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Original article

Detection of *Bordetella avium* by TaqMan real-time PCR in tracheal swabs from wildlife birds

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Abstract

Bordetella avium, the causing agent of bordetellosis, a highly contagious infection of the respiratory tract in young poultry, causes significant losses in poultry farming throughout the world. Wildlife birds can be a reservoir of various pathogens that infect farm animals. For this reason the studies were conducted to estimate the prevalence of *Bordetella avium* in wildlife birds in Poland. Tracheal swab samples were collected from 650 birds representing 27 species. The bacterial DNA was isolated directly from the swabs and screened for *Bordetella avium* by TaqMan real-time PCR.

The assay specificity was evaluated by testing DNA isolated from 8 other bacteria that can be present in avian respiratory tract, and there was no amplification from non-*Bordetella avium* agents. Test sensitivity was determined by preparing standard tenfold serial dilutions of DNA isolated from positive control. The assay revealed to be sensitive, with detection limit of approximately 4.07x10^2 copies of *Bordetella avium* DNA. The genetic material of *Bordetella avium* was found in 54.54% of common pheasants, in 9.09% of Eurasian coots, in 3.22% of black-headed gulls and in 2.77% of mallard ducks.

The results of this study point to low prevalence of *Bordetella avium* infections in wildlife birds. The results also show that described molecular assay proved to be suitable for the rapid diagnosis of bordetellosis in the routine diagnostic laboratory.

Key words: *Bordetella avium*, TaqMan real-time PCR, wildlife birds

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Introduction

Bordetella avium (*B. avium*), a Gram-negative, non-fermentative, strictly aerobic, motile bacterium from the genus *Bordetella* causes bordetellosis, a highly contagious infection of the respiratory tract in young poultry, which is characterized by high morbidity (up to 100% birds in the flock) and relatively low mortality (Jackwood et al. 1995). *Bordetella avium* is also associated with the Lockjaw Syndrome in psittacine birds (Grespan et al. 2012). Bordetellosis causes significant losses in poultry farming throughout the world, mainly by predisposing birds to secondary infections. This disease affects mainly young turkeys, whereas in chickens, only several strains are able to induce clinical disease. Bordetellosis usually has a milder course in chickens than in turkey poults (Rimler 1985, Jackwood et al. 1995).

Four toxins produced by *Bordetella avium*: endotoxin, tracheal cytotoxin, heat-labile dermonecrotic toxin and osteotoxin, were isolated to analyze the pathogenesis of *B. avium* infections (Rimler 1985). The above substances have been researched extensively, but their role in the pathogenesis of bordetellosis has not been fully explained. Deformation of tracheal rings and damage to articular cartilages, which are observed in some field cases of *B. avium* infections, could be associated with osteotoxin activity (Yersin et al. 1991).

Bordetella avium has to adhere to tracheal epithelial cells to colonize the respiratory tract of turkeys (Arp et al. 1988, Temple et al. 2010). Hemagglutinin produced by *B. avium* as well as autotransporter proteins and fimbriae play the main role in this process (Arp et al. 1988, Hellwig et al. 1988, Moore and Jackwood 1994, Loker et al. 2011, Stockwell et al. 2011). Interestingly, the fimbrial locus of *B. avium* is regulated in response to temperature (37°C), and the bacterium is able to adhere to the host's respiratory epithelium only under such conditions (Loker et al. 2011).

Wildlife birds can be a reservoir of various pathogens that infect farm animals and humans (Stenzel et al. 2008, Benskin et al. 2009, Bancerz-Kisiel et al. 2012). Despite the above, very little is known about the incidence of *Bordetella avium* in populations of wildlife birds. A US study revealed that *Bordetella avium* can affect mallards, Canada geese, wild turkeys and birds kept in Zoos (Hopkins et al. 1990, Raffel et al. 2002, Hollamby et al. 2003).

The golden standard in diagnosis of bacterial infections is bacterial culture. In the literature there are reports about molecular diagnosis of bacterial infections including *Bordetella* sp. (Register et al. 2005, Koidl et al. 2007). The described methods requires multiplication of bacteria before the DNA extraction, so if the sample contains bacteria, it should be alive. Live bacteria is not required for successful DNA extraction for molecular methods, which is the main advantage of this diagnostic procedure. The quantity of bacterial genetic material in field samples usually is low, therefore a very sensitive diagnostic method is required for obtaining the reliable results. In the light of the above the main aim of this study was to develop a sensitive method for molecular diagnosis of *Bordetella avium* directly from avian tracheal swabs. Having regard to the fact that wildlife birds can be a reservoir and vector of *B. avium* for poultry, the second aim of this investigation was to estimate the prevalence of *B. avium* in wildlife birds in Poland.

Materials and Methods

Experimental material was collected in 2010-2014 from wildlife birds that were harvested by hunters or caught by ornithologists during ringing actions. In 2010, waterfowl kept in a free-range aviary in the Gdańsk Zoo (Poland) were also tested. Tracheal swab samples were collected from birds with the use of the commercial transport system (ESwab Collection and Transport System, Copan Diagnostic, USA). A total of 650 birds representing 27 species were analyzed. The list of examined birds is presented in Table 1.

The bacterial DNA was extracted directly from the swab transport medium, without prior culture of bacteria. Before attempting to isolate DNA from the swabs, the entire swab transport medium was transferred to a microtube and centrifuged (5430 R centrifuge, Eppendorf, Germany) at 240xg for 15 minutes at 4°C. The supernatant was discarded and the cellular pellet was suspended in 170 µL phosphate buffered saline solution (PBS). DNA was extracted by the magnetic method using the automated workstation (Janus, Perkin Elmer, USA) and the commercial kit (NucleoMag Tissue Kit, Macherey-Nagel, Germany) in accordance with the manufacturer's instructions. The concentrations of eluted DNA were measured using the spectrophotometer (NanoDrop 2000, Thermo Scientific, USA) and DNA was stored at -80°C for further analysis.

Screening for *Bordetella avium* was performed by TaqMan real-time PCR. The reaction was carried out with the use of the commercial kit (TaqMan Fast Universal PCR MasterMix, Life Technologies, USA) and the following primers: BArecAFwd 5'-CGGTTCGCTGGGCTTGG-3', BArecARev 5'-CACGCGGCAGCCCGC-3' and BarecA Hex CATCGCGCTGGGTG BHQ-1 probe amplifying

Table 1. List of samples collected in 2010-2014 and the results of TaqMan real-time PCR screening for the presence of *Bordetella avium* genetic material in tracheal swab from wildlife birds.

Scientific name	Common name	Number of samples		Number of samples positive for *B. avium*	
		n	%	n	%
Alca torda	Razorbill	1	0.15	0	0
Anas crecca	Euroasian teal	14	2.15	0	0
Anas platyrhynchos	Mallard duck	397	61.1	11	2.77
Anser anser	Greylag goose	3	0.46	0	0
Anser albifrons	White frontem goose	2	0.31	0	0
Anser fabalis	Bean goose	8	1.23	0	0
Aquila pomarina	Lesser spotted eagle	1	0.15	0	0
Aythya fuligula	Tufted duck	3	0.46	0	0
Buteo buteo	Common buzzard	1	0.15	0	0
Chroicocephalus ridibundus	Black-headed gull	31	4.77	1	3.22
Clangula hyemalis	Long-tailed duck	1	0.15	0	0
Corvus corone cornix	Hooded crow	3	0.46	0	0
Corvus monedula	Jackdaw	1	0.15	0	0
Cygnus olor	Mute swan	43	6.62	0	0
Fulica atra	Euroasian coot	11	1.7	1	9.09
Gavia stel lata	Red-throated loon	1	0.15	0	0
Haliaeetus albicilla	White-tailed eagle	1	0.15	0	0
Larus argentatus	Herring gull	15	2.31	0	0
Larus canus	Common gull	4	0.61	0	0
Melanita fusca	Velvet scoter	4	0.61	0	0
Pelecanus onocrotalus	Great white pelican	13	2	0	0
Pernis apivorus	Honey buzzard	1	0.15	0	0
Phalacrocorax carbo	Black cormorant	49	7.54	0	0
Phasianus colchicus	Common pheasant	22	3.39	12	54.54
Phoenicopterus ruber	American flamingo	16	2.47	0	0
Pica pica	Euroasian magpie	3	0.46	0	0
Podiceps cristatus	Great crested grebe	1	0.15	0	0
	Total:	650	100		

a ~50 bp region of the *B. avium RecA* gene (GenBank accession no. AY124330).

The reaction was performed in the thermocycler (MX 3005P, Stratagene /Agilent technologies, USA) at the final volume of 20 µL, comprising 10 µL of the TaqMan Fast Universal PCR MasterMix, 1.8 µL of each primer (10 µM), 2 µL of the labeled probe (2.5 µM) and 4.4 µL of purified DNA. The following thermal cycling parameters were applied: 95°C for 30 s, 40 cycles of a two-stage temperature profile of 95°C for 15 s and 60°C for 1 min.

Test specificity was evaluated by testing DNA isolated from other pathogens that can be present in avian respiratory tract: *Pasteurella multocida* (ATCC 12945), *Ornithobacterium rhinotracheale* (ATCC 51463), *Klebsiella pneumoniae* (ATCC 13882), *Escherichia coli* (ATCC 10536), *Mycoplasma (M.) gallisepticum* (ATCC 19610), *Mycoplasma iowae* (ATCC 33552), *Mycoplasma synoviae* (ATCC 25204) and *Mycoplasma meleagridis* (ATCC 25294).

Assay sensitivity was determined by preparing the standard curve. The first step of this procedure was amplification of a 740 bp fragment of Ba *Rec*A gene. The amplified fragment contained a sequences complementary for BArecA primers and probe. The reaction was carried out in a thermocycler (Mastercycler, Eppendorf, Germany) using the commercial reagent kit (HotStarTaq Plus Master Mix Kit, Qiagen, Netherlands) and a pair of primers: B.aviumrecA740bpF 3'-CCGCTTATGCATGACCGTTT-5' and B.aviumrecA740bpR 3'-TTCAGGGTTGCCGAACATCA-5'. The contents of the reaction mixture were as follows: 10 µL of HotStarTaq Plus Master Mix, 0.1 µL of each primer (100 µM), 2 µL of loading dye[i], 2 µL of template DNA and 5.82 µL of water. The PCR conditions were as follows: 95°C for 5 minutes; then 30 cycles: 94°C for 60 s, 60°C for 60 s and 72°C for 90 s; final elongation after the last cycle at 72°C for 10 minutes. The resulting product was purified from the residues of buffer and nucleotides using commercial kit (Clean-Up, A&A Biotechnology, Poland) and next the concentration of amplicon was measured using a spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). Afterwards, the gene copy number

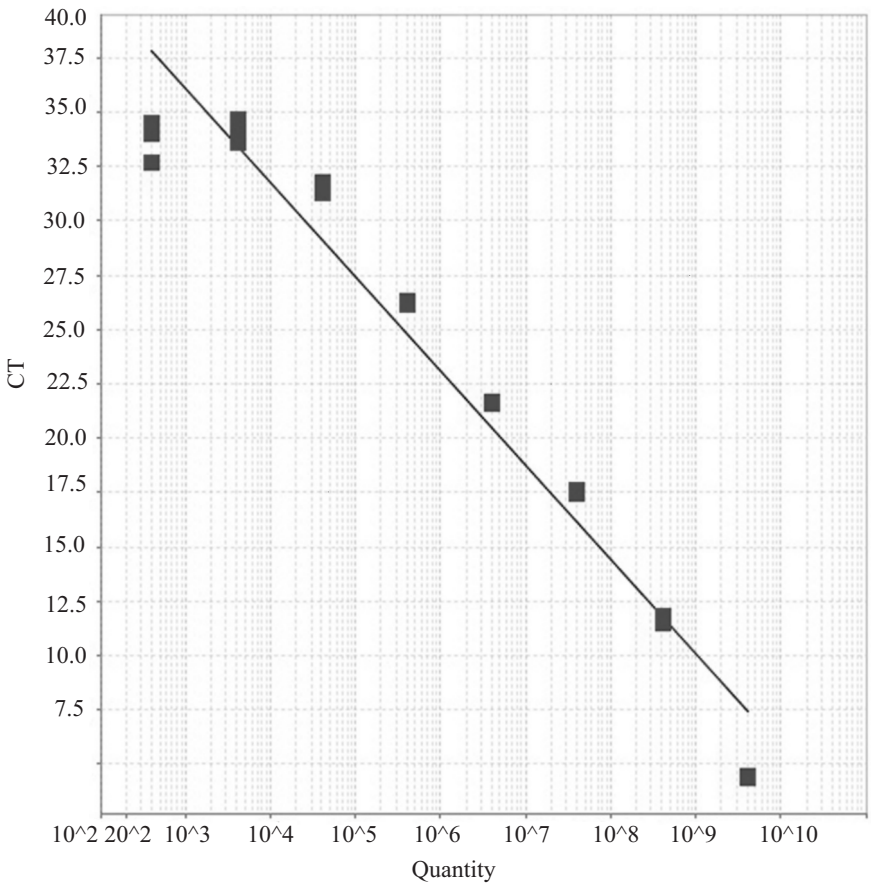

Fig. 1. The standard curve derived from tenfold serial dilutions of DNA isolated from positive control (*Bordetella avium*, ATCC 35086).

resulting from the concentration and size of the amplicon was calculated using ds DNA calculator software (dsDNA copy number calculator, Rhode Island Genomic and Sequencing Center, USA).

The next step was to prepare standard tenfold serial dilutions of amplicon used as a template DNA. Aliquots of each dilution (initial dilution: 10^10, final dilution: 10^2) were subjected to the TaqMan real-time PCR assay to determine the lowest number of DNA copies that can be detected with the assay.

During examination of field samples the fluorescence curves were analyzed with thermocycler software. The results were expressed by determination of the threshold of detection, or crossing point (Cp), which marked the cycle at which fluorescence of the sample became significantly different from the baseline signal. Each DNA sample was submitted to TaqMan real-time PCR amplification in duplicate. The samples were recognized as positive when the Cp value was lower than 35 cycles in both replicates. One positive control and two negative controls were added to each run. Positive control consisted of DNA isolated directly from a laboratory culture of *Bordetella avium* (ATCC 35086), whereas negative controls were two sterile water samples, one submitted to the extraction and amplification protocols and the other submitted only to the amplification protocol. This was done to verify that there was no contamination in any step of the technical procedure.

Results

The assay revealed to be highly sensitive and specific. Test specificity has shown that there was no amplification from non-*B. avium* agents sequences. The detection limit in sensitivity evaluation was 4.07x10^2 copies of *Bordetella avium* DNA. The standard curve was linear over a wide range of dilutions (Slope: -4.33; R2: 0.953; Efficiency: 70.1%) (Fig. 1).

Samples positive for *Bordetella avium* are characterized in Table 1. A total of 25 birds (3.84%) of the examined population, tested positive for *B. avium*. The genetic material of *B. avium* was found in 12 out of 22 examined common pheasants (54.54%), in 1 out of 11 Eurasian coots (9.09%), in 1 out of 31 black-headed gulls (3.22%) and in 11 out of 397 mallard ducks (2.77%). None of the examined Zoo birds tested positive for *Bordetella avium.*

Discussion

A rapid and reliable diagnostic method is essential for the correct treatment of each infectious disease. For many years culture has been considered as a gold standard for the detection of several bacteria, but this method often lacks sensitivity and also requires a few days of examination. The molecular methods have been reported to be more sensitive and specific than traditional diagnosis of bacterial infections (Dragsted et al. 2004).

In our study, a new molecular method for the detection of *Bordetella avium* based on automated DNA extraction and TaqMan real-time PCR was evaluated. The results have shown that assay is specific and sensitive with detection limit of approximately $4.07x10^2$ copies of *Bordetella avium* DNA.

A US study on the prevalence of *Bordetella avium* in wildlife birds revealed that the bacterium can colonize large range of avian species (Hopkins et al. 1990, Raffel et al. 2002). The antibodies against this bacterium were detected in 45% of the serum samples collected from 62 species of wildlife birds. The seroprevalence was the highest in waterfowl birds like great blue herons (*Ardea herodias*), canada geese (*Branta canadiensis*), muscovy ducks (*Carina moschata*), mallards (*Anas platyrhynchos*) and birds belonging to the order *Passeriformes*: blue jays (*Cyanocitta cristata*) and american crows (*Corvus brachyrhynchos*). The antibodies against *B. avium* were also found in *Galliformes* birds like ring necked pheasants (*Phasianus colchicus*) and wild turkeys (*Meleagris gallopavo*) (Raffel et al. 2002). *Bordetella avium* was also isolated from wildlife birds in Europe. Szabó et al. (2015) performed antimicrobial susceptibility tests of *B. avium* strains isolated from wildlife waterfowl (geese and ducks of unknown species) and partridge (*Perdix perdix*).

Turkeys (*Meleagris gallopavo*) are the typical hosts for *B. avium*, but this species is not encountered in the wild in Poland. In our study, *Bordetella avium* was most prevalent in the common pheasant, also a member of the order *Galliformes*. All pheasants that tested positive for *B. avium* were harvested by hunters in the same hunting district where pheasants are released into the wild by the same commercial aviary every year. All hunted birds originated from the same, infected, but probably asymptomatic flock. We were unable to obtain samples from that flock, therefore, our hypothesis remains unverified.

The presence of *Bordetella avium* genetic material in tracheal swabs from wild waterfowl indicates that those birds could be a *B. avium* carriers. Infections spread easily among aquatic birds during migration when birds congregate in large flocks. Migratory birds often stop over for short periods of time in the same, often very shallow bodies of water, which contributes to the transmission of pathogens not only between individuals of the same species, but also between different species of birds. The above could explain the presence of *Bordetella avium* genetic material in mallard ducks, as well as in Eurasian coots and black-headed gulls. The above results partially correspond with investigation of Raffel et al. (2002) who found that isolation rate of *B. avium* from tracheas of wildlife birds is quite low. Those authors have found this bacterium only in 9/ 128 tracheal swab samples collected from 24 avian species (7 obtained from mallards and one from canada goose and wild turkey, respectively).

The results of this study indicate that the used method is a quick, convenient and not requiring a bacterial culture, way for diagnosis *B. avium* from clinical samples of birds. This assay can be a sensitive, specific and fast alternative method in the routine diagnostic laboratory. Rapid laboratory diagnosis of turkey coryza can be a useful tool for practicing veterinarians, because the prevalence of *Bordelella avium* in domesticated turkeys bred in Poland is very high (44 – 100% of flocks depending on the age) which was confirmed by serological screening (Śmiałek et al. 2015). The obtained results also point to low prevalence of *Bordetella avium* infections in wildlife birds. However, wild birds can be a reservoir of this bacterium and they can pose a threat for domestic poultry, in particular when biosecurity requirements are not restricted.

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