Bulletin UASVM, Veterinary Medicine 68(1)/2011
pISSN 1843-5270; eISSN 1843-5378

Molecular Detection of *Pasteurella multocida* Strains from Oral Swabs in Pets

Simona IVANA[1,3)], Viorica CHIURCIU[6)], Dana Magdalena CAPLAN[3)], Camelia BALA[2)] Lucian IONIŢĂ[4)], Carmen IONIŢĂ[4)], Iudith IPATE[5)], Cristina Pârvu[7)], Alexandru Popescu[1)]

[1)] "Asclepius" Research Center, *93, Gheorghe Şerban street, district 2, Bucharest Corresponding author: Simona Ivana, Faculty of Veterinary Medicine, 105, Splaiul Independentei, 050097, Bucharest, Romania, phone: 073.538.93.99, email:* simonaivana@yahoo.com
[2)] Bucharest University, 36-46, Bd. M. Kogălniceanu, district 5, 050107, Bucharest
[3)] *"Cantacuzino" National Researches-Development Institute for Microbiology-Immunology, 104, Splaiul Independentei, district 5, Bucharest*
[4)] *Faculty of Veterinary Medicine, University of Agronomical Science and Veterinary medicine Bucharest, 105, Splaiul Independentei, 050097, Bucharest, Romania*
[5)] Acad. David Davidescu Studies and Researches Center for Agriculture and Forestry Biodiversity, 13, 13 Septembrie bd., district 5, Bucharest, Romania.
[6)] Romvac Company, Voluntari, Romania
[7)] *"Carol Davila" Medicine and Pharmacy University, Bucharest*

Abstract. The molecular detection by means of the polymerase chain reaction (PCR) represents a much more sensitive, specific and fast detection method of Pasteurella and confirms the infection. Although the isolation and identification of the pathogenic agent represent the most reliable method of demonstration of its existence, the results of the bacteriologic examination are frequently false negative, owing to the fact that P. multodica is either rather easily destroyed during the transportation or eliminated from the culture medium by the bacteriological suprainfection germs (nasal flora or contaminants).Pasteurellae were identified both in clinically healthy cats and in ill cats. These dates suggest that the contaminated cats, regardless of their clinical state, may transmit de bacteria to humans by bite or scratch. In this work, cats kept as pets from Bucharest were studied with suspect to their contamination with Pasteurella. Of 44 samples taken from the oral cavity of cats, 22 Pasteurella spp. strains could be isolated, 18 of which belonged to the Pasteurella multocida. The organisms were isolated both from healthy and from infected cats.

Keywords: molecular detection, *Pasteurella multocida*, PCR, PFGE, zoonosis

INTRODUCTION

In carnivorous pets, the Pasteurella carriage at the level of the oral cavity is often not associated with a well-defined clinic-lesional picture, their contamination being usually suspected to be due to the complications occurred at the level of bite or scratch wounds caused in humans. On the other hand, not from all the human infections may be isolated Pasteurellae and, moreover as it was mentioned above, in human cases develop themselves as opportunistic germs on the background of the course of diseases with an well-known etiology.

In this context, in the human pathology caused by Pasteurella it is compulsory to make the differential diagnosis with a series of diseases which may have also other etiologies. Numerous bite wounds are not indexed and therefore the real epidemiological situation of the incidence and prevalence of the microorganisms which infect these wounds is not known. The infections may be caused by almost any group of pathogenic agents: bacteria, viruses, rickettsia, spirochetes and fungi.

Traditionally, the diagnosis of Pasteurella infection was based on clinical symptoms, cultivation or serological testing. Although the isolation and identification of the pathogenic agent represent the most reliable method of demonstration of its existence in the sample that makes the object of the study, the method requires a long period and its costs are excessive. The results of the bacteriological examination as frequently false negative, owing to the fact that P. multocida is rather easily destroyed during its transportation to the laboratory of microbiology or it is eliminated from the culture medium by the bacterial suprainfection germs (the nasal flora or contaminants). The serological tests were used when the infection was suspected in organs which could not be investigated bacteriologically or when the cultures did not furnish conclusive results. However, a positive serological test for P. multocida may indicate the current infection, as well as the former exposure of the organism to the microorganism. As many animal have been exposed to the microorganism, a current diagnosis of pasteurellosis cannot be based only on the results of the serological tests. The molecular detection by PCR represents a much more sensitive, specific and rapid method of Pasteurella identification and confirmation of the infection [6, 7].

MATERIALS AND METHODS

In the present study, 44 specimens sampled from the oral cavity of cats were investigated. Before the sampling, relevant epidemiological informations were obtained from the owners of the cats and the animals were examinated clinically. In order to assure the uniformity of the epidemiological data, an unique questionnaire was used and the clinical examination included the classic semiological stages. All the cats that made the object of the research was assessed at the Biotehnological Research Center "Asclepius" and University Hospital of the Faculty of Veterinary Medicine, Bucharest.

The specimens were taken with the sampling device and they were transported on the Amies conservation and transport medium to the Reference Center for Zoonoses – Cantacuzino Institute. Afterwards, the specimens were seeded by dispersion on agar media with 5% defibrinated ram blood, on lactose-treated medium (AABTL medium – preparated at the Cantacuzino Institute) and on MacConkey agar medium.

The characteristics of the Pasteurella genus were identified by examination of the aspect of colonies, by the motility test, by the study of Gram-stained swears and by biochemical tube-tests, using media prepared at the "Cantacuzino" Institute.

A special attention was paid to the non-hemolytic aspect after incubation on the blood-agar medium, to the presence of Gram-negative, non-motile coccobacilli, with positive test for indol, catalase and oxidase, and to the strains which produced acid from glucose and saccharose and which were lysine-decarboxylase negative.

For the macrorestriction analysis, isolates identified by conventional methods as being Pasteurella multocida were cultivated in gelose – 5% ram blood. The bacterial suspension in Tris-EDTA buffer, adjusted to 4U McFarland value was mixed with an equal volume of 1% agarose gel Seakem Gold (Lonza) and cast in the form of small 1 mm-thick plates. They were incubated overnight in a lytic buffer containing 50 mM tris, 50 mM EDTA, 1% sarcosil and 0.5 mh proteinase K, after which they were washed several times in sterile distilled water and

TE buffer. Until the moment of enzymatic restriction, the slab containing the genetic material were kept in a TE buffer at the temperature of 4°C [1, 3].

The genomic material (total DNA) was digested with 40 U ApaI and 30 SalI, the digestion conditions indicated by the producer (Roche Diagnostics) being respected. The DNA fragments which resulted were separated by a CHEP Mapper (Bio Rad) system in 1% agarose SeaKem Gold gel. The electrophoresis conditions were the following: Tris-Borat-EDTA 0,5x buffer, at 14°C, 6V/cm of electric field, an angle of 120° and a linear ramp. For the separation of the macrorestriction ApaI fragments migration programme lasted 20 hours, with a pulse spacing of 1-30 seconds. The SalI macrorestriction fragments were separated within 17 hours, with a pulse spacing of 1.5-17 seconds [2].

Concomitently with the Pasteurella isolates, a marker of molecular weight, respectively Lambda Ladder PFG Marker (New England Biolabs), was migrated, which permitted to appreciate the size of the restriction fragments.

The PFGE gel was colored in an ethidium bromide solution (1 mg/ml), decolorized in sterile distilled water and photographed in UV light.

The profiles of bands which resulted were interpreted by means of the Fingerprinting II (BioRad) programme, the analysis of the clonal characteristics of the isolates being based on the Dice coefficient and the UPGMA method (unweighted-pair group method using arithmetic average linkages) [4, 5].

RESULTS AND DISCUSSION

For the current research were selected 44 feline *(Felis catus)* specimens, patients recorded in the register of the University Hospital of the Faculty of Veterinary Medicine, Bucharest.

The analysis of the epidemiological evaluation records and the clinical diagnosis pointed out the heterogenicity of the group which made the object of the study, but with a relatively uniform distribution of the cases of Pasteurella infection or Pasteurella carriage.

The contamination of the cats with Pasteurella, with or without clinical manifestations (Chart 2) was demonstrated with the same frequency in the young, the adult and the aged animals. In contrast with our data, literature reports mention that the young animals are more susceptible to the Pasteurella infections, but normally during the first weeks of life they are not affected [8, 9].

From the 44 samples investigated, taken from the oral cavity of cats, 22 Pasteurella strains were isolated (table 2). For the identification of the Pasteurella species, the following characteristics were studied: the Gram negative, microaerofilic, non-hemolytic, non-motile, without growth on the Mac Conkey agar, oxidaso-positive and sacharose fermentative coccobacilli, without gas production (table 1). Other biochemical properties included the indole reaction, the catalase reaction, the nitrate reduction, the ornitine-decarboxylase and the urease production. Taking into account these data, the following species were isolated: : *P. multocida* (18 strains), *P. stomatis* (1 strain) and *P. speciae B* (3 strains).

Table 1.

Biochemical differentiation of the isolated Pasteurella strains

Test	No.(%) of positive strains		
	P. multocida (n=18)	*P. stomatis* (n=1)	*P. speciae B* (n=3)
Acid from:			
Mannitol	18 (100)	0	3 (100)
Sorbitol	18 (100)	0	0
Trehalose	5 (22.72)	1 (100)	3 (100)
D-xylose	12 (54.54)	0	3 (100)
Gas from glucose	0	0	0
Ornitin-decarboxylase	18 (100)	0	3 (100)
Urease	0	0	0

Table 2

Aerobic bacteria isolated from cats

Species	No.(%) of strains isolated from cat
Pasteurella spp	22(40.0)
P. multocida	18(32.72)
P. stomatis	1(1.81)
P. speciae B	3(5.45)
Staphylococcus spp	11(20.0)
Streptococcus spp	10(18.18)
Enterococcus spp	3(5.45)
Corynebacterium spp	8(14.54)
Enterobacteriaceae	1(1.81)
Enterobacter	1(1.81)
Acinetobacter spp	2(3.63)
Bacillus spp	3(5.45)

The present research was aimed at the elaboration of a protocol permitting the optimum typing of the autochthonous isolates of *Pasteurella multocida*, a representative species in the framework of the genus, pathogenic for humans, in the transmission of which the contact with pets (dogs and cats) is the main factor involved. The electrophoresis in pulsed-field gel was chosen as typing method, considered by many researchers as the "gold standard" of bacterial typing methods. It proved its efficiency also for the various species of the Pasteurella genus and therefore it is preferred to other methods, such as the ribotyping and the REP-PCR. Owing to the high discriminatory capacity of PFGE, results may be obtained which can elucidate the equivocal situations occurring in the classical epidemiological investigation, painting out the heterogeneity/clonality of bacterial isolates.

The PFGE protocol was developed after the consultation of the specialty literature, as it is an adaptation based on the experience and the tehnico-material equipment of the team of researches working at the Laboratory of Microbiology in the National "Cantacuzino" Institute. Its optimization was aimed at the study of the parameters related to the

concentration of bacteria included in the slab, to the restriction enzymes (type and concentration/sample) and the migration programme [11].

Two *P. multocida* isolates could not be typed, in spite of the repeated attempts, owing to the degradation of the genetic material before its controlled restriction. The profiles of bands generated by Apa I could be most easily analyzed, the lower number of bands, well separated, being well integrated in the interval of development of the lambda markers. *Sal*I produced significantly more bands, the computer-assisted gel processing stage (the definition of the bands) being thus considered more difficult. For the macrorestriction with *Sal*I, the DNA≤48,5kb (the smallest marker fragment), could not be taken into consideration in the clonal analysis, a fact which represents another drawback in the comparison of PFGE profiles. Taking into account the good discrimination between isolates based on the clarity of the profiles of *Apa*I generated bands, a correct interpretation of the genetic affinity degree was possible. However, a limitation of this experimental was the absence, among the isolates of the collection under study, of strains from owners of pets, suspected to be infected with Pasteurella. All the *P. multocida* strains analyzed were obtained exclusively from cats. Thus, the analysis of their clonal characteristics materialized itself in the assessment of the polymorphism degree of a sample of a *P. multocida* population circulating among pets, without the existence of any epidemiological relationship between the investigated isolates. The comparison of the profiles of PFGE bands by means of Fingerprint II programme and thir interpretation on the basis of the Dice coefficient and of the UPGMA analysis method revealed a heterogeneous *P. multocida* population [10].

CONCLUSIONS

- The typing of bacterial isolates justifies its utility not only the diagnosis or the therapeutic strategy, but especially in the monitoring of their zoonotic pathogenic agent, aimed at an optimum control of the transmission to the human host. Therefore, we recommend the extension of the research and the enrichment of the collection of isolates, the foundation of an autochthonous data bank for Pasteurella being laid in this way.
- The Pasteurella species was identified both in clinically healthy and in infected cats. These data (see findings) suggest that the contaminated cats, regardless of their clinical state, may transmit the bacteria to humans by bite or scratch. From 44 samples obtained, 22 Pasteurella strains could be isolated 18 of which were P. multocida. The profiles of PFGE bands showed a significant heterogeneity of Pasteurella isolates.

REFERENCES

1 WEBER DJ, WOLFSON JS, SWARTZ MN. Pasteurella multocida infections. Report of 34 cases and review of the literature. Medicine (Baltimore); 63(3):133-54. 1984 [Medline].

2 SHEWEN P.E., AND RICE CONLON J.A. Pasteurella, p. 216-225. In C.L. Gyles and C.O. Thoen (ed.), Pathogenesis of bacterial infections in animals, 2nd Ed., Iowa State University Press, Ames, USA. 1993.

3 RYCROFT A.N., AND GARSIDE L.H. Actinobacillus species and their role in animal disease. Vet. J. 159, 18-36. 2000.

4 CHRISTENSEN H., BISGAARD M., AELBAEK B., OLSEN J.E. Reclassification of Bisgaard taxon 33, with proposal of Volucribacter psittacicida gen.nov., sp. nov. and

Volucribacter amazonae sp. nov. as new members of the Pasteurellaceae. Int. J. Syst. Evol. Microbiol. 54, 813-818. 2004.
5 CHRISTENSEN H., FOSTER G., CHRISTENSEN J.P., PENNYCOTT T., OLSEN J.E., BISGAARD M. Phylogenetic analysis by 16S rDNA gene sequence comparison of avian taxa of Bisgaard and characterization and description of two new taxa of Pasteurellaceae. J. Appl. Microbiol. 95, 354-363. 2003.
6 MIFLIN, J. K. AND BLACKALL, P. J. Development of a 23 S rRNA-based PCR assay for the identification of Pasteurella multocida. Lett. Appl. Microbiol. 33: 216–221. 2001.
7 CITRON D.M., WARREN Y.A., FERNANDEZ H.T., et all. Broth Microdilution and Disk Diffusion Tests for Susceptibility Testing of Pasteurella Species Isolated from Human Clinical Specimens J. Clin. Microbiol., 2005, 43(5), 2485-2488.
8 CHRISTIDOU A., MARAKI S., GITTI Z. And TSELENTIS Y. Review of Pasteurella multocida Infections over a Twelve-Year Period in a Tertiary Care Hospital. Am. J. Infectious Dis., 2005, 1(2), 107-110.
9 HOLST E., ROLLOF J., LARSSON L., and NIELSEN J.P. Characterization and Distribution of Pasteurella Species Recovered from Infected Humans. J. Clin. Microbiol., 1992, 30 (11), 2984-2987.
10 TALAN D.A., CITRON D.M., ABRAHAMIAN F.M., et all. Bacteriologic Analysis of Infected Dog and Cat Bites. New Engl. J. Med., 1999, 340 (2), 85-92.
11 TSENG H.-K., SU S.-C., LIU C.-P., LEE C.-M. Pasteurella multocida bacteremia due to non-bite animal exposure in cirrhotic patients: report of two cases J. Microbiol. Immunol. Infect., 2001, 34, 293-296.