

Prevalence of respiratory pathogens detected in dogs with kennel cough in Poland

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Abstract

Kennel cough is a multifactorial disease occurring all over the world; however, its epidemiology is still not fully understood. To the authors' knowledge, no studies monitoring the occurrence of infectious agents responsible for kennel cough have been carried out in Poland. Therefore, the objective of our study was to determine which of the four pathogens most frequently isolated in other countries are predominant in north-eastern Poland. Swabs from the upper respiratory tract and tracheal lavage fluids from dogs (n = 40) exhibiting symptoms of this disease were analysed. Canine herpesvirus, canine parainfluenza virus, canine adenovirus type 2 and *Bordetella bronchiseptica* were identified by polymerase chain reaction. At least one of the above-listed infectious agents was found in all dogs. The predominant pathogen within the area under our study, both in mono- and co-infections, was canine herpesvirus (32/40), whereas canine adenovirus type 2 occurred least frequently (4/40). The effectiveness of detection of selected pathogens from both types of study material was also compared. Tracheal lavage fluid was more suitable for the isolation of canine herpes virus, canine parainfluenza virus, and *Bordetella bronchiseptica*. Swabs from the upper respiratory tract were more suitable for the isolation of canine adenovirus type 2.

Canine, canine herpesvirus, canine parainfluenza virus, respiratory disease

Kennel cough is a highly contagious pandemic disease in dogs of all breeds and ages. The disease is spread through airborne droplets, and therefore it primarily affects animals kept in large groups (veterinary clinics, animal shelters, kennels, working dogs, and sports competitions), but may also occur in animals kept at home (Mochizuki et al. 2008; Pouswan et al. 2010; Wesse and Stull 2013). Kennel cough is mainly manifested as infections of the upper respiratory tract, although, in some cases it may involve the lower respiratory tract. Two forms of the disease are recognised: mild and severe. The mild form is usually manifested by a characteristic dry cough, excessive mucus production, and retching or vomiting. Severe kennel cough occurs far less frequently and is additionally manifested by fever, depression, emaciation, and in some cases leads to the death of the animal (Chalker et al. 2003; Buonavoglia and Martella 2007). The course of the disease is, to a large extent, determined by the condition of the host (mainly immune status), environmental factors, and the type of aetiologic agent (Buonavoglia and Martella 2007; Ellis et al. 2011). It is believed that the disease is caused by a complex of viruses and bacteria which usually co-exist. This can be proven by findings from experimental infections of dogs with a single infectious agent, where either the absence or mild symptoms of the disease are observed without the natural full spectrum of kennel cough symptoms. Major infectious agents responsible for mild kennel cough include: canine parainfluenza virus (CPIV), canine adenovirus types 1 and 2 (CAV-1, CAV-2), canine distemper virus (CDV), *Bordetella bronchiseptica*, and *Mycoplasma* sp.. *Streptococcus equi* subsp.

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zoepidemicus and *Mycoplasma cynos* were most frequently isolated from dogs with the severe form of kennel cough (Foley et al. 2002; Erles and Brownile 2005; Gore et al. 2005; Buonavoglia and Martella 2007; Ellis and Krakowka 2012; Bhardwaj et al. 2013). Canine herpesvirus (CHV) is also one of the more frequently found pathogens, yet its significance in the aetiology of the disease is not fully understood, and is still very controversial. Canine herpesvirus is probably latent in dogs, and is activated under the influence of various factors. This may produce pathological symptoms in the respiratory tract or reproductive system (Miyoshi et al. 1999; Erles et al. 2004; Krogenæs et al. 2012). In some dogs the influenza virus, two types of coronavirus, canine reovirus type 1-3, *Pasteurella* sp. or *Pseudomonas* sp. are also isolated (Edinboro et al. 2004; Erles et al. 2004; Buonavoglia and Martella 2007; Bhardwaj et al. 2013).

Even though preventive vaccination campaigns against some of the above-mentioned pathogens are organised, new reservoirs of the disease are still reported worldwide. This situation may indicate that kennel cough is probably caused by additional pathogens not covered by the vaccination programme. Moreover, the spread of this disease in animal shelters, due to the housing conditions, may be a consequence of either a too short or totally eliminated period of isolation of vaccinated dogs from symptomatic ones, which prevents the complete development of immunity in vaccinated animals (Erles et al. 2003; Edinboro et al. 2004).

Monitoring studies are carried out worldwide to identify infectious agents responsible for kennel cough in dogs. Researchers investigate which pathogens are predominant within a certain area, and whether or not new pathogens emerge. Hence, the aim of our study was to determine by polymerase chain reaction (PCR) which of the four most common pathogens can be detected from swabs and tracheal lavage fluid in dogs with kennel cough symptoms in north-eastern Poland.

Materials and Methods

This study was conducted upon the approval of the local Ethics Committee (Decision no. 87/2013/N).

The study was performed in the north-east region of Poland on 40 dogs of different breeds, ages and sexes from private owners between September 2014 and January 2015. All animals demonstrated the clinical signs of kennel cough (nasal discharge, dry cough, temperature, dyspnoea, depression, and anorexia). In most cases the subjected animals came from adoptions and their vaccination history was not known.

Prior to the sampling procedure, animals were sedated with 1–2 mg/kg bw i.v. xylazine (Vetaxyl 20 mg/ml, Vet-Agro, Lublin, Poland), and then general anaesthesia was induced with 2–3 mg/kg bw propofol bolus (Scanofol 10 mg/ml, ScanVet, Skiereszewo, Poland).

Swabs

Swabs were taken using sterile cotton buds (sterile rods in a test tube A020 9/170, manufactured by Hagmed, Rawa, Poland) from nasal passages, the laryngeal region, and the tracheal orifice.

Tracheal lavage

A relevant-size endotracheal tube (Curity I.D. 4.0–9.0) was inserted under general anaesthesia into the animal's trachea. Next, a sterile catheter was inserted inside the tube until resistance was met (1.3–4.0/500 mm). Saline (0.9% NaCl) heated to body temperature was introduced via the catheter (5–20 ml per dog, depending on the dog's body weight). The lavage fluid was then removed by suction using a syringe attached to the catheter, and transferred into sterile plastic containers.

DNA and RNA isolation

Viral DNA or RNA and bacterial DNA from nasal swabs or transtracheal wash was extracted by using the QIAamp cadior Pathogen mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Eluted DNA and RNA concentrations were measured using the BioSpectrometer® (Eppendorf, Hamburg, Germany) and stored at -20 °C or -80 °C, respectively, for further analysis.

Reverse transcription and nested PCR- CPiV

Reverse transcription and first PCR were carried out with Transcriptor One-Step RT-PCR kit (Roche Diagnostics, Meylan, France) in the nexus gradient thermocycler (Eppendorf, Hamburg, Germany). The 50 µl reaction mixture contained: 10 µl 5 × reaction buffer, 1 µl primer PNP1 (final concentration 0.4 µM), 1 µl primer

PNP2 (final concentration 0.4 μ M), 34 μ l RNase-free water, 1 μ l Transcriptor Enzyme Mix, and 3 μ l RNA. Cycling conditions were as follows: 50 $^{\circ}$ C for 60 min, 95 $^{\circ}$ C for 3 min, followed by 35 cycles of 95 $^{\circ}$ C for 60 s, 50 $^{\circ}$ C for 40 s, 68 $^{\circ}$ C for 60 s and final extension of 68 $^{\circ}$ C for 10 min.

Nested PCR was carried out with HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) in the nexus gradient thermocycler (Eppendorf, Hamburg, Germany). The 20 μ l reaction contained: 10 μ l HotStarTaq Plus Master Mix \times 2, 1 μ l primer PNP3 (final concentration 0.4 μ M), 1 μ l primer PNP4 (final concentration 0.4 μ M), 2 μ l CoralLoad Concentrate \times 10, 4 μ l RNase-free water, and 2 μ l of the amplicon from the first PCR. Cycling conditions were as follows: 95 $^{\circ}$ C for 5 min, followed by 35 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, 72 $^{\circ}$ C for 60 s and final extension of 72 $^{\circ}$ C for 10 min. All PCR reactions were performed with positive and negative control. Primer sequences and product size are summarized in Table 1. All primers were synthesized by Genomed S.A. (Warsaw, Poland).

Table 1. Primer sequences and product length. Canine adeno virus type 2 (CAV-2), canine herpes virus (CHV), canine parainfluenza virus (CPiV).

Pathogens and primers	Sequence (5'-3')	Product length (bp)	References
<i>CPiV</i>			
PNP1	AGTTTGGGCAATTTTTTCGTCC	667	Erles et al. 2004
PNP2	TGCAGGAGATATCTCGGGTTG		
PNP3	CGTGGAGAGATCAATGCCTATGC	182	Erles et al. 2004
PNP4	GCAGTCATGCACTTGAAGTCACTA		
<i>CAV2</i>			
CAV- F1	TGTCAACAAGGTTTTGTCTTTT	254	Erles et al. 2004
CAV-R1	TTTTCAAGGGAGGTGCGT		
<i>CHV</i>			
CHV1	AAGAGCTCGTGTAGTGAAAAT	494	Erles et al. 2004
CHV2	TAAACCCGCTGGATGATAC		
<i>Bordetella bronchiseptica</i>			
237BBFla 4 F	TGGCGCCTGCCCTATC	237	Hozbor et al. 1999
237BBFla 2 R	AGGCTCCAAGAGAGAAAGGCTT		

PCR – DNA viruses and bacteria

For CHV, CAV2 and *Bordetella bronchiseptica* PCR was also carried out with HotStarTaq Plus Master Mix Kit (Qiagen, Hilden, Germany) in the nexus gradient thermocycler (Eppendorf, Hamburg, Germany). The reaction mixture and PCR condition used were the same as mentioned above except for the annealing temperature: 47 $^{\circ}$ C for CHV, 46 $^{\circ}$ C for CAV2 and 50 $^{\circ}$ C for *Bordetella bronchiseptica*. All PCR reactions were performed with positive and negative control. Primer sequences and product size are summarized in Table 1. All primers were synthesized by Genomed S.A. (Warsaw, Poland).

Analysis of PCR products

Ten microlitres of PCR products were electrophoresed on 2% agarose gel in the presence of Midori Green Advance (Nippon Genetics, Düren, Germany), at 120 V for 60 min. Obtained results were read using the Quantum ST5 Gel Documentation System (Vilber, Eberhardzell, Germany). To confirm the specificity of the obtained amplicons, single PCR products from all four pathogens were randomly chosen and purified using a CleanUp kit (A&A Biotechnology, Gdynia, Poland) for sequencing (Genomed, Warsaw, Poland).

Results

At least one of the four pathogens was detected from all dogs exhibiting kennel cough symptoms. In most tested animals (65%) co-infections were found, caused by 2 or more detected pathogens. Most frequently (45%), simultaneous infection was confirmed by the presence of genetic material of two pathogens. Only 2.5% of the animals confirmed the presence of DNA or RNA of all four pathogens, whereas mono-infections were found in approximately 35% (Fig. 1) of the dogs.

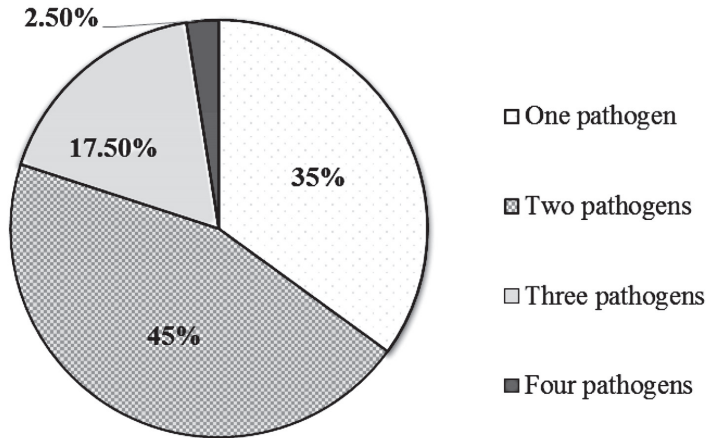


Fig. 1. The frequency of mono- and co-infections

In the case of mixed infections, most frequently the presence of genetic material from CHV plus CPIV (approximately 28%), and CHV plus *B. bronchiseptica* (12.5%) was confirmed. A combination of CHV and CAV-2 was not identified in any of the analysed samples. The combination of CHV, CPIV and *B. bronchiseptica* (12.5%) was the most frequent infection caused by three pathogens (Table 2).

The CHV DNA was detected in as many as 32 out of 40 dogs. The second most frequently detected pathogen was CPIV ($n = 27$). The presence of *B. bronchiseptica* DNA was found in 13 dogs, while CAV-2 genetic material was detected from only 4 animals (Fig. 2).

Two types of biological material were used in the experiment: a swab from the upper respiratory tract, and tracheal lavage fluid, from which both DNA and RNA were isolated simultaneously, and then PCR was performed to confirm the presence of the selected

Table 2. Prevalence of canine respiratory pathogens in co-infections. Canine adeno virus type 2 (CAV-2), canine herpes virus (CHV), canine parainfluenza virus (CPIV) and *Bordetella bronchiseptica* (*B. bronchiseptica*).

Pathogens	Dogs with kennel cough $n = 40$ (%)
Two pathogens	
CHV, CPIV	11 (27.5%)
CHV, <i>B. bronchiseptica</i>	5 (12.5%)
CHV, CAV-2	0 (0%)
CPIV, <i>B. bronchiseptica</i>	1 (2.5%)
CPIV, CAV-2	1 (2.5%)
Three pathogens	
CHV, CPIV, <i>B. bronchiseptica</i>	5 (12.5%)
CPIV, CAV-2, <i>B. bronchiseptica</i>	1 (2.5%)
CHV, CPIV, CAV-2	1 (2.5%)

pathogen. The CHV, CPIV and *B. bronchiseptica* in infected dogs were detected more frequently from the lavage fluid than from the swabs. Only for CAV-2 the situation was reversed: out of the 4 dogs in which the presence of the virus was found, CAV-2 was also detected only in one case from the lavage fluid (Fig. 3).

Discussion

Kennel cough is a common disease all over the world. It is believed that it can be caused by either one infectious agent or a complex thereof. In our

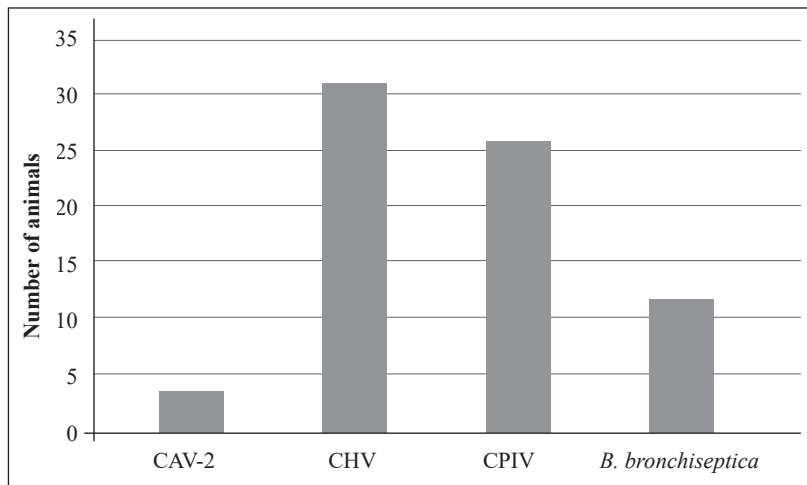


Fig. 2. Prevalence of canine respiratory pathogens in dogs. Canine adeno virus type 2 (CAV-2), canine herpes virus (CHV), canine parainfluenza virus (CPIV) and *Bordetella bronchiseptica* (*B. bronchiseptica*).

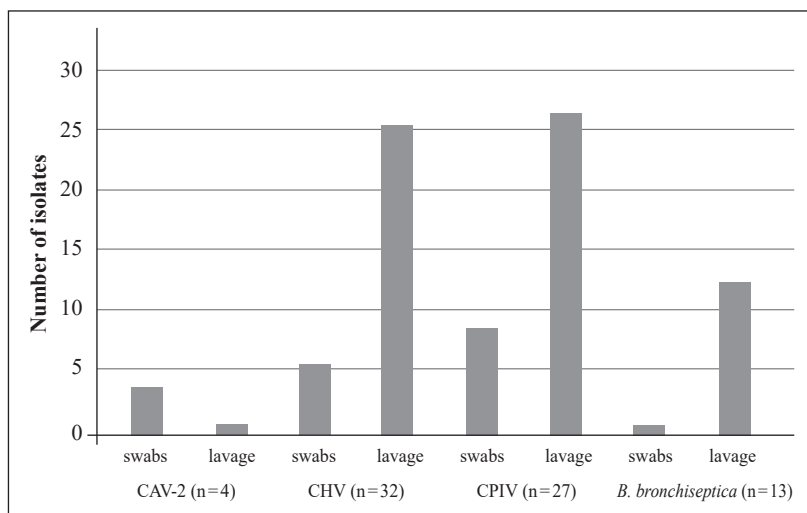


Fig. 3. The frequency of respiratory pathogens isolated from swabs and lavage fluid. Canine adeno virus type 2 (CAV-2), canine herpes virus (CHV), canine parainfluenza virus (CPIV) and *Bordetella bronchiseptica* (*B. bronchiseptica*).

experiment swabs and lavage fluids taken from dogs exhibiting kennel cough symptoms were analysed for the presence of the genetic material of four selected pathogens (CHV, CPIV, CAV-2 and *Bordetella bronchiseptica*). In all animals, at least one of the infectious agents was detected, and in most cases (65%) co-infections were confirmed. These results are in contrast to those obtained in studies carried out in the UK, Japan, and Thailand, where co-infections accounted for no more than 15%, and in Germany, where they accounted

for approximately 37% of animals with kennel cough symptoms (Erles et al. 2004; Mochizuki et al. 2008; Posuwan et al. 2010; Schulz et al. 2014). One of the reasons for the discrepancy between findings may be the fact that in our study co-infections were primarily caused by the combination of CHV and another pathogen, which may indicate a chronic form of the disease. This theory is confirmed by the results of serological and molecular tests performed by Erles et al. (2004), in which the occurrence of particular viruses depending on the disease stage was assessed. The data reported indicate that CHV was much more frequently found in dogs not earlier than in the third or fourth week of the disease. Another reason for the discrepancy between the results may be the choice of pathogens analysed by particular research teams. In our study we focused on the prevalence of four infectious agents, including CHV, which could have had a significant effect on our findings. In experiments conducted in Japan and Thailand, the prevalence of CHV in dogs with kennel cough was not investigated at all. The identification of CHV in the analysed material could be the reason for the higher percentage of co-infection cases in our study (Mochizuki et al. 2008; Posuwan et al. 2010).

As already mentioned, CHV was the virus most frequently detected in dogs within the area under study, and it was found in approximately 80% (32 dogs) of the animals we tested. These results are partially consistent with those obtained in other countries. In the UK CHV was identified as the second most prevalent pathogen in animals with kennel cough (Erles et al. 2004), but in Germany the presence of this virus was confirmed neither in healthy dogs nor in those exhibiting clinical symptoms (Schulz et al. 2014). Even though CHV is mentioned as one of the infectious agents of kennel cough, its role and importance in the aetiology of this disease is not fully understood, and is still very controversial. This is associated with the occurrence of this virus in a latent form, also in clinically healthy dogs, as indicated by the results of serological tests performed in the United Kingdom, Belgium, and the Netherlands (Erles et al. 2005). Such studies have not been carried out in Poland, hence the absence of information on the prevalence of CHV in healthy animals in our country. However, the results obtained in our study suggest that CHV may play an important role in the pathogenesis of kennel cough. This hypothesis can be supported by the case of a nosocomial outbreak of kennel cough caused by CHV infection in dogs treated with immunosuppressants at an animal medical centre reported by Kwakami et al. (2010).

The second most often detected virus in our study was the canine parainfluenza virus. Out of the 40 dogs under study, CPIV RNA was confirmed in as many as 27 dogs (approximately 70%). Numerous studies monitoring the aetiological factors of kennel cough have shown that CPIV virus is very frequently detected in dogs with this disease around the world. Therefore, the high prevalence of CPIV RNA in the analysed samples should not come as a surprise (Erles et al. 2004; Mochizuki et al. 2008; Posuwan et al. 2010; Schulz et al. 2014). On the other hand, a vaccine containing an attenuated CPIV strain is commercially available in Poland, so the high prevalence of this virus in such a large number of dogs with kennel cough may be considered alarming. A similar problem was noted in the UK and Finland, where, despite regular vaccinations against CPIV, it was found in clinical trials (Erles et al. 2004; Viitanen et al. 2015). This may mean that the vaccine used in Europe does not sufficiently stimulate the immune system, allowing the virus to initiate the disease process in favourable situations. However, in our study, the vaccination history is unknown, and therefore we find it difficult to refer to those results.

The third pathogen we analysed was *Bordetella bronchiseptica*. The DNA of this bacterium was found in approximately 30% of the examined dogs. It follows from the available literature that *Bordetella bronchiseptica* is one of the more frequently isolated pathogens in animals with kennel cough, but its prevalence may vary at different latitudes. In a study carried out by Bhardwaj et al. (2013) in India, this microorganism was only

found in 2 out of 68 dogs exhibiting kennel cough symptoms. A different situation was reported in Germany, where the presence of *Bordetella bronchiseptica* was confirmed in as many as 78% symptomatic dogs, and in most cases it was the only isolated infectious agent (Schulz et al. 2014). The results obtained in our study may indicate that this bacterium is an important aetiological factor of kennel cough, although not the most frequent one in Poland.

The fourth pathogen analysed was canine adenovirus type 2. It was the pathogen least frequently detected from clinical samples in our experiment, and the results are comparable with those obtained in other countries. The occasional presence of CAV-2 may be explained by the fact that preventive vaccinations against this pathogen are applied in Poland, and, as indicated by the observations of other researchers, such protection in the case of CAV-2 is highly effective, gradually minimising the role of this virus in the aetiology of kennel cough. Similar observations were made regarding the canine distemper virus, which is also listed as an infectious agent of kennel cough, yet due to the use of effective vaccines it is less and less frequently found in dogs around the world (Miyoshi et al. 1999; Erles et al. 2004; Mochizuki et al. 2008; Posuwan et al. 2010; Schulz et al. 2014; Viitanen et al. 2015).

Two biological materials were used in our experiment: tracheal lavage fluid and swabs from the upper respiratory tract. The results we have presented indicate that the analysed pathogens were more frequently detected from the lavage fluid. Swabs proved to be a better analytical material only for CAV-2. This may indicate a different tropism of the analysed pathogens. Therefore, in studies monitoring the infectious agents of kennel cough it is important to take samples from various areas, where possible, to ensure the most reliable analysis.

To our best knowledge, no studies monitoring the prevalence of infectious agents responsible for kennel cough in Poland have been carried out so far. The aim of our experiment was to analyse the four aetiological factors most frequently isolated in other countries, and to obtain preliminary data on the predominant ones in the study area. The study indicated that CHV is the most prevalent pathogen in both mono- and co-infections as regards kennel cough in north-eastern Poland. This fact may indicate an increase in the importance of this virus in the aetiology of kennel cough. However, these results should be interpreted with caution, and further research should be extended to include a greater area and a wider research panel. Studies monitoring the infectious agents of a particular disease may allow the development of appropriate prevention programmes tailored to the area under analysis, and the detection of new, emerging pathogens.

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