Original article

Phenotypic and genotypic characterization of *Bordetella bronchiseptica* strains isolated from pigs in Poland

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Abstract

A total of 209 *Bordetella bronchiseptica* (*Bbr*) strains isolated from pigs were examined. Phenotypic study included: biochemical characterization (motility, catalase, oxidase, urease activity, nitrate reduction and growth on MacConkey agar) and antimicrobial susceptibility (disc diffusion method). Genotypic studies based on detection of three genes encoded virulence factors, such as: flagella (*fla*), dermonecrotoxin (*dnt*), and exogenous ferric siderophore receptor (*bfrZ*), using PCR.

Most of the *Bbr* strains tested had a homogeneous biochemical profile. 97.6% of them provided suitable results in biochemical tests. All *Bbr* isolates tested showed high resistance to penicillin (100%), linco-spectin (100%) and ceftiofur (97.9%). Over 57% and 43% of *Bbr* strains were resistant to ampicillin and amoxicillin, respectively. All *Bbr* isolates showed high sensitivity to most chemotherapeutics used such as enrofloxacin (97.9%), tetracycline (97.9%), oxytetracycline (97.9%), amoxicillin with clavulonic acid (95.8%), florfenicol (90.4%), and gentamicine (77.6%). Over of 94% of *Bbr* strains were moderately susceptible to norfloxacine.

Molecular analysis confirmed that almost all evaluated *Bbr* strains (94.7%) possessed the *fla* gene. A lower percentage of isolates had the *dnt* gene (72.7%) and the lowest percentage of strains (51.7%), had the *bfrZ* gene.

Key words: swine, *Bordetella bronchiseptica*, phenotypic and genotypic characterization, antimicrobial susceptibility

Introduction

Bordetella bronchiseptica (*Bbr*) was isolated for the first time by Ferry in 1910 from the respiratory tract of dogs (Goodnow 1980). The bacterium was renamed many times before receiving its final name. *Bordetella bronchiseptica* is an upper respiratory tract pathogen of many animal species such as: swine, dogs, cats, rabbits, horses, rats and guinea pigs. *Bordetella bronchiseptica* was also isolated from wild animals such as: bears, leopards, foxes, seals, koalas and raccoons (Ross et al. 1967, Woode and McLeod 1967, Bemis et al. 1977, Musser et al. 1987, Winn et al. 2006, Bemis et al. 2010). Occasionally it affects man.

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Depending on the health of the pig herd *Bbr* alone can cause a mild to moderate form of atrophic rhinitis (AR) with nonprogressive turbinate bone atrophy; however, in combination with dermonecrotoxic *Pasteurella multocida* (*Pm*) strains it can cause a more severe progressive form of AR (Jong De 2006, Pomorska-Mól et al. 2011, Register 2012). Other *Bordetella* spp. which belong to the *Alcaligenaceae* family do not cause diseases of animals (*Bordetella hinzii, B. holmesii, B. trematum, B. petrii*), although *B. hinzii* is isolated from the respiratory tract of poultry. In contrast *Bordetella hinzii, B. holmesii* and *B. trematum* are connected with human opportunistic infections (Bemis et al. 2010).

Many studies suggested that three species: *Bbr*, *Bordetella pertussis* (*Bp*) and *Bordetella parapertussis* (*Bpp*) are closely related (Parkhill et al. 2003, Parton 2005). These studies were based on a phenotypic and genotypic comparison between *Bordetella* species. Phenotypic analyses included biochemical properties and antigenic structure. Genotypic analyses were related to DNA-DNA hybridization analyses, mean G+C contents of their genomic DNA, sequence similarity of 23S rRNA and phage typing, as well as the comparison of the whole genome of each analyzed species. Phylogenetic analysis and the results of insertion sequence (IS) typing demonstrated that both *Bp* and *Bpp* are derived from *Bbr*. Comparision of genomic sequences of reference strains confirmed that the nucleotide sequence similarity is very high in conservative regions. This suggests that these strains have evolved from a *Bbr*-like ancestor (Gerlacha et al. 2001, Parkhill et al. 2003, Parton 2005, Sebaihia et al. 2006).

Bbr is small, Gram-negative, coccobacilli. The bacterium grows in obligatory aerobic conditions, optimally at 35°-37°C. *Bbr* is a nonfermentative species with the ability to move, which is facilitated by peritrichous flagella (Goodnow 1980). *Bbr* has catalase, oxidase, and urease activity (in the case of urease, up to 4h). The bacterium also has the ability to cause decarboxylation of ornithine and nitrate reduction. Colonies of *Bbr* grow on routinely used agar media in 2-3 days, they grow more rapidly than other species of the *Bordetella* genus and are more resistant to variable physical and chemical conditions (Bemis et al. 1977, Parton 2005).

Some virulence factors have an important role in the pathogenicity of *Bbr*. Fimbriae, for example, are associated with bacterial attachment and colonization of nasal ephitelium cells. Dermonecrototoxin is necessary for the induction of clinical signs in swine (Kume et al. 1986, Pullinger et al. 1996, Parton 2005), while iron is associated with the metabolism of *Bbr* and determines the bacterium's ability to colonize and proliferate (Brickman et al. 2007).

The aim of this study was to analyze the phenotypic and genotypic properties of Polish *Bbr* strains isolated from nasal swabs of pigs.

Materials and Methods

Bordetella bronchiseptica strains

In total 209 *Bbr* strains were analyzed. They were isolated at the National Veterinary Research Institute in Pulawy between 2007-2011 from 912 nasal swabs of pigs from 106 farms located in 13 Polish provinces: Wielkopolskie (35), Śląskie (17), Kujawsko-Pomorskie (13), Mazowieckie (10), Zachodnio-Pomorskie (8), Lubelskie (7), Warmińsko-Mazurskie (4), Łódzkie (3), Podlaskie (3), Lubuskie (2), Świętokrzyskie (2), Dolnośląskie (1) and Małopolskie (1). Figure 1. shows the results of isolation of *Bbr* from provinces and farms.

As a positive control *Bbr* vaccine strain (B16) and two reference *Bbr* strains from ATCC (4617 and 10580) were used. As a negative control, *Bordetella parapertussis* (ATCC 15311) and *Bordetella pertussis* (ATCC 8467) were included.

Culture conditions

The strains were isolated on G20G medium, supplemented with penicillinum crystallisatum (10 mg/ml, Polfa Tarchomin S.A.), gentamicin (0.25 mg/ml, Polfa Tarchomin S.A.), nitrofurantoine (10 mg/ml, Sigma Aldrich), and nystatinum (10 mg/ml, Sigma Aldrich). All plates were incubated at 37°C for 48-72 h. Blue-green suspected colonies were subcultured on agar medium with 5% horse blood.

Biochemical characterisation and classification

Identification of *Bbr* isolates was carried out using standard biochemical tests. Gram staining, motility, catalase and oxidase tests, urease activity, nitrate reduction, and growth on MacConkey agar were used as criteria for identification of *Bbr*.

Antimicrobial susceptibility

Isolates of *Bbr* were tested for their *in vitro* sensitivity to a panel of 14 commonly used chemotherapeutics by the disc diffusion method, following the

Fig. 1. Results of isolation of *Bbr*. Legend: red dot – provinces from which samples were tested, first number – number of farm tested from individual province, second – number of samples tested, third – number of *Bbr* isolates.

Table 1. Sequence of primers used for amplification of *fla*, *dnt* and *bfrZ* genes of *Bbr*.

Species	Primer	Gene	Sequence 5'→3'	Primer length (nt)	PCR product length (bp)
Bbr	FlaF	*fla*	AGG CTC CCA AGA GAG AAA GGC T	22	237
	FlaR		TGG CGC CTG CCC TAT C	16	
	dntF	*dnt*	GCG CTA CTT GGG ATA ATA GA	20	224
	dntR		ATA AAG ATG AAT CGG CAT TG	20	
	BfrZf	*bfrZ*	GCA ATG ACC TGA ACC TGT ATT T	22	368
	BfrZr		CAT GGG CAT GTT CTT CTT GT	20	

recommendations and criteria provided by the Clinical and Laboratory Standards Institute (CLSI). The chemotherapeutics were: amoxicillin (25 μg/ml), amoxicillin with clavulanic acid (30 μg/ml), ampicillin (10 μg/ml), ceftiofur (30 μg/ml), doxicilin (30 μg/ml), enrofloxacin (5 μg/ml), florfenicol (30 μg/ml), gentamicin (10 μg/ml), linco-spectin (109 μg/ml), neomycin (30 μg/ml), norfloxacin (10 μg/ml), oxytetracycline (30 μg/ml), penicillin (10 μg/ml) and tetracycline (30 μg/ml). All antibiotic discs used in this study were supplied by Oxoid.

For analysis of antimicrobial susceptibility of *Bbr* isolates Müeller-Hinton medium with 5% sheep blood was used.

DNA extraction

DNA extraction from *Bbr* isolates was performed according to the Genomic Mini isolation kit protocol (A&A Biotechnology, Poland). Genetic material was used directly in PCR or stored at -80ºC for further analysis.

Primers

Three sets of primers were used in PCR for detection of genes encoding selected virulence factors of *Bbr* such as flagella (*fla*), dermonecrotoxin (*dnt*) and exogenous ferric siderophore receptor (*bfrZ*). Primers which amplify a 237 bp fragment of the *fla* gene were designed by Hozbor et al. (1999).

Primers which amplify *dnt* and *bfrZ* genes were designed by us, based on the analysis of their gene sequence available from GeneBank (BB3978 and BB4744, respectively), using the LaserGene computer programme. The specification of primers used is shown in Table 1.

Reaction conditions

All PCRs were performed in 0.2 ml individual PCR Tubes™ (BioRad, UK) in a T3-thermocycler (Biometra, Germany). Reactions were performed using an AmpliTaq Gold set containing 10xPCR Gold Buffer, 25 mM MgCl₂ solution, polymerase AmpliTaq Gold 5 U/mL (Applied Biosystem, Roche), 10 mM dNTPs (Fermentas) and 20 pM each of primer and water free from DNase and RNase. The total volume of reaction mixture was 25 µl, which included 2.5 µl of DNA and 22.5 µl of reagent mix. Each reaction mixture was subjected to an amplification regimen consisting of 10 min of initial denaturation at 95ºC, and followed by 40 cycles of 30 s of denaturation at 95ºC, 30 s of annealing at 56ºC, and 30 s of extension at 72ºC. Final elongation was done for 10 min at 72ºC.

Detection of PCR products

PCR products were separated by electrophoresis in 2% agarose gel with ethidium bromide at a concentration of 1 µl/ml. The electrophoresis was done in 1xTAE buffer, at 350 mA. 10 µl of reaction mixture and 2 µl of loading buffer 6xDNA Loading Dye (Fermentas) were inserted into each well. The molecular weight of the products obtained was determined by comparison to the molecular weight of marker GeneRuler™ 100 bp DNA Ladder Plus (Fermentas). The agarose gels were photographed under UV light using an EC3 Chemi HR 410 Imaging System (UK).

Results

Isolates of *Bbr* strains were obtained from 22.9% of nasal swabs taken from 3 groups of pigs: with AR, suspected of having the disease or of unknown health. All *Bbr* strains showed a high level of homogeneity. They had typical morphology, were Gram (-) and produced catalase. Over 99% of them had the ability to move (99.5%), produced urease (98.6%), gave positive results in an oxidase production test (99.5%) and reduced nitrate (97.6%). *Bbr* showed typical growth on MacConkey agar (99.5%).

Results of antimicrobial susceptibility show that all *Bbr* isolates were resistant to penicillin and linco-spectin. Over 97.9% of *Bbr* strains were also resistant to ceftiofur. In contrast, over 95% of strains were sensitive to amoxicillin with clavulanic acid (95.8%), oxytetracycline, tetracycline and enrofloxacin (97.9% each). The percentage of strains sensitive to florfenicol was 90.4%. 94.4% of strains were moderately susceptible to norfloxacin.

The highest diversity in susceptibility was noticed in the group of β-lactams, commonly using chemotherapeutics. Within this group, all *Bbr* isolates were resistant to penicillin and highly sensitive to amoxicillin with clavulanic acid. Just 4.2% of *Bbr* strains were moderately susceptible to amoxicillin with clavulanic acid. A high percent of *Bbr* strains were resistant to ampicillin (57.4%) and 29.9% of strains were susceptible to this antibiotic. Over 43% of *Bbr* strains were sensitive to amoxillin (43.9%), while 39% of strains were resistant to it.

All *Bbr* strains were resistant to ceftiofur, linco-spectin and penicillin. Figure 2. shows the results of antimicrobial susceptibility of *Bbr* strains.

Additionally to phenotypic analysis of *Bbr* strains, genotypic characterisation was done. The presence of three genes was tested in *Bbr* strains by specific PCRs. 198 of 209 *Bbr* isolates (94.7%) from nasal swabs possessed the *fla* gene, which was indicated by presence of 237 bp product. This was the highest percentage of *Bbr* isolates which possessed this gene. In a lower number of tested *Bbr* strains the *dnt* gene was detected. 152 isolates were positive for the presence of this gene (72.7%). The *bfrZ* gene was
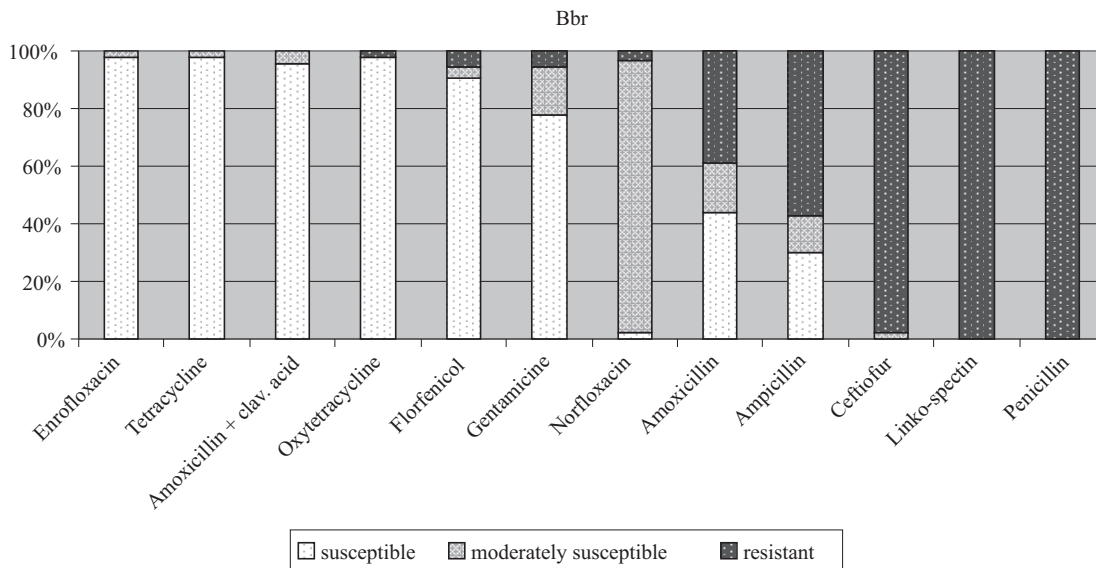
Fig. 2. Antimicrobial susceptibility of *Bbr* strains

detected in the lowest number of *Bbr* strains. The gene was found only in 108 isolates, which was a little more than a half of all tested *Bbr* strains (51.7%).

Discussion

Classic bacteriological methods are still the "gold standard" in laboratory diagnostics. The success of isolation of *Bbr* strains mainly depends on the time between sample acquisition and further laboratory procedures, sample storage conditions and the presence of appropriate medium in swabs during transport (Markowska-Daniel and Stępniewska 2009).

Samples taken from the nasal cavity of pigs are specific. The pig is a digging animal species and has contact with other pigs and their secretions. Therefore the pig nasal cavity harbours many atypical bacterial species. As a consequence, competitive growth of bacteria could make the isolation of *Bbr* strains on blood agar more difficult and thereby extend the time of bacteriological examination.

Colony morphology on solid medium is commonly used as a microbial identification criterion in laboratory diagnosis. Isolation of *Bbr* directly from nasal swabs on blood agar medium is a little troublesome because of the small size of the *Bbr* colony and the fact that their growth might be blocked by mixed bacterial flora. Therefore, it is important to use selective media during *Bbr* isolation. Smith – Baskerville medium is in common use in the isolation of *Bbr* from biological material, especially when the number of *Bbr* present is low. The medium contains three antibiotics: 20 µg/ml penicillin, 20 µg/ml furaltadone, and 0.5 µg/ml gentamicin (Smith and Baskerville 1979,

Lariviere et al. 1993, Register 2012). In our study G20G medium was used, which, similarly to Smith – Baskerville, contains penicillin and gentamicin, but additionally includes nitrofurantoine and nystatinum, which prevents the growth of fungal pathogens.

For identification of *Bbr* species, Lariviere et al. (1993), Bemis et al. (1997), Register and Ackerman (1997), Register and DeJong (2006) and Zhao et al. (2010) used a catalase, oxydase and urease test, growth on MacConkey medium, nitrate reduction and colony morphology analysis. Analogous tests were use in our studies. Most of the *Bbr* strains tested have a homogeneous biochemical profile. 97.6% of them obtained suitable results in biochemical tests.

The next part of this study was the evaluation of the sensitivity of *Bbr* isolates to commonly used chemotherapeutics. There are only few reports connected with drug resistance of *Bbr* strains.

Among swine respiratory pathogens examined such as: *Bbr*, *Pm*, *Mannheimia haemolitica* (*M. haemolitica*), *Actinobacillus pleuropneumoniae* (*App*), *Streptococcus suis* (*S. suis*) the highest MIC values for various concentrations of florfenicol for *Bbr* was achieved by Priebe and Schwarz (2003). Those results suggest low efficiency of florfenicol in low concentrations against *Bbr*, in comparison to other pathogens tested.

Kadlec et al. (2004) conducted studies on the susceptibility of *Bbr* isolates from pigs collected between 2001-2003 in Germany. Their results showed high sensitivity of *Bbr* isolates tested to tetracycline, enrofloxacin and gentamicin. These antibiotics in a concentration of 0.5-2 µg/ml inhibited the growth of 90% of *Bbr* strains. Polish *Bbr* isolates were also sensitive to these three antibiotics. Kadlec et al.

(2004) also showed that the MICs values to ampicillin and ceftiofur for *Bbr* strains were the highest (MIC90≤16). The ceftiofur from the cephalosporin group of antibiotics, could be used against a wide range of pathogens of the pig respiratory tract and is in common use against *Salmonella spp.*, *Pm*, *App*, *S. suis* and *Escherichia coli*, whereas it is not effective against *Bbr* (Burton et al. 1996). Among our *Bbr* isolates significant resistance to ampicillin and ceftiofur was also observed.

Mortensen et al. (1989) showed high sensitivity of *Bbr* strains to tetracycline. This antibiotic in a concentration of 2 μg/ml inhibits the growth of 90% of *Bbr* strains isolated from people and pigs. For American and German *Bbr* strains MIC90 values to ampicillin and SxT were similar, but to gentamicin and tetracycline MIC values were higher in the case of strains obtained from the USA.

Assignment of *Bbr* based on MIC values for different antibiotics into susceptible, moderately susceptible and resistant strains is problematic, because the range of antimicrobial susceptibility published by CLSI contains data only for florfenicol.

The efficiency of some chemotherapeutics on *Bbr* may depend on the growth phase of the bacterium. Ishikawa et al. (1988) showed that *Bbr* in the C growth phase was more resistant to ampicillin than in the X phase. Bannatyne and Cheung (1984) showed that strains which lost some functional structural antigens were more resistant to antibiotic in comparison to the wild type.

Isolation of *Bbr* is time-consuming, and may take 3-7 days. Commercial ELISA tests for *Bbr* detection are not available. Serological tests, which use *Bp* antigen cannot differentiate infection caused by *Bpp* from *Bbr*. Furthermore, Jenkins (1977) who tested cross reactivity of ELISA, showed that 2.7% and 13% serum samples from growing pigs and mature hogs, respecively, reacted with *Pm* antigen. Boot et al. (1993) suggested that ELISA be used in cases of monitoring infection caused by *Bbr* in rabbits and guinea pigs.

A more sensitive, specific and accurate method used in diagnosis of *Bbr* infection is PCR.

In our study the specific sequence of the *fla* gene was amplified in PCR. The main reason why the *fla* gene was used was the fact that *Bbr* strains are ciliated bacteria and have the ability to move, in comparison to *Bp* and *Bpp*, which are nonmotile. Hozbor et al. (1999) used primers which amplify the 237 bp fragment of this gene. Evaluation of its specificity show that they were specific only for *Bbr*. The specificity was tested also with such pathogens as *Staphylococcus aureus*, *M. hyopneumoniae*, *Aspergillus fumigatus*, *Candida albicans*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae* and *Chlamydia pneumoniae*. PCR results showed that this gene was present in 94.7% of *Bbr* strains.

For detection of dermonecrotoxin traditional bacteriological methods, such as cytotoxic tests using cell lines, or biological test (laboratory animals) can be used but they are time consuming, and therefore PCR seems to be a good tool. As was shown by Stępniewska and Markowska (2010) primers for amplification were specific only for *Bbr* and the sensitivity of the elaborated test was high. It is important that PCR based on the *dnt* gene can be use directly to nasal swabs with no need of bacteria isolation.

In the pathogenicity of *Bbr* the siderrophore receptor gene is no less important than dermonecrotoxin or flagella. The *bfrZ* gene is only present in the genome of *Bbr*. The lowest number of *Bbr* strains with this gene may suggest that other siderrophore receptor genes were expressed.

Our results show the common presence of *Bbr* strains in Polish pig herds. The presence of *fla*, *dnt* and *bfrZ* genes in 94.7%, 72.7% and 51.5% respectively in *Bbr* strains confirm a high level of pathogenictity among Polish *Bbr* isolates. It is suggested that *fla*, *dnt* and *bfrZ* genes can be use as markers of pathogenicity of Bbr strains (Stępniewska and Markowska 2012).

According to our best knowledge there are no other reports on phenotypic and genotypic characterisation of Polish isolates of *Bbr* from nasal swabs of pigs.

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