Case report   UDK 636.7:[616.233-002:616-07

LABORATORY DIAGNOSIS OF *BORDATELLA BRONCHISEPTICA* TRACHEOBRONCHITIS IN DOG

Dubravka Milanov[1]*, Milan Đilas[2], Maja Velhner[1], Nevenka Aleksić[3]

[1] Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia
[2] Institute of Public Health of Vojvodina, Novi Sad, Serbia
[3] Faculty of Veterinary Medicine, Belgrade, University of Belgrade, Serbia

Abstract

In the present paper the laboratory isolation and identification of *Bordatella bronchiseptica,* the causative agent of canine tracheobronhitis, is described. A dog which suffered persistent cough, loss of appetite and fever was previously unsucceffully treated with antibiotics, which is why nasal swabs were taken and sent for microbiological assessment. The isolation of the causative agent was performed on routinely used standard solid growth mediums. The final identification of the isolate was done with MALDI-TOF (matrix-assisted laser desorption/ionization - time of flight) and real-time PCR (polymerase chain reaction) assays. Therapy based on the results of the antibiogram lead to successful recovery. The necessity of cooperation of veterinary clinicians and veterinary microbiologists for timely and reliable identification of the microbe(s) and selection of antimicrobials based on the results of the susceptibility testing is emphasized. The significance of the collaboration between microbiological veterinary laboratories and those dealing with human material is underlined. These can provide precise identification of zoonotic agents.

Key words: *Bordatella bronchiseptica*, dog, tracheobronchitis, MALDI TOF, real-time PCR

[1]* Corresponding author: dubravka@niv.ns.ac.rs

LABORATORIJSKA DIJAGNOZA TRACHEOBRONHITISA PSA ČIJI JE UZROČNIK *BORDATELLA BRONCHISEPTICA*

Dubravka Milanov[1*], Milan Đilas[2], Maja Velhner[1], Nevenka Aleksić[3]

[1] Naučni institut za veterinarstvo „Novi Sad", Novi Sad, Srbija
[2] Institut za javno zdravlje Vojvodine, Novi Sad, Srbija
[3] Fakultet veterinarske medicine Beograd, Univerzitet u Beogradu, Srbija

Kratak sadržaj

U ovom radu prikazujemo laboratorijsku izolaciju i identifikaciju *Bordatella bronchiseptica,* uzročnika traheobronhitisa psa. Pas koji je imao uporan kašalj, ispoljavao gubitak apetita i imao hipertermiju, prethodno je bez uspeha bio lečen antibioticima, zbog čega su brisevi nosa poslati na mikrobiološki pregled. Izolacija uzročnika izvedena je na podlogama koje se uobičajeno koriste u mikrobiološkim laboratorijama. Identifikacija izolata do vrste izvedena je primenom MALDI-TOF (matrix-assisted laser desorption/ionization - time of flight) i real-time PCR (polymerase chain reaction) metoda. Posle primene terapije na osnovu antibiograma, pas je uspešno izlečen. Istaknuta je neophodnost saradnje veterinara kliničara i veterinarskih mikrobioloških laboratorija u cilju blagovremene i pouzdane identifikacije uzročnika i odabira terapije na osnovu rezultata ispitivanja osetljivosti izolata na antibiotike. Dodatno ukazujemo na značaj povezanosti mikrobioloških laboratorija humane i veterinarske medicine u preciznoj identifikaciji zoonotskih agenasa.

Ključne reči: *Bordatella bronchiseptica*, pas, traheobronhitis, MALDI TOF, real-time PCR

INTRODUCTION

Bacterial species of the *Bordetella* genus are inhabitants of the respiratory system in both healthy and diseased animals and humans (Markey et al., 2013). The most infamous among these is *B. pertussis*, the primary aetiological agent of whooping cough, which can also be caused by *B. parapertussis*. A similar ailment in dogs is caused by *B. bronchiseptica*, and results in infectious tracheobronchitis, which may attack any dog breed at any age (Ford, 2006). In laymen it is referred to as kennel or canine cough and among professionals as canine croup. The etymology originates from the idea that it primarily affects dogs dwelling in kennels (but also other places where there are many

animals in limited areas), and that it resembles the synonymous childhood diseases. The pathogen is transmitted by direct contact or via Flügge droplets (Vieson et al., 2012), but contaminated fomites may also serve as a source of infection (Datz, 2003). Following an incubation which lasts generally from two days to two weeks, the infected dogs start coughing; in the beginning the cough is a dry, paroxismal cough, but later transforms into productive, with nasal discharge, conjunctivitis and fever (Shelton et al., 1977). The most severe complications develop in young dogs due to the immaturity of their immune systems. Aged animals, those with impaired immunity and pregnant bitches are at higher risk. The disease may lead to tracheal collapse (Oskouizadeh et al., 2011). *B. bronchiseptica* has been identified in rabbits with bronchopneumonia, causing even septicaemia, in cats, horses, guinea pigs and rats suffering from respiratory ailments, and as an opportunistic agent contributing to atrophic rhinitis in pigs (Pittman, 1984; Datz, 2003). Although relatively rare in humans, it was found in people with endocarditis, peritonitis, meningitis and infected wounds, as well as in immunocompromised persons suffering from respiratory infections (Hadley et al., 2009; Woolfrey and Moody, 1991).

Several virulence factors play role in the pathogenesis of respiratory infections which develop owing to *B. bronchisepica*. For example, fimbriae (FIM), filamentous haemagglutinin (FHA) and pertactin (PTN) mediate the attachment to specific receptors in the respiratory system (Datz, 2003). Since adhesion is a prerequisite for invasion, flagella also may contribute to the adherence to eukaryotic cells (Savelkoul et al., 1996). *B. bronchiseptica* (and *B. avium*) are motile by peritrichous flagella (*B. pertussis* and *B. parapertussis* are nonmotile) (Markey et al., 2013).

Not unlike other gram-negative bacteria, the outer membrane of *Bordetella* species contains a lipopolysaccharide endotoxin (Woolfrey and Moody, 1991). In addition, they produce several toxins: tracheal cytotoxin (TCT), dermonecrotic toxin (DNT), osteotoxin, and adenylate cyclase toxin (ACT) (Markey et al., 2013). TCT disrupts ciliated cells (Cookson et al., 1989), DNT is capable of damaging tissues and suppresses both humoral and cellular immunity (Magyar et al., 2000), ACT inhibits the phagocytic function of neutrophils and macrophages (Datz, 2003) and ACT interferes with the activities of epithelial cells (Woolfrey and Moody, 1991). Pertussis toxin (PTX) is an exoprotein produced only by *B. pertussis*, although the corresponding genes are found also in *B. parapertussis* and *B. bronchiseptica* (Masin et al., 2015).

Isolation and identification of *B. bronchiseptica*.

From a four-month-old dog with symptoms of respiratory disease samples of nasal discharge were taken with two flexible nasal swabs. One sample was sent to the laboratory of the Scientific Veterinary Institute "Novi Sad" for the isolation of the microbes and the other was delivered to the Institute of Public Health of Vojvodina to be subjected to real time-PCR (polymerase chain reaction) assay.

On arrival, the nasal swab was streaked on to Columbia blood agar base (CM0331, Oxoid, UK) with 5% defibrinated ovine blood and MacConkey agar (CM0007, Oxoid, UK). The plates were incubated at 37°C in aerobic conditions. After 24h of incubation, the blood agar was covered in very small (0.5-1 mm in diameter), convex, smooth, non-haemolytic colonies, which turned to opaque in the next 24h (Figure 1. A). On the MacConkey agar plate (Figure 1. B) the isolate gave rise to minute, pale colonies.
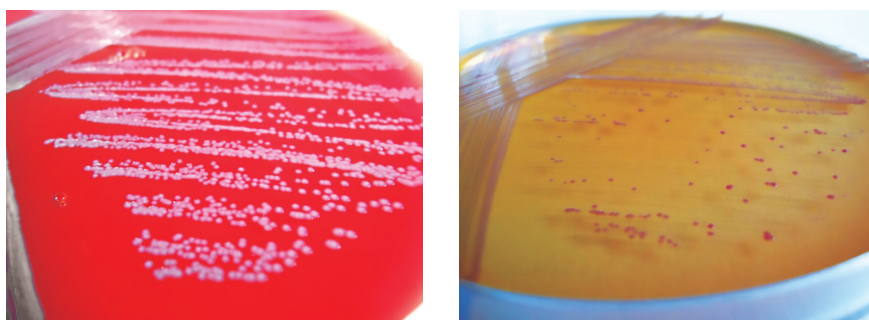

A.   **B.**

Figure 1. Colonies of isolate on blood (A) and MacConkey agar (B) after 48h incubation at 37°C.

The isolate did not ferment carbohydrates (glucose, sucrose and arabinose), but was positive for catalase, urease and oxidase production, and citrate utilization. When the slides were stained with Gram, small Gram-negative coccobacilli were revealed with light microscopy. Based on these characters, the isolate was identified as *B. bronchiseptica*, and was sent to the Institute of Public Health for confirmation by MALDI TOF (matrix-assisted laser desorption/ ionization - time of flight).

The isolate was prepared using the standard Bruker's direct transfer sample preparation procedure for MALDI-TOF MS. A single bacterial colony was spot-

ted directly onto a MALDI target plate (Bruker Daltonics, Germany), allowed to dry and overlaid with 1.0 μL of matrix solution (Bruker Matrix HCCA; α-Cyano-4-hydroxycinnamic acid). MALDI-TOF mass spectrometry was performed on Microflex LT/SH Biotyper system (Bruker Daltonics, Germany) under the control of flexControl software ver. 3.4 (Bruker Daltonics, Germany) (Fig 2). Spectra in the mass range of 2 to 20 kDa were collected by accumulating 240 laser shots (laser frequency, 60 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.15 kV; lens voltage, 6 kV) at 30–40% of maximum laser power.
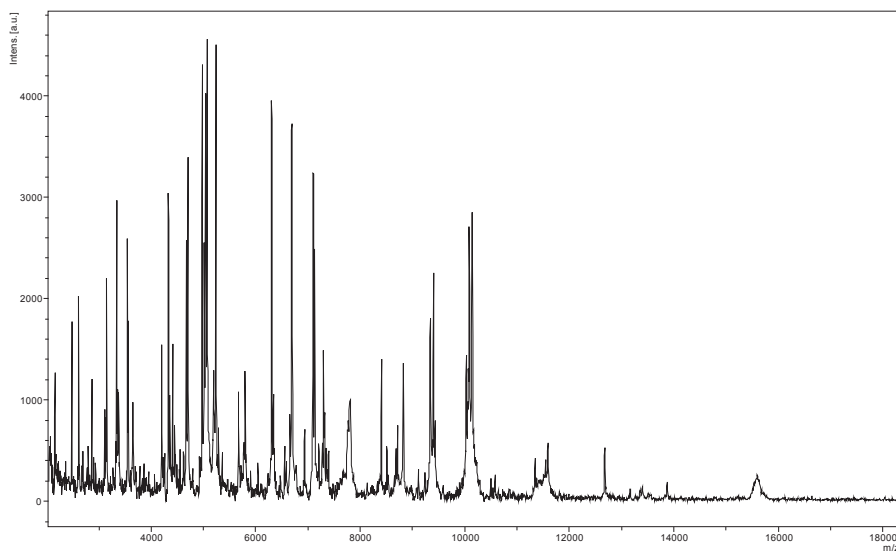

Figure 2. Spectra of *Bordatella bronchiseptica* isolate generated by MALDI-TOF Bruker flexControl software.

RT-PCR. *Bordetella* species determination in the PCR with hybridization fluorescent detection includes three stages: DNA extraction from the samples, PCR amplification of pathogen genome specific region and real-time hybridization fluorescent detection. In real-time PCR, the amplified product is detected with fluorescent dyes, linked to oligonucleotide probes which bind specifically to the amplified product during thermocycling. DNA was extracted with the DNA-Sorb-A kit (Sacace, Italy). Real-time PCR was done with a commercial kit on SaCycler-96 system (Sacace, Italy). *Bordetella pertussis /B. parapertussis/ B. bronchiseptica* Real-TM PCR kit is an *in vitro* nucleic acid amplification test for detection and differentiation of these three species. It can be used for both clinical materials (nasal and oropharyngeal swabs) and mi-

crobial cultures with real-time hybridization fluorescence detection. During the amplification stage, three simultaneous reactions take place – amplification of the conservative region of *ptxA* gene that codes pertussistoxin located in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* genomes; identification of specific regions in genomes of *B. pertussis* and *B. bronchiseptica*, as well as amplification of nucleic acid sequence of the Internal Control (IC) sample. The target regions are detected by different detection channels (FAM for IC, JOE/HEX for *ptxA*, ROX for *B. pertussis* and Cy5 for *B. bronchiseptica*). Sample was tested positive for *B. bronchiseptica*.

The susceptibility of *B. bronchiseptica* isolates to antibiotics was assessed with the standard disc-diffusion test on Müller-Hinton agar medium and presented in Table 1.

Table 1. A*ntibiotic susceptibility* of *B. bronchiseptica* isolate

Antibiotics (abbreviation & dose)	Inhibition zone (mm)
Penicillin (P 10 U)	0
Amoxicillin (AX 25 mg)	0
Ampicillin (AM 10mg)	25
Amoxicillin/clavulanic acid (AMC 30mg)	30
Cefpodoxime (CPD 10mg)	0
Ceftazidime (CAZ 30mg)	32
Cefotaxime (CTX 30mg)	25
Ceftriaxone (CRO 30mg)	30
Erythromycin (E 15mg)	25
Tetracycline (TE 30 mg)	35
Streptomycin (S10 mg)	0
Trimethoprim/sulphamethoxazole (SXT 1.25/23.75 mg)	17
Neomycin (N 30mg)	12
Gentamicin (CN 10mg)	24
Enrofloxacin (ENR5mg)	28
Ciprofloxacin (CIP 5mg)	35
Nalidixic acid (NAL 30mg)	34

No breakpoints have been set for any of the antimicrobials tested for *B. bronchiseptica* susceptibility (Morrissey et al., 2016), which is why it is impossible to catagorize the isolates even based on their MIC values (Kadlec et al., 2006).

COMMENT

Besides parainfluenza virus and canine adenovirus type 2, *B. bronchiseptica* is the most frequent causative agent of canine respiratory diseases (Datz, 2003; Vieson et al., 2012). Although bordetella infection in the dog is usually mild and results in a self-limiting disease, it can be fatal for young animals. In this case, a four-month-old dog of mixed breed was treated empirically, with the combination of antibiotics: penicillin and streptomycin. Both were administered i.m., on five consecutive days: benzyl penicillin 20,000 IU/kg and streptomycin 150 mg/kg. The laboratory testing was required due to the absence of the response to the therapy, but not before two weeks had passed from the onset of the symptoms. The results of *in vitro* investigation of the nasal swabs confirmed the clinical suspicion of antibiotic resistance, which is understandable. Streptomycin seems generally ineffective against *B. bronchiseptica in vitro* (Woolfrey and Moody, 1991). Despite the wide use of penicillin, ampicillin and amoxicillin for canine respiratory infections, they have been proven ineffective against *B. bronchiseptica*, except when the latter is combined with clavulanate (Lappin et al., 2017). Resistance to penicillin has been reported in canine isolates (Markey et al., 2013). In addition, penicillin does not penetrate well into bronchial secretions, which impairs its efficacy. The susceptibility of *B. bronchiseptica* is intrinsically low to some β-lactams (e.g. penicillins and first-generation cephalosporines) owing to the production of β-lactamase and/or low membrane permeability to cephalosporines (Prüller et al., 2015; Morrissey et al., 2016). By contrast, aminoglycosides appear to be highly effective against *B. bronchiseptica*: in severe infections when animals do not respond to parenteral therapy, aerosolized gentamicin may be helpful (Vieson et al., 2012). The most commonly used antibiotics are amoxicillin/clavulanic acid and cephalexin (Vieson et al., 2012). Tetracyclines are also highly efficacious in treating bordetellosis.

To conclude, antibiotics should be selected based on culture and sensitivity tests. Definitive diagnosis of *B. bronchiseptica* infection in dogs should be confirmed by microbiological findings in nasal or pharyngeal swabs. History and clinical signs can only imply that it is infectious tracheobronchitis caused by *B. bronchiseptica* we are dealing with. Frequently, it is necessary to cooperate with public health diagnostic laboratories, which are provided with more sophisticated equipment and are capable of performing more precise diagnostic procedures, as it was in this case. In addition, this collaboration can result in better insight into the epidemiology of zoonoses. The initiation of therapy should be guided by the clinical signs: in critically ill animals, empiric therapy

should start on the collection of swabs, but in those more stable, antimicrobial therapy should be postponed until the results of the antibiogram arrive, which takes two to three days. Treatment should last about two weeks, or seven days beyond the resolution of health problems (Leekha et al., 2011). When deciding on antibiotic therapy, veterinary surgeons must always have in mind the threatening possibility of resistance development and by giving adequate therapy not contribute to its emergence and spread.

ACKNOWLEDGMENTS

REFERENCES

1. Cookson BT., Cho HL., Herwaldt LA., Goldman WE.: Biological activities and chemical composition of purified tracheal cytotoxin of *Bordetella pertussis*. *Infection and Immunity*, 57, 7, 2223–2229, 1989.
2. Datz C.: *Bordatella* infections in dogs and cats: pathogenesis, clinical signs, and diagnosis. *Compendium*, 25, 12, 896-900, 2003.
3. Ford RB. Canine infectious tracheobronchitis. In: Infectious diseases of the dog and cat. C. Greene C.E. (Ed.). St. Louis, MO: Saunders Elsevier. 54–61, 2006.
4. Hadley K., Montante Torres A., Moran J., Schiller B.: *Bordetella bronchiseptica* peritonitis -Beware of the dog! *Peritoneal Dialysis International*, 29, 670–678, 2009.
5. Kadlec K., Wallmann J., Kehrenberg C., Schwarz S.: In-vitro susceptibility of German *Bordatella bronchiseptica* isolates from pigs, Proceedings of the 19th IPVS Congress, Copenhagen, Denmark, 2, 475, 2006.
6. Lappin MR., Blondeau J., Boothe D., Breitschwerdt EB., Guardabassi L., Lloyd DH., Papich MG., Rankin SC., Sykes JE., Turnidge J., Weese JS.: Antimicrobial use guidelines for treatment of respiratory tract disease in dogs and cats: Antimicrobial guidelines working group of the International Society for Companion animal infectious diseases. *Journal of Veteterinary Internal Medicine*, 31, 279-294, 2017.
7. Leekha S., Terrell CL, Edson, RS.: General principles of antimicrobial therapy. *Mayo Clinic Proceedings*, 86, 2, 156-167, 2011.
8. Magyar T., Glavits R., Pullinger GD., Lax A.J.: The pathological effect of the

Bordetella dermonecrotic toxin in mice. *Acta Veterinaria Hungarica*, 48, 4, 397–406, 2000.

9. Markey B., Leonard F., Archambault M., Cullinane A., Maguire D.: *Bordatella* species, Chapter 28, In: *Clinical Veterinary Microbiology*, Elsevier Health Sciences, Amazon. Com. E-Book, 2013, 359-367.

10. Masin J., Osicka R., Bumba L., Sebo P., Locht C.: Chapter 6. *Bordatella* protein toxins. In: Comprehensive Sourcebook of Bacterial Protein Toxins (Fourth Edition), 2015, 161-194.

11. Morrissey I., Moyaert H., de Jong A., El Garch F., Klein U., Ludwig C., Thiry J., Youala M.: Antimicrobial susceptibility monitoring of bacterial pathogens isolated from respiratory tract infections in dogs and cats across Europe: ComPath results. *Veterinary Microbiology*, 191, 44-51, 2016.

12. Oskouizadeh K., Selk-Ghafari M., Zahraei-Salehi T., Dezfolian O.: Isolation of *Bordetella bronchiseptica* in a dog with tracheal collapse. *Comparative Clinical Pathology*, 20, 5, 527-529, 2011.

13. Pittman M.: Gram-negative aerobic rods and cocci, genus *Bordetella*, In: N. R. Krieg, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, Vol. 1. Williams & Wilkins, Baltimore, 388–393, 1984.

14. Prüller S., Rensch U., Meemken D., Kaspar H., Kopp P.A., Klein G., Kehrenberg C.: Antimicrobial Susceptibility of *Bordetella bronchiseptica* isolates from swine and companion animals and detection of resistance genes. *PLoS One*, 10, 8, e0135703, 2015.

15. Savelkoul PHM., De Kerf DPG., Willems RJ., Mooi FR., Van Der Zeijst BAM., Gaastra W.: Characterization of the fim2 and fim3 fimbrial subunit genes of *Bordetella bronchiseptica*: roles of Fim2 and Fim3 fimbriae and flagella in adhesion. *Infection and Immunity*, 64, 12, 5098-5105, 1996.

16. Shelton ME., Draper DD., Farrington DO.: Control of a tracheobronchitis epizootic associated with *Bordetella bronchiseptica* in a closed dog colony. *Veterinary medicine, small animal clinician*, 72, 2, 189-193, 1977.

17. Vieson MD., Piñeyro P., LeRoith T.: A review of the pathology and treatment of canine respiratory infections. *Veterinary Medicine: Research and Reports*, 3, 25-39, 2012.

18. Woolfrey BF., Moody JA.: Human infections associated with *Bordetella bronchiseptica*. *Clinical Microbiology Reviews*, 4, 3, 243-255, 1991.