020a: https://www.bvl.bund.de/SharedDocs/Downloads/01_Lebensmittel/04_Zoonosen_Monitoring/Zoonosen_Monitoring_Bericht_2019.pdf?__blob=publicationFile&v=5. 29 December 2020.

BVL. Resistenzsituation bei klinisch wichtigen tierpathogenen Bakterien. 2020b: https://www.bvl.bund.de/SharedDocs/Berichte/07_Resistenzmonitoringstudie/Bericht_Resistenzmonitoring_2018.pdf?__blob=publicationFile&v=4. 29 December 2020.

BVL. Die Bestimmung der Kennzahlen zu den Therapiehäufigkeiten. 2021: https://www.bvl.bund.de/DE/Arbeitsbereiche/05_Tierarzneimittel/01_Aufgaben/05_AufgAntibiotikaResistenz/02_KennzahlenTherapiehaeufigkeit/KennzahlenTherapiehaeufigkeit_node.html. 28 August 2021.

Carter GR. The type specific capsular antigen of *Pasteurella multocida*. Can J Med Sci 1952; 30: 48-53.

Carter GR. Improved hemagglutination test for identifying type A strains of *Pasteurella multocida*. Appl Microbiol 1972; 24: 162-3.

Caswell JL, Middleton DM, Sorden SD, Gordon JR. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. Vet Pathol 1998; 35: 124-31.

Cernicchiaro N, White BJ, Renter DG, Babcock AH, Kelly L, Slattery R. Associations between the distance traveled from sale barns to commercial feedlots in the United States and overall performance, risk of respiratory disease, and cumulative mortality in feeder cattle during 1997 to 2009. J Anim Sci 2012; 90: 1929-39.

Chigerwe M, Tyler JW, Schultz LG, Middleton JR, Steevens BJ, Spain JN. Effect of colostrum administration by use of oroesophageal intubation on serum IgG concentrations in Holstein bull calves. Am J Vet Res 2008; 69: 1158-63.

CLSI. CLSI.Generation, Presentation, and Application of Antimicrobial Susceptibility Test Data for Bacteria of Animal Origin; A Report. CLSI document VET05-R,Wayne, PA: Clinical and Laboratory Standards Institute. 2011;

CLSI (2018) Performance Standards for Antimicrobial Disc and Dilution Susceptibility Tests for Bacteria isolated from Animals. 4th edition. CLSI supplement VET08. Clinical and Laboratory Standards Institute.

CLSI. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 5th ed. CLSI supplement VET01S. Clinical and Laboratory Standards Institute. 2020;

Coetzee JF, Cernicchiaro N, Sidhu PK, Kleinhenz MD. Association between antimicrobial drug class selection for treatment and retreatment of bovine respiratory disease and health, performance, and carcass quality outcomes in feedlot cattle. J Anim Sci 2020; 98

Confer AW. Update on bacterial pathogenesis in BRD. Anim Health Res Rev 2009; 10: 145-8.

Correia S, Poeta P, Hébraud M, Capelo JL, Igrejas G. Mechanisms of quinolone action and resistance: where do we stand? J Med Microbiol 2017; 66: 551-9.

Dabo SM, Confer AW, Quijano-Blas RA. Molecular and immunological characterization of *Pasteurella multocida* serotype A:3 OmpA: evidence of its role in P. multocida interaction with extracellular matrix molecules. Microb Pathog 2003; 35: 147-57.

Dabo SM, Taylor JD, Confer AW. *Pasteurella multocida* and bovine respiratory disease. Anim Health Res Rev 2007; 8: 129-50.

Davies J, Wright GD. Bacterial resistance to aminoglycoside antibiotics. Trends Microbiol 1997; 5: 234-40.

Davies RL, MacCorquodale R, Caffrey B. Diversity of avian *Pasteurella multocida* strains based on capsular PCR typing and variation of the OmpA and OmpH outer membrane proteins. Vet Microbiol 2003a; 91: 169-82.

Davies RL, MacCorquodale R, Baillie S, Caffrey B. Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. J Med Microbiol 2003b; 52: 59-67.

De Alwis MC. Haemorrhagic septicaemia--a general review. Br Vet J 1992; 148: 99-112.

De Alwis MC. Haemorrhagic Septicaemia. . 1999: https://www.aciar.gov.au/publication/books-and-manuals/haemorrhagic-septicaemia. 9 January 2022.

DeAngelis PL, Gunay NS, Toida T, Mao WJ, Linhardt RJ. Identification of the capsular polysaccharides of Type D and F *Pasteurella multocida* as unmodified heparin and chondroitin, respectively. Carbohydr Res 2002; 337: 1547-52.

Desmolaize B, Rose S, Warrass R, Douthwaite S. A novel Erm monomethyltransferase in antibiotic-resistant isolates of *Mannheimia haemolytica* and *Pasteurella multocida*. Mol Microbiol 2011a; 80: 184-94.

Desmolaize B, Rose S, Wilhelm C, Warrass R, Douthwaite S. Combinations of macrolide resistance determinants in field isolates of *Mannheimia haemolytica* and *Pasteurella multocida*. Antimicrob Agents Chemother 2011b; 55: 4128-33.

Dubrovsky SA, Van Eenennaam AL, Karle BM, Rossitto PV, Lehenbauer TW, Aly SS. Bovine respiratory disease (BRD) cause-specific and overall mortality in preweaned calves on California dairies: The BRD 10K study. J Dairy Sci 2019; 102: 7320-8.

Dudek K, Nicholas RAJ, Szacawa E, Bednarek D. *Mycoplasma bovis* Infections-Occurrence, Diagnosis and Control. Pathogens 2020; 9

Edwards TA. Control methods for bovine respiratory disease for feedlot cattle. Vet Clin North Am Food Anim Pract 2010; 26: 273-84.

Eidam C, Poehlein A, Leimbach A, Michael GB, Kadlec K, Liesegang H, Daniel R, Sweeney MT, Murray RW, Watts JL, Schwarz S. Analysis and comparative genomics of ICEMh1, a novel integrative and conjugative element (ICE) of *Mannheimia haemolytica*. J Antimicrob Chemother 2015; 70: 93-7.

El Garch F, de Jong A, Simjee S, Moyaert H, Klein U, Ludwig C, Marion H, Haag-Diergarten S, Richard-Mazet A, Thomas V, Siegwart E. Monitoring of antimicrobial susceptibility of respiratory tract pathogens isolated from diseased cattle and pigs across Europe, 2009-2012: VetPath results. Vet Microbiol 2016; 194: 11-22.

Ewers C, Lübke-Becker A, Bethe A, Kiebling S, Filter M, Wieler LH. Virulence genotype of *Pasteurella multocida* strains isolated from different hosts with various disease status. Vet Microbiol 2006; 114: 304-17.

Frost LS, Leplae R, Summers AO, Toussaint A. Mobile genetic elements: the agents of open source evolution. Nat Rev Microbiol 2005; 3: 722-32.

Gagea MI, Bateman KG, Shanahan RA, van Dreumel T, McEwen BJ, Carman S, Archambault M, Caswell JL. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. J Vet Diagn Invest 2006; 18: 29-40.

Galdiero M, Folgore A, Nuzzo I, Galdiero E. Neutrophil adhesion and transmigration through bovine endothelial cells *in vitro* by protein H and LPS of *Pasteurella multocida*. Immunobiology 2000; 202: 226-38.

Gershwin LJ, Gunther RA, Hornof WJ, Larson RF. Effect of infection with bovine respiratory syncytial virus on pulmonary clearance of an inhaled antigen in calves. Am J Vet Res 2008; 69: 416-22.

Grissett GP, White BJ, Larson RL. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. J Vet Intern Med 2015; 29: 770-80.

Hansen LM, Hirsh DC. Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. Vet Microbiol 1989; 21: 177-84.

Hansen LM, McMurry LM, Levy SB, Hirsh DC. A new tetracycline resistance determinant, Tet H, from *Pasteurella multocida* specifying active efflux of tetracycline. Antimicrob Agents Chemother 1993; 37: 2699-705.

Harper M, Boyce JD, Adler B. The key surface components of *Pasteurella multocida*: capsule and lipopolysaccharide. Curr Top Microbiol Immunol 2012; 361: 39-51.

Harper M, John M, Turni C, Edmunds M, St Michael F, Adler B, Blackall PJ, Cox AD, Boyce JD. Development of a rapid multiplex PCR assay to genotype *Pasteurella multocida* strains by use of the lipopolysaccharide outer core biosynthesis locus. J Clin Microbiol 2015; 53: 477-85.

Headley SA, Okano W, Balbo LC, Marcasso RA, Oliveira TE, Alfieri AF, Negri Filho LC, Michelazzo MZ, Rodrigues SC, Baptista AL, Saut JPE, Alfieri AA. Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. J Vet Diagn Invest 2018; 30: 249-51.

Hilton WM. BRD in 2014: where have we been, where are we now, and where do we want to go? Anim Health Res Rev 2014; 15: 120-2.

Holschbach CL, Aulik N, Poulsen K, Ollivett TL. Prevalence and temporal trends in antimicrobial resistance of bovine respiratory disease pathogen isolates submitted to the Wisconsin Veterinary Diagnostic Laboratory: 2008-2017. J Dairy Sci 2020; 103: 9464-72.

Ishizaki H, Kariya Y. Road transportation stress promptly increases bovine peripheral blood absolute NK cell counts and cortisol levels. J Vet Med Sci 2010; 72: 747-53.

Jacques M, Kobisch M, Bélanger M, Dugal F. Virulence of capsulated and noncapsulated isolates of *Pasteurella multocida* and their adherence to porcine respiratory tract cells and mucus. Infect Immun 1993; 61: 4785-92.

Jatuponwiphat T, Chumnanpuen P, Othman S, T EK, Vongsangnak W. Iron-associated protein interaction networks reveal the key functional modules related to survival and virulence of *Pasteurella multocida*. Microb Pathog 2019; 127: 257-66.

Kadlec K, Watts JL, Schwarz S, Sweeney MT. Plasmid-located extended-spectrum β-lactamase gene blaROB-2 in *Mannheimia haemolytica*. J Antimicrob Chemother 2019; 74: 851-3.

Katsuda K, Kohmoto M, Mikami O, Uchida I. Antimicrobial resistance and genetic characterization of fluoroquinolone-resistant *Mannheimia haemolytica* isolates from cattle with bovine pneumonia. Vet Microbiol 2009; 139: 74-9.

Kehrenberg C, Salmon SA, Watts JL, Schwarz S. Tetracycline resistance genes in isolates of *Pasteurella multocida*, *Mannheimia haemolytica*, *Mannheimia glucosida* and *Mannheimia varigena* from bovine and swine respiratory disease: intergeneric spread of the tet(H) plasmid pMHT1. J Antimicrob Chemother 2001; 48: 631-40.

Kehrenberg C, Tham NT, Schwarz S. New plasmid-borne antibiotic resistance gene cluster in *Pasteurella multocida*. Antimicrob Agents Chemother 2003; 47: 2978-80.

Kehrenberg C, Schwarz S. Plasmid-borne florfenicol resistance in *Pasteurella multocida*. J Antimicrob Chemother 2005; 55: 773-5.

Kehrenberg C, Catry B, Haesebrouck F, de Kruif A, Schwarz S. tet(L)-mediated tetracycline resistance in bovine *Mannheimia* and *Pasteurella* isolates. J Antimicrob Chemother 2005; 56: 403-6.

Kehrenberg C, Wallmann J, Schwarz S. Molecular analysis of florfenicol-resistant *Pasteurella multocida* isolates in Germany. J Antimicrob Chemother 2008; 62: 951-5.

Klima CL, Alexander TW, Hendrick S, McAllister TA. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. Can J Vet Res 2014; 78: 38-45.

Klima CL, Holman DB, Cook SR, Conrad CC, Ralston BJ, Allan N, Anholt RM, Niu YD, Stanford K, Hannon SJ, Booker CW, McAllister TA. Multidrug Resistance in Pasteurellaceae Associated With Bovine Respiratory Disease Mortalities in North America From 2011 to 2016. Front Microbiol 2020; 11: 606438.

Kong LC, Gao D, Gao YH, Liu SM, Ma HX. Fluoroquinolone resistance mechanism of clinical isolates and selected mutants of *Pasteurella multocida* from bovine respiratory disease in China. J Vet Med Sci 2014; 76: 1655-7.

Lago A, McGuirk SM, Bennett TB, Cook NB, Nordlund KV. Calf respiratory disease and pen microenvironments in naturally ventilated calf barns in winter. J Dairy Sci 2006; 89: 4014-25.

Larson RL, Step DL. Evidence-based effectiveness of vaccination against *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni* in feedlot cattle for mitigating the incidence and effect of bovine respiratory disease complex. Vet Clin North Am Food Anim Pract 2012; 28: 97-106, e1-7, ix.

Lerminiaux NA, Cameron ADS. Horizontal transfer of antibiotic resistance genes in clinical environments. Can J Microbiol 2019; 65: 34-44.

Lopez A, Thomson RG, Savan M. The pulmonary clearance of *Pasteurella hemolytica* in calves infected with bovine parainfluenza-3 virus. Can J Comp Med 1976; 40: 385-91.

Lubbers BV, Hanzlicek GA. Antimicrobial multidrug resistance and coresistance patterns of *Mannheimia haemolytica* isolated from bovine respiratory disease cases--a three-year (2009-2011) retrospective analysis. J Vet Diagn Invest 2013; 25: 413-7.

Mehinagic K, Pilo P, Vidondo B, Stokar-Regenscheit N. Coinfection of Swiss cattle with bovine parainfluenza virus 3 and *Mycoplasma bovis* at acute and chronic stages of bovine respiratory disease complex. J Vet Diagn Invest 2019; 31: 674-80.

Melchner A, van de Berg S, Scuda N, Feuerstein A, Hanczaruk M, Schumacher M, Straubinger RK, Marosevic D, Riehm JM. Antimicrobial Resistance in Isolates from Cattle with Bovine Respiratory Disease in Bavaria, Germany. Antibiotics (Basel) 2021; 10

Michael GB, Kadlec K, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, Murray RW, Watts JL, Schwarz S. ICEPmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes. J Antimicrob Chemother 2012a; 67: 84-90.

Michael GB, Kadlec K, Sweeney MT, Brzuszkiewicz E, Liesegang H, Daniel R, Murray RW, Watts JL, Schwarz S. ICEPmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: structure and transfer. J Antimicrob Chemother 2012b; 67: 91-100.

Miyachiro MM, Contreras-Martel C, Dessen A. Penicillin-Binding Proteins (PBPs) and Bacterial Cell Wall Elongation Complexes. Subcell Biochem 2019; 93: 273-89.

Mizan S, Henk A, Stallings A, Maier M, Lee MD. Cloning and characterization of sialidases with 2-6' and 2-3' sialyl lactose specificity from *Pasteurella multocida*. J Bacteriol 2000; 182: 6874-83.

Modi SR, Lee HH, Spina CS, Collins JJ. Antibiotic treatment expands the resistance reservoir and ecological network of the phage metagenome. Nature 2013; 499: 219-22.

Müller M, Lochner N. Hämorrhagische Septikämie bei Wildtieren und Rindern in Nordbayern. Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit 2017: https://www.lgl.bayern.de/tiergesundheit/tierkrankheiten/bakterielle_pilzinfektionen/haemorrhagische_septikaemie/index.htm. 13 December 2021.

Muniandy N, Edgar, J., Woolcock, JB., Mukkur, T.K.S., . Virulence, purification, structure, and protective potential of the putative capsular polysaccharide of *Pasteurella multocida* type 6:B. Pasteurellosis in Production Animals International Workshop Pasteurellosis in production animas 1992: 47-54.

Murray GM, Cassidy JP, Clegg TA, Tratalos JA, McClure J, O'Neill RG, Sammin DJ, Casey MJ, McElroy M, Earley B, Bourke N, More SJ. A retrospective epidemiological analysis of risk factors for a primary necropsy diagnosis of bovine respiratory disease. Prev Vet Med 2016; 132: 49-56.

Negrete-Abascal E, Tenorio VR, de la Garza M. Secretion of proteases from *Pasteurella multocida* isolates. Curr Microbiol 1999; 38: 64-7.

Nordlund KV, Halbach CE. Calf Barn Design to Optimize Health and Ease of Management. Vet Clin North Am Food Anim Pract 2019; 35: 29-45.

Noyes NR, Benedict KM, Gow SP, Booker CW, Hannon SJ, McAllister TA, Morley PS. *Mannheimia haemolytica* in feedlot cattle: prevalence of recovery and associations with antimicrobial use, resistance, and health outcomes. J Vet Intern Med 2015; 29: 705-13.

OIE. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2018. https://www.oie.int/standard-setting/terrestrial-manual/access-online/: 2018: 19 November 2020.

Panciera RJ, Confer AW. Pathogenesis and pathology of bovine pneumonia. Vet Clin North Am Food Anim Pract 2010; 26: 191-214.

Paul Ehrlich Institute. Immunulogische Arzneimittel für Rinder. 2021: https://www.pei.de/DE/arzneimittel/tierarzneimittel/rinder/rinder-node.html. 24 November 2021.

Payne SM. Iron acquisition in microbial pathogenesis. Trends Microbiol 1993; 1: 66-9.

Periasamy S, Praveena PE, Singh N. Effects of *Pasteurella multocida* lipopolysaccharides on bovine leukocytes. Microb Pathog 2018; 119: 225-32.

Petruzzi B, Briggs RE, Tatum FM, Swords WE, De Castro C, Molinaro A, Inzana TJ. Capsular Polysaccharide Interferes with Biofilm Formation by *Pasteurella multocida* Serogroup A. mBio 2017; 8

Pillai DK, Cha E, Mosier D. Role of the stress-associated chemicals norepinephrine, epinephrine and substance P in dispersal of *Mannheimia haemolytica* from biofilms. Vet Microbiol 2018; 215: 11-7.

Portis E, Lindeman C, Johansen L, Stoltman G. A ten-year (2000-2009) study of antimicrobial susceptibility of bacteria that cause bovine respiratory disease complex--*Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*--in the United States and Canada. J Vet Diagn Invest 2012; 24: 932-44.

Praveena PE, Periasamy S, Kumar AA, Singh N. Pathology of experimental infection by *Pasteurella multocida* serotype A: 1 in buffalo calves. Vet Pathol 2014; 51: 1109-12.

Prudhomme M, Attaiech L, Sanchez G, Martin B, Claverys JP. Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. Science 2006; 313: 89-92.

Pruimboom IM, Rimler RB, Ackermann MR, Brogden KA. Capsular hyaluronic acid-mediated adhesion of *Pasteurella multocida* to turkey air sac macrophages. Avian Dis 1996; 40: 887-93.

Rimler RB, Rhoades KR. Serogroup F, a new capsule serogroup of *Pasteurella multocida*. J Clin Microbiol 1987; 25: 615-8.

Risseti RM, Zastempowska E, Twarużek M, Lassa H, Pantoja JCF, de Vargas APC, Guerra ST, Bolaños CAD, de Paula CL, Alves AC, Colhado BS, Portilho FVR, Tasca C, Lara GHB, Ribeiro MG. Virulence markers associated with *Trueperella pyogenes* infections in livestock and companion animals. Lett Appl Microbiol 2017; 65: 125-32.

Robert Koch Institute. Von AMR bis VRE: wichtige Begriffe und Abkürzungen im Überblick. 2020: https://www.rki.de/DE/Content/Infekt/Antibiotikaresistenz/Grundwissen/Glossar.htmlVon 9 January 2020.

Ruffolo CG, Tennent JM, Michalski WP, Adler B. Identification, purification, and characterization of the type 4 fimbriae of *Pasteurella multocida*. Infect Immun 1997; 65: 339-43.

Sanderson MW, Dargatz DA, Wagner BA. Risk factors for initial respiratory disease in United States' feedlots based on producer-collected daily morbidity counts. Can Vet J 2008; 49: 373-8.

Schoch CL, Ciufo S, Domrachev M, Hotton CL, Kannan S, Khovanskaya R, Leipe D, McVeigh R, O'Neill K, Robbertse B, Sharma S, Soussov V, Sullivan JP, Sun L, Turner S, Karsch-Mizrachi I. NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database (Oxford) 2020; 2020

Schott C, Cai H, Parker L, Bateman KG, Caswell JL. Hydrogen peroxide production and free radical-mediated cell stress in *Mycoplasma bovis* pneumonia. J Comp Pathol 2014; 150: 127-37.

Schunicht OC, Booker CW, Jim GK, Guichon PT, Wildman BK, Hill BW. Comparison of a multivalent viral vaccine program versus a univalent viral vaccine program on animal health, feedlot performance, and carcass characteristics of feedlot calves. Can Vet J 2003; 44: 43-50.

Selbitz H-J, Truyen U, Valentin-Weigand P (2015) Tiermedizinische Mikrobiologie, Infektions- und Seuchenlehre, 10 edn. Enke Verlag, Stuttgart

Sköld O. Sulfonamide resistance: mechanisms and trends. Drug Resist Updat 2000; 3: 155-60.

Snowder GD, Van Vleck LD, Cundiff LV, Bennett GL. Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. J Anim Sci 2006; 84: 1999-2008.

Snyder E, Credille B. *Mannheimia haemolytica* and *Pasteurella multocida* in Bovine Respiratory Disease: How Are They Changing in Response to Efforts to Control Them? Vet Clin North Am Food Anim Pract 2020; 36: 253-68.

Soike D, Schulze C, Kutzer P, Ewert B, van der Grinten E, Schliephake A, Ewers C, Bethe A, Rau J. [Acute pasteurellosis in fallow deer, cattle and pigs in a region of Eastern Germany]. Berl Munch Tierarztl Wochenschr 2012; 125: 122-8.

Stanford K, Zaheer R, Klima C, McAllister T, Peters D, Niu YD, Ralston B. Antimicrobial Resistance in Members of the Bacterial Bovine Respiratory Disease Complex Isolated from Lung Tissue of Cattle Mortalities Managed with or without the Use of Antimicrobials. Microorganisms 2020; 8

Stern AL, Van der Verren SE, Kanchugal PS, Näsvall J, Gutiérrez-de-Terán H, Selmer M. Structural mechanism of AadA, a dual-specificity aminoglycoside adenylyltransferase from *Salmonella enterica*. J Biol Chem 2018; 293: 11481-90.

StIKo Vet. Leitlinie zur Impfung von Rindern und kleinen Wiederkäuern. 2018: https://www.openagrar.de/servlets/MCRFileNodeServlet/openagrar_derivate_00013734/Impfleitlinie_Wiederkaeuer_2018-05-31_Schluetersche.pdf. 24 November 2021.

Svensson C, Liberg P. The effect of group size on health and growth rate of Swedish dairy calves housed in pens with automatic milk-feeders. Prev Vet Med 2006; 73: 43-53.

Sweeney MT, Lubbers BV, Schwarz S, Watts JL. Applying definitions for multidrug resistance, extensive drug resistance and pandrug resistance to clinically significant livestock and companion animal bacterial pathogens. J Antimicrob Chemother 2018; 73: 1460-3.

Theurer ME, Larson RL, White BJ. Systematic review and meta-analysis of the effectiveness of commercially available vaccines against bovine herpesvirus, bovine viral diarrhea virus, bovine respiratory syncytial virus, and parainfluenza type 3 virus for mitigation of bovine respiratory disease complex in cattle. J Am Vet Med Assoc 2015; 246: 126-42.

Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJ. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. J Clin Microbiol 1998; 36: 1096-100.

Townsend KM, Boyce JD, Chung JY, Frost AJ, Adler B. Genetic organization of *Pasteurella multocida* cap Loci and development of a multiplex capsular PCR typing system. J Clin Microbiol 2001; 39: 924-9.

Universität Leipzig. Veterinärmedizinischer Informationsdienst für Arzneimittelanwendung, Toxikologie und Arzneimittelrecht. 2020: https://vetidata.de/public/quicksearch/do.php. 14 December 2020.

Valarcher JF, Taylor G. Bovine respiratory syncytial virus infection. Vet Res 2007; 38: 153-80.

Vázquez-Laslop N, Mankin AS. How Macrolide Antibiotics Work. Trends Biochem Sci 2018; 43: 668-84.

Virtala AM, Gröhn YT, Mechor GD, Erb HN. The effect of maternally derived immunoglobulin G on the risk of respiratory disease in heifers during the first 3 months of life. Prev Vet Med 1999; 39: 25-37.

Wang Y, Goodman SD, Redfield RJ, Chen C. Natural transformation and DNA uptake signal sequences in *Actinobacillus actinomycetemcomitans*. J Bacteriol 2002; 184: 3442-9.

Watts JL, Yancey RJ, Jr., Salmon SA, Case CA. A 4-year survey of antimicrobial susceptibility trends for isolates from cattle with bovine respiratory disease in North America. J Clin Microbiol 1994; 32: 725-31.

Welsh RD, Dye LB, Payton ME, Confer AW. Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994--2002. J Vet Diagn Invest 2004; 16: 426-31.

White DJ, Jolley WL, Purdy CW, Straus DC. Extracellular neuraminidase production by a *Pasteurella multocida* A:3 strain associated with bovine pneumonia. Infect Immun 1995; 63: 1703-9.

White PA, Rawlinson WD. Current status of the aadA and dfr gene cassette families. J Antimicrob Chemother 2001; 47: 495-6.

WHO. GLOBAL ACTION PLAN ON ANTIMICROBIAL RESISTANCE. 2015: https://www.who.int/publications/i/item/9789241509763. 28 August 2021.

WHO. Critically Important Antimicrobials for Human Medicine. 2019: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf. 15 December 2020.

Wildman BK, Perrett T, Abutarbush SM, Guichon PT, Pittman TJ, Booker CW, Schunicht OC, Fenton RK, Jim GK. A comparison of 2 vaccination programs in feedlot calves at ultra-high risk of developing undifferentiated fever/bovine respiratory disease. Can Vet J 2008; 49: 463-72.

Woolums AR, Karisch BB, Frye JG, Epperson W, Smith DR, Blanton J, Jr., Austin F, Kaplan R, Hiott L, Woodley T, Gupta SK, Jackson CR, McClelland M. Multidrug resistant *Mannheimia haemolytica* isolated from high-risk beef stocker cattle after antimicrobial metaphylaxis and treatment for bovine respiratory disease. Vet Microbiol 2018; 221: 143-52.

Zhang PY, Xu PP, Xia ZJ, Wang J, Xiong J, Li YZ. Combined treatment with the antibiotics kanamycin and streptomycin promotes the conjugation of *Escherichia coli*. FEMS Microbiol Lett 2013; 348: 149-56.

IX. ACKNOWLEDGEMENTS

I would like to start by thanking Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D. for providing me with this exciting and practice-oriented topic, as well as for the professional supervision of this dissertation work.

I would especially like to thank my mentor, PD Dr. Julia M. Riehm, who gave me the opportunity to do this work at the Bavarian Health and Food Safety Authority in Oberschleißheim. The competent supervision contributed substantially to the success of this dissertation work.

I would also like to thank Sarah van de Berg and Durdica Marosevic, who supported me in the statistical analysis. Without them, a statistical analysis in this form would not have been possible.

I would also like to thank all the co-authors, the laboratory staff and all the other people who have contributed in any way to the success of this dissertation work.

I would like to thank Dr. Ulrich Güthle and the whole team of the Tierarztpraxis Geilsheim, Wassertrüdingen, for giving me the opportunity to work as a practical veterinarian during my time as doctoral researcher.

Finally, I would like to thank my family, my friends and all those who have accompanied me on my journey through life.