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Characterization of antibiotic resistance plasmids from *Bordetella bronchiseptica*

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Of 52 antibiotic-resistant *Bordetella bronchiseptica* isolates from cats, ten carried plasmids. Only two of these plasmids, pLV1400 and pLV1401, were self-transmissible to *Escherichia coli* K12; both plasmids encoded resistance to ampicillin, tetracycline, sulphonamides, streptomycin and mercuric chloride, and were of incompatibility group P (IncP). Transferable tetracycline resistance has not been reported in *B. bronchiseptica* previously. The plasmids were identical in size (*c.*51 kb), restriction endonuclease digestion pattern and gene sequences (*trf*A and *kor*A) within the IncP replicon. The *trf*A and *kor*A sequences differed from those of the archetypal IncP plasmids RP4 and R751. Although the two *B. bronchiseptica* isolates were from epidemiologically and geographically separated cats, pulsed-field gel electrophoresis of their *Xba*I- or *Dra*I-digested chromosomal DNA indicated that they were genotypically identical. The plasmid-encoded ampicillin resistance was mediated by a penicillinase of molecular weight 49,000, and pI 8.45 which was inhibited by clavulanate (IC_{50} = 0.1 mg/L) and tazobactam (IC_{50} = 0.42 mg/L) but not by parachloromercuribenzoate or EDTA. The high-level tetracycline resistance was mediated by a class C efflux mechanism that has not been described previously in this genus. The presence of transferable multidrug resistance on a promiscuous plasmid may limit options for therapy of respiratory tract infection in companion and farm animals.

Introduction

Bordetella bronchiseptica is an important respiratory tract pathogen of dogs,[1,2] pigs[3,4] and laboratory animals.[5,6] Recently, it has also been increasingly reported as a cause of upper respiratory tract infection and bronchopneumonia in cats.[7–9] In addition, there has been a dramatic increase in reports of *B. bronchiseptica* infections in humans,[10,11] particularly amongst immunocompromised patients in whom it may cause peritonitis[12] and bacteraemia[13] as well as respiratory tract infections.[14] A zoonotic potential of *B. bronchiseptica* has also been described.[15,16]

Antimicrobial chemotherapy is the mainstay of management of *B. bronchiseptica* infections and a number of studies have addressed the antimicrobial susceptibility of this organism. These studies have mostly used isolates from pigs[17,18] or humans,[19,20] although some have used isolates from companion animals.[21,22]

The most commonly recommended antimicrobials for treatment of respiratory tract infection in companion animals are tetracycline, ampicillin and co-trimoxazole.[23,24] Plasmids encoding resistance to ampicillin, sulphonamides and streptomycin have previously been found in porcine isolates of *B. bronchiseptica*.[25–29] It is not known whether *B. bronchiseptica* is transmissible between farm and companion animals nor are there data on resistance plasmids in *B. bronchiseptica* isolates from companion animals. Finally, tetracycline and its derivatives form the mainstay of treatment of canine infectious tracheobronchitis (kennel cough), yet no information is available regarding transferable tetracycline resistance in *B. bronchiseptica* from companion animals. The emergence of transferable resistance among *B. bronchiseptica* isolates to tetracycline and other

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antibiotics commonly used in both human and veterinary medicine could have significant implications and limit therapeutic options.

Materials and methods

Isolates

Of 147 *B. bronchiseptica* isolates from oropharyngeal swabs from cats with and without respiratory disease throughout England and Wales, 52 were chosen for further study.[22] These isolates were chosen as representative of each of the different resistance profiles detected in the above study, so no fully sensitive isolates were included. Isolates were stored at −70°C in 15% glycerol broth. *Escherichia coli* NCTC 10418 was used as a control for antimicrobial susceptibility and *E. coli* K-12 (Nal^R, Lac^+) as recipient in mating experiments.

Antimicrobial susceptibility

Antimicrobial susceptibility of parental and transconjugant strains was initially tested by a controlled disc diffusion method. Subsequently, MICs were determined by incorporating doubling dilutions of antimicrobials in Isosensitest agar (Oxoid, Basingstoke, UK) as described previously.[22] Mercury resistance was determined by a breakpoint method by incorporating $HgCl_2$ (50 μM) in nutrient agar.

Plasmid studies

Attempts to transfer tetracycline resistance to *E. coli* K-12 were made in broth culture according to the method of Datta.[30] Transconjugants were selected by plating on to MacConkey agar containing nalidixic acid (30 mg/L) and tetracycline (10 mg/L). Plasmid DNA was extracted from donors and transconjugants by the method of Birnboim & Doly.[31] Plasmid DNA was separated by electrophoresis through 0.8% agarose gels and stained with ethidium bromide (Sigma, Poole, UK). Plasmid bands were visualized using a Polaroid MP-3 camera (Polaroid, MA, USA). Purified plasmid DNA was digested with *Eco*RI, *Bam*HI or *Hin*dIII according to the manufacturer's instructions (Boehringer, Lewes, UK). Agarose gel electrophoresis was performed as above and *Hin*dIII-digested bacteriophage λ DNA (Sigma) was used as a molecular weight marker. Incompatibility grouping of plasmids was carried out using DNA hybridization and biotinylated probes as described previously.[32] The *trf*A and *kor*A regions of the IncP replicon were amplified by the PCR using *Taq* DNA polymerase (2.5 U) and the oligonucleotide primers *trf*A1 and 2 and *kor*A1 and 2[33] respectively. Reactions were performed for 30 cycles (94°C for 1 min, 55°C or 56°C (for *trf*A and *kor*A, respectively) for 1 min, 72°C for 2 min) followed by 1 cycle of 72°C for 10 min. Concentrations of primer and dNTPs (dATP, dCTP, dGTP and dTTP) were 40 pmol and 50 μM respectively. Amplification products were visualized following electrophoresis on 1.2% agarose TBE gels containing ethidium bromide (1 mg/L). PCR products were purified using Microspin S-400 HR columns (Pharmacia, St Albans, UK) and sequenced on both strands using an ABI 373A DNA automated sequencer (Applied Biosystems Inc., Warrington, UK) with the primers that were used in the PCR. The *kor*A and *trf*A regions were chosen since they are important in replication and transcription of IncP plasmids. *kor*A is involved in coordination of transcription of many operons on the plasmid and prevents lethal overexpression. *trf*A activates replication of the plasmid from the vegetative origin of replication (*oriV*).

The DNA sequences reported here have been deposited in the EMBL database under the following accession numbers: pLV1400 *trf*A, Y10538; pLV1400 *kor*A, Y10540; pLV1401 *trf*A, Y10539; pLV1401 *kor*A, Y10541. They were compared with other IncP sequences using the programs FASTA and PILEUP from the University of Wisconsin GCG suite of sequence analysis programs.[34]

Antibiotic resistance

The nature of the tetracycline resistance gene was determined by DNA hybridization using biotin-labelled oligonucleotide probes for *tet*A, *tet*B and *tet*C.[35] β-Lactamases were initially detected using the chromogenic cephalosporin, nitrocefin (Oxoid). β-Lactamase extraction, substrate and inhibition profiles, molecular weight determination and isoelectric point determination on parental *B. bronchiseptica* and transconjugant *E. coli* were as described previously.[36]

Pulsed-field gel electrophoresis

Chromosomal DNA from *B. bronchiseptica* isolates was separately digested using *Xba*I and *Dra*I restriction endonucleases and separated by pulsed-field gel electrophoresis (PFGE) using an LKB Gene Navigator (Pharmacia) as described by Gueirard *et al.*[15] Gels were stained and photographed as above. Isolates with three or more differences in banding patterns were considered different.[37]

Results

Plasmids

Of the 52 *B. bronchiseptica* isolates studied, ten contained plasmids. Eight of these isolates contained plasmids of approximately 20 kb but in no case was the plasmid transferrable to *E. coli* K-12. The remaining two *B. bronchiseptica* isolates each carried a single plasmid of

approximately 51 kb. The MICs for the ten plasmid-containing isolates are shown in Table I. Breakpoints for resistance are based on the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS)[38] except for sulphadiazine where the breakpoint suggested by Mengelers *et al.*[18] for porcine respiratory tract pathogens was used rather than the NCCLS one which is based on human urinary tract pathogens.

The two plasmids of approximately 51 kb were readily transferrable to *E. coli* K-12 and transferred resistance to tetracycline (MIC > 250 mg/L), doxycycline (MIC > 4 mg/L), ampicillin (>256 mg/L), streptomycin (>1024 mg/L), sulphadiazine (>250 mg/L) and mercuric chloride (>50 μM). The two plasmids were designated pLV1400 and pLV1401. Restriction endonuclease digestion with *Eco*RI, *Bam*HI or *Hin*dIII of the two plasmids extracted from both parental *B. bronchiseptica* and transconjugant *E. coli* K-12 produced identical profiles. On DNA hybridization using eight different biotinylated Inc probes pLV1400 and pLV1401 were found to be of incompatibility group P. None of the plasmids from the eight remaining *B. bronchiseptica* isolates could be assigned to an incompatibility group.

In order to determine the relatedness of these IncP replicons with existing IncP plasmids two separate regions (*trf*A and *kor*A) were amplified by PCR and sequenced. Amplicons from *trf*A (241 kb) and *kor*A (294) regions were obtained by PCR from both pLV1400 and pLV1401. No amplification was observed following PCR of sterile distilled water. The *trf*A and *kor*A regions of pLV1400 and pLV1401 were identical. Comparisons of the *trf*A and *kor*A regions of pLV1400 were made with those of the known IncP plasmids R751 and RP4.[39–42] These demonstrated that pLV1400 differed in both genes from both of the archetypal IncP plasmids but that they were more closely related to R751 than RP4 (Table II). Furthermore, pLV1400 and pLV1401 are more closely related to R751 than R751 is to RP4. Thus pLV1400 and pLV1401 appear identical in terms of molecular weight and restriction endonuclease digestion pattern and in the genetic make-up of their incompatibility loci.

Antibiotic resistance

Both pLV1400 and pLV1401 encoded high level tetracycline resistance (MIC > 256 mg/L) and both were subjected to dot-blot DNA hybridization with biotinylated probes for various tetracycline resistance genes. Both pLV1400 and pLV1401 hybridized to the *tet*C probe, and to none of the others.

Both plasmids also encoded production of a β-lactamase. This β-lactamase had a molecular weight of approximately 49,000 (as determined by Ultragel column chromatography and SDS–polyacrylamide gel electrophoresis) and an isoelectric point of 8.45. By the disc inactivation method the β-lactamases expressed by both pLV1400 and pLV1401 were shown to hydrolyse penicillin, cloxacillin, ampicillin and ticarcillin, but not cephradine,

Table I. MICs of eight antimicrobial agents for 10 plasmid-containing feline *B. bronchiseptica*

Antimicrobial agent	MIC (mg/L) range	mode	MIC_{50}	MIC_{90}	% Resistant
Ampicillin	6–>256	12	12	>256	90
Co-amoxiclav	1–8	4	4	8	0
Doxycycline	0.25–16	0.5	0.5	>4	20
Enrofloxacin	>0.12–1	0.5–1	0.5	1	0
Streptomycin	4–>1024	12	12	>1024	20
Sulphadiazine	2–>256	>256	>256	>256	70
Tetracycline	1.5–>256	2	2	>256	20
Trimethoprim	4–250	64	64	250	80

Table II. DNA relatedness of *trf*A and *kor*A region of IncP plasmids

	% Identity (across number of bases) *trfA*	*korA*
pLV1400/1 and R751	98.3 (174)	90.6 (213)
pLV1400/1 and RP4	83.6 (171)	76.0 (216)
R751 and RP4	82.2 (174)	76.9 (216)

cefuroxime, cefotaxime or cephaloridine. The inhibition profiles of the β-lactamases encoded on the two plasmids were the same (Table III). The enzymes were inhibited by clavulanic acid and tazobactam, but not by cloxacillin, parachloromercuribenzoate or EDTA. The IC_{50}s for tazobactam and clavulanic acid were 0.42 mg/L and 0.1 mg/L.

The β-lactamases are thus plasmid-encoded penicillinases.

Molecular epidemiology

The isolates of *B. bronchiseptica* carrying pLV1400 and pLV1401 were from cats in widely separated parts of northwest England with no known epidemiological connections. The former was the only cat in the household and the latter from a breeder with three other cats who were also excreting *B. bronchiseptica*, but which did not have resistance plasmids. Chromosomal DNA extracted from each isolate was digested with *Xba*I and *Dra*I and subjected to PFGE. The restriction digestion patterns of the *B. bronchiseptica* isolates carrying pLV1400 and pLV1401 were identical. The restriction digestion patterns of the chromosomal DNA from the three other *B. bronchiseptica* isolates from the second household differed from the plasmid containing isolates by four bands but were similar to each other.

Discussion

B. bronchiseptica is a significant cause of respiratory disease in veterinary practice; it is commonly encountered and is typically of high morbidity and low mortality. Nevertheless, because of its high infectivity, and because clinical signs often persist, antimicrobial chemotherapy is advised for clinical cases. In companion animal practice, tetracycline is the antimicrobial of choice.[43] It is therefore a matter of concern that we have found plasmid-encoded high level tetracycline resistance, which has not been documented previously.

Of the 52 multiresistant isolates of *B. bronchiseptica* examined, only ten (19%) carried plasmids compared with 11/27[28] and 6/14[29] in previous studies. However, the latter studies used porcine rather than feline isolates. Eight of our plasmid containing isolates carried 20 kb plasmids that were not self-transmissible or related to any particular antibiotic resistance phenotype. The two remaining isolates had self-transferable 51 kb plasmids that encoded resistance to tetracycline, sulphadiazine, streptomycin, ampicillin and mercuric chloride. The two plasmids appeared to be identical not only in resistance phenotype but also in restriction endonuclease digestion pattern and IncP locus. Previous studies of porcine *B. bronchiseptica* isolates have demonstrated conjugative plasmids of 55–58 kb that encode resistance to various combinations of ampicillin, streptomycin, sulphonamides and mercuric chloride.[26–28,44] In several cases the plasmids were also of incompatibility group P.[26,27] Incompatibility group P plasmids characteristically have a wide host range among Gram-negative bacteria in contrast to other incompatibility groups which tend to be restricted to a single species or group of related species. On PCR amplification and sequencing of two regions of the IncP replicon (*trf*A and *kor*A), our two plasmids were found to be identical. On comparison with the only other IncP *trf*A and *kor*A sequences available (on RP4 and R751), those on our plasmids differed from both but were more closely related to those of R751.

Transferable tetracycline resistance has not previously been demonstrated in *B. bronchiseptica*; indeed some have concluded that plasmids from *B. bronchiseptica* can be characterized by their lack of a tetracycline resistance marker.[44] However, an IncP plasmid encoding resistance to streptomycin, sulphonamide and tetracycline has been detected in *B. avium*.[45] The tetracycline resistance on our plasmids was apparently encoded in the *tet*C gene. Tetracycline resistance classes A–E have an active efflux mechanism and are found among the members of the family Enterobacteriaceae and genera *Haemophilus*, *Vibrio*, *Aeromonas* and *Moraxella*[46] but have not previously been described in the genus *Bordetella*. *tet*C is not common in other bacteria; for example, it was not the major tetracycline resistance determinant in surveys of Gram-negative bacteria from polluted and unpolluted marine sediments,[47] being found in only 6% of tetracycline resistant isolates.

The plasmid-encoded resistance to ampicillin was due to production of a β-lactamase. The β-lactamase was a penicillinase with very poor activity against cephalosporins. It had a molecular weight of *c.*49,000 and a pI of 8.45 and was inhibited by clavulanate and tazobactam but not by cloxacillin, parachloromercuribenzoate or EDTA. These characteristics put it into class 2a or 2d of the Bush classification:[48] it does not fit completely into either class and will require sequence data for full assignment. A similar penicillinase has been described mediated by an R-plasmid from a porcine *B. bronchiseptica* isolate.[49] This enzyme had a molecular weight of 46,000 and a pI of 8.3.

Table III. Inhibition profiles of the plasmid-encoded β-lactamases derived from *B. bronchiseptica*

Inhibitor	Inhibition (%)
Clavulanic acid (10 mg/L)	>96
Tazobactam (10 mg/L)	96
Cloxacillin (10^{-2}M)	28
Parachloromercuribenzoate (10^{-4}M)	16
EDTA (10^{-3}M)	0

pLV1400 and pLV1401 are identical in molecular weight, restriction digestion profile and incompatibility group, and have the same resistance phenotype, tetracycline resistance determinants and β-lactamase. Has the plasmid spread between two different *B. bronchiseptica* isolates? Although there were no epidemiological data to suggest that the two parental *B. bronchiseptica* isolates could be the same, no differences in banding pattern could be detected in PFGE of restiction digested chromosomal DNA. This suggests that the same plasmid was present in two *B. bronchiseptica* isolates that were genetically identical. How this could have occurred is unclear since the two cats were from different parts of the region and had not been in contact.

This study has demonstrated the presence of high level tetracycline resistance in *B. bronchiseptica*. The fact that this resistance was encoded on a promiscuous IncP plasmid that also encoded resistance to sulphonamide, streptomycin and ampicillin is disturbing in its potential for further dissemination among other members of the species, genus and other Gram-negative bacteria.

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