Detection and organisation of 
antimicrobial resistance genes in 
*Bordetella bronchiseptica* isolates from pigs

THESIS

Submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY (PhD)

at the University of Veterinary Medicine Hannover

by

Kristina Kadlec

from Prague

Hannover 2006.
Supervisor: Prof. Dr. S. Schwarz
Co-Supervisor: Dr. C. Kehrenberg, Ph.D.

Tutorial group: Prof. Dr. S. Schwarz
Prof. Dr. G.-F. Gerlach
Dr. J. Wallmann

Internal evaluation: Prof. Dr. S. Schwarz, Institute for Animal Breeding, Federal Agricultural Research Centre (FAL), Neustadt-Mariensee
Prof. Dr. G.-F. Gerlach, Institute for Microbiology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Hannover
Dr. J. Wallmann, Federal Office of Consumer Protection and Food Safety (BVL), Berlin

External evaluation: S. Simjee, Ph.D., Elanco Animal Health, Basingstoke, UK

Examination: 09.11.2006

This work was supported by the H. Wilhelm Schaumann foundation.
pro dědu
Parts of the thesis have already been published or will be published:

**Kadlec K, Wallman J, Kehrenberg C, and Schwarz S.**
Antimicrobial susceptibility of *Bordetella bronchiseptica* from porcine respiratory tract infections.

**Kadlec K, Kehrenberg C, and Schwarz S.**
Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*.

**Kadlec K, Kehrenberg C, and Schwarz S.**
tet(A)-mediated tetracycline resistance in porcine *Bordetella bronchiseptica* isolates is based on plasmid-borne Tn1721 relics.

**Kadlec K, Kehrenberg C, and Schwarz S.**
Efflux-mediated resistance to florfenicol and/or chloramphenicol in *Bordetella bronchiseptica*: identification of a novel chloramphenicol exporter.

**Kadlec K, Wiegand I, Kehrenberg C, and Schwarz S.**
Studies on the mechanisms of β-lactam resistance in *Bordetella bronchiseptica*.
Further aspects have been presented at national or international conferences as oral presentations or as posters:

Kadlec K, Wallman J, Kehrenberg C, and Schwarz S.
Untersuchungen zur in-vitro Empfindlichkeit von porcinen Bordetella bronchiseptica Isolaten gegenüber antimikrobiellen Wirkstoffen.

Kadlec K, Wallman J, Kehrenberg C, and Schwarz S.
In-vitro susceptibility of porcine Bordetella bronchiseptica isolates to antimicrobial agents.

Kadlec K, Kehrenberg C, and Schwarz S.
Trimethoprimresistenz bei Bordetella bronchiseptica.

Kadlec K, Kehrenberg C, and Schwarz S.
Cassette-borne trimethoprim resistance among Bordetella bronchiseptica isolates from pigs.
**Kadlec K, Kehrenberg C, and Schwarz S.**  
Truncated Tn1721 mediates resistance to tetracycline in porcine *Bordetella bronchiseptica* isolates.  

**Kadlec K, Wallman J, Kehrenberg C, and Schwarz S.**  
A four-year survey on in-vitro susceptibility of porcine *Bordetella bronchiseptica* isolates from Germany.  

**Kadlec K, Wiegand I, Kehrenberg C, and Schwarz S.**  
Genetic basis of ampicillin resistance in *Bordetella bronchiseptica*.  

**Kadlec K, Wiegand I, Kehrenberg C, and Schwarz S.**  
Grundlagen der β-Laktamresistenz bei *Bordetella bronchiseptica*.  

**Kadlec K, Wallman J, Kehrenberg C, and Schwarz S.**  
In-vitro susceptibility of German *Bordetella bronchiseptica* isolates from pigs.  
Contents

Chapter 1  Introduction  11
  1.  General considerations  13
  2.  The respiratory tract pathogen *Bordetella bronchiseptica*  13
    2.1  The genus *Bordetella*  14
    2.2  The species *Bordetella bronchiseptica*  15
  3.  Susceptibility testing  17
    3.1  Phenotypical susceptibility testing  17
    3.2  Genotypical susceptibility testing  20
  4.  Selected antimicrobial agents  21
    4.1  Trimethoprim and sulphonamides  22
    4.2  Tetracycline  24
    4.3  Phenicols  26
    4.4  ß-Lactams  29
  5.  Horizontal gene transfer of resistance genes  32
    5.1  Plasmids  32
    5.2  Transposons  33
    5.3  Gene cassettes and integrons  34
  6.  Aims of the present study  36

Chapter 2  Antimicrobial susceptibility of *Bordetella bronchiseptica* from porcine respiratory tract infections  45

Chapter 3  Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*  55

Chapter 4  *tet*(A)-mediated tetracycline resistance in porcine *Bordetella bronchiseptica* isolates is based on plasmid-borne Tn1721 relics  69

Chapter 5  Efflux-mediated resistance to florfenicol and/or chloramphenicol in *Bordetella bronchiseptica*: identification of a novel chloramphenicol exporter  77
Chapter 6  Studies on the mechanisms of β-lactam resistance in *Bordetella bronchiseptica*  93

Chapter 7  General discussion  111
1. General considerations  113
2. Antimicrobial susceptibility of porcine *Bordetella bronchiseptica* in comparison to other porcine respiratory tract pathogens  114
   2.1 Trimethoprim and sulfamethoxazole/trimethoprim  116
   2.2 Tetracyclines  118
   2.3 Chloramphenicol and florfenicol  120
   2.4 β-Lactam antibiotics  122
   2.5 Macrolides  126
3. Resistance genes in *Bordetella bronchiseptica*  126
   3.1 Trimethoprim and sulphonamide resistance genes  127
   3.2 Tetracycline resistance genes  130
   3.3 Phenicol resistance genes  133
   3.4 β-Lactam resistance genes  136
4. Localization of resistance genes on mobile genetic elements  138
   4.1 Gene cassettes and class 1 integrons  139
   4.2 Transposons  140
   4.3 Plasmids  141
5. General comparison within the genus *Bordetella*  141
   5.1 *B. bronchiseptica* isolated from cats  141
   5.2 Susceptibility and resistance in *Bordetella* spp. from human infections  142
6. General conclusion  143

Chapter 8  Summary  151

Chapter 9  Zusammenfassung  157
Chapter 1

Introduction
1. **General considerations**

   Up to now antimicrobial resistance genes coding for various resistance mechanisms have been described not only in microorganisms producing antibiotics, but also in environmental, commensal, and pathogenic bacteria. At the beginning of this Ph.D. project, very little has been known about antimicrobial resistance in *Bordetella bronchiseptica*. Internationally accepted, veterinary-specific breakpoints to classify *B. bronchiseptica* isolates as resistant, intermediate or susceptible are so far only approved for a single antimicrobial agent, namely florfenicol.\(^{22,23}\) Although a few studies on antimicrobial susceptibility testing of *B. bronchiseptica* isolates have been published and resistance properties transferable to *Escherichia coli* have been described,\(^{43,132,138,141,162}\) no sequences of resistance genes from *B. bronchiseptica* have been available. Solely, a single tetracycline resistance gene, \(\text{tet}(C)\),\(^{138}\) was identified by Southern blot hybridization.

   This Ph.D. project dealt with the detection and organisation of antimicrobial resistance genes in *B. bronchiseptica* isolates from pigs and was divided into two parts. Initially, *B. bronchiseptica* isolates collected in 2000-2003 were tested for their susceptibility to several antimicrobial agents. Based on these data, the second part of the project aimed at the detection of resistance genes to different antimicrobial agents and focussed especially on their localization on mobile genetic elements and their possibilities to be transferred horizontally.

2. **The respiratory tract pathogen *Bordetella bronchiseptica***

   *B. bronchiseptica* is a Gram-negative bacterium and belongs to the genus *Bordetella* within the family *Alcaligenaceae* which forms together with the family *Ralstoniaeae* the order *Burkholderiales*. In the division *Proteobacteria*, the *Burkholderiales, Neisseriales*, and *Nitrosomonadales* belong to the class of *Betaproteobacteria*.
2.1 The genus *Bordetella*

The genus *Bordetella* comprises nine species (Figure 1), among which *B. parapertussis* consists of two different lineages.\(^6,34,73,90\) The most well-known species are *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, because they are the most important human and animal pathogens within this genus. Isolates of these three species have been completely sequenced, and *B. bronchiseptica* is considered as the common ancestor. The sequenced strain has more than 1,000 additional open reading frames and does not harbour insertion sequences in the genome.\(^29,34,99,110\)

![Figure 1. Phylogenetic relationship of *Bordetella* spp. based on 16S rRNA (modified from Ko\(^73\))](image)

*B. pertussis* is the causative agent of whooping cough. It infects only humans and causes severe respiratory disease with the typical whooping sound mainly in children of less than one year of age.\(^109\) Two populations have been described from *B. parapertussis*, one adapted to humans, the other adapted to sheep.\(^108,145\) Mattoo et. al. even divide *B. parapertussis* in two species: *B. parapertussis*\(_{hu}\) for the human-adapted species and *B. parapertussis*\(_{ov}\) for the...
As an *in vitro* example for the adaptation to the different hosts, these two populations showed different adherence behaviour to epithelial cells from the human or the ovine respiratory tract.\textsuperscript{85} In humans, *B. parapertussis* causes pertussis-like disease with similar respiratory tract symptoms, but milder than those seen in whooping cough. Ovine-adapted isolates have been isolated from sheep with pneumonia and can be distinguished by macrorestriction analysis and by multilocus sequence typing (MLST) from the human-adapted isolates.\textsuperscript{34}

*B. avium* and *B. hinzii* are two species that are commonly isolated from birds.\textsuperscript{50,147} *B. avium* causes respiratory tract infections in birds and has been described to cause rhinotracheitis or oryza in turkeys.\textsuperscript{51,139} *B. hinzii* is a commensal bacterium of fowl.\textsuperscript{90,147} In contrast to *B. avium, B. hinzii* has been isolated rarely as pathogen from humans.\textsuperscript{25,90} In one case it has been isolated from a human patient suffering from septicaemia without exhibiting symptoms of a respiratory disease.\textsuperscript{25}

*B. holmesii* and *B. trematum* have been isolated from the respiratory tract and from wounds of humans, although their pathogenicity remains unknown.\textsuperscript{146} *B. petrii* was isolated from the environment\textsuperscript{151} and the first clinical isolate was identified in a patient with mandibular osteomyelitis in 2005.\textsuperscript{42} The last species that has been described so far is *B. ansorpii*, named after the Asian Network for Surveillance of Resistant Pathogens (ANSORP). *B. ansorpii* was isolated from an epidermal cyst of a female patient receiving cancer therapy.\textsuperscript{73}

### 2.2 The species *Bordetella bronchiseptica*

*B. bronchiseptica* causes respiratory tract infections in a variety of mammals and rarely in birds. Differences in susceptibility to infection have been seen for different mammalian species: pigs, dogs, and guinea pigs are highly susceptible, rats, rabbits, and horses are moderately susceptible, and humans and chickens have a low susceptibility to the infection with *B. bronchiseptica*.\textsuperscript{7} A study about the adherence of *B. bronchiseptica* to cells from the respiratory tract of different species underlines some of these differences in susceptibility: *B.*
bronnchiseptica showed a markedly reduced adherence to human- or chicken-derived epithelial cells in comparison to cells derived from rabbits or hamsters.\textsuperscript{144}

Although \textit{B. bronchiseptica} is considered as a zoonotic agent, \textit{B. bronchiseptica} infections in humans are rarely observed.\textsuperscript{160} Most of the patients showed respiratory disease, such as pneumonia or pertussis-like symptoms.\textsuperscript{102,131,160} In selected cases, \textit{B. bronchiseptica} can also cause systemic diseases, e.g. septicaemia in a 70-year-old man.\textsuperscript{60} Most frequently, \textit{B. bronchiseptica} infections in humans are seen in immunocompromised individuals with increasing numbers of cases in AIDS patients or in elderly people.\textsuperscript{30,84,102,131,160} However, severe infections can also occur in immunocompetent adults.\textsuperscript{53,82} In the majority of those cases, contact to infected animals was reported.\textsuperscript{160}

The clinical disease in animals is commonly associated with respiratory symptoms like sneezing, coughing, muco-purulent oculonasal discharge, and dyspnoe. In dogs and cats, \textit{B. bronchiseptica} is most frequently associated with canine infectious tracheobronchitis, also known as kennel cough,\textsuperscript{70,91,152} and feline infectious upper respiratory tract disease.\textsuperscript{136,155} In commercially reared rabbits \textit{B. bronchiseptica} together with \textit{Pasteurella multocida} may cause an economically important respiratory disease known as snuffles.\textsuperscript{31} Transmission of \textit{B. bronchiseptica} is usually from host to host by direct contact or airborne by droplet infection, but can also be due to contact with infectious material.\textsuperscript{27} Hence, \textit{B. bronchiseptica} infections may preferentially develop under conditions where animals are kept at high density, e.g. in intensive animal production systems or animal shelters.\textsuperscript{11}

In pigs, \textit{B. bronchiseptica} can cause a wide variety of symptoms ranging from mild rhinitis to severe pneumonia.\textsuperscript{15} Moreover, infections with \textit{B. bronchiseptica} may predispose pigs to infections with other respiratory tract pathogens, in particular toxigenic \textit{P. multocida} which then can cause the severe progressive form of atrophic rhinitis.\textsuperscript{36,37,119} This disease is characterized by progressive degenerative changes in nasal turbinate bones, which finally lead - by atrophy of the conchae - to a characteristic lateral deformation of the snout.\textsuperscript{20,87,133} In pigs, respiratory disease is the most important health concern for swine producers in Germany and has been reported to be the leading cause of mortality in nursery and grower-finisher units in 1995 in the USA.\textsuperscript{14,52} Studies conducted in the Northwestern part of Germany revealed that \textit{B. bronchiseptica} is widespread in the pig production and is a frequent cause of rhinitis in piglets.\textsuperscript{123,124} Results of a study on the aetiology of bacterial porcine pneumonia in Germany
recorded pneumonia as the main diagnosis in 24.4% and as the second diagnosis in 14.3% of 6560 necropsy cases.\textsuperscript{1} \textit{B. bronchiseptica} was one of the three most common pathogens and was isolated in 6.0% of these cases with pneumonia.\textsuperscript{1} Results from Austria showed that \textit{B. bronchiseptica} was involved in 27.8% of 854 cases of pneumonia.\textsuperscript{74}

To treat respiratory disease in pigs three possibilities are given: change in management, vaccination and/or antibiotic treatment.\textsuperscript{14} The improvement of the conditions for the animals reduces respiratory disease problems, but cannot eradicate a primary pathogen. Despite the fact that vaccination is used to prevent atrophic rhinitis in pigs with combined vaccines against \textit{B. bronchiseptica} and \textit{P. multocida} - Respiporc\textsuperscript{®} and Porcilis ART\textsuperscript{®} are used in Germany - antimicrobial agents are frequently used to treat pigs with respiratory infections. It was shown, that the clearance rate of \textit{B. bronchiseptica} was low and even with antibiotic treatment complete clearance was not achieved.\textsuperscript{149}

3. Susceptibility testing

The susceptibility of bacteria to antimicrobial agents \textit{in vitro} is commonly determined phenotypically, although genotypical testing is also possible. Several different methods for phenotypical susceptibility testing are available, some of which yield qualitative, others quantitative results.

3.1 Phenotypical susceptibility testing

In principle three methods for phenotypical susceptibility testing of bacteria to antimicrobial agents are performed: disk diffusion, E-test, and dilution systems. The aim of all methods is to classify bacteria into the categories “resistant”, “intermediate” or “susceptible” to the antimicrobial agent used. The result of \textit{in vitro} susceptibility testing is expected to have a prognostic value with regard to the \textit{in vivo} efficacy of the antimicrobial agent(s) applied. Thus, quantitative results - given as the minimum inhibitory concentration (MIC) - are preferred since they indicate how susceptible or how resistant a bacterial pathogen is. The
MIC value describes the lowest concentration of the antimicrobial agent(s) that inhibits visibly growth of bacteria under standardized test conditions.

In disk diffusion systems, a defined bacterial suspension (= inoculum) is spread on a plate containing a defined solidified medium (e.g. Mueller Hinton agar). Commercially available disks, which contain the respective antimicrobial agent(s) in a defined concentration, are placed on this inoculated plate. After incubation for defined times (e.g. 18-20 h) at a suitable temperature (e.g. 35°C), the zones of growth inhibition around each disk are measured (Figure 2); the zone diameter allows a classification into the categories “resistant”, “intermediate” or “susceptible”. With agar diffusion only qualitative results can be obtained. The calculation of a MIC value from the zone diameter via regression analysis is strictly forbidden.

The E-test (Figure 3) is an agar diffusion method that allows determination of MIC values. Instead of disks, commercially available strips that contain a concentration gradient of the antimicrobial agent(s) are placed on the inoculated agar. A scale showing the different concentrations of the antimicrobial agent(s) is shown on these strips. The MIC is set at the next higher concentration at which the elliptical inhibitory zone meets the strip. Figure 3 shows, that the interpretation of results can be difficult: whereas a clear inhibitory zone is seen on the right side of the agar plate, the result of the other three strips is not that clear. This method has been used for screening *B. pertussis* isolates for their susceptibility to erythromycin.48

![Figure 2. Disk diffusion test](image1)

![Figure 3. E-test with four different agents; arrows indicate the MICs.](image2)
Dilution systems also determine the MIC of bacteria to antimicrobial agents. Usually a two-fold dilution series of the antimicrobial agent(s) is used. The testing can be either performed by plating the bacteria on agar plates, which contain different concentrations of antimicrobial agent(s) (agar dilution), or as broth dilution, where the antibiotic is added to liquid medium. MIC determination in liquid medium can be performed in tubes (broth macrodilution, Figure 4) or with microtitre plates (broth microdilution, Figure 5).

![Figure 4. Broth macrodilution](image1)

![Figure 5. Broth microdilution; growth can be seen as white plug, e.g. in well H12](image2)

For all test systems several aspects are important to achieve reliable in vitro results. These aspects include (i) the correct choice of the antibiotics to be tested, (ii) the lege artis performance of in vitro susceptibility testing, and (iii) interpretation of results and the application of breakpoints to classify the isolate tested as susceptible or resistant. To reduce the number of antibiotics to be tested, representatives of classes of antimicrobial agents or indicator drugs can be used. As an example, tetracycline is used for susceptibility testing as a class representative of tetracyclines and the qualitative results obtained for tetracycline are also regarded as being valid for chlortetracycline, oxytetracycline, and doxycycline. Known resistance mechanisms have to be taken into account for the selection of the antimicrobial agents to be tested. Different media, growth conditions, and inoculum densities can lead to different results, e.g. inoculum effects have been described in susceptibility testing of Enterobacteriaceae to cephalosporins. Finally, breakpoints determined for one group of bacteria (e.g. specific bacterial species, genera or families as
indicated in the CLSI documents M31-A2 and M31-S1)\(^{22,23}\) cannot be applied to other bacteria, and breakpoints determined in one test system cannot be used for interpretation in a different test system.\(^{22,33,86,127}\)

Guidelines have been developed to achieve reproducible results. The aim of these guidelines is to have standard conditions and to achieve identical results in different laboratories. Information is provided for the testing conditions concerning the medium, the inoculum density, and the growth conditions. The guidelines also provide information on the interpretation of the results. To guarantee correct results, control strains of different bacterial species are available for quality assurance.\(^{22,71,118}\) These control strains and their acceptable ranges of results are given in the guidelines. Comparisons of different methods have shown, that the results obtained with the different test systems can correspond to each other,\(^{56,137}\) although a comparison is difficult due the different conditions used in the systems. Even in the comparison of different methods in one laboratory, errors that influence the interpretation of results are observed.\(^{111,118,127}\) For the testing of bacteria originating from animals guidelines from the Clinical and Laboratory Standards Institute (CLSI) in the USA and the calibrated dichotomous sensitivity (CDS) disk diffusion method, used in Australia, are the only available standards.\(^{153}\)

Although some studies on susceptibility of \textit{B. bronchiseptica} to antimicrobial agents have been published, in most of them the number of isolates was low (ranging from 10 to 50 isolates) and/or different testing methods were used, e.g. agar dilution and E-test in a study with 152 isolates.\(^{137,160}\) The guideline from the CLSI used throughout this project gives only breakpoints for florfenicol for \textit{B. bronchiseptica}.\(^{22,23}\)

### 3.2 Genotypical susceptibility testing

Genotypical methods aim at the detection of specific resistance genes. For this, the bacterial pathogen causing the infection has to be identified and resistance genes for the antimicrobial agents which are available for treatment have to be detected. Rapid methods, such as PCR analysis, which yield results within a few hours, are used.\(^{10,26}\)
However, the genotypical techniques also bear some problems and thus, do not give satisfactory results in a lot of cases. Mutations in genes of the bacterial genome leading to resistance cannot be detected directly, e.g. an upregulation of \textit{ampC}, which is located on the chromosome of \textit{Enterobacteriaceae} can lead to resistance to several \textit{β}-lactam antibiotics and can be caused by different mutations in the regulator gene \textit{ampR}.$^{26,159}$ Another example is the occurrence of fluoroquinolone-resistance mediating mutations in the genes for DNA gyrase (\textit{gyrA}, \textit{gyrB}) and topoisomerase IV (\textit{parC}, \textit{parE}). To detect mutations in such genes, further approaches, e.g. sequence analysis, are necessary. All genotypical tests can only detect known resistance genes since gene sequences deposited in the databases are a pre-requisite to generate specific primers for PCR analysis. In turn, this means that genes, for which no nucleotide sequences are available, cannot be detected by PCR approaches. Moreover, even if a resistance gene is detected by PCR, this does not necessarily mean that the gene is functionally active and confers resistance in the causative pathogen.$^{26,157}$ There are several examples in which point mutations within a resistance gene result in its functional inactivity without interfering with its detection by PCR.$^{78,93}$ On the other hand, genotypic resistance testing can be the method of choice, if the aim is to detect a specific pathogen, e.g. in an outbreak situation, in combination with a specific resistance gene, e.g. the detection of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) carrying the gene \textit{mecA}$^{148}$

4. \textbf{Selected antimicrobial agents}

For the treatment and control of respiratory tract infections some antimicrobial agents are used more commonly than others. For the treatment of bacterial infections in pigs trimethoprim and sulphonamides, tetracyclines, and \textit{β}-lactams are used most frequently. Other agents are approved explicitly for respiratory tract infections. In Germany florfenicol (Nuflor®) and ceftiofur (Excenel®) are licensed for the treatment of respiratory tract infections in pigs and tilmicosin (Pulmotil®) is licensed for treatment of pneumonia in piglets and fattening pigs.
4.1 Trimethoprim and sulphonamides

Trimethoprim (Figure 6) is a synthetic broad spectrum antimicrobial agent and interferes with folate synthesis in Gram-negative and Gram-positive bacteria. It acts bacteriostatic by a competitive and strong binding to the dihydrofolate reductase (DHFR) (Figure 8). Although DHFRs from eukaryotic cells can also bind trimethoprim, the affinity of the drug to the bacterial enzymes is higher.\textsuperscript{54,55}

![Figure 6. The chemical structure of trimethoprim](image)

Sulphonamides are also synthetic substances and a large number of different sulphonamides has been already synthesized, most of which differ in their molecule structure and their kinetic properties (Figure 7). Sulphonamides also inhibit the folate synthesis pathway and act bacteriostatically. The enzyme dihydropteroate synthase uses sulphonamides as a substrate competitively to p-aminobenzoic acid (Figure 8).

![Figure 7. The chemical structure of a) sulphonamides in general and b) sulfamethoxazole](image)

If both agents are used together, their mode of action is bactericidal and this synergistic effect is the reason why most of the preparations on the market are a combination of trimethoprim and sulphonamides, the so-called potentiated sulphonamides. Potentiated sulphonamides are most commonly used for the treatment of urinary tract or respiratory tract...
infections in animals, but they are also used in human medicine. The combination trimethoprim/sulfamethoxazole is recommended by the WHO for the treatment of *Pneumocystis carinii* infections in HIV infected patients.

The most common resistance mechanism to trimethoprim is the expression of a trimethoprim-resistant DHFR.\textsuperscript{54,55,134} This DHFR is expressed additionally to the original enzyme and the gene coding for this additional enzyme is very often located on mobile genetic elements, like plasmids, transposons or gene cassettes.\textsuperscript{54,55,134} To date, over 25 different DHFRs conferring trimethoprim resistance are known.\textsuperscript{64} A second trimethoprim resistance mechanism is to use alternative folate pathways either by usage of external supply of thymidine or by the use other thymidylate synthases than DHFR.\textsuperscript{55,94} The third possibility are mutational changes in the DHFR. These mutations result in a decreased binding of trimethoprim to the DHFR or can lead to an overproduction of a trimethoprim-sensitive DHFR.\textsuperscript{39,54,55,134}
Similar mechanisms have been described for sulphonamide resistance, whereas only three genes (sul1, sul2, sul3) are currently known to code for sulphonamide-resistant dihydropteroate synthases. The gene sul1 was described as part of the 5’ region of class 1 integrons.

Respiratory tract pathogens, such as *Haemophilus influenzae*, *Streptococcus pneumoniae* or *Moraxella catarrhalis*, carry chromosomally located genes for trimethoprim or sulphonamide resistance; in a bovine *P. multocida* isolate a plasmid-borne gene, dfrA20, coding for a new DHFR has been described recently.

### 4.2 Tetracycline

Since the discovery of chlortetracycline produced by *Streptomyces aureofaciens* in 1945 several tetracyclines have been isolated from the natural producers or have been chemically synthesized (Figure 9).

![Figure 9. The chemical structure of tetracyclines](image)

Tetracyclines have a broad spectrum activity and were the most frequently used antibiotics in veterinary medicine in the EU and Switzerland in 1997. Tetracyclines used for therapy have a bacteriostatic effect by binding reversibly to the ribosome; thereby they inhibit bacterial protein synthesis.

The most common resistance mechanism of Gram-negative bacteria is the efflux of tetracyclines. All genes conferring tetracycline resistance have been named *tet* genes followed by a letter from the alphabet or a number, e.g. *tet(A)* or *tet(34)*, so far 38 different genes are known. Different classes of tetracycline specific exporters have been identified. According to their phylogenetic classification, many different groups of efflux proteins have
been defined, one of them contains the efflux proteins commonly found in Gram-negative bacteria\(^{12,21,79,121,122}\). In Gram-negative bacteria, a repressor gene (*tetR*) is commonly associated with the efflux gene. The TetR protein blocks the expression of the *tet* gene in the absence of tetracycline. In the presence of tetracycline, a tetracycline-Mg\(^{2+}\) complex binds to the TetR protein. Under these conditions TetR cannot bind to the *tet* gene associated promoter and allows the expression of the *tet* gene. Thus, *tetR* leads to an inducible expression of the *tet* resistance gene.\(^{49,122}\) Tet(B) is the most widespread efflux protein and confers - in contrast to other tetracycline efflux proteins - also resistance to minocycline.\(^{21}\) The second tetracycline resistance mechanism is the protection of the ribosomal target structure. The protection encoded by *tet* genes, e.g. *tet*(M) and *tet*(O), is not yet completely understood. Current data suggest, that the deduced proteins are elongation factors utilizing energy from GTP hydrolysis, which release the tetracycline bound to the ribosome and enable the ribosome to go back to the conformational state. Once back in the normal conformation, the protein synthesis can proceed.\(^{114}\) A third mechanism, the enzymatic inactivation of tetracyclines, has also been described. Three genes have been identified so far, *tet*(X), *tet*(34), and *tet*(37).\(^{21,115,121}\)

Furthermore, alterations in membrane permeability can contribute to tetracycline resistance. Mutations in the 16S rRNA were identified to confer tetracycline resistance by disturbing the binding of tetracycline to the ribosome in *Propionibacterium acnes* and in *Helicobacter pylori*.\(^{116}\) One gene, *tet*(U), confers tetracycline resistance by a so far unknown mechanism.\(^{21,116,122}\)

Investigation of bacteria isolated prior to the use of tetracyclines suggest that resistance is often selected by the use of this drug.\(^{116}\) Most of the *tet* genes are located on mobile genetic elements.\(^{21}\) In Gram-negative bacteria, they are very often located on large conjugative plasmids, which also harbour other resistance genes.\(^{21}\) Transposons, carrying tetracycline resistance genes, have been described in many bacteria, for example in *Enterobacteriaceae* where Tn10 harbouring *tet*(B) and Tn1721 carrying *tet*(A) were identified.\(^{21}\)

In isolates from the respiratory tract from pigs, *tet*(B) has been detected in *Haemophilus parasuis*, *tet*(A), *tet*(B), *tet*(H), *tet*(L) and *tet*(O) in *Actinobacillus pleuropneumoniae*;\(^{76}\) and *tet*(B), and *tet*(H) have been detected in *P. multocida*.\(^{68,69}\) In porcine *B. bronchiseptica* tetracycline resistant isolates have been reported, but genes were not identified.\(^{97,104}\) In feline *B. bronchiseptica* isolates, the gene *tet*(C) was identified on two 51-kb conjugative plasmids.
These plasmids conferred also resistance to sulphadiazine, streptomycin, ampicillin, and mercuric chloride.\textsuperscript{138}

4.3 Phenicol

While chloramphenicol is not licensed for food-producing animals anymore, the fluorinated chloramphenicol derivative florfenicol (Figure 10) is licensed for the treatment of respiratory tract infections in pigs due to \textit{A. pleuropneumoniae} and \textit{P. multocida} since late 2000. Azidamfenicol and thiamphenicol are other phenicols, which are only approved for human medicine.\textsuperscript{126} Chloramphenicol has been banned from use in food-producing animals in the European Union in 1994 because of the occurrence of a dose-unrelated aplastic anaemia in patients. However, it is still approved and used in pets. As a last choice agent, it is also used for the treatment of life threatening infections in humans.\textsuperscript{125}

\begin{center}
\begin{tabular}{ l l l }
\hline
                  & \chem{\text{R}_1} & \chem{\text{R}_2} & \chem{\text{R}_3} \\
\hline
Chloramphenicol  & \text{–NO}_2      & \text{–OH}       & \text{=Cl}_2  \\
Azidamfenicol    & \text{–NO}_2      & \text{–OH}       & \text{N=N=N} \\
Thiamphenicol    & \text{–SO}_2\text{CH}_3 & \text{–OH} & \text{=Cl}_2 \\
Florfenicol      & \text{–SO}_2\text{CH}_3 & \text{–F}       & \text{=Cl}_2  \\
\hline
\end{tabular}
\end{center}

\textbf{Figure 10.} The chemical structure of phenicols\textsuperscript{126}
Phenicols bind reversibly to the 50S subunit of the bacterial ribosome and thereby inhibit bacterial protein synthesis. Chloramphenicol and florfenicol show broad spectrum activity and act bacteriostatically on Gram-negative and Gram-positive bacteria.

Mechanisms conferring florfenicol resistance described so far, confer resistance to both phenicols. In contrast, resistance genes are known, which confer only chloramphenicol, but not florfenicol resistance. The most common resistance mechanism to chloramphenicol in Gram-negative bacteria is the expression of a chloramphenicol acetyltransferase (CAT), which inactivates chloramphenicol, but not florfenicol.\(^\text{40,125,126}\) The CATs can be distinguished into two major groups: type A and type B CATs. Based on sequence variations the type A CATs can be subdivided into at least 16 groups based on their phylogeny. The proteins of each phylogenetic group share > 80% sequence identity. In total, more than 40 type A CATs have been described so far.\(^\text{125}\) Type B CATs are structurally different to type A CATs and based on their phylogeny five groups can be distinguished.\(^\text{125}\) In Gram-negative bacteria the expression of CATs is constitutive.\(^\text{125}\)

The second resistance mechanism is the active efflux of chloramphenicol. The chloramphenicol-specific exporter CmlA has been identified so far in \textit{E. coli}, \textit{Salmonella enterica}, \textit{Klebsiella pneumoniae}, \textit{Pseudomonas aeruginosa}, and \textit{Enterobacter aerogenes}. The amino acid sequences of the so far known CmlA proteins are very similar. Solely, CmlA2 from \textit{Enterobacter} has 83% identity to the other CmlA proteins, which share 97-100% identity.\(^\text{18,125}\) In \textit{Streptomyces} spp., \textit{Rhodococcus} spp., and \textit{Corynebacterium} spp., different chloramphenicol transporters have been identified. Multidrug transporters in Gram-negative bacteria have been described to export chloramphenicol, e.g. AcrAB-TolC from \textit{E. coli} and MexAB-OprM from \textit{P. aeruginosa}.\(^\text{125}\)

The other group of phenicol exporters, occasionally described as CmlA-like proteins, export chloramphenicol as well as florfenicol, and their resistance genes have been named \textit{flo} or \textit{floR}.\(^\text{18,125}\) The FloR proteins share 89-100% identity and are about 50% homologous to CmlA.\(^\text{18,125}\) The \textit{floR} genes have been identified so far only in Gram-negative bacteria: \textit{S. enterica}, \textit{E. coli}, \textit{K. pneumoniae}, \textit{Vibrio cholerae}, \textit{P. multocida}, \textit{Pasteurella trehalosi}, \textit{Pasteurella piscicida}, \textit{Stenotrophomonas maltophilia}, \textit{Photobacterium damselae}, and \textit{Acinetobacter baumanii}.\(^\text{125}\)
In Gram-positive bacteria, only one gene, *fexA*, coding for a chloramphenicol and florfenicol transporter, has been identified in *Staphylococcus lentus*.\textsuperscript{18,67,125} Furthermore, a different mechanism has been identified for a novel pentaresistance phenotype, which also includes combined resistance to chloramphenicol and florfenicol: The gene *cfr*, coding for a rRNA methyltransferase, modifies the ribosome at the drug binding site and thereby confers resistance to these phenicols as well as to other antimicrobial agents binding in a similar region at the ribosome, such as lincosamides, pleuromutilins, oxazolidinones, and streptogramin A antibiotics.\textsuperscript{65,83} The genes *cfr* and *fexA* coding for combined chloramphenicol and florfenicol resistance have been detected only in staphylococci until now.\textsuperscript{62}

The majority of the genes conferring resistance to chloramphenicol or to chloramphenicol and florfenicol have been detected on mobile genetic elements. Most of the genes are located on plasmids and some are part of transposons, e.g. *catA1* is localized on Tn9 and has been identified on multi-resistance plasmids of different Gram-negative bacteria.\textsuperscript{125} Genes coding for type B CATs and *cmlA* have been detected on gene cassettes.\textsuperscript{18,125} In contrast to other gene cassettes, the *cmlA* cassette includes its own promoter and its expression is regulated by translational attenuation.\textsuperscript{125} The gene *floR* has been described to be located on the chromosome or on plasmids and a functionally active transposon Tn*floR* has been identified in *E. coli*.\textsuperscript{35,125}

In Gram-negative respiratory tract pathogens, the following phenicol resistance genes have been identified so far: *catA2* in *H. influenzae* and *K. pneumoniae*, *catA3* in *P. trehalosi, Mannheimia* spp., and *K. pneumoniae*, *catB2* in *P. multocida, catB3* in *K. pneumoniae, floR* in *Pasteurella* spp. and *K. pneumoniae*, as well as *cmlA4* and *cmlA7* in *K. pneumoniae*.\textsuperscript{24,61,106,125,150} According to the CLSI-approved breakpoints, porcine *B. bronchiseptica* isolates have been classified as florfenicol-resistant, but the genetic basis has not been identified.\textsuperscript{66,111}
4.4 β-Lactams

Agents from the class of β-lactam antibiotics are widely used in human and veterinary medicine. β-Lactam antibiotics are subdivided into four groups: penicillins, cephalosporins, monobactams, and carbapenems. Only penicillins and cephalosporins (Figure 11) are licensed for the treatment of animals, of which penicillins are often used in combination with a β-lactamase inhibitor like clavulanic acid. Both groups have a wide spectrum of activity and act bactericidal. They inhibit the bacterial cell wall synthesis by binding to the penicillin-binding proteins (PBP). The PBPs are proteins - mainly transpeptidases and carboxypeptidases, also called murein synthases - that are involved in the transpeptidation of peptidoglycans and essential for the formation of the bacterial cell wall.\textsuperscript{107}

![Chemical structure of a) ampicillin and b) cephalothin](image)

Figure 11. Chemical structure of a) ampicillin and b) cephalothin

The most common resistance mechanism in Gram-negative bacteria is the expression of a β-lactamase. This enzyme deactivates β-lactams by hydrolysing the β-lactam ring.\textsuperscript{57} Hundreds of different β-lactamases have been described so far, a continuously updated list is available at http://www.lahey.org/studies/webt.asp. The major structural difference in these enzymes is that they can have either a metal ion or a serine residue at the active site. β-lactamases can either hydrolyse specific β-lactam antibiotics, or can have a broad spectrum of activity (extended spectrum β-lactamases, ESBLs). The difference of single point mutations can be sufficient for a change in the substrate spectrum. β-Lactamases resistant to the β-lactam inhibitors (= inhibitor resistant β-lactamases, IRBLs) have also been detected.\textsuperscript{13} The enzymes have been named arbitrarily, e.g. by their activity spectrum, by the name of the bacterial host or even by the name of the patient from whom the resistant bacterium was
isolated.\textsuperscript{57,159} Different classifications schemes have been suggested, and the classification from Bush, Jacoby and Medeiros (BJM),\textsuperscript{17} which divides the β-lactamases into four major groups, is now commonly used.\textsuperscript{16,17,57}

BJM group one consists of chromosomally encoded species-specific β-lactamases from Gram-negative bacteria e.g. AmpC from \textit{Enterobacteriaceae}. These enzymes have been shown to confer resistance when overexpressed and hydrolyse penicillins and cephalosporins. In some species the expression of AmpC is inducible.\textsuperscript{159} Plasmid-encoded resistance genes have been described which are derived from these species-specific enzymes, e.g. \textit{bla}\textsubscript{CMY-2} from the AmpC of \textit{Citrobacter freundii}.\textsuperscript{16,17,57} Group two comprises the β-lactamases which have a serine residue as active site. This group is divided into subgroups 2a - 2f and contains penicillinases from Gram-positive bacteria as well as the most common β-lactamases from Gram-negative bacteria, namely TEM-, SHV-, PSE-, and OXA-type β-lactamases.\textsuperscript{16,17,57} Genes coding for these β-lactamases can be located on the chromosome or on mobile genetic elements, such as transposons or on gene cassettes.\textsuperscript{154} The transposon Tn3, for example, carries the ampicillin resistance gene \textit{bla}\textsubscript{TEM-1}.\textsuperscript{100} Group three includes the metallo-β-lactamases, such as VIM-1 and IMP-1. In contrast to other β-lactamases, members of this group are inhibited by EDTA and their activity is not influenced by clavulanic acid or tazobactam.\textsuperscript{16,17,57} While enzymes of this group encoded by chromosomally located genes have been detected in species of minor clinical relevance, such as \textit{Aeromonas hydrophila} or \textit{Bacillus cereus}, genes from pathogens with clinical importance have been detected on large conjugative plasmids or were located on gene cassettes integrated in integrons.\textsuperscript{16,17,57} Group four comprises enzymes, that do not fit into the other groups and/or are not characterized sufficiently to be classified, e.g. SAR-2 from \textit{E. coli}.\textsuperscript{16,17,57}

Other resistance mechanisms are mutations in the genes for the target enzymes, the PBPs, or the reduced intracellular concentration of β-lactams. Mutations of in PBP genes have been described in Gram-negative bacteria, such as \textit{H. influenzae}, \textit{Enterococcus} spp., and \textit{P. aeruginosa}.\textsuperscript{107} However, this resistance mechanism is more important in Gram-positive bacteria. In \textit{S. pneumoniae}, resistance determining β-lactamases are not so important, but several mutations in genes oncoding PBPs have been detected, some of them conferring β-lactam resistance. In methicillin resistant \textit{S. aureus} (MRSA) PBP 2a confers resistance and is encoded by the \textit{mecA} gene, which is located on different types of the staphylococcal cassette
chromosome mec (SSCmec).\textsuperscript{107} Because of their structure of the cell wall, Gram-negative bacteria show in general a lower permeability for β-lactams than Gram-positive organisms, resulting in a lower intracellular concentration.\textsuperscript{96} In addition, the majority of the produced β-lactamases - many Gram-negative bacteria express a species-specific β-lactamase - is released into the periplasmatic space and not directly into the environment. In the Gram-negative bacterial cell, the low permeability and the hydrolysis work together, resulting in a steady state level of diffusion and subsequent hydrolysis in the periplasmatic space of the fraction of β-lactams, that crosses the outer membrane.\textsuperscript{96} The most important uptake mechanism for β-lactams is the diffusion via porins. In agreement to this hypothesis, porin-deficient mutants of \textit{Enterobacteriaceae} have been shown to be resistant to penicillins and cephalosporins by a reduced uptake of the agents.\textsuperscript{96} In several Gram-negative bacteria, porin deficiency has been shown to contribute to β-lactam resistance, e.g. in \textit{P. aeruginosa}, \textit{K. pneumoniae} or \textit{Enterobacter} spp.\textsuperscript{107} The lower intracellular drug concentration cannot only be achieved by reduced influx, but also by an increased efflux of β-lactams. Active efflux by efflux pumps of the resistance-nodulation-division (RND) family is supposed to play a role in β-lactam resistance in \textit{Enterobacteriaceae} and has been shown to contribute to carbapenem resistance in \textit{P. aeruginosa}, \textit{H. influenzae},\textsuperscript{59,159} and \textit{Bacteroides fragilis}.\textsuperscript{72,96,98,112}

The spread of bacteria carrying β-lactamase genes from animals to humans - directly or via the food chain - has been suggested, e.g. for \textit{S. Typhimurium} carrying \textit{bla}\textsubscript{OXA}}\textsuperscript{3} or for \textit{Haemophilus} with \textit{bla}\textsubscript{ROB-1}.\textsuperscript{38,58,92} In respiratory tract pathogens from pigs, a TEM-type β-lactamase and a ROB-1 enzyme have been detected so far in \textit{Pasteurella} spp.\textsuperscript{103,130} and in \textit{A. pleuropneumoniae}.\textsuperscript{38,58} \textit{B. bronchiseptica} isolates have shown a low susceptibility to different β-lactams.\textsuperscript{4,5} Plasmid-borne ampicillin resistance could be transferred from \textit{B. bronchiseptica} to \textit{E. coli}.\textsuperscript{43,132,138,141,162} β-Lactamases were detected in porcine and feline isolates.\textsuperscript{138,162} In these two studies, the activity to different β-lactams was determined and activity profiles of the respective enzyme showed, that oxacillin was hydrolysed better than penicillin.\textsuperscript{138,162} In 2005, investigations on the species-specific β-lactamase from \textit{B. bronchiseptica} and the nucleotide sequence of its gene, \textit{bla}\textsubscript{BOR-1}, were published. The β-lactamase BOR-1 conferred amoxycillin resistance to \textit{E. coli} (MIC 512 mg/L). The \textit{B. bronchiseptica} isolate showed MICs of 8 mg/L for amoxycillin.\textsuperscript{77}
5. Horizontal gene transfer of resistance genes

Several types of mobile genetic elements have been described to date, which play an important role in acquisition, maintenance, and spread of antimicrobial resistance genes. In this regard, plasmids, transposons, and gene cassettes are the most important elements. Mobile genetic elements can be disseminated horizontally among bacteria of the same species, but also among those of different species or even different genera. Bacteria of different species and different genera are also able to take up free DNA under special environmental conditions. This natural transformation has been described for pathogenic genera, such as Haemophilus, Campylobacter or Pseudomonas. Transfer of DNA between different host cells can also occur by transduction via bacteriophages, which act as vehicles. Under natural conditions, conjugation is a frequently used transfer mechanism for plasmids and transposons. Conjugation is a process, in which a cell to cell junction is established and a pore is formed, through which DNA can pass from a donor cell into a recipient cell.

5.1 Plasmids

Plasmids are circular double-stranded DNA molecules, that can replicate independently from the host cell. Broad host range plasmid, like RSF1010 or RK2, are able to replicate in several bacterial hosts. Besides transformation and transduction, conjugation is a common mechanism for horizontal transfer of plasmids. Genes required for conjugation are clustered in a so-called tra gene complex of ≥ 15 kb in size. Thus, conjugative plasmids should be at least 20 kb large. Smaller plasmids can be mobilized during conjugative transfer.

In B. bronchiseptica resistance genes were located on large, conjugative plasmids. Transfer into E. coli recipients revealed that these plasmids conferred resistance to sulphonamides, streptomycin, and penicillin. Non-conjugative plasmids conferring resistance to sulphonamides, streptomycin, and ampicillin have been also described in porcine B. bronchiseptica isolates. Smaller plasmids were rarely detected, like pBBR1 with a size of 2.6 kb, and did not carry resistance genes. Only a few B. bronchiseptica plasmids have been further characterised and/or sequenced. Plasmid pBBR1 was sequenced and combines mobilisation genes common in Gram-positive bacteria with replication genes from Gram-
negative bacteria.² In other respiratory tract pathogens mainly smaller plasmids of <10 kb have been described. The size of plasmids carrying resistance genes from *Pasteurellaceae* usually ranged from 2 to 8 kb.⁶⁹

### 5.2 Transposons

Whereas plasmids have their own replication systems,³² transposons do not possess replication genes and therefore have to integrate into chromosomal or plasmid DNA to be replicated. Based on their structure, composite, and complex transposons are differentiated. Composite transposons have derived from a structure with long terminal direct or inverted repeats, originating from insertion sequences (IS). These IS elements were the first transposable elements identified.⁹ They consist of a transposase gene and terminal inverted repeats of variable length.⁸⁸ In composite transposons, they can still function as independent elements, but after fusion processes they can lose this ability.⁹ Examples for composite transposons are Tn⁵¹¹³ with resistance genes to aminoglycosides or Tn⁷²⁸ with resistance genes to trimethoprim and aminoglycosides. Complex transposons usually have short inverted repeats of 15 to 40 bp and an internal repeat, which separates the part responsible for resistance functions from the part responsible for transposition functions. An example for this type of transposon is Tn³ conferring resistance to ampicillin.⁴⁴,⁴⁷ Tn/721 conferring tetracycline resistance, belongs also to this type of transposons. The class of conjugative transposons also belongs to these complex transposable elements. Conjugative transposons form a circular intermediate and can promote their transfer from one cell to another.⁸ Integration of transposons can occur in many sites of the bacterial chromosome or of plasmids, but for some of them site-specific insertion has been described. Tn7 inserts in *E. coli* near the gene *glmS*.⁸,²⁸ During insertion many transposable elements produce a target site duplication, such as Tn5 which is flanked by a 5-bp repeat after integration.

Whereas no transposons carrying resistance genes have been described in *Bordetella*, bacteria of the family *Pasteurellaceae* have been shown to carry transposons conferring tetracycline resistance. Complete and truncated copies of Tn10 carrying *tet*(B) have been detected in porcine *P. multocida* isolates.⁵⁸ The *tet*(H)-carrying transposon Tn5706 has been so far identified only in *Pasteurellaceae*.⁶⁹
5.3 Gene cassettes and integrons

Among the newer mobile genetic elements, gene cassettes are of major importance. Most gene cassettes consist of an antimicrobial resistance gene and the 59-base-element carrying the attachment site (attC). This attachment site is needed for the integration into a specific site of an integron (e.g. attI1 in class 1 integrons). Integrons can integrate or excise gene cassettes site-specifically. Integrons have a strong promoter (Pc), which transcribes the genes from all inserted gene cassettes. Due to these abilities, integrons have been described as “gene capture systems” or as “natural cloning and expression vectors”. The conserved structure of integrons differs and results in a classification of these elements. In class 1 integrons, which are the most common ones, the integrase gene intI1 is located in the 5’-conserved segment (CS). The 3’-CS consists of a truncated resistance gene to quaternary ammonium compounds qacEA1 and the sulphonamide resistance gene sul1 (Figure 12).

![Diagram](image)

**Figure 12.** Schematic presentation class 1 integrons; a) class 1 integron without a gene cassettes; b) the integration of a second resistance gene cassette is shown – more detailed explanation is given in the text (modified from Carattoli).
Although only gene cassettes - but not integrons - can move on their own, integrons are often located on mobile genetic elements like transposons or plasmids. The transposon Tn21 carries a class 1 integron with a single gene cassette, the aadA1 cassette coding for streptomycin and spectinomycin resistance.\textsuperscript{80} The transposon Tn2603 is a derivative of Tn21 and its integron harbours also a bla\textsubscript{OXA-1} cassette. Class 2 integrons are commonly found on the transposon Tn7.\textsuperscript{28}

In \textit{B. bronchiseptica} integrons and associated gene cassettes had not been described so far. A plasmid pJR2 was sequenced from an avian \textit{P. multocida} isolate.\textsuperscript{61} This plasmid carried a truncated integrase gene \textit{intI1} and two gene cassettes, one of which with the resistance gene aadA1 and the other one with a β-lactamase gene. Genes from the 3’-CS were absent. Besides this report, no integrons have been identified in \textit{Pasteurellaceae} so far.\textsuperscript{63} Integrons have been described in various bacterial isolates from the normal intestinal flora from pigs,\textsuperscript{140} but not from porcine respiratory tract pathogens.
6. **Aims of the present study**

This study will give an overview on the susceptibility situation of porcine *B. bronchiseptica* isolates in Germany [chapter 2] and will provide details on the occurrence and the localization of selected resistance genes in *B. bronchiseptica* isolates [chapters 3-6].

Based on the data from the susceptibility testing, isolates with high MICs to trimethoprim/sulfamethoxazole were investigated for the corresponding resistance genes. As trimethoprim resistance genes are often located on gene cassettes and the sulphonamide resistance gene *sul1* have been described to be part of class 1 integrons this part of the study focussed on the occurrence of integrons conferring resistance to trimethoprim and sulphonamides [chapter 3].

Two isolates carrying different plasmids were chosen to identify tetracycline resistance genes on mobile genetic elements [chapter 4].

Florfenicol is the only antimicrobial agent for which the CLSI gives veterinary-specific breakpoints to classify *B. bronchiseptica* isolates as susceptible, intermediate or resistant. Resistant isolates were also investigated for chloramphenicol resistance genes, because all so far known florfenicol resistance genes also confer chloramphenicol resistance. In addition, chloramphenicol-resistant, but florfenicol-susceptible isolates were tested for the presence of chloramphenicol resistance genes [chapter 5].

Transferable β-lactam resistance has been described earlier in *B. bronchiseptica*, so the aim of this part of the study was to detect genes conferring resistance to β-lactams. For this purpose isolates with high MIC values to ampicillin were chosen [chapter 6].
Introduction

References


Chapter 2

Antimicrobial susceptibility of
*Bordetella bronchiseptica* isolates from porcine respiratory tract infections

Kristina Kadlec, Corinna Kehrenberg, Jürgen Wallmann
and Stefan Schwarz

The extent of contribution from Kristina Kadlec to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: B
MICs of 349 *Bordetella bronchiseptica* isolates from respiratory tract infections of swine were determined by broth microdilution. The lowest MIC at which 90% of isolates tested are inhibited (MIC$_{90}$) was that of tetracycline and enrofloxacin (0.5 µg/ml), whereas the highest MIC$_{90}$s were those of tilmicosin and cephalothin (32 µg/ml) as well as streptomycin (256 µg/ml).

Porcine respiratory diseases represent the leading cause of mortality in nursery and finishing units (12). *Bordetella bronchiseptica* is often involved in porcine respiratory tract infections along with viruses and other bacteria (1). It has been shown that infections with *B. bronchiseptica* predispose pigs to secondary infections with toxigenic strains of *Pasteurella multocida* and thus play an important role in the pathogenesis of severe atrophic rhinitis (1, 8). Various antimicrobial agents are licensed and used for the control of bacteria involved in porcine respiratory diseases and atrophic rhinitis, including aminopenicillins, cephalosporins, aminoglycosides, tetracyclines, macrolides, lincosamides alone or in combination with spectinomycin, potentiated sulfonamides, fluoroquinolones, pleuromutilins, and florfenicol. In contrast to well-studied pathogens such as *P. multocida* (for a review see reference 5), comparatively little is known about the antimicrobial susceptibility of porcine *B. bronchiseptica* isolates (3, 5, 6, 9-11, 14, 16, 20).

Between 2000 and 2003, 349 *B. bronchiseptica* isolates were collected from cases of bronchopneumonia and/or atrophic rhinitis of swine in Germany. This study includes 78 isolates from 2000, 98 from 2001, 91 from 2002, and 82 from 2003. All isolates were collected from diseased animals on the basis of one isolate per herd. The animals had not been treated with antimicrobial agents in the 3 weeks prior to sample collection. Samples included nasal swabs sent to the diagnostic labs by veterinarians and lung tissue obtained during postmortem inspections at diagnostic laboratories. Microbiological sample processing and biochemical confirmation of species assignment followed standard procedures (7). All bacterial isolates were investigated for their in vitro susceptibility to antimicrobial agents by the microdilution broth method with microtiter plates (Sensititre, Westlake, Ohio) that
contained the antimicrobial agents in serial twofold dilutions. The layouts of the microtiter plates corresponded to those used in the German resistance monitoring program for veterinary pathogens (GERM-VET). The antimicrobial agents and concentrations tested are shown in Table 1. Performance and evaluation of the susceptibility tests followed the recommendations given in document M31-A2 of the National Committee for Clinical Laboratory Standards (13). Specifically, an inoculum that corresponded to a 0.5 McFarland standard was prepared in cation-supplemented Mueller-Hinton broth and then further diluted to yield a final concentration of $10^5$ CFU/ml. After incubation for 16 to 20 h at 35°C the wells of the microtiter plates were inspected macroscopically for growth. The reference strain *Escherichia coli* ATCC 25922 served for quality control purposes (13).

The distribution of the MICs of the *B. bronchiseptica* isolates tested in this study is shown in Table 1. A year-by-year comparison of the data obtained for each antimicrobial agent revealed virtually no variations in the MICs at which 50 and 90% of isolates tested are inhibited (MIC$_{50s}$ and MIC$_{90s}$, respectively) over the 4-year period. The maximum difference seen was two dilution steps in the MIC$_{50s}$ of cephalothin and trimethoprim and in the MIC$_{90s}$ of trimethoprim/sulfamethoxazole.

Using National Committee for Clinical Laboratory Standards-approved *B. bronchiseptica*-specific breakpoints for florfenicol (susceptible, $\leq 2$ µg/ml; intermediate, 4 µg/ml; resistant, $\geq 8$ µg/ml), 10 (2.9%) isolates were classified as resistant and another 61 (17.5%) as intermediate. This confirms the results of two florfenicol-specific monitoring studies conducted in Germany in 2000/2001 (16) and 2002/2003 (4). The MICs for chloramphenicol for all florfenicol-resistant strains were also high ($\geq 128$ µg/ml). A comparison of the MICs of ampicillin and amoxicillin/clavulanic acid suggested that the presumable β-lactamases which may account for the high MICs of ampicillin are susceptible to inhibition by clavulanic acid. Different distributions of MICs were recorded for the three aminoglycoside antibiotics gentamicin, neomycin and streptomycin. While the MICs of streptomycin for 336 (96.3%) of the isolates were $\geq 64$ µg/ml, those of gentamicin ranged between 0.25 and 4 µg/ml, with the MICs for 343 (98.3%) isolates 1 or 2 µg/ml. In the case of neomycin, the MICs for 345 (98.9%) isolates were 2 to 8 µg/ml, while distinctly higher MICs of 64 and 128 µg/ml were seen for single isolates. With tetracycline, the MICs for 346 isolates were $\leq 2$ µg/ml and that for the remaining 3 isolates was 64 µg/ml. Although
sulfonamides were not included in the test panels, a comparison of the MICs of trimethoprim and trimethoprim/sulfamethoxazole suggested that sulfonamides had some effect against isolates for which MICs of trimethoprim were elevated. The overall MICs of both cephalosporins tested for the *B. bronchiseptica* isolates in this study were high: ceftiofur, MIC of ≥16 µg/ml; and cephalothin, MIC of 32 µg/ml. A similar situation was seen with tilmicosin, with MIC50 and MIC90 of 16 and 32 µg/ml, respectively. In contrast, a low MIC50 and MIC90 of 0.25 and 0.5 µg/ml, respectively, were recorded for enrofloxacin.

Comparison of the results of this study with those of other studies is often problematic for several reasons: (i) different methodologies were used for susceptibility testing, including agar dilution (9, 10, 14, 17, 18), E-test (17, 18), and disk diffusion (6, 15, 19, 20); (ii) different antimicrobial agents were tested (6, 9, 10, 14); (iii) the evaluation of the results followed different guidelines (6, 9, 10, 14, 19); and/or (iv) isolates from animals other than pigs were tested (15, 17, 18). However, three studies from the United States (2, 3, 11) were suitable for comparisons with our data. In the first study (11), the range of MICs as well as the MIC90 of various antimicrobial agents were determined in 1988 from 48 porcine *B. bronchiseptica* isolates collected in the United States. The results for ampicillin, gentamicin, chloramphenicol, cephalothin, and trimethoprim/sulfamethoxazole corresponded closely to those of the present study, whereas the values for tetracycline were lower in the current study of German isolates (11). The second study described the in vitro susceptibility to tilmicosin of porcine respiratory tract pathogens collected between 1994 and 1998 in the United States (3). There was a close similarity between their observed range and MIC90 of tilmicosin and those found in the present study. The third study dealt with the in vitro susceptibility of porcine respiratory tract pathogens to ceftiofur and revealed that *B. bronchiseptica* isolates are rather insensitive to ceftiofur; the MICs for these isolates were ≥8 µg/ml (2). This was in good accordance with our observation that the ceftiofur MIC for 345 (98.9%) of the 349 *B. bronchiseptica* isolates was ≥8 µg/ml.

The classification of *B. bronchiseptica* isolates as susceptible, intermediate, or resistant based on the MIC data presents some problems. Interpretive criteria that can be used explicitly for *B. bronchiseptica* are currently only available for florfenicol, but not for the other antimicrobial agents tested in this study. Nevertheless, the data presented in this study allow a reliable estimate of the resistance status of German *B. bronchiseptica* isolates from.
<table>
<thead>
<tr>
<th>Antimicrobial agent and year&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of isolates for which MIC (µg/ml) is&lt;sup&gt;b&lt;/sup&gt;:</th>
<th>MIC (µg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.015  0.03  0.06  0.12  0.25  0.5  1  2  4  8  16  32  64  128  256</td>
<td>50%  90%</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0 0 0 8 2 7 56 2 0 2 1 0 8 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>0 0 0 2 0 5 78 7 1 3 2 0 8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0 0 0 1 3 6 36 41 1 0 3 0 16 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0 0 0 1 1 13 30 31 5 1 0 0 8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0 0 0 12 6 31 200 81 7 6 6 0 8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid (2:1)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0 0 0 8 3 47 20 0 0 0 0 2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>0 0 0 2 0 49 45 2 0 0 0 2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0 0 0 3 5 42 40 1 0 0 0 2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0 0 0 1 9 31 38 2 1 0 0 2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0 0 0 14 17 169 143 5 1 0 0 2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0 0 0 0 0 4 31 34 1 2 2 0 3 1 4 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>0 0 0 0 0 0 23 67 5 1 1 1 0 0 4 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0 0 0 0 1 35 43 10 0 0 0 1 1 4 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0 0 0 0 1 11 30 33 3 0 0 2 2 1 2 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0 0 0 0 0 16 119 177 19 3 3 3 6 3 4 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Florfenicol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td>67 7 2 2 0 2 4</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td>71 26 1 0 0 2 4</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td>73 17 0 1 0 2 4</td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td>67 11 0 0 4 2 4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>278 61 3 3 4 2 4</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>29 39 7 3 0 0 0 0 0 0 0 0 0 0 0 0 2 4</td>
<td>0.25 0.5</td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>5 65 27 1 0 0 0 0 0 0 0 0 0 0 0 0 0 2 4</td>
<td>0.25 0.5</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>6 63 17 1 1 0 0 0 0 0 3 0 0 0 0 0 0 0 2 4</td>
<td>0.25 0.5</td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>8 60 11 2 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 2 4</td>
<td>0.25 0.5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48 227 62 7 2 0 0 0 0 0 0 0 0 0 0 0 0 0.25 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>2001</td>
<td>2002</td>
</tr>
<tr>
<td>----------------</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Gentamicin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2001</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Neomycin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2001</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Streptomycin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2001</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2002</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2003</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Nalidixic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>1</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>2001</td>
<td>1</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>2002</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>2003</td>
<td>3</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>5</td>
<td>11</td>
<td>36</td>
</tr>
<tr>
<td><strong>Enrofloxacin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2001</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2002</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>2003</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>1</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td><strong>Trimethoprim</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Antimicrobial susceptibility

<table>
<thead>
<tr>
<th>Antimicrobial agent and year&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of isolates for which MIC (µg/ml) is&lt;sup&gt;b&lt;/sup&gt;:</th>
<th>MIC (µg/ml)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trimethoprim/sulfamethoxazole</strong>&lt;sup&gt;d&lt;/sup&gt; (1:19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>31 6 4 3 1 4 14 10 1 1 2 1</td>
<td>0.12 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>36 20 8 0 0 3 11 15 4 0 1 0</td>
<td>0.12 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>47 8 1 1 4 14 10 3 2 1 0</td>
<td>0.06 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>52 7 1 1 6 7 2 6 0 0 0 0</td>
<td>0.06 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>166 41 14 4 8 18 41 41 8 3 4 1</td>
<td>0.12 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tilmicosin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0 0 0 0 2 12 32 22 10</td>
<td>8 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>0 0 0 0 1 1 8 54 34</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0 0 0 0 0 10 5 51 25</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0 0 0 1 0 10 13 35 23</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0 0 0 1 3 33 58 162 92</td>
<td>16 32</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ceftiofur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0 0 0 0 0 0 0 0 0 5 73</td>
<td>16 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>0 0 0 0 0 0 0 0 0 1 97</td>
<td>16 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0 0 0 0 0 0 0 0 0 1 78 12</td>
<td>8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0 0 0 0 0 0 1 2 57 22</td>
<td>8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0 0 0 0 0 0 0 1 3 141 204</td>
<td>16 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cephalothin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td>0 0 0 0 2 8 8</td>
<td>8 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td>0 0 0 0 0 8 8</td>
<td>8 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>0 0 0 0 0 0 8 16</td>
<td>8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2003</td>
<td>0 0 0 0 0 0 8 16</td>
<td>8 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0 0 0 0 0 8 16</td>
<td>8 16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 78, 98, 91, and 82 for 2000, 2001, 2002, and 2003, respectively.

<sup>b</sup> MICs equal to or lower than the lowest concentration tested are given as the lowest concentration; whereas MICs equal to or higher as the highest concentration tested are given as the highest concentration.

<sup>c</sup> 50% and 90%, MIC<sub>50</sub> and MIC<sub>90</sub>, respectively.

<sup>d</sup> The MIC values of amoxicillin/clavulanic acid (2:1) are expressed as MIC values of amoxicillin.

<sup>e</sup> The MIC values of trimethoprim/sulfamethoxazole (1:19) are expressed as MIC values of trimethoprim.
porcine respiratory diseases based on testing a large number of isolates and using internationally accepted methods. In addition to other data such as pharmacokinetic and pharmacodynamic parameters or clinical efficacy, the MIC data of this study may help to establish breakpoints for antimicrobial agents for which no breakpoints approved for \textit{B. bronchiseptica} are currently available.

Kristina Kadlec is supported by a scholarship of the H. Wilhelm Schaumann foundation.

We thank Thomas R. Shryock and the NCCLS Subcommittee on Veterinary Antimicrobial Susceptibility Testing as well as Joseph W. Carnwath for helpful discussions.

References


Chapter 3

Molecular basis of resistance to trimethoprim, chloramphenicol and sulphonamides in *Bordetella bronchiseptica*

Kristina Kadlec, Corinna Kehrenberg and Stefan Schwarz

The extent of contribution from Kristina Kadlec to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Objectives: To date, little is known about the molecular basis of antimicrobial resistance in *Bordetella bronchiseptica*, an important respiratory tract pathogen in pigs, dogs and cats. The aim of this study was to identify genes coding for trimethoprim resistance present in porcine *B. bronchiseptica* and to determine their localisation, transferability, and association with other resistance genes.

Methods: Six *B. bronchiseptica* isolates with elevated MICs for trimethoprim were investigated by PCR for the presence of trimethoprim resistance genes and their association with class 1 integrons. The amplicons obtained were cloned and sequenced. Plasmid localisation of these integrons was confirmed by transformation and conjugation. Isolates carrying the same integron were compared for their genetic relatedness by *Xba*I and *Spe*I pulsed-field gel electrophoresis (PFGE).

Results: Five *B. bronchiseptica* isolates carried a class 1 integron with two gene cassettes, one carrying the trimethoprim resistance gene *dfrA1* and the other the chloramphenicol resistance gene *catB3*. This integron was present on a common conjugative plasmid in four of the five isolates and on the chromosome in the remaining isolate. All five *B. bronchiseptica* isolates proved to be related on the basis of their PFGE patterns. Another isolate had a class 1 integron with a *dfrB1* and a *catB2* cassette on a structurally different conjugative plasmid. The sulphonamide resistance gene *sul1* was detected in the 3’-conserved segment of both types of integrons.

Conclusions: This is the first report of trimethoprim, chloramphenicol and sulphonamide resistance genes and class 1 integrons in *B. bronchiseptica* isolates.
Introduction

*Bordetella bronchiseptica* is often involved in respiratory tract infections of food-producing animals, such as pigs and rabbits, but also companion animals, such as dogs and cats.\(^1\) Although *B. bronchiseptica* is also considered as a zoonotic agent, *B. bronchiseptica* infections in humans are rarely observed, and if so, they are most frequently seen in immunocompromised individuals.\(^2,3\) Antimicrobial agents are commonly used to control *B. bronchiseptica* infections, however, very little is known about the antimicrobial resistance of these bacteria.\(^4\) The antimicrobial susceptibility of *B. bronchiseptica* isolates from pigs has been monitored since 2002 in a single national resistance monitoring program in the veterinary field, the GermVet programme.\(^5\) However, *B. bronchiseptica* isolates have been collected for drug-specific monitoring programs since 2000 in Germany.\(^6\) A first large scale analysis of 349 porcine *B. bronchiseptica* isolates collected during the 4-year period 2000-2003 has recently been published.\(^7\) It showed that the vast majority of the isolates had MICs of trimethoprim in the range between ≤2 and 16 mg/L, whereas a small number of isolates exhibited distinctly higher MICs of ≥64 mg/L. These high-level trimethoprim resistant *B. bronchiseptica* isolates were considered as the most suitable candidates for the detection of trimethoprim resistance genes. In the present study, we investigated these isolates for the trimethoprim resistance genes present, their location on plasmids or on the chromosome, their transferability and their physical linkage to other resistance genes.

Material and methods

Isolates and susceptibility testing

The six isolates included in this study were obtained during 2000–2003 from diagnostic laboratories in Germany on the basis of one isolate per herd. All isolates were from pigs suffering from respiratory tract infections.\(^7\) The initial susceptibility testing was performed by broth microdilution according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, formerly known as NCCLS) document M31-A2.\(^8\) Since the highest test concentration of trimethoprim in the microtitre plate panels used in the previous study
was 64 mg/L, the isolates that grew at 64 mg/L were additionally tested for growth in the presence of 128 and 256 mg/L trimethoprim by broth macrodilution with *Escherichia coli* ATCC25922 as quality control strain.\(^7\) Susceptibility testing of the transformants and transconjugants was performed by either broth dilution or disc diffusion.\(^8\)

**DNA preparation and PCR analysis**

Isolation of plasmids and whole cell DNA followed standard protocols.\(^9\) To detect the most common trimethoprim resistance genes by PCR, four recently described primer sets each of which allowed the amplification of 2 – 3 closely related *dfrA* or *dfrB* genes were used.\(^10\) Integrase genes of classes 1 and 2, gene cassettes and sulphonamide resistance genes were detected by previously reported PCR assays.\(^10\)-\(^16\) All primers used are listed in Table 1. The amplicons obtained were confirmed and compared by restriction analysis. To confirm the linkage between the sequenced variable part of the class 1 integrons, the integrase and the sulfonamide resistance gene *sul1*, two combinations of primer pairs were used: (i) the 5′-CS primer for class 1 integrons was combined with the *sul1* reverse primer and used at an annealing temperature of 55°C, and (ii) the forward primer for the class 1 integrase gene was used with the reverse primer for the detected *dfrA* or *dfrB* gene at an annealing temperature of 50°C.

**Conjugation, transformation, cloning and sequence analysis**

Conjugation experiments were performed by filter mating with the rifampicin resistant *E. coli* HK225 as recipient strain.\(^16\) Transconjugants were selected on LB agar plates containing rifampicin (100 mg/L) and trimethoprim (20 mg/L). A donor:recipient ratio of 1:5 was used in this approach. For transformation, competent *E. coli* JM109 cells (Stratagene, Amsterdam, The Netherlands) were used and transformants were selected on LB agar supplemented with 20 mg/L trimethoprim.\(^18\) Amplicons representing the variable parts of the class 1 integrons were cloned into pCR Blunt II TOPO and transformed into *E. coli* TOP10 cells (Invitrogen, Groningen, The Netherlands).\(^18\) The complete sequence of both amplicons was determined by primer walking. Sequence comparisons were carried out using the BLAST\(^6\) programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST/; last accessed 25 May 2005) and with the
chapter 3  Trimethoprim, chloramphenicol and sulphamamide resistance


Table 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Gene/amplified region</th>
<th>Amplicon size (bp)</th>
<th>Forward (fw)/ Reverse (rv)</th>
<th>Sequence (5' → 3')</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>dfrB1, dfrB2</td>
<td>205</td>
<td>fw</td>
<td>CAAAGTAGCGATGAAGCC</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>CAGGATAAAATTTCAGACTTCCG</td>
<td></td>
</tr>
<tr>
<td>dfrA5, dfrA14</td>
<td>383</td>
<td>fw</td>
<td>GATTGGTGCGCTCCCA</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>TCATAAAACCAACTTCCGAA</td>
<td></td>
</tr>
<tr>
<td>dfrA7, dfrA17</td>
<td>345</td>
<td>fw</td>
<td>CAGAAAATGGCATTACG</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>TCACCTTCAACCTCAACG</td>
<td></td>
</tr>
<tr>
<td>dfrA1, dfrA15, dfrA16</td>
<td>414</td>
<td>fw</td>
<td>GATATTCCATGGACAGCAAG</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>ACCCTTTGGCGAGATTGG</td>
<td></td>
</tr>
<tr>
<td>variable part of class 1 integrons</td>
<td>variable</td>
<td></td>
<td>5'-CS GGATCCAAAGCACAGAAG</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-CS AAGCAGACTTGACCTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>class 1 integrase</td>
<td>450</td>
<td>fw</td>
<td>CGGAATGGCGAGCACATC</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>CAGGGTTTCTCAGACAGTGTCCG</td>
<td></td>
</tr>
<tr>
<td>sul1</td>
<td>840</td>
<td>fw</td>
<td>CTAGGCATGATCTAACCTCCTCATGTC</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>ATGGTGACGGTTTGCCACCTAAGGCC</td>
<td></td>
</tr>
<tr>
<td>sul2</td>
<td>704</td>
<td>fw</td>
<td>ACAGTTTCTCCAGTGAGGCC</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>CTGGTGTGCGGGAGATGACG</td>
<td></td>
</tr>
<tr>
<td>variable part of class 2 integrons</td>
<td>variable</td>
<td></td>
<td>5'-CS CGGGATCCGGGACAGCGATCGTGTTAGTA</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-CS GATGCCATCGCAAGTGACAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>class 2 integrase</td>
<td>401</td>
<td>fw</td>
<td>ATTAGGCCTGGGCGAGATTAG</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv</td>
<td>CGTCATCTCAGACCATGAGCG</td>
<td></td>
</tr>
</tbody>
</table>

Pulsed-field gel electrophoresis

For pulsed-field gel electrophoresis (PFGE) with XbaI and SpeI, a standard protocol was used. Whole cell DNA of *Staphylococcus aureus* 8325 digested with SmaI and of *Salmonella* Typhimurium LT2 digested with XbaI served as size markers. PFGE was performed in a CHEF DR III system (Bio-Rad, Munich, Germany) using 0.5 x Tris-borate-EDTA buffer as running buffer and 5.6 V/cm. The pulse times were increased from 7 to 20 s for the first 11h and from 30 to 50 s for the following 13h.
Results

Antimicrobial susceptibility and detection of trimethoprim resistance genes

Of the 349 *B. bronchiseptica* isolates originally tested, six isolates proved to be high-level resistant to trimethoprim with MICs of 128 mg/L (one isolate) or ≥512 mg/L (five isolates). These isolates also had high MICs of 16/304–64/1216 mg/L for the combination trimethoprim/sulfamethoxazole (1:19), suggesting that all six isolates were also resistant to sulphonamides. Moreover, the six isolates also exhibited elevated MICs of 16–32 mg/L for chloramphenicol, whereas their florfenicol MICs were ≤2 mg/L. The PCR assay with consensus primers for the simultaneous detection of the trimethoprim resistance genes *dfrA1*-*dfrA15*-*dfrA16* yielded the expected amplicon of 414 bp in the five *B. bronchiseptica* isolates with MICs of ≥512 mg/L trimethoprim. *ClaI* digestion of the amplicon was used to discriminate between these three *dfrA* genes since there was one *ClaI* site in *dfrA1*, two *ClaI* sites in *dfrA15* and no *ClaI* site in the amplicon specific for *dfrA16*. *ClaI* fragments of ~0.26 and ~0.15 kb which are indicative for *dfrA1* were detected in all five amplicons. The remaining *B. bronchiseptica* isolate that exhibited a MIC of 128 mg/L trimethoprim yielded an amplicon of 205 bp which was obtained with consensus primers for the genes *dfrB1* and *dfrB2*. Owing to its small size this amplicon was not subjected to restriction analysis, but was sequenced.

Characterisation of class 1 integrons and associated gene cassettes

Since the genes *dfrA1* and *dfrB1/dfrB2* have previously been found on gene cassettes located in class 1 or class 2 integrons, the six *B. bronchiseptica* isolates were investigated for the presence of class 1 and class 2 integrons and associated gene cassettes. All six isolates carried a class 1 integron, but were negative for class 2 integrons. Amplicons of 450 and 840 bp, which were specific for the integrase gene and the sulphonamide resistance gene *sul1* of class 1 integrons, respectively, were detected by PCR. In addition, two different sized amplicons were obtained by PCR analysis of the variable part located between the 5’-CS and the 3’-CS region.

Each of the five isolates with trimethoprim MICs of ≥512 mg/L yielded an amplicon of 1445 bp which comprised two gene cassettes flanked by short sequences of the 5’-CS and 3’-CS region.
CS regions. Restriction analysis of all five amplicons with \textit{Cla}I and \textit{Bcl}I revealed the same fragment patterns. Therefore, one of the amplicons, namely that of \textit{B. bronchiseptica} isolate 668, was chosen for sequence analysis. It showed that this integron harboured a first gene cassette of 577 bp which contained the trimethoprim resistance gene \textit{dfrA1} and a second cassette of 715 bp with the chloramphenicol resistance gene \textit{catB3}. The \textit{dfrA1} gene codes for a trimethoprim-resistant class A dihydrofolate reductase consisting of 157 amino acids. The corresponding 59-base element was 95 bp in size. The gene \textit{catB3} codes for a type B chloramphenicol acetyltransferase (CAT) of 210 amino acids. The 59-base element of the \textit{catB3} cassette was 60 bp in size (Figure 1a).

The sixth \textit{B. bronchiseptica} isolate with the trimethoprim MIC of 128 mg/L also harboured an integron with two gene cassettes. The first cassette of 411 bp contained the gene \textit{dfrB1} which codes for a small trimethoprim resistant class B dihydrofolate reductase of 78 amino acids. The 59-base element of this cassette was 57 bp in size. The second gene cassette harboured a \textit{catB2} gene which codes for another variant of type B CATs. The corresponding CatB2 protein consisted also of 210 amino acids and the 59-base element of the \textit{catB2} cassette was 72 bp in size (Figure 1b).

\textit{Localisation and transferability of the integrons}

The integron harbouring the \textit{dfrA1-catB3} gene cassettes was located on plasmids of ~24 kb in four of the five isolates. Since the fifth isolate was plasmid-free, it was assumed that the integron was located in the chromosomal DNA. Comparative restriction analysis using the endonucleases \textit{Dra}I, \textit{Pvu}I, \textit{Pvu}II, \textit{Cla}I, and \textit{Hind}III showed indistinguishable fragment patterns consisting of 2 to 4 fragments among the four plasmids. Therefore, a common designation, pKBB668, was chosen for this type of plasmid. Plasmid pKBB668 was transferred into \textit{E. coli} JM109 where it expressed its resistance properties. Conjugation experiments with \textit{E. coli} HK225 as recipient confirmed that plasmid pKBB668 was conjugative and transferred from \textit{B. bronchiseptica} to \textit{E. coli} at a frequency of ~10^{-5} per recipient. The presence of the class 1 integron and its gene cassettes was confirmed by PCR using plasmid DNA from \textit{E. coli} JM109::pKBB668 transformants and \textit{E. coli} HK225::pKBB668 transconjugants. Plasmid pKBB668 mediated no resistance properties other than those associated with the class 1 integron. The integron with the \textit{dfrB1-catB2} gene
Figure 1. Schematic presentation of the class 1 integrons with two gene cassettes with (a) the class 1 integron found in five *B. bronchiseptica* isolates (EMBL accession number: AJ844287), and (b) the class 1 integron detected in one *B. bronchiseptica* isolate (EMBL accession number AJ879564). The reading frames of the antimicrobial resistance genes are shown as arrows, the conserved segments of the class 1 integron as boxes. The beginning and the end of the integrated cassettes are shown in detail below. The translational start and stop codons are underlined. The 59-base elements are shown in bold type, the putative IntI1 integrase binding domains 1L, 2L, 2R and 1R are indicated by arrows. The numbers refer to the positions of the bases in the respective EMBL database entries.
chapter 3  Trimethoprim, chloramphenicol and sulphonamide resistance

cassettes was also located on a conjugative plasmid, designated pKBB958. This plasmid was distinctly larger and structurally different from pKBB668. In addition to the integron-associated resistance properties, the 38 kb plasmid pKBB958 also mediated tetracycline resistance. Again, all resistance properties were expressed in E. coli JM109 transformants or E. coli HK255 transconjugants. Plasmid pKBB958 showed conjugal transfer into E. coli at a high frequency of $10^{-3}$ per recipient.

**Genomic relatedness of dfrA1-catB3-carrying B. bronchiseptica isolates**

To assess the genomic relatedness of the five isolates that harboured the integron with the dfrA1-catB3 gene cassettes, PFGE was conducted. The results confirmed that all five B. bronchiseptica isolates were related with isolates 2, 3 and 5 being indistinguishable by their XbaI patterns and isolate 4 differing by two bands. Isolate 1 differed from the others by four bands (Figure 2). Upon SpeI analysis (data not shown) isolates 2, 3 and 5 exhibited the same pattern whereas isolates 1 and 4 had an additional band. Comparison of these fragment patterns with those of unrelated B. bronchiseptica isolates from pigs and that of the type strain NCTC452 revealed differences of at least eight fragments.

**Discussion**

The finding that B. bronchiseptica isolates from porcine respiratory tract infections carry class 1 integrons with gene cassettes for different trimethoprim and chloramphenicol resistance genes suggests a resistance gene flow between porcine respiratory tract pathogens and other bacteria, such as enteric bacteria and pseudomonads. The dfrA1-catB3 gene cassettes detected in B. bronchiseptica isolates have also been detected in class 1 integrons of plasmid pAPEC-O2-R from E. coli (accession no. AY214164) and in Pseudomonas aeruginosa (accession no. AB195796), which, however, carried additional aadA4 or aacA4 gene cassettes. The combination of the two gene cassettes dfrB1-catB2 present on the second type of class 1 integron detected in this study has previously been identified on plasmid pSp39 (accession no. AY139601) from an uncultured bacterium from a wastewater treatment plant and plasmid pMVH202 from Klebsiella pneumoniae and E. coli (accession nos. AY987853, AY970968).
These integrons also contained an additional blaVIM-1, aacA4, and/or aadA1 gene cassettes. These comparisons showed that the gene cassettes dfrA1-catB3 and dfrB1-catB2 have only rarely been detected in the same integron, and if so, always together with other gene cassettes.

**Figure 2.** XbaI PFGE patterns from isolates carrying the same class 1 integron. Isolates nos. 1–4 carry the integron on plasmid pKBB668, isolate 5 is plasmid-free. Lanes 1–5, isolates 1–5; lane R, *B. bronchiseptica* NCTC452; lane M1, XbaI pattern of *Salmonella Typhimurium* LT2; lane M2, SmaI pattern of *S. aureus* 8325.
A closer look at the \textit{dfrB1} cassette detected in the present study revealed that this cassette was 74 bp shorter than the prototype \textit{dfrB1} cassette (accession no. U36276).\textsuperscript{20} This difference in size was based on the loss of a 72 bp tandem duplication and two single base pairs in the part upstream of the \textit{dfrB1} gene in the respective cassette from \textit{B. bronchiseptica}. The \textit{dfrB1} cassette described in the present study was indistinguishable from the \textit{dfrB1} cassettes found on plasmids pMVH202 or pSp39. Surprisingly, the DfrB1 proteins of pMVH202 and pSp39 were described to be 97 amino acids in size while that of DfrB1 from \textit{B. bronchiseptica} was found to be 78 amino acids. This difference is most likely the result of a search for the largest possible open reading frame within the \textit{dfrB1} cassette. In this case, an ATG codon (position 111-113 in Figure 1b) was recognized as the putative translational start codon of the \textit{dfrB1} gene. However, the intact DfrB1 protein from \textit{E. coli} plasmid R67 had been purified and shown by protein sequencing to be 78 amino acids in size.\textsuperscript{21} Hence, the start codon at positions 168-170 (Figure 1b) is most likely the true translational start codon of the \textit{dfrB1} gene.

Since the same type of plasmid-borne class 1 integron was detected in isolates from different farms in the Northern part of Germany, there are two general possibilities: spread of a resistant clone or horizontal dissemination of the plasmid-borne integron into members of different clonal lineages. PFGE strongly suggested a clonal relationship between the five isolates rather than a horizontal spread of the conjugative plasmid pKBB668 between unrelated \textit{B. bronchiseptica} isolates. The dissemination of closely related \textit{B. bronchiseptica} isolates within a particular geographic area might be explained by the purchase of piglets already carrying these resistant \textit{B. bronchiseptica} isolates and originating from the same pig breeder by different commercial pig growers. Another possibility is the transmission via living and non-living vectors. Since three of the farms from which the isolates in question were obtained were located <100 km apart from each other and known to be under support of the same veterinarian, a farm-to-farm spread of the \textit{B. bronchiseptica} isolates by the veterinarian cannot be excluded. Exchange of pigs between these herds as well as close contacts between people working on these farms could not be confirmed.

Among the antimicrobial agents licensed for the control of bacteria involved in porcine respiratory diseases and atrophic rhinitis, older and comparatively cheaper antimicrobials, such as tetracyclines and the combination trimethoprim/sulphonamides, are often preferred.
over newer and more expensive agents such as 3rd generation cephalosporins, tilmicosin or florfenicol. This might explain why plasmids such as pKBB668 and pKBB958 which mediate resistance to trimethoprim, sulphonamides and chloramphenicol (and in the case of pKBB958 also to tetracyclines), are acquired by and stably maintained in *B. bronchiseptica*. The observation that a gene cassette for chloramphenicol resistance – an antimicrobial agent that was banned from use in food animals – is still present in both types of integrons might be explained by co-selection in the presence of selective pressure imposed by the use of sulphonamides and trimethoprim.

In conclusion, the data presented in this study underline that there is a potential resistance gene flow between porcine respiratory tract pathogens and enteric and environmental bacteria, which also includes class 1 integrons and their associated gene cassettes.

**Acknowledgements**

We thank Vera Nöding and Roswitha Becker for excellent technical assistance, Geovana Brenner Michael for a strain carrying a class 1 integron as well as for helpful discussions, Jürgen Wallmann for the cooperation with the susceptibility testing, and Reiner Helmuth for kindly providing a strain harbouring a class 2 integron. K. K. is supported by a scholarship of the H. Wilhelm Schaumann foundation.

**References**


Chapter 4

*tet(A)-mediated tetracycline resistance
in porcine *Bordetella bronchiseptica* isolates is based
on plasmid-borne Tn1721 relics

Kristina Kadlec, Corinna Kehrenberg and Stefan Schwarz

The extent of contribution from Kristina Kadlec to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Sir,

*Bordetella bronchiseptica* is often involved in respiratory tract infections of farm animals (i.e. pigs and rabbits) and pets (i.e. cats and dogs). Infections in humans are seen mainly in older and immunocompromised patients. Although antimicrobial agents are commonly applied to control *B. bronchiseptica* infections,\(^1\) little is known about the molecular basis of antimicrobial resistance in these bacteria. Although tetracycline resistance in *B. bronchiseptica* was already described in 1981 to be associated with a non-conjugative plasmid,\(^2\) it took until 1997 when the first and so far only tetracycline resistance gene, *tet*(C), was identified in *B. bronchiseptica* isolates from cats.\(^3\) In the present study, we investigated two isolates from pigs for the molecular basis of tetracycline resistance with particular reference to the type of *tet* gene present, its location on a mobile genetic element and the possibility of horizontal transfer.

A recent survey revealed that tetracycline resistance was seen in < 1% of 349 porcine *B. bronchiseptica* isolates.\(^1\) A tetracycline MIC value of 64 mg/L had been reported for the two isolates, nos. 958 and V4037/8.\(^1\) PCR screening for *tet* genes, plasmid profiling, conjugation, cloning and sequencing followed previously described protocols.\(^4,5\) PCR confirmed that both *B. bronchiseptica* isolates carried a *tet* gene of hybridization class A. Both *tet*(A)-carrying isolates were subjected to macrorestriction analysis.\(^4\) Their *Xba*I fragment patterns differed by ten bands and thus confirmed that the two *B. bronchiseptica* isolates were unrelated.

In isolate no. 958, the *tet*(A) gene was located on the conjugative 38 kb plasmid pKBB958, previously described to harbour also a class 1 integron with genes for resistance to trimethoprim, chloramphenicol and sulphonamides.\(^4\) Using tetracycline (20 mg/L) as selective agent, transfer of plasmid pKBB958 into *Escherichia coli* recipient strains HK225, JM109 and JM101 was achieved by conjugation and transformation. Restriction analysis showed that the *tet*(A) gene was located on a 16 kb *Xba*I fragment of which we sequenced a 4338 bp segment. Comparisons revealed that 3445 bp were homologous to the *tet*(A)-carrying prototype transposon Tn\(1721\).\(^6\) The transposon Tn\(1721\) consists of 11139 bp and is divided by two terminal and one central 38 bp repeat into two parts (Figure 1).\(^6\) Homology (>99%) started immediately downstream of the internal 38 bp repeat of Tn\(1721\) and ended upstream of the truncated transposase gene Δ*tnpA* in the right-hand portion of the transposon (Figure 1). While the part including *tetR* and *tet*(A) was indistinguishable from that of Tn\(1721\) (accession
no. X61367), minor variations were detected in the non-coding regions. The sequences flanking this Tn1721-homologous part exhibited neither similarities to other sequences deposited in the databases, nor sequence features that might give a hint to the processes that led to the truncation of Tn1721.

In isolate no. V4037/8, the tet(A) gene was located on a non-conjugative plasmid of ~24 kb, designated pKBB4037, which conferred only tetracycline resistance. Cloning of restriction fragments of pKBB4037 produced an EcoRI self-ligand which replicated and conferred tetracycline resistance in E. coli JM101. Sequencing of this self-ligand revealed the presence of open reading frames for a resolvase Res, two partition proteins ParA1 and ParC and a plasmid replication protein Rep within the initial 4936 bp (Figure 1). The 295-amino-acid resolvase protein showed 82% identity to a putative plasmid-borne 283-amino-acid resolvase from Pseudomonas aeruginosa (accession no. AAP22618). The 210-amino-acid ParA1 protein is 95% identical to ParA1 located on plasmid pXcB from Xanthomonas citri (AAO72130) while the 106-amino-acid ParC protein showed 55% identity to the 128-amino-acid plasmid-borne ParC proteins from P. aeruginosa (CAI46991) and Pseudomonas alcaligenes (AAD40336). The N-terminal 395 amino acids of the 488-amino-acid plasmid replication protein Rep exhibited 79% identity to a 452-amino-acid hypothetical protein from Nitrosomonas eutropha C71 (ZP_00671175). Lesser degrees of identity of 72 % and 59% were seen with plasmid replication protein of P. aeruginosa (CAI46990) and Aeromonas hydrophila (ABD64829). The adjacent 5490 bp segment was virtually identical to Tn1721 (>99% homology) and included almost the entire right-hand portion of the transposon with the tetracycline resistance gene region, the truncated ΔtnpA gene, and the right terminal 38 bp repeat. A stretch of 739 bp which included most of the right terminal repeat of Tn1721 and the sequences downstream of the Tn1721-homologous segment closely resembled an internal segment of the transposase gene of Tn5393 (ΔtnpA*). The nucleotide sequences of the tetracycline resistance gene region and their flanking areas of plasmids pKBB958 and pKBB4037 have been deposited in the EMBL database under accession nos. AM183165 and AJ877266.
Figure 1. Comparison of Tn1721 (accession no. X61367) and the sequenced parts of the resistance plasmids pKBB958 (AM183165) and pKBB4037 (AJ877266) from B. bronchiseptica. A distance scale in kb is given below each map. The genes \( \text{tetR}, \text{tet(A)}, \text{mcp}, \text{tnpR}, \text{tnpA}, \Delta \text{tnpA}, \text{res}, \text{parA1}, \text{parC} \) and \( \Delta \text{tnpA}^* \) are presented as arrows with the arrowhead indicating the direction of transcription. The \( \Delta \) symbol indicates a truncated, functionally inactive gene. The black boxes represent the terminal or internal 38 bp repeats of Tn1721. The grey shaded areas indicate the homologous parts between the B. bronchiseptica plasmids and Tn1721.
Truncated forms of the transposon Tn1721 have been described in various Gram-negative bacteria.\(^5,7\) In all those cases, the tetracycline resistance gene region was intact whereas the genes coding for transposition functions were deleted in part or completely. Although the Tn1721 relics found in the two plasmids from \(B.\) bronchiseptica differed from all previously described ones, we also noticed the presence of an intact resistance gene region and the lack of the transposase part.

In contrast to feline \(B.\) bronchiseptica isolates, where a plasmid-borne \(tet(C)\) gene was identified,\(^3\) we found a different type of \(tet\) gene, \(tet(A)\), in two unrelated porcine \(B.\) bronchiseptica isolates. This is to the best of our knowledge the first report of a \(tet(A)\) gene in \(B.\) bronchiseptica and extends the knowledge of the distribution of this transposon-associated \(tet\) gene (http://faculty.washington.edu/marilynr/tetweb2.pdf). Although \(tet\) genes of various classes have been detected in animal isolates of the family Pasteurellaceae,\(^8\) which share the same habitat with \(B.\) bronchiseptica, the \(tet(A)\) gene has not yet been detected in these bacteria. Its presence on a conjugative plasmid, however, may further the dissemination of the \(tet(A)\) gene not only to other \(Bordetella\) isolates, but also to other Gram-negative respiratory tract pathogens.

Acknowledgements

We thank Jürgen Wallman, Frederique Pasquali and Petra Lüthje for helpful discussions. K. K. is supported by a scholarship of the H. Wilhelm Schaumann foundation.
References


Chapter 5

Efflux-mediated resistance
to florfenicol and/or chloramphenicol
in *Bordetella bronchiseptica:*
identification of a novel chloramphenicol exporter

Kristina Kadlec, Corinna Kehrenberg and Stefan Schwarz

The extent of contribution from Kristina Kadlec to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B

2. Performing of the experimental part of the study: C

3. Analysis of the experiments: C

4. Presentation and discussion of the study in article form: C
Objectives: Twenty florfenicol- and/or chloramphenicol-resistant *Bordetella bronchiseptica* isolates of porcine and feline origin were investigated for the presence of *floR* and *cml* genes and their location on plasmids.

Methods: The *B. bronchiseptica* isolates were investigated for their susceptibility to antimicrobial agents by broth micro- or macrodilution and for their plasmid content. Hybridization experiments and PCR assays were conducted to identify resistance genes. Transformation and conjugation studies were performed to show their transferability. Representatives of both types of genes including their flanking regions were sequenced. Moreover, inhibitor studies with the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) were performed.

Results: The gene *floR* was found in the chromosomal DNA of nine of the 18 florfenicol/chloramphenicol-resistant isolates. Sequence analysis revealed that the deduced FloR protein sequence differed by a single amino acid exchange from FloR of *Vibrio cholerae*. A chloramphenicol-resistant, but florfenicol-susceptible isolate carried a novel plasmid-borne *cml* gene, designated *cmlB1*. The CmlB1 protein revealed only 73.8-76.5% identity to known CmlA proteins. The gene *cmlB1* was not part of a gene cassette. The results of inhibitor studies with PAβN suggested that a so far unidentified efflux system might play a role in phenicol resistance of the remaining florfenicol- and/or chloramphenicol-resistant isolates.

Conclusion: This is to the best of our knowledge the first report of a *floR* gene in *B. bronchiseptica* isolates. The identification of the first member of a new subclass of *cml* genes, *cmlB1* from *B. bronchiseptica*, extends our knowledge on specific chloramphenicol exporters.
**Introduction**

*Bordetella bronchiseptica* is frequently involved in respiratory tract infections of food-producing animals and companion animals.\(^1\) Antimicrobial agents are commonly used to treat these infections in animals. Initial studies of antimicrobial resistance in *B. bronchiseptica* from pigs revealed a decreased susceptibility to most of the antimicrobial agents currently approved for the treatment of respiratory tract infections, such as tilmicosin and ceftiofur with \(\text{MIC}_{90}\) values of 16 mg/L and 32 mg/L, respectively.\(^2\) For other antimicrobial agents, such as florfenicol, the corresponding \(\text{MIC}\) values were distinctly lower.\(^2\) Florfenicol is a fluorinated chloramphenicol derivative, which – after the EU-ban of chloramphenicol use in food-producing animals in 1994 – has been approved for the treatment of respiratory tract infections in cattle in 1995 and in pigs in 2000. In contrast, chloramphenicol is still approved for use in dogs, cats and other non food-producing animals and – based on its favourable susceptibility situation – is used for the control of a wide variety of infections in these animals.

Although florfenicol-resistant *B. bronchiseptica* isolates from respiratory tract infections in pigs have been detected during recent years,\(^3,4\) the genetic basis for this resistance in *B. bronchiseptica* had not been elucidated. In the present study, we analyzed isolates classified as florfenicol/chloramphenicol-resistant or only chloramphenicol-resistant for the presence of known florfenicol and/or chloramphenicol resistance genes. In addition, an efflux pump inhibitor was used to assess whether efflux may play a role in phenicol resistance of *B. bronchiseptica*.

**Material and methods**

*Bacterial isolates and susceptibility testing*

A total of 496 *B. bronchiseptica* isolates from animals suffering from respiratory tract infections in Germany, including 349 isolates from pigs collected between 2000 and 2003,\(^2\) as well as 105 isolates from pigs, 8 isolates from cats and 34 from dogs, all collected between 2004 and 2006, were investigated for their susceptibility to florfenicol and chloramphenicol.
Biochemical species identification was confirmed by genus- and species-specific PCR analysis.\textsuperscript{5} Macrorestriction analysis with \textit{XbaI} was performed as described previously.\textsuperscript{6} MIC determination by broth micro- or macrodilution followed the recommendations of the Clinical and Laboratory Standards Institute (CLSI) as laid down in documents M31-A2 and M31-S1.\textsuperscript{7,8} The reference strains \textit{Escherichia coli} ATCC 25922 and/or \textit{Staphylococcus aureus} ATCC 25923 were included for quality control purposes. To induce phenicol resistance gene expression, the strains were grown overnight either on MH-agar plates containing chloramphenicol (0.5 mg/L) or florfenicol (0.5 mg/L). MIC determinations have been performed at least twice on independent occasions.

\textit{Detection of phenicol resistance genes}

PCR assays for the genes conferring combined resistance to chloramphenicol and florfenicol, namely \textit{floR}, \textit{fexA} and \textit{cfr}, but also for the most common chloramphenicol resistance genes \textit{catA1}, \textit{catA2}, and \textit{catA3} were performed according to previously described protocols.\textsuperscript{6,9-11} For the detection of the chloramphenicol resistance gene \textit{cmlA} previously described primers were used at an annealing temperature of 60°C.\textsuperscript{12} In addition, all isolates were investigated for class 1 integrons and their associated \textit{catB} gene cassettes.\textsuperscript{6} For the gene \textit{floR}, additional new primers were designed to amplify the entire \textit{floR} gene and used with the annealing temperature of 50°C. The forward primer (5’-AGGGTTGATTTCGTCATGACCA-3’) contained the start codon and the reverse primer (5’-CGGTAGACGACTGGCGACT-3’) the stop codon of the \textit{floR} gene. To detect circular forms of the \textit{floR}-carrying transposon Tn\textit{floR}, the primers floRcirc1 and floRcirc2 were used.\textsuperscript{13} In addition, PCR assays were conducted for the \textit{oqxAB} operon\textsuperscript{14} which has recently been described to mediate the efflux of chloramphenicol in addition to that of olaquindox.

\textit{Plasmid profiling, transfer experiments and Southern blot hybridization}

Plasmid profiles were prepared by alkaline lysis as described.\textsuperscript{6} Conjugation into \textit{E. coli} HK225 was performed.\textsuperscript{6} Electrotransformation into \textit{B. bronchiseptica} B543 and into \textit{E. coli} HB101 was carried out as described previously for \textit{Pasteurella}\textsuperscript{15} with the Gene Pulser II electroporation system (Bio-Rad, Munich, Germany). Transfer was confirmed by MIC determination and by plasmid isolation with subsequent restriction analysis and PCR assays.
Southern blot hybridization was performed with a PCR-generated floR gene probe using either EcoRI- or SacI-digested whole cell DNA or uncut plasmid profiles as target DNA. Probe labelling was achieved with the DIG-High Prime DNA labelling and detection system. Hybridization and signal detection followed the recommendations given by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

Sequencing of resistance gene regions

For sequence analysis, the floR and cmlA PCR amplicons were cloned into the vector pCR-blunt (Invitrogen, Groningen, The Netherlands) as described previously. To sequence the flanking regions of floR, chromosomal DNA was first digested with the restriction enzyme AgeI and the fragments re-ligated with T4 DNA ligase. Subsequently, inverse PCR with the floRcirc primers was conducted. The resulting amplicon was cloned into pCR-blunt and sequenced. To sequence the flanking regions of the plasmid-borne cmlA-like gene, the plasmid was digested with EcoRI and KpnI and the fragments cloned into pBluescript II SK+ (Stratagene, Amsterdam, The Netherlands). The clones were confirmed by plasmid profiling, restriction analysis, PCR-directed detection of the cmlA-like gene, and expression of chloramphenicol resistance. Sequences were deposited in the EMBL database under accession nos. AM296480 (floR) and AM296481 (cmlB1).

Inhibition of efflux mechanisms

The efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) was used for inhibition studies. First, the strain-specific susceptibility to the efflux inhibitor was determined by broth macrodilution. Then, MICs for chloramphenicol and florfenicol, but also nalidixic acid were determined in parallel in the presence and absence of the inhibitor. An inhibitor concentration of 80 mg/L was used, representing ¼ of the MIC for PAβN.
Results and Discussion

Susceptibility testing

Of all the isolates analyzed, only 18 *B. bronchiseptica* isolates – 17 from pigs and one from a cat – were classified as florfenicol-resistant by CLSI criteria with MICs for florfenicol of ≥ 8 mg/L. All florfenicol-resistant isolates (nos. 1-18) exhibited MICs for chloramphenicol of ≥ 16 mg/L (Table 1). In addition, two isolates (nos. 19 and 20) were chloramphenicol-resistant, but not florfenicol-resistant, and had MICs for chloramphenicol of 128 mg/L and 32 mg/L, respectively. The high MIC for chloramphenicol of 128 mg/L in isolate no. 19 suggested the presence of a specific chloramphenicol resistance gene. The remaining isolates (nos. 21-23) were used for control purposes and showed low MIC values of 4 mg/L for chloramphenicol and florfenicol (Table 1).

**FloR-mediated florfenicol/chloramphenicol resistance**

In isolates nos. 3 to 11 (Table 1), the gene *floR* was detected by PCR. Plasmid profiling revealed that the *floR*-carrying strains were plasmid-free. Hybridization studies confirmed that this gene was located in the chromosomal DNA in all nine cases. Since all these *B. bronchiseptica* isolates shared indistinguishable or closely related XbaI macrorestriction patterns, one of these isolates, isolate no. 5, was chosen for further analysis. Analysis of a 1638-bp region including the *floR* gene and 66 bp in its upstream and 356 bp in its downstream flanking region revealed a single bp exchange as compared to the corresponding sequence of *Vibrio cholerae* (accession no. AY822603). This bp exchange resulted in an amino acid exchange of His202 in *B. bronchiseptica* versus Arg202 in *V. cholerae*. As compared to FloR proteins so far found in other respiratory tract pathogens, FloR from *B. bronchiseptica* differed by four amino acid exchanges each from FloR of *Pasteurella multocida* [Leu178, His202, Pro207 and Phe228 in *B. bronchiseptica* versus Arg178, Arg202, Ala207 and Tyr228 in *P. multocida*] and from FloR of *Pasteurella trehalosi* [Ile32, Met147, His202, and Met225 in *B. bronchiseptica* versus Met32, Ile147, Arg202, and Ile225 in *P. trehalosi*]. Although the determined up- and downstream flanking regions of *floR* were identical to the sequence of Tn*floR,* a circular intermediate, which might confirm the mobility of *floR*, could not be detected in any of the nine *floR*-carrying isolates.
Table 1. MICs of the *B. bronchiseptica* isolates for florfenicol (FFC), chloramphenicol (CHL), and nalidixic acid (NAL) determined in the absence (-) or presence (+) of the efflux pump inhibitor PAβN, PFGE patterns, and the phenicol resistance genes detected in the isolates.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Year</th>
<th>MIC [mg/L]</th>
<th>PFGE pattern(^3)</th>
<th>Phenicol resistance gene present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CHL</td>
<td>FFC</td>
<td>NAL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- PAβN + PAβN</td>
<td>- PAβN + PAβN</td>
<td>- PAβN + PAβN</td>
</tr>
<tr>
<td>1</td>
<td>2003</td>
<td>128</td>
<td>4</td>
<td>128</td>
</tr>
<tr>
<td>2</td>
<td>2003</td>
<td>128</td>
<td>8</td>
<td>128</td>
</tr>
<tr>
<td>3</td>
<td>2000</td>
<td>256</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>2006</td>
<td>256</td>
<td>128</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>2003</td>
<td>256</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>2000</td>
<td>256</td>
<td>n.d.(^1)</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>2002</td>
<td>256</td>
<td>n.d.</td>
<td>16</td>
</tr>
<tr>
<td>12(^2)</td>
<td>2005</td>
<td>64</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>13</td>
<td>2006</td>
<td>32</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>14</td>
<td>2006</td>
<td>32</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>15</td>
<td>2001</td>
<td>32</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>16</td>
<td>2006</td>
<td>32</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>17</td>
<td>2006</td>
<td>32</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>18</td>
<td>2006</td>
<td>16</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>19</td>
<td>2001</td>
<td>128</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>2003</td>
<td>32</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>2000</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>22</td>
<td>2000</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>23</td>
<td>2006</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^1\) not determined  
\(^2\) Isolate no. 18 was from a cat suffering of an upper respiratory tract infection, while all other isolates were from pigs  
\(^3\) A new letter was given, if the pattern differed by three or more bands. Patterns indicated as e.g. A1 or A2 differed by only one or two bands from pattern A.
In the remaining nine florfenicol-resistant isolates, the \textit{floR} gene was not detectable by PCR or by specific hybridization. In addition, none of the other two so far known florfenicol resistance genes, \textit{cfr} and \textit{fexA}, were detectable. PCR assays for genes, conferring resistance to chloramphenicol only, did not yield amplicons in any of the 18 florfenicol-resistant isolates, thus confirming that no additional chloramphenicol resistance gene is present in these isolates.

\textit{CmlB1-mediated chloramphenicol resistance}

Solely in isolate no. 19, which had a MIC for chloramphenicol of 128 mg/L, a \textit{cmlA}-like gene was detected. Plasmid profiling as well as conjugation and transformation experiments revealed its localization on a non-conjugative plasmid of ca. 50 kb. Sequencing of the entire gene and analysis of the deduced amino acid sequence showed that the corresponding gene product differed distinctly from the amino acid sequences of all so far known CmlA proteins. Based on a multi-sequence alignment with all CmlA amino acid sequences currently deposited in the databases, the 421-amino acids protein from \textit{B. bronchiseptica} showed identities of only 73.7 to 76.5 \% to the different CmlA proteins with least identity to the CmlA4 protein of \textit{Salmonella enterica} serovar Agona\textsuperscript{21} and the highest identity to CmlA5 from \textit{Acinetobacter baumannii}.\textsuperscript{22} Based on this level of identity, the chloramphenicol exporter from \textit{B. bronchiseptica} was considered as the first representative of a novel class of CmlA-like proteins different from the CmlA proteins. Therefore, it was designated CmlB1. A phylogenetic tree (Figure 1) confirmed the evolutionary distance of the CmlB1 protein from the different CmlA variants. In this regard, it should be noted that the CmlA protein sequences as deposited in the databases varied in size between 390 and 437 amino acids with most of the CmlA proteins having a size of 419 amino acids. The \textit{cmlA} genes coding for proteins of 418 and 419 amino acids have been reported to start with GTG start codon.\textsuperscript{21} This unusual start codon has also been identified in the novel \textit{cmlB1} gene. A closer look at the reading frames for the 390-amino acids proteins (accession nos. AAY43147, AAY43150, ABH07981, ABB71444, CAD31707) strongly suggested that these \textit{cmlA} genes also have the GTG start codon rather than the proposed ATG start codon and thus code for a protein of 419 amino acids as well. A wrong annotation of the start codon (ATC at positions 43516-43518) in the nucleotide sequence of the \textit{cmlA5} gene of \textit{A. baumannii} (CT025832) resulted in the
uncommon size of 437 aa of the respective gene product (CAJ77046). Most likely, the CmlA5 gene of *A. baumannii* also starts with GTG (at positions 43570-43572) and codes for a 419-amino acids protein.

![Phylogenetic tree of CmlA amino acid sequences](image)

**Figure 1.** Phylogenetic tree of the CmlA amino acid sequences deposited in the databases. Branch lengths are scaled according to amino acid exchanges observed in a multi-sequence alignment produced with the DNAMAN software (Lynnon-BioSoft, Ontario, Canada). The numbers at the major branch points refer to the percentage of times that a particular node was found in 10000 bootstrap replications. The bacterial source and the database accession number are given for each CmlA protein.
The analysis of a 2291-bp region encompassing the cmlB1 gene revealed a 582 bp region in the upstream part which differed only by one bp from the respective part in the whole genome sequence of B. bronchiseptica strain RB50 (BX640441). Immediately downstream of the cmlB1 gene, an incomplete reading frame was detected which resembled the N-terminus of a transposase from Marinobacter aquaeolei VT8 (ZP_00818190). Although many cmlA genes are part of gene cassettes located in class 1 integrons, no structures resembling the 5’- and 3’-conserved segments of integrons were detectable up- and downstream of the cmlB1 gene. Moreover, no 59-base element was detectable downstream of the translational termination codon of cmlB1.

In the area immediately upstream of the cmlB1 gene, a putative regulatory region comprising a small reading frame for a 9-amino acid peptide and two pairs of imperfect inverted repeated (IR) sequences of 12 and 10 bp, respectively, were detected. Such an arrangement has also been described for the cmlA1 gene of Tn1696 and assumed to play a role in the chloramphenicol-inducible expression of the cmlA1 gene by attenuated translation. The IR1 sequence in the cmlB1 upstream region was detected immediately after the translational stop codon of the small reading frame whereas the IR4 sequence comprised the start of the cmlB1 gene. Calculation of the mRNA stabilities suggested that IR1:IR2 (ΔG = 90.3 kJ/mol) and IR3:IR4 (ΔG = 79.4 kJ/mol), but also IR2:IR3 (ΔG = 74.4 kJ/mol) may be able to form stable mRNA secondary structures. In addition, the small reading frame also contained a ribosome stall sequence 5’-AAGAAAGCAGAC-3’ which was indistinguishable from that in the small reading frame upstream of the inducibly expressed chloramphenicol resistance gene of the staphylococcal plasmid pC194. All these sequence features may support the assumption that cmlB1 expression is also regulated by translational attenuation. MIC determination of the original cmlB1-carrying B. bronchiseptica isolate and its B. bronchiseptica B543 and E. coli HB101 transformants revealed an up to 16-fold increase in the MICs for chloramphenicol and an up to 8-fold increase in the MICs for florfenicol after pre-incubation in subinhibitory concentrations of chloramphenicol or florfenicol (Table 2).
Table 2. The MICs for chloramphenicol (CHL) and florfenicol (FFC) of the cmlB1-carrying isolates determined with and without induction by CHL or FFC.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>CHL MIC [mg/L] not induced</th>
<th>induced with CHL</th>
<th>FFC MIC [mg/L] not induced</th>
<th>induced with FFC</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. bronchiseptica B1115</td>
<td>64</td>
<td>256</td>
<td>256</td>
<td>8</td>
</tr>
<tr>
<td>B. bronchiseptica B543</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>B. bronchiseptica B543::pKBB1115</td>
<td>16</td>
<td>256</td>
<td>128</td>
<td>8</td>
</tr>
<tr>
<td>E. coli HB101</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>E. coli HB101::pKBB1115</td>
<td>8</td>
<td>32</td>
<td>32</td>
<td>2</td>
</tr>
</tbody>
</table>

1 The test strains comprise the original cmlB1-carrying B. bronchiseptica isolate B1115, but also the recipient strains B. bronchiseptica B543 and E. coli HB101 and their transformants harbouring the cmlB1-carrying plasmid pKBB115.

Inhibition of efflux-mediated phenicol resistance

To investigate efflux inhibition, we used three of the nine floR-carrying isolates, all nine florfenicol-resistant but floR-negative isolates, the two chloramphenicol-resistant isolates and as controls three isolates with lower MICs of 4 mg/L for chloramphenicol and florfenicol. The MIC values for the antimicrobial agents in the absence and in the presence of the efflux inhibitor PAβN are shown in Table 1. In isolates carrying floR, a 2- to 4-fold decrease in the MICs for both phenicols was seen in the presence of PAβN. In contrast, floR-negative florfenicol-resistant isolates showed a distinctly more pronounced susceptibility to both phenicols in the presence of PAβN, as illustrated by an 8- to 32-fold decrease in the corresponding MICs. A very similar situation was seen with the MICs of the B. bronchiseptica isolates classified as intermediately susceptible to florfenicol (MIC 4 mg/L) (Table 1).

Since PAβN interferes with multi-drug efflux systems of the resistance-nodulation-division (RND) family, it may be possible that one or more such systems, which are widespread among Gram-negative bacteria,28 are also present in B. bronchiseptica and may play a role in phenicol resistance. In other bacteria, such as Salmonella enterica, it has been shown that the MIC for florfenicol dropped distinctly in the presence of the efflux pump...
inhibitor PAβN.\textsuperscript{29} Efflux systems of the RND family, like AcrAB-TolC, can also export other antimicrobials such as the quinolone nalidixic acid.\textsuperscript{28} In good accordance with the results for florfenicol and chloramphenicol, the MICs for nalidixic acid of the \textit{B. bronchiseptica} isolates also dropped by three to seven dilution steps in the presence of PAβN (Table 1). While the isolates nos. 1, 2, 12-18, and 20 showed MICs to nalidixic acid of 64 mg/L and 128 mg/L, the isolates with the phenicol-specific efflux pumps FloR or CmlB1 and the isolates nos. 21-23 used for control purposes had lower MICs of 16 mg/L for nalidixic acid. In the presence of PAβN, a MIC of 1-2 mg/L for nalidixic acid was determined for these \textit{B. bronchiseptica} isolates, concluding that they may also harbour one or more not further specified efflux system(s) – putatively also of the RND family – exporting phenicols and/or nalidixic acid. Enhanced expression of RND systems in resistant isolates have been described for the AcrAB-TolC tripartite pump from \textit{E. coli} and \textit{S. enterica}.\textsuperscript{28} In \textit{S. enterica} these pumps conferred lower susceptibility to chloramphenicol, florfenicol and quinolones, but not to ampicillin or streptomycin.\textsuperscript{30,31} In the genome of the completely sequenced \textit{B. bronchiseptica} isolate RB50,\textsuperscript{23} several putative efflux proteins have been identified. One cluster of genes shows homology to genes encoding a RND efflux system common in \textit{Enterobacteriaceae}: a gene encoding for an AcrA homologue (CAE34795), followed by two genes encoding proteins similar to AcrB (CAE34794, CAE24793), and followed by a gene encoding a protein similar to TolC (CAE34792). Further work is needed to clarify whether these putative efflux proteins from \textit{B. bronchiseptica} act as a multi-drug transporter and – if so – what is the substrate spectrum of this efflux system.

In conclusion, the results of this study showed that at least two different phenicol-specific efflux pumps of the MF superfamily, encoded by the genes \textit{floR} and \textit{cmlB1}, but also a not further specified efflux system confer resistance to phenicols in \textit{B. bronchiseptica}. These data complement recent findings on chloramphenicol resistance genes \textit{catB2} and \textit{catB3}, coding for chloramphenicol inactivating enzymes, in porcine \textit{B. bronchiseptica}.\textsuperscript{6}

\textbf{Transparency declaration}

None to declare
Acknowledgements

Kristina Kadlec is supported by a scholarship of the H. Wilhelm Schaumann foundation.

References


Chapter 6

Studies on the mechanisms of β-lactam resistance in *Bordetella bronchiseptica*

Kristina Kadlec, Irith Wiegand, Corinna Kehrenberg
and Stefan Schwarz

The extent of contribution from Kristina Kadlec to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: C
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
**Objectives:** Little is currently known about β-lactam resistance in *Bordetella bronchiseptica*. So far, only a single β-lactamase gene, *bla*$_{\text{BOR-1}}$, has been identified. In a previous study, high MIC values for ampicillin, cephalothin and ceftiofur were determined among 349 porcine *B. bronchiseptica* isolates. The aim of this study was to identify gene(s) associated with elevated MICs for β-lactams and their transferability.

**Methods:** Selected isolates were investigated by PCR for commonly found *bla* genes and class 1 integrons; selected amplicons were sequenced. Plasmid location of resistance genes was confirmed by conjugation. β-Lactamases were characterized by SDS-PAGE and isoelectric focusing. The genomic relatedness of the isolates was investigated by *Xba*I macrorestriction analysis. Inhibition studies with the efflux pump inhibitors were conducted. The permeability of cephalosporins into intact cells was measured exemplarily for one isolate.

**Results:** Of the 349 porcine *B. bronchiseptica*, eight isolates carried a class 1 integron with a *bla*$_{\text{OXA-2}}$ gene cassette on a single conjugative plasmid of ca. 50 kb. In addition, one plasmid-free isolate also carried this class 1 integron. Besides *bla*$_{\text{BOR-1}}$, no other β-lactamase was detected in the remaining isolates with high MIC values for ampicillin. Inhibition experiments suggested that efflux does not play a role in β-lactam resistance. Instead, membrane permeability for cephalosporins was reduced as shown for *B. bronchiseptica* isolate B543.

**Conclusion:** This is to the best of our knowledge the first report of a mobile *bla* gene in *B. bronchiseptica*. Reduced membrane permeability in *B. bronchiseptica* seems to decrease susceptibility against cephalosporins.
Introduction

*Bordetella bronchiseptica* is often involved in respiratory tract infections of mammals and plays an important role in farm animals such as pigs and rabbits as well as in pets, e.g. cats and dogs. *B. bronchiseptica* infections may preferentially develop under conditions where animals are kept at high density, e.g. in intensive animal production systems or animal shelters. Infections with *B. bronchiseptica* may predispose pigs to infections with other respiratory tract pathogens, in particular toxigenic *Pasteurella multocida* which then can cause the severe progressive form of atrophic rhinitis. *B. bronchiseptica* is a zoonotic agent and *B. bronchiseptica* infections causing pneumonia or pertussis-like symptoms in humans are rarely observed. If so, they are most frequently seen in immunocompromised individuals and/or persons with intensive contact to infected animals.

Little is known about β-lactam resistance of *B. bronchiseptica*. High minimum inhibitory concentrations (MICs) to penicillins and cephalosporins have been described for *B. bronchiseptica*. Plasmid-associated resistance to penicillin has also been observed. An oxacillin hydrolysing protein was described in 1974 and a β-lactamase with the molecular weight of 46 ± 3 kDa and the isoelectric point (pI) at pH 8.3 was detected in 1975 in porcine *B. bronchiseptica* isolates. Similar studies on isolates from cats were done more than 20 years later, where a penicillinase of 49 kD and a pI at pH 8.45 was detected. However, in none of these studies the corresponding β-lactamase gene was identified. In 2005, the first β-lactamase gene sequence from *B. bronchiseptica* was published for the chromosomally located species-specific *blaBOR-1* from a human isolate with a MIC for amoxillin of 8 mg/L.

In the present study, 19 isolates from pigs were investigated for the molecular basis of ampicillin resistance with a focus on the association of resistance genes with mobile genetic elements and the possibility of horizontal transfer of the resistance genes. Moreover, the role of efflux in β-lactam resistance was investigated and the diffusion of cephalosporins into intact *B. bronchiseptica* cells was investigated exemplarily for one isolate.
Material and methods

Isolates and susceptibility testing

From 349 porcine *B. bronchiseptica* isolates collected in Germany in 2000 to 2003, the results of susceptibility testing against 15 different antimicrobial agents or combination of agents have been published. As reported, 19 isolates showed MICs of ≥ 32 mg/L for ampicillin and were included in this study. To better describe the β-lactam resistance phenotype, these 19 isolates and 7 further *B. bronchiseptica* isolates which exhibited lower MICs for ampicillin (1-16 mg/L) were tested for susceptibility to additional β-lactams. The 19 isolates with high MIC values for ampicillin, the isolate B543 – used for permeability experiments – and three isolates with lower MICs to ampicillin were chosen to investigate whether efflux mechanisms may play a role. Susceptibility testing was done by broth micro- or macrodilution or by disk diffusion according to the guideline M31-A2 of the Clinical and Laboratory Standards Institute (CLSI).

Detection of β-lactamases

For biochemical β-lactamase characterization, cells were grown to an OD$_{600}$ of 1.0 and then harvested by centrifugation at 4°C. Crude protein extracts were either prepared as described previously using ultrasound treatment for cell disruption or lysozyme treatment (final concentration 0.2 mg/ml) for 15 minutes at room temperature with three additional freeze and thaw steps. The protein content in the crude β-lactamase extracts was determined using bovine serum albumin as standard. In addition, the extract of each isolate was loaded on a SDS-PAGE with 13% (w/v) acrylamide and on an isoelectric focusing (IEF) gel with a pH range of 3.0–10.0 (Bio-Rad, Munich, Germany). Gels were stained with 1mM nitrocefin to detect β-lactamase activity.

Genetic basis of ampicillin resistance

Isolation of plasmids by alkaline lysis and whole cell DNA by phenol/chloroform extraction followed previously described standard protocols. To detect the most common ampicillin resistance genes by PCR, previously described primer sets were used for the detection of
beta-Lactam resistance

\( \text{bla}_{\text{TEM}}^{17}, \text{bla}_{\text{PSE}}^{18}, \text{bla}_{\text{SHV}}^{19,20}, \text{bla}_{\text{ROB}}^{21} \) and chromosomally and plasmid-encoded AmpC beta-lactamase genes in \textit{Enterobacteriaceae}.\(^{22}\) PCRs for the \( \text{bla}_{\text{Bor}}^{13} \) the species-specific beta-lactamase gene from \textit{B. bronchiseptica}, were also done and DMSO was added to a final concentration of 9% (v/v) to the reaction mixture. As beta-lactamase genes are often located on gene cassettes or associated with class 1 integrons, PCRs for conserved regions of class 1 integrons were performed.\(^{16}\)

Conjugation experiments were performed by filter mating with the rifampicin resistant recipient strain \textit{E. coli} HK225 as described previously.\(^{16}\) In addition, \textit{B. bronchiseptica} V1645/2 was used as recipient which has a high MIC for neomycin of 128 mg/L suitable for counter-selection purposes. Transformation into \textit{E. coli} recipient strains JM109 and JM101 (Stratagene, Amsterdam, The Netherlands) followed a previously described protocol.\(^{16}\) For both experiments, LB or blood agar plates containing ampicillin (30 mg/L) were used.

To sequence selected PCR products, cloning experiments were performed into the vector pCR Blunt and the recombinant plasmids were transformed into competent \textit{E. coli} TOP10 cells (Invitrogen, Groningen, The Netherlands). Sequence comparisons were carried out using the BLAST\textsuperscript{®} programs blastn and blastp (http://www.ncbi.nlm.nih.gov/BLAST/) and with the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequence has been deposited in the European Molecular Biology Laboratory (EMBL) database under accession number AJ877267.

Macrorestriction analysis with \textit{XbaI} and pulsed-field gel electrophoresis (PFGE) of the fragment patterns followed a described previously protocol.\(^{16}\)

\textit{Inhibition of efflux mechanisms}

The efflux pump inhibitors Phe-Arg-beta-Naphtylamide (PA\(\beta\)N) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were used for inhibition profiles.\(^{23,24}\) The MICs were determined by macrodilution according to the CLSI guidelines\(^{14}\) and PA\(\beta\)N was added to each tube with a final concentration of 20 – 80 mg/L or CCCP with 4 – 0.5 mg/L, representing \(\frac{1}{4}\) of the strain-specific MIC for these substances.\(^{25}\)
**Diffusion of cephalosporins into intact cells**

We investigated the membrane permeability of one representative *B. bronchiseptica* isolate (B543) to the cephalosporin cefoxitin by using the Zimmermann and Rosselet technique, which requires that the strain under investigation harbours a suitable β-lactamase. The test is based on the fact that β-lactamases in the periplasm of a Gram-negative bacterium act in cooperation with the permeability barrier represented by the outer membrane. At equilibrium, the rate of drug entry equals the rate of hydrolysis within the periplasm. In addition to *B. bronchiseptica* B543, the rifampicin resistant *E. coli* strain HK225 was used for comparison. A plasmid carrying the β-lactamase gene *bla*<sub>CMY-2</sub> was transferred into both strains. The corresponding β-lactamase CMY-2 is capable of hydrolysing cephalosporins of different generations, including the tested antibiotics cefoxitin and cephalothin, but not ceftiofur. The plasmid carrying *bla*<sub>CMY-2</sub> also harbours other resistance genes, including *tet*(A) for tetracycline resistance, and was isolated from the *E. coli* clinical isolate no. 56 provided by K. J. Sherwood (Institut für Medizinische Mikrobiologie, Immunologie und Parasitologie, Universität Bonn, Germany). The plasmid was transferred into *E. coli* HK225 by filter mating as described previously with selection on LB with 50 mg/L cefoxitin and 100 mg/L rifampicin. The plasmid carrying *bla*<sub>CMY-2</sub> was also transferred to *B. bronchiseptica* B543 via electroporation as previously described for *Pasteurella* with the Gene Pulser II electroporation system (Bio-Rad, Munich, Germany) and selection was done on blood agar plates containing 20 mg/L tetracycline. In order to confirm the successful transfer of the plasmid into the transformants and transconjugants, PCR for *bla*<sub>CMY-2</sub> was performed using recently described primers. Furthermore, the strains were tested for the expression of the β-lactamase by determination of the specific β-lactamase activity. β-Lactamase activity was quantified spectrophotometrically by measuring the change in absorbance at 485 nm using 50μM nitrocefin (Oxoid, Basingstoke, UK) as substrate and 0.01M TrisHCl buffer (pH 7.0) as test buffer.

The test for diffusion of cephalosporins into intact *B. bronchiseptica* B543 and *E. coli* HK225 cells was performed as described previously with slight modifications. In brief (Figure 2a), overnight cultures were diluted 1:20 for *E. coli* HK225 and *E. coli* HK225::*bla*<sub>CMY-2</sub> and 1:10 for *B. bronchiseptica* B543 and *B. bronchiseptica* B543::*bla*<sub>CMY-2</sub> and grown in 100 ml cation-adjusted Mueller Hinton bouillon (CAMHB, Oxoid, Wesel,
Germany) supplemented with 5 mM MgCl$_2$ to an optical density (OD) of 0.8 at 650 nm. Cells were harvested, washed twice with ice-cold phosphate buffer (0.1 M, pH 7), resuspended in 30 ml of the same buffer supplemented with 5 mM MgCl$_2$ per 1 g cells. Of this bacterial suspension, 1 ml was dried at 105°C to constant weight. At room temperature, 500 µl of 5 mM cefoxitin was added to 4.5 ml cell suspension. Immediately after addition of the cephalosporin (= time point 0) and after 15, 30 and 60 minutes aliquots of 1 ml were removed, filtered (0.2 µm pore-size filter units) and the filtrates were frozen at -20°C. The same approach was performed with 10 mM cephalothin.

The cephalosporin concentration in the filtrates was measured by a bioassay using *Klebsiella pneumoniae* IV-2-3 as the test organism. For the bioassay 20 ml CAMH agar (Oxoid, Wesel, Germany) cooled down at 50°C was mixed with 80 µl of the *K. pneumoniae* suspension (0.5 McFarland) before pouring the plates. Cavities in the bacteria-supplemented agar were produced with sterile cylinders of 10 mm in diameter. Each cavity was loaded with 100 µl sample, all samples were measured at least three times. For calibration, five sets of two-fold dilutions (2 mM to 0.004 mM) of cefoxitin and cephalothin were used. After 16 to 20 h incubation at 37°C, the inhibition zones were measured and the cephalosporin concentration in the filtrates was determined via the calibration curves. In addition, nitrocefin hydrolysis by the filtrates was measured as described above to check for leaked β-lactamase activity.

**Results**

*Molecular and biochemical basis of ampicillin resistance*

The species-specific *bla*$_{BOR-1}$ gene was detected by PCR analysis in all 19 tested isolated independently of their MIC values for ampicillin. The *bla*$_{BOR-1}$ gene of four isolates with different PFGE patterns and with different MICs for ampicillin (32-128 mg/L) were cloned and sequenced. The *bla*$_{BOR-1}$ genes showed the same nucleotide sequence with 99% identity to the originally described *bla*$_{BOR-1}$ sequence.$^{13}$ Cloning of the complete *bla*$_{BOR-1}$ gene into *E. coli* and subsequent susceptibility testing revealed that all clones were resistant to ampicillin with MICs of $\geq$ 256 mg/L, in comparison to the recipient *E. coli* TOP10 which had a MIC of
4 mg/L. No \( \text{bla}_{\text{TEM}} \), \( \text{bla}_{\text{PSE}} \), \( \text{bla}_{\text{SHV}} \), \( \text{bla}_{\text{ROB-1}} \) or \( \text{bla}_{\text{AmpC}} \) genes could be detected in any of these 19 isolates by PCR.

**Figure 1.** Class 1 integron found in nine *B. bronchiseptica* isolates. The reading frame of the antimicrobial resistance gene \( \text{bla}_{\text{OXA-2}} \) is shown as arrow, the conserved segments of the class 1 integron as boxes. The beginning and the end of the integrated cassette are shown in detail below. The translational start and stop codons are underlined. The 59-base element is shown in bold type, the putative IntI1 integrase binding domains 1L, 2L, 2R and 1R are indicated by arrows. The numbers refer to the positions of the basepairs in the EMBL database entry (AJ877267).

Of the 19 isolates, nine carried a class 1 integron with a single \( \text{bla}_{\text{OXA-2}} \) gene cassette. This \( \text{bla}_{\text{OXA-2}} \) cassette was indistinguishable in its nucleotide sequence from previously described gene cassettes carrying \( \text{bla}_{\text{OXA-2}} \). The \( \text{bla}_{\text{OXA-2}} \) gene coded for a protein of 275 amino acids of which the first 21 amino acids represent a leader peptide that is removed during the maturation process. In contrast to most other gene cassettes, the translational termination codon of the \( \text{bla}_{\text{OXA-2}} \) gene was located downstream of the 1L and 2L integrase binding sites within the 59-base element (Figure 1). This class 1 integron was located on a plasmid of ca. 50 kb in eight isolates. Since this plasmid from each of the eight isolates exhibited the same *Eco*RI and *Pst*I restriction pattern, a common designation pKBB282 was given. Plasmid pKBB282 proved to be conjugative and conferred resistance to ampicillin in *E. coli* HK225 recipients with a 4- to 8- fold increase in the MIC to 16-32 mg/L and in *B.*
bronchiseptica V1645/2 with an 8-fold increase in the MIC to 128 mg/L. The same integron was also detected in one of the remaining plasmid-free isolates (Table 1). PCR assays with the primer sets intI1/5’-CS and 3’-CS/sul1 revealed the expected products for all nine isolates and thus, confirmed the presence of a complete class 1 integron. The corresponding OXA-2 β-lactamase had a molecular weight of 29 kDa and an pI at > pH 8. The calculated pI value for the mature protein was 9.07 (Compute pI/Mw tool; ExPASy, Switzerland).

Table 1. Characteristics of the 19 B. bronchiseptica isolates investigated in this study

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>MIC for [mg/L]</th>
<th>plasmid</th>
<th>bla\textsubscript{OXA-2}</th>
<th>PFGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ampicillin</td>
<td>Amoxycillin / clavulanic acid (2/1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B135</td>
<td>32</td>
<td>8/4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>B282</td>
<td>128</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>B413</td>
<td>64</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>B864</td>
<td>64</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>B942</td>
<td>64</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>B1222</td>
<td>64</td>
<td>8/4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>H1941</td>
<td>128</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>H1942</td>
<td>64</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>H2978</td>
<td>128</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>V3213/2</td>
<td>128</td>
<td>4/2</td>
<td>pKBB282</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>32</td>
<td>4/2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>129</td>
<td>128</td>
<td>4/2</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>452</td>
<td>128</td>
<td>4/2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1465</td>
<td>32</td>
<td>4/2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1468</td>
<td>32</td>
<td>4/2</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1478</td>
<td>32</td>
<td>1/0.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1676</td>
<td>32</td>
<td>8/4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1823</td>
<td>32</td>
<td>8/4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>1897</td>
<td>64</td>
<td>16/8</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

All isolates carrying \textit{bla}\textsubscript{OXA-2} showed closely related macrorestriction patterns: four of them exhibited the most common pattern A and the other five isolates had pattern A* differing from pattern A in one band only. While five \textit{bla}\textsubscript{OXA-2}-negative isolates also showed the most common pattern A, the remaining five \textit{bla}\textsubscript{OXA-2}-negative isolates differed from pattern A by at least two XbaI fragments (Table 1).
No other β-lactamases could be identified in the nine \textit{bla}_{OXA-2}\textsuperscript{-}positive isolates as well as in the remaining ten \textit{bla}_{OXA-2}\textsuperscript{-}negative isolates by SDS-PAGE and IEF. Although the carriage of the \textit{bla}_{BOR-1} gene was confirmed for all 19 isolates, no band corresponding to the BOR-1 β-lactamase with the calculated weight of the mature enzyme of 29.6 kDa (Compute pI/Mw tool; ExPASy, Switzerland) could be observed on the SDS-PAGE stained with nitrocefin. With a calculated pI value of 9.97 (Compute pI/Mw tool; ExPASy, Switzerland), BOR-1 was not expected to be seen on the IEF gels used.

\textit{Additional susceptibility testing}

Although MIC values for ampicillin varied over more than six dilution steps, all selected 20 isolates showed a similar susceptibility profile for the other β-lactam antibiotics tested (Table 2). All isolates showed low MICs to piperacillin, piperacillin/tazobactam and meropenem. However, high MIC values were observed for cephalosporins and the respective inhibitor combinations.

\textbf{Table 2.} MICs to different β-lactam antibiotics of 26 isolates with varying ampicillin MIC values

<table>
<thead>
<tr>
<th>β-lactam</th>
<th>number of isolates with MIC of \textit{[mg/L]}\textsuperscript{1}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤ 2</td>
</tr>
<tr>
<td>ampicillin</td>
<td>2</td>
</tr>
<tr>
<td>cefoxitin</td>
<td>n.t.</td>
</tr>
<tr>
<td>cefepime</td>
<td>1</td>
</tr>
<tr>
<td>cefepime + 4 mg/L clavulanic acid</td>
<td>1</td>
</tr>
<tr>
<td>ceftazidime</td>
<td>1</td>
</tr>
<tr>
<td>ceftazidime + 4 mg/L clavulanic acid</td>
<td>3</td>
</tr>
<tr>
<td>cefotaxime</td>
<td>0</td>
</tr>
<tr>
<td>cefotaxime C + 4 mg/L clavulanic acid</td>
<td>0</td>
</tr>
<tr>
<td>cefpodoxime</td>
<td>0</td>
</tr>
<tr>
<td>cefpodoxime + 4 mg/L clavulanic acid</td>
<td>0</td>
</tr>
<tr>
<td>piperacillin</td>
<td>26</td>
</tr>
<tr>
<td>piperacillin + 4 mg/L tazobactam</td>
<td>26</td>
</tr>
<tr>
<td>aztreonam</td>
<td>0</td>
</tr>
<tr>
<td>meropenem</td>
<td>26</td>
</tr>
</tbody>
</table>

\textsuperscript{1} MICs equal to or lower than the lowest concentration tested are given as the lowest concentration; whereas MICs equal to or higher as the highest concentration tested are given as the highest concentration.

\textsuperscript{2} not tested
Inhibition of efflux pumps

The MIC values for ampicillin and cefoxitin either remained unchanged or decreased by not more than two dilution steps in the presence of the two different efflux pump inhibitors PAβN and CCCP in any of the isolates.

Diffusion of cephalosporins into intact cells

Both test strains, *B. bronchiseptica* B543::*bla*<sub>CMY-2</sub> and *E. coli* HK225::*bla*<sub>CMY-2</sub>, produced high levels of the introduced CMY-2 β-lactamase. Crude protein extracts of the respective parental strains showed only marginal specific β-lactamase activities towards nitrocefin with 0.02 and 0.01 µmol/min/mg protein, whereas the activities were ca. 1000-fold increased in the protein extracts of both strains transformed with the *bla*<sub>CMY-2</sub>-carrying plasmid.

The bioassay with the filtrates of the intact cells revealed that neither the two recipients nor *B. bronchiseptica* B543::*bla*<sub>CMY-2</sub> showed hydrolysis of cefoxitin, whereas for *E. coli* HK225::*bla*<sub>CMY-2</sub> cefoxitin hydrolysis a rate of 95.9 nmol/min/mg dry cells was measured (Figure 2). Similar results were achieved with cephalothin; the cephalothin hydrolysis rate for *E. coli* HK225::*bla*<sub>CMY-2</sub> was 42.57 nmol/min per mg dry cells.

Discussion

In this study, the gene for a plasmid-located β-lactamase (OXA-2) was sequenced for the first time for *B. bronchiseptica*. After the primary description of OXA-2<sup>31</sup> and the first sequence of *bla*<sub>OXA-2</sub> located on the plasmid R46 from *Salmonella* Typhimurium,<sup>32</sup> the *bla*<sub>OXA-2</sub> gene has been detected in a variety of bacterial species, e.g. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

As frequently seen in Enterobacteriaceae, the *bla*<sub>OXA-2</sub> gene in this study was part of a gene cassette in a class 1 integron located on a conjugative plasmid. This plasmid proved to be transferable to *E. coli*. In contrast to other *bla*<sub>OXA-2</sub>-carrying multiresistance plasmids like R46<sup>33</sup> or pB10,<sup>34</sup> no additional resistance except the sulphonamide resistance gene *sul1*, located in the 3’-conserved segment of class 1 integrons, were detected on plasmid pKBB282.
Figure 3. Diffusion of cephalosporins into intact cells; a) schematic presentation of the method – more detailed information is given in the text; b) and c) bioassay with the filtrates of b) *E. coli* HK225::*bla*<sub>CMY-2</sub> and c) *B. bronchiseptica* B543::*bla*<sub>CMY-2</sub>
As recently described for trimethoprim, chloramphenicol on tetracycline resistance genes in *B. bronchiseptica*, the *bla*_{OXA-2} gene is another example for a resistance gene from the respiratory tract pathogen *B. bronchiseptica* which is frequently seen in Enterobacteriaceae, but not in other porcine respiratory tract pathogens.

Although this is the first proof of an OXA-2 enzyme in nine porcine *B. bronchiseptica* isolates, the data from the literature points to a further distribution of class D oxacillinases in *Bordetella bronchiseptica*. The β-lactamase described in *B. bronchiseptica* from pigs in 1974 showed much better oxacillin than benzylpenicillin or ampicillin hydrolysis – this has been also shown for *bla*_{OXA-2}. The two enzymes described later on in *B. bronchiseptica*, one from a porcine isolate and the other from a feline isolate, also hydrolyzed oxacillin efficiently. The molecular weight for the two enzymes that were given in theses studies was approximately 49 kDa. This high molecular weight is unusual for β-lactamases. In both studies column chromatography was used for the determination of the molecular weight and it seems likely that both enzymes were purified as dimers. Dimerisation has been confirmed for the OXA-10 β-lactamase and it was also suggested earlier that other class D β-lactamases, such as OXA-2, form active dimers.

Only nine out of 19 isolates with high MIC values for ampicillin carried *bla*_{OXA-2}, thus, other mechanisms must contribute to reduced ampicillin susceptibility in the remaining ten isolates. Active efflux from the cells is unlikely to play a relevant role in ampicillin resistance, because ampicillin MICs did not change distinctly in the presence of efflux pump inhibitors.

A species-specific β-lactamase from *B. bronchiseptica*, BOR-1, has been described in a human isolate with a MIC value for amoxicillin of 8 mg/L. In the present study, the gene *bla*_{BOR-1} was sequenced from four porcine isolates with different MIC values and different macrorestriction patterns. The sequences were identical and the gene conferred high level amoxicillin resistance to *E. coli*. The significant reduction of the amoxicillin MIC value by clavulanic acid in all ten *bla*_{OXA-2}-negative *B. bronchiseptica* isolates points towards the involvement of a β-lactamase in the high MIC values for amoxicillin. Moreover, hydrolysis of nitrocefin by crude protein extracts proved that all isolates produced an active β-lactamase (data not shown). Since BOR-1 has been shown to be sensitive to inhibition by clavulanic acid and as no other β-lactamases were detected in the isolates, we conclude that different expression levels of *bla*_{BOR-1} might lead to differences in the amoxicillin susceptibility.
In accordance with the high MIC values previously determined for the two cephalosporins cephalothin and ceftiofur, B. bronchiseptica isolates with different ampicillin MICs exhibited the same high MICs for the cephalosporins tested. No significant reduction of resistance was seen in the presence of the the β-lactamase inhibitor clavulanic acid. For E. coli expressing the cloned bla\textsubscript{BOR-1} gene, Lartigue et al. observed no differences in MICs compared to the parent strain for cephalothin, cefoxitin, cefotaxime, cefuroxime, ceftazidime, cefepime and cefpirome. In addition, we also noticed that the MIC for ceftiofur was not increased (data not shown). As B. bronchiseptica only produces the narrow spectrum enzyme BOR-1, other mechanisms have been proposed to play a role in the low susceptibility to cephalosporins.

One possibility for low susceptibility to cephalosporins is an efflux mechanism. Efflux mechanisms have been shown to contribute to β-lactam resistance in Gram-negative bacteria, but appear to have no impact on the MICs of the tested B. bronchiseptica isolates.

Another option is reduced membrane permeability, which has been described as a possible factor contributing to reduced susceptibility against β-lactams and has also been suggested by Lartigue et al. for B. bronchiseptica. In order to investigate the permeability of cephalosporins into intact cells, the broad-spectrum AmpC enzyme CMY-2 was chosen. Cefoxitin hydrolysis by intact B. bronchiseptica cells expressing CMY-2 was not observed during the time period of the experiment, indicating a very low permeability for this antibiotic. Similar results were observed for cephalothin. As low to negligible permeability for the two representative cephalosporins, we postulate that reduced outer membrane permeability plays a relevant role in cephalosporin resistance of B. bronchiseptica.

In conclusion, the detected and sequenced β-lactamase gene \textit{bla}\textsubscript{OXA-2} conferred ampicillin resistance in porcine B. bronchiseptia isolates. In contrast, low susceptibility of porcine B. bronchiseptica isolates to cephalosporins was not based on the production of a β-lactamase, but seems to be due to low membrane permeability of this pathogen.
Acknowledgements

Kristina Kadlec is supported by a scholarship of the H. Wilhelm Schaumann foundation. We thank Patrice Nordmann and Laurent Poirel for the blaBOR-1 positive control as well as Kimberley J. Sherwood for providing the *E. coli* isolate 56. The authors thank Inge Luhmer-Becker for excellent technical assistance and Noha Khalaf for helpful discussions.

References


Chapter 7

General discussion
1. General considerations

*Bordetella bronchiseptica* is an important respiratory tract pathogen from pigs and other domestic and wild animals. In contrast to other pathogens of the genus *Bordetella*, such as *Bordetella pertussis* and *Bordetella parapertussis*, which have adapted to humans only, *B. bronchiseptica* can also cause infections in humans. In pigs, *B. bronchiseptica* is associated with a wide variety of symptoms, ranging from mild rhinitis to severe pneumonia. In commercial swine production respiratory diseases in pigs are often multifactorial diseases, in which bacterial and viral pathogens are involved and in which environmental and management conditions also play an important role. *B. bronchiseptica* is - besides *Actinobacillus pleuropneumoniae* and *Mycoplasma hyopneumoniae* - a primary causative bacterial agent and thereby predisposes infected pigs to secondary infections. *Pasteurella multocida* is the most common opportunistic pathogen and complicates primary infections by the aforementioned bacterial and viral pathogens. In a study from Germany *B. bronchiseptica* was isolated from pigs with pneumonia at a frequency of 6.0% and was accompanied by *P. multocida* in 33.9% of the cases. The multifactorial character of porcine respiratory tract infections has serious implications for the control of this disease. Antimicrobial agents are commonly applied for therapeutic interventions. Although antimicrobial agents are not active against viral pathogens, but only against bacterial pathogens. Moreover, the different bacterial pathogens involved in porcine respiratory tract infections exhibit different intrinsic and acquired resistance properties. While intrinsic resistance properties, such as β-lactam resistance in *Mycoplasma* spp., are genus-specific resistance properties, acquired resistance is a strain-specific resistance property and thus may vary over time depending on the selective pressure as imposed by the use of antimicrobial agents. Based on these latter considerations, most antimicrobial agents approved for the control of respiratory tract infections in pigs are more or less efficient against the one or the other pathogen. In this regard, it is necessary to have valid data on the antimicrobial susceptibility of the respiratory tract pathogens commonly found in pigs. Such data require the use of a sufficiently high number of epidemiologically unrelated bacterial isolates, the performance of *in vitro* susceptibility testing according to an internationally accepted
generations and the evaluation of the test results by following approved veterinary-specific breakpoints – as far as these are available.

2. Antimicrobial susceptibility of porcine *Bordetella bronchiseptica* in comparison to other porcine respiratory tract pathogens

In comparison to other respiratory tract pathogens from pigs, comparatively little is known about the susceptibility of *B. bronchiseptica* to antimicrobial agents. A first large-scale study on the susceptibility of 349 German *B. bronchiseptica* isolates from pigs with respiratory disease was performed [chapter 2]. The pigs, from which the isolates originated, had not been treated with antimicrobial agents before the samples were taken. The minimum inhibitory concentrations (MICs) of the isolates to 15 antimicrobial agents or combinations of agents were determined by broth microdilution according to the international guideline M31-A2 of the Clinical and Laboratory Standards Institute (CLSI). The working group „Antimicrobial Resistance“ of the German Veterinary Society (DVG e.V.) also recommended to follow this guideline when testing bacteria of animal origin for their susceptibility to antimicrobial agents. The MIC values determined in this study confirmed previously published results for the one or the other antimicrobial agent determined for *B. bronchiseptica* isolates from other countries. In many cases, however, the comparison of the results was hampered by the use of different methodologies [chapter 2]. Usually, only studies that use the same methodology and breakpoints, follow the same sampling plan and include sufficiently high numbers of isolates allow reliable comparisons.

In the following subchapters, data for different groups of antimicrobial agents tested are presented for *B. bronchiseptica* and other porcine respiratory tract pathogens. Again, a direct comparison of the data is hampered by the use of different methodologies in the different studies. In addition, different breakpoints for some of the antimicrobial agents tested (as given in the respective guidelines) are occasionally used, which - in turn - may result in different percentages of resistant isolates. Furthermore, the sampling plans for the different studies may vary to a large extent and thus have also an impact on the results. Finally, the numbers of isolates tested also differ distinctly from study to study and it is noteworthy that percentages
of resistant isolates as well as MIC\textsubscript{90} values are particularly influenced by decreasing numbers of isolates tested. Taking into account all these considerations, comparisons between the different studies mentioned in the following subchapters need to be done with extreme caution.

A particular problem with assessing antimicrobial resistance or susceptibility of \textit{B. bronchiseptica} is the use of correct breakpoints. Only for a single antimicrobial agent, namely florfenicol, \textit{B. bronchiseptica}-specific, CLSI-approved clinical breakpoints are available. In the CLSI document M31-S1,\textsuperscript{27} which contains the latest update on approved breakpoints for bacteria of animal origin, more or less non-specific breakpoints - most of which are adopted from human medicine - are available for a number of antimicrobial agents. For some antimicrobial agents, such as gentamicin or cephalothin, no limitations for the use of the breakpoints are given with regard to bacteria or disease conditions, whereas for another small group of antimicrobial agents the limitations for the use of the approved breakpoints only exclude specific groups of microorganisms, e.g. chloramphenicol and tetracycline (organisms other than streptococci), amoxycillin/clavulanic acid (organisms other than staphylococci), and trimethoprim/sulfamethoxazole (organisms other than \textit{Streptococcus pneumoniae}). Moreover, approved breakpoints for bacteria from porcine respiratory tract infections are available for tilmicosin and ceftiofur, but the list of bacteria for which these breakpoints are applicable does not include \textit{B. bronchiseptica}. In the case of enrofloxacin, breakpoints are available only for bovine, but not for porcine respiratory tract pathogens. Finally, there are also antimicrobial agents, such as trimethoprim, neomycin or streptomycin, for which no breakpoints are available at all. Thus, no approved breakpoints for the classification of the isolates are currently available for many of the antimicrobial agents tested in the study presented as [chapter 2]. As a consequence, no percentages of resistant \textit{B. bronchiseptica} isolates were calculated for all antimicrobial agents tested, except for florfenicol.

Based on the fact that - with the aforementioned exception - no approved clinical breakpoints applicable to \textit{B. bronchiseptica} are available, percentages of resistant isolates - as mentioned in the studies cited in the following subchapters - must be considered very carefully. Since the interpretive criteria for the assignment of an isolate as resistant or susceptible are not given in detail in most of these studies, it is impossible to validate these
results in retrospective. As a consequence, percentages of resistant isolates are cited as they appeared in the corresponding original publications.

The data from the German national resistance monitoring\(^{109}\) can be compared directly to the data from \[\text{chapter 2}\], because the same method was used, the same antimicrobial agents were tested and the results for \textit{B. bronchiseptica} are similar to the results from this study.

### 2.1 Trimethoprim and sulfamethoxazole/trimethoprim

Compared to other porcine Gram-negative respiratory tract pathogens, results for trimethoprim showed in a study from the Netherlands that – in good accordance with the data of this study – for \textit{B. bronchiseptica} isolates MIC\(_{50}\) and MIC\(_{90}\) values in agar dilution tests were > 8 mg/L, whereas for \textit{P. multocida} and \textit{A. pleuropneumoniae} isolates MICs of ≤ 2 mg/L were determined (Table 1).\(^{69}\)

#### Table 1. Susceptibility testing of respiratory tract pathogens to trimethoprim

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference no. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. bronchiseptica}</td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>4</td>
<td>16</td>
<td>[chapter 2]</td>
</tr>
<tr>
<td>\textit{B. bronchiseptica}</td>
<td>138</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>≤ 2</td>
<td>16</td>
<td>109 (2004)</td>
</tr>
<tr>
<td>\textit{P. multocida}</td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>≤ 2</td>
<td>64</td>
<td>109 (2004)</td>
</tr>
<tr>
<td>\textit{B. bronchiseptica}</td>
<td>10</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>≥ 8</td>
<td>≥ 8</td>
<td>69 (1990)</td>
</tr>
<tr>
<td>\textit{P. multocida}</td>
<td>10</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.06</td>
<td>0.5</td>
<td>69 (1990)</td>
</tr>
<tr>
<td>\textit{A. pleuropneumoniae}</td>
<td>20</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.25</td>
<td>2</td>
<td>69 (1990)</td>
</tr>
</tbody>
</table>

For the combination of trimethoprim with sulfamethoxazole (1:19) the isolates from the Netherlands showed – similar to the German isolates - a MIC\(_{50}\) of 0.06/1 mg/L and a MIC\(_{90}\) of 4/64 mg/L (Table 2). In contrast, MICs from \textit{P. multocida} and \textit{A. pleuropneumoniae} were ≤ 0.12/2 mg/L.\(^{69}\) \textit{A. pleuropneumoniae} and \textit{P. multocida} isolates from North America and Denmark showed also low MICs (Table 2).\(^{84}\)
### Table 2. Susceptibility testing of respiratory tract pathogens to trimethoprim/sulfamethoxazole (1:19)

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference no. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MIC₅₀</td>
<td>MIC₉₀</td>
<td></td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.12/2.38</td>
<td>4/76</td>
<td>[chapter 2]</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>138</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>≤0.03/0.59</td>
<td>2/38</td>
<td>8.0</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.12/2.38</td>
<td>32/608</td>
<td>27.8</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>74</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.06/1.19⁺</td>
<td>0.25/4.75⁺</td>
<td>84 (2004)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>83</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.03/0.59⁺</td>
<td>0.13/2.38⁺</td>
<td>84 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>931</td>
<td>diverse</td>
<td>similar CLSI</td>
<td>microdilution</td>
<td>&lt;0.25/4.75⁵</td>
<td>8/152</td>
<td>8.0</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>833</td>
<td>diverse</td>
<td>similar CLSI</td>
<td>microdilution</td>
<td>&lt;0.25/4.75⁵</td>
<td>&lt;0.25/4.75⁵</td>
<td>8.0</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>10</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.06/1¹</td>
<td>4/64¹</td>
<td>69 (1990)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>10</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.06/1¹</td>
<td>0.12/2²</td>
<td>69 (1990)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>20</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.03/0.5³</td>
<td>0.03/0.5³</td>
<td>69 (1990)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>17</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>17.6</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td>Pasteurella spp.³</td>
<td>49</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>4.1</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>14</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>18</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>16.7</td>
<td>113</td>
<td>2004</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>70</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>100.0</td>
<td>92</td>
<td>2005</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>50</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>64.0</td>
<td>92</td>
<td>2005</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>81</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>17.2</td>
<td>92</td>
<td>2005</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>46</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>50.0</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>138</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>46.4</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>35</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>2.9</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>32</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>9.4</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>287</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>23.0</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>599</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>7.7</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>99</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>45.5</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>234</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>38.5</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td><em>P. haemolytica</em></td>
<td>44</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>9.1</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>128</td>
<td>Germany</td>
<td>n.i.¹</td>
<td>disk diffusion</td>
<td>58.6</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>1001</td>
<td>Germany</td>
<td>n.i.¹</td>
<td>disk diffusion</td>
<td>19.0</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>102</td>
<td>Germany</td>
<td>n.i.¹</td>
<td>disk diffusion</td>
<td>22.6</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>33</td>
<td>Germany</td>
<td>n.i.¹</td>
<td>disk diffusion</td>
<td>27.3</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>82</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>59.8</td>
<td>44</td>
<td>1998</td>
</tr>
<tr>
<td><em>P. haemolytica</em></td>
<td>11</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>90.9</td>
<td>44</td>
<td>1998</td>
</tr>
</tbody>
</table>

- Sulfadiazine was used instead of sulfamethoxazole.
- The most frequently occurring MIC (mode) is given instead of the MIC₅₀.
- Instead of trimethoprim/ sulfamethoxazole was used in a ratio 1:16 instead of 1:19.
- Only 14 out of 49 isolates were from pigs.
- Interpretative criteria of the producer of the disks (Sanofi).
- no information about the used methodology

Three more recent reports from Switzerland¹¹²,¹¹⁶ and Korea,⁹² in which disk diffusion method was used, revealed the highest resistance rates for *B. bronchiseptica* and the lowest rates for *A. pleuropneumoniae*. In the studies from Switzerland also low rates were determined for *Haemophilus parasuis*. One study with low numbers of isolates revealed high
resistance rates for *P. multocida* and *Pasteurella haemolytica*. For the latter one the old nomenclature from the cited articles is used. In contrast, in the study from Austria high rates where low resistance rates were recorded for *P. multocida* and *P. haemolytica* and distinctly higher rates for *A. pleuropneumoniae, H. parasuis*, and *B. bronchiseptica*. One study from Germany described higher resistance rates for *H. parasuis, A. pleuropneumoniae* and a lower rate for *P. multocida*, while *B. bronchiseptica* had the highest resistance rate of 58.6%.

In summary, *B. bronchiseptica* shows comparatively low susceptibility to trimethoprim and to the combination trimethoprim/sulphonamides as compared to the resistance rates of other porcine respiratory tract pathogens.

### 2.2 Tetracyclines

In comparison to *B. bronchiseptica*, other respiratory tract pathogens showed higher MICs for tetracycline in studies, in which the CLSI guidelines were used (Table 3). Using agar dilution, *B. bronchiseptica* and *A. pleuropneumoniae* isolates from the Netherlands showed MICs for tetracycline in the range from 0.25-1 mg/L and only single isolates had higher MICs. In this Dutch study with low numbers of isolates *P. multocida* isolates were divided into a tetracycline susceptible (s) and a resistant (r) population. An European project called Antibiotic Resistance in bacteria of animal origin - II (ARBAO-II), which collects susceptibility data from various European countries, reports 4 - 22% resistant *A. pleuropneumoniae* from about 900 isolates in 2002 and 5 - 37% in the year 2003; although the aim of the project was to establish broth microdilution in all participating laboratories - data from studies with different methods had been collected. A description of the project and the collected data can be found at http://www.dfvf.dk/Default.asp?ID=9753.

Data obtained with disk diffusion reported no resistant *B. bronchiseptica* isolates in one study and 9.1% and 4.7% in two others. These results correspond well with the low resistance rate of 1.3% in feline and no resistant isolates in canine *B. bronchiseptica* isolates as reported by Speakman et al. In one study very low resistance rates were detected for the four pathogens tested isolates (0 – 3.1%). A study from Germany detected also low rates of 1% (*A. pleuropneumoniae*), 3.3% (*P. multocida*) and 9.1% (*H. parasuis*). In a study from
Austria, resistance rates for *P. multocida* (5.7%), *H. parasuis* (6.4%), *A. pleuropneumoniae* (12.1%) and *P. haemolytica* (25%) were reported. A publication with German isolates reports 15.9% resistant *P. multocida* and 54.5% resistant *P. haemolytica* isolates. In accordance to these results, in isolates from cattle higher MICs were determined for *P. haemolytica* (MIC$_{50}$ of 32 mg/L and MIC$_{90}$ of 64 mg/L) in comparison to *P. multocida* (MIC$_{50}$ of 2 mg/L and MIC$_{90}$ of 16 mg/L).

Table 3. Susceptibility testing of respiratory tract pathogens to tetracycline

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.25 0.5</td>
<td>[chapter 2]</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>138</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.25 0.5</td>
<td>1.4 109 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.5 2</td>
<td>5.7 109 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>74</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>1 16</td>
<td>84 (2004)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>83</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>8 32</td>
<td>84 (2004)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>20</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.5 4</td>
<td>76 (1989)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>17</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.5 0.5</td>
<td>76 (1989)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>19</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>32 32</td>
<td>76 (1989)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>20</td>
<td>Netherlands</td>
<td>Dutch</td>
<td>agar dilution</td>
<td>0.5 0.5</td>
<td>76 (1989)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>17</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>Etest</td>
<td>0.25 0.5</td>
<td>0.0 113 (2000)</td>
</tr>
<tr>
<td><em>Pasteurella spp.</em></td>
<td>49</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td>112 (2004)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>12</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>Etest</td>
<td>1.0 1.5</td>
<td>0.0 113 (2000)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>13</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>Etest</td>
<td>0.5 1.0</td>
<td>0.0 113 (2000)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>46</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>138</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>2.9</td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>35</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>2.9</td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>32</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>3.1</td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>287</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>9.1</td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>599</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>5.7</td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>99</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>12.1</td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>234</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>6.4</td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>P. haemolytica</em></td>
<td>44</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>25.0</td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>128</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>4.7</td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>1003</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>3.3</td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>102</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>1.0</td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>33</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>9.1</td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>82</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>15.9</td>
<td>44 (1998)</td>
</tr>
<tr>
<td><em>P. haemolytica</em></td>
<td>11</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>54.5</td>
<td>44 (1998)</td>
</tr>
</tbody>
</table>

*a* Only 14 out of 49 isolates were from pigs.

*b* Interpretative criteria of the producer of the disks (Sanofi).

*c* no information about the used methodology
These results from tetracycline susceptibility of respiratory tract pathogens indicate that tetracycline is a useful drug to treat *B. bronchiseptica* infections as well as respiratory tract infections caused by other bacteria.\(^{57}\)

### 2.3 Chloramphenicol and florfenicol

In German isolates *P. multocida* has lower phenicol MICs than *B. bronchiseptica* (Table 4).\(^{109}\)

#### Table 4. Susceptibility testing of respiratory tract pathogens to chloramphenicol

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference no. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>4</td>
<td>8</td>
<td>[chapter2]</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>138</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>2</td>
<td>8</td>
<td>109 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.5</td>
<td>1</td>
<td>109 (2004)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>17</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td></td>
<td>112 (2004)</td>
</tr>
<tr>
<td><em>Pasteurella spp.</em></td>
<td>49</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>2.0</td>
<td></td>
<td>112 (2004)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>14</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td></td>
<td>112 (2004)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>18</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td></td>
<td>112 (2004)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>70</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>30.0</td>
<td></td>
<td>92 (2005)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>50</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>10.0</td>
<td></td>
<td>92 (2005)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>81</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>4.9</td>
<td></td>
<td>92 (2005)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>46</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>2.2</td>
<td></td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>138</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>5.1</td>
<td></td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>35</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td></td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>32</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0.0</td>
<td></td>
<td>116 (2001)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>287</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>2.4</td>
<td></td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>599</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>1.2</td>
<td></td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>99</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>1.0</td>
<td></td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>234</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>0.4</td>
<td></td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>P. haemolytica</em></td>
<td>44</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>4.5</td>
<td></td>
<td>60 (1992)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>120</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>10.0</td>
<td></td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>920</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>3.9</td>
<td></td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>98</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>4.1</td>
<td></td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>H. parasuis</em></td>
<td>31</td>
<td>Germany</td>
<td>n.i.</td>
<td>disk diffusion</td>
<td>6.5</td>
<td></td>
<td>4 (1998)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>82</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>1.2</td>
<td></td>
<td>44 (1998)</td>
</tr>
<tr>
<td><em>P. haemolytica</em></td>
<td>11</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>54.5</td>
<td></td>
<td>44 (1998)</td>
</tr>
</tbody>
</table>

* Only 14 out of 49 isolates were from pigs.
* Interpretative criteria of the producer of the disks (Sanofi).
* no information about the used methodology
Disk diffusion tests for chloramphenicol susceptibility revealed in three studies low resistance rates (0 – 5%) for *B. bronchiseptica*, *P. multocida*, *M. haemolytica*, *A. pleuropneumoniae* and *H. parasuis*.\(^{60,112,116}\) In Korean isolates 30% of the *B. bronchiseptica*, 10% of the *P. multocida* and 4.9% of the *A. pleuropneumoniae* isolates tested were considered as resistant.\(^92\) One study from Germany revealed low chloramphenicol resistance rates of \(\leq 10\)% for all porcine respiratory tract pathogens tested.\(^4\) Solely, a study from Germany identified 54.5 % of the 11 *P. haemolytica* isolates tested as chloramphenicol resistant. This is surprisingly high since chloramphenicol has been banned from use in food producing animals in the European Union in 1994.

MIC values were also determined for florfenicol, which revealed that over a four-year monitoring in Germany no resistant *P. multocida* or *A. pleuropneumoniae* isolates were identified, but 25.6%\(^{79}\) [this percentage has been corrected in the meantime to 6.3%\(^{87}\)] and 0.8%\(^{55}\) *B. bronchiseptica* isolates from 2002/2003 and 2003/2004, respectively, were classified as resistant. In total only 4 resistant *P. multocida* isolates (0.9%) were reported.\(^{109}\) The same high susceptibility was described for *A. pleuropneumoniae* and *P. multocida* isolates from Korea, where a single *B. bronchiseptica* isolate had the MIC of 4 mg/L – the authors considered it as resistant, but according to the CLSI interpretive criteria this MIC means intermediate susceptible.\(^92\)

Table 5. Susceptibility testing of respiratory tract pathogens to florfenicol

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference no. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>2</td>
<td>4</td>
<td>2.9 [chapter 2]</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>138</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>(\leq 2)</td>
<td>4</td>
<td>1.4 109 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>2</td>
<td>2</td>
<td>0.9 109 (2004)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>160</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>4</td>
<td>8</td>
<td>6.3 79,87 (2003)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>212</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.25</td>
<td>0.5</td>
<td>0.0 79 (2003)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>45</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.25</td>
<td>0.5</td>
<td>0.0 79 (2003)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>131</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>2</td>
<td>2</td>
<td>0.8 55 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>103</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0 55 (2004)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>63</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.25</td>
<td>0.25</td>
<td>0.0 55 (2004)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>82</td>
<td>Germany</td>
<td>DIN</td>
<td>microdilution</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0 44 (1998)</td>
</tr>
<tr>
<td><em>M. haemolytica</em></td>
<td>11</td>
<td>Germany</td>
<td>DIN</td>
<td>microdilution</td>
<td>(&lt;0.25)</td>
<td>1.0</td>
<td>0.0 44 (1998)</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em></td>
<td>70</td>
<td>Korea</td>
<td>CLSI</td>
<td>microdilution</td>
<td>1.0</td>
<td>2.0</td>
<td>1.4 92 (2005)</td>
</tr>
<tr>
<td><em>P. multocida</em></td>
<td>50</td>
<td>Korea</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0 92 (2005)</td>
</tr>
<tr>
<td><em>A. pleuropneumoniae</em></td>
<td>81</td>
<td>Korea</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0 92 (2005)</td>
</tr>
</tbody>
</table>
The data in Table 5 confirm that florfenicol, which has been licensed in 2000 as an injectable antimicrobial agent for the treatment of respiratory tract infections in pigs, is still highly effective against the target bacteria *P. multocida* and *A. pleuropneumoniae*. However for *B. bronchiseptica*, which is very often associated with *P. multocida* infections, resistant isolates have been identified occasionally. In cattle one florfenicol resistant *P. multocida* isolate from the UK has been described in 2005 and one resistant *Pasteurella trehalosi* isolate from France in 2006. Thus, it can be assumed that resistance genes might also be identified in target bacteria from pigs in the near future.

### 2.4 β-Lactam antibiotics

In good accordance with the results in the study presented in [chapter 2], decreased susceptibility of *B. bronchiseptica* to β-lactam antibiotics was also observed in other studies (Table 6). In contrast to 32.8% ampicillin-resistant *B. bronchiseptica* isolates, resistance rates of ≤ 10% were recorded for *P. multocida*, *H. parasuis*, *A. pleuropneumoniae* and *P. haemolytica* isolates from Austria. Three studies described high resistance rates for *P. multocida* of 19.8%, 46%, and 68%, whereas 37.5%, 100%, and 93% of the *B. bronchiseptica* isolates were identified as ampicillin resistant, respectively. In a report from Switzerland, 58.8% of the 17 *B. bronchiseptica* isolates were considered as resistant to ampicillin, whereas none of the *Pasteurella*, *A. pleuropneumoniae*, or *H. parasuis* isolates was considered resistant. The ampicillin MIC values 16 mg/L for *A. pleuropneumoniae* and 0.5 mg/L for *P. multocida* were determined in one study including North American and European isolates. In contrast to the - in part - very high percentages of resistance to ampicillin, isolates from porcine respiratory tract infections commonly proved to be susceptible to amoxycillin when combined with the β-lactamase inhibitor clavulanic acid.

Concerning their resistance to cephalosporins, *B. bronchiseptica* isolates show a distinctly decreased susceptibility as compared to other porcine respiratory tract pathogens. Examples are given for the two cephalosporins cephalothin (Table 7) and ceftiofur (Table 8).
### Table 6. Susceptibility testing of respiratory tract pathogens to ampicillin

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference no. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>8</td>
<td>16</td>
<td>[chapter 2]</td>
</tr>
<tr>
<td>P. multocida</td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>≤0.12</td>
<td>0.5</td>
<td>109 (2004)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>74</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.13</td>
<td>0.5</td>
<td>84 (2004)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>83</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.13</td>
<td>16</td>
<td>84 (2004)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>969</td>
<td>diverse</td>
<td>similar</td>
<td>CLSI microdilution</td>
<td>≤0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>79 (1994)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>839</td>
<td>diverse</td>
<td>similar</td>
<td>CLSI microdilution</td>
<td>≤0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>≤0.5</td>
<td>79 (1994)</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>17</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>58.8</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td>Pasteurella spp.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>14</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td>H. parasuis</td>
<td>18</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0</td>
<td>112</td>
<td>2004</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>70</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>100.0</td>
<td>92</td>
<td>2005</td>
</tr>
<tr>
<td>P. multocida</td>
<td>50</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>46.0</td>
<td>92</td>
<td>2005</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>81</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>28.4</td>
<td>92</td>
<td>2005</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>46</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>93.5</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td>P. multocida</td>
<td>138</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>68.1</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>35</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td>H. parasuis</td>
<td>32</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>0</td>
<td>116</td>
<td>2001</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>287</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>32.8</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td>P. multocida</td>
<td>599</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>1.5</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>99</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>4.0</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td>H. parasuis</td>
<td>234</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>4.3</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td>P. haemolytica</td>
<td>44</td>
<td>Austria</td>
<td>FDA/WHO</td>
<td>disk diffusion</td>
<td>9.1</td>
<td>60</td>
<td>1992</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>120</td>
<td>Germany</td>
<td>n.i.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>disk diffusion</td>
<td>37.5</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td>P. multocida</td>
<td>887</td>
<td>Germany</td>
<td>n.i.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>disk diffusion</td>
<td>19.8</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>68</td>
<td>Germany</td>
<td>n.i.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>disk diffusion</td>
<td>4.4</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td>H. parasuis</td>
<td>12</td>
<td>Germany</td>
<td>n.i.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>disk diffusion</td>
<td>0.0</td>
<td>4</td>
<td>1998</td>
</tr>
<tr>
<td>P. multocida</td>
<td>82</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>1.2</td>
<td>44</td>
<td>1998</td>
</tr>
<tr>
<td>P. haemolytica</td>
<td>11</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>18.2</td>
<td>44</td>
<td>1998</td>
</tr>
</tbody>
</table>

<sup>a</sup> The most frequently occurring MIC (mode) is given instead of the MIC<sub>50</sub>.  
<sup>b</sup> Only 14 out of 49 isolates were from pigs.  
<sup>c</sup> Interpretative criteria of the producer of the disks (Sanofi).  
<sup>d</sup> No information about the used methodology.

In one study conducted in Switzerland in 2004 53% *B. bronchiseptica* isolates were classified as resistant and another 41% as intermediate, whereas all *P. multocida, M. haemolytica, A. pleuropneumoniae* and *H. parasuis* isolates were susceptible (Table 7).  

Very similar results were seen in a second study from Switzerland published in 2001 and in the German study from 2004.  

113
### Table 7. Susceptibility testing of respiratory tract pathogens to cephalothin

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference no. (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>8</td>
<td>32</td>
<td>[chapter 2]</td>
</tr>
<tr>
<td>P. multocida</td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.25</td>
<td>0.5</td>
<td>109 (2004)</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>17</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>52.9</td>
<td>112 (2004)</td>
</tr>
<tr>
<td>Pasteurella spp.</td>
<td>49</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>0.0</td>
<td>112 (2004)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>14</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>0.0</td>
<td>112 (2004)</td>
</tr>
<tr>
<td>H. parasuis</td>
<td>18</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>0.0</td>
<td>112 (2004)</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>46</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>47.8</td>
<td>116 (2001)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>138</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>0.0</td>
<td>116 (2001)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>35</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>0.0</td>
<td>116 (2001)</td>
</tr>
<tr>
<td>H. parasuis</td>
<td>32</td>
<td>Switzerland</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td></td>
<td>0.0</td>
<td>116 (2001)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>82</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>0.0</td>
<td>18.2</td>
<td>44 (1998)</td>
</tr>
<tr>
<td>P. haemolytica</td>
<td>11</td>
<td>Germany</td>
<td>DIN</td>
<td>disk diffusion</td>
<td>0.0</td>
<td>18.2</td>
<td>44 (1998)</td>
</tr>
</tbody>
</table>

* Only 14 out of 49 isolates were from pigs.

* Interpretative criteria of the producer of the disks (Sanofi).

The extended-spectrum cephalosporin ceftiofur is licensed for the treatment of respiratory tract infections of pigs and cattle. The studies, in which MICs were determined with microdilution, show distinctly lower MICs of < 0.125 mg/L for other respiratory tract pathogens than for *B. bronchiseptica* (≥ 8 mg/L).<sup>80,84,109</sup> In good accordance, MICs determined for bovine *P. multocida* and *M. haemolytica* isolates from 1991<sup>78</sup> and for porcine *P. multocida* and *A. pleuropneumoniae* in 1994<sup>80</sup> and 1995<sup>84</sup> revealed, that all isolates were highly susceptible to ceftiofur (MICs ≤ 1 mg/L, MIC<sub>90</sub> ≤ 0.125 mg/L). To establish breakpoints for bacteria associated with respiratory disease in pigs, susceptibility testing for *B. bronchiseptica* isolates was performed and MICs of 8 - ≥ 32 mg/L were determined; 64% of these isolates had an MIC of ≥ 32 mg/L. In the same study all other bacteria tested had MICs of ≤ 2 mg/L (Table 8).<sup>18</sup>

Among Korean porcine isolates tested for their susceptibility to ceftiofur by disk diffusion, 98.6% (*B. bronchiseptica*), 30% (*P. multocida*) and 5% (*A. pleuropneumoniae*) were considered as resistant.<sup>92</sup>
Table 8. Susceptibility testing of respiratory tract pathogens to ceftiofur

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Country</th>
<th>Guideline</th>
<th>Method</th>
<th>MIC in mg/L</th>
<th>Resistance rate in %</th>
<th>Reference (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MIC₉₀</td>
<td>MIC₅₀</td>
<td></td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>349</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>16</td>
<td>16</td>
<td>[chapter 2]</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>138</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>≥ 8</td>
<td>≥ 8</td>
<td>109 (2004)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>442</td>
<td>Germany</td>
<td>CLSI</td>
<td>microdilution</td>
<td>0.004</td>
<td>0.06</td>
<td>109 (2004)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>74</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>&lt; 0.03</td>
<td>&lt; 0.03</td>
<td>84 (2004)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>83</td>
<td>USA/Denmark</td>
<td>CLSI</td>
<td>microdilution</td>
<td>&lt; 0.03</td>
<td>&lt; 0.03</td>
<td>84 (2004)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>90</td>
<td>diverse</td>
<td>similar CLSI</td>
<td>microdilution</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>80 (1994)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>166</td>
<td>diverse</td>
<td>similar CLSI</td>
<td>microdilution</td>
<td>&lt;0.125</td>
<td>&lt;0.125</td>
<td>80 (1994)</td>
</tr>
<tr>
<td>B. bronchiseptica</td>
<td>70</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>98.6</td>
<td>92</td>
<td>92 (2005)</td>
</tr>
<tr>
<td>P. multocida</td>
<td>50</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>30.0</td>
<td>92</td>
<td>92 (2005)</td>
</tr>
<tr>
<td>A. pleuropneumoniae</td>
<td>81</td>
<td>Korea</td>
<td>CLSI</td>
<td>disk diffusion</td>
<td>4.9</td>
<td>92</td>
<td>92 (2005)</td>
</tr>
</tbody>
</table>

In the studies, in which MICs were determined with broth microdilution, the *B. bronchiseptica* isolates tested showed much higher MICs to cephalosporins, whereas other respiratory tract pathogens were highly susceptible to the cephalosporins cephalothin and ceftiofur. *B. bronchiseptica* has been investigated together with other Gram-negative nonfermentative bacteria for susceptibility to β-lactam antibiotics.⁶,⁷ Compared to these organisms (*Pseudomonas* spp., *Alcaligines* spp. and *Moraxella* spp.) *B. bronchiseptica* has the lowest MIC₉₀ value of 16 mg/L and the MIC₅₀ value of 16 mg/L is in the middle of the observed range (0.5 - > 64 mg/L). In conclusion, *B. bronchiseptica* does not fit into this susceptibility profile of Gram-negative nonfermentative bacteria either.

In summary, the available results reveal that *B. bronchiseptica* is less susceptible to β-lactams than other respiratory tract pathogens. Ampicillin, which is tested as the class representative for aminopenicillins, was at least active against some *B. bronchiseptica* isolates. However, *B. bronchiseptica* and the other respiratory tract pathogens were susceptible to amoxicillin in combination with clavulanic acid, a β-lactamase inhibitor, concluding that the resistance could be due to the production of a β-lactamase. Amoxicillin/clavulanic acid has also been shown to be efficient against *B. bronchiseptica* and for the treatment of pneumonia in pigs⁶⁷, but the development of resistance is possible: Mutations of β-lactamases that lead to an inhibitor-resistant activity of β-lactamases have been already described in *Enterobacteriaceae* such as *E. coli*, *Klebsiella*, *Proteus* and *Citrobacter*.¹⁵
2.5 Macrolides

Tilmicosin is the only macrolide tested in this study, because it is approved for the treatment of respiratory tract infections in pigs. In contrast, the macrolide erythromycin is the most important antimicrobial agent for the treatment of *Bordetella* infections in humans. A good agreement of tilmicosin and erythromycin MICs has been shown for *A. pleuropneumoniae* and *P. multocida*. In this study the 349 porcine *B. bronchiseptica* isolates had a MIC\(_{50}\) value of 16 mg/L and a MIC\(_{90}\) value of 32 mg/L and only four isolates exhibited MIC values of ≤ 2 mg/L. These high MIC values of *B. bronchiseptica* have also been observed in a study from the USA (MIC\(_{50}\) of 32 mg/L and MIC\(_{90}\) of 64 mg/L). For *P. multocida* a MIC\(_{50}\) of 3.13 and a MIC\(_{90}\) of 6.25 mg/L was reported. In vivo studies revealed that *P. multocida* and *H. parasuis* clearance was achieved under tilmicosin treatment, whereas *B. bronchiseptica* was not eliminated from the tilmicosin-treated animals.

In conclusion, tilmicosin cannot be recommended for the treatment of *B. bronchiseptica*, but it is a good antimicrobial agent for the successful elimination of other respiratory tract pathogens in pigs.

3. Resistance genes in *Bordetella bronchiseptica*

Although almost no clinical breakpoints are available for the classification of *B. bronchiseptica* isolates as resistant or susceptible, the MIC values determined in [chapter 2] reflected in a way the susceptibility of the isolates to the antimicrobial agents tested. In some cases, a bimodal distribution of the MICs became apparent with the majority of the isolates reflecting the “susceptible” subpopulation and a small number of isolates representing the “resistant” population. It should be noted that in this case “susceptible” and “resistant” must not be interpreted as responding or non-responding to therapeutic interventions with the respective antimicrobial agent. The “resistant” subpopulation was considered to represent those candidates most likely carrying a gene or a mutation that confers resistance to the respective antimicrobial agent.
At the beginning of this study, very little information on resistance genes present in *B. bronchiseptica* isolates has been available. Only one resistance gene, the tetracycline resistance gene *tet*(C) from feline *B. bronchiseptica*\(^9\) had been identified by Southern blot hybridization. During the course of this study, the sequence of the chromosomal β-lactamase gene *bla*\(_{BOR-1}\) has been published.\(^63\) Studies with the cloned *bla*\(_{BOR-1}\) gene in *E. coli* showed that this gene confers resistance to penicillins, such as ampicillin and amoxycillin, but not to cephalosporins. In this Ph.D. thesis genes conferring trimethoprim or sulphonamide resistance [chapter 3], tetracycline resistance [chapter 4], florfenicol and/or chloramphenicol resistance [chapters 3, 5] or β-lactam resistance [chapter 6] were detected and characterized.

### 3.1 Trimethoprim and sulphonamide resistance genes

Although no breakpoints for trimethoprim and *B. bronchiseptica* are available, the few isolates with high MICs of 32 mg/L and ≥ 64 mg/L were considered as the most promising candidates for the detection of trimethoprim resistance genes. Isolates with high trimethoprim MICs of ≥ 64 mg/L showed also high MICs for the combination of trimethoprim and sulfamethoxazole (≥ 8/152 mg/L). Since 32 mg/L represented the highest test concentration in the micropanels used, additional MIC determination via broth macrodilution was performed and revealed MICs of 128 and 256 mg/L for the isolates with MICs of trimethoprim of ≥ 64 mg/L [chapter 3]. Seven isolates showed trimethoprim MICs of 32 mg/L and neither any of the trimethoprim resistance genes studied nor any of the two most common sulphonamide resistance genes *sul1* and *sul2*\(^39\) could be detected by PCR in these isolates (data not shown).

In contrast, PCR assays revealed, that the trimethoprim resistance gene *dfrA1* was present in five of the six isolates with MIC values of 128 or 256 mg/L. This gene codes for a class A dihydrofolate reductase with a size of 157 aa. The sixth isolate carried the gene *dfrB1* coding for a class B dihydrofolate reductase with a size of 78 aa. Both genes were located on gene cassettes and integrated into a class 1 integron. The integron with the *dfrA1* cassette harboured also a gene cassette carrying the chloramphenicol resistance gene *catB3*. The integron with *dfrB1* harboured also a *catB2* gene cassette. The genes *catB2* and *catB3* code for type B chloramphenicol acetyltransferases, which inactivate chloramphenicol and thus
confer chloramphenicol resistance. All four different gene cassettes showed a complete 59-base element, which is important for the excision and capture of gene cassettes. Parts of the conserved segments, namely the integrase gene \textit{intI} and the sulphonamide resistance gene \textit{sul1}, which codes for a sulphonamide-resistant dihydropteroate synthase, could be detected by PCR. The sequences were deposited in the EMBL database with the accession numbers AJ844287 and AJ9879564.

The gene cassette harbouring \textit{dfrA1} is widespread in \textit{Enterobactericeae} and has been sequenced also from other \textit{Gammaproteobacteria} such as \textit{Acinetobacter}, \textit{Pseudomonas} and \textit{Vibrio}. The \textit{dfrA1} cassette is one of the few gene cassettes that have also been detected in class 2 integrons so far. Although \textit{cat} genes coding for type B CATs are not as widespread as \textit{cat} genes coding for type A CATs, \textit{catB} genes have been detected in a variety of bacteria, including \textit{Gammaproteobacteria} and \textit{Enterobactericeae}. The \textit{catB} genes located on gene cassettes are constitutively expressed from the common promoter of class 1 integrons. Whereas \textit{catB3} cassettes have been sequenced from several species, including - besides \textit{Enterobactericeae} - \textit{Aeromonas} spp. (accession nos. AY740681, AY751518 and DQ462519) and \textit{A. baumannii} (accession nos. DQ532122, AF445082 and AY922989), only eight \textit{catB2} cassette sequences have been deposited in the databases. In \textit{A. baumannii} (accession no. DQ176450) the gene cassette is located in a class 2 integron, but the gene is truncated and the 59-base element is missing. In addition to the \textit{B. bronchiseptica} isolate from this study, another \textit{catB2} cassette has been sequenced from a respiratory tract pathogen of animal origin. This cassette was located on plasmid pJR1 from an avian \textit{P. multocida} isolate (accession no. AY232670).

The combination of a \textit{dfrA1} and \textit{catB3} cassette, however, has been detected very rarely. The same gene cassettes have been detected in a class 1 integron from \textit{Pseudomonas aeruginosa} that carried an additional \textit{aacA4} cassette coding for an aminoglycoside 6'-N-acetyltransferase and conferring tobramycin resistance (accession no. AB195796). The combination of \textit{dfrA1} and \textit{catB3} has also been sequenced from the plasmid pAPEC-O2-R from \textit{E. coli}. The class 1 integron of this isolate harbourered a third gene cassette carrying an \textit{aadA} gene, which codes for an aminoglycoside-3'-adenyltransferase and confers resistance to streptomycin and spectinomycin. Whereas the gene was named \textit{aadA4} in the database entry (accession no. AY214164), it was called \textit{aadA5} in the corresponding article. Database
comparisons revealed that both names have been used for an identical resistance gene: in one database entry from August 1999 the gene was named *aadA4* (accession no. AF180469), although in an earlier entry from March 1999 a gene with 100% identity was named *aadA5* (accession no. AF137361). The latter gene has been described to confer streptomycin and spectinomycin resistance. One year later in 2000, a gene coding for an aminoglycoside-3'-adenyltransferase with 95% identity to *aadA5* was published as *aadA4* (accession no. Z50802).

In contrast to *dfrA1*, database searches for *dfrB1* cassettes with the BLAST® program (available at http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) revealed only four identical gene cassettes (last accessed July 2006). One of them was detected in *E. coli* isolated from meat samples in Norway and the integron harboured also an *aadA1* cassette (accession no. DQ104737). In the remaining three database entries, the integrons carried also a *catB2* cassette in addition to other gene cassettes. Class 1 integrons harbouring *dfrB1* and *catB2* have been described in an uncultured bacterium from a wastewater treatment plant, on plasmid pMVH202 isolated from *Klebsiella pneumoniae* from a human stool sample and in *E. coli* isolated from a patient with urinary tract infection. In the clinical samples, the same plasmid containing a class 1 integron with five gene cassettes was detected in *K. pneumoniae* and *E. coli* (accession nos. AY987853 and 970968). In addition to *dfrB1* and *catB2* the integron harboured *aadA1, aacA4* and *blaVIM-1*, coding for a metallo β-lactamase. In the environmental bacterium from the wastewater treatment plant, the class 1 integron harboured also an *aadA1* cassette coding for streptomycin and spectinomycin resistance (accession no. AY139601). The detection of integrons from environmental samples has been also reported from bacteria isolated from aquatic environments and from soil samples. In one study, the *aadA1* cassette was the most prevalent gene cassette in samples from the North Sea. Class 1 and class 2 integrons harbouring the *dfrA1* gene have also been detected in bacteria from the aquatic environment.

In this study both types of class 1 integrons were detected on two different conjugative plasmids. One of the five isolates with the *dfrA1-catB3* integron was plasmid-free, concluding that the integron and its associated resistance gene cassettes were located on the chromosome. The possibility of horizontal transfer of the resistance genes to other bacterial species was shown: Both plasmids replicated in *E. coli* recipient strains and conferred the expected
resistance properties. The *B. bronchiseptica* isolates carrying the same class 1 integron showed closely related macrorestriction patterns, concluding that the resistance properties could be due to clonal spread of one resistant isolate rather than to horizontal transfer of the plasmid.

Although chloramphenicol has been banned from use in food producing animals in the European Union, chloramphenicol resistance genes were detected in porcine *B. bronchiseptica* isolates. The localization on the described class 1 integron can explain this finding: the genes were co-transferred with genes conferring resistance to trimethoprim and sulphonamides, two antimicrobial agents, which are frequently used in pig production. In contrast to the detection of common trimethoprim resistance genes in *B. bronchiseptica*, none of the known trimethoprim resistance genes was so far described in *Pasteurella* spp., but a new different type of trimethoprim resistant dihydrofolate reductase *dfrA20* was present in a bovine *P. multocida* isolate. The finding of class 1 integrons and of plasmids, that are able to replicate in *E. coli*, indicates a potential gene flow between the respiratory tract pathogen *B. bronchiseptica* and enteric or environmental bacteria.

### 3.2 Tetracycline resistance genes

To look for tetracycline resistance genes, two isolates were investigated, which had much higher MICs of 64 mg/L than the rest of the isolates tested (≤ 2 mg/L) [chapter 4].

In these two *B. bronchiseptica* isolates the tetracycline resistance gene tet(A) and its repressor tetR were detected. The tet(A) gene codes for an efflux protein of the major facilitator superfamily (MFS). This efflux protein confers tetracycline resistance by pumping tetracycline out of the cell. Tetracycline resistance genes (tet genes) are very often located on mobile genetic elements. For tet(A)-tetR, the location on the non-conjugative transposon Tn1721 has been described. In both *B. bronchiseptica* isolates, the tet(A)-tetR gene area was located on a plasmid and the flanking sequences of the resistance gene region revealed perfect homologies to Tn1721, although in both cases not the complete Tn1721 sequence was detected. Tn1721 consists of two parts, one transposase region and the other one the resistance determining region. The transposon shows three 38-bp repeats, two
terminal repeats and one repeat in the middle, dividing the transposase encoding part from the resistance determining part. A truncation of Tn1721 has been described in detail in *Salmonella enterica* and in *E. coli* isolates. In two of the three described Tn1721 relics, the resistance region was complete, whereas in the third case the bases coding for the last nine amino acids of tet(A) were not present on the plasmid pTOJO1 (accession no. AJ307714). This truncation resulted in a different open reading frame coding for a protein of 398 aa instead of the Tet(A) of 399 aa. However, this alteration did not influence the function of this protein to confer tetracycline resistance. The plasmid pTOJO1 was a product of a previously described plasmid, carrying streptomycin and sulphonamide resistance, in which the tetracycline resistance region of 2354 bp was integrated; recombination sites were identified on both sides of the integrated segment. In the second report, an internal part of the transposase region was deleted whereas both terminal repeats of Tn1721 were still present and integration sites were identified by the presence of the characteristic 5-bp direct repeats at the termini of the integrated Tn1721. In the last study, also 5-bp repeats were identified at the integration sites, but the left terminal repeat had been replaced by a shortened internal repeat.

In the first *B. bronchiseptica* isolate, tet(A) was located on a conjugative, ca. 38 kb plasmid pKBB958, which harboured also a class 1 integron carrying the gene cassettes dfrB1 and catB2. The homologous part to Tn1721 comprised 3445 bp (accession no. AM183165). In contrast to the previous report of pTOJO1, plasmid pKBB958 from *B. bronchiseptica* did not show recombination sites, which could explain how the Tn1721 element was truncated.

The second *B. bronchiseptica* isolate carried the tet(A) gene on the non-conjugative plasmid pKBB4037 of about 24 kb. The homologous region to Tn1721 of 5490 bp comprised the whole resistance determining part of Tn1721, including the right terminal repeat, but neither the central repeat nor the left terminal repeat (accession no. AJ877266). Integration sites as described previously for Tn1721 could not be identified. During cloning experiments with EcoRI fragments of this plasmid, a self-ligand of pKBB4037 was produced. This plasmid of 10.5 kb replicated in *E. coli* and conferred tetracycline resistance. The self-ligand was completely sequenced. The proteins encoded upstream of the truncated Tn1721 were not completely identical to proteins from the database, but showed homologies to proteins sequenced from *Pseudomonas* spp., *Xanthomonas citri* or *Nitrosomonas eutropha* (Table 9).
Table 9. Comparison of the proteins sequenced from pKBB4037 to proteins from the database

<table>
<thead>
<tr>
<th>Encoded protein</th>
<th>Position (AJ877266)</th>
<th>Protein length</th>
<th>Amino acid identity to database match</th>
<th>Database match from</th>
<th>Accession no. of database match</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resolvase</td>
<td>245-1132</td>
<td>295 aa</td>
<td>82% to a putative resolvase (283 aa)</td>
<td>Pseudomonas aeruginosa</td>
<td>AAP22618</td>
</tr>
<tr>
<td>ParA1</td>
<td>1221-1853</td>
<td>210 aa</td>
<td>95% to ParA1 (210 aa)</td>
<td>Xanthomonas citri</td>
<td>AA072130</td>
</tr>
<tr>
<td>ParC</td>
<td>2283-2603</td>
<td>106 aa</td>
<td>55% homology to ParC (128 aa)</td>
<td>Pseudomonas aeruginosa; Pseudomonas alcaligenes</td>
<td>CAI46991; YP_245485</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>51% to a hypothetical protein NeutDRAFT_0034 (107 aa)</td>
<td>Nitrosomonas eutropha C71</td>
<td>ZP_00671176</td>
</tr>
<tr>
<td>Rep</td>
<td>2795-4261</td>
<td>488 aa</td>
<td>72.2% to a hypothetical protein NeutDRAFT_0033 (452 aa)</td>
<td>Nitrosomonas eutropha C71</td>
<td>ZP_00671175</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63.8% to RepA (453 aa)</td>
<td>Pseudomonas aeruginosa</td>
<td>CAI46990</td>
</tr>
</tbody>
</table>

The downstream region of the truncated Tn1721 showed identity to an internal part of the transposase gene from transposon Tn5393 (accession no. M96392). This transposon carries the genes strA and strB and confers streptomycin resistance. A truncated Tn1721 in combination with a truncated Tn5393 has been described in the plasmid pB10 derived from an uncultured bacterium isolated from a wastewater treatment plant. In contrast to the sequence of pKBB4037, an inverted repeat of the insertion sequence IS1071 is located between the two transposon relics in pB10 (accession no. AJ564903).

In contrast to other tetracycline resistance genes, tet(A) had not been detected in porcine respiratory tract pathogens so far. In P. multocida isolates from pigs, the genes tet(B) and tet(H) have been detected. The gene tet(B) was located on the chromosome either on the transposon Tn10 or on relics of Tn10. The gene tet(H) has also been detected on the chromosome on a truncated transposon Tn5706. In two porcine isolates, a Tn5706 relic containing intact tet(H)-tetR genes was located on plasmid pMHT1. This plasmid has been also detected in a bovine P. multocida isolate and in Mannheimia spp. isolated from cattle. In H. parasuis also tet(B) was identified on a small plasmid of about 5.1 kb; in this case tetR was not present resulting in a constitutive expression of tet(B). In human Haemophilus isolates Tn10 and a transferable tetracycline resistance had been described previously; in accordance to the isolates from pigs a mutation in tetR led to a constitutive expression of tet(B). In A. pleuropneumoniae, the tetR was also absent on tet(B) carrying plasmids.
Furthermore, *A. pleuropneumoniae* isolates from pigs carried *tet* (H) with its *tetR* and *tet* (L) on plasmids and *tet* (O) on the chromosome.\(^{13}\)

### 3.3 Phenicol resistance genes

Veterinary-specific breakpoints for florfenicol have been approved by the CLSI\(^{27}\) for porcine *B. bronchiseptica*. Thus all 10 resistant isolates with MICs of \(\geq 8\) mg/L were analysed for the presence of phenicol resistance genes. These ten isolates and the six isolates carrying cassette-borne *catB* genes had MICs for chloramphenicol of \(\geq 16\) mg/L, subsequently the remaining two isolates with MIC values for chloramphenicol of \(\geq 16\) mg/L were also investigated for the molecular basis of their phenicol resistance [chapter 5]. In addition, seven porcine isolates and one feline isolate, which had been isolated in the years 2004 to 2006 and identified as florfenicol resistant were also included in this study.

The chloramphenicol resistance genes *catB2* and *catB3* coding for chloramphenicol acetyltransferases type B had been identified in class 1 integrons from *B. bronchiseptica* [chapter 3], but neither these *catB* genes nor class 1 integrons conferring resistance to chloramphenicol and/or florfenicol could be detected in the 20 isolates investigated in [chapter 5]. In Gram-negative bacteria, the efflux protein FloR conferring florfenicol and chloramphenicol resistance has been described.\(^{88}\) This protein also belongs to the major facilitator superfamily (MFS) of efflux proteins.\(^{16}\) The resistance gene *floR* encoding this transporter was detected by PCR in nine *B. bronchiseptica* isolates. A small transposon Tn*floR*, carrying the resistance gene *floR*, has been described in *E. coli*\(^{51}\) and the location of the gene *floR* on plasmids has been observed.\(^{20,28,50,52}\) In this study, Southern blot hybridisation revealed that all *floR* genes were located on the chromosome and an active Tn*floR* could not be identified by inverse PCR. A transfer of the resistance gene to *E. coli* was not achieved. Other resistance genes conferring combined florfenicol and chloramphenicol resistance known so far, namely *fexA*\(^{56}\) and *cfr*\(^{54}\), have been identified in Gram-positive bacteria\(^{51}\) and were not detected in the *B. bronchiseptica* isolates [chapter 5].

In one isolate with a high chloramphenicol MIC of 64 mg/L an internal segment of a *cmlA*-like gene coding for a MFS efflux protein was detected by PCR. Further analysis of the
complete gene and its flanking regions revealed that the gene had less than 80% homology to so far known chloramphenicol exporters of the CmlA subfamily. The gene from *B. bronchiseptica* was therefore named *cmlB1* [chapter 5] and its product was considered as the first representative of a new subclass of CmlB proteins. The new gene *cmlB1* was located on a conjugative plasmid of ca. 50 kb. This plasmid conferred chloramphenicol resistance in *E. coli* recipients and increased the MICs for chloramphenicol and florfenicol when reintroduced into a *B. bronchiseptica* recipient. Sequence analysis of the flanking regions of *cmlB1* revealed, that the gene was not located on a gene cassette, a structure representing the 59-base element could not be identified. Database search identified 29 *cmlA* gene sequences (accessed July 2006) from *E. coli*, *Salmonella* spp., *K. pneumoniae*, *Ps. aeruginosa*, one sequence derived from *Enterobacter aerogenes* and one from *A. baumanii*. While most of them were located on gene cassettes within class 1 integrons, one *cmlA* gene cassette was integrated in a class 2 integron (accession no. AY509004). Most of these integrons harboured also *aadA* gene cassettes and/or a gene cassette with a gene coding for a β-lactamase, only a few carried a trimethoprim resistance gene. In three database entries the *cmlA* gene was not sequenced completely. Two sequenced *cmlA* genes were interrupted or truncated by an insertion element and thus should be functionally inactive. One of these and one intact *cmlA* gene were not located on a gene cassette, but both were flanked by other resistance genes. The truncated *cmlA* was located close to the sulphonamide resistance gene *sul3*. The *A. baumanii* isolate carried several resistance genes, including two intact *cmlA* genes, one of which was located in a class 1 integron also described in *Salmonella* and the other one was located between a class 1 integron harbouring a single trimethoprim cassette and a tetracycline resistance gene region coding for Tet(A).

It could be shown that in the remaining isolates an efflux mechanism is involved in chloramphenicol and florfenicol resistance. MICs were determined with and without the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN). In the isolates carrying MFS transporters, a reduction of the MICs by one or two dilution steps could be seen with the inhibitor. In contrast the isolates without known resistance genes showed a decrease of three to seven dilution steps in the presence of PAβN. These results indicate that an efflux mechanism of the resistance-nodulation-division (RND) family could be responsible for the efflux of chloramphenicol and florfenicol. RND efflux pumps are inhibited by PAβN and
have been described as multi-drug transporters, which can export also chloramphenicol in Gram-negative bacteria. A study on genes responsible for drug efflux has been done in *E. coli*: The susceptibility to several antimicrobial agents was determined in the wild-type strain W3110 and in mutants, in which genes coding for efflux systems were deleted. Results revealed, that intrinsic resistance was mainly due to the RND pump AcrAB in combination with the outer membrane protein TolC. In *Salmonella* Typhimurium DT104, the same phenomenon has been observed for *acrB* and *tolC* deletion mutants of isolates with chloramphenicol MICs of 256 mg/L as well as for one isolate with the MIC of 8 mg/L. A similar efflux system could be responsible for chloramphenicol and florfenicol resistance in *B. bronchiseptica*. A cluster of genes, which show homology to genes encoding this RND efflux system has been identified in the genome of *B. bronchiseptica* RB50: a gene encoding for an AcrA homologue (CAE34795) in combination with two genes encoding proteins similar to AcrB (CAE34794, CAE24793) and a gene coding for a protein similar to TolC (CAE34792). This hypothesis of a species-specific efflux system is supported by the fact that also *B. bronchiseptica* isolates with lower MICs of 4 mg/L for chloramphenicol and florfenicol showed a reduction of three to four steps in MICs determined in the presence of PAβN.

Although *B. bronchiseptica* is not a target bacterium for florfenicol treatment, it is frequently associated with other respiratory tract pathogens, such as *P. multocida*. While only a few florfenicol-resistant pathogens from the family Pasteurellaceae had been reported, florfenicol-resistant *B. bronchiseptica* isolates had been already observed. The resistance mechanism in *B. bronchiseptica* had not been identified so far. In summary efflux mechanisms have been detected to be responsible for florfenicol and chloramphenicol resistance in *B. bronchiseptica* [chapter 5]. These results show again, that genes common in *Enterobacteriaceae*, especially in *E. coli* and *Salmonella* also code for resistance in *B. bronchiseptica*. In contrast to other resistance gens identified in *B. bronchiseptica* so far, the gene *floR* has also been described in *Pasteurella* spp.from cattle. The second phenicol efflux gene identified in *B. bronchiseptica*, *cmlB1*, codes for resistance to chloramphenicol. Chloramphenicol resistance encoded by *cml* genes has been described in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* but not yet in *Bordetella* or in *Pasteurellaceae*. For the identification of the efflux mechanism of the remaining isolates,
further investigation is needed, but based on the results of this study, an efflux system of the RND type can be assumed to be present.

3.4 \(\beta\)-Lactam resistance genes

The occurrence of transferable, plasmid-borne ampicillin resistance in porcine \(B.\ bronchiseptica\) isolates has been observed previously, and it could be shown that the plasmid conferred resistance to \(E.\ coli\) recipient strains.\(^{37,102-104}\) A \(\beta\)-lactamase encoded from one of these plasmids was characterised: it had a molecular weight of about 46 kDa and an isoelectric point of approximately pH 8.3. This \(\beta\)-lactamase hydrolysed several penicillins with the best activity for oxacillin, but had low rates for hydrolysis of cephalosporins - one of the tested cephalosporins was cephalothin.\(^{119}\) The isolates for the present study were chosen in accordance with the results of the susceptibility testing, which showed that MICs to the cephalosporins cephalothin and ceftiofur were high for all isolates tested – leading to the conclusion that no single isolates had better resistance properties than others. In contrast, ampicillin MICs for the isolates differed and ranged from 1 mg/L to 128 mg/L [chapter 2]. For the detection of \(\beta\)-lactam resistance genes, isolates with high MICs for ampicillin of \(\geq 32\) mg/L were chosen [chapter 6]. The \(\beta\)-lactamase gene \(bla_{OXA-2}\) was identified. It conferred ampicillin resistance, but not resistance to cephalosporins in \(E.\ coli\). This resistance gene was identified in nine out of 19 isolates. These nine isolates had MIC values for ampicillin of 64 or 128 mg/L. The gene \(bla_{OXA-2}\) was located on a gene cassette and integrated into a class 1 integron. The presence of the conserved segments (CS) of the class 1 integron was confirmed by PCR. The integrase in the 5’-CS and the sulfonamide resistance gene \(sul\), which codes for a sulfonamide resistant dihydropteroate synthase and is part of the 3’-CS of class 1 integrons were detected. In eight of the nine isolates the integron was located on a conjugative plasmid of ca. 50 kb. The plasmid conferred resistance to \(E.\ coli\) recipients and in one \(B.\ bronchiseptica\) recipient the MIC for ampicillin increased from 8 mg/L to 128 mg/L. The corresponding protein OXA-2 had a molecular weight of about 29 kDa and an isoelectric point of pH 8. This \(\beta\)-lactamase belongs to the Bush, Jacoby, Medeiros group 2d.\(^{19}\) The members of this group hydrolyse
oxacillin better than other penicillins. The finding of an OXA-type β-lactamase fits to the previously described enzyme from *B. bronchiseptica*. In the isolates which did not carry *bla*OXA-2, no other β-lactamase was identified.

The species-specific β-lactamase *bla*BOR-1 from *B. bronchiseptica* has been described recently. This enzyme was sequenced from a *B. bronchiseptica* isolate, which had an ampicillin MIC of 8 mg/L - which is the MIC₅₀ and the most common MIC in the 349 isolates tested [chapter 2]. When cloned into *E. coli*, it conferred high-level resistance to ampicillin (MIC 512 mg/L) to its new host while the *E. coli* recipient remained highly susceptible to cephalosporins. Four *bla*BOR-1 PCR products amplified from *B. bronchiseptica* isolates with different ampicillin MICs were cloned and sequenced in this study. The four sequences were identical and showed only a single nucleotide exchange compared to the reported sequence. These results showed, that the species-specific β-lactamase gene *bla*BOR-1 is highly conserved in *B. bronchiseptica*. Possibilities for further investigations of the *B. bronchiseptica* isolates could be to determine the expression of the species-specific β-lactamase BOR-1. The derepression or the upregulation of the species-specific β-lactamase from *E. coli* AmpC has been described to result in high expression of the enzyme and subsequently in resistance of *E. coli* isolates. In this study, detection of the β-lactamases with nitrocefin was performed and BOR-1 was not seen on the protein gels. This observation has also been made in other species which carry a species-specific β-lactamase. Depending on the amount of total protein extract loaded onto the gel, either a weak band or no band could be seen. If the β-lactamase was overexpressed, it would have been visible on the protein gels stained with nitrocefin. One other difficulty has also to be taken into account: the β-lactamase BOR-1 has an isoelectric point of pH >9 and the used gels for isoelectric focussing range from 3-10, concluding that the protein might be close to or at the terminus of visible range. To exclude this possibility also protein gels were prepared as SDS-PAGE in which BOR-1 with a size of 32 kDa would have been detected. However, no BOR-1-specific band was seen on these gels suggesting that this enzyme was expressed at a level too low for detection in crude protein extracts.

Because β-lactamases could not explain the higher MICs for all of the 19 *B. bronchiseptica* isolates, other mechanisms were also investigated. Efflux mechanisms have been described to contribute to β-lactam resistance. Selected isolates with high and lower MICs to ampicillin were investigated. MICs with and without efflux inhibitors were
determined for ampicillin and no difference could be seen – showing, that efflux mechanisms are not involved in the decreased susceptibility of *B. bronchiseptica* to β-lactams. Low permeability of the outer membrane has been described to contribute to β-lactam resistance, too. Reduced membrane permeability has been shown exemplarily in one *B. bronchiseptica* isolate for the cephalosporins cefoxitin and cephalothin, concluding that this mechanism plays a role in low susceptibility to β-lactams of *B. bronchiseptica* isolates.

One possibility that could be investigated in following studies is the mutation of penicillin binding proteins (PBP). Whereas the mutated PBP2a is known in methicillin resistant *Staphylococcus aureus* (MRSA), this mechanism of altered PBP has been recently described also in the Gram-negative human respiratory tract pathogen *Haemophilus influenzae*. While in one report also a β-lactamase was expressed and the mutated PBP only contributed to resistance, another study described high MICs for isolates without a β-lactamase. A third study, also from 2006, showed increased MICs for isolates with different mutations in PBP3.

In summary in nine from 19 *B. bronchiseptica* isolates with high MICs of ≥ 32 mg/L for ampicillin the β-lactamase *bla*OXA-2 was identified on a gene cassette in a class 1 integron. In eight from these nine isolates the resistance genes were located on a conjugative 50 kb plasmid, which conferred resistance to ampicillin. The high MICs of the remaining isolates cannot be explained by a β-lactamase. In addition, the low susceptibility to cephalosporins of all *B. bronchiseptica* isolates tested cannot be explained by resistance genes. While efflux mechanism do not seem to play a role in *B. bronchiseptica*, low membrane permeability could be shown and does very likely contribute to the decreased susceptibility of *B. bronchiseptica* to β-lactam antibiotics.

### 4. Localization of resistance genes on mobile genetic elements

All resistance genes detected in the *B. bronchiseptica* isolates could be transferred to *E. coli* recipient strains and proved to be functionally active by conferring the respective resistance properties in these new hosts. The sequences of the resistance genes and their
General discussion

flanking regions were analysed [chapters 3-6] and most of the genes were located on mobile genetic elements.

4.1 Gene cassettes and class 1 integrons

Trimethoprim (dfrA1 and dfrB1), chloramphenicol (catB2 and catB3) and ampicillin (blaOXA-2) resistance genes were identified on gene cassettes, which were integrated in class 1 integrons [chapters 3,6]. In the study representing [chapter 3], class 1 integrons were detected for the first time in the respiratory tract pathogen B. bronchiseptica. Class 1 integrons are able to mobilize and capture gene cassettes carrying resistance genes. Integrons occur frequently in Enterobacteriaceae and have been described also in bacteria of the normal intestinal flora of pigs. Spread of Salmonella isolates carrying integrons from animals to humans via the food chain has been reported. Class 1 integrons have not been detected in common porcine respiratory tract pathogens. However, class 1 integrons have been described in avian P. multocida isolates that carried a plasmid harbouring several resistance genes. In these isolates the gene for the integrase of class 1 integrons intI was truncated. In [chapters 3,6], two types of integrons with different gene cassettes carrying resistance genes to trimethoprim and chloramphenicol and another type harbouring a single gene cassette coding for an OXA-2 β-lactamase were sequenced from porcine B. bronchiseptica isolates. While the trimethoprim resistance gene dfrA1 has been frequently found in Enterobacteriaceae, dfrB1 and the two chloramphenicol resistance genes are not so common.

The identification of a β-lactamase of the OXA-type corresponds to the observation, that oxacillin hydrolysing enzymes have been described earlier in B. bronchiseptica, however, without determination of the type of bla gene. In general, integrons are very often located on plasmids and in the studies representing [chapters 3, 6], the three different types of class 1 integrons and their associated gene cassettes were located on conjugative plasmids. It could be shown, that these conjugative plasmids replicate in E. coli and confer the expected resistance properties. For the plasmid harbouring the class 1 integron with the blaOXA-2 gene cassette, transformation back into a B. bronchiseptica isolate, that did not carry any plasmid,
was performed. With the acquisition of the respective plasmid, the MIC for ampicillin increased from 8 mg/L to 256 mg/L for the *B. bronchiseptica* isolate used as recipient in this approach. This experiment confirmed that the cassette-borne *bla*<sub>OXA-2</sub> gene is functionally active in *B. bronchiseptica*.

### 4.2 Transposons

In addition to integrons and plasmids, transposons which carry a wide variety of resistance genes have been identified as mobile genetic elements. So far, no transposons associated with antimicrobial resistance have been detected in *B. bronchiseptica*.

In [chapter 4], the tetracycline resistance gene *tet*(A) has been identified on plasmids in two different *B. bronchiseptica* isolates. This *tet* gene is known to be located on the 11.9-kb non-conjugative transposon Tn1721. Analysis of the sequences up- and downstream of the two plasmid-borne *tet*(A) genes identified incomplete copies of Tn1721. Although both truncated Tn1721 copies differed from one another, the part responsible for the transposition functions was deleted in both cases. As a consequence, both Tn1721 relics lost their ability to transpose. The tetracycline resistance gene *tet*(A) has been identified in different members of the family *Enterobacteriaceae*, also located on either complete or truncated Tn1721 elements. Although *tet*(A) was located on truncated transposons in both *B. bronchiseptica* isolates, horizontal spread to other bacteria is possible, due to their localization on plasmids, one of which proved to be conjugative. This conjugative plasmid also harboured one of the class 1 integrons described in [chapter 3].

For the chloramphenicol and florfenicol resistance gene *floR* a transposon Tn*FloR* has been described in *E. coli* and the location on plasmids has been observed. In this study neither the same structure nor the localization of the gene *floR* on a mobile genetic element could be identified [chapter 5].
4.3 Plasmids

In contrast to Pasteurella and Mannheimia, where the resistance genes are commonly located on small mobilizable plasmids, B. bronchiseptica carries its resistance properties on large plasmids.\textsuperscript{37,64,90,102,118} In these studies the resistance genes had not been identified, but resistance properties had been shown by transfer of the plasmid. Other studies describe a correlation between the carriage of plasmids and resistance properties in B. bronchiseptica.\textsuperscript{48} Another interesting finding is, that B. bronchiseptica isolates from the present study mostly carried only single plasmids. Mainly large plasmids of ca. 50 kb in size were detected while smaller plasmids of 10 kb - not associated with antimicrobial resistance - were also detected (data not shown). While this localization of resistance genes corresponds to previous reports on B. bronchiseptica plasmids.\textsuperscript{64} This finding is in contrast to the situation in other porcine respiratory tract pathogens, such as Pasteurella spp., where usually more than one plasmid was isolated and the majority of plasmids was 2 - 6 kb in size\textsuperscript{46} or as A. pleuropneumoniae in which resistance plasmids with a size of ca. 6 kb\textsuperscript{13,38} and 8 kb\textsuperscript{47} have been described.

5. General comparison within the genus Bordetella

5.1 B. bronchiseptica isolated from cats

This study has shown similarities and differences to the earlier investigated feline B. bronchiseptica isolates. In contrast to different resistance genes coding for resistance to tetracycline in feline and porcine isolates, the β-lactamase located on the same plasmid as \textit{tet}(C)\textsuperscript{95} seems to be similar to the one detected in porcine isolates in this project [chapter 6]: In the feline isolate the substrate profile of the β-lactamase was analysed and revealed, that most probably an oxacillinase, a β-lactamase belonging to the Bush, Jacoby and Medeiros\textsuperscript{19} group 2d, is located on the same plasmid. To draw further conclusions on whether B. bronchiseptica isolates from different sources, e.g. from companion animals or human patients, carry the same or different resistance genes, further investigations on B. bronchiseptica isolates from other sources have to be done.
5.2 Susceptibility and resistance in *Bordetella* spp. from human infections

In human medicine, mainly macrolides are used for the treatment of *Bordetella* infections. The causative agents for disease in humans, *B. pertussis* and *B. parapertussis*, show a relatively high susceptibility to erythromycin,\(^{43}\) which is tested as the class representative for macrolide antibiotics. Although susceptibility testing has shown that the susceptibility of *B. bronchiseptica* to erythromycin was low, the use of macrolides has nevertheless been recommended in human infections with *B. bronchiseptica*.\(^{49,91}\) Resistance to macrolides have been investigated in *B. pertussis*, but it is likely that the mechanisms of resistance could be similar in other species of the genus *Bordetella*. In *B. pertussis*, mutations of 23S rRNA have been described as the only resistance mechanism so far\(^8\) and erythromycin resistance based on mutations in the 23s rRNA has been found in a variety of bacteria.\(^{108}\)

So far unpublished data obtained during this Ph.D. project suggested that efflux may also play a role in macrolide resistance. Using the efflux pump inhibitor PAβN, the MIC for erythromycin decreased from 64 mg/L to 0.5 mg/L in the feline *B. bronchiseptica* isolate, suggesting that an efflux pump of the RND-type might be involved. Efflux pumps of the RND family, that include erythromycin in their substrate profile have been described in other Gram-negative bacteria, such as *E. coli*\(^{73}\) and *E. aerogenes*\(^{24}\) (AcrAB-ToIC system), *Ps. aeruginosa* (MexAB-OprM and MexXY-OprM system), *Burkholderia pseudomallei* (AmrAB-OprA system), *Campylobacter jejuni* and *Campylobacter coli*\(^{21}\) (CmeAB-CmeC system), and *Stenotrophomonas maltophilia* (SmeDE-SmeF system).\(^{66}\) In the respiratory tract pathogen *H. influenzae*, the disruption of one of the genes HI0894 or HI0895, which are homologous to *acrA* and *acrB* from *E. coli*, resulted in a strain highly susceptible to erythromycin.\(^{85}\) In this feline *B. bronchiseptica* isolate, the susceptibility to chloramphenicol, florfenicol and nalidixic acid was also higher in the presence of the efflux inhibitor PAβN [chapter 5]. It remains to be answered, if the same efflux pump is responsible for the transport of these structurally different antimicrobial agents or if different efflux mechanisms export macrolides, phenicols and quinolones.
6. **General conclusion**

The results of this Ph.D. thesis showed that porcine *B. bronchiseptica* isolates carry resistance genes on mobile genetic elements. Some of the identified resistance genes or similar genes have been described in *Enterobacteriaceae*, but most of them not yet in porcine respiratory tract pathogens. In conclusion this study showed, that porcine *B. bronchiseptica* isolates carry resistance genes on large conjugative plasmids. Horizontal transfer to a different species was shown, the plasmids replicated in *E. coli* and conferred resistance to the recipients. The resistance genes harboured by *B. bronchiseptica* were identical or similar to isolates from the gastro-intestinal tract or from the environment, but differed from the resistance genes occurring in *Pasteurellaceae*. Based on the mobile character of the identified resistance genes, it is likely that *B. bronchiseptica* has acquired these genes from enterobacterial hosts, in which these genes commonly occur.
References


87. Schwarz S. 2006. personal communication.


Chapter 8

Summary
Kristina Kadlec: Detection and Organization of antimicrobial resistance genes in *Bordetella bronchiseptica* isolates from pigs

*Bordetella bronchiseptica* is a Gram-negative respiratory tract pathogen. In pigs, it causes various symptoms ranging from mild rhinitis to severe pneumonia. Moreover, *B. bronchiseptica* infections predispose pigs to infections with other pathogens, e.g. toxigenic *Pasteurella multocida*. Antimicrobial agents are frequently used to control respiratory tract infections in swine production. In contrast to other well-studied respiratory tract pathogens, little is known about antimicrobial resistance and resistance genes in *B. bronchiseptica*. The aims of this study were to 1) provide an overview on antimicrobial resistance of German *B. bronchiseptica* isolates from pigs, 2) detect genes that confer resistance to selected antimicrobial agents in *B. bronchiseptica*, and 3) identify their localization on mobile genetic elements and their transferability.

In the first part of the study 349 *B. bronchiseptica* isolates, which had been collected from pigs with respiratory tract disease between 2000 and 2003, were investigated for their susceptibility to 15 antimicrobial agents or combinations of agents by microdilution according to CLSI recommendations [chapter 2]. Interpretation of these results was hampered by the lack of breakpoints for a classification of the minimum inhibitory concentrations (MICs) into one of the three categories “susceptible”, “intermediate” or “resistant”. Such approved *B. bronchiseptica*-specific breakpoints are currently available only for a single antimicrobial agent, namely florfenicol. For florfenicol, 2.9% of the isolates were resistant and 17.5% were classified as intermediately susceptible. The *B. bronchiseptica* isolates differed in their antimicrobial susceptibility from other porcine respiratory tract pathogens, such as *Pasteurella multocida* or *Actinobacillus pleuropneumoniae*.

For the following parts of the study, isolates were chosen for further investigations on the basis of their MICs [chapters 3-6]. Six isolates showed high MICs to trimethoprim and genes coding for trimethoprim-resistant dihydrofolate reductases (*dfr* genes) were identified [chapter 3]. These genes were located on gene cassettes. In all six isolates, a class 1 integron harboured the *dfr* cassette in combination with a *catB* cassette coding for a type B chloramphenicol acetyltransferase, which confers resistance to chloramphenicol, but not to florfenicol. In one isolate, a *dfrB1* cassette and a *catB2* cassette were sequenced. The
remaining five genetically related isolates carried the resistance gene cassettes *dfrA1* and *catB3*. Both types of class 1 integrons were located on conjugative plasmids.

In two tetracycline-resistant isolates, the tetracycline resistance gene *tet(A)* coding for a tetracycline exporter was identified on two different conjugative or non-conjugative plasmids [chapter 4]. The resistance gene *tet(A)* and its repressor gene *tetR* were part of truncated transposon Tn1721 relics in both cases. The conjugative plasmid harboured also the *dfrB1-catB2* integron and sequencing of the regions flanking region the *tetR-tet(A)* part identified a stretch of 3445 bp, which were homologous to Tn1721. The non-conjugative tetracycline resistance plasmid showed homology to Tn1721 for 5490 bp. From this latter plasmid, an *Eco*RI self-ligand was produced, sequenced completely and a new gene for plasmid replication was identified. The encoded replication protein (Rep) showed 72.2% and 63.8% homology to Rep proteins from *Nitrosomonas eutropha* and *Pseudomonas aeruginosa*, respectively.

According to the CLSI-approved breakpoints, ten of the 349 *B. bronchiseptica* isolates were classified as florfenicol-resistant. In addition, seven porcine florfenicol-resistant isolates collected in 2004-2006 and one feline florfenicol-resistant isolate from 2005 were included [chapter 5]. Nine of the 18 florfenicol-resistant isolates carried the gene *floR* in the chromosomal DNA. This gene codes for an efflux protein of the major facilitator (MF) superfamily and confers resistance to chloramphenicol and florfenicol. The isolates carrying *floR* were plasmid-free and showed similar macrorestriction patterns.

The remaining nine florfenicol/chloramphenicol-resistant but *floR*-negative isolates showed distinctly decreased MICs to florfenicol and chloramphenicol, when exposed to the efflux pump inhibitor Phe-Arg-β-naphthylamide, concluding that a not further specified efflux mechanism is responsible for florfenicol/chloramphenicol resistance in these isolates [chapter 5].

Moreover, a novel gene, *cmlB1*, coding for a chloramphenicol exporter of the MF superfamily, was detected in an isolate with a high MIC for chloramphenicol of 128 mg/L [chapter 5]. The gene *cmlB1* was located on a conjugative 50 kb plasmid. The corresponding gene product showed 73.8-76.5% homology to the known CmlA proteins. In contrast to most of the sequenced *cmlA* variants, the gene *cmlB1* was not located on a gene cassette.
The *B. bronchiseptica* isolates from this study showed a low susceptibility to β-lactams. For the detection of β-lactam resistance genes, 19 isolates with high MICs to ampicillin were used [chapter 6]. In nine isolates with ampicillin MICs of 64 and 128 mg/L, the β-lactamase gene *bla*<sub>OXA-2</sub> was identified. This gene was located on a gene cassette in a class 1 integron. The gene *bla*<sub>OXA-2</sub> was detected in eight of the isolates on a 50 kb conjugative plasmid, which conferred ampicillin resistance in *E. coli* hosts, and in the chromosomal DNA in the ninth isolate. The nine *bla*<sub>OXA-2</sub>-positive isolates showed related macrorestriction pattern, whereas other isolates with high ampicillin MICs differed in their patterns. The species-specific β-lactamase from *B. bronchiseptica* BOR-1, which also mediates ampicillin resistance in *E. coli*, was detected in *B. bronchiseptica* isolates with different ampicillin MICs. No further β-lactamases could be detected by isoelectric focusing or SDS-PAGE. Susceptibility testing in the presence of efflux inhibitors revealed, that efflux mechanisms are not involved in β-lactam resistance. Studies on the membrane permeability of a selected *B. bronchiseptica* isolate showed, that the cephalosporins cefoxitin and cephalothin are not able to efficiently enter the cell. Thus, a low membrane permeability may also account for the low susceptibility of *B. bronchiseptica* to β-lactam antibiotics.

In porcine *B. bronchiseptica* isolates the resistance genes identified differ distinctly from those detected in other respiratory tract pathogens of pigs, such as *P. multocida*. The majority of genes was located on mobile genetic elements. Class 1 integrons, which are common in *Enterobacteriaceae* but not in *Pasteurellaceae*, were detected. Plasmid-borne genes could be transferred to *E. coli* recipient strains and conferred the expected resistance properties in these bacteria. This observation points towards an exchange of resistance genes between *Enterobacteriaceae* and *B. bronchiseptica*. 
Zusammenfassung

Chapter 9

Zusammenfassung
Kristina Kadlec: Identifizierung and Organisation antimikrobieller Resistenzgene von *Bordetella bronchiseptica* Isolaten vom Schwein


Für die weiteren Untersuchungen hinsichtlich der molekularen Grundlagen der antimikrobiellen Resistenz wurden Isolate auf der Basis der MHK-Werte ausgewählt [Kapitel 3-6]. Sechs Isolate zeigten hohe MHK-Werte für Trimethoprim und Gene (*dfr* Gene), die für resistenzvermittelnde Dihydrofolat-Reduktasen kodieren, wurden identifiziert [Kapitel 3].


Die übrigen neun florfenicol-/chloramphenicolresistenten Isolate, die floR-negativ waren, zeigten deutlich reduzierte MHK-Werte gegenüber Florfenicol und Chloramphenicol in Gegenwart des Effluxinhibitors Phe-Arg-β-Naphthylamid [Kapitel 5]. Diese Inhibition deutete darauf hin, dass wahrscheinlich in diesen Isolaten ein noch nicht weiter spezifizierter Effluxmechanismus für Florfenicol-/Chloramphenicolresistenz verantwortlich ist.
Ferner wurde in einem Isolat mit einem hohen MHK für Chloramphenicol von 128 mg/L ein neues Gen, \textit{cmlB1}, identifiziert \textbf{[Kapitel 5]}. Es kodiert für einen Chloramphenicolexporter vom MFS-Typ. Das Gen \textit{cmlB1} war auf einem konjugativen Plasmid von ca. 50 kb lokalisiert. Das entsprechende CmlB1-Protein zeigte 73,8-76,5\% Homologie zu den bislang bekannten CmlA-Proteinen. Im Gegensatz zu den meisten der sequenzierten \textit{cmlA}-Varianten, war das Gen \textit{cmlB1} nicht auf einer Genkassette lokalisiert.


Die Resistenzgene, die in porcinen \textit{B. bronchiseptica}-Isolaten identifiziert wurden, unterschieden sich deutlich von denen anderer Erreger respiratorischer Erkrankungen, wie \textit{P. multocida}. Ein Großteil der Gene war auf mobilen genetischen Elementen lokalisiert. Klasse 1 Integrons, die häufig in \textit{Enterobacteriaceae} aber nicht in \textit{Pasteurellaeae} vorkommen, wurden nachgewiesen. Plasmid-lokalierte Gene konnten in \textit{E. coli} übertragen werden und vermittelten die erwarteten Resistenzeigenschaften auch in diesen Bakterien, was auf einen Austausch von Resistenzgenen zwischen \textit{Enterobacteriaceae} und \textit{B. bronchiseptica} hindeutet.
Danksagung

Mein Dank gilt ganz besonders Herrn Prof. Dr. Stefan Schwarz für die Überlassung des Themas dieser Arbeit und die Einarbeit in das Gebiet der Molekularbiologie. Vor allem danke ich ihm auch für die engagierte Betreuung und Unterstützung bei dem Projekt, durch die mir zahlreiche Möglichkeiten gewährt wurden und die vergangenen Jahre für mich eine sehr spannende und lehrreiche Zeit waren.

Frau Dr. Corinna Kehrenberg, Ph.D., danke ich für die Betreuung des Projektes, ihre Hilfsbereitschaft und die zahlreichen wissenschaftlichen Diskussionen.

Der H. Wilhelm Schaumann Stiftung danke ich für die Bereitstellung eines Stipendiums für das Projekt und für die finanzielle Unterstützung beim Kongress in den USA.

Herrn Prof. Dr. sc. agr. Dr. habil. Dr. h. c. F. Ellendorf danke ich für die freundliche Aufnahme im Institut für Tierzucht, für die Bereitstellung des Arbeitsplatzes und für seine Unterstützung bei der Finanzierung.

Bei Frau Dr. Irith Wiegand bedanke ich mich für die Einarbeitung in die Arbeit mit β-Laktamasen, ihr Interesse, ihr Fachwissen und ihre Ideen; sowie ihr und Herrn Prof. Dr. H.-G. Sahl für meinen Gastaufenthalt im Institut für Medizinische Mikrobiologie und Immunologie, Arbeitsgruppe Pharmazeutische Mikrobiologie an der Uni Bonn.

Ich danke Herrn Dr. Jürgen Wallmann für die Zusammenarbeit und ihm und Prof. Dr. Gerald-F. Gerlach für die Betreuung des Ph.D.-Projektes. Vielen Dank an die Ph.D. Kommission und an Frau Faber für die Unterstützung im Ph.D.-Studium, sowie an die Gesellschaft der Freunde der TiHo.


Ich bedanke mich beim Schwesternhaus und allen seinen BewohnerInnen!

Ich danke allen, die mich durch meine Studienzeit begleitet haben. Ein ganz herzliches Dankeschön an Frau Dr. Soumaya Lhafi und Anke Albert für alles und noch viel mehr!

Mein Dankeschön gilt - von ganzem Herzen - Daniel Meenderink. Ich danke auch seiner und vor allem meiner Familie.