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

Kristina Kadlec, Corinna Kehrenberg and Stefan Schwarz*

*Institut für Tierzucht, Bundesforschungsanstalt für Landwirtschaft (FAL), Hölystrasse 10, 31535 Neustadt-Mariensee, Germany*

Received 25 May 2005; returned 23 June 2005; revised 24 June 2005; accepted 25 June 2005

**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 localization, transferability and association with other resistance genes.

**Methods:** Six *B. bronchiseptica* isolates with elevated MICs of 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 localization of these integrons was confirmed by transformation and conjugation. Isolates carrying the same integron were compared for their genetic relatedness by XbaI and SpeI 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.

Keywords: *dfrA1*, *dfrB1*, *catB2*, *catB3*, *sul1*, class 1 integrons, conjugation, respiratory tract infections

**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. 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. Antimicrobial agents are commonly used to control *B. bronchiseptica* infections; however, very little is known about the antimicrobial resistance of these bacteria. The antimicrobial susceptibility of *B. bronchiseptica* isolates from pigs has been monitored since 2002 in a single national resistance monitoring programme in the veterinary field, the GermVet programme. However, *B. bronchiseptica* isolates have been collected for drug-specific monitoring programmes since 2000 in Germany. A first large-scale analysis of 349 porcine *B. bronchiseptica* isolates collected during the 4-year period 2000–2003 has recently been published. 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 this 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.
Materials 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. The initial susceptibility testing was performed by broth microdilution according to the recommendations of the Clinical Laboratory Standards Institute (CLSI; formerly known as the NCCLS) document M31-A2. 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 microdilution with Escherichia coli ATCC 25922 as a quality control strain. Susceptibility testing of the transformants and transconjugants was performed by either broth dilution or disc diffusion.

DNA preparation and PCR analysis

Isolation of plasmids and whole-cell DNA followed standard protocols. To detect the most common trimethoprim resistance genes by PCR, four recently described primer sets each of which allowed the amplification of two to three closely related dfrA or dfrB genes were used. Integrase genes of classes 1 and 2, gene cassettes and sulphonamide resistance genes were detected by previously reported PCR assays. All primers used are listed in Table 1.

Pulsed-field gel electrophoresis

For pulsed-field gel electrophoresis (PFGE) with XhoI and SpeI, a standard protocol was used. Whole-cell DNA of Staphylococcus aureus B325 digested with SmalI and of Salmonella Typhimurium LT2 digested with XhoI served as size markers. PFGE was performed in a CHEF DR III system (Bio-Rad, Munich, Germany) using 0.5x 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 11 h, and from 30 to 50 s for the following 13 h.

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) Sequence (5'→3')</th>
<th>Reference no.</th>
</tr>
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<tbody>
<tr>
<td>dfrB1, dfrB2</td>
<td>205</td>
<td>fw CAAAGTACCGTGAAGCCCA</td>
<td>10</td>
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<tr>
<td></td>
<td></td>
<td>rv CAAGATACCGTGAAGCCCA</td>
<td></td>
</tr>
<tr>
<td>dfrA5, dfrA14</td>
<td>383</td>
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<tr>
<td></td>
<td></td>
<td>rv CTCGAAAAAACACTTCGAAGG</td>
<td></td>
</tr>
<tr>
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<td>fw CAGAAAATGGCCTATCAG</td>
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<tr>
<td></td>
<td></td>
<td>rv TCACCTTCAACCTCAACG</td>
<td></td>
</tr>
<tr>
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<td>414</td>
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<tr>
<td></td>
<td></td>
<td>rv ACCCTTTCGCCAGATTTG</td>
<td></td>
</tr>
<tr>
<td>Variable part of class 1 integrons</td>
<td>variable</td>
<td>5'-CS GGCATCCAGCAGCAAAG</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3'-CS AAGCAAGCTTGGACCGTA</td>
<td></td>
</tr>
<tr>
<td>Class 1 integrase</td>
<td>450</td>
<td>fw CCGAATTGCGGAGCGACATG</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv CAAAGTTCGGGACAGTTGCC</td>
<td></td>
</tr>
<tr>
<td>sul1</td>
<td>840</td>
<td>fw CTAGGCATGATCAGGACCGA</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv ATGTTGACGGGATTTGCGCGA</td>
<td></td>
</tr>
<tr>
<td>sul2</td>
<td>704</td>
<td>fw CAGTTTCTCCGATGGACGCC</td>
<td>16</td>
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<td>rv CTAGTTTCTCCGATGGACGCC</td>
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</tr>
<tr>
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<td>variable</td>
<td>5'-CS GGGATCCGGGACCGCATGCGTTTGA</td>
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<tr>
<td></td>
<td></td>
<td>3'-CS GATGCGATCGGACGCGACGTTTGA</td>
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<tr>
<td>Class 2 integrase</td>
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<td>fw ATTAGGCAGGCGGCGGACGAG</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rv CTGTCAGCAGGCGGACGAG</td>
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</table>
Antimicrobial resistance of *B. bronchiseptica*

**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 16304–64/1216 mg/L for the combination trimethoprim/sulfamethoxazole (1:19), suggesting that all six isolates were also resistant to sulfonamides. 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 trimethoprim MICs of ≥512 mg/L. 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 of dfrA1, were detected in all five amplicons. The remaining *B. bronchiseptica* expressed its resistance properties. Conjugation experiments with *E. coli* HK225 as recipient confirmed that plasmid pKBB668 was conjugal and transferred from *B. bronchiseptica* to *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 *E. coli* JM109:pKBB668 transconjugants and *E. coli* HK225:pKBB668 transconjugants. Plasmid pKBB668 mediated no resistance properties other than those associated with the class 1 integron. The integron with the dfrB1-catB2 gene 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 transconjugants 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 *B. bronchiseptica* 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. Y214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 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. AY214164) and in *Pseudomonas aeruginosa* (accession no. AB195796), which, however, carried additional adaA4 or aacA4 gene cassettes.
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 additional bla*VIM*-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.
Antimicrobial resistance of *B. bronchiseptica*

A closer look at the *dfrB1* cassette detected in the present study revealed that this cassette was 74 bp shorter than the prototype *dfrB1* cassette (accession no. U36276). 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 *dfrB1* gene in the respective cassette from *B. bronchiseptica*. The *dfrB1* cassette described in the present study was indistinguishable from the *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 *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 *dfrB1* cassette. In this case, an ATG codon (position 111–113 in Figure 1b) was recognized as the putative translational start codon of the *dfrB1* gene. However, the intact DfrB1 protein from *E. coli* plasmid R67 had been purified and shown by protein sequencing to be 78 amino acids in size. Hence, the start codon at positions 168–170 (Figure 1b) is most likely the true translational start codon of the *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 *B. bronchiseptica* isolates. The dissemination of closely related *B. bronchiseptica* isolates within a particular geographic area might be explained by the purchase of piglets already carrying these resistant *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 *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 trimethoprimsulphamycin, are often preferred over newer and more expensive agents such as third-generation cephalosporins, tilmicosin or florfenicol. This might explain why plasmids such as pKBB668 and pKBB958, which mediate resistance to trimethoprim, sulphamethoxazole 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 sulphamethoxazole 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 2 integron. K. K. is supported by a scholarship of the H. Wilhelm Schaumann foundation.

### References


