Characterisation and comparison of *Pasteurella multocida* isolated from different species in the Czech Republic: capsular PCR typing, ribotyping and dermonecrotoxin production

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ABSTRACT: The aim of this study was to characterise and compare *Pasteurella multocida* isolates originating from pigs (n = 43), calves (n = 31), rabbits (n = 27), and to a lesser extent from other hosts (n = 6). A total of 107 *P. multocida* isolates were obtained from various locations in the Czech Republic. They were analysed by capsular PCR typing and ribotyping, and tested for the production of dermonecrotoxin. Most frequently, serogroup A isolates (n = 74) were found, followed by serogroup D (n = 25) and serogroup F (n = 8) isolates. From a total of fifteen different ribotypes (1–15) generated by restriction endonuclease *Msp*I, four ribotypes (1, 3, 4, and 7) were predominant. The prevalence of predominant ribotypes was different in isolates originating from different hosts. Ribotype 1 was characteristic for rabbit isolates, ribotype 3 was primarily found in pig isolates, and ribotype 7 dominated among calf isolates. Sixteen (mainly porcine) isolates produced dermonecrotoxin but significant correlation among ribotypes and dermonecrotoxin production was not observed.

Keywords: pasteurellosis; serotyping; RFLP; pasteurella multocida toxin

The Gram-negative bacterium *Pasteurella multocida* is a causative agent of infectious diseases which affects a broad host range such as most mammals including humans, and birds (Adlam and Rutter, 1989). Five capsular serogroups (A, B, D, E, and F) of *P. multocida* have been identified so far (Carter, 1967; Rimler and Rhoades, 1987). It is known that besides the geographical distribution these serogroups are more or less specific with regard to the host and the disease induced (Quinn et al., 1994; Boyce et al., 2000). Serogroups A and D are worldwide spread serogroups which can be found in a wide range of domestic animals (e.g. from fowl to calves, pigs, sheep, goats, and rabbits) in which they cause various infections (Confer, 1993; Quinn et al., 1994). Serogroups B and E have been found predominantly in tropic areas where they induce hemorrhagic septicemia in cattle and wild ruminants (Carter, 1961; Penn and Nagy, 1976; Townsend et al., 1997). Serogroup F was first isolated from turkeys in the U.S.A. (Rimler and Rhoades, 1987), and isolates of this serogroup have usually originated from birds situated in North America (Rhoades and Rimler, 1987; Wilson et al., 1993; Rimler, 1994; Wilson et al., 1995) and less frequently in other parts of the world (Hinz and Luders, 1991; Jonas et al., 2001). The serogroup F has been known as a causative agent of fowl cholera (Rimler, 1987; Jonas et al., 2001) but, in recent time, it has also been found in some mammalian species in different world areas (Moreno et al., 2003; Davies et al., 2003c, 2004; Jaglic et al., 2004, Catry et al., 2005).

Serotyping and biochemical characterisation have indicated a high heterogeneity within the *P. multocida* species (Brogden and Packer, 1979; Mutters et al., 1985; Blackall et al., 1997), which was later

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confirmed by a new-generation of typing system based on molecular techniques. Outer membrane and whole cell proteins were shown to be a suitable target for isolate typing (Dabo et al., 1999a; Davies et al., 2003b). Multi-locus enzyme electrophoresis was also used as a precise tool in characterisation of *P. multocida* (Blackall et al., 1999). DNA-based typing methods, mainly the restriction endonuclease techniques showing a similar discriminatory power (Blackall and Miflin, 2000), have already been established in detailed analysis of *P. multocida* isolates by many authors (Gardner et al., 1994; Bowles et al., 2000; El Tayeb et al., 2004).

P. multocida isolates can also be differentiated based on their ability to produce dermonecrotoxin (DNT). DNT exhibits osteolytic (DiGiacomo et al., 1993; Sternerkock et al., 1995) and mitogenic activity (Lax and Grigoriadis, 2001; Pullinger et al., 2001) which results into serious lesions (Chrisp and Foged, 1991; Schimmel et al., 1992). DNT was recorded mainly in isolates originating from pigs (Nielsen et al., 1986).

Due to the great variability of *P. multocida* in general and lack of knowledge on isolates circulating in the Czech Republic, the aim of this work was to characterise a panel of various *P. multocida* isolates originating from different hosts and obtained from different locations in the Czech Republic by capsular PCR typing, ribotyping and DNT production determination.

MATERIAL AND METHODS

Bacterial strains and culture media. Four reference strains of *P. multocida* (14070/82, W-9217, 45/78, and 93 IOWA) obtained from the Collection of Animal Pathogenic Microorganisms (CAPM, Czech Republic), and 107 *P. multocida* isolates originating from 89 different locations in the Czech Republic and isolated from pigs (n = 43), calves (n = 31), rabbits (n = 27), fowl (n = 3), a cat (n = 1), a manul (*Otocolobus manul)* (n = 1), and a human (n = 1) were included in this study (Table 1). The rabbit isolates have already been capsular-typed and 8 of them belonging to serogroup F characterised by ribotyping in our previous work (Jaglic et al., 2004). The isolates were limited to one per location (farm) except for those which were of different serogroups. The bacterial strains were grown at 37°C on blood agar (Blood Agar Base No. 2, HiMedia, India) containing 5% of sheep blood.

Identification of *P. multocida* and capsular PCR typing. The suspected colonies were classified as *P. multocida* by means of PCR using species-specific primers (Townsend et al., 1998). Capsular typing was performed by multiplex capsular PCR with the capsule-specific primers pairs (CAPA, CAPB, CAPD, CAPE, and CAPF) designed by Townsend et al. (2001). The capsular PCR conditions have been already described elsewhere (Jaglic et al., 2004).

Ribotyping. For ribotyping, chromosomal DNA was purified using QIAGEN Blood & Cell Culture DNA Kit (QIAGEN, Germany) according to the manufacturer's instruction. The DNA was digested for 12 hours using restriction endonuclease (RE) *Msp*I (New England BioLabs, USA) and ribotyping was performed by an adapted RFLP method described elsewhere (Pavlik et al., 1999). Briefly, after electrophoresis in 0.8% agarose, DNA was transferred to Hybond N nylon membrane (Amersham Biosciences, UK) using vacuum blotting (BioRad, USA). The 16S rDNA probe was amplified from genomic DNA of *P. multocida* reference strain X-73 using the oligonucleotides UNB51 (5´-GAG TTT GAT CCT GGC TCA-3´) corresponding from positions 8 to 27 and UNB800 (5´-GGA CTA CCA GGG TAT CTA AT-3´) corresponding from positions 806 to 787 of the *Escherichia coli* 16S rDNA gene sequence (Kirschner and Bottger, 1998). Prior to labelling, the PCR product was purified using a QIAquick PCR Purification Kit (QIAGEN, Germany). The probe labelling, hybridisation and signal development were carried out using ECL Direct Nucleic Acid Labelling System (Amersham Biosciences, UK). Restriction endonuclease patterns (ribotypes) were compared by the Gel Base software (Applied Maths, Belgium). A similarity matrix was constructed according to the UPGMA algorithm using Dice coefficient.

Determination of the dermonecrotoxin production. DNT production was assayed using an ELISA test (PMT ELISA Kit ref. K0009, DakoCytomation, Denmark), according to the manufacturer's instructions.

RESULTS

Detailed characters of *P. multocida* isolates and reference strains examined are shown in Table 1.

Among 107 isolates identified as *P. multocida*, three capsular serogroups A, D, and F were found by capsular PCR typing. Isolates belonging to the

Table 1. A. Characterisation of *Pasteurella multocida* isolated from different hosts

No. of isolates	Host	Site of isolation	Herd status	Year of isolation	Serogroup	Ribotype	DNT
1	pig	nose	data not available	2001	A	3	+
1	pig	lungs	bronchopneumonia	2001	A	3	+
1	pig	lungs	bronchopneumonia	2001	A	3	–
1	pig	lungs	bronchopneumonia	2001	A	4	+
3	pig	lungs	bronchopneumonia	2001	A	4	–
1	pig	lungs	bronchopneumonia	2001	A	7	+
5	pig	lungs	bronchopneumonia	2001	A	7	–
1	pig	nose	data not available	2001	D	3	+
5	pig	lungs	bronchopneumonia	2001	D	3	–
1	pig	lungs	bronchopneumonia	2001	D	7	+
2	pig	lungs	bronchopneumonia	2002	A	3	+
1	pig	nose	PAR[1]	2002	A	3	+
1	pig	tonsil	bronchopneumonia	2002	A	3	–
1	pig	lungs	bronchopneumonia	2002	A	4	–
1	pig	nose	PAR[1]	2002	A	4	–
3	pig	lungs	bronchopneumonia	2002	A	7	–
1	pig	nose	PAR[1]	2002	A	7	–
2	pig	lungs	bronchopneumonia	2002	D	3	+
3	pig	lungs	bronchopneumonia	2002	D	3	–
2	pig	nose	PAR[1]	2002	D	3	–
1	pig	nose	PAR[1]	2003	D	3	+
1	pig	nose	PAR[1]	2003	D	3	–
1	pig	lungs	bronchopneumonia	2004	A	3	+
1	pig	lungs	bronchopneumonia	2004	A	8	–
1	pig	lungs	bronchopneumonia	2004	A	9	–
1	pig	lungs	bronchopneumonia	2004	D	3	+
2	calf	lungs	bronchopneumonia	2002	A	7	–
1	calf	data not available	data not available	2002	A	8	–
1	calf	lungs	bronchopneumonia	2002	A	11	–
1	calf	data not available	data not available	2002	D	3	–
1	calf	lungs	bronchopneumonia	2002	D	10	–
2	calf	lungs	bronchopneumonia	2004	A	4	–
1	calf	data not available	data not available	2004	A	4	–
1	calf	nose	bronchopneumonia	2004	A	4	–
1	calf	lungs	bronchopneumonia	2004	A	7	+
5	calf	lungs	bronchopneumonia	2004	A	7	–
2	calf	nose	bronchopneumonia	2004	A	7	–
4	calf	data not available	data not available	2004	A	7	–
1	calf	lungs	bronchopneumonia	2004	A	9	–
1	calf	nose	bronchopneumonia	2004	A	11	–
1	calf	nose	bronchopneumonia	2004	A	12	–
1	calf	data not available	data not available	2004	A	12	–

Table 1 continued

No. of isolates	Host	Site of isolation	Herd status	Year of isolation	Serogroup	Ribotype	DNT
1	calf	data not available	data not available	2004	A	13	–
1	calf	lungs	bronchopneumonia	2004	D	3	–
2	calf	lungs	bronchopneumonia	2004	D	10	–
1	calf	nose	bronchopneumonia	2004	D	10	–
1	rabbit	data not available	data not available	1967	A	6	–
1	rabbit	lungs	rhinitis, pneumonia	1997	A	1	–
1	rabbit	blood	rhinitis, pneumonia	1997	A	5	–
1	rabbit	blood	rhinitis, pneumonia	1997	A	6	–
1	rabbit	data not available	data not available	1999	F	1	–
1	rabbit	nose	data not available	2000	A	4	–
1	rabbit	lungs	rhinitis, pneumonia	2000	F	1	–
1	rabbit	nose	rhinitis, pneumonia	2000	F	1	–
1	rabbit	uterus	acute septicemia	2001	A	1	–
2	rabbit	lungs	data not available	2001	A	1	–
2	rabbit	nose	data not available	2001	A	4	–
1	rabbit	data not available	data not available	2001	A	4	–
1	rabbit	conjunctiva	data not available	2001	D	3	–
1	rabbit	lungs	data not available	2001	D	3	–
3	rabbit	lungs	data not available	2001	F	1	–
1	rabbit	nose	rhinitis, pneumonia	2001	F	2	–
1	rabbit	data not available	data not available	2002	A	1	–
1	rabbit	lungs	rhinitis, pneumonia	2003	F	1	–
1	rabbit	lungs	rhinitis, pneumonia	2004	A	1	–
1	rabbit	nose	rhinitis	2004	A	1	–
1	rabbit	nose	data not available	2004	A	1	–
1	rabbit	heart	data not available	2004	A	1	–
1	rabbit	lungs	data not available	2004	A	4	–
1	hen	ovary	chronic fowl cholera	2004	A	14	–
1	hen	liver	chronic fowl cholera	2004	A	14	–
1	hen	joint	chronic fowl cholera	2004	A	14	–
1	cat	nose	rhinitis	2004	A	14	–
1	manul	nose	rhinitis	2004	A	15	–
1	human	throat	pharyngitis	2004	A	3	+

[1]randomly selected isolates from monitored herds with history of progressive atrophic rhinitis

B. Characterisation of *Pasteurella multocida* reference strains

Strain Code	CAPM No.	Source	Serogroup	Ribotype	DNT
14070/82	6272	pig	A	3	+
W-9217	6077 T	pig	A	7	–
45/78	6271	pig	D	3	+
93 IOWA	6080	cattle	D	3	–

serogroup A had the highest incidence (n = 74) and they were the most common in each of the host. Twenty five isolates were classified as members of the serogroup D and they were found in pigs, calves, and rabbits. The serogroup F isolates (n = 8) were found in rabbits only, and two of them are available in the Czech Collection of Animal Pathogens Microorganisms (CAPM 6423 and CAPM 6431).

Fifteen distinct ribotypes (1–15), which showed clear patterns of five bands, were identified among all of *P. multocida* isolates (Figure 1A). Among these ribotypes four ribotypes (1, 3, 4, and 7) were predominant. Most frequently (27.1%), ribotype 3 (n = 29) was observed, which was mainly detected in isolates originating from pigs (as a dominant ribotype of pig isolates), or in isolates belonging to the serogroup D. Ribotype 7 (n = 25), the most common ribotype in isolates originating from calves, was also recorded in pig isolates, and almost all isolates of ribotype 7 belonged to the serogroup A. These two ribotypes were also detected in the reference strains examined. The most frequently found ribotype in rabbit isolates was ribotype 1 (n = 16), which was not recorded in any other host, and it was detected in isolates of the serogroups A and F. Ribotype 4 (n = 15) occurred in pig, calf, and rabbit isolates, which belonged to the serogroup A. Other ribotypes were found less frequently or sporadically, and most of them were only recorded in one of the hosts or serogroups.

A high divergence among ribotypes was found in isolates that belonged to the serogroup A (13 different ribotypes) where ribotype 7 was observed as the most common ribotype. In isolates of the serogroups D and F only a few ribotypes were found with domination of ribotypes 3 and 1, respectively. An occurrence of *P. multocida* serogroups and ribotypes in connection with different hosts is shown in Table 2.

A dendrogram of genetic relatedness is presented in Figure 1B. Two major clusters (I and II) were found at a genetic similarity of 0.5. Cluster II was divided to four subclusters (IIa, IIb, IIc, and IId).

Table 2. Prevalence of *Pasteurella multocida* serogroups and ribotypes in isolates originated from different hosts

Ribotype	Pigs		Calves		Rabbits			Others	Total
	A	D	A	D	A	D	F	A	
1	–	–	–	–	9	–	7	–	16
2	–	–	–	–	–	–	1	–	1
3	8	16	–	2	–	2	–	1[a]	29
4	6	–	4	–	5	–	–	–	15
5	–	–	–	–	1	–	–	–	1
6	–	–	–	–	2	–	–	–	2
7	10	1	14	–	–	–	–	–	25
8	1	–	1	–		–	–	–	2
9	1	–	1	–	–	–	–	–	2
10	–	–	–	4	–	–	–	–	4
11	–	–	2	–	–	–	–	–	2
12	–	–	2	–	–	–	–	–	2
13	–	–	1	–	–	–	–	–	1
14	–	–	–	–	–	–	–	4[b]	4
15	–	–	–	–	–	–	–	1[c]	1
Total	26	17	25	6	17	2	8	6	107

[a]isolate from human

[b]three isolates from fowl (*Gallus gallus domesticus*) and one isolate from cat

[c]isolate from manul (*Otocolobus manul*)

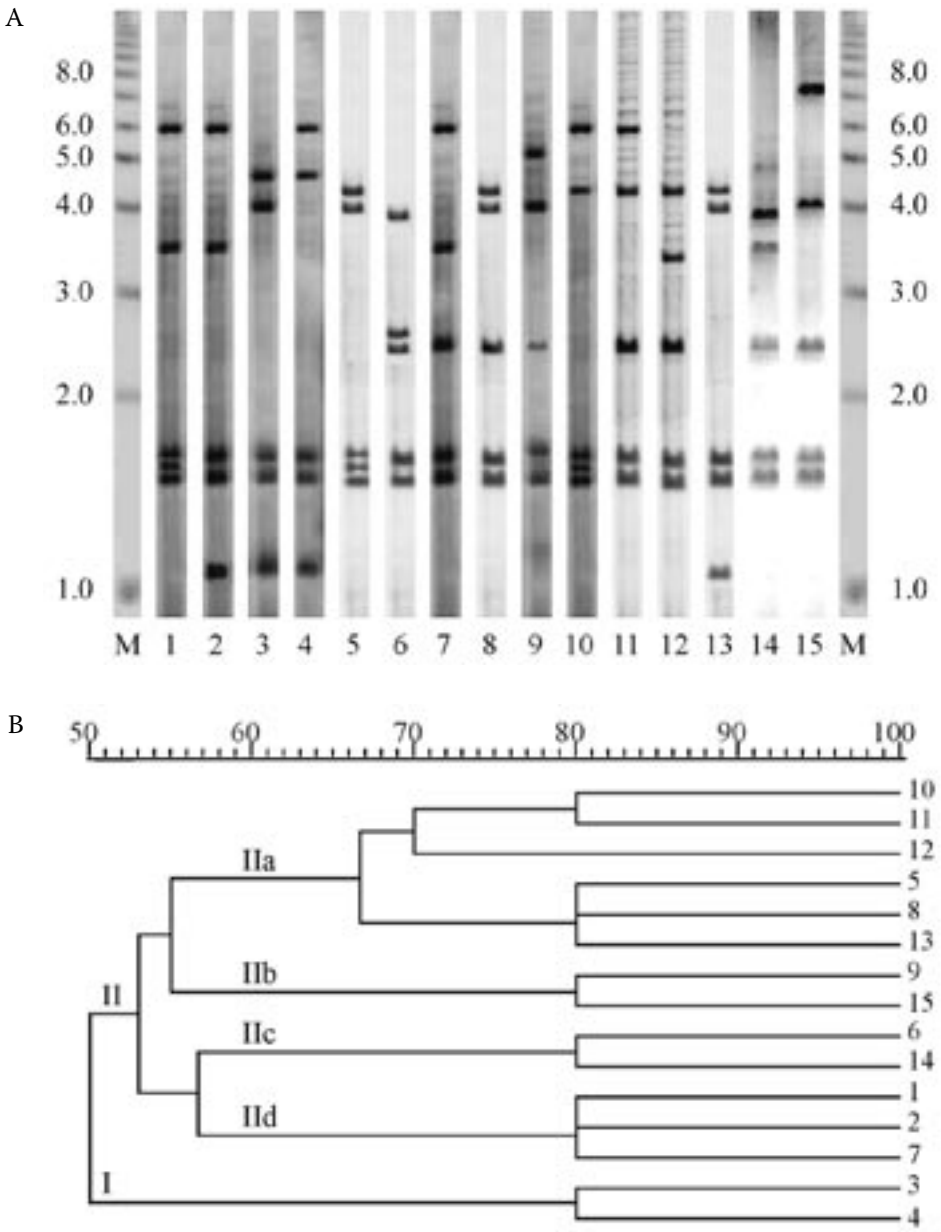

Figure 1. **A**. Ribotypes of *Pasteurella multocida* isolates and reference strains found after digestion with restriction endonuclease *Msp*I and hybridisation with the 16S-rDNA probe. M = 1 kb molecular weight marker (ABgene, Epsom, United Kingdom). Lanes 1–15: ribotypes 1 – 15, respectively. **B**. Dendrogram of genetic distance among 15 ribotypes clustered by UPGMA strategy. The two ribotype clusters (I and II) and four subclusters (IIa – IId) are marked

Ribotypes 3 and 4 were found in cluster I, remaining ribotypes were found in cluster II. The isolates of serogroups A and D were found in the both cluster I and II, whereas the isolates of serogroup F (all isolated from rabbit) were found only in subcluster IId. Clustering of the ribotypes using the UPGMA strategy has shown that most of the porcine (30 of 43) isolates belonged to cluster I, and most of the calf (25 of 31) and rabbit (20 of 27) isolates belonged to cluster II (Figure 1B).

DNT production was confirmed in 16 isolates (14.9%), mainly in those which originated from pigs (n = 14) of which eight isolates were serogroup A, and six were serogroup D. The remaining two toxigenic isolates originating from calf and human were serogroup A (Table 3).

Table 3. Occurrence of dermonecrotoxin positive *Pasteurella multocida* isolates in connection with the host origin, serogroups and ribotypes

Ribotype	Pigs		Calves	Human	Total
	A	D	A	A	
3	6	5	–	1	12
4	1	–	–	–	1
7	1	1	1	–	3
Total	8	6	1	1	16

DISCUSSION

This study was undertaken to examine the diversity of Czech *P. multocida* isolates originating from different hosts and different locations. As it shown in Table 1, a relatively high diversity among them was found what confirms that at least two different typing methods should be used in detailed epizootiological studies. The fact that some ribotypes occurred in different serogroups or the serogroups consisted of different ribotypes indicates that the combination of capsular typing and ribotyping provides better discriminatory power of *P. multocida* (Table 2).

Capsular typing revealed that 74 of 107 *P. multocida* isolates (69.1%) were serogroup A, which was dominant in each of the hosts. This finding is in general agreement with earlier studies which report that serogroup A is the most common, followed by other serogroups (Dabo et al., 1999a; Dziva et al., 2001; Davies et al., 2003a). Serogroup F rabbits isolates included in this study have been already described in our previous work (Jaglic et al., 2004). Nevertheless, a relatively high incidence (8 of 27) of these isolates in rabbits was unexcepted since the occurrence of serogroup F in a rabbit host has not been described so far.

The identified serogroups also differed to a degree of isolates RFLP diversity. A high number of different ribotypes (13 of 15) was detected in serogroup A isolates. Although the limited number of the serogroup D and F isolates was examined, it could be presumed that these serogroups were quite uniformed as in both of them one ribotype was clearly dominant.

RE *Msp*I used in this study is an isoschizomer of RE *Hpa*II, which has been shown to allow differentiation and good discrimination of *P. multocida* isolates (Zhao et al., 1992; Blackall and Miflin, 2000; El Tayeb et al., 2004). Ribotyping using RE *Msp*I revealed ribotype patterns which consisted of five distinct hybridization bands. Five bands ribotype patterns generated by RE *Hpa*II were also predominant in porcine isolates examined by Bowles et al. (2000).

The ribotype clustering revealed that most of the porcine isolates belonged to cluster I, and most of the calf and rabbit isolates belonged to cluster II. However, relationship among individual clusters (subclusters) and host origin was not absolute since for example in cluster I, besides the porcine isolates, approximately one third of isolates originated also from other hosts.

Most of the ribotypes detected in our study (11 of 15) occurred in more than one isolate. However, most of the isolates (85 of 107) had one of the four predominant ribotypes. The prevalence of the predominant ribotypes was different in isolates originating from different hosts. The most commonly detected ribotype in isolates originating from pigs was ribotype 3 (55.6%). Ribotype 7 dominated in calf isolates (45.2%), but it was also relatively frequent in porcine isolates. Most of the rabbit isolates (59.3%) were of ribotype 1, which was not observed in any other host. In general, the rabbit isolates showed the highest host specificity since 74.0% of them had ribotype patterns which were not found in isolates originating from other hosts. It therefore seems that some clones might be host adapted or, at least, different ribotypes dominate in different hosts. Similar host specificity of *P. multocida* isolates has been also observed in several host species (Davies et al., 2004) or dairy cattle (Dabo et al., 1999b).

Other ribotypes were found less frequently, and they were mainly detected in only one of the host (Table 2). For example, ribotype 10 was found in only four serogroup D calf isolates obtained from separated outbreaks of severe bronchopneumonia accompanied by a relatively high mortality rate. Ribotype 14, which was found in fowl isolates originating from breeding flocks suffering from chronic pasteurellosis was also recorded in a single cat isolate. Relationship among *P. multocida* isolated from closely located fowl, cats, and dogs was investigated by Muhairwa et al. (2001). However, in our case we did not have any information about potential contact between the cat and the fowl. On the other hand, the most common ribotype found in the porcine isolates (including toxigenic isolates) was also

found in one toxigenic isolate originating from a human, who was in close contact with affected pigs. Donnio et al. (1999) analysed toxigenic isolates of porcine and human origin, and based on the fact that some of them were closely related, they suggested possible colonisation of people from a porcine reservoir. Nevertheless, the limited number of our isolates makes it difficult to make any definitive conclusions with these findings.

DNT production was tested using ELISA, which has been shown to be a sensitive and specific technique (Bowersock et al., 1992). A significant correlation among DNT production and ribotypes was not observed in this study. Most of the porcine toxigenic isolates (11 of 14), as well as two toxigenic reference strains, belonged to ribotype 3, however, the remaining 13 of 24 porcine ribotype 3 isolates were found to be non-toxigenic. However, a close association between DNT production and a single ribotype has been already reported (Donnio et al., 1999). Gardner et al. (1994) also described a significant relationship between DNT production and a single specific ribotype, but only in isolates within the one geographic area and not in isolates originated from remote locations. Whether DNT production relates to certain ribotype patterns remains an opened question.

In this study we have shown that certain diversity exists among *P. multocida* isolates originating from different hosts in the Czech Republic. Based on the characterisation and comparison of the isolates, we found that infection or colonisation of various hosts is usually caused by different *P. multocida* clones which might be host adapted. This observation was the most noticeable in the isolates of rabbit origin since most of them had profiles which were not detected in other hosts. Furthermore, according to identified ribotype patterns we can consider that isolates of serogroup A were more variable than isolates of serogroups D and F. DNT positive isolates were much more common in pigs than in any other host. Although the toxigenic isolates could not be characterised by an unique ribotype pattern, domination of ribotype 3 among them was apparent.

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