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Bull Vet Inst Pulawy 56, 483-487, 2012
DOI: 10.2478/v10213-012-0085-2

OCCURRENCE OF GENES ENCODING VIRULENCE FACTORS IN *BORDETELLA BRONCHISEPTICA* STRAINS ISOLATED FROM INFECTED AND HEALTHY PIGS

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Received: July 13, 2012 *Accepted: November 26, 2012*

Abstract

The objective of the study was to determine genotypic profiles of *Bordetella bronchiseptica* (*Bbr*) strains, based on the occurrence of genes encoding virulence factors, such as flagella (*fla*), dermonecrotoxin (*dnt*), and exogenous ferric siderophore receptor (*bfrZ*), using PCR. 209 tested *Bbr* strains were obtained from Polish swine herds with different health status (with progressive atrophic rhinitis - PAR, suspected for PAR, and unknown). In total, seven different *Bbr* genotypes were determined. In 39.2% of *Bbr* isolates all three genes were present. In 41.1% of the isolates only two genes were detected. The most common genotype *dnt+bfrZ-fla+* was present in 60 (28.5%) *Bbr* strains, 65% of them were obtained from farms with PAR. Twenty five (12%) *Bbr* isolates were identified as *dnt-bfrZ+fla+* genotype and, as above, they were more frequently isolated from clinical cases of disease (84%). Among 31 (14.8%) strains only *fla* gene was evident, and in nine (4.3%) only *dnt* gene was present. There were no *Bbr* strains with *bfrZ* gene only. These results confirm the heterogenicity among *Bbr* strains.

Key words: swine, atrophic rhinitis, *Bordetella bronchiseptica*, virulence factors, genes.

Bordetella bronchiseptica (*Bbr*) is a worldwide spread pathogen of pigs' respiratory tract. When infecting the pig alone it may cause a mild form of atrophic rhinitis (AR) called NPAR (nonprogressive atrophic rhinitis), whereas, when the bacteria invade the respiratory tract in cooperation with *Pasteurella multocida* (*Pm*) it may cause progressive changes characteristic for PAR (progressive atrophic rhinitis) (8, 14).

Bordetella bronchiseptica and other members of the genus *Bordetella* produce a high variety of virulence factors, which have been associated with bacterial attachment and colonisation, such as: pertactin, fimbriae, and filamentous haemagglutinin. The dermonecrototoxin (DNT) or the adenylate cyclase haemolysin can be responsible for many pathogenesis aspects such as tissue damage, alteration of host clearance mechanisms, and suppression of antibody responses (8, 16). Bordetellae exhibit an essential requirement for iron. Highly developed iron transport systems underscore the importance of iron for *Bordetella* species, which is associated with metabolic, as well as, enzymatic activities and determine their ability to colonisation and proliferation.

The aim of the study was to determine the prevalence of genes encoding fimbriae (*fla*), dermonecrotoxin (*dnt*), and the exogenous ferric siderophore receptor (*bfrZ*) in *Bbr* strains isolated from pigs with typical clinical signs of AR and from the animals suspected of PAR. It is worth mentioning here that no studies devoted to this subject have been conducted yet.

Material and Methods

Bacterial strains, growth conditions. In total 209 *Bbr* strains were analysed. Samples were obtained directly from nasal swabs of pigs raised in 106 herds located in 13 provinces of Poland. A hundred and twenty six *Bbr* strains were obtained from 31 herds with PAR, 60 strains from 37 herds where PAR was suspected, and 23 strains from 38 herds with unknown health status for PAR.

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) wereused. The strains were isolated by streaking onto 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 37ºC for 48-72 h. Blue-green suspected colonies were subcultured on agar medium with 5% horse blood. The identification was carried out by standard bacteriological methods. Gram

staining, motility, catalase and oxidase tests, urease activity, nitrate reduction, and growth on MacConkey agar were used as criteria to identify *Bbr*.

DNA extraction from colonies selected according to the criteria mentioned above, was performed according to the protocol of Genomic Mini isolation kit (A&A Biotechnology, Poland). Genetic material was used directly in PCR or stored at -80°C for further analysis.

Primers. The oligonucleotide primers FlaF/FlaR were designed by Hozbor *et al.* (6). Primers dntF/dntR and BfrZf/BfrZr were designed by us, based on the analysis of sequence of *dnt* and *bfrZ Bbr* genes from GeneBank (BB3978 and BB4744, respectively). To select the best sequence of primers, computer programme LaserGene was used. The specification of primers used is shown in Table 1.

Reaction conditions. All PCR amplifications were performed in 0.2 ml individual PCR Tubes™ (BioRad, UK) in a T3-thermocycler (Biometra, Germany) using a following programme: 10 min of initial denaturation at 95ºC, and 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.

Reactions were performed with AmpliTaq Gold set (Applied Biosystem, Roche). The total volume of reaction mixture was 25 µl, which include 2.5 µl of DNA and 22.5 µl of reagents mix. Table 2 shows the concentration of each PCR reagent.

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

Results

The presence of genes of virulence factors in studied *Bbr* strains is shown in Table 3.

In tested *Bbr* isolates, *fla* gene was present with the highest frequency (94.7%). The percentage of positive results was from 93.6% to 100% in each group of the herds. This would be expected because of the fact that *Bbr* is ciliated bacterium.

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

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

Table 2
Mixture composition for PCR

PCR reagents	Gene		
	fla	*dnt*	*bfrZ*
2.5 µl 10xPCR gold buffer	x	x	x
2.5 µl $MgCl_2$ solution	x	x	x
0.5 µl 10 mM dNTPs	x	x	x
0.8 µM primer set	x	x	
1.2 µM primer set			x
0.13 µl polymerase AmpliTaq Gold 5 U/mL	x	x	x
Water free from DNase and RNase	x	x	x

Table 3
The presence of genes coding DNT, BfrZ, and Fla in *Bbr* strains isolated from different swine herds

***Bbr* isolates analysed from the herds with different health status**	**Number**	**Genes**		
		dnt	***bfrZ***	***fla***
With PAR	126	83 (65.8%)	58 (46%)	118 (93.6%)
Suspected for PAR	60	53 (88.3%)	32 (53.3%)	57 (95%)
Unknown healthy status	23	16 (69.6%)	18 (78.3%)	23 (100%)
Total	209	152 (72.7%)	108 (51.7%)	198 (94.7%)

Table 4
Genetic profil of *Bbr* strains isolated from different swine herds

Presence/absence of gene			**Number (%)**	***Bbr* isolates analysed from the herds with different health status**		
				with PAR	**suspected for PAR**	**unknown healthy status**
dnt +	*bfrZ* +	*fla* +	82 (39.2%)	37 (45.1%)	30 (36.6%)	15 (18.3%)
dnt +	*bfrZ* -	*fla* +	60 (28.7%)	39 (65%)	20 (33.3%)	1 (1.7%)
dnt -	*bfrZ* -	*fla* +	31 (14.8%)	21 (67.7%)	6 (19.4%)	4 (12.9%)
dnt -	*bfrZ* +	*fla* +	25 (12%)	21 (84%)	1 (4%)	3 (12%)
dnt +	*bfrZ* -	*fla* -	9 (4.3%)	7 (77.8%)	2 (22.2%)	0
dnt +	*bfrZ* +	*fla* -	1 (0.5%)	0	1 (100%)	0
dnt -	*bfrZ* -	*fla* -	1 (0.5%)	1 (100%)	0	0
dnt -	*bfrZ* +	*fla* -	0	0	0	0
	Total		209	126 (60.3%)	60 (28.7%)	23 (11%)

The presence of *dnt* gene was detected in 72.7% of *Bbr* strains. In case of this gene, the greatest variability occurred within each herd. The mentioned gene was detected in strains isolated from 65.8% to 88.3% in herds with PAR, suspected PAR, or of unknown healthy status.

bfrZ gene was detected in 51.7% of *Bbr* strains. The highest number of these strains was obtained from herds with PAR and suspected of the disease (46% and 53.3%, respectively).

In total, seven different genotypes among isolated *Bbr* strains were evidenced. Table 4 shows the genotypes of the examined *Bbr* strains.

PCR tests developed to detect *dnt*, *bfrZ*, and *fla* genes in *Bbr* were used to analyse the genetic profile of the Polish *Bbr* isolates. In 82 (39.2%) *Bbr* strains, all three genes were present. Thirty seven (45.1%) strains were obtained from pigs with clinical symptoms of PAR and 30 (36.6%) from herds where PAR was suspected. The lowest number of strains in which the genes were present simultaneously was in the herd with unknown health status.

In 41.1% of *Bbr* isolates only two genes were detected. The most common genotype *dnt+bfrZ-fla+* was present in 60 (28.7%) *Bbr* strains. In this case, the majority of strains were obtained from herds with PAR and suspected of the disease (65% and 33.3%, respectively). Only a single *Bbr* isolate was evidenced from the third group of the tested herds.

Twenty five (12%) *Bbr* isolates were identified as *dnt-bfrZ+fla+* genotype and they were more frequently isolated from clinical cases of the disease (84%). One strain was obtained from herd with unknown health status. Among *Bbr* genotypes with two detected genes, there was only one *Bbr* strain with *dnt+bfrZ+fla-* genotype. Among 31 (14.8%) strains only *fla* gene was evident, and in 9 (4.3%) only *dnt* gene was present. No *Bbr* strains with *bfrZ* gene only were found.

Discussion

Bordetella bronchiseptica is a respiratory pathogen, which causes illnesses in pigs. The bacterium produces a few virulence factors that could influence the bacterial adherence and colonisation ability. Surface structures that are important in pathogenesis include filamentous haemagglutinin, pertactin, and fimbriae, and dermonecrotoxin or adenylate cyclase toxin. Production of these virulence factors is regulated at the transcriptional level by a two-component sensory transduction system encoded by the *bvg* locus. The

expression of most of the virulence genes is coordinated by the mentioned regulatory system in response to the environmental changes of temperature or in the presence of the nicotinic acid, sulfate ions, or $MgSO_4$, regardless of growth temperature (15, 16, 20).

Many genes, which are involved in pathogenesis are regulated when Bvg system is active (Bvg^+). Phase Bvg^- seems to be expressed when the bacteria is in the environment. The third possible phase option is intermediate (Bvg^i), with the lack of expression of virulence-repressed genes and expression of only a few virulence-activated genes (5, 7, 9).

Iron is indispensable for growth of most of bacteria but generally is not easily accessible for them. According to Pradel *et al*. (18), in an oxic environment at neutral pH, the concentration of free Fe^{3+} in solution is less than 10^{-18}M, which is far below the minimal concentration required to support bacterial growth. In the host organism, the iron is trapped in proteins such as transferrin and lactoferrin or globulins or is distributed within the cytoplasm, as cytochromes or Fe/S-containing enzymes. The iron is an essential metal for successful colonisation of the host tissues by bacteria. *Bordetella* sp. internalises iron through TonB-dependent outer-membrane ferric complex receptors. *Bordetella bronchiseptica* encodes full set of these receptors while *B. pertussis* and *B. parapertussis* genoms contain more pseudogenes (3, 12, 13, 17, 22). Not all analysed strains have this gene, which may suggest that other siderrophore receptor genes were expressed, or the Bvg+ phase was active and concentrated on expression of virulence-activated genes, such as dermonecrotoxin or fimbriae.

Dermonecrotoxin is positively regulated by *bvg*, heat-labile toxin that induces mucosal damage in swine nasal tissue *in vitro* and causes degenerative changes in osteoblasts and the periosteum. Several studies have shown that DNT production is necessary for the induction of clinical signs in swine (10, 16, 19) and because of this, a rapid PCR test was developed to determine the presence of *dnt* gene in *Bbr* isolates and nasal swabs (23).

Brockmeier *et al.* (4) do not rule out the possibility that virulence factors other than DNT are essential in the pathogenesis of AR. The studies with dnt mutant of *Bbr* strains showed that they were not completely avirulent. Pigs infected with these mutants still have clinical symptoms of illness such as sneezing, coughing, and inflammation in the nasal cavity. The undertaken studies showed that even when isolates were identified as *dnt* negative *Bbr* strains, they were isolated from all types of herds. It suggests that compilation of many factors is needed for AR. The changes caused by DNT form actin stress fiber and may positively affect colonisation of the upper respiratory tracts by Pm dermonecrotic strains.

No less important than DNT are fimbriae, which are usually involved in adhession. They are important for both, pathogenesis and immunity. *Bbr* is motile and encodes full flagellar operon. In the genomes of *B. pertussis* and *B. parapertussis*, there is a lack of expression of these factors, which reflects adaptation of the bacteria to specific host niches, and probably enhances the virulence by reducing the number of targets available for recognition by the immune system (1, 2, 11, 21).

Due to the fact that *Bbr* is perceived as a comensal microorganism widely present in pig herds, it was suspected that a high number of isolated *Bbr* strains will have *fla* gene. Almost all isolated *Bbr* strains in our studies had *fla* gene. Fimbriae only provide adherence to the walls of nasal epithelium, which is not synonymous to the ability to cause the disease. A much better marker of pathogenicity of *Bbr* strains is *dnt* gene. It could be used to provide selection among *Bbr* strains of higher virulence. *bfrZ* gene is not regulated by Bvg system. Confirmation of this gene in strains may indicate an increasing activity in the acquisition and capture of the available forms of iron from the environment. During the infection process, the immune cells and erythrocytes provide good source of iron for *Bbr*, therefore, the presence of *bfrZ* gene may indicate the next phase of pathogenic process.

To sum up, Polish isolates of *Bbr* demonstrated different genotype profiles. The presence of *dnt*+ and *fla*+ or *bfrZ*+ and *fla*+ genes in most of the strains isolated from the tested herds with PAR indicate their influence on pathogenicity of *Bbr* strains. On the other hand, strains lacking one or combination of tested genes may explain the variation in their virulence and differences in clinical signs of the disease observed in the examined farms.

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