RESULTS OF THE RESEARCH

Pasteurella multocida is a widespread veterinary pathogen with zoonotic potential. This bacterial species infects a wide range of animal species and is the causative agent of numerous, economically important diseases. As a primary pathogen, it is involved in the aetiology of fowl cholera, atrophic rhinitis in pigs and haemorrhagic septicaemia in buffalo and cattle. As a secondary invader, it is associated with pneumonia in swine and ruminants as well as various respiratory tract diseases in rodents.

The main goal of the project was to investigate the prevalence, distribution, epidemiology, and host adaptation of *P. multocida* from different host species including humans. Further aims were to examine the diversity of phenotypic and genotypic properties of the isolates, learning more about their genetic variety, virulence factors, and to determine the antimicrobial resistance pattern of the strains together with its genetic background.

During the whole research, we were continuously expanding our formerly established *P. multocida* strain collection with new isolates from various animal species and also from humans. The strains represented a wide range of host species, different geographical regions and covered a longer period of time. In total, we had 120 strains from cattle, 35 strains from small ruminants, 298 strains from swine, 137 strains from rabbits, 15 strains from companion animals, and 31 strains from humans.

Basic properties, phenotypic and genotypic characterisation

The identity of all isolates was confirmed by using a multiplex PCR (polymerase chain reaction) system (species, capsular type and the sequence encoding *P. multocida* toxin). The somatic serovar of the strains was determined by the agarose gel diffusion precipitin test. Besides it, a multiplex PCR system has also been established in our laboratory that categorise *P. multocida* into eight distinct genotypes according to LPS outer core biosynthesis loci (L1 – L8). Moreover, we developed a serovar-specific PCR scheme for the molecular differentiation of *P. multocida* Heddleston serovar 3 and 4 strains. Finally, using conventional biochemical tests we determined the biovars of the strains that enabled the subspecies categorisation as well. However, the latter showed inaccuracy sometimes, therefore, we developed a rapid, multiplex PCR scheme to differentiate amongst the three subspecies of *P. multocida* based on the detection of key genes associated with *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, and *P. multocida* subsp. *gallicida.* This method could be useful for epidemiological investigations. In general, the strains examined displayed great diversity in their fundamental characteristics.

To identify potential virulence-associated genes, type I and type IV fimbrial subunits (*fimA*, *ptfA*), autotransporter adhesins (*hsf-1*, *hsf-2*), tight adherence protein D (*tadD*) and filamentous haemagglutinin (*pfhA*) coding genes were detected by PCR. The prevalence of genes encoding iron acquisition proteins (*hgbA*, *hgbB*, *tbpA*) and the neuraminidase gene *nanH* was also investigated by PCR. Combination of the tested virulence-associated genes classified our *P. multocida* isolates into 13 different virulence gene profiles (VGPs) (Table 1). The comparative analysis of the VGPs of our *P. multocida* isolates confirmed the diversity of this bacterial species, and these groups showed an association with the host species, except for human isolates. This may be explained by the fact that man is not a natural host for *P. multocida*, and thus humans presumably acquire the infection through contact with household pets or

commercial farm animals. 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.

Table 1.
Virulence gene profiles (VGPs) of *P. multocida* strains based on the presence of virulence associated genes.

Virulence gene profile	Host species	*hgbA*	*nanH*	*hgbB*	*tbpA*	*pfhA*	*hsf1*	*hsf2*	*tadD*	*ptfA**
VGP 1	cattle	+	+	-	+	+	-	+	+	B
VGP 2	cattle	+	+	-	+	-	-	+	+	B
VGP 3	cattle	+	+	-	+	-	-	+	-	B
VGP 4	sheep	+	+	-	+	-	-	+	-	A
VGP 5	sheep, goat	+	-	-	+	-	-	-	-	A
VGP 6	swine, human	+	+	-	-	+	-	+	+	B
VGP 7	swine	+	+	-	-	-	-	+	+	B
VGP 8	swine	+	+	+	-	-	-	+	+	B
VGP 9	swine, human	+	+	+	-	-	+	+	-	B
VGP 10	human	+	+	-	-	+	+	+	-	B
VGP 11	human	+	-	+	-	-	+	+	-	B
VGP 12	human, cat	+	+	+	-	-	-	+	-	B
VGP 13	human, cat	-	+	+	-	-	-	+	-	B

**ptfA* allelic variant

Additionally, prevalence of *P. multocida* toxin (PMT) was also analysed. The *toxA* gene was detected in all ovine isolates, and in a high proportion of caprine and porcine strains, but was not present in isolates from humans, cats and cattle. Our data strongly suggest that isolates from small ruminants form a distinct group within the species, and this view is supported by the high proportion of PMT producing strains and the fact that they belong to unique VGP groups.

OmpA is one of the major outer membrane proteins of *P. multocida* and have an effect on the virulence of *P. multocida*. The *ompA* gene sequence was analysed in a selection of 94 strains of *P. multocida* from different host species representing various geographic region, serovar and VGP. We revealed differences in *ompA* sequence types of *P. multocida* in association with host species and virulence gene profile, which may indicate host preference and clonality among *P. multocida* isolates (Figure 1).

Multi-locus sequence typing (MLST) classified the 20 *P. multocida* strains of human origin into 16 different sequence types, and we assigned 11 new sequence types. The most prevalent subspecies among strains from human infections was *P. multocida* subsp. *septica* (80%). Comparison with isolates in the MLST database indicated that *P. multocida* subsp. *septica* strains form a distinct phylogenetic group, regardless of the host species (Figure 2). The common characteristics shared by *P. multocida* strains isolated from humans and cats support the view that domestic cats serve as a major reservoir for *P. multocida* causing human infections. Our results also confirmed that *P. multocida* subsp. *septica* and subsp. *multocida* strains are forming two distinct clusters within the species with a considerable diversity within each cluster.

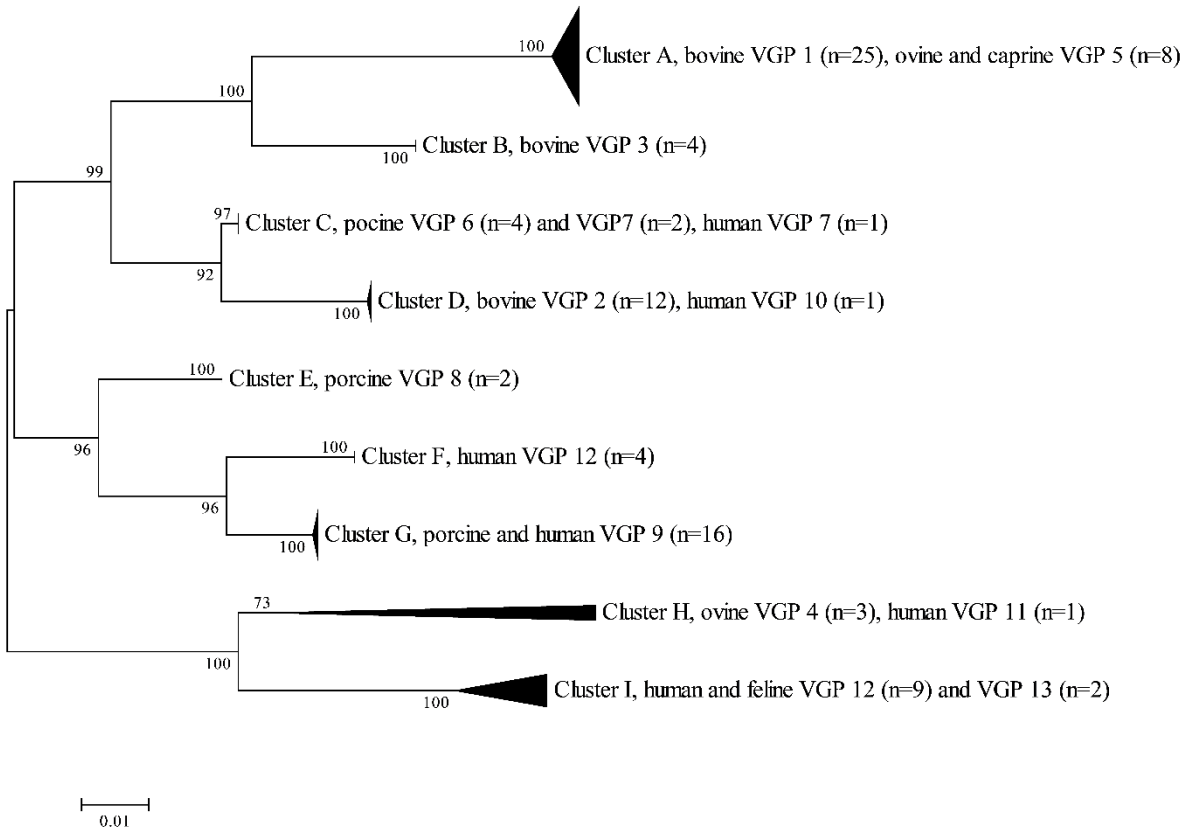

Figure 1.
Collapsed phylogenetic tree of partial *ompA* genes. The branches of the tree are indicated by the prevalence of their representatives, and are shown as triangles or vertical lines. Host species, *ompA* cluster, virulence gene profiles (VGPs) and the number of isolates is displayed next to the branches. The evolutionary history was inferred using the neighbour-joining method. Evolutionary analyses were conducted in MEGA7 software.

The typing scheme we used is likely to be useful for epidemiological studies and our results demonstrate a high level of diversity in virulence gene content among *P. multocida* strains from different host species.

Haemorrhagic septicaemia

Haemorrhagic septicaemia outbreaks caused by *P. multocida* are generally sporadic, but their frequency seems to be on the increase recently in Europe, including our country as well. So far, we have detected it in dairy cattle and also in backyard pigs. Although these cases were unexpectedly produced by life, the characterization of the isolates suited well into the topic of our research. Our latest case occurred in a fallow deer population in a hunting ground causing large-scale mortality. Pathological findings included subcutaneous oedema, swollen and haemorrhagic lymph nodes, confluent petechial epicardial and endocardial haemorrhages, ventricular dilatation, mild splenomegaly and foamy to watery rumen contents. The *P. multocida* strain isolated from all examined samples was identified by classical and molecular bacteriology methods as a serotype B:2 strain exhibiting sequence type ST64, clearly clustering

this isolate with other HS-causing strains. More detailed characterization of the isolates, including determination of virulence factor and antibiotic susceptibility profiles, is currently underway.

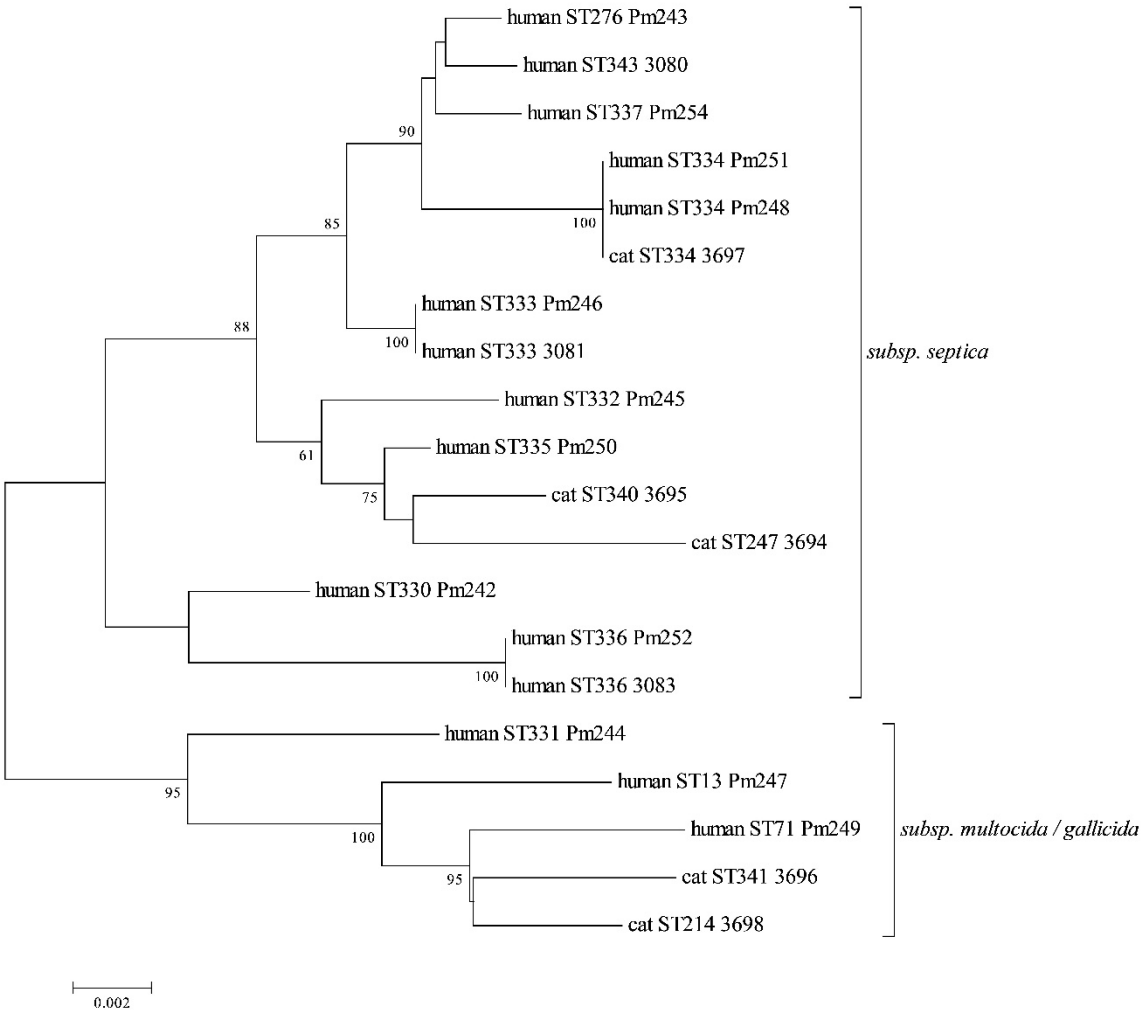

Figure 2.
MLST based evolutionary relationships between *P. multocida* isolates obtained from cats and humans. The evolutionary history was inferred using the Neighbour-Joining method. Evolutionary analyses were conducted using MEGA7 software

Antimicrobial resistance

For preliminary screening of the antimicrobial sensitivity of our *P. multocida* strains to various antibiotics, the traditional disk diffusion test was used. Based on the results, we determined the minimal inhibitory concentrations (MIC) for 15 antibiotics for 155 *P. multocida* strains of bovine, ovine, caprine and porcine origin isolated from 1988 to 2017 representing the whole geographical area of Hungary. MIC determination proved to be more accurate and reproducible than the disk diffusion test, we were able to quantify differences of sensitivity patterns between strains from various host species that might provide useful data for the optimization of therapeutic use of antibiotics, which is a delicate issue of recent times.

More than 90% of the tested strains were sensitive to amoxicillin, ceftiofur, tetracycline, doxycycline, florfenicol, and tilmicosin. All 155 strains were resistant to clindamycin. 82% of the strains showed moderate sensitivity to erythromycin (Figure 2). Out of the 155 strains, 19 proved to be multidrug-resistant (MDR) strains (resistant to at least one agent in three or more antimicrobial categories) (Figure 3).

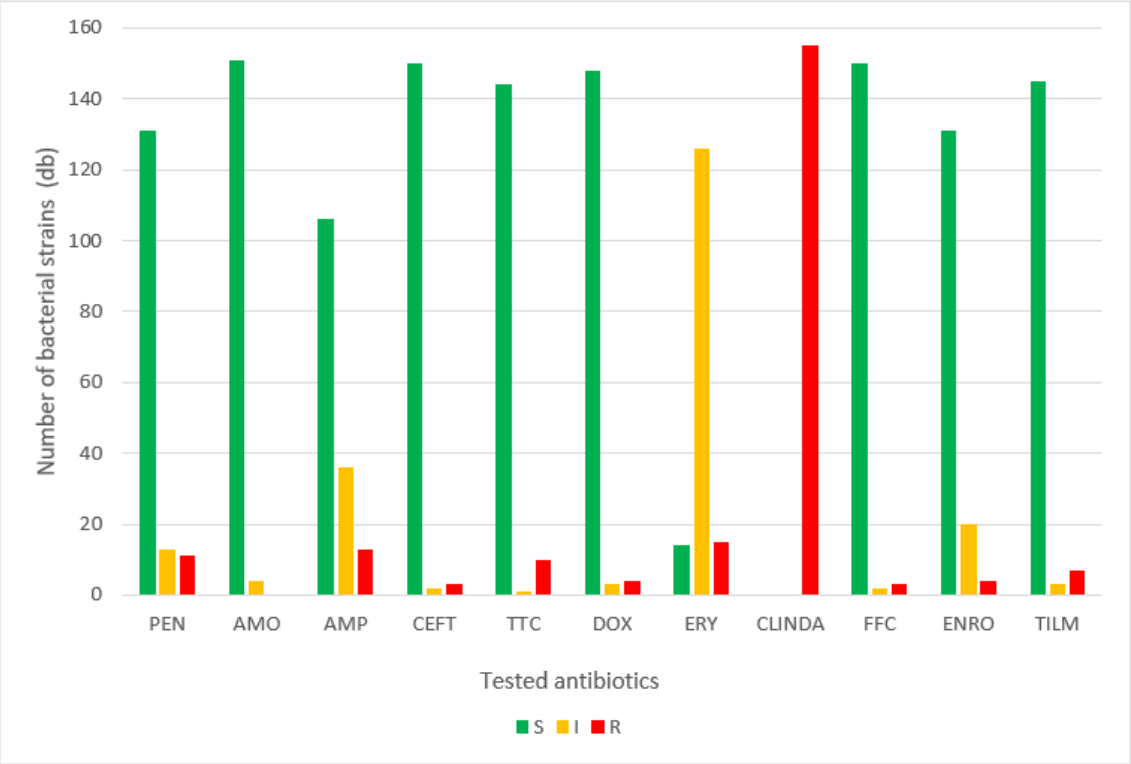

Figure 2.
Antibiotic sensitivity of the tested *Pasteurella multocida* strains (n= 155). Abbreviations: S: susceptible, I: intermediate, R: resistant, PEN: penicillin, AMO: amoxicillin, AMP: ampicillin, CEF: ceftiofur, TTC: tetracycline, DOX: doxycycline, ERY: erythromycin, CLINDA: clindamycin, FFC: florfenicol, ENRO: enrofloxacin, TILM: tilmicosin

Differences in antibiotic susceptibility patterns could be detected among strains isolated from various host species. Ovine and caprine isolates showed resistance to fewer antibiotics than strains from cattle or swine, and lower MIC values could be identified in their case. Isolates of bovine and porcine origin showed more diverse resistance conditions and increased MIC values, and multidrug-resistant strains could also be recognised. Resistance to chloramphenicol, enrofloxacin and nalidixic acid was also found in several bovine isolates, but not in strains from other host species. Our results strongly suggest that the number of multidrug resistant strains shows an increasing tendency. The antibiotic sensitivity patterns fluctuate in the various host species, presumably due to differences in treatment practices.

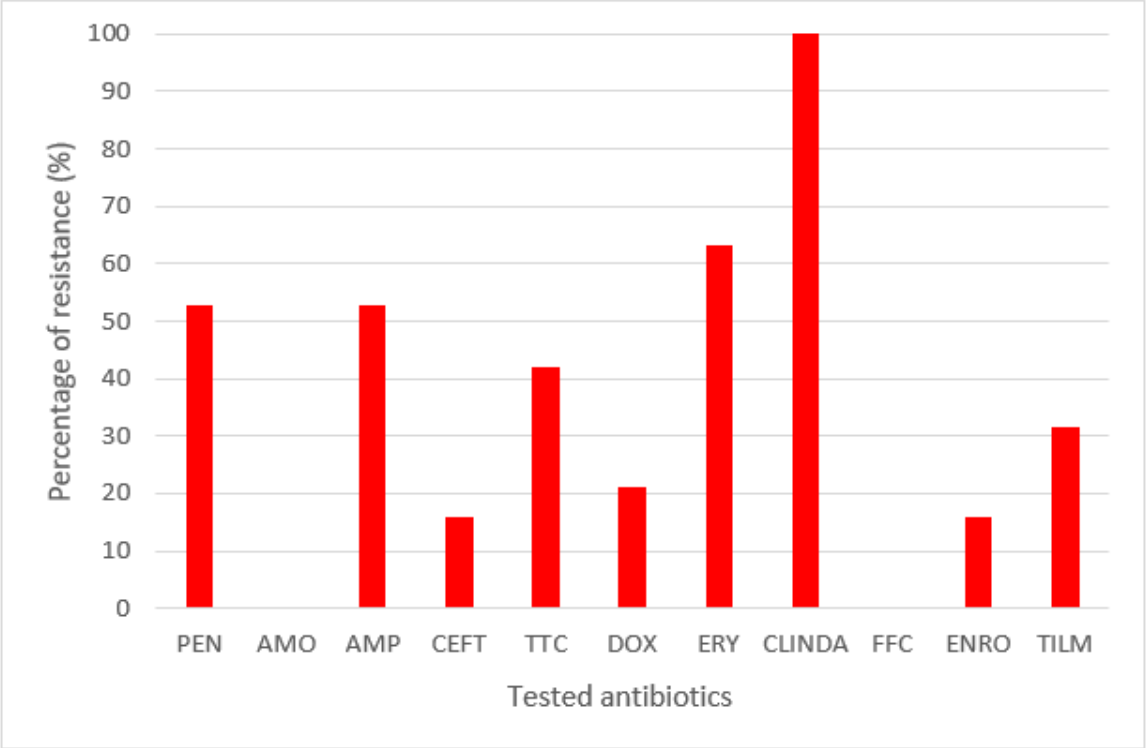

Figure 3
Percentage occurrence of resistance per antibiotic for MDR strains, n= 19. Abbreviations: MDR: multidrug resistance, PEN: penicillin, AMO: amoxicillin, AMP: ampicillin, CEF: ceftiofur, TTC: tetracycline, DOX: doxycycline, ERY: erythromycin, CLINDA: clindamycin, FFC: florfenicol, ENRO: enrofloxacin, TILM: tilmicosin

For strains exhibiting phenotypic antimicrobial resistance, we studied the genetic background responsible for the development of resistance (encoded on a plasmid or on the chromosome). Only 11% of the strains carried plasmid. These strains were isolated from sheep, swine and cattle. We also initiated to detect resistance genes using PCR reactions described in previous studies. We successfully detected streptomycin resistance genes *strA* and *strB*, the sulphonamide resistance gene *sul2*, the chloramphenicol resistance gene *catAIII*, the *tetB* gene responsible for tetracycline resistance, and the *msr(E)-mph*(E) genes causing macrolide resistant phenotype. We have developed a PCR for the identification of mutations in the quinolone-resistance-determining regions of topoisomerase IV (*parC)* and DNA gyrase (*gyrA)* genes, and also in the 23S rRNA gene associated with macrolide and lincosamide resistance phenotype.

Macrolides are commonly used to control respiratory tract infections in ruminants, but the susceptibility of *P. multocida* strains has shown a decrease to macrolide antibiotics in the last decade. Looking for the reasons behind this phenomenon, we assessed the prevalence of macrolide resistance of 100 *P. multocida* isolates from ruminant hosts and studied the resistance genotypes with newly designed PCR reactions. Susceptibility to erythromycin and tilmicosin was tested using minimal inhibitory concentration (MIC) test strips. We elaborated a PCR for the detection of *macAB* genes, and a PCR plus restriction enzyme-based technique was also developed for detecting a 23S rRNA gene mutation at position 2059. Five bovine isolates with notably increased MICs (≥256 µg/ml for erythromycin and ≥32 µg/ml for tilmicosin) carried resistance genes *msr*(E) and *mph*(E) or the A2059G point mutation in the 23S rRNA gene. Over 73% strains from small ruminants and all bovine isolates were MacAB PCR positive. Bovine

strains were less sensitive to macrolide antibiotics than isolates from small ruminants, and an increase in the prevalence of macrolide resistance in bovine *P. multocida* isolates has also been observed over time.

The emergence of simultaneous resistance to multiple classes of antibiotics presents an increasing threat. Plasmid-borne multidrug-resistance and integrative conjugative elements have formerly been reported in *P. multocida*. We described an alternative strategy for the development of multidrug-resistance observed in a *P. multocida* strain isolated from calf pneumonia. We identified genes integrated into the chromosomal DNA that conferred resistance to streptomycin (*strA*), tetracycline (*tetB*), chloramphenicol (*catAIII*), and sulphonamides (*sulII*). We also detected mutation in the quinolone-resistance-determining regions of *parC*. Strain Pm238 was plasmid-free with no chromosome-borne integrative and conjugative elements. These results suggest that *P. multocida* can accumulate multiple resistance determinants on the chromosome as single genes.

Whole genome sequencing

Complete genome sequencing of selected strains has also been performed, and analysis of whole genome sequences is in progress to identify antimicrobial resistance and virulence associated genes. Based on antimicrobial resistance gene databases, we identified antimicrobial resistance genes present in the tested isolates, which showed correlation with the resistance phenotype. The identification of transferable elements associated with resistance genes detected in these *P. multocida* strains is also being investigated, which is important because genes located on mobile genetic elements may contribute to the spread of antimicrobial resistance through horizontal gene transfer processes.

This approach made it also possible to determine the sequence type of the strains according to both presently available MLST systems. We discovered new allele types, and thus we have so far described further 17 new sequence types. Then, we have examined the presence of the following virulence genes of: *nanH, hgbA, hgbB, fimA, tbpA, ptfA, hsf-1, hsf-2, pfhA, tadD* both with the Geneious Prime program and with suitable PCR protocols, and thus we are going to revise and expand our VGP scheme.

General comments

The data generated by this research may be useful for epidemiological investigations, and the deeper characterization of *P. multocida* utilising our results might serve the better understanding of this bacterium and may help the prudent antibiotic use in animal husbandry and the selection of more efficacious vaccine candidates against the economically important diseases caused by this pathogen.

A PhD thesis was prepared based on the results of this project, which was successfully defended (Barbara Ujvári: Emlősökből izolált *Pasteurella multocida* törzsek összehasonlító jellemzése, 2021).