Molecular epidemiologic analyses of bacteria of the genera *Pasteurella* and *Mannheimia* to establish valid diagnostic tools based on Multiplex polymerase chain reactions

Summary

In this experimental thesis, an extensive collection of the important veterinary pathogenic genera *Pasteurella* and *Mannheimia* from various hosts and disease complexes were characterized with pheno- and genotypical methods. The molecular analyses were necessary to replace the currently established ambiguous phenotypical identification methods with valid, unambiguous molecular tools. With these tools, future molecular epidemiological studies are enabled, which finally will lead to a valid analysis of the actual prevalence as well as the pathogenic role of these important veterinary pathogens.

To our knowledge, with a total of 289 *P. multocida ssp.*-, 25 *P. sensu stricto*- as well as 104 *M. species*, for the first time such a large collection has been analysed, facilitating the establishment of valid diagnostical tools. Via PCR and DNA-DNA-hybridization, *Pasteurella* ssp. and spp. were investigated for the presence of 16 virulence associated genes [*psl*, *ompH*, *oma87*, *ptf*, *pfha*, *nanB*, *nanH*, *toxA*, *tbpA*, *tonB*, *hgbA*, *hgbB*, *sodA*, *sodC*, *iga*, *escv*], one species-specific [*kmt*] and five capsule genes [*capA*, *capB*, *capD*, *capE*, *capF*], while *M. haemolytica* and additional *Mannheimia* species were screened for the presence of 12 virulence associated genes [*lktA*, *pomA*, *gcp*, *trBA*, *tonB*, *irp*, *sodA*, *sodC*, *escv*, *iga*, *adh*].

Nearly all *P. multocida* ssp. strains showed a combination of genes, coding for outer membrane proteins (*psl*, *ompH*, *oma87*), colonisation factors (*ptf*, *nanB*, *nanH*), superoxide dismutase (*sodC*, *sodA*) as well as iron acquisition systems (*tonB*, *hgbA*, *hgbB*). In contrast, *tbpA* (31.5 %), coding for an iron acquisition system which seems to be specific for strains from ruminants, *pfha* (37.0 %), coding for filamentous hemagglutinine, as well as *toxA* (12.5 %), coding for dermonecrotoxin, were found to be less prevalent. Isolates of *P. sensu stricto* possessed mostly none of these genes, or, depending on the species investigated, *ompH*, *oma87*, *psl* and *tonB* only. Thus, genotypically these species could be easily differentiated from *P. multocida* ssp.. 86.9 % of the *P. multocida* ssp. harboured a combination of three genes encoding adhesins, namely *ptf*, *nanH* and *nanB*. In addition, 39.0 % of those strains reacted positive for *pfhA*, a gene which was rarely seen in strains of capsule type D (4.8 %).

The simultaneous expression of fimbriae, sialidases and hemagglutinine in *P. multocida* ssp. is a fundamental requirement for successful infection of the host and host tissues, respectively, and may be one reason for the wide host spectrum of *Pasteurella*. Additionally, the finding that genes *hgbA*, *hgbB* and *tonB*, involved in iron acquisition, were found in combination in 76.5 % of the strains, as well as the presence of *tbpA* in 20.4 % of them should theoretically give *P. multocida* ssp. an advantage in multiplication in different hosts. Thus, the data generated also give relevant information for future experimental studies, investigating the role of adhesins as well as iron acquisition systems during pathogenesis of different *Pasteurella*-associated disease complexes. Furthermore, the suitability of those gene products as vaccine candidates should be investigated.

Biotyping of 89 *M. haemolytica* strains classified 66 strains as members of biogroup 1, while 23 strains belonged to other biogroups. Six strains gave such a variable biochemical profile, that only 16S-rRNA gene sequencing led to their classification as *M. varigena* (5) and *M. ruminalis* (1), respectively. With the exception of *lktA*, *pomA* and *trBA*, which were nearly regularly identified in all strains of *M. haemolytica*, the other genes under investigation (like *tonB*, *irp*, *gcp*, and *sodA*) were found with higher prevalence in biogroup 1 strains, which are prone to be more virulent. As the latter genes were identified only recently, these new data also give reason to more detailed analyses of their possible role during the infectious process.

Based on these extensive analyses, two Multiplex PCRs were established successfully. With these diagnostic tools, the currently used conventional and partly ambiguous phenotypical methods for the identification of *P. multocida* ssp. und *M. haemolytica* can be replaced. Due to the confirmation of the regional importance of *P. multocida* ssp. *multocida* capsule type A and D strains, as well as the notification requirement of *toxA* positive strains, primers for the detection of those three genes as well *lktA* and *pomA* for the identification of *M. haemolytica* were integrated into one Multiplex-PCR. As both species may be present in mixed culture in biological samples, for example in cases of enzootic bronchopneumonia, we reasoned a differentiation to be useful. The establishment of a second Multiplex-PCR, enabling the simultaneous identification of *oma87*, *ptf*, *pfha*, *capF*, *tbpA*, *hgbA*, *hgbB*, *tonB*, *nanH* as well as *nanB*, will be useful in future epidemiological studies to identify colonization of various host species with *P. multocida* ssp.. Especially the recognition of the epidemiological shift, which seems to have taken place concerning *P. multocida* ssp., we sensed this tool to be highly useful. Indeed, the question of a horizontal gene transfer of the phage encoded *toxA*

not only between strains of a distinct capsule type, but also between strains isolated from various host animals is thrilling. In contrast to current knowledge, our data revealed that *toxA* is also found in capsule type A strains not only isolated from swine but also from bovines. Furthermore, the fact that several strains were isolated from pigs suffering from atrophic rhinitis, but being negative for *toxA*, indicates that these animals may be infected with various different *P. multocida* strains. This epidemiological highly important finding was unknown so far, most probably due to limitations of the currently used conventional diagnostic tools.

Another interesting finding was the fact, that *P. multocida* strains of capsule type F could be isolated from cattle as well as from cats. Due to the literature, such strains can only be found in poultry. In contrast, our results indicate a change in the adaption of strains with certain capsules to distinctive hosts. This epidemiological important finding should be investigated more detailed in the future – a perspective enabled by the Multiplex-PCRs established in the current work.

Finally, phylogenetic analyses were performed by sequencing the variable outer membrane protein gene *ompH* of *Pasteurella*. Due to the finding that *ompH* was present not only in *P. multocida* ssp., but in various *P. sensu stricto* isolates additionally, a total of 12 strains were included in this investigation. Although the gene product, an outer membrane protein, is under selective pressure, gene sequence analyses are in accordance with results of phylogenetical and taxonomical studies of *Pasteurellaceae* species, based on 16S-rRNA sequence data. Thus, analysing *ompH* is a useful complementation of phylogenetic investigations of *Pasteurella* species, clarifying the evolutionary development of these microorganisms.