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Original article

Occurence of *Bordetella bronchiseptica* in domestic cats with upper respiratory tract infections

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Abstract

Bordetella bronchiseptica is a widespread Gram-negative pathogen occurring in different mammal species. It is known to play a role in the etiology of infectious atrophic rhinitis of swine, canine kennel cough, respiratory syndromes of cats, rabbits and guinea pigs, and sporadic human cases have also been reported. The aim of this article is to present the occurrence of infections caused by these bacteria in domestic cats with respiratory symptoms, as well as to conduct a molecular analysis of the flaA gene *B. bronchiseptica* for the purpose of ascertaining whether cats become infected with one or more bacteria strains. *B. bronchiseptica* was isolated from the respiratory system of 16 out of 35 domestic cats with symptoms of respiratory tract infections. Polymorphism analysis of polymerase chain reaction products of *B. bronchiseptica* flaA was performed to reveal the possible differences in nucleotide sequences of the flagellin gene.

The phylogenetic analysis of nucleotide sequences obtained during PCR indicated that the isolates of bacteria from our own studies are characterised by 100% homology of the analysed fragment of the flaA gene, which suggests maintenance of a single genotype of these microorganisms in the cat population. Moreover, the bacteria revealed full homology with reference strain *B. bronchiseptica* ATCC 4617, and 99.4% homology with strain *B. parapertussis* ATCC 15311. This indicates that the PCR optimised for the *Bordetella* spp. flaA gene, combined with sequencing of amplicons obtained in PCR, is an effective diagnostic method allowing differentiation of *Bordetella* spp. type microorganisms.

Key words: *Bordetella bronchiseptica*, PCR, microbiological examination, domestic cat

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Introduction

Upper respiratory infections are one of the most common syndromes affecting cats in shelters, boarding facilities, and multiple cat households. The infectious agents implicated as primary causes of rhinitis in cats include feline herpesvirus 1 (FHV-1), feline calicivirus (FCV), *B. bronchiseptica, Chlamydia felis*, Mycoplasma spp., and some strains of *Pasteurella* spp. (Johnson et al. 2005, Maggs and Clarke 2005, Pesavento et al. 2008, Veir et al. 2008). *B. bronchiseptica* is a primary pathogen of domestic cats, particularly in high population density conditions. This Gram-negative bacteria is closely related to *B. pertussis*, and *B. parapertussis*. Sequence analysis has shown that *B. parapertussis* and *B. pertussis* are independent derivatives of *B. bronchiseptica*-like ancestors. During their evolution, there was large-scale gene loss and inactivation; host adaptation seems to be a consequence of loss, not gain, of function, and differences in virulence may be related to loss of regulatory or control functions. *B. bronchiseptica* causes chronic respiratory infections in cats, dogs, rabbits, pigs and humans (Parkhill et al. 2003).

Since the registers kept by veterinary clinics indicate that the problem of bordetellosis in cats has recently increased, and the disease itself, in spite of immediate treatment administration, often takes a chronic course and, moreover, may constitute a threat to human health, the objective of this article is to **present the occurrence** of infections caused by these bacteria in domestic cats with respiratory symptoms, as well as to conduct a molecular analysis of the flaA gene *B. bronchiseptica* for the purpose of ascertaining whether cats become infected with one or more bacteria strains.

Materials and Methods

Animals used in the study

The study covered 35 domestic cats of various breeds (21 females and 14 males aged 4 months to 14 years). The animals came from the Lubelskie Voivodeship and they exhibited symptoms of upper respiratory tract infection (nasal discharge and cough). All the animals had been vaccinated against the basic contagious diseases (panleucopenia, calicivirosis, herpesvirosis, feline leukaemia) and received regular antiparasitic prophylaxis.

The research material was collected from all animals (swabs and broncho-alveolar lavage-BAL) for bacteriological and virological examinations. Virological examinations was performed only for viruses that are responsible for respiratory tract infections (FHV-1, FCV). Bacteriological examinations were carried out in the laboratory of the Clinic of Infectious Diseases of the Faculty of Veterinary Medicine in Lublin, whereas virological tests were performed in a commercial diagnostic laboratory.

Bacteriological examination

The experimental material was swabs from upper respiratory tracts (nose, throat) and BAL fluids collected during an endoscopic examination. Bacteria were cultured on the following media: agar 1% with the addition of 5% defibrinated horse blood (Blood Agar Base), Charcol Agar with supplement addition (Bordetella Selective Supplement FD004), MacConkey Agar with FK (Emapol) and Champman base (Mannitol Salt LAB AGAR).

The cultures were incubated at 37°C for 24-48 hours under aerobic conditions. The identification of bacteria cultures was performed on the basis of colony and cell morphology. Initial determination was made with the use of a bacterial cytochromoxidase detection test (OXItest Mikro La by Erba Lachema).

Biochemical identification was performed with the NEFERMtest 24 kit used in the diagnostics of Gram-negative non-fermenting bacteria (Mikro La Test by Erba Lachema).

Molecular analysis

The *B. bronchiseptica* culture isolates identified during bacteriological examination on nasal and pharyngeal swabs and BAL fluids were subjected to molecular examination. DNA isolation was performed using a DNA Genomic mini isolation kit (A&A Biotechnology, Gdańsk, Poland) according to the procedure as provided by the manufacturer. Purified DNA was suspended in 100 µl of Tris buffer to be used in further analysis. The amplification of *B. bronchiseptica* flaA gene was performed using the primers Fla2: 5' AGGCTCCCAAGAGAGAAAGGCTT 3', and Fla3: 5' CACCTGCCCCATCTCC 3'. The primers made it possible to amplify a gene fragment of 165 base pairs (Hozbor et al. 1999). The composition of the PCR mix was determined during the optimisation of concentrations of its particular components (MgCl2, dNTP, starters, Taq Polymerase). The most optimal results were obtained with a primer concentration of 50 pm/µl and final concentrations of dNTP-100 µM, $MgCl_2$-1.6 mM, Taq polimerase – 2.5 units. The PCR was conducted in the Biometra apparatus (Gottingen, Germany). It covered 35 cycles, each consisting of

a stage of DNA denaturation at 94°C for 1 min, annealing of primers at 57°C for 30 sec, and strand extension at 72°C for 40 sec. The reaction was conducted using positive and negative control. The DNA obtained from the reference bacteria strain *B. bronchiseptica* ATCC 655422™ was used as positive control, whereas the total DNA isolated from nasal swabs collected from a healthy dog served as negative control. Products of the PCR were analysed using the electrophoresis method in 1.5% agarose gel, in TBE buffer, at a voltage of 10 V/cm. The gel was stained with ethidium bromide (1 µg/ml) for 15 min. The size of products was determined according to the weight standard DNA ladder 100 bp (Fermentas, Lithuania).

The amplicons obtained were purified on columns using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified products were sequenced in the Laboratory of DNA Sequencing and Oligonucleotide Synthesis of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw. The sequencing results were received via e-mail and developed using Lasergene MegAlign software (Madison USA). The developed sequences of our own isolates were then compared to one another and then to the standard sequence of the *B. bronchiseptica* flaA gene from isolates from China (EU 327790), Argentina (AJ 012319) and the USA (L 13034) from the NCBI GenBank.

Results

Results of bacteriological examination

In the examined group of cats, mixed bacterial flora was isolated from nasal, pharyngeal swabs and BAL fluids sampled from 34 out of 35 examined animals.

In one case no microorganism growth was observed. The inoculation of swabs sampled from one animal resulted in the growth of unidentified microorganisms. In the case of two cats saprophytic flora was isolated from the throat and nose (*Micrococcus* spp., *Neisseria* spp.). In the case of other animals, the most frequently isolated microorganisms from pharyngeal and nasal swabs were *B. bronchiseptica* (in 16 out of 35 tested cats), next staphylococci (in 12 out of 35 animals), streptococci (in 5 out of 35 cats), *E. coli* (in 2 out of 35 cats), *Proteus* spp. (in 1 out of 35 cats) and *Pasteurella* spp. (in 1 out of 35 tested cats). In 7 cases the bacteriological examination indicated infections caused by more than one species of bacteria.

The presence of *B. bronchiseptica* bacteria in the inoculation of BAL fluids was indicated only in 5 animals.

Results of molecular analysis

The PCR examination on the pharyngeal and nasal swabs carried out in a commercial laboratory did not reveal FHV-1 and FCV infections in any of the cats.

The DNA of *B. bronchiseptica* bacteria isolated in 16 cats revealing the symptoms of inflammation of upper respiratory tracts, as well as of two reference strains (*B. bronchiseptica* ATCC 4617 and *B. parapertussis* ATCC 15311) was subjected to amplification with the use of Fla2 and Fla3 primers. The genetic material from a fragment of *Bordetella* spp. flaA gene was observed in all 18 samples.

Clear sequences of DNA *B. bronchiseptica* amplification products were obtained with respect to all the examined samples marked with numbers from 1 to 16 (Fig. 1) as well as for two standard strains marked as 17 (*B. bronchiseptica* ATCC 4617) and 18 (*B. parapertussis* ATCC 15311). Comparison of nucleotide sequences of the isolates obtained in our own studies with the use of DNA Star MegAlign software allowed the level of their mutual homology to be determined as being in the range 99.4-100.0%.

The analysis of the said sequences allowed two polymorphic groups marked A and B to distinguished. Group A contained isolates no. 1-17, characterised by a 100% similarity of the following nucleotide sequence: TCCCCCGCACATTTCC GAACTTCACT TTTTTTGCTTAAGTCCGTCGCAAACCTGCCG TAATCCAGGCAACAAAGGAAATCGCGGCGC TGTGCAAGCGAAAGTCCGATGTTACAGATG GGCGGCCTAGCTGCCCGGTTTGAAGAAG-CCTTTCTCTCTTGGGAGCCT, whereas group B was constituted by isolate no. 18, whose nucleotide sequence showed a similarity with the remaining isolates at a level of 99.4%. The difference of 0.6% between the sequence of isolate 18 and the sequences of isolates from group A resulted from a substitution of adenine with guanine in position 99 in the sequence of isolate 18. A graphic representation of this similarity takes the form of a phylogenetic tree (Fig. 1).

The sequences of our own isolates of phylogenetic groups A and B were compared with the use of DNA Star MegAlign software with correspondent sequences of *B. bronchiseptica* isolates available in the PubMed NCBI database:

– EU 327790 from the territory of China,
– AJ 012319 from the territory of Argentina,
– L 13034 from the territory of the United States of America.

The assumed point of reference was the nucleotide sequence of the representatives of group A, characterised by a 100% homology.

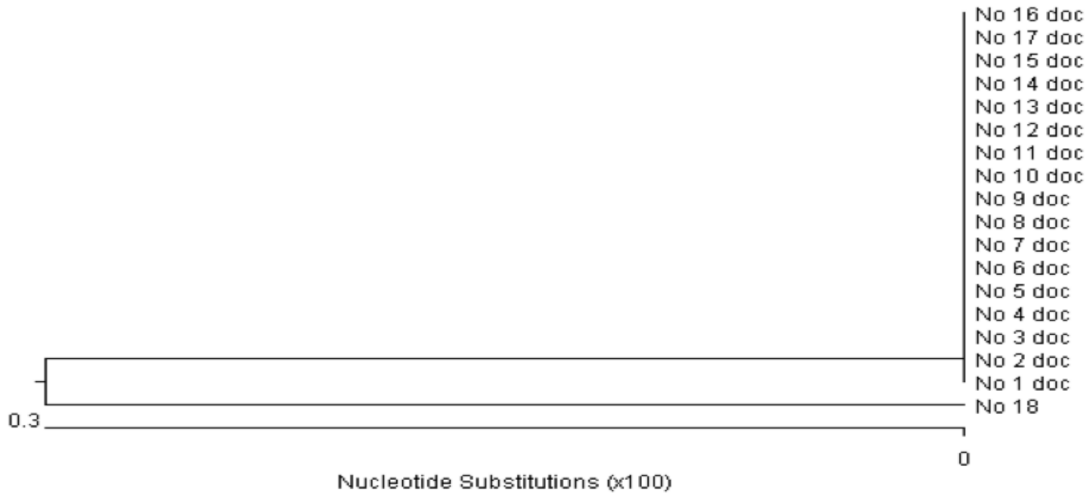

Fig. 1. Phylogenetic tree depicting the level of similarity of nucleotide sequencesof a fragment of the flaA gene *Bordetella* spp. obtained from isolates of our own bacteria and two reference strains (no. 17 and 18).

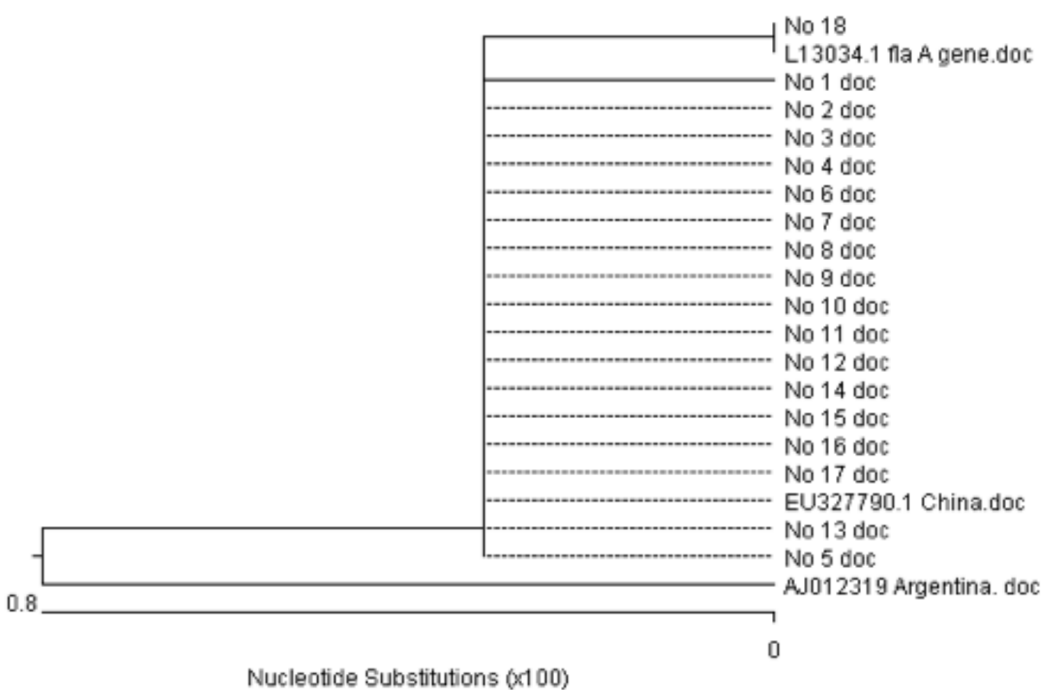

Fig. 2. Dendrogram from the sequence analysis of the flaA gene of global *Bordetella* spp. isolates available in the database and the sequence of A and B group isolates.

The similarity of sequences of the examined world isolates available in the GenBank with the sequences of our own isolates also proved to be very high – within the range of 98.2-100%.

The results of the phylogenetic analysis of our own isolate sequences and the sequences available in the GenBank are shown in Fig. 2.

Discussion

In small animal veterinary medicine the issue of bacterial infections of respiratory tracts is often marginalised with the treatment being limited to the implementation of antibiotic therapy with broad spectrum chemotherapeutic agents. The omission of a di-

agnostic process in respiratory infections and treatment implementation without a conclusive diagnosis may be fatal in consequence (Chappuis 1985).

Our own studies indicate that in the groups of domestic cats with bacterial respiratory tract infections the most serious problem is infections caused by *B. bronchiseptica.* Their course is also significantly more severe as compared with infections induced by other microorganisms (Garbal 2015). Research conducted by Adler et al. (2007) on 460 cats with symptoms of severe infections of upper respiratory tracts confirmed the presence of *B. bronchiseptica* only in 0.4% of affected animals. The most common microorganisms isolated by research authors from pharyngeal swabs included: *Pasteurella* spp. (32.5%), *Staphylococcus* spp. (18.5%), *Escherichia coli* (17.0%), *Streptococcus* spp. (9.1%), *Pseudomonas* spp. (6.9%) and *Klebsiella* spp. (3.0%). Importantly, the type of isolated microflora was largely dependent on the geographical location of the tested animals.

There are numerous factors predisposing the development of infections caused by *Bordetella* spp. Undoubtedly, one of them includes the age of infected animals. According to the studies by Welsh (1996), the most severe course of bordetellosis is noted in young animals, and in cats these are commonly animals aged below 8 weeks, and the disease itself may end in death in 64% of cases.

Bordetellosis occurence is strictly correlated with the conditions in which animals are kept. A large concentration of animals is conducive to the spread of infection. As is apparent from the studies performed by Binns et al. (1999) on 740 cats, in the population studied the infections caused by *B. bronchiseptica* were observed with the following frequency: in shelters 19.5%, in breeding facilities 9.0%, among cats kept in breeding facilities for scientific purposes 13.5%, whereas in individual specimens kept in households such infections were not detected. The above results are in conflict with the authors' own observations. Although it is possible to agree that shelters and breeding facilities are places where bordetellosis spreads quickly and easily, the fact that infections caused by the microorganisms in question were not found in any domestic animals seems somewhat surprising. In the course of our own studies *B. bronchiseptica* was isolated in as many as 16 out of 35 domestic cats revealing symptoms of upper respiratory tract infections. Such discrepancies can be explained by the fact that the animals qualified for research in the quoted article never left their habitats, whereas those analysed in our own studies were often free-walking animals, which guaranteed their contact with various pathogens. Indeed, the animals' exposure to various infectious agents (viruses, bacteria, fungi, parasites) constitutes another element increasing the risk of bordetellosis; nonetheless it should be remembered that the disease does not always accompany such infections/invasions, and often is a result only of an infection induced by *B. bronchiseptica* (Binns 1999).

If we reverse the course of the above considerations, we will note that the same infections caused by *B. bronchiseptica* predispose the occurrence of secondary infections (Wagener et al. 1984). Even asymptomatic infections caused by *B. bronchiseptica* and resulting from the colonisation of the mucous membrane of respiratory tracts by these bacteria with accompanying viral or bacterial infections, significantly impair the functions of the respiratory system, thus resulting in an aggravation of the disease process.

Apart from environmental and age-related factors, a large impact on the development of infections caused by *B. bronchiseptica* may also be exerted by certain genetic conditioning, namely genetic defects, which predispose to the occurrence of certain anomalies in the respiratory system, and also facilitate the development of infections, as is the case with people with emphysema with alpha 1-antiprotease deficit (Dye 1992).

The PCR technique was used in the identification of *B. bronchoseptica* isolated in 16 cats with symptoms of inflammation of upper respiratory tracts. The test was based on the detection of the flaA gene which was formerly used to distinguish *B. bronchiseptica* strains and shows a relatively high polymorphism (Friedman et al. 2006). The phylogenetic analysis of nucleotide sequences obtained through the polymerase chain reaction indicated that the isolates of bacteria from our own studies are characterised by a 100% homology of the analysed fragment of the flaA gene, which suggests maintenance of a single genotype of these microorganisms in the cat population. Moreover, the bacteria revealed full homology with the reference strain *B. bronchiseptica* ATCC 4617. The comparison of the nucleotide flaA gene sequences obtained during examinations of our own isolates of *B. bronchiseptica* with an analogous fragment of a flaA gene of the reference strain *B. parapertussis* ATCC 15311 allowed both these bacteria species to be differentiated. The degree of homology of the examined gene between both microorganisms reached 99.4%. This indicates that the PCR optimised for the flaA ***Bordetella*** spp. gene, combined with sequencing of amplicons obtained in PCR, is a diagnostic technique allowing differentiation of *Bordetella* type microorganisms. This is particularly important with regard to the fact that in numerous diagnostic procedures improperly selected starters, designed, for instance, for a different gene, enabled amplification of the genetic material of

non-pathogenic microorganisms and resulted in false positive results of the PCR being obtained in the situation when the specified bacteria were not in fact responsible for disease development (Register and Nicholson 2007).

The research conducted to date aimed at working around this problem consisted of the indication of potential genes which could serve as markers of infections with particular *Bordetella* spp., the detection of which would indicate the participation of a clearly defined bacteria in the disease process (Guldemir et al. 2011). The most intense research was conducted on multicopy insertion sequences (IS's), particularly on IS481 and IS1001, which are quite commonly used in the detection of *Bordetella* bacteria. They are observed in numerous representatives of this genus, in particular *B. pertussis* and *B. parapertussis.* Since they were also detected in the genome of *B. holmesii* and *B. bronchiseptica*, they cannot serve as intrinsic markers of a single species (Tizolova et al. 2013).

The results of this study indicate that *B. bronchiseptica* respiratory tract infections in cats are still a significant problem in veterinary practice, and one of the elements of effective elimination of these infections is their proper diagnosis. Correct *Bordetella* spp. identification with the use of molecular biology methods is important not only for the purposes of epidemiology and taxonomy, but is also significant from the practical point of view, namely for defining efficient immunoprophylaxis. A detailed determination of which *Bordetella* genotypes are the most common in cats and dogs may be of value in the preparation of a vaccine with antigens containing complete cells or proteins of these particular microorganisms. Correct antigen selection has a decisive effect on the effectiveness of immunity-boosting products (Ellis et al. 2001).

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