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Genetic relatedness and antimicrobial susceptibility of porcine respiratory tract pathogens *Streptococcus suis, Bordetella bronchiseptica* and *Pasteurella multocida*

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“Is it not a strange fate that we should suffer so much
fear and doubt for so small a thing?
So small a thing!”

Boromir,
The Lord of the Rings – The Fellowship of the Ring,
J. R. R. Tolkien, 1954

Table of contents

List of figures and tables

Figures of the introduction

Figures and tables of publication I

Figures and tables of publication II

Tables of publication III

Tables of the unpublished data

Figures and tables of the appendix

List of abbreviations

Ala	alanine
Asn	asparagine
BALF	bronchoalveolar lavage fluid
bp	base pairs
bvg	*Bordetella virulence gene* locus
BVL	Federal Office of Consumer Protection and Food Safety
CLSI	Clinical and Laboratory Standards Institute
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
F-factor	fertility factor
Gln	glutamine
Gly	glycine
HMM	high molecular mass
ICE	integrative and conjugative element
kb	kilo base pairs
LMM	low molecular mass
Lys	lysine
MIC	minimal inhibitory concentration
MLS_B	macrolide-lincosamide-streptogramin B
MLST	multi-locus sequence typing
Mob	mobilization protein
oriT	origin of transfer
oriV	origin of vegetative replication
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
Phe	phenylalanine
PMT	*Pasteurella multocida*-toxin
RND	resistance-nodulation-division family
Ser	serine
ST	sequence type
SXT	trimethoprim/sulfamethoxazole
T4CP	type IV coupling protein
T4SS	type IV secretion system
Thr	threonine
X	any amino acid

1 Introduction

The high antimicrobial consumption in veterinary medicine has become an issue of great public concern. Reports on the increase of multidrug-resistant bacteria in animals, humans and the environment result in discussions of the antimicrobial treatment in veterinary medicine. After the 16th amendment of the German veterinary medicine act came into force in July 2014, a further reduction of antimicrobial consumption was noted in veterinary medicine [1, 2]. As a result of improved education and antibiotic stewardship measures for veterinarians and farmers, the sales figures of antimicrobial agents in veterinary medicine in Germany decreased from 1,706 t in 2011 to 733 t in 2017. This corresponds to a decrease by 57.03 % [2]. However, these sales figures cannot be attributed to specific animal species since the majority of veterinary antimicrobial agents is approved for use in various animal species. Thus, it is unknown how which amounts of which antimicrobial agents are used in Germany for the treatment of specific animals or specific diseases in a given animals species. Moreover, it is also not known whether there are regional differences in the preferential use of certain antimicrobial agents in veterinary medicine. Despite this significant decrease of sales figures for antimicrobial agents in veterinary medicine, the German National Resistance Monitoring program GE*RM*-Vet, conducted by the Federal Office of Consumer Protection and Food Safety (BVL), still identifies a considerable proportion of resistant bacteria from food-producing as well as from companion animals [3-6]. The analysis of trends in antimicrobial resistance must be conducted with extreme caution, as ‘resistance’ commonly refers to a specific antimicrobial agent and a specific bacterial pathogen in a specific disease condition of a given animal species. Thus, resistance to pirlimycin among *Staphylococcus aureus* from mastitis in dairy cows differs completely from resistance to ampicillin among *Escherichia coli* from intestinal infections of pigs. Nevertheless, bacteria which exhibit resistance to antimicrobial agents are still a major problem and require adequate control measures.

This project was based on the monitoring of 30 porcine farrow-to-finish farms for their frequency of respiratory tract therapies with antimicrobial agents within a one-year period, the detection of three defined respiratory tract pathogens, their relatedness as well as their antimicrobial susceptibilities/resistances.

1.1 Structure of German pig farms

The German pig industry follows a hierarchal system, as represented in Figure 1. The purebred pigs of the nucleus farms, on the top of the hierarchal pyramid, produce pigs for the multiplier farms. In multiplier farms, gilts are reproduced and sold to farrow-to-nursery farms. In farrow-to-nursery farms, these gilts, later designated as sows, reproduce piglets that become fattening pigs [7]. The last three levels of pig farming (farrow-to-nursery, nursery-to-finish and finish farms) can be combined in a farrow-to-finish farm (closed system) or remain as independent farms. The advantage of the closed farm system is a decreased import of pigs that may bring along a variety of porcine pathogens from their origins. In closed farms, no piglets from external farms are purchased. Besides, gilts may be bred within closed farms to replace older sows. Nevertheless, new gilts of farrow-to-nursery or farrow-to-finish farms can also be purchased from external sources of multiplier farms [7, 8]. Whenever animals are purchased from other farms, they may bring along their farm-specific spectrum of bacteria to which these pigs are usually well-adapted. However, when mingled with animals in the new farm, a transfer of bacteria between pigs from both farms will occur and may result in respiratory or enteric diseases. These diseases are supported by stress factors, such as transport, change of the diet in the new farm or fights for social ranking. The disadvantage of closed farms is a fast spread of pathogens through all production stages, since the separation within those is supposed to be less restrictive [7].

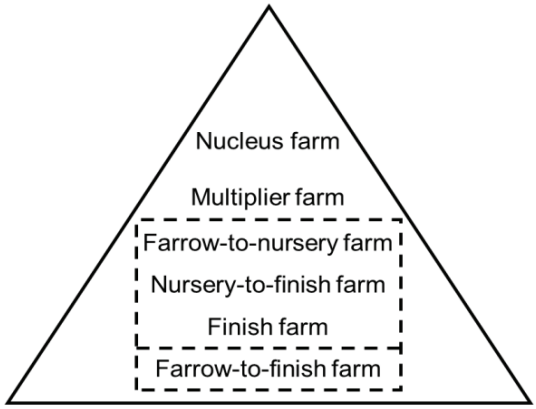

Figure 1 Hierarchal structure of German pig industry.

Pig farms are organized according to the “all-in, all-out” policy. “All-in, all-out” means that a group of pigs is moved as an entire group into a new barn, which was cleaned and disinfected before. In this new barn, the next production period starts. Farrow-to-finish farms consist of different barns that harbor pigs in their different production stages. Sows are moved through different barns while changing between the breeding, gestation and farrowing period (Figure 2). They are inseminated during the breeding period in a specific barn. The whole group of sows is moved into the gestation barn after positive pregnancy test, for approximately 115 days. In their gestation period, sows are kept in groups within the barn, where they stay in close contact. Next, the sows are moved again into the farrowing pen just before giving birth. There, the sows deliver a litter of piglets with whom they spend the time of lactation together. After the lactation time of 21 or 28 days, the breeding period of the sows starts again. Then, the piglets are separated from the sows and moved into the nursery barn. Before starting the

nursery and fattening periods, piglets with similar weights are rearranged together into boxes. This method is used to reach better results in weight gain. At this time, the piglets have a weight of approximately 7 kg and are designated as weaners. The growing and finishing period starts after the end of the nursery period, when the weaners reach a weight of approximately 28 kg [7, 8]. Fattening pigs are slaughtered at a weight of 120 kg after a life cycle of 190 to 200 days [8, 9]. Pig farms are structured on the basis of the black-and-white principle. The black area describes the polluted, pathogen-containing outer part of a pig farm. In contrast, the white area is defined as the inner part of the pig farm. Before farm workers or veterinarians enter pig stables, they have to shower and change their clothes and rubber boots [8]. In farms with a high hygienic standard, farm workers change their clothes even before entering a new barn with a different pig group. In general, the contact between humans and pigs is minimized as much as possible to reduce pathogen transfer [8].

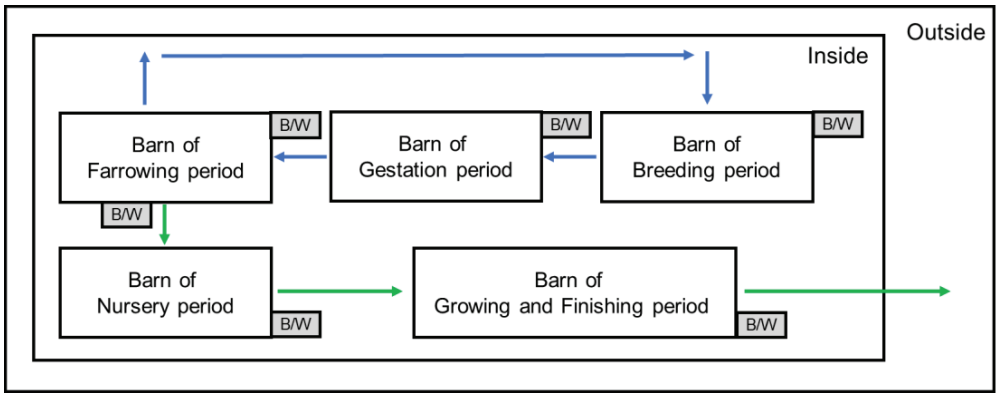

Figure 2 Schematic structure of a closed farm (farrow-to-finish farm), indicating the way of the sows with blue and the way of the piglets with green arrows. Black-and-white areas (B/W) are shown as grey shaded boxes.

Pathogens can be transferred directly or indirectly. **Direct transmission** describes fecal-oral, airborne, cutaneous, prenatal or perinatal contacts between animals that result in pathogen transfer [10]. **Indirect transmission** occurs when pathogens are attached to objects that come into contact with animals. This might be through food, water, soil, dust, inanimate objects like buckets or rubber boots, arthropods or rodents which serve as vectors. Moreover, humans who come into contact with pigs can also transfer pathogens [10].

Good hygienic conditions in intense pig farming and vaccination programs are the first lines of defense to avoid respiratory tract infections. Infections in livestock populations, especially in pig and poultry farms, are commonly treated as group medications via feed or drinking water [11]. In this doctoral thesis, the term pathogen is used to describe disease-causing bacteria, even though viruses, parasites, yeasts and fungi can also act as causative agents of diseases.

In order to characterize the relatedness of selected pathogens in this study, we used whole genome sequencing, pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) methods. The PFGE DNA fragment patterns allow conclusions about the genetic relatedness of different isolates, especially during disease outbreaks, based on their genomic structure. Isolates that show an indistinguishable DNA fragment pattern are supposed to be members of the same clone [12]. **Clones** are defined as ' bacterial cultures isolated independently from different sources, in different locations, and perhaps at different times, but showing many identical phenotypic and genetic traits that the most likely explanation for this identity is a common origin ' [13]. Antimicrobial susceptibility testing of bacterial pathogens provides helpful information on the basis of which the veterinarian can choose the most suitable antimicrobial for therapeutic interventions. In Germany, β-lactams, tetracyclines, fluoroquinolones, macrolides, aminoglycosides, sulfonamides and phenicols are approved antimicrobial classes of therapeutic interventions of pigs suffering from respiratory tract infections [14]. In practice, amoxicillin is the most frequently prescribed antimicrobial agent for respiratory tract infections in weaner pigs, followed by tetracyclines and sulfonamides [15].

1.2 Respiratory tract pathogens

Respiratory tract infections in pigs are multifactorial diseases, commonly induced by viruses, and only in rare cases by bacteria. Bacteria might appear as secondary invaders after the damaging of the respiratory tissue. Major viral respiratory tract pathogens in pigs are swine influenza virus, porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2). *Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae,* toxin-producing *Pasteurella multocida* and *Bordetella bronchiseptica* are listed as primary infectious bacterial pathogens [14]. In contrast, non-toxin producing *P. multocida, Streptococcus suis* and *Haemophilus parasuis* are considered to be secondary pathogens [14].

1.2.1 General characteristics of Streptococcus suis

S. suis isolates are Gram-positive coccoid bacteria, belonging to the order *Lactobacillales* and the family *Streptococcaceae.* Streptococci are categorized according to their type of hemolysis, their colony size and some of them, especially the pyogenic group, are classified into Lancefield groups due to their C-polysaccharides [16, 17]. Streptococci are grouped into β-hemolytic and non-β-hemolytic streptococci, both including a wide variety of mammalian and avian pathogens and are known to have zoonotic potential. The group of β-hemolytic streptococci is also designated as pyogenic group, comprising *Streptococcus*

pyogenes, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus equi subsp. *equi, Streptococcus equi* subsp. *zooepidemicus* and *Streptococcus canis.* Whereas the non-β-hemolytic streptococci include *S. suis, Streptococcus pneumoniae* and the viridans group of streptococci. Streptococci of the non-β-hemolytic group are considered to be α- or γ-hemolytic and are designated as the non-pyogenic group, although not all species belonging to this classification accomplish these characteristics [16]. *S. suis* was formerly classified into 35 serotypes according to its capsular polysaccharide antigens [18, 19]. However, recent examinations of 16S rRNA sequence similarities reclassified *S. suis* into just 29 serotypes [20, 21]. For a closer differentiation and typing of virulent and avirulent *S. suis* isolates, King *et al.* (2002) established a multi-locus sequence typing (MLST) scheme consisting of seven housekeeping genes, which revealed a phylogenetically diverse species. *S. suis* is usually a commensal, that is transferred by direct oral or nasal contact as well as by indirect contact and colonizes the mucosa of the upper respiratory tract, especially the tonsils, in pigs [19, 23, 24]. Infections are caused by virulent serotypes. *S. suis* serotype 2, followed by serotypes 1, 1/2, 7 and 9 are designated as the most virulent ones, since they were confirmed in many porcine infections in Europe [25]. However, the **virulence factors** of *S. suis* are crucial for host infection. The streptococcal capsule mediates protection against phagocytosis [26]. The hyaluronidase supports the bacterial dissemination within the host [27]. Muramidase-released protein, extracellular factor and their weight variants, which are indicated to be heavier or lighter than the original proteins due to changes in their amino acid sequence, are considered to be associated with virulence [25, 28-31]. Some *S. suis* isolates express suilysin, which is a hemolytic toxin that damages the hosts' erythrocyte membrane and thereby leading to the release of their intracellular substances, like iron, which are necessary for the pathogens' survival [32, 33]. **Infections** with virulent *S. suis* can result in meningitis, pneumonia, arthritis, septicemia, endocarditis, polyserositis or abortion in pigs [19]. Infections in humans are described in people working or living in close contact to pigs, especially on the Asian continent in slaughterhouses, pig farms or laboratories. However, *S. suis* is also detected in other mammals, including dogs, cats, ruminants and horses, as well as in birds [19, 34-37].

1.2.2 Important antimicrobial resistance properties of S. suis

Resistance to **β-lactams** in general can be either mediated by (1) β-lactamases, (2) changes in the penicillin-binding proteins (PBPs) or (3) reduction of the intracellular antimicrobial concentration [38]. However, so far no acquired resistance genes like β-lactamases or alternate PBPs, such as *mecA* in *S. aureus*, have been detected in *S. suis* [39, 40]. Furthermore, a decreased intracellular accumulation of β-lactams through efflux pumps

has been described only for Gram-negative bacteria [41]. Consequently, alterations of single amino acids in the penicillin-binding proteins (PBPs) are discussed to have effects on β-lactam resistance in *S. suis* [42, 43]. PBPs are important proteins for the synthesis of the Gram-positive peptidoglycan cell wall. They are membrane-bound enzymes consisting of three parts, a domain at the outer surface, a transmembrane anchor and a cytoplasmatic tail. PBPs are classified into proteins with high molecular mass (HMM) and low molecular mass (LMM). The HMM PBPs are divided into bifunctional class A proteins that exhibit glycosyltransferase as well as transpeptidase functions and monofunctional class B proteins with only transpeptidase activity. LMM proteins include class C PBPs with DD-carboxypeptidase function, which, however, are supposed to have no effect in β-lactam resistance [41, 44]. Different bacterial species have variable numbers of PBPs [45]. Based on the lack of information about PBPs of *S. suis,* the names of the five PBPs (PBP1a, 2a, 1b, 2x and 2b) as well as their conserved motifs were adopted from *S. pneumoniae* [39, 42, 45-47]. Six PBPs are known to occur in *S. pneumoniae*. Class A summarizes three proteins, named PBP1a, PBP2a and PBP1b. The class B proteins in *S. pneumoniae* are PBP2x and PBP2b, while PBP3 is a class C protein [45]. Ser-X-X-Lys, Ser-X-Asn and Lys-Thr/Ser-Gly represent the conserved motifs at the catalytic site of the PBPs [43, 46, 48]. β-Lactams bind covalently at the catalytic Ser of the Ser-X-X-Lys motif and cause an inhibition of the peptidoglycan synthesis with a bactericidal effect on growing bacteria [46]. The (a) development of mosaic genes or (b) accumulation of point mutations that may cause amino acid alterations in indigenous PBPs can result in a reduced affinity to β-lactams. (a) Mosaic genes are composed of various domains: these domains can be part of individual genes. During homologous recombination events, closely related species may exchange sequences coding for protein domains. These recombinations can result in the formation of genes that code for proteins consisting of two indigenous domains and a new domain obtained from another species [49]. For example, PBPs of penicillin-resistant *S. pneumoniae* distinctly differ from those of penicillin-susceptible *S. pneumoniae.* Different parts of the PBPs of a penicillin-resistant *S. pneumoniae* are similar to regions of PBPs from penicillin-resistant *S. mitis* or *S. oralis* isolates [50-54]. (b) Point mutations within the genes for PBPs may result in amino acid alterations that could have effects on the protein structure. Amino acid substitutions nearby or within the active center as well as changes in charge might have the greatest effects in mediating β-lactam resistance [47, 55-58]. Furthermore, amino acid alterations in PBP2x and PBP2b are associated with low-level β-lactam resistance, whereas additional substitutions in PBP1a are considered to confer high-level β-lactam resistance in *S. pneumoniae* [58, 59].

Many *S. suis* isolates are resistant to **tetracyclines** through acquired resistance genes. The mechanisms of tetracycline resistance are distinguished as (1) reduction of intracellular

tetracycline concentration, (2) protection of the ribosomal target site, or (3) enzymatic inactivation [60]. Until now, only genes that code for efflux pumps and ribosomal protection proteins have been detected in *S. suis* [61-64]. Tetracyclines bind at the ribosome, inhibit the tRNA attachment during translation, and stop the protein biosynthesis. Ribosome protective proteins are expressed in the presence of tetracyclines. They bind to the ribosome as well, but do not interrupt translation and simultaneously prevent drug binding [60]. Ribosomal protection proteins of *S. suis* are encoded by *tet*(O), *tet*(M), *tet*(W) and *tet*(O/W/32/O) [43, 63, 64]. Efflux pumps transport tetracyclines actively against the concentration gradient out of the cell. Thereby, protons provide the necessary energy due to their transfer into the cell along the concentration gradient. In *S. suis, tet*(L), *tet*(B) and *tet*(40) were identified to encode such efflux pumps [43, 63, 64].

S. suis is considered to be mainly susceptible to **florfenicol**, although florfenicol resistance has already been detected in single *S. suis* isolates [65, 66]. Phenicols inhibit the bacterial protein biosynthesis due to binding at the 50S ribosomal subunit [67]. Florfenicol is the fluorinated derivate of chloramphenicol and approved to treat food-producing animals. In contrast, chloramphenicol is banned from use in food-producing animals [68]. In general, resistance to chloramphenicol and/or florfenicol is mediated by a (1) reduction of the intracellular phenicol concentration, (2) modification of target sites, or (3) enzymatic inactivation [67]. The gene *fexA* encodes an efflux pump that confers resistance to chloramphenicol as well as florfenicol and was detected on the plasmid pStrcfr of *S. suis* [69, 70]. On the same plasmid, the gene *cfr* was located [70]. This gene encodes a RNA methyltransferase and mediates resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A [70, 71]. Chloramphenicol acetyltransferase genes (*cat*) are classified into type A or B, which depends on their structure [67]. The chloramphenicol acetyltransferase mediates resistance only to chloramphenicol and *catA* was already identified in *S. suis* [72].

Resistance to **macrolides** and **lincosamides** is also mediated by a (1) reduction of intracellular antimicrobial concentration, (2) modification of ribosomal target sites or (3) enzymatic inactivation. Macrolides, lincosamides and streptogramin B agents have a different chemical structure but attack the same ribosomal site for bacterial protein translation. Therefore, resistance against one agent frequently leads to cross-resistance against the other two. This fact is designated as MLS_B resistance [73]. Antimicrobial binding at the 50S subunit of the ribosome causes an inhibition of the bacterial protein translation [73]. In *S. suis,* the major facilitator superfamily- or ATP-binding transporters, encoded by *mef*(A) and *msr*(D), respectively, mediate an increased antimicrobial efflux of macrolides and lincosamides [43, 63, 64, 73]. The ribosome methylase encoded by the gene *erm*(B) represents the most common

MLS_B resistance gene in *S. suis* [43, 63, 64]. This erythromycin rRNA methyltransferase methylates an adenine residue in the ribosomal 23S rRNA and prevents the binding of MLS_B agents [74]. Unlike, *erm*(C) has been detected in *S. suis* only rarely [64]. The macrolide phosphorylase encoded by the gene *mph*(C), inactivates macrolides and has only rarely been identified in *S. suis* [64].

1.2.3 General characteristics of Bordetella bronchiseptica

B. bronchiseptica are Gram-negative, obligate aerobic, rod-shaped coccobacilli [75]. They belong to the order *Burkholderiales* and the family *Alcaligenaceae* in the β-class of *Proteobacteria* [76]. The genus *Bordetella* comprises eight species that are mammalian or avian pathogens, with *B. bronchiseptica, B. pertussis* and *B. parapertussis* in a close genetic relation [77]. *B. bronchiseptica* colonizes the upper respiratory tract. *B. bronchiseptica* virulence is regulated by the *Bordetella virulence gene* locus (*bvg*) [78]. Good environmental conditions like a temperature of 37°C, low concentration of nicotinic acid or sulphate anions result in the activation of the *bvg* locus [79, 80]. **Virulence factors** that are expressed in this *bvg*+ phase facilitate *B. bronchiseptica* to infect the respiratory tract. In this phase the filamentous hemagglutinin, fimbriae, adenylate cyclase or dermonecrotic toxin are expressed [78, 81]. The dermonecrotic toxin impairs the osteoblast differentiation, which causes nasal atrophy in turbinate pig bones [82]. The name of the dermonecrotic toxin originates from its induction of necrosis in guinea pig skin after intradermal injection [83]. The *bvg*- phase is characterized by a reduced expression of virulence factors, which enables *B. bronchiseptica* to rest under limited nutritional conditions [79-81, 84]. Unlike to *B. pertussis* and *B. parapertussis*, *B. bronchiseptica* is motile through peritrichous flagellation that is expressed in the *bvg*- phase [76, 85]. Overall, *B. bronchiseptica* infects the respiratory tract of various animals and is transmitted through direct contact due to aerosol droplets of *B. bronchiseptica* carrying hosts, but also through indirect contact via infectious air over short distances [81, 86]. Pigs are one of the most predisposed species for *B. bronchiseptica* **infections**, especially in areas of intensive animal farming [75]. *B. bronchiseptica* can induce bronchopneumonia. In addition, they can also cause a mild form of atrophic rhinitis, but often serve as primary invader to predispose the respiratory tissue for a subsequent infection with toxin-producing *P. multocida* [75, 76, 87]. *B. bronchiseptica* along with the canine parainfluenza virus are the main pathogens of kennel cough in dogs [75]. *B. bronchiseptica* can colonize humans and cause pneumonia in immunocompromised persons [88]. Furthermore, bronchopneumonia, caused by *B. bronchiseptica,* was also reported in guinea pigs, rabbits, cats and horses [76, 78].

1.2.4 Important antimicrobial resistance properties of B. bronchiseptica

β-Lactam resistance in *B. bronchiseptica* is mediated by a species-specific chromosomally located β-lactamase producing bla_{BOR-1} gene that can be inhibited by clavulanic acid [89]. Additionally, some *B. bronchiseptica* isolates harbor a not species-specific class 1 integron with a bla_{OXA-2} gene cassette, which is located on the chromosome or plasmids [90]. Kadlec *et al.* (2007) assumed that active efflux pumps do not play a role in ampicillin resistance, since their inhibition had no effect on bacterial susceptibility. The same study supposed a β-lactamase-independent cephalosporin resistance based on reduced outer membrane permeability [90].

B. bronchiseptica is supposed to be susceptible to **tetracyclines**, although no clinical breakpoints approved by the Clinical and Laboratory Standards Institute (CLSI) are currently available [6, 91]. Nevertheless, *B. bronchiseptica* can harbor acquired, plasmid-located tetracycline resistance genes. These genes, *tet*(A) or *tet*(C), encode efflux pumps and commonly mediate a minimal inhibitory concentration (MIC) of ≥64 mg/L [92, 93]. The *tet*(A) gene is part of transposon Tn*1721* and is coupled to the repressor gene *tetR*, which results in tetracycline-inducible expression of the Tet(A) efflux pump [93, 94].

The gene *floR* of *B. bronchiseptica* is chromosomally located and encodes an efflux pump that mediates resistance to chloramphenicol and **florfenicol** [95]. Furthermore, *B. bronchiseptica* isolates were identified, which harbored the plasmid-borne gene *cmlB1*, which codes only for a chloramphenicol efflux pump. Chloramphenicol resistance in *B. bronchiseptica* can also be mediated by the genes *catB2* and *catB3*, which code for class B chloramphenicol acetyltransferases. These genes were identified on different class 1 integrons, which were located either in the chromosomal DNA or on plasmids [96].

Gram-negative bacteria are thought to usually have a reduced susceptibility to **macrolides**, **lincosamides** and **streptogramins** of the B group (MLS_B), due to their decreased outer membrane permeability for these classes of antimicrobials [97]. Moreover, many Gram-negative bacteria are associated with multi-drug efflux pumps that transfer harmful agents, e.g. antimicrobials, detergents or salts out of the cell. Among them, efflux pumps belonging to the resistance-nodulation-division (RND) family, play an important role. RND pumps obtain their energy from a proton-antiporter system [97, 98]. Resistance to MLS_B antibiotics in Gram-negatives and Gram-positives can result from mutations that cause alterations in the V domain of the 50S ribosomal subunit of the 23S rRNA. These mutations confer a reduced affinity of the ribosome to MLS_B [73, 99, 100].

1.2.5 General characteristics of Pasteurella multocida

P. multocida belongs to the family *Pasteurellaceae* within the order *Pasteurellales.* This family includes many animal and/or human pathogenic genera, e.g. *Mannheimia* spp., *Actinobacillus* spp., *Haemophilus* spp. and *Histophilus* spp. [101]. *P. multocida* are Gram-negative, rod-shaped coccobacilli [101]. *P. multocida* typing is based on their capsule, consisting of hydrophilic polysaccharides that are linked to the cell membrane. Carter (1955) established capsule typing by passive hemagglutination of erythrocytes with different *P. multocida* capsule antigens. Hemagglutination has resulted into five different capsular serotypes, designated as A, B, D, E and F. The capsules of serotype A consists mainly of hyaluronic acid, those of serotype B of arabinose, mannose and galactose, the capsules of serotype D of heparin, and those of serotype F of chondroitin, whereas the capsules of serotype E are largely uncharacterized. Serotype C has not been confirmed yet and does not belong to the classification system anymore [102-107]. *P. multocida* colonizes mucosal surfaces, especially the respiratory and oropharyngeal tract in animals and is transferred between them through direct contact [103, 108]. *P. multocida* might act as primary pathogen or as secondary opportunistic pathogen by chance after a primary viral or bacterial infection, e.g. with *B. bronchiseptica*, has occurred [109, 110]. The capsule represents an important **virulence factor** that mediates adherence at cell surfaces and provides protection against phagocytosis and the host complement response [111]. Fimbriae, e.g. type IV, are responsible for adhesion at and colonization of the epithelium of the respiratory tract [112, 113]. Some *P. multocida* isolates of serotypes D and sometimes type A, possess the *toxA* gene, which encodes the *P. multocida*-toxin (PMT) that belongs to the dermonecrotic toxin family [103, 114]. Furthermore, PMT has similar properties like the described dermonecrotic toxin of *B. bronchiseptica* [115]. Consequently, PMT also causes deformation of the pigs' nasal bones. *P. multocida* **infections** are known to occur in many animals, e.g. pigs, cattle, rabbits, dogs, cats, birds and reptiles. *P. multocida* is also considered as a zoonotic pathogen [101, 108]. Human infections occur especially as local injuries through bites and scratches of cats or dogs, as pneumonia when humans are living in close contact to animals or rarely as systemic infections, especially in immunocompromised humans [116, 117]. PMT-producing *P. multocida* triggers the progressive form of atrophic rhinitis in pigs, which is indicated by purulent nasal discharge, sneezing and distorted turbinate bones [14]. Non-toxin producing serotype A isolates are associated with porcine pneumonia [101].

1.2.6 Important antimicrobial resistance properties of P. multocida

P. multocida isolates are considered to have a high susceptibility to antimicrobial agents, although a number of acquired resistance genes have been detected during the past 20 years [6, 118]. The resistance genes of *P. multocida* are commonly located on mobile genetic elements, mainly on plasmids. Plasmids of *P. multocida* that harbor antimicrobial resistance genes range in sizes between 4.3 to 11 kilobases (kb) [118-120]. The acquisition of resistance gene clusters can result in the development of multidrug-resistant bacteria through antimicrobial co-selection. A transfer of resistance genes commonly occurs within the *Pasteurellaceae* family, but also to other Gram-negative as well as Gram-positive bacteria [118, 121, 122].

β-Lactam resistance of *P. multocida* is usually mediated by β-lactamases. So far *bla* genes encoding ROB-1, TEM-1 and PSE-1 β-lactamases were detected [123-125]. Recently, an extended-spectrum β-lactamase gene, bla_{ROB-2}, was detected on a plasmid of a *Mannheimia haemolytica* isolate for the first time. Plasmid transformation experiments resulted in the plasmid uptake into *P. multocida* and mediated β-lactam resistance to them [126].

Energy-depended substrate specific efflux pumps of the major facilitator superfamily commonly meditate **tetracycline** resistance in *P. multocida* [118]. While genes coding for ribosomal protective proteins are less frequent. The most prevalent efflux pump detected in *P. multocida* is encoded by the gene *tet*(H), which is coupled to a tetracycline depended repressor *tetR* [127, 128]. The *tet*(H) gene has first been described to be part of the composite transposon Tn*5706*, which was located on a plasmid. However, *tet*(H) genes have also identified in the chromosomal DNA and as parts of integrative and conjugative elements (ICEs), such as ICE*Pmu1* [129]. The genes *tet*(B), *tet*(G) and *tet*(L), all coding for tetracycline exporters, have also been found in *P. multocida* [125, 130, 131]. The gene *tet*(M) is the only *tet* gene that encodes a ribosomal protective protein in *P. multocida* [132].

Resistance to **florfenicol** and chloramphenicol of *P. multocida* is mediated by a plasmid-located gene *floR* [133]. Resistance is to non-fluorinated phenicols can be conferred by the chloramphenicol acetyltransferase genes *catA1*, *catA3* and *catB2* [125, 134].

Aminoglycosides bind at the 30S subunit of the ribosome and inhibit protein translation. Aminoglycoside resistance can be either mediated by a (1) reduction of intracellular antimicrobial concentration, (2) the modification of the target site or (3) enzymatic inactivation. Enzymatic inactivation represents the only resistance mechanism against aminoglycosides in *P. multocida*. The drug specific enzymes adenylate, acetylate or phosphorylate aminoglycosides and cause a structural aminoglycoside modification that makes the aminoglycosides unable to bind at the ribosome [118]. The streptomycin resistance mediating

genes *strA* and *strB* are frequently detected in *P. multocida* [135]. Aminoglycoside-3''-phosphotransferase is encoded by *strA*, while *strB* codes for an aminoglycoside-6-phosphotransferase. Furthermore, the genes *aadA1, aadA14* and *aadA25* mediate resistance to the combination of streptomycin and spectinomycin. The *aadB* and *aacA4* genes confer gentamicin resistance and *aphA1* as well as *aphA3* mediates resistance to the combination of kanamycin and neomycin in *P. multocida* [125, 129, 136, 137].

Sulfonamides and **trimethoprim** (SXT) competitively inhibit the enzymes dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR) of the folic acid biosynthesis pathway, respectively [138]. The antimicrobial effect is based on the prevention of bacterial growth caused by the lack of the essential folic acid. The genes *sul2, dfrA14* and *dfrA20* are the only detected sulfonamide and trimethoprim resistance genes in *P. multocida* and all three genes are commonly located on plasmids [120, 139, 140]. The gene *sul2* encodes an insensitive form of DHPS that mediates resistance to sulfonamides, whereas *dfrA14* and *dfrA20* code for a DHFR that confers trimethoprim resistance. Moreover, *sul2* is often plasmid associated together with *strA* and/or *strB* within the family of *Pasteurellaceae* [129, 135, 139, 141].

1.3 Horizontal gene transfer among bacteria

Vertical gene transfer is the transmission of the complete genome from one generation to the next. The complement DNA strand is transferred during cell division from a parent to a daughter cell. In contrast, horizontal gene transfer describes the transmission of pieces of the genetic information within a generation [142]. Transformation, transduction and conjugation/mobilization are processes of horizontal gene transfer.

Transformation is defined as the uptake of free DNA that is located in the environment of competent bacteria. These incorporated DNA segments can originate from viruses or bacteria [143]. This process allows intra- and inter-species gene transfer, including an uptake of antimicrobial resistance genes. External DNA fragments can be integrated as plasmids, whole genes or just parts of genes into the host genome via recombination events [143].

Transduction describes the gene transfer, mediated by bacteriophages. Phages represent viruses that are specialized to infect bacteria or archaea [143]. Lytic bacteriophages reproduce themselves only in the lytic cycle, whereas temperate bacteriophages multiply themselves during the lysogenic and lytic cycle. During the lytic cycle, phages decompose host DNA into nucleotides to use them for their own phage DNA reproduction. During assembling of new phage particles, pieces of bacterial DNA – also including plasmids – may be packed

into phage capsids by accident. This process is called generalized transduction. In contrast, specialized transduction occurs during the lysogenic cycle. Thereby, phage DNA is integrated into the bacterial chromosome. In the first phase of this cycle, phage reproduction is coupled to the host cell division. After extracellular stimulation, the lysogenic cycle changes to a lytic cycle. Bacteriophage DNA, also occasionally including flanking bacterial genes, is excised from the chromosome and integrated into newly assembled bacteriophage particles [143, 144].

For **conjugation** a cell-to-cell contact is needed for the formation of a pore to transfer bacterial DNA, e.g. plasmids, transposons or ICEs, from a donor cell to a recipient cell [143]. Genetic information for potential conjugation is encoded on conjugative plasmids and transposons, as well as on ICEs. Conjugation may occur between bacteria of the same or different species and genera, especially within biofilms [143]. Plasmids are extrachromosomal, mostly circular elements that consist usually of double-stranded DNA. They are capable of autonomous replication. Plasmids can have a size between 1 to 400,000 kb. Bacteria may harbor a different number of plasmids that are distinguished in low- and high-copy plasmids. Low-copy plasmids are commonly large plasmids with only one or few copies per bacterial cell, whereas high-copy plasmids are smaller plasmids with usually >100 copies per bacterial cell [142, 145]. **Conjugative plasmids** harbor the transfer (*tra*) gene complex, which carries the information for all necessary conjugation proteins. Conjugative and mobilizable plasmids have an origin of transfer (*oriT*) at which the separation of the two DNA strands starts during conjugation/mobilization. In contrast, the origin of vegetative replication (*oriV*) represents the start of the replication of non-conjugative plasmids [143, 146]. Furthermore, conjugative plasmids often encode a relaxase, a type IV coupling protein (T4CP) and a type IV secretion system (T4SS) [143, 147]. The relaxase binds at the *oriT* within the donor cell and induces the cleavage of the double-stranded DNA. The connected complex of relaxase, DNA-strand and T4CP form the relaxosome and become linked to the T4SS. The T4SS forms a protein cylinder that connects the donor cell with the recipient. During conjugation, one single DNA strand remains in the donor cell, while the other one is transported across the pore forming T4SS into the recipient. The complementary strands are synthesized during conjugation in both cells through the DNA polymerases [143, 146]. While conjugative plasmids are self-transmissible, **mobilizable plasmids** are only transmissible by the help of conjugative elements located within the same cell. Mobilizable plasmids only harbor *mob* genes, e.g. *mobA*, *mobB* and *mobC*, that encode components of the relaxosome forming unit, but no parts of the secretion system [148, 149]. MobA is the relaxase that forms a complex with *oriT*. MobC supports the relaxase to cleave the double stranded-DNA at the *oriT* site into a single strand. Furthermore, MobB stimulates MobC to get active and stabilize the relaxosome [149]. Finally, the carrier of plasmids commonly benefits from the additional plasmid-located genes, which might code for

antimicrobial resistances, toxins or virulence factors. Those genes might provide advantage in bacterial survival during challenging environmental conditions.

1.4 VASIB project

VASIB is a collaborative project between different partners of the University of Veterinary Medicine Hannover, the Friedrich-Loeffler-Institute, the Vet team Reken and the Freie Universität Berlin. The title is an acronym deduced from the German project title and includes the projects' aim to reduce the antimicrobial consumption in pig production by integrating epidemiological information from consulting expertise in clinic, hygiene, microbiology and pharmacology. For this reason, 30 porcine farrow-to-finish farms, with a history of recurring respiratory tract infections were investigated. The environment of the weaners with an age of four to ten weeks was evaluated. Therefore, dust, water and feed samples were taken to measure antimicrobial residues. Furthermore, blood and bronchoalveolar lavage fluid (BALF) samples were taken from the weaners. While blood was examined for the concentration of antimicrobial agents, BALFs were analyzed for respiratory tract pathogens and antimicrobial residues. The farrow-to-finish farms were sampled at different time points A to F:

- (A) The weaners were healthy and four of them were sampled.
- (B) The weaners showed respiratory tract problems and three of these were sampled before starting the antimicrobial treatment.
- (C) The same three weaners as in B were tested at the last day of antimicrobial treatment, five to seven days after time point B.
- (D) The weaners showed respiratory tract symptoms yet again and three pigs were sampled before the antimicrobial treatment.
- (E) The same three weaners from time point D were tested at the last day of antimicrobial treatment. The animals of time points B/C and D/E were not the same, since the collection time between them were four to seven months.
- (F) Four healthy pigs were sampled at the final visit, approximately one year after visit A.

The aims of the microbiological part of the VASIB project and also of this thesis were to:

- examine the antimicrobial resistance profiles of the pathogens in each farm through determination of the MICs;

- determine the degree of relationship of members of each bacterial species within selected farms at different collection time points as well as between farms;
- detect antimicrobial resistance mechanisms of bacteria by studying resistance-mediating plasmids, acquired resistance genes or amino acid alterations in resistance-mediating proteins;
- clarify possible resistance gene transfers or shifts in the bacterial resistance profiles immediately after the antimicrobial treatment.

2 Publications

2.1 Publication I

Niemann L, Müller P, Brauns J, Nathaus R, Schäkel F, Kipschull K, Höltig D, Wendt M, Schwarz S, Kadlec K. 2018.

Antimicrobial susceptibility and genetic relatedness of respiratory tract pathogens in weaner pigs over a 12-month period.

https://doi.org/10.1016/j.vetmic.2018.03.030

Vet Microbiol **219**:165-170.

2.2 Publication II

Niemann L, Feudi C, Eichhorn I, Hanke D, Müller P, Brauns J, Nathaus R, Schäkel F, Höltig D, Wendt M, Kadlec K, Schwarz S. 2019.

Plasmid-located *dfrA14* gene in *Pasteurella multocida* isolates from three different pig-producing farms in Germany.

https://doi.org/10.1016/j.vetmic.2019.01.016

Vet Microbiol **230**:235-240.

2.3 Publication III

Niemann L, Eichhorn I, Müller P, Brauns J, Nathaus R, Schäkel F, Höltig D, Wendt M, Kadlec K, Schwarz S. 2019.

Draft genome sequences of three porcine *Streptococcus suis* isolates which differ in their susceptibility to penicillin.

https://doi.org/10.1128/mra.01711-18

Microbiol Resour Announc **8**: e01711-18.

2.4 Unpublished data

Publication I gives an impression on the relatedness and antimicrobial susceptibility of *S. suis*, *B. bronchiseptica* and *P. multocida* only in Farm 1 and Farm 2, while **Publications II** and **III** display specific resistance mechanisms on certain farms. In order to gain a more precise overview on the antimicrobial susceptibility of the pathogens, we added the unpublished MIC data tables 1, 2 and 3 of *S. suis*, *B. bronchiseptica* and *P. multocida*, respectively, from all farms examined in this study.

Unpublished data Table 1 MIC distribution of ***S. suis*** isolates (n=257) from all examined farms to important antimicrobials of this project. The vertical green lines indicate susceptible and the red lines resistant isolates according to CLSI breakpoints.

Antimicrobial class	**Antimicrobial agent**																	**susceptible**		**intermediate**		**resistant**		**MIC_{50} [mg/L]**	**MIC_{90} [mg/L]**
		0.015	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**[n]**	**[%]**	**[n]**	**[%]**	**[n]**	**[%]**		
β-Lactam	Penicillin	76	34	11	24	46	42	11	3	6	2	2	-	-	-			191	74.3	42	16.3	24	9.3	0.12	0.5
	Ampicillin		164	59	17	4	1	7	2	-	1	1	1	-	-			245	95.3	7	2.7	5	1.9	≤0.03	0.12
	Amoxicillin/ clavulanic acid[a]		181	50	5	8	1	7	2	-	2	-	1	-	-			-	-	-	-	-	-	≤0.03	0.06
Tetracycline	Tetracycline				-	6	11	35	24	12	1	10	29	102	21	2	4	17	6.6	35	13.6	205	79.8	64	128
	Doxycycline			20	55	7	-	2	-	12	82	75	2	-	-	2		-	-	-	-	-	-	8	16
Phenicol	Florfenicol				-	-	1	143	113	-	-	-	-	-	-	-	-	257	100.0	0	0.0	0	0.0	1	2
Pleuromutilin	Tiamulin		-	-	15	11	15	56	52	17	19	32	22	17	1			-	-	-	-	-	-	2	32

[a] Concentration of amoxicillin is presented, concentration ratio of amoxicillin/clavulanic acid 2:1.

Unpublished data Table 2 MIC distribution of ***B. bronchiseptica*** isolates (n=20) with one isolate per farm that harbored *B. bronchiseptica*. Important antimicrobials of this project are listed in the table. The vertical green lines indicate susceptible and the red lines resistant isolates according to CLSI breakpoints.

Antimicrobial class	**Antimicrobial agent**																	**susceptible**		**intermediate**		**resistant**		**MIC_{50} [mg/L]**	**MIC_{90} [mg/L]**
		0.015	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**[n]**	**[%]**	**[n]**	**[%]**	**[n]**	**[%]**		
β-Lactam	Penicillin	-	-	-	-	-	-	-	-	-	-	-	-	20				-	-	-	-	-	-	≥64	≥64
	Ampicillin		-	-	-	-	-	-	-	-	2	18	-	-	-			0	0.0	0	0.0	20	100.0	16	16
	Amoxicillin/ clavulanic acid[a]		-	-	-	-	-	-	1	18	1	-	-	-	-			-	-	-	-	-	-	4	4
Tetracycline	Tetracycline				-	4	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5
	Doxycycline			2	14	3	1	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	0.12	0.25
Phenicol	Florfenicol				-	-	-	2	7	11	-	-	-	-	-	-	-	9	45.0	11	55.0	0	0.0	4	4
Pleuromutilin	Tiamulin		-	-	-	-	-	-	-	-	1	-	-	7	12			-	-	-	-	-	-	≥128	≥128

[a] Concentration of amoxicillin is presented, concentration ratio of amoxicillin/clavulanic acid 2:1.

Unpublished data Table 3 MIC distribution of ***P. multocida*** isolates (n=39) with one or two isolates per farm that were distinguishable due to their serotype. Important antimicrobials of this project are listed in the table. The vertical green lines indicate susceptible and the red lines resistant isolates according to CLSI breakpoints.

Antimicrobial class	Antimicrobial agent	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	susceptible [n]	susceptible [%]	intermediate [n]	intermediate [%]	resistant [n]	resistant [%]	MIC_{50} [mg/L]	MIC_{90} [mg/L]
β-Lactam	Penicillin	2	16	15	3	2	1	-	-	-	-	-	-	-				38	97.4	1	2.6	0	0.0	0.06	0.12
	Ampicillin		1	8	24	6	-	-	-	-	-	-	-	-	-			39	100.0	0	0.0	0	0.0	0.12	0.25
	Amoxicillin/ clavulanic acid[a]		-	-	18	19	2	-	-	-	-	-	-	-	-			-	-	-	-	-	-	0.25	0.25
Tetracycline	Tetracycline				-	2	25	9	3	-	-	-	-	-	-	-	-	27	69.2	9	23.1	3	7.7	0.5	1
	Doxycycline			-	1	26	9	2	1	-	-	-	-	-	-	-		-	-	-	-	-	-	0.25	0.5
Phenicol	Florfenicol				-	6	33	-	-	-	-	-	-	-	-	-	-	39	100.0	0	0.0	0	0.0	0.5	0.5
Pleuromutilin	Tiamulin		-	-	-	-	-	-	-	3	4	12	20	-	-			-	-	-	-	-	-	32	32

[a] Concentration of amoxicillin is presented, concentration ratio of amoxicillin/clavulanic acid 2:1.

3 Discussion

This doctoral thesis is focused on the determination of the antimicrobial susceptibility of the aforementioned respiratory tract pathogens in the context of 30 farrow-to-finish farms. In addition, the genetic relatedness of selected *S. suis*, *B. bronchiseptica* and *P. multocida* isolates was investigated. The genetic relatedness of the pathogens on farm level as well as between farms and the antimicrobial susceptibility is presented in **Publication I**. **Publications II** and **III** provide detailed information on some *P. multocida* and *S. suis* isolates, exhibiting SXT resistance and differences in penicillin susceptibility, respectively.

The examined weaners frequently harbored *S. suis*, whereas *P. multocida* and *B. bronchiseptica* were present in lower numbers. This observation was in agreement with the results of other studies, in which *S. suis* was detected with a prevalence of 98 % in the porcine upper respiratory tract [150, 151]. In contrast, the average isolation rate of *B. bronchiseptica* from porcine lung samples was 18.6 % [152]. The studies of MacInnes *et al.* (2008) and Zhao *et al.* (2011) looked only for toxigenic *P. multocida* and the latter one determined a low herd prevalence of PMT-producing *P. multocida*. The study of Zhao *et al.* (2011) detected toxin-producing *P. multocida* only in low numbers, but always in association with *B. bronchiseptica*. However, the *P. multocida* isolates of the VASIB study were investigated only for their serotype and not for PMT production. The porcine respiratory tract is colonized by mixed cultures of commensal bacteria such as *S. suis*, *H. parasuis* or *Mycoplasma hyorhinis* [14]. Especially *A. pleuropneumoniae* and *Mycoplasma hyopneumoniae,* but also toxin-producing *P. multocida* or *B. bronchiseptica* may act as primary bacterial pathogens. Therefore, the identification of the causative pathogen, which triggers the respiratory tract infection, may be difficult. Furthermore, different bacterial species show different intrinsic and acquired antimicrobial resistances, which complicates the selection of effective antimicrobial agents that act against the causative respiratory tract pathogens. In the VASIB study, pigs from 20 of the 30 examined farms showed respiratory tract symptoms. This resulted in the therapeutic application of antimicrobial agents. In four of the aforementioned 20 farms, respiratory tract diseases reoccurred at a later stage. In total, antimicrobial agents were prescribed and applied 24 times on farms for five or seven days. Doxycycline was used most frequently (14 times). Amoxicillin was prescribed eight times, whereas each combination of amoxicillin/clavulanic acid and tiamulin/florfenicol was applied only once. In order to gain more information about the effects of the prescribed antimicrobial agents, we focused on the MIC results of *S. suis*, *B. bronchiseptica* and *P. multocida* towards tetracyclines, β-lactams, tiamulin and florfenicol. Moreover, we looked in retrospect on the pathogen MICs towards the prescribed antimicrobials and tried to interpret the results as well as indicate better antimicrobial therapy options, since the 30 porcine farrow-to-finish farms reflect economical working and real-life farm conditions.

A modified clinical score system of Höltig *et al.* (2008) was used to determine the effect of the antimicrobial therapy on the health status of the pigs. This score value identified a successful antimicrobial treatment in 21 of the 24 farms with porcine respiratory tract infection (Table S1) [153]. In three farms, no improvement or even a deterioration of the health status of the pigs occurred after doxycycline (n=2) and amoxicillin (n=1) therapy. The cross-sectional study of van Rennings *et al.* (2015) about the antimicrobial treatment within a one-year period identified amoxicillin (2.2 t) as the most frequently prescribed antimicrobial agent in weaner pigs suffering from respiratory tract infections in German pig farms in 2011. Tetracycline (1.1 t) and chlortetracycline (0.64 t) were the second and third most frequently used antimicrobial agents, followed by sulfadiazine (0.52 t) [15]. Even the BVL reported on the highest sales of penicillins (269 t), followed by tetracyclines (188 t), polypeptide antibiotics (74 t) and sulfonamides (62 t) in the total amount of prescribed veterinary antimicrobials, in Germany in 2017 [2]. These reports are in accordance with the antimicrobials used in the VASIB study, since doxycycline belongs to the antimicrobial class of tetracyclines and amoxicillin and amoxicillin/clavulanic acid to the β-lactams. This study represents a snapshot of 30 examined porcine farrow-to-finish farms. In ten farms, weaners showed no respiratory tract infections, whereas other farms had up to two veterinary visits, B/C and D/E, during which antimicrobial agents were prescribed and applied.

3.1 Genetic relatedness of *S. suis* at farm level and between different farms

In the following explanations, isolates are designated as members of the same clones, when they shared indistinguishable PFGE patterns as confirmed by cluster analysis using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) analysis [12, 154, 155]. PFGE patterns were designated as closely related with an identity of ≥80 % according to the UPGMA analysis [12, 154, 155]. Closely related isolates might have developed over time from a common ancestor [12, 13]. Unrelated PFGE patterns differed in up to seven fragments [12]. **Publication I** showed the results of the macrorestriction with subsequently PFGE analyses.

Up to two or more *S. suis* isolates with different PFGE patterns were detected within a pig barn at the same time point (**Publication I**). The study of Vela *et al.* (2003) already confirmed the presence of a variety of *S. suis* isolates among diseased pigs within the same herd. Diseased pigs of that study had symptoms of meningitis, septicemia, pneumonia, pericarditis or arthritis. In contrast, the pigs of the VASIB study were apparently healthy or showed mild respiratory tract symptoms. The results of the VASIB study revealed that weaners, who lived in separate boxes of the same barn were positive for the same *S. suis* clones that also were detected in pigs of other boxes. Thus, these isolates might be transferred

through direct or indirect transmission events. The weaners seem to become infected with *S. suis* during birth by contact with the maternal genital tract [157]. Moreover, nasal contact between the pigs is known to be the most important transmission route, since *S. suis* is commonly located in the upper respiratory tract. *S. suis* may also be transferred via contact with contaminated skin wounds and feces [19]. Berthelot-Hérault and colleagues (2001) confirmed experimentally the airborne transmission of pathogens from a *S. suis* infected pig herd to a specific pathogen-free herd that had no contact, but was located in a distance of 40 cm. Hence, the study of Berthelot-Hérault *et al.* (2001) may explain airborne *S. suis* spread across pig boxes. Furthermore, *S. suis* may be transferred via contaminated dust, water or feed [19]. The study of Dee and Corey (1993) displayed the survival of *S. suis* at farm surfaces and veterinary equipment. The highest survival rate was measured on manure contaminated rubber or plastic at room temperature, thus rubber boots, buckets or needles are described as probable sources of pathogen transfer. However, one of these possible transfer routes may be responsible for the appearance of *S. suis* clones in weaners without direct contact at the moment of the sampling. Moreover, the pigs had been rearranged after the end of the farrowing period, and were newly assembled into groups for the nursery as well as fattening periods [8]. Here, weaners of different litters were brought together with the consequence of a probable isolate exchange. This might explain the presence of different *S. suis* clones in different pig boxes. Single pigs harbored up to two or three *S. suis* isolates with different PFGE patterns (**Publication I**). This leads to the conclusion that unrelated *S. suis* isolates are able to coexist at the same time in the same sampling site within the same pig. The majority of the detected *S. suis* isolates seems to be commensals, since only 14.0 % (n=36) of all examined isolates (n=257) belonged to the most pathogenic serotypes 1, 2, 7 and 9 (data not shown), concluding that the other isolates belong to the less pathogenic serotypes [158]. This might be an explanation for the occurrence of only slight respiratory tract symptoms of the pigs. It has been shown that less virulent or avirulent isolates cause mild diseases or result in apparently healthy carrier pigs [19, 23]. The *S. suis* isolates, which were obtained within the one-year sampling period at the different time points (A - F), revealed PFGE patterns that differed from those of isolates of earlier sampling time points (**Publication I**). Allgaier *et al.* (2001) determined the relatedness of *S. suis* based on macrorestriction analysis from healthy and diseased pigs and suggested a higher clonal relatedness of virulent *S. suis* strains causing meningitis or arthritis. A higher PFGE diversity was observed among the less virulent isolates that caused pneumonia or were detected in apparently healthy carrier pigs [159]. This genetic diversity of *S. suis* was confirmed in the VASIB study as shown in the different *S. suis* PFGE patterns seen at Farms 1 and 2 in **Publication I**. Besides, the isolates of the present study were obtained from BALF samples of apparently healthy carrier pigs and those with mild respiratory tract symptoms. Similar to the observation of Allgaier *et al.* (2001), we observed also a higher PFGE diversity

among *S. suis* isolates obtained from these pigs. The occurrence of unrelated *S. suis* isolates at earlier sampling time points raises the question how the previously not identified isolates were imported into the farms. Since the 30 examined farms in this project were farrow-to-finish farms, no weaners from other farms were purchased before the start of a new production stage. That excludes an import of new isolates from bought fattening pigs. However, the different pig barns undergo a constant pig flow, since the sows give approximately 2.34 litters a year and thus, the different groups of piglets will change the barns according to their production stage [9]. Moreover, the examined pigs of this study were at an age of four to ten weeks. The sampling (A - F) occurred at the end of the nursery or start of the fattening period in different barns. In conclusion, the pigs in different fattening cycles and at time points A, B/C, D/E or F should have their own bacterial population. This still leaves the question, how *S. suis* isolates with different PFGE patterns in the same sampled pigs of time points B/C or D/E occurred. New isolates might have existed in undetectable numbers in the pigs' habitat and after changes of environmental conditions, like antimicrobial therapy, these new isolates might have replaced others. However, in Farm 2 some closely related isolates were identified at different sampling time points. In addition, clones were detected at different sampling times within six to twelve month periods of time between the samplings within single farms (**Publication I***,* Figure S1 and Figure S2). This indicates a long-term survival of these isolates on single farms. As already introduced, the closed farm system is characterized by a low bacterial import from the outside. However, the spread of bacteria within closed farms is supposed to be much higher, due to lower barrier restrictions between the different rearing phases of the pigs [7]. Another reason for the appearance of clones or closely related isolates in different pig herds on farm level might be the bacterial transfer between sows and their piglets. Sows of a production cycle, especially during the gestation period, live in a close contact to each other [8]. Hence, bacteria can be transferred across the whole group of sows. Weaners originating from sows of the same production cycle may have been perinatally colonized with bacteria from their parents [157]. Some other reasons for the occurrence of closely related isolates over a long time period might be caused by farm specific deficiencies in hygiene that enable a transmission or survival of the bacteria. Hygienic deficiencies may be insufficient concentrations of detergents or disinfectant agents or a reduced effect of the disinfectant agent after an insufficient cleaning, thus protein waste may remain in a pig barn. This protein waste may coagulate and enclose pathogens after their contact with disinfectants. Protein enclosed pathogens are protected against antiseptic effects and survive the cleaning and disinfection steps.

The comparison of *S. suis* PFGE patterns of different farms revealed a wide variety of macrorestriction patterns among the isolates (data shown in parts in Figure S1 and Figure S3) as already indicated in other studies [156, 159-161]. Nevertheless, members of the same

clones were also identified in different farms in the VASIB study (Figure S3). However, the same clone was detected only in a maximum of two farms. Hence, each farm couple, Farms 2 and 14, Farms 3 and 25 as well as Farms 20 and 30 showed the presence of their own *S. suis* clones. Vela *et al.* (2003) detected isolates with similar PFGE patterns on several farms and suggested that those isolates might have a higher pathogenic potential. However, in the six examined farms that harbored these three *S. suis* clones, a mild respiratory tract infection occurred in several weaners. Moreover, only the clone of Farms 20 and 30 belonged to the pathogenic serotype 7. The clones of Farms 2 and 14 as well as Farms 3 and 25 did not belong to the pathogenic serotypes 1, 2, 7 or 9 [25, 158]. Berthelot-Hérault *et al.* (2002) identified a high genetic diversity, according to the macrorestriction patterns, among *S. suis* isolates of serotypes 2 and 7 obtained from diseased pigs and humans. However, a higher similarity of the PFGE patterns of each serotype 1/2, 3 and 9 was observed in the same study [160]. Blume *et al.* (2009) examined serotypes 2 and 9 of diseased pigs and recognized a high genetic diversity among them. Nevertheless, three different PFGE profiles of serotype 9 and two different profiles of serotype 2 were frequently isolated in different herds in several Spanish provinces, indicating a wide distribution of genetically related pathogenic isolates [161]. However, clones that were identified in different farms in the VASIB study might indicate possible transfer ways. Since no new weaners were purchased from other farms or markets, the replacement of sows within farms might be the reason for the import of new pathogens. Sow longevity in intense animal farms depends individually on their litter size, age, gestation rate, udder and leg fitness [162]. In case, sows of the same multiplier farms were purchased by different farrow-to-finish farms, they might be carriers of bacteria with similar PFGE patterns and peri- or postnatally transmit these bacteria to their piglets. Only six of the 30 examined farms in the VASIB project, produced their own gilts. Each of the remaining 24 farms purchased their gilts from one or two multiplier farms. Unfortunately, whether or not the different farrow-to-finish farms obtained their gilts from the same or from different multiplier farms was not included in the questionnaire that was prepared for the farm workers at the first sampling at time point A.

3.2 Genetic relatedness of *B. bronchiseptica* at farm level and between different farms

In **Publication I**, all *B. bronchiseptica* isolates obtained from the same pig showed indistinguishable PFGE patterns. This is in contrast to the results observed for *S. suis*, where an individual pig may harbor *S. suis* with different PFGE patterns. Furthermore, only one *B. bronchiseptica* clone was detected during a certain time period in all examined pigs of the

same farm (**Publication I**). Binns *et al.* (1998) examined *B. bronchiseptica,* especially from cats, by macrorestriction analysis. They grouped the isolates, according to their PFGE patterns, into 17 strains (A-Q), each of them differed by at least four fragments from the others [163]. Furthermore, the strain designation included subtypes that differed in up to three fragments to a strain. Most *B. bronchiseptica* of the cats belonged to strains A and B, which showed a low diversity in their PFGE patterns. If we follow the classification scheme of Binns and colleagues (1998) for strain and subtype classification, the *B. bronchiseptica* isolates from Farms 1 and 2 of **Publication I** would be classified as representing the same strain. Furthermore, the one clone of Farm 1 and the two clones of Farm 2 would belong to three different subtypes of that strain, since they differed in up to one or two fragments from each other. Binns *et al.* (1998) determined that cats, who had lived in the same household harbored *B. bronchiseptica* isolates of the same strain. This observation is also confirmed by the results of the VASIB study, since the isolates of an individual farm all represented the same strain. In addition, the isolates of both farms that were investigated in **Publication I** also represented the same strain.

Furthermore, we compared the *B. bronchiseptica* PFGE patterns of seven different farms (Figure S4). These seven farms include the isolates of Farms 1 and 2 (**Publication I**), which were obtained over a one-year period and the isolates of five farms, which were collected at sampling time point A. This analysis revealed the occurrence of one individual clone per farm. Nevertheless, these individual clones from the different farms were closely related to each other (Figure S4). Shin *et al.* (2007) examined porcine *B. bronchiseptica* isolates and classified them, similar to Binns *et al*. (1998), into three strains (A-C) with their subtypes. Most isolates of his classification belonged to strains A and B. So, they also observed a low genetic diversity among porcine *B. bronchiseptica* isolates [164]. We can confirm this low genetic diversity, since even the *B. bronchiseptica* isolates of the seven different farrow-to-finish farms revealed closely related PFGE patterns. Furthermore, the *B. bronchiseptica* isolates of the study from Shin *et al.* (2007) originated from two adjacent provinces in Korea. The *B. bronchiseptica* isolates of the one province were classified only as strain A, whereas the isolates of the other province represented strains A and B [164]. This result might indicate that in certain geographical areas a limited number of strains occur. However, Shin and colleagues (2007) pointed out that a larger number of *B. bronchiseptica* isolates from different areas is needed to confirm this hypothesis. Nevertheless, the results of the present study would agree with this finding, since the closely related isolates of the seven different porcine farrow-to-finish farms originated from the Western part of Germany. Based on the close genetic relationships of the *B. bronchiseptica* isolates seen in all seven farms, it is likely that the isolates of the different farms originate from a common ancestor. In conclusion, the results of the VASIB study as well

as other studies showed that *B. bronchiseptica* isolates often displayed related PFGE patterns, which may be indicative of a comparatively low heterogeneity of porcine *B. bronchiseptica* isolates [96, 163-165].

3.3 Genetic relatedness of *P. multocida* at farm level and between different farms

PFGE analysis of *P. multocida* isolates showed different patterns at each sampling time point as exemplarily shown for Farm 2 of **Publication I**. In contrast to the situation seen for *S. suis*, each investigated pig carried only a single *P. multocida* clone (**Publication I**). This observation is in accordance with a French study that detected isolates with indistinguishable PFGE patterns from the same pig [166]. While the PFGE results of Farm 2 suggest only one clone per time point within a pig barn, further examinations yielded other results. Namely, isolates with different PFGE patterns were detected at the same sampling time point among different animals within the same farm (Figure S5). In addition, five of the 29 farms, in which *P. multocida* was found among the pigs, harbored isolates belonging to serotypes A and D or A and B at the same time (data not shown), suggesting the presence of at least two genetically different *P. multocida* isolates at the same time within a pig herd, but not within the same animal. These results are similar to the result of Marois *et al.* (2009), who identified four different *P. multocida* PFGE patterns at the same time within a farm, but also not within the same animal.

The comparison of PFGE patterns of *P. multocida* isolates from different farms illustrates the occurrence of diverse isolates per farm (Figure S6). Nevertheless, some isolates were closely related in their PFGE patterns (identity of 82.5 % to 97 %) to isolates of other farms as shown in **Publication II** and Figure S6. Similar to this, the French study of Marois *et al.* (2009) identified isolates with indistinguishable PFGE patterns in three different farms. However, the closely related isolates in different farms of the VASIB study belonged to the same serotype A or D, respectively. However, not all isolates belonging to the same serotype showed closely related PFGE patterns (Figure S6). Some studies about the genetic relatedness of *P. multocida* of porcine, human or avian origin assumed a limited number of clonal lineages within their specific hosts, especially within certain regions [166-169]. As already mentioned for *B. bronchiseptica*, *P. multocida* isolates were classified in those studies into strains and subtypes [163]. The Danish study of Pors *et al.* (2011) examined 139 *P. multocida* isolates and grouped them into 13 PFGE strains each with subtypes and observed the highest prevalence of serotype A and sequence type (ST) 13. We examined only the isolates of **Publication II** for their ST and detected ST13 as well. The remaining isolates were not detected for their ST in

the present study. From the 101 *P. multocida* isolates examined in the VASIB study, 69.3 % (n=70) belonged to serotype A, whereas 27.7 % (n=28) were type D and 3.0 % (n=3) type B. Isolates of the VASIB study originated from apparently healthy and diseased pigs, whereas the isolates examined in the Danish study were obtained from bronchopneumonia lesions of pigs at slaughter houses [167]. Bronchopneumonia is most frequently associated with serotype A, that may explain the higher occurrence in the Danish study. *P. multocida* isolates presented in **Publication II** were identified as serotype A and ST13, assuming a higher prevalence of this lineage in pigs. However, these isolates of this lineage were not only detected in diseased pigs, they were also identified in clinically healthy pigs in the VASIB study, which might provide hints towards their commensal character. However, *P. multocida* were not investigated in the VASIB study for the presence of virulence genes and conclusions about their virulence are only speculative. In conclusion, some studies seem to indicate the existence of certain clonal lineages of *P. multocida* within specific geographical areas, as noticed in Denmark or Vietnam [166, 167]. This observation may explain the occurrence of a limited number of *P. multocida* lineages in Germany, which is confirmed by the presence of closely related isolates in different farms of the VASIB project (Figure S6). Thus, porcine *P. multocida* isolates display a moderate genetic heterogeneity, which is in contrast to the broad genetic diversity observed in porcine *S. suis* and the great genetic homogeneity seen in porcine *B. bronchiseptica*.

3.4 Antimicrobial susceptibility of *S. suis*

S. suis isolates with indistinguishable PFGE patterns exhibited MIC values of antimicrobial agents that were rather similar and differed from each other in only +/- one dilution step (**Publication I**). Accordingly, isolates with closely related, but not identical PFGE patterns showed differences in their MIC values as well. Isolates with PFGE patterns that were more different, and hence not related with each other showed rather different MICs. This led us to the conclusion, that an antibiogram for only one *S. suis* isolate per pig farm is not sufficient to extrapolate the susceptibility to antibiotics of the *S. suis* isolates of the entire farm. Genetically different *S. suis* isolates were present at the same time within a farm as well as within an animal and these isolates may exhibit different antimicrobial susceptibility patterns. However, performing macrorestriction analysis before generating an antibiogram is too expensive and too time consuming. In order to capture a more complete picture of the variable antimicrobial susceptibility of *S. suis* isolates, diagnostic laboratories should pay attention on differences in morphology of capsule expression or α-hemolysis and then decide to conduct antibiograms of all phenotypically different *S. suis* isolates.

The distribution of *S. suis* MIC values observed in Farm 1 (n=7) and Farm 2 (n=15) of **Publication I** corresponds to the MICs of all examined farms in which *S. suis* (n=257) was detected in the VASIB project (unpublished data Table 1). The MIC_{90} of 128 mg/L indicated that the vast majority of the tested *S. suis* isolates was resistant to **tetracyclines** (unpublished data Table 1). This result is comparable with the results of the GE*RM*-Vet and VetPath studies, where the *S. suis* isolates from diseased pigs showed tetracycline MIC_{90} values of 64 mg/L [5, 65]. Tetracycline CLSI breakpoints of *S. suis* are applicable only for injectable formulations [91]. However, antimicrobial agents in pig industry are usually applied through feed or water. There is no information about how the CLSI-approved clinical breakpoints and the tetracycline concentration in the respiratory tract tissue after oral tetracycline application correlate with each other. Hence, the bacteriostatic effect of tetracyclines may fail, if the antimicrobial concentration is too low in the respiratory tract tissue. Moreover, doxycycline was the most prescribed antimicrobial agent in the VASIB study. However, there is no doxycycline-specific clinical breakpoint for *S. suis* from swine. Although tetracycline is considered as the class representative, it is questionable whether the tetracycline breakpoints can be applied for doxycycline. In staphylococci, the doxycycline-specific breakpoints for *Staphylococcus pseudintermedius* are one dilution step lower than the tetracycline-specific breakpoints for *Staphylococcus* spp. (including *S. pseudintermedius*). In the VASIB study doxycycline MIC_{50} and MIC_{90} were 8 mg/L and 16 mg/L, respectively. Bearing in mind that the *S. suis*-specific tetracycline breakpoint for resistance is at ≥2 mg/L, most of the *S. suis* isolates in the VASIB study may be regarded as doxycycline-resistant. Isolates classified as tetracycline- and doxycycline-resistant (n=205) harbored tetracycline resistance genes *tet*(O) with 57.6 %, *tet*(M) 14.1 %, *tet*(L) 2.4 % and *tet*(W) 3.9 %. In 22.0 % of the *S. suis* isolates, none of the examined tetracycline resistance genes was detected, assuming the presence of another tetracycline resistance-mediating gene. The presence of tetracycline resistance genes might indicate reduced effects on pig recovery during doxycycline therapy. However, the doxycycline therapy was only in two farms unsuccessful, as indicated by the modified diagnostic respiratory score system [153]. Besides, in pigs of twelve of the fourteen farms, where doxycycline was applied, tetracycline-resistant *S. suis* were detected at the last day of antimicrobial treatment (Table S1). Anyway, doxycycline usage leads to a high selection pressure under which isolates with tetracycline resistance genes can persist.

MIC_{90} values of 0.12 mg/L confirmed an overall good susceptibility of *S. suis* (n=257) to **ampicillin.** The *S. suis*-specific clinical breakpoint for ampicillin is at ≤0.5 mg/L. For the combination amoxicillin/clavulanic acid, a MIC_{90} value of 0.06/0.03 mg/L was determined (unpublished data Table 1). The aforementioned MIC_{90} values from the VASIB study are in accordance with the MIC_{90} values obtained in the VetPath study, i.e. 0.06 mg/L and

0.06/0.03 mg/L, respectively [65]. The GE*RM*-Vet study only determined the MIC_{90} of ampicillin with ≤0.5 mg/L [5]. In contrast, most of the isolates of the VASIB study were classified as intermediate to penicillin with a MIC_{90} value of 0.5 mg/L (unpublished data Table 1), whereas no penicillin MICs were determined in the VetPath and GE*RM*-Vet studies [6, 65, 91]. In addition, 24 isolates originating from different farms and animals in the VASIB study showed a penicillin-resistance phenotype. Reduced penicillin susceptibility of *S. suis* isolates was already reported almost 30 years ago and is still discussed [43, 64, 170]. In order to gain more information on *S. suis* with reduced penicillin susceptibility, three isolates that were susceptible, intermediate and resistant to penicillin were subjected to whole genome sequence analysis. Their sequences were investigated for structural variations in the PBPs and the results presented in **Publication III**. The lowest number of amino acid alterations in their PBPs, as compared to *S. suis* BM407 (accession number FM252032.1), were identified in the penicillin-susceptible isolate IMT40343, whereas the penicillin-intermediate isolate IMT40201 and the penicillin-resistant isolate IMT40738 exhibited distinctly more amino acid alterations. These findings suggest that even in *S. suis*, alterations in their PBPs mediate reduced susceptibility to penicillins as already detected in *S. pneumoniae* [58, 59]. Furthermore, the lowest amino acid alterations were identified in PBP1a, PBP1b and PBP2a of IMT40343 and IMT40201. The penicillin-resistant isolate IMT40738 and the intermediate isolate IMT40201 had the highest number of amino acid substitutions in PBP2x and PBP2b, while IMT40738 had additional amino acid alterations in PBP2a. Limited information exists about the effects of amino acid alterations in the PBPs of *S. suis* [42, 43]. As a consequence, the information from the well-examined human pathogen *S. pneumoniae* was transferred to *S. suis*. Mosaic genes as identified in *S. pneumoniae* were not confirmed in the examined PBPs of *S. suis* in our study [50-52]. *S. pneumoniae* exhibits a moderate β-lactam resistance while having substitutions in PBP2x or PBP2b, whereas high level β-lactam resistance in *S. pneumoniae* is additionally mediated by alterations in PBP1a [58, 59]. Since *S. suis* isolate IMT40738 is penicillin-resistant with a MIC of 2 mg/L and intermediate to ampicillin and ceftiofur, we suggest a moderate β-lactam resistance. The *S. suis* isolate IMT40201 exhibited an intermediate MIC to penicillin and was classified as susceptible to ampicillin and ceftiofur. This leads us to the conclusion that amino acid alterations in PBP2x and PBP2b in *S. suis* reduce the penicillin susceptibility. Moreover, additional alterations in other PBPs, like in PBP2a in *S. suis* of this study or in PBP1a in *S. pneumoniae,* seem to mediate a reduced susceptibility to the remaining β-lactams [55, 58]. Moreover, no amino acid alterations were detected in the conserved motifs of all PBPs in this study. This is in contrast to other studies of *S. pneumoniae,* where an alteration within the conserved motif Ser-X-X-Lys of PBP1a was described, which led to a reduced susceptibility to penicillin [58]. Smith *et al.* (1998) identified a substitution from Thr^{371} to Ala or Ser after the catalytic Ser^{370} and suggested herein the cause for a reduced β-lactam

affinity. Hu *et al.* (2011) observed a substitution in PBP2x close to a conserved motif in a β-lactam-resistant *S. suis* and assumed an effect on its reduced β-lactam affinity. Smith *et al.* (1998) also suggested another important substitution of four amino acids in *S. pneumoniae* downstream of the Lys-Thr/Ser-Gly motif of PBP1a that could led to a reduced penicillin affinity, but we could also not detect this alteration in the isolates of our study. The lack of finding similar amino acid substitutions, which were already described in other studies, led to the suggestion that some other amino acid alterations within the PBPs of *S. suis* may confer a change in charge and reduce the affinity to β-lactams. However, to have an evidence for that, transformation and cloning experiments would be necessary. Besides, the serotypes of the sequenced isolates indicate that these *S. suis* belong to the less pathogenic, rather commensal flora of the respiratory tract. Hence, the more frequently occurring commensals might accumulate mutations in their PBPs and represent a source of resistance genes for pathogenic isolates.

The *S. suis* isolates showed a **florfenicol** MIC_{90} value of 2 mg/L (unpublished data Table 1). Since 2 mg/L was the highest florfenicol MIC value measured, all *S. suis* isolates of the VASIB study were classified as florfenicol-susceptible according to the CLSI breakpoints [91]. The same MIC_{90} value was detected in the VetPath study, indicating a high susceptibility of *S. suis* to florfenicol and no presence of florfenicol resistance genes. In veterinary medicine, florfenicol formulations are approved for pigs via injection, as well as feed and water supplement with an average withdrawal period of 20 days after the last antimicrobial application. Nevertheless, these formulations are only approved for the treatment of respiratory tract infections in swine caused by florfenicol-susceptible isolates of *A. pleuropneumoniae* and *P. multocida* [68].

As already displayed in **Publication I**, **tiamulin** showed a wide range of MICs with a MIC_{90} of 32 mg/L (n=257) (unpublished data Table 1). Unfortunately, no tiamulin CLSI breakpoints are available for *S. suis*, but the high MIC values to tiamulin indicate a reduced effect on *S. suis* and hence, tiamulin should not be used for therapeutic interventions of respiratory tract infections caused by *S. suis*.

3.5 Antimicrobial susceptibility of *B. bronchiseptica*

MIC values of *B. bronchiseptica* are difficult to interpret, since CLSI breakpoints are available for only four antimicrobial agents – ampicillin, tulathromycin, tildipirosin, and florfenicol. The MIC results of the selected *B. bronchiseptica* isolates of the 30 examined farms corresponded with the results of Farm 1 and 2 in **Publication I**. The macrorestriction analysis revealed only one *B. bronchiseptica* clone on farm level at a certain time point. Since the clones

differed in their MIC values in only +/- one dilution step, we claim that the MIC testing for only one *B. bronchiseptica* clone within a pig herd and time point is sufficient. However, as already indicated for *S. suis*, closely related isolates differed in their MIC ranges of some antimicrobial agents, such as SXT (data not shown).

The **tetracycline** MIC values of *B. bronchiseptica* of Farms 1 and 2 (**Publication I**) coincide with the tetracycline MIC_{90} of 0.5 mg/L (n=20) of *B. bronchiseptica* in all farms screened in this study (unpublished data Table 2). Moreover, this tetracycline MIC_{90} value is lower than the MIC_{90} values seen in the GE*RM*-Vet and VetPath studies, both 2 mg/L [6, 65]. Doxycycline MIC_{90} was 0.25 mg/L (n=20) in the VASIB study (unpublished data Table 2). We assume a broad tetracycline and doxycycline susceptibility among our isolates. Other studies examined *B. bronchiseptica* isolates with much higher MICs of at least 64 mg/L and detected the tetracycline resistance genes *tet*(A) or *tet*(C) [92, 93]. We did not test for these genes, since the tetracycline MICs of our isolates were too low to be indicative for the presence of a *tet* gene.

B. bronchiseptica exhibit **β-lactam** resistance due to the production of a species-specific β-lactamase as well as reduced outer membrane permeability. The lower amoxicillin/clavulanic acid MIC_{90} of 4/2 mg/L (n=20, unpublished data Table 2) of *B. bronchiseptica* correlates with the fact, that the species-specific β-lactamase plays an important role in resistance and is inhibited by clavulanic acid [89]. Agents without a β-lactamase inhibitor like ampicillin, penicillin or ceftiofur have no effect on *B. bronchiseptica* as shown by the comparatively high MIC values in **Publication I** and in the unpublished data Table 2. Furthermore, the ampicillin MIC values are indicated as resistant according to CLSI breakpoints (unpublished data Table 2). The high β-lactam MIC values were confirmed in the GE*RM*-Vet and VetPath studies [6, 65]. In summary, the use of β-lactams for the control of *B. bronchiseptica* infections without lactamase inhibitors should be avoided. Nevertheless, β-lactams, especially amoxicillin, are besides doxycycline the most frequently prescribed antimicrobials in this study and are indicated as frequently used antimicrobials in other surveys [1, 2, 15]. However, amoxicillin and even amoxicillin/clavulanic acid had no effect on *B. bronchiseptica*, which was verified in Farms 1 and 2, where *B. bronchiseptica* was still detected at the last day of antimicrobial treatment with amoxicillin or amoxicillin/clavulanic acid. Furthermore, the extensive use of β-lactams may promote the development of β-lactam resistance in other former highly β-lactam susceptible bacteria such as *S. suis* (**Publication I** and **III**). However, even though *B. bronchiseptica* was verified after the antimicrobial treatment, the herd-health of Farms 1 and 2 improved. This indicates that *B. bronchiseptica* was not the main causative pathogen of the disease.

The *B. bronchiseptica* isolates of the VASIB study exhibited **florfenicol** MICs that classified them as susceptible (≤2 mg/L) or intermediate (4 mg/L, unpublished data Table 2). This is in accordance with the florfenicol MICs observed in the studies of VetPath, Priebe and Schwarz (2003) or Kadlec *et al.* (2004) and excludes the presence of an acquired resistance gene coding for florfenicol resistance. *B. bronchiseptica* isolates that harbor *floR* exhibit higher florfenicol MICs of ≥8 mg/L [95].

Tiamulin MIC values of *B. bronchiseptica* are quite high with ≥128 mg/L (**Publication I**, unpublished data Table 2). We assume no bacteriostatic effect on *B. bronchiseptica*, since their cell wall might confer a reduced antimicrobial influx.

3.6 Antimicrobial susceptibility of *P. multocida*

P. multocida isolates with different PFGE patterns were present at every sampling in Farm 2 (**Publication I**). Sometimes up to two different *P. multocida* isolates per time point were detected on a single farm (Figure S5). The MIC values of *P. multocida* isolates of Farms 1 and 2 in **Publication I** give a good overview on the MIC profiles of *P. multocida* detected in the farms of the VASIB study. Hence, we suggest that an antibiogram for one representative isolate per sampling time point and farm may be sufficient. A second antibiogram should be performed when a genetically different *P. multocida* isolate is expected due to differing phenotypic characteristics.

Most *P. multocida* isolates were susceptible to **tetracyclines**, but some exhibited MIC values that classified them as intermediate or resistant as already indicated in **Publication I** [91]. The tetracycline MIC_{90} of selected *P. multocida* isolates (n=39) was 1 mg/L (unpublished data Table 3) in the VASIB study. The highest MIC that classified *P. multocida* as tetracycline-resistant in the present study was 2 mg/L (n=3). The isolates of the VetPath study had a MIC_{90} of 2 mg/L [65]. Overall, we assume the presence of multi-drug efflux pumps, since the most common acquired tetracycline resistance gene *tet*(H) was not detected in the resistant *P. multocida* isolates of this study [127, 128]. Further, the detected MIC values of 1 mg/L (n=9) and 2 mg/L (n=3) indicate the absence of a tetracycline resistance gene, which mediates distinctly higher MICs of at least 32 mg/L [130]. No CLSI breakpoints which allow a classification of the doxycycline susceptibility/resistance of *P. multocida* are available [91]. In the GE*RM*-Vet study, a doxycycline MIC_{90} of 0.5 mg/L was determined for *P. multocida* from weaners [6]. The same doxycycline MIC_{90} was identified in the VASIB study (unpublished data Table 3). Doxycycline MIC values were up to one or two dilution steps lower than the tetracycline values (unpublished data Table 3). Thus, a slightly better effect of doxycycline on *P. multocida* is expected.

The *P. multocida* isolates revealed a high susceptibility to **β-lactams** according to CLSI breakpoints [91]. The penicillin MIC_{90} of 0.25 mg/L in GE*RM*-Vet correspond to the MIC_{90} of 0.12 mg/L (n=39, unpublished data Table 3) in the VASIB study [6]. Ampicillin and amoxicillin/clavulanic acid MIC_{90} values of the VASIB study were 0.25 mg/L and 0.25/0.12 mg/L (unpublished data Table 3), respectively. The VetPath study showed partly higher MIC_{90} values of 0.5 mg/L for amoxicillin and 0.5/0.25 mg/L for amoxicillin/clavulanic acid [65]. The examined pigs in our study were both, apparently healthy and diseased, whereas the VetPath and GE*RM*-Vet studies exclusively investigated isolates from diseased pigs and examined a higher number of isolates (n≥150) [6, 65]. Nevertheless, the MIC results of the VASIB study as well as other studies like VetPath or GE*RM*-Vet revealed a broad β-lactam susceptibility of *P. multocida* and β-lactam usage during a single infection with *P. multocida* is supposed to be an effective therapeutic option.

The **florfenicol** MIC_{90} of *P. multocida* in the VASIB study was 0.5 mg/L (n=39) and the isolates were classified as susceptible (unpublished data Table 3) [91]. The GE*RM*-Vet and VetPath projects obtained also MIC_{90} values that indicate *P. multocida* as susceptible to florfenicol and give no presumption on the presence of florfenicol resistance genes. Since florfenicol formulations are approved for the treatment of infections caused by *A. pleuropneumoniae* and *P. multocida,* florfenicol represents a favorable option to treat respiratory tract infections caused by *P. multocida*.

The **tiamulin** MICs in this study ranged from 4 mg/L to 32 mg/L with a MIC_{90} of 32 mg/L (n=39, unpublished data Table 3), but no CLSI breakpoints are available [91]. Furthermore, tiamulin formulations are only approved for gastrointestinal tract infections as well as respiratory tract infections with *Mycoplasma* spp. or *A. pleuropneumoniae* in pigs [68]. A treatment of respiratory tract infections caused by *P. multocida*, but also by *S. suis* as well as *B. bronchiseptica*, with tiamulin is not recommended, since the respective bacteria exhibit higher MICs to tiamulin and a tiamulin off-label use would be necessary to treat those infections.

Next to β-lactams, tetracyclines and polypeptide antibiotics, sulfonamides are also frequently prescribed antimicrobials in veterinary medicine [1, 2, 15]. In therapy, sulfonamides are commonly used in combination with trimethoprim to take advantage of their antimicrobial synergistic effects and reduced adverse drug reactions [138]. **SXT** is approved especially for primary and secondary gastrointestinal and respiratory tract infections with SXT-susceptible pathogens [68]. **Publication II** describes the occurrence of *P. multocida* isolates from three different farms with reduced SXT susceptibility. CLSI breakpoints of SXT susceptibility for *P. multocida* are not available [91]. However, with regard to the common membership of *P. multocida* and *Haemophilus influenzae* to the family *Pasteurellaceae*, human CLSI

breakpoints of *H. influenzae* were used to deduce the SXT susceptibility of *P. multocida.* According to that, isolates with SXT MICs of ≥4/76 mg/L were classified as resistant [65]. The SXT MIC_{90} of the *P. multocida* isolates in the VASIB study was 0.25/4.75 mg/L (n=39, data not shown). Nevertheless, isolates with SXT resistance were identified in three farms as described in **Publication II**. The 152 *P. multocida* isolates of the VetPath project had a SXT MIC_{90} value of 0.5/9.5 mg/L and the isolates of the GE*RM*-Vet study 16/304 mg/L (n=150) [6, 65]. Further examinations of the SXT-resistant *P. multocida* in the VASIB study revealed a resistance plasmid pIMT41689_PM of 6 kb in all of these isolates (n=9). The small plasmid size is in accordance with other resistance plasmids of the family *Pasteurellaceae* [135]. Further examinations of the SXT-resistant isolates from the three different farms revealed their close relatedness, since they exhibited macrorestriction patterns with an identity of 100 % on farm level as well as 92.4 % and 94.7 % between the farms (**Publication II**). Moreover, the isolates of the three farms were classified as serotype A and ST13. Serotype A and ST13 are frequently associated with porcine bronchopneumonia [101, 167, 173]. These facts may indicate that the SXT-resistant isolates have a common ancestor and belong to the same clonal lineage. The structure of the 6 kb SXT resistance plasmid of *P. multocida* is described in **Publication II**. The trimethoprim resistance gene *dfrA14* belongs to the *dfrA* gene family and encodes the 157 amino acid large, trimethoprim-insensitive DHFR, which is quite common on mobile genetic elements of Gram-negatives [138]. The *dfrA14* gene is detected in *P. multocida* for the first time in the VASIB study. However, *dfrA14* integrates quite commonly at a secondary site within the streptomycin resistance gene *strA*, which was already identified in other Gram-negatives, including *Enterobacteriaceae* and *Pasteurellaceae* [174, 175]. Furthermore, the region between s*ul2* and *strA* also seems to be associated with recombination events, as detected on plasmids or chromosomal DNA of *Pasteurella aerogenes, Mannheimia varigena* and *Mannheimia* sp. with the combination of *sul2-catA3-strA*, or *P. multocida* with *sul2-tetR-tet*(H)-*strA-strB* [139, 141]. The insertion of *dfrA14* into *strA* leads to the truncation and functional inactivity of *strA*. Furthermore, the also plasmid located *strB* gene exhibited a mutation that resulted in a stop codon already after 237 amino acids instead of 278 amino acids. The insertion and mutation resulted in dysfunctional streptomycin resistance proteins as verified by streptomycin MICs of 4 - 8 mg/L of the SXT-resistant isolates (data not shown) in this study. In contrast, intact streptomycin resistance genes confer much higher MICs of ≥64 mg/L [129]. The *sul2* gene encodes a sulfonamide-insensitive DHPS that is frequently linked to *strA-strB* within members of the *Pasteurellaceae* family [135]. Furthermore, the genes *mobA*, *mobB* and *mobC* were located on plasmid pIMT41689_PM (**Publication II**). These genes encode proteins for the relaxosome formation during mobilization. A similar plasmid pM3224T with an identity to pIMT41689_PM of 99.9 % was identified in *A. pleuropneumoniae,* which belongs also to the *Pasteurellaceae* family [176]. We

suggest the presence of a conjugative element, which would enable a plasmid exchange between *P. multocida* and *A. pleuropneumoniae* within their common habitat of the porcine respiratory tract. It is rather unlikely that two so closely related plasmids have developed independent from each other.

3.7 Concluding remarks

The respiratory tract of pigs is usually colonized by a mixed flora of various bacteria with different antimicrobial susceptibilities. In this thesis, we focused on the relatedness and antimicrobial susceptibilities of *S. suis*, *B. bronchiseptica* and *P. multocida*. However, *H. parasuis*, *A. pleuropneumoniae* or *M. hyorhinis* may also colonize the respiratory tract and need to be monitored for their antimicrobial susceptibilities, too. In this project, most porcine herds as well as the examined individual animals within those herds revealed mild respiratory tract symptoms. In addition, most pigs improved their respiratory fitness after the antimicrobial treatment, despite the presence of some isolates with a reduced susceptibility or resistance to the prescribed antibiotics. This may indicate their marginal role in causing respiratory symptoms. Other bacteria or viruses that colonize the respiratory tract may have caused the infection. Nevertheless, antimicrobial treatment results in a selection pressure on different bacterial species, which may either cause diseases or are present as commensal flora. Under a selective pressure, commensals, facultative and obligate pathogens can acquire resistance genes. The antimicrobial resistance profiles of the aforementioned pathogens were determined in each farm. However, the presented analyses revealed no acquisition of new antimicrobial resistance genes immediately after the antimicrobial therapy. Moreover, the examined pathogens differed in their susceptibility to the prescribed antimicrobials, except for florfenicol. This may indicate that florfenicol is an effective alternative to treat respiratory tract infections caused by *S. suis*, *P. multocida* and *B. bronchiseptica*. However, florfenicol is only approved for porcine respiratory tract infections caused by *A. pleuropneumoniae* and *P. multocida* and an off-label use in some other indications would be necessary. Macrorestriction analyses revealed a large diversity of PFGE patterns of *S. suis* accompanied by variable susceptibility patterns of the corresponding isolates. In contrast, a limited diversity of PFGE patterns was observed for *B. bronchiseptica* and accordingly, the MICs of the respective isolates were also rather similar. The *P. multocida* isolates showed a genetic relatedness to some extent but were not as diverse as the *S. suis* isolates. Nevertheless, the MICs of the different *P. multocida* isolates had mostly a narrow range of distribution. Finally, one antibiogram for *B. bronchiseptica*, up to two for morphological different *P. multocida* and at least antibiograms of two or three morphologically diverse *S. suis* per farm and sampling time point should be

recommended for diagnostic laboratories in order to get a representative overview of the antimicrobial susceptibility of the pathogens.

4 Summary

Streptococcus suis is a Gram-positive bacterium and a commensal colonizing the porcine respiratory tract. However, some serotypes have a higher virulence and can cause pneumonia, meningitis, septicaemia, arthritis, endocarditis, polyserositis or abortion in pigs. *Bordetella bronchiseptica* and *Pasteurella multocida* are Gram-negative bacteria that can cause bronchopneumonia or atrophic rhinitis. Bacterial infections of the porcine respiratory tract are commonly treated with antimicrobials.

In this thesis, bronchoalveolar lavage fluid samples (BALFs) of weaners of four to ten weeks of age and originating from 30 farrow-to-finish farms were investigated for the aforementioned pathogens. Furthermore, the influence of antimicrobial treatment during respiratory tract infection on such pathogens within a one-year period was monitored. The genetic relatedness of selected *S. suis*, *B. bronchiseptica* and *P. multocida* isolates on farm level and between different farms was investigated by macrorestriction with subsequent pulsed-field gel electrophoresis (PFGE). In addition, the antimicrobial susceptibility and resistance mechanisms of the pathogens were determined. However, a valid recommendation to the susceptibility of the pathogens to all examined antibiotics is sometimes difficult, since CLSI approved breakpoints are not available for all tested antimicrobial agents.

This study revealed a great diversity of PFGE patterns among *S. suis* isolates. Single pigs can harbor *S. suis* isolates with completely different macrorestriction patterns, which also differ in their antimicrobial susceptibilities. *B. bronchiseptica* showed a greater homogeneity in their PFGE patterns and *P. multocida* displayed related and different PFGE patterns. One or two closely related *B. bronchiseptica* isolates with quite similar minimal inhibitory concentrations (MICs) existed over a one-year period within one farm. One or two *P. multocida* isolates with different PFGE patterns can exist on one farm at a certain time point, nevertheless most *P. multocida* isolates had similar MICs. Antimicrobial agents were prescribed 24 times for five to seven days in the farms of this project. Doxycycline was most frequently prescribed (n=14 times) in this study, followed by amoxicillin (n=8), amoxicillin/clavulanic acid (n=1) and tiamulin/florfenicol (n=1).

S. suis isolates were frequently tetracycline-resistant, since they harbored resistance genes such as *tet*(O), *tet*(M), *tet*(L) or *tet*(W). *S. suis* exhibited higher MICs to tiamulin, was susceptible to florfenicol and to aminopenicillins alone and in combination with clavulanic acid. However, individual *S. suis* isolates showed distinctly higher MICs to penicillin. Therefore, three *S. suis* isolates, which were susceptible, intermediate or resistant to penicillin were subjected to whole genome sequencing. The analyses of the whole genome sequences revealed genes encoding five penicillin-binding proteins (PBPs) for all three isolates. The

penicillin-resistant isolate had the most amino acid alterations in PBP2x, PBP2b and PBP2a, whereas the penicillin-intermediate isolate had the most amino acid substitutions only in PBP2x and PBP2b compared to the penicillin-susceptible isolate. However, none of the isolates exhibited amino acid alterations in the conserved motifs, which form the catalytic centre of the PBPs and interact with penicillin.

Tetracyclines may have bacteriostatic effects on *B. bronchiseptica*, since these bacteria exhibit low doxycycline and tetracycline MIC_{90} values of 0.25 mg/L and 0.5 mg/L, respectively. The *B. bronchiseptica* isolates showed an overall reduced susceptibility to β-lactams except for amoxicillin/clavulanic acid. *B. bronchiseptica* isolates had a tiamulin MIC_{90} of ≥128 mg/L and were either susceptible or intermediate towards florfenicol.

The *P. multocida* isolates were overall β-lactam- and florfenicol-susceptible. Some isolates showed tetracycline MICs of 1 mg/L (n=9) and 2 mg/L (n=3) that classified the respective isolates as intermediate and resistant, respectively. The tiamulin MIC_{90} was 32 mg/L. Regardless of the antimicrobial treatment on the farms, trimethoprim/sulfamethoxazole (SXT)-resistant *P. multocida* isolates were detected on three different farrow-to-finish farms. A closer examination of these isolates revealed their close genetic relatedness and the existence of a *dfrA14*-carrying, 6 kb plasmid. The *dfrA14* gene mediates trimethoprim resistance and was detected in *P. multocida* for the first time. However, a similar plasmid with a nucleotide sequence identity of 99.9 % to that of *P. multocida* was already identified in *Actinobacillus pleuropneumoniae*. Hence, we suspect that a plasmid exchange of the pathogens within the common habitat of the porcine respiratory tract has taken place in the past.

The MIC testing revealed a broad susceptibility of *S. suis* and *P. multocida* to florfenicol as well as an intermediate susceptibility of *B. bronchiseptica*, which might identify florfenicol as an effective therapeutic alternative to tetracyclines or β-lactams. Besides, the frequent use of β-lactams, tetracyclines and sulfonamides in veterinary medicine increases the selection pressure on certain pathogens as shown in this thesis. Tetracycline resistance genes seems to become conserved in the streptococcal genome due to the frequent use of doxycycline. Former fully β-lactam susceptible isolates, such as *S. suis*, accumulate amino acid alterations in their PBPs, which mediate a lower affinity to penicillin. The development of a SXT resistance plasmid and its presence in already two members of the family *Pasteurellaceae*, demonstrates the dissemination of such plasmids within this family.

5 Zusammenfassung

Genetische Verwandtschaft und antimikrobielle Empfindlichkeit der porzinen Atemwegsinfektionserreger *Streptococcus suis*, *Bordetella bronchiseptica* und *Pasteurella multocida*

Streptococcus suis ist ein Gram-positives Bakterium und besiedelt den Respirationstrakt von Schweinen als Kommensale. Einige Serotypen haben jedoch eine höhere Virulenz, durch welche Pneumonien, Meningitis, Septikämien, Arthritis, Endokarditis, Polyserositis oder Abort bei Schweinen ausgelöst werden können. *Bordetella bronchiseptica* und *Pasteurella multocida* sind Gram-negative Bakterien, welche Bronchopneumonien oder Rhinitis atrophicans auslösen können. Bakterielle Infektionen des Respirationstrakts von Schweinen werden gewöhnlich mit Antibiotika behandelt.

In dieser Arbeit wurden bronchoalveoläre Lavageflüssigkeiten (BALF) von vier bis zehn Wochen alten Absetzferkeln aus 30 Ferkelerzeugerbetrieben auf die vorab genannten Pathogene hin untersucht sowie die antimikrobielle Behandlung von Atemwegserkrankungen innerhalb eines Jahres erfasst. Die genetische Verwandtschaft ausgewählter *S. suis*, *B. bronchiseptica* und *P. multocida* Isolate wurde auf Betriebsebene sowie zwischen verschiedenen Betrieben mit Hilfe der Makrorestriktionsanalyse und anschließender Pulsfeld-Gelelektrophorese (PFGE) analysiert. Zusätzlich wurde die antimikrobielle Empfindlichkeit der Pathogene sowie deren Resistenzmechanismen untersucht. Die Bewertung der Empfindlichkeit einiger Pathogene gestaltete sich teilweise schwierig, da nicht für alle getesteten Antibiotika seitens CLSI anerkannte Grenzwerte vorhanden sind.

Diese Studie zeigt eine große genetische Diversität zwischen den untersuchten *S. suis* Isolaten. Einzelne Schweine können *S. suis* Isolate mit unterschiedlichen Makrorestriktionsmustern und verschiedenen antimikrobiellen Empfindlichkeiten tragen. *B. bronchiseptica* zeigte eine größere genetische Homogenität hinsichtlich der Makrorestriktionsmuster, wohingegen *P. multocida* eine etwas breitere Verwandtschaft der Isolate aufweist. Pro Betrieb konnten in einem Zeitraum von einem Jahr ein oder zwei eng verwandte *B. bronchiseptica* Isolate mit ähnlichen minimalen Hemmstoffkonzentrationen (MHKs) nachgewiesen werden. Im Vergleich dazu, konnten mitunter zwei *P. multocida* Isolate mit unterschiedlichen Makrorestriktionsmustern im gleichen Betrieb zu einem bestimmten Zeitpunkt existieren, welche jedoch ähnliche MHK Werte aufweisen. Die antimikrobielle Behandlung auf den Betrieben dauerte fünf bis sieben Tage. Insgesamt wurde auf den Farmen 24-mal ein Antibiotikum angewendet. Doxycyclin war mit 14 Anwendungen das am häufigsten verwendete Antibiotikum in dieser Studie, gefolgt von Amoxicillin (8 Anwendungen) sowie Amoxicillin/Clavulansäure und Tiamulin/Florfenicol (je eine Anwendung).

S. suis Isolate waren meist resistent gegenüber Tetrazyklinen, da sie Resistenzgene wie z.B. *tet*(O), *tet*(M), *tet*(L) oder *tet*(W) trugen. *S. suis* zeigte erhöhte MHK Werte gegenüber Tiamulin, war jedoch empfindlich gegenüber Aminopenicillinen und deren Kombination mit Clavulansäure. Jedoch zeigten einige *S. suis* Isolate auch deutlich höhere Penicillin MHK-Werte. Aus diesem Grund wurden drei Isolate, welche empfindlich, intermediär und resistent gegenüber Penicillin waren, ausgewählt und einer Gesamtgenomsequenzierung unterzogen. Die Analyse der Gesamtgenomsequenzen ergab für die drei Isolate jeweils fünf Gene, die für Penicillin-bindende Proteine (PBPs) kodierten. Im Vergleich zu dem Penicillin-sensiblen Isolat wies das Penicillin-resistente Isolat die meisten Aminosäureänderungen in PBP2x, PBP2b und PBP2a auf, wohingegen das Penicillin-intermediäre Isolat die meisten Aminosäureänderungen in PBP2x und PBP2b aufwies. Keines der Isolate zeigte Aminosäureveränderungen in den konservierten Sequenzmotiven, welche das katalytische Zentrum der PBPs bilden und mit Penicillin interagieren.

Die untersuchten *B. bronchiseptica* Isolate zeigten niedrige Doxycyclin und Tetrazyklin MHK_{90} Werte mit jeweils 0.25 mg/L und 0.5 mg/L, welche einen bakteriostatischen Effekt haben könnten. Die *B. bronchiseptica* Isolate besitzen eine umfassende β-Laktam Resistenz, ausgenommen gegen die Kombination von Amoxicillin/Clavulansäure. In dieser Studie hatten die *B. bronchiseptica* Isolate einen Tiamulin MHK_{90} von 128 mg/L und zeigten MHK-Werte, die sie als empfindlich oder intermediär gegenüber Florfenicol klassifizieren.

P. multocida war empfindlich gegenüber β-Laktamen und Florfenicol. Einzelne *P. multocida* Isolate zeigten mit 1 mg/L (n=9) und 2 mg/L (n=3) Tetrazyklin MHK Werte, die sie als intermediär bzw. resistent klassifizieren. Der Tiamulin MHK_{90} der untersuchten Isolate lag bei 32 mg/L. Unabhängig von den Antibiotikabehandlungen in den Betrieben wurden Trimethoprim/Sulfamethoxazol (SXT)-resistente *P. multocida* Isolate in drei verschiedenen Ferkelerzeugerbetrieben detektiert. Eine genauere Untersuchung dieser Isolate ergab eine enge genetische Verwandtschaft zwischen diesen Isolaten und das Vorhandensein eines 6 kb großen Plasmides, welches die Gene *sul2* und *dfrA14* trug. Das *dfrA14* Gen vermittelt Resistenz gegenüber Trimethoprim und wurde in dieser Studie zum ersten Mal bei *P. multocida* nachgewiesen. Ein ähnliches Plasmid mit einer Sequenzübereinstimmung von 99.9 % wurde bereits bei *Actinobacillus pleuropneumoniae* identifiziert. Man könnte daher einen Plasmidaustausch zwischen den beiden Bakterienspezies in ihrem gemeinsamen Habitat, dem Respirationstrakt des Schweins, in der Vergangenheit annehmen.

Unsere Untersuchungen verdeutlichten, dass *S. suis* und *P. multocida* eine hohe Empfindlichkeit gegenüber Florfenicol besitzen, während *B. bronchiseptica* auch intermediäre MHK Werte aufweist. Daher kann Florfenicol als eine wirkungsvolle Therapiealternative zu Tetrazyklinen und β-Laktamen angesehen werden. Außerdem führt der häufige Gebrauch von

β-Laktamen, Tetrazyklinen und Sulfonamiden in der Veterinärmedizin zu einem erhöhten Selektionsdruck gegenüber einigen Pathogenen, wie in dieser Studie gezeigt wurde. Gene, welche Tetrazyklinresistenz vermitteln, scheinen durch den häufigen Therapieeinsatz von Doxycyclin bereits im Streptokokkengenom konserviert zu sein. Zudem weisen Isolate, wie z.B. *S. suis,* die zuvor vollständig empfindlich gegenüber Penicillin waren, eine Ansammlung von Aminosäureveränderungen in ihren PBPs auf. Diese Veränderungen können zu einer geringeren Affinität der Isolate gegenüber Penicillinen führen. Die Entstehung eines SXT-Resistenzgene-tragenden Plasmids sowie dessen bereits vorliegende Existenz in zwei Mitgliedern der Familie *Pasteurellaceae* verdeutlicht die mögliche weitere Verbreitung solcher Plasmide innerhalb dieser Familie sowie deren klinische Relevanz.

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7 Appendix

Table S1 Information of the prescribed antimicrobials on the different farms with their treatment success determined by a modified respiratory health score according to Höltig and colleagues (2008). The total score represents the herd fitness, identified by the median score of 15 and 10 examined pigs at time points B/D and C/E, respectively. The scores of pigs 1-3 indicate the health of the three BALF animals. A score of ≤0.18 reflects healthy and ≥0.18 respiratory diseased animals. The pathogens *B. bronchiseptica*, *S. suis* and *P. multocida* represent the number of colonies isolated from the BALFs. The dash within the columns indicates no isolation of the required isolates.

Farm	Time Point	Antimicrobial Therapy[a]	Total Score	Score Pig 1	Score Pig 2	Score Pig 3	*B. bronchiseptica*	*S. suis*	*P. multocida*
1	B	AMX	0.89	1	1	0.33	8	8	-
1	C		0.42	0.33	0.5	0.33	3	-	1
2	B	AUG	0.72	0.72	1.39	0.5	4	12	8
2	C		0.33	0.17	0.17	0.5	8	3	4
3	B	AMX	0.56	0.89	0.89	0.33	-	7	4
3	C		0.33	0.33	0.17	1.22	12	6	6
6	B	DOX	1.78	1.78	1.78	1.78	-	12	8
6	C		0.42	1.17	0.5	0.33	-	12	-
6	D	DOX	1.89	1.89	1.72	1.72	-	8	-
6	E		0.67	0.72	1.22	1.56	-	-	4
9	B	DOX	1.22	1.39	1.22	1.39	-	5	-
9	C		0.36	0.33	0.33	0.83	-	7	-
11	B	DOX	0.72	0.56	1.06	0.56	-	-	-
11	C		0.5	1.45	0.39	0.5	-	-	-
11	D	AMX	0.56	0.56	0.72	0.56	8	8	-
11	E		0.39	0.67	0.5	0.33	4	4	-
12	B	DOX	0.72	0.22	0.56	1.22	-	8	-
12	C		0.33	0.33	0	0.17	-	4	-
12	D	DOX	1.06	0.89	1.39	0.89	-	8	4
12	E		0.72	0.72	0.72	0.72	-	4	1
13	B	AMX	0.56	1.22	0.67	0.56	-	12	10
13	C		0.67	1.39	0.83	0.67	2	5	2
14	B	DOX	0.72	0.39	0.89	0.72	-	12	-
14	C		0.72	0	0.72	n.d.[b]	-	7	-
18	B	DOX	0.72	0.56	1.06	0.56	-	8	-
18	C		0.39	0.39	0.56	0.17	-	7	-
18	D	DOX	0.89	1.11	0.89	0.72	-	11	-
18	E		0.92	0.56	0.56	0.39	-	8	-
19	B	AMX	0.56	0.72	1.06	0.39	8	4	2
19	C		0.53	0.89	0.5	0.95	-	11	-
20	B	AMX	1.06	0.89	1.06	0.89	12	7	8
20	C		0.5	0.17	0.33	0.5	12	-	4
21	B	TIA/FFN	0.89	1.06	0.89	0.89	3	10	-
21	C		0.53	0.67	1.56	0.83	8	11	-
22	B	DOX	0.56	0.56	0.72	0.72	-	4	-
22	C		0.33	0.17	0.33	0.5	-	7	-
24	B	DOX	1.06	1.28	1.06	0.89	8	8	5
24	C		0.75	1.22	0.83	1.22	12	4	4
25	B	DOX	0.89	1.06	0.89	0.72	12	8	-
25	C		0.75	0.89	0.56	0.67	-	7	-
26	B	DOX	0.72	1.22	0.72	1.06	4	12	4
26	C		0.36	0.89	0.39	0.33	4	8	4
27	B	DOX	0.89	1.22	1.89	0.72	-	10	9
27	C		0.56	1.39	1	0.33	-	12	4
29	B	AMX	0.89	1.45	1.39	1.06	-	1	-
29	C		0.25	0.17	0.33	0.17	-	-	-
30	B	AMX	0.56	0.56	0.72	0.89	-	4	1
30	C		0.33	0.33	0.17	0.33	-	4	1

[a] AMX amoxicillin; AUG amoxicillin/clavulanic acid; DOX doxycycline; TIA/FFN tiamulin/florfenicol

[b] not determined

Figure S1 *S. suis* PFGE patterns of Farms 14, 29 and 30 obtained from several sampling time points with indistinguishable PFGE profiles per farm. *S. suis* was digested with SmaI as described in **Publication I** and PFGE analysis was performed with BioNumerics v7.5 (bioMérieux, Applied Math, Sint-Martens-Latem, Belgium) by a cluster analysis with a tolerance of 1 % and optimization of 0.5 %. The dendrogram indicates the similarity of the analysed isolates.

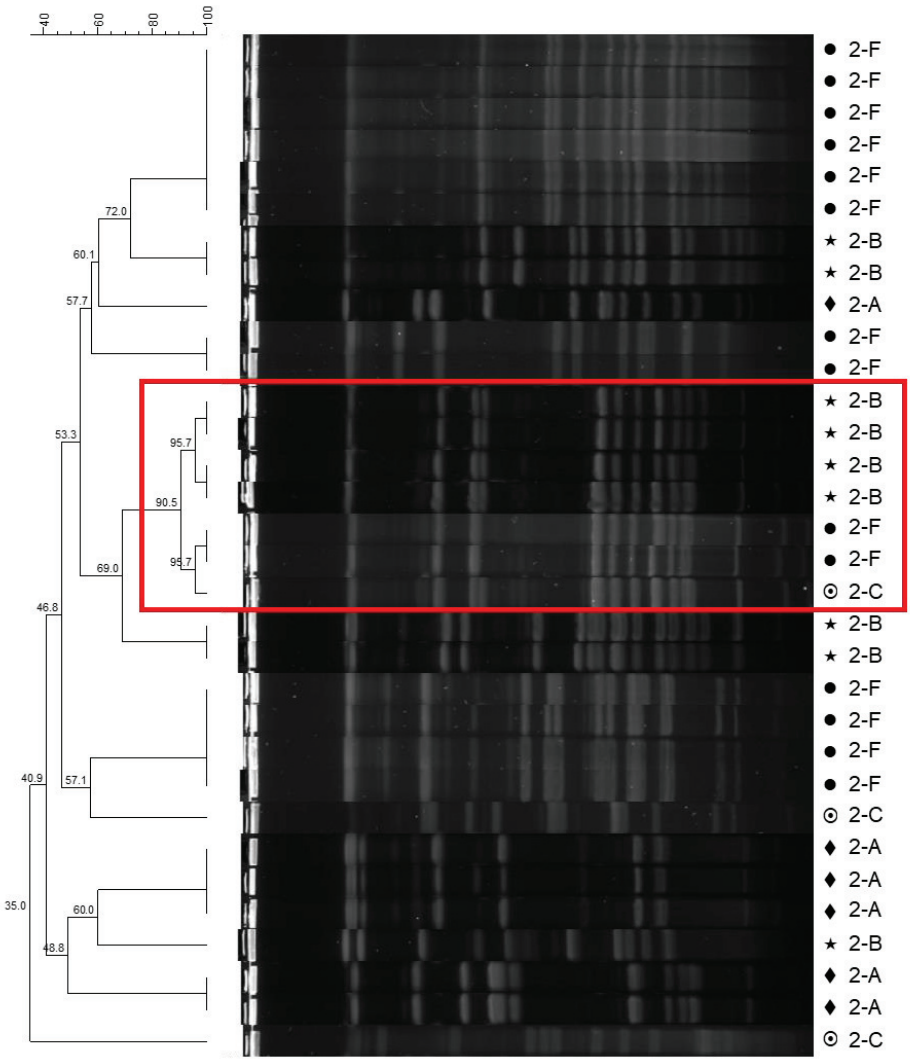

Figure S2 *S. suis* PFGE patterns of Farm 2 with closely related isolates at several time points indicated in the red framed box. Digestion experiment and PFGE analysis was performed as described in Figure S1.

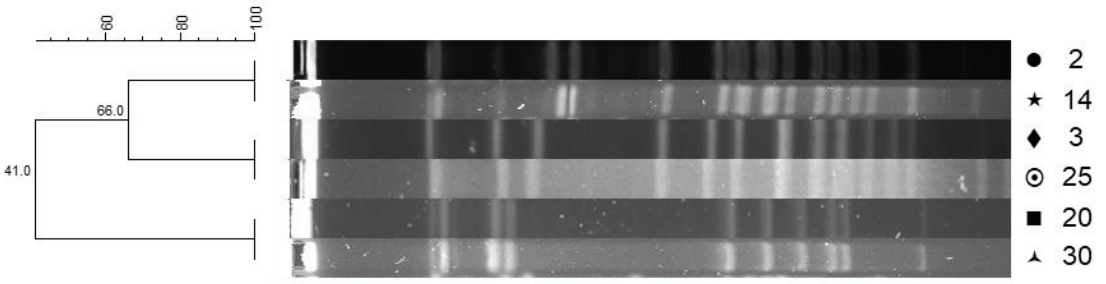

Figure S3 *S. suis* PFGE patterns of Farms 2, 3, 14, 25, 20 and 30, each with indistinguishable profiles between two farms. Digestion experiment and PFGE analysis was performed as described in Figure S1.

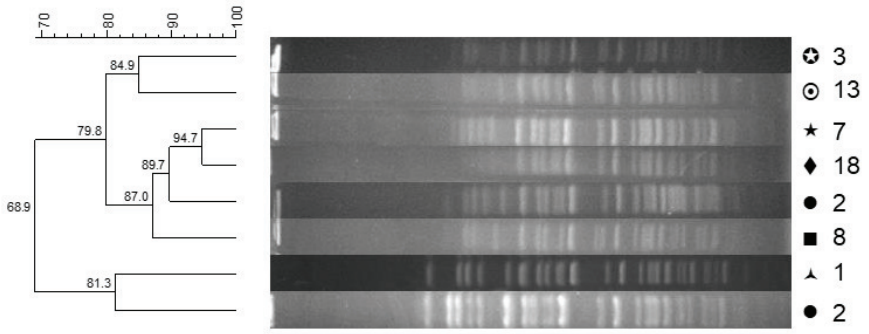

Figure S4 *B. bronchiseptica* PFGE patterns of different farms showed in parts closely related profiles. *B. bronchiseptica* was digested with XbaI as described in **Publication I** and PFGE analysis was performed as explained in Figure S1.

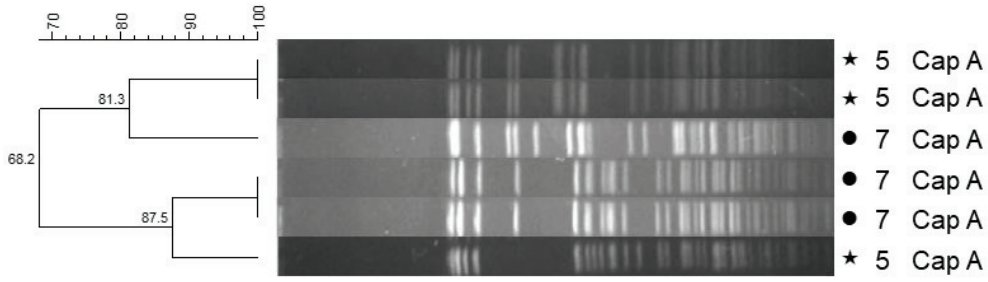

Figure S5 *P. multocida* PFGE patterns of Farms 5 and 7 obtained at the same sampling time point A. Macrorestriction revealed different patterns within a farm at the same time. Information about the serotype is provided behind the farm numbers. *P. multocida* was digested with SmaI as described in **Publication I** and PFGE analysis was performed as clarified in Figure S1.

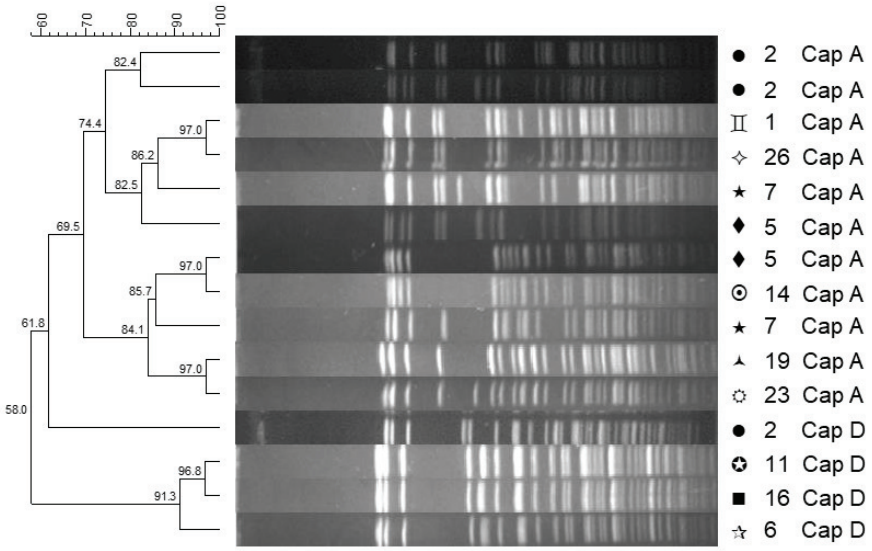

Figure S6 *P. multocida* PFGE patterns of different farms with in parts closely related isolates between the farms. Information about the serotype is provided behind the farm numbers. *P. multocida* was digested with SmaI as described in **Publication I** and PFGE analysis was performed as clarified in Figure S1.

List of publications

Parts of this thesis have already been published:

Niemann L, Müller P, Brauns J, Nathaus R, Schäkel F, Kipschull K, Höltig D, Wendt M, Schwarz S, Kadlec K (2018): Antimicrobial susceptibility and genetic relatedness of respiratory tract pathogens in weaner pigs over a 12-month period.
Veterinary Microbiology **219**, 165 – 170.

Niemann L, Feudi C, Eichhorn I, Hanke D, Müller P, Brauns J, Nathaus R, Schäkel F, Höltig D, Wendt M, Kadlec K, Schwarz S (2019): Plasmid-located *dfrA14* gene in *Pasteurella multocida* isolates from three different piglet-producing farms in Germany.
Veterinary Microbiology **230**, 235 – 240.

Niemann L, Eichhorn I, Müller P, Brauns J, Nathaus R, Schäkel F, Höltig D, Wendt M, Kadlec K, Schwarz S (2019): Draft genome sequences of three porcine *Streptococcus suis* isolates which differ in their susceptibility to penicillin.
Microbiology Resource Announcements **8**, e01711-18.

Further aspects have been presented at national and international conferences as posters:

Niemann L, Müller P, Brauns J, Nathaus R, Schäkel F, Schwarz S, Kadlec K.
Antimicrobial susceptibility and genetic relatedness of respiratory tract pathogens before and after antibiotic treatment.
Proceedings of the 7th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE), Braunschweig, Germany (2017), Poster 35.

Niemann L, Müller P, Brauns J, Nathaus R, Kipschull K, Schäkel F, Höltig D, Wendt M, Schwarz S, Kadlec K.
Antimicrobial resistance of respiratory tract pathogens in piglet-producing farms in Germany before and after antimicrobial therapy.
Proceedings of the Conference of the *Deutsche Veterinärmedizinische Gesellschaft* (DVG), division Bacteriology and Mycology, Hannover, Germany (2018), Poster 31.

Niemann L, Eichhorn I, Kadlec K, Schwarz S.
Alterations in penicillin-binding proteins of penicillin-resistant *Streptococcus suis.*
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Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Berlin, den 24.06.2019 Lisa Niemann