Summary of Ph.D. thesis

COMPARATIVE CHARACTERIZATION OF *PASTEURELLA MULTOCIDA* STRAINS ISOLATED FROM MAMMALS

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Introduction and aims

Pasteurella multocida is a widespread Gram-negative bacterium that can infect several avian and mammalian species. Settled on the mucous membranes of the airways, it is responsible for the development of a variety of diseases, often characteristic of host species. The major diseases it is responsible for are fowl cholera, atrophic rhinitis of swine, and haemorrhagic septicaemia of cattle and buffalo. As an opportunistic pathogen it causes pneumonic diseases in ruminants (cattle, sheep, goats), horses and pigs, and snuffles of rabbits. *P. multocida* causes significant economic losses in Hungarian livestock nowadays, therefore the minimization of predisposing factors and the choice of treatments and vaccines based on detailed knowledge of virulent *P. multocida* strains occurring in the affected regions have significant importance.

P. multocida strains show a high degree of variability in their phenotypic and genotypic characteristics. The first step of the characterization of bacterial strains is species identification by traditional biochemical methods, biovar determination based on the

fermentation of sugars and sugar alcohols, and serological classification based on capsule and lipopolysaccharide antigens. The primary aim of our work was the detailed characterization of Hungarian *P. multocida* isolates using serological, biochemical and molecular biological methods. Bacterial identification by a species-specific polymerase chain reaction (PCR) using oligonucleotides designed for the unique (*kmt1*) gene is a fast and reliable method. Additional PCR and sequence-based technics can be used to differentiate strains, to investigate phylogenetic relationships, and to epidemiological research. In addition to the investigation of the diversity of strains, we also wanted to explore the differences between isolates from different host species. Our additional aim was to identify the virulent strain type(s) responsible for the development of diseases.

Nowadays, the spread of antibiotic resistance is becoming more and more threatening, so the study of antibiotic resistance of *P. multocida* and the underlying genetic background were also included in our aims. *P. multocida* has a number of virulence factors, including toxin production responsible for atrophic rhinitis in pigs and adhesins that play a prominent role in host colonization. The investigation of the structures

responsible for adhesion is one of the main directions of *P. multocida* research nowadays, so in our work we also paid attention to the study of virulence genes that can be related to the above processes.

Our results can also provide useful information on virulence factors and host specificity of the strain types present in domestic pig and cattle herds. In addition to expanding scientific knowledge, the results of the research may also be a prerequisite for the novel and efficient diagnosis of diseases causing significant economic losses, as well as the development of new control methods based on detailed knowledge of the isolates.

Materials and Methods

Bacterial isolates

A total of 185 isolates of *P. multocida* recovered from different host species were used in this study. These strains were isolated from cattle, small ruminants (sheep and goat), pigs, cats and humans in Hungary from 1988 to 2018. Strains of human origin were isolated from patients at the Szent-Györgyi Albert Medical and Pharmaceutical Centre, University of Szeged, Hungary.

Identification of strains

The strains were stored in 20% skim milk powder solution (Skim milk powder, BD Difco) at -70°C. The isolates were cultured on Columbia agar (LAB M Ltd., Bury, UK) plates supplemented with 5% sheep blood under aerobic conditions at 37 °C for 24 h. Colonies were incubated in brain-infusion broth (Merck) for biochemical tests, and were cultured on dextrose starch medium (Dextrose Strach agar, BD Difco) for determination of somatic serovar. Plates were incubated at 37 °C for 24 h.

Phenotypic examinations

Biochemical tests were performed to phenotypically characterize the isolates. Biovar determination was based on ornithine decarboxylase activity and carbohydrate fermentation patterns. Production of ornithine decarboxylase was determined in the presence of 1% L-ornithine (Millipore, Ornithine Decarboxylase Broth). Lactose, maltose, arabinose, trehalose, xylose, dulcitol and sorbitol tests were performed using sterile solutions of sugars.

Somatic serovar was determined using the gel diffusion precipitin test. The heat-stable antigens used in the assay were produced by the method of Heddleston et al. (1972).

Antibiotic susceptibility was determined by the disk diffusion method for all *P. multocida* strains. Strains were tested according to the recommendation of CLSI (CLSI, 2018a). For the strain Pm238, antibiotic resistance conditions were further identified by determining the minimum inhibitory concentrations (MIC) using MIC test strips (Liofilchem, Roseto, Italy).

Genotypic examinations

A multiplex PCR reaction was used to confirm the species classification of the strains. With this method, we were able to simultaneously detect the species-specific gene (*kmt1*), the toxin gene, and the gene encoding capsular type A. (Townsend et al., 1998b; Gautam et al., 2004; Register and DeJong, 2006). The combined multiplex PCR reaction described above was used to determine capsular type A. Multiplex capsular PCR was used to detect other capsular types (B, D, E, F) (Townsend et al., 2001). The LPS genotype of the strains was determined by LPS PCR (Harper et al., 2015).

Within the species *P. multocida*, the two major phylogenetic lineages were separated by PCR-RFLP (fragment length polymorphism based on polymerase chain reaction) of the 16S ribosomal RNA gene (Sellyei et al., 2012).

The I and IV fimbrial subunit genes (*fimA*, *ptfA*), autotransporter adhesins (*hsf1*, *hsf2*), part of the secretory apparatus responsible for assembling Flp pilus (*tadD*), and filamentous haemagglutinin (*pfhA*) coding genes were identified by PCR (Sellyei et al., 2010; Tang et al., 2009). The genes encoding iron-acquisition proteins (*hgbA*, *hgbB*, *tbpA*) and the *nanH* neuraminidase were

detected by the method of Ewers et al. (2006) and Atashpaz et al. (2009).

The chloramphenicol (*catAIII*), sulfonamide (*sulII*), and streptomycin (*strA*) resistance genes were detected as described by Kehrenberg et al. (2001), and the gene responsible for the development of tetracycline resistance (*tetB*) was also detected by the method of Aminov et al. (2002). The PCR targeting the *parC* gene region was performed by the scheme of Katsuda et al. (2009). PCR reactions targeting genes associated with the ICE*Pmu*1 mobile genetic element were performed according to Klima et al., 2014. Plasmid isolation was performed using the Quiagen Plasmid Mini kit (Hilden, Germany).

Phylogenetic analysis of the *ompA* outer membrane protein gene was performed on 94 *P. multocida* strains selected based on their geographical distribution, serotype, and virulence gene profile. Amplification of the *ompA* gene region was carried out as described by Katoch et al. (2014). Sequencing of PCR products was performed by Macrogen Europe (Amsterdam, The Netherlands). Alignment of partial *ompA* gene sequences was done using the BioEdit program (version 7.2.3) (Hall, 2011). Nucleotide sequence data were further analysed with MEGA7 software (Kumar et al., 2016). Distances

were determined using the Neighbor-Joining algorithm (Saitu and Nei, 1987).

Strains isolated from haemorrhagic septicaemia cases were characterized using the Multi-host MLST scheme (Davies et al., 2004). MLST of strains isolated from feline and human cases was accomplished according to the RIRDC MLST scheme as decribed by Subaaharan et al. (2010). Sequencing of PCR products was performed by Macrogen Europe (Amsterdam, The Netherlands). Alignment of DNA sequences was performed with BioEdit (version 7.2.3) (Hall, 2011). Nucleotide sequence data were further analysed with MEGA7 software (Kumar et al., 2016). Distances were determined using the Neighbor-Joining algorithm (Saitu and Nei, 1987).

Results

Phenotypic characterisation

Capsular typing detected two capsular genotypes (A and D) for the bovine, ovine, caprine and porcine strains of *P. multocida*. The isolates from human cases were capsular serogroup A or F, and all strains from cats belonged to capsular type A.

LPS typing identified L3 and L6 LPS genotypes for the isolates from cattle and sheep. Only L3 LPS genotype was found in *P. multocida* isolates from goats and swine. In isolates from human cases, five categories of LPS genotypes (L1, L3, L4, L5 and L7) were detected. For the feline *P. multocida* isolates, L1, L3 and L7 LPS genotypes were found.

When capsular types were combined with LPS genotypes, the most commonly identified type was A:L3 for the isolates of bovine, ovine and porcine origin. For *P. multocida* strains from goats, D:L3 was the most common one, while A:L1 was the dominant type for isolates from humans and cats.

Genotypic characterisation

The *toxA* gene was detected in all ovine isolates, and in a high proportion of caprine and porcine strains (40% and 55%), but was not present in isolates from humans, cats and cattle. Among the caprine *P. multocida* strains, *toxA* gene was found only in pneumonic, capsular type D isolates. In porcine pneumonic isolates, we found a lower prevalence of the *toxA* gene (38.8%) as compared to strains recovered from nasal swabs (68.2%). In relation to the capsular type, we found a higher prevalence of *toxA* in capsular type A strains than in capsular type D isolates, both in porcine pneumonic (40%) and nasal swab (71.4%) isolates.

Type I fimbrial subunit encoding gene *fimA* was found in all *P. multocida* strains included in the study, therefore it was not analysed further. Based on the presence or absence of the other nine virulence associated genes, we distinguished 13 different virulence-associated gene profiles (VGP). With a few exceptions, each VGP was associated with only one host species. Bovine *P. multocida* isolates were exclusively grouped into VGP 1, 2, or 3, with the dominance of VGP 1 (61.7%). The ovine strains were assigned to VGP 4 and 5, of which VGP 5 was the more common (67%), and caprine isolates

were grouped into VGP 5. Porcine *P. multocida* strains were assigned to four VGPs (6–9). *P. multocida* isolates from cats were assigned into VGP 12 (82%) and 13 (18%). *P. multocida* strains from humans were more diverse, they belonged to six different VGPs (6, 9–13). In four of the six VGPs, they were grouped together with other host species (pigs, cats).

The *ompA* genes of the 94 *P. multocida* strains examined represented 25 unique, but closely related sequence types forming 9 clusters of allelic variants (Cluster A–I). OmpA clusters correlated with VGPs.

P. multocida isolates from human cases

A total of 15 isolates from human cases were further examined. Type A was the most prevalent capsular type detected among the isolates (14/15 strains), while the capsule biosynthesis gene *fcbD* (serogroup F) was found only in one case. Among *P. multocida* human clinical isolates, Heddleston serovar 1 (53%) and 3 (40%) were the most frequently found serovars, while in one case Heddleston serovar 6 was detected. The *toxA* gene was not shown out in none of the isolates. The most prevalent subspecies among the strains isolated from human infections was *P. multocida* ssp. *septica* (12/15 strains,

80%). Carbohydrate fermentation patterns and ornithine decarboxylase activity recognized six different biovars among the isolates. Biovars 2, 3 and 7 were the most prevalent biovars; however, biovars 6, 9 and 10 were also detected in individual cases. Based on the RIRDC MLST data we assigned 9 new STs, and three STs were represented by more than one strain (ST333, ST334 and ST336). Comparison with the isolates in the RIRDC MLST database indicated that *P. multocida* ssp. *septica* and ssp. *multocida* strains formed a distinct phylogenetic group within the species *P. multocida*.

Type B:2 *P. multocida* isolates

In August 2013, an unusual disease was detected among backyard pigs in Hungary. Three bacterial strains were isolated from pigs at different plots, which were identified as *P. multocida* ssp. *multocida*. Each of the strains belonged to biovar 3, and proved to be capsular type B and somatic serovar 2. Based on the data obtained by the Multi-host MLST, a new sequence type (ST61) was described. Using the RIRDC MLST scheme, the strains were classified into sequence type 122, which is characteristic of strains causing haemorrhagic septicaemia.

In July 2016, haemorrhagic septicaemia were observed in calves. Two isolates of *P. multocida* from the herd were identified as *P. multocida* ssp. *multocida,* and examined in detail. They were classified into biovar 3, which had capsular type B and serovar 2 characteristic of haemorrhagic septicaemia cases. Based on the data obtained by the Multi-host MLST, we described a new sequence type (ST64) that differs in one allele (*aroA*) from ST61 identified in backyard pigs. Comparison to isolates in the multi-host MLST database revealed a clearly separated cluster of HS-causing type B:2 strains.

Antimicrobial susceptibility tests

The vast majority of our strains were sensitive to florfenicol (98.7%), chloramphenicol (93.7%), ampicillin (91.8%), penicillin (91.1%), enrofloxacin (88.6%), ceftiofur (88%), tulathromycin (79.1%), tetracycline (86.7%) tilmicosin (86.1%) and doxycycline (82.9%). Gentamicin, erythromycin, streptomycin and nalidixic acid were less effective (44.9%, 23.4%, 27.2 and 13.9% resistance), while the proportion of moderately susceptible strains was also high for erythromycin (71.5%) and gentamicin (32.3%). A large number of strains were resistant to

apramycin (87.3%), clindamycin (97.5%), and sulfonamides (97.5%).

A multidrug-resistant *P. multocida* strain isolated from a calf has been studied in detail. The strain was classified into capsular type A, Heddleston serovar 3, and biovar 9. By RIRDC MLST, the strain was found to be sequence type 79. Based on MIC tests, the isolate was found to be resistant to streptomycin, tetracycline, doxycycline, erythromycin, clindamycin, chloramphenicol, sulfamethoxazole, enrofloxacin, and nalidixic acid. The isolate had no plasmid. On the other hand, according to the antibiotic resistance phenotype described above, we were able to successfully detect genes responsible for the development of resistance to chloramphenicol (*catAIII*), sulfonamide (*sulII*), streptomycin (*strA*), and tetracycline (*tetB*) by PCR reactions. PCR reactions targeting genes associated with the ICE*Pmu*1 mobile genetic element were also negative. A point mutation responsible for the development of quinolone resistance was also identified by sequence analysis of the topoisomerase IV (*parC*) gene region, which resulted an amino acid substitution at codon 84 (Glu → Lys).

Discussion

Virulence gene profile determination and *ompA* sequence analysis

In the present study, isolates of *P. multocida* from a broad range of host species were characterised comprehensively by determining their capsular type and LPS genotype. In total, eight capsule - LPS genotype combinations were detected in our isolates, with A:L3 being the most common.

PMT is responsible for the characteristic pathological lesions of progressive atrophic rhinitis in swine (Magyar and Lax, 2002). Therefore, the high incidence (55.0%) of PMT among the isolates from swine was not surprising. Toxigenic strains were isolated in a larger proportion from nasal swabs (68.2%), however, a remarkable percentage of *toxA*-positive isolates could also be identified from cases of pneumonia (38.8%).

Furthermore, *toxA* gene was also present in high ratios in ovine and caprine (100% and 40%, respectively) *P. multocida* isolates. Although its presence in isolates from small ruminants has been described before (Ewers et al., 2006; Shayegh et al., 2009; García-Alvarez et al.,

2017), a 100 percent incidence has not been previously reported in sheep. Moreover, the role of PMT in small ruminants, contrary to swine, has not been established yet, and further efforts are needed to explore the potential pathological effect of toxin production in *P. multocida* infections in sheep and goat.

Studying virulence associated genes is important in order to be able to understand the pathogenicity of *P. multocida*, and it also seems to be a promising tool for characterisation of isolates. Using analysis based on virulence associated genes, we could identify several different virulence gene profiles (VGP) among *P. multocida* strains tested in this study. The comparative analysis of the VGPs of our *P. multocida* isolates confirmed the diversity of this bacterial species, revealing the existence of at least 13 different profiles, and these groups showed a remarkable association with the host species. Recent publications (Harper et al., 2006; Katoch et al., 2014) reported that *ompA* gene variability had an effect on the virulence of *P. multocida*. In the present study, we discovered differences in *ompA* sequence types of *P. multocida* in association with host species and virulence gene profiles, which may indicate host preference and clonality among *P. multocida* isolates.

Based on our findings, VGP determination together with *ompA* sequence analysis is a valuable tool for the investigation of the diversity and host preference of *P. multocida*.

P. multocida isolates from human cases

In accordance with the data in previous studies (Boyce et al, 2010; Ewers et al., 2006), type A proved to be the most prevalent capsular serogroup among *P. multocida* strains from humans, and to the best of our knowledge, this is the first detection of capsular type F in a human case. The dominant serovars among human *P. multocida* isolates were A:1 and A:3, which widely occur in all host species (Boyce et al., 2010). Interestingly, we identified the rarely reported Heddleston serovar 6 too.

Subspecies differentiation is considered an important epidemiological marker of *P. multocida*. In nearly all hosts, ssp. *multocida* is the most frequently detected subspecies (Fegan et al., 1995; Blackall et al., 1997; Ekundayo et al., 2008). As an exception, strains from cats and dogs usually belong to ssp. *septica* (Kuhnert et al., 2000). In this study, ssp. *septica* was also found to be the predominant subspecies in the human clinical isolates.

Our MLST results confirmed the previous finding (Blackall et al., 1998) that *P. multocida* ssp. *septica* and ssp. *multocida* strains are forming two distinct clusters, with a number of newly described STs, and considerable diversity within each cluster.

Type B:2 *P. multocida* isolates

In August 2013, we came across with a disease caused by *P. multocida* type B in backyard pig herds which had not been identified in Hungary before. Subsequently, in July 2016, we also identified *P. multocida* strains of capsular type B from cases of bovine haemorrhagic septicaemia.

Neither the classical nor the molecular methods showed any difference between the *P. multocida* isolates from backyard pigs, suggesting that the pathogen that appeared in different herds may have come from a common source. Furthermore, based on the results of the MLST, it can be stated that the tested type B:2 strains are closely related to the haemorrhagic septicaemia causing strains isolated from different host species previously analysed. Compared to strains isolated in Europe, we can conclude that the disease-causing strain in Spain (Cardoso-Toset et al., 2013) is not the same as the

isolates from the cases described in Hungary. The haemorrhagic septicaemia causing isolate found by Soike et al. (2011) in Germany was identified as the worldwide RIRDC 122 sequence type (Petersen et al., 2014), which also includes strains isolated from the Hungarian case. Consequently, the epidemiological link between the German and Hungarian cases cannot be clearly justified on the basis of the present results, but it also cannot be ruled out.

Antimicrobial susceptibility tests

Due to the spread of antibiotic resistance, the study of pathogenic bacteria is of great importance, so the susceptibility of *P. multocida* to antibiotics has also been analysed in several previous studies. Data in these publications vary depending on the geographical location of the isolation. Due to the spread of multidrug resistance in *P. multocida*, continuous monitoring of antibiotic susceptibility of isolates should be considered. The emergence of multidrug-resistant isolates similar to strain Pm238 demonstrates that a single clone can acquire resistance genes in multiple steps. Our results also suggest that *P. multocida* may become multidrug-resistant in several ways, which may increasingly

compromise the therapeutic efficacy of antibiotics and support the spread of the pathogen. Better understanding of the mechanisms underlying multidrug resistance in *P. multocida* and preventing the spread of resistance between pathogenic bacteria are among the most important challenges in both human and veterinary medicine today.

Overview of the new scientific results

1. We were the first to determine the very high prevalence of *P. multocida* toxin-producing strains in small ruminants in Hungary.

2. Examining the occurrence of virulence-associated genes, we described 13 different virulence gene profiles, which, with few exceptions, were specific to only one host species.

3. With sequence analysis of the *ompA* gene, a total of 25 unique sequence types were established, forming nine clusters. These were associated with virulence gene profiles and host species of the strains.

4. We were the first to perform a detailed comparative study of *P. multocida* strains isolated from human cases of Hungarian origin, which showed a high degree of similarity with feline isolates, thus referring to the zoonotic origin.

5 For the first time in pigs and after a longer interval in cattle, the occurrence of type B:2 *P. multocida* strains was diagnosed in Hungary. We studied the isolates in detail,

comparing the data with the properties of recently emerging type B:2 *P. multocida* strains in Europe.

6. Antibiotic susceptibility of a large number of *P. multocida* strains from different mammalian species was determined. Examining the genetic background of antibiotic resistance in a multidrug-resistant *P. multocida* strain, we found that the strain probably accumulated resistance genes in several consecutive gene transfer steps rather than by conjugation transfer of a plasmid or multidrug-resistant transferable element.

Scientific publications

Publications on the topic of the thesis in peer-reviewed journals

Ujvári, B., Szeredi, L., Pertl, L., Tóth, G., Erdélyi, K., Jánosi, S., Molnár, T., Magyar, T., 2015. First detection of *Pasteurella multocida* type B: 2 in Hungary associated with systemic pasteurellosis in backyard pigs. Acta Veterinaria Hungarica, 63, 141-156.

IF: 0,871

Ujvári, B., Szeredi, L., Pertl, L., Erdélyi, K., Tóth, G., Jánosi, S., Molnár, T., Magyar, T., 2016. B: 2 típusú *Pasteurella multocida* törzsek okozta megbetegedés előfordulása sertésekben. Irodalmi összefoglaló és esetismertetés. Magyar Állatorvosok Lapja, 138, 333-346.

IF: 0,189

Magyar, T., Ujvári, B., Szeredi, L., Virsinger, N., Albert, E., Német, Z., Csuka, E., Biksi, I., 2017. Re-emergence of bovine haemorrhagic septicaemia in Hungary. Acta Veterinaria Hungarica, 65, 41-49.

IF: 1,042

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IF: 1,059

Ujvári, B., Weiczner, R., Deim, Z., Terhes, G., Urbán, E., Tóth, A. R., Magyar, T. 2019. Characterization of *Pasteurella multocida* strains isolated from human infections. Comparative Immunology, Microbiology and Infectious Diseases, 63, 37-43.

IF: 1,573

Ujvári B., Makrai L., Magyar T. 2019. Virulence gene profiling and *ompA* sequence analysis of *Pasteurella multocida* and their correlation with host species. Veterinary Microbiology. 233, 190-195.

IF: 3,03

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IF: 0,107

Publications on other topics in peer-reviewed journals

Magyar, T., Gyuris, É., Ujvári, B., Metzner, M., Wehmann, E. 2019. Genotyping of *Riemerella anatipestifer* by ERIC-PCR and correlation with serotypes. Avian Pathology, 48, 12-16.
IF: 2,338

Nemes, Cs., Schauta, M., Simonyai, E., Turbók, J., Ujvári, B., Magyar T. 2020. *Riemerella anatipestifer* okozta agyburokgyulladás előnevelt pulyka állományban (esetismertetés). Magyar Állatorvosok Lapja, 142, 87-93.
IF: 0,107

Ujvári, B., Szeredi, L., Magyar, T. 2020. Detection of *Frederiksenia* sp. isolated from a cat with nephritis. Acta Veterinaria Hungarica, 68, 140–146.
IF: 0,991

Ujvári, B., Orbán, B., Incze, Zs., Psáder, R., Magyar, T. 2020. Occurrence of *Pasteurellaceae* and *Neisseriaceae* bacteria in the pharyngeal and respiratory tract of dogs and cats. Acta Veterinaria Hungarica, In Press.
IF: 0,991

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