Comparative analysis of *Pasteurella multocida* strains isolated from bovine respiratory infections

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Bovine respiratory disease (BRD) is the leading cause of significant economic losses in the intensive beef industry worldwide. Beside numerous risk factors *Pasteurella multocida*, which is regarded as a secondary pathogen, may play a role in the development of the disease. Previous studies of strainsfrom swine pneumonia revealed that there are a few clones associated with clinical disease, suggesting that some strains may be more virulent than others. This linkage may be true in the BRD, however composition of *P. multocida* populations in the herds are slightly characterized. Thus, we decided to perform phenotypic and genotypic characterisation of strains isolated from calves with respiratory infection at 31 different herds in Hungary. The results demonstrated the presence of two dominant strain types. At the identical taxonomic background (*P. multocida* subsp. *multocida*) with slight phenotypic variability they could be separated by trehalose fermentation capacity, α-glucosidase activity and molecular fingerprint patterns of ERIC- and M13-PCR. Independent prevalence and geographical origin of the strain types may refer to their significance in the illness, but their comparison with strains isolated from healthy individuals is taken into consideration.

Keywords: *Pasteurella multocida*, bovine respiratory disease, ERIC-PCR, M13-PCR, trehalose fermentation capacity, α-glucosidase activity

Introduction

The economic losses caused by Bovine Respiratory Disease (BRD) far exceed those due to gastrointestinal problems in calf rearing. It is a major health problem in cattle herds both locally and internationally [9]. The BRD is a multi-factorial disease; virus (BVDV, BRSV, PI3) or bacterial infections (*Pasteurella multocida*, *Mannheimia heamolytica*, *Histphilus somni*, *Trueperella pyogenes*, *Mycoplasma bovis*) beside many other predisposing factors may be involved in its development. *P. multocida* has been known to be associated with this disease since the early 1950s [2]. As *P. multocida* is a common inhabitant of the upper airways, it has long been considered to be a secondary pathogen and its exact role in the infection has not been cleared. Recently, the recurrence of BRD outbreaks despite vaccination against other pathogens, and the high *P. multocida* isolation rate from serious illnesses drew attention to its putative significance [8, 10, 24]. The respiratory diseases caused by *P. multocida* affect both calves (ECP - enzootic calf pneumonia) and young cattle (shipping fever). The differentiation of the two forms is slightly arbitrary. While ECP manifests within the first 6 months of life (calves contract disease from carrier dams or other herd members), the shipping fever occurs following exhaustive transporting to the stores of livestock-markets, where animals from different herds mingle, and their immune response is diminished due to stress and other predisposing factors. In the two (types of) diseases the clinical symptoms are similar: fever, lethargy, anorexia, coughing, nasal discharge and dyspnoea. They are manifestations of the chronic pneumonia and/or pleuritis induced by *P. multocida.* Most of the calves suffering from pneumonia will die despite treatment or have to be removed from

the herd, while in heifers mortality is less significant, the disease mainly manifests in a reduction of weight gain, milk yield, and problems of the meat quality or fertility [9].

The epidemiology of *P. multocida* strains associated with BRD represents a poorly studied research topic so far. There is little knowledge about whether the various commensal strains or pathogenic clone(s), differ from each other in their innate abilities induce disease, or how they take part in it; moreover there is no information about the relationship of strains causing infections in different age groups. Therefore detailed phenotypic and genotypic characterisation of *P. multocida* strains isolated from diseased animals of Hungarian cattle herds was carried out in order to explore the diversity of the bacterial population.

Materials and Methods

Bacterial strains

The studied 31 *P. multocida* strains were collected from different Hungarian cattle herds between 2006 and 2011 (Fig. 1). Twenty-six strains were isolated from lungs of heifers and five from nasal swabs (P930, and P931) and lungs (P929, P932, and P933) of calves.

Following bacteriological identification, the strains were stored in 20% skim milk powder solution (LAB M Ltd., Bury, Lancashire, UK) at -70˚C. For detailed examinations they were streaked on Columbia agar plate (LAB M Ltd., Bury, Lancashire, UK) supplemented with 5% defibrinated sheep blood. The plates were incubated for 24 hours at 37˚C, then separated colonies were inoculated onto brain-heart infusion broth (LAB M Ltd., Bury, Lancashire, UK) for biochemical studies and streaked on dextrose-starch agar plate (LAB M Ltd., Bury, Lancashire, UK) for serological examinations.

Phenotypical characterisation

Biochemical features

In the biochemical tests beside indole production, urease-, ornithine-decarboxilase-, and α-glucosidase activities and sugar (arabinose, glucose, lactose, sucrose, trehalose, maltose és xylose) or sugar-alcohol (dulcitol, sorbitol) fermentation abilities [12] were detected. Based on the results the strains were grouped in biovars [1, 4, 16]. The ingredients of solutions and methods were described previously by Varga et al. [22].

Serological features

The capsular type of the strains was identified by PCR according to the method of Townsend at al. [20]. The somatic serogroups were studied with agar gel precipitation test [5].

Molecular characterisation

For molecular examination, the bacterial DNA was extracted by Chelex' method [14]. The basic features were detected by species, toxin, and capsule A specific multiplex PCR [16, 19]. Capsule types beyond A were identified by multiplex capsular PCR [20]. The subgroups of the strains were classified with PCR-RFLP on the 16S rRNA gene [13]. The relationship of strains was examined with ERIC (enterobacterial repetitive intergenic consensus) - [23] and M13 PCRs [3, 17].

The reaction mixtures for ERIC and M13 PCRs were prepared in 25 μl: 1 × PCR buffer (Fermentas), 3.5 mM $MgCl_2$, 200 nM dNTP-mix, 25 pmol primers, 2.5 U Dream Taq (Fermentas), and 5 μl template DNA. The reaction conditions were: pre-denaturation 3 minutes at 93 °C, then 30 cycles at 93 °C 30 sec, 50 °C 1 min, and 72 °C 70 sec, and the final polymerisation step for 5 minutes at 72 °C.

The PCR fragments were detected by gel electrophoresis on 2% or 1.5% agarose gel. The molecular patterns were evaluated with the Hyper Ladder II DNA molecular marker (50-2000 bp, Bioline, Massachusetts, US). The representation of the generated distance matrix using

UPGMA (unweighted pair-group method with arithmetic mean) algorithm was carried out with TREECON software package [21].

Results and Discussion

Although, the association of *P. multocida* with respiratory diseases in various host species (swine, poultry, and rabbits) is well-known, the correlation of this pathogen with BRD is poorly studied [6, 18]. In this study detailed phenotypic and genotypic characterization was carried out on 31 bovine *P. multocida* strains isolated predominantly from lungs of cattle suffered from pneumonia in different Hungarian herds. The results showed that the strains possessed similar serological, biochemical and genetic features without reference to their origin. Serologically they mainly belonged to serogroups A3 (14/31), A3,4 (7/31) or A4 (4/31), which are considered to be typical for strains causing pneumonia in both cattle and pigs [11, 17]. However, some strains belonging to serogroups D and A1 were detected as well. These serogroups are known to be associated with diseases in swine or poultry (fowl cholera), respectively. Interestingly, the fermentation properties of the strains were fairly uniform in contrast to the diversity of strains from other hosts (swine, rabbits, and poultry). Eighty percent of all strains belonged to two biochemical variants (biovar 2 and 3). These biovars differed from each other only in their trehalose fermentation ability. The dominance of these two types is characteristic among strains from other host species as well. In small number, five other biovars (1, 12, 4, 7, and 9) were detected, differing from the two dominant types only in some biochemical features (Table I).

Notably, the presence of α-glucosidase activity, which has not been studied in this context earlier, correlated with the trehalose fermentation ability of the strains, except for P1185 and P1006. This biochemical feature was considered earlier as a tool for the differentiation of *P.*

multocida subsp. *multocida* and *septica*, the two dulcitol-negative subspecies [7]. However, the results of molecular studies have not supported this coherence clearly.

In the 16S ribosomal RNA gene PCR-RFLP assay, aiming the differentiation of subspecies, the strains displayed identical profiles, which is typical of *P. multocida* subspecies *multocida* [13]. For mapping of the genetic relationship of these highly similar strains, different molecular fingerprint methods are required. In this study the M13 minisatellite marker assay based on the comparative study of molecular methodological approaches of Taylor et al. [18] and the ERIC (enterobacterial repetitive intergenic consensus) -PCR, based on own experience [15], were chosen. The results of genotypic studies were correlated with each other and with the results of phenotypic characterisation as well (Fig. 2). Both approaches sorted the strains into two major sub-populations. In each group some features seem to be characteristic consistently. Biovar 2 strains fermented trehalose and had α-glucosidase activity, belonged to the same ERIC-PCR group and presented B pattern in M13 PCR; while biovar 3 strains were unable to ferment trehalose, missed α-glucosidase activity, and displayed M13 A pattern.

It is worth considering that all strains in the first group originated from lungs, while strains isolated from the nasal cavity and non-respiratory tract as commensals (milk, vagina, or fetus – unpublished data) belonged to the latter group.

Outside of the two main types, the M13 minisatellite marker PCR identified a few subgroups with various molecular profiles. The various molecular types could be associated with different biochemical characteristics, that is biovars: biovar 9 with B2, the toxin-producing strains with A1 and A2, and strains with capsule type D or F (not presented) with B1.

Conclusion

In our study the *P. multocida* strains isolated from the respiratory tract of diseased cattle were highly similar in phenotypic and genotypic features, as well, regardless of their geographical origin. The detected lack of diversity, usual for other hosts, alludes to the potential significance of each strain type. The used various methods confirmed irrespectively the presence of two dominant strain types within the bovine *P. multocida* population. For the clarification of their role in the disease process comparison with strains isolated from healthy animals is required.

For such studies, the 16S rRNA gene based PCR-RFLP, and examination of some phenotypic features (trehalose fermentation, and the α-glucosidase activity), along with high-resolution molecular methods are recommended for strain categorization.

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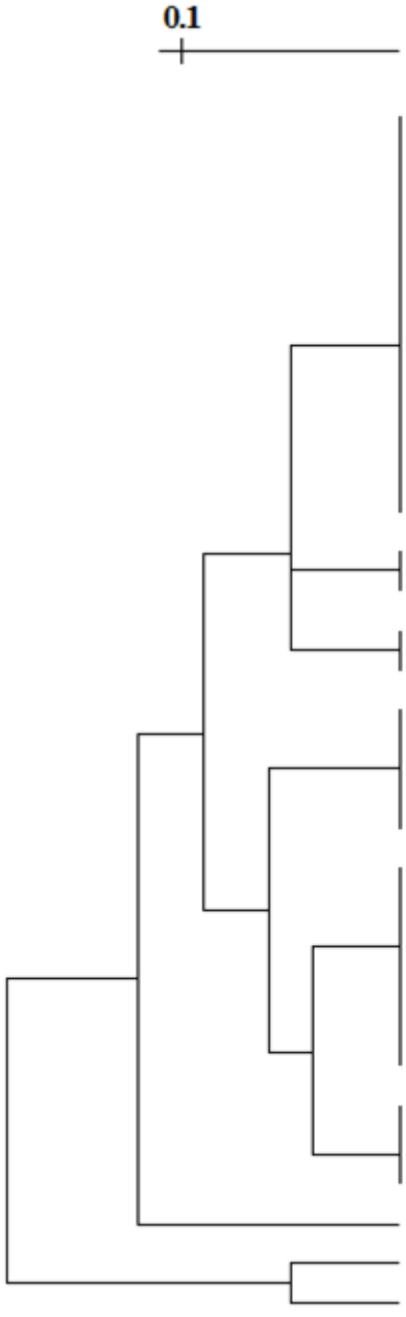
235 **Figure 1.** Geographical localisations of the sampled bovine populations in Hungary



	Isolation place	year	Serogroup	Biovar	M13 PCR
>P798	Rum	2007	A3	2	B
>P835	Kőröstetétlen	2007	A3,4	2	B
>P757	Kömlő	2007	A3	2	B
>P769	Ballószög	2007	A3	9	B2
>P1203	Cegléd	2011	A3	2	B
>P726	Sóshartyán	2006	A3	2	B
>P744	Hajdúnánás	2007	A-	9	B2
>P932	Pincehely	2006	A1	2	B
>P1006	Kunszentmárton	2009	A4	2	B
>P1142	Lajosmizse	2010	A3	2	B
>P1185	Tiszasüly	2010	A4	2	B
>P687	Budapest	2006	A3,4	2	B
>P1087	Püspökhatvan	2009	A3,4	2	B
>P842	Komárom	2007	A3	2	B
>P904	Lajoskomárom	2008	A3,4	2	B
>P1180	Gecse	2010	A3,4	3	A
>P1211	Rácalmás	2011	A4	3	A
>P896	Kisigmánd	2008	A-	12	A
>P930	Tarhos	2008	A4	3	A
>P931	Tiszanána	2008	A3	3	A
>P933	Hidashát	2005	A3,4	3	A
>P737	Vácszentlászló	2007	A3	3	A
>P788	Dénesfa	2007	A3	3	A
>P838	Dömös	2007	A3	3	A
>P857	Besnyőtelek	2008	A3	3	A
>P929	Törökszentmiklós	2008	D-	3	B1
>P1153	Kisújszállás	2010	D3	3	B1
>P1182	Etyek	2010	D-	3	B1
>P732	Alap	2007	A3,4	1	B
>P897	Bugyi	2008	A3	7	A1
>P1202	Nyársapát	2011	A3	4	A2

Figure 2. Comparision of genotypic (M13-, and ERIC PCR) and phenotypic features (serological and biochemical) of studied bovine *P. multocida* strains. The similarity dendogram was constructed by unweighted pair group method with averages (UPGMA) based on ERIC-PCR patterns.

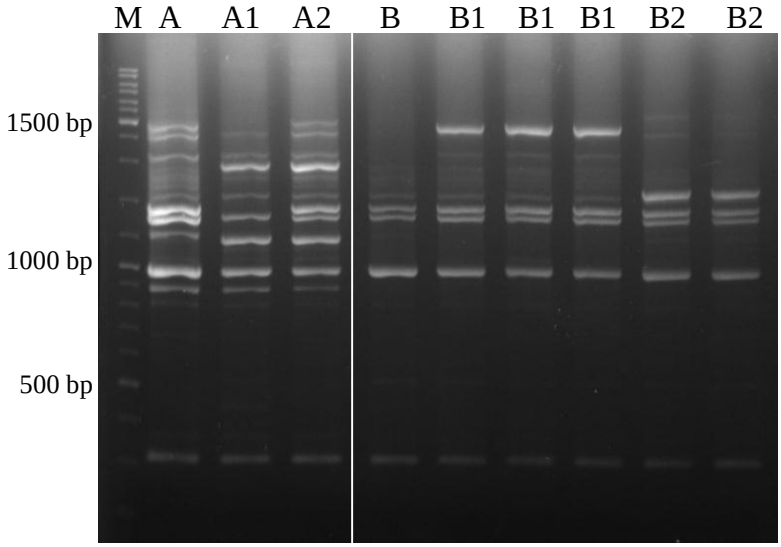


Figure 3 Detected fingerprint profiles generated by M13 PCR

pattern A and B – most frequent profiles

pattern A1 and A2 – profiles of toxin-producing strains

pattern B1 – profiles of strains with capsule type D

Table I

Fermentation patterns of the studied *P. multocida* strains

Biovars	3	1	12	2	4	7	9
No. of strains	(12)	(1)	(1)	(13)	(1)	(1)	(2)
ODC	+			+			-
Fermentation							
D(-)Arabinose	-			-			
Lactose	-		+	-			
Maltose	-			-			
Trehalose	**-**			**+**			
D(+)xylose	+	-		+	-		
Dulcitol	-			-			
D(-)Sorbitol	+			+		-	