

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

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

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

28

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Introduction

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

50 the herd, while in heifers mortality is less significant, the disease mainly manifests in a
51 reduction of weight gain, milk yield, and problems of the meat quality or fertility [9].
52 The epidemiology of *P. multocida* strains associated with BRD represents a poorly studied
53 research topic so far. There is little knowledge about whether the various commensal strains
54 or pathogenic clone(s), differ from each other in their innate abilities induce disease, or how
55 they take part in it; moreover there is no information about the relationship of strains causing
56 infections in different age groups. Therefore detailed phenotypic and genotypic
57 characterisation of *P. multocida* strains isolated from diseased animals of Hungarian cattle
58 herds was carried out in order to explore the diversity of the bacterial population.

59

60

Materials and Methods

61 **Bacterial strains**

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

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

72

73 **Phenotypical characterisation**

74 **Biochemical features**

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

80

81 **Serological features**

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

84

85 **Molecular characterisation**

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

92 The reaction mixtures for ERIC and M13 PCRs were prepared in 25 μ l: 1 \times PCR buffer
93 (Fermentas), 3.5 mM MgCl₂, 200 nM dNTP-mix, 25 pmol primers, 2.5 U Dream Taq
94 (Fermentas), and 5 μ l template DNA. The reaction conditions were: pre-denaturation 3
95 minutes at 93 °C, then 30 cycles at 93 °C 30 sec, 50 °C 1 min, and 72 °C 70 sec, and the final
96 polymerisation step for 5 minutes at 72 °C.

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

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

102

103 **Results and Discussion**

104 Although, the association of *P. multocida* with respiratory diseases in various host
105 species (swine, poultry, and rabbits) is well-known, the correlation of this pathogen with BRD
106 is poorly studied [6, 18]. In this study detailed phenotypic and genotypic characterization was
107 carried out on 31 bovine *P. multocida* strains isolated predominantly from lungs of cattle
108 suffered from pneumonia in different Hungarian herds. The results showed that the strains
109 possessed similar serological, biochemical and genetic features without reference to their
110 origin. Serologically they mainly belonged to serogroups A3 (14/31), A3,4 (7/31) or A4
111 (4/31), which are considered to be typical for strains causing pneumonia in both cattle and
112 pigs [11, 17]. However, some strains belonging to serogroups D and A1 were detected as
113 well. These serogroups are known to be associated with diseases in swine or poultry (fowl
114 cholera), respectively. Interestingly, the fermentation properties of the strains were fairly
115 uniform in contrast to the diversity of strains from other hosts (swine, rabbits, and poultry).
116 Eighty percent of all strains belonged to two biochemical variants (biovar 2 and 3). These
117 biovars differed from each other only in their trehalose fermentation ability. The dominance
118 of these two types is characteristic among strains from other host species as well. In small
119 number, five other biovars (1, 12, 4, 7, and 9) were detected, differing from the two dominant
120 types only in some biochemical features (Table I).

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

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

126 In the 16S ribosomal RNA gene PCR-RFLP assay, aiming the differentiation of subspecies,
127 the strains displayed identical profiles, which is typical of *P. multocida* subspecies *multocida*
128 [13]. For mapping of the genetic relationship of these highly similar strains, different
129 molecular fingerprint methods are required. In this study the M13 minisatellite marker assay
130 based on the comparative study of molecular methodological approaches of Taylor et al. [18]
131 and the ERIC (enterobacterial repetitive intergenic consensus) -PCR, based on own
132 experience [15], were chosen. The results of genotypic studies were correlated with each other
133 and with the results of phenotypic characterisation as well (Fig. 2). Both approaches sorted
134 the strains into two major sub-populations. In each group some features seem to be
135 characteristic consistently. Biovar 2 strains fermented trehalose and had α -glucosidase
136 activity, belonged to the same ERIC-PCR group and presented B pattern in M13 PCR; while
137 biovar 3 strains were unable to ferment trehalose, missed α -glucosidase activity, and
138 displayed M13 A pattern.

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

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

146

147 **Conclusion**

148

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

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

159

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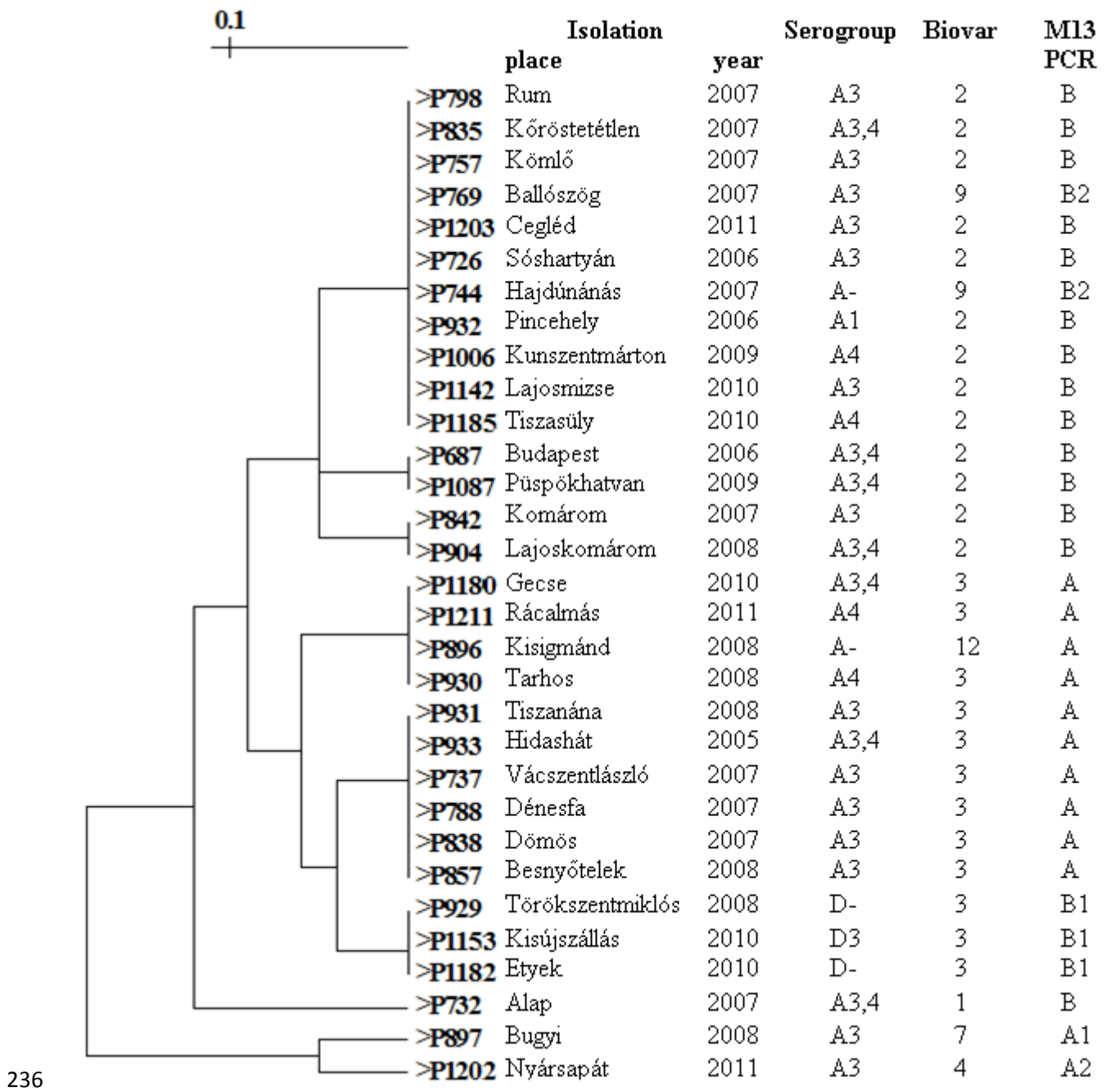
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Figure 1. Geographical localisations of the sampled bovine populations in Hungary



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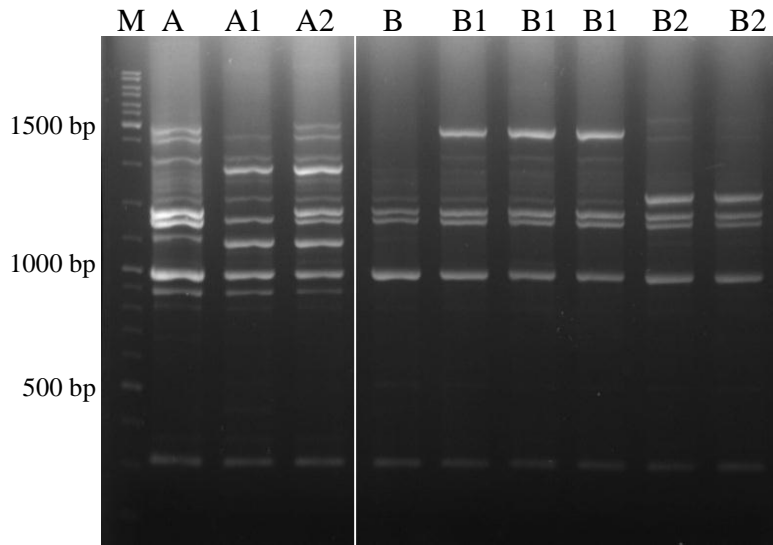
240 **Figure 2.** Comparison of genotypic (M13-, and ERIC PCR) and phenotypic features

241 (serological and biochemical) of studied bovine *P. multocida* strains. The similarity

242 dendrogram was constructed by unweighted pair group method with averages (UPGMA)

243 based on ERIC-PCR patterns.

244



245

246 **Figure 3** Detected fingerprint profiles generated by M13 PCR

247 pattern A and B – most frequent profiles

248 pattern A1 and A2 – profiles of toxin-producing strains

249 pattern B1 – profiles of strains with capsule type D

250

251

252

Table I

253

Fermentation patterns of the studied *P. multocida* strains

254

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

260