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Characterisation of *Pasteurella multocida* strains isolated from different animals with traditional and molecular methods

Thesis of PhD dissertation

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Introduction

Pasteurella multocida is a facultative pathogenic bacterium that can infect a wide range of wild and domesticated mammals and avian species. The pathogenic potential of *P. multocida* in vertebrate animals was recognized over a century ago. *P. multocida* can colonize mucous membranes of the upper respiratory and urogenital tract without any symptoms. In conjunction with predisposing factors it can produce primary pasteurellosis such as haemorrhagic septicaemia of cattle and buffalo, fowl cholera in various avian species, and atrophic rhinitis in swine. Secondary infection with *P. multocida* may conduce to complicated pneumonia in ruminants, horses and pigs and snuffles in rabbits. Sporadic human infections are associated mostly with cat and dog bites and scratches and with licking of either intact or injured skin by pet animals. Inhalation of the microorganism is a rare route of infection in humans.

Diseases caused by *P. multocida* impose a huge economic burden on the livestock industry. This has led to intensive research to understand host-adaptation mechanisms and virulence factors in order to develop effective vaccines. Additionally, the desire for rapid diagnostic tests to either complement or substitute traditional methods and gather more information over general features of this bacterium (identification of species, capsular type, somatic serotype, toxicity) is present.

Genotypic and phenotypic characterisations have indicated a high heterogeneity within *P. multocida*. This diversity suggests some closely related strains (groups of strains) with properties different form the rest of the *P. multocida* population. Difference among these groups of strains could give an explanation to the varied pathogenic potential of strains and to diverse susceptibility against *P. multocida* in different hosts.

Our aim was to study the sorting rules regarding the heterogeneity of *P. multocida* strains that could ensure delineation of unique groups of related strains that would be separated either by the origin of host or their pathogenicity. Detailed observation of these groups of strains could provide the opportunity to characterise virulence and host-adaptation factors of *P. multocida* much more correctly.

Aims of study:

The main aim of this study was to identify and characterise groups of related strains within the heterogenic *P. multocida* population. Parts of the work were:

- Characterisation of numerous *P. multocida* strains isolated from swine, rabbits and poultry using conventional and newer phenotypic methods. In the course of these observations we tried to find features that could indicate relation of strains belonged to the same group.

- Verification of the existence of these groups and demonstration of a possible link with host by whole-genome examination (ERIC-PCR) of strains isolated from different avian species.

- Further characterisation of strain groups with gene-analysis for identification of molecular marker(s) that could be used for studying field isolates.

Materials and methods

***P. multocida* strains:**

One-hundred and forty five *P. multocida* strains isolated from swine, 76 strains from rabbits and 61 strains from poultry (17 goose, 20 duck, 15 turkey, 4 chicken, 3 pheasant, 2 Muskovy duck [*Cairina moschata*]) were studied. The strains represented various geographic locations in Hungary.

Methods

Phenotypic characterisation

1, Biochemical features

Phenotypic properties of *P. multocida* strains were primary examined by conventional biochemical and carbohydrate fermentation tests. In its scope oxidase, catalase, urease, ornithine decarboxylase activity were checked and glucose, lactose, sucrose, dulcitol, sorbitol, trehalose, maltose and xylose fermentation were determined. Biochemical characteristics were used to define subspecies (subsp. *multocida,* subsp. *septica,* subsp. *gallicida*) and biovars (1-14) of our strains (Varga et al. 2007, Sellyei et al. 2008a).

2, Antigenic features

Capsular typing was performed by multiplex capsular PCR. Somatic serotypes were identified by agar gel diffusion precipitation test.

3, Antimicrobial resistance

Antimicrobial susceptibility of our strains was determined by disk diffusion test based on 'Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacterial Isolated from Animals (M31-A3)' of Clinical and Laboratory Standards Institute (CLSI). Sensitivity of the *P. multocida* strains was evaluated against 14 widely used compounds (apramycin, neomycin; penicillin; florfenicol, chloramphenicol; enrofloxacin, flumequine; erythromycin; oxolinic acid; doxycycline, tetracycline; sulphamethoxazole-trimethoprim; colistin; sulphonamides) (Sellyei et al. 2009).

Genotyping

Sixty-one strains isolated from poultry were used for genetic characterisation.

1, Whole-genome examination

For studying the relationship of the strains enterobacterial repetitive intergenic consensus sequence (ERIC) PCR was carried out. Fingerprinting II Informatix^{TM} Software (Bio-Rad) was

used to compare the ERIC-PCR fingerprint profiles of the tested *P. multocida* strains (Sellyei et al. 2008b).

2, **Observation of genes**

Strain groups delineated by ERIC-PCR were characterized with the analysis of certain genes.

PCR-RFLP

Five gene region encoding cell surface exposed molecules (*hya*DC, *oma*87, *omp*H, *ptf*A-*hof*B, *omp*A) were investigated by PCR-restriction fragment length polymorphism (RFLP) method using *Dra*I, *Bsr*DI, *Pvu*II restriction enzymes.

Sequencing

Forty strains isolated from poultry, belonging to various somatic serotypes, ERIC-PCR groups and geographic origin, were selected for the sequencing study of the *ptf*A-*hof*B gene region (BigDye Terminator v1.1 Cycle Sequencing Kit, ABI Prism 3100 Genetic Analyzer capillary sequencer).

Allele-specific PCR

The nucleotide sequence alignment (MultAlin V5.4.1) showed marked differences in the 3' end of the *ptf*A gene. Two major sequence variants were identified, referred to as type A and type B. The low genetic heterogeneity of this gene region provided opportunity for developing an allele specific PCR assay (Sellyei et al. 2010).

Results

Biochemical features

The subspecies of *P. multocida* was determined with differential carbohydrate (dulcitol and sorbitol) fermentation tests. The dominance of *P. multocida* subsp. *multocida* strains was typical in all host species. It suggests high degree of flexibility of *P. multocida* subsp. *multocida* strains. The ratio of *P. multocida* subsp. *multocida* and *P. multocida* subsp. *septica* strains came near to literature data (80%-20%) except for strains isolated from geese. In case of these strains prevalence rate of subspecies *multocida* and *septica* was close to equal. *P. multocida* subsp. *gallicida* strains were detected in small number in swine.

Studying wide-range of biochemical features indicated high phenotypic heterogenicity except for strains isolated from ducks that were mostly uniform. Ten biochemical fermentation patterns (biovars) could be detected in three types of host. Biovars 3, 6, 2 and 1 were the most common. Strains from different hosts could be separated in two groups. In one group (strains from swine, goose, turkey), the majority of the strains represented biovar 3 (or in lesser number biovar 2), while in the other group (strains from duck and rabbits) biovars 1 and 6 were dominant. A few biochemical features or biovars, like lacking of ornithine decarboxylase (biovars 9 and 13) among strains from mammals or the presence of lactose fermentation (biovar 12) among strains from swine, were typical in strains from certain hosts.

Antigenic features

Three capsule types (A, D, F) could be identified among our *P. multocida* strains. Significant presence of capsule type A was detected among strains from all host (93,5%, 63% and 41% among strains from poultry, rabbits and swine, respectively). At the same time, the number of strains with capsule type D showed a decrease among pigs (59%) as compared to earlier findings. Capsule type F that is considered typical for strains isolated from turkey proved to be well represented among strains from rabbits (25%).

Capsule type and somatic serotype together establish the traditional antigenic characterisation of *P. multocida* strains. Seven different serotypes (A:1; A:3; A:3,5; A:4,5; F:1; F:4,5,[7]; F:10) were present among strains isolated from poultry in our study. The serological typing showed that the majority of the strains isolated from poultry belonged to serotype A:1 and A:3. Few unique serotypes (F:4,5,[7]; F:10) occurred only among strains from turkey. The available diagnostic background of the strains suggests that serotype A:1 strains might represent a rather virulent type while serotype A:3 may belong to a less virulent type.

Antimicrobial resistance

Generally, *P. multocida* is susceptible to most of the widely used commercial antimicrobial agents. Antimicrobial resistance can evolve in the strains by molecular transmission of mobile genetic elements carried resistance mechanisms from other bacteria. The antimicrobial resistance pattern of *P. multocida* is pointed to the sensitivity of strains to genetic changes. Fifty-four percent of strains isolated from poultry possessed resistance to at least one of the antimicrobial agents tested in our study. Most of them (82%) were multi-resistant (resistant against 2-8 antimicrobial agents). The resistance to derivates of nalidix acid and sulphonamides was the most frequent. The resistance to neomycin or tetracycline also was high (29% and 32%, respectively). While resistance to doxycycline and penicillin (7%) or to enrofloxacin and florfenicol (3,5%) were not considerable. The different antimicrobial resistance patterns showed an accumulation of resistance in certain hosts (duck and goose) and also a progress by time.

Relationship among *P. multocida* strains isolated from poultry

The computer-assisted comparison of ERIC-PCR fingerprint profiles generated similarity dendogram by the unweighted pair group arithmetic average (UPGMA) linkage method. At an arbitrary 75% similarity cut-off level, the strains could be grouped into four larger clusters (I, II, III and IV) that showed correlation with the host species (Muscovy duck, galliformes, anseriformes). The dissimilarity was the highest between strains isolated from Muscovy duck (cluster I) and the strains isolated from anseriformes (cluster III) and galliformes (cluster II and IV), however, even this difference remained unrecognized by phenotypic markers. Separation according to geographic origin was also detected in the ERIC-PCR groups.

Clusters II and IV contained a mixture of strains isolated from galliformes (chicken, turkey and pheasant). These strains were localized on the region between rivers Danube and Tisza and the territory east of river Tisza or the Transdanubian region of the country, respectively. Strains isolated from duck and goose formed cluster III and its subclusters were collected from the region between rivers Danube and Tisza and the territory east of river Tisza. Subclusters of cluster III separated according to host (III/1, 4b – from duck; III/2, 3, 4a – from goose) and geographic origin Geographic regions of subclusters III/1 and 4b contained strains mostly from duck overlapped each other (between the rivers Danube and Tisza).

Subclusters of cluster III including strains from anseriformes primary delineated by similarity fenogram showed cohesion of serotype A:1 and A:3 strains independently from the host.

Observation on genes

The groups of strains was further characterised with PCR-RFLP on 5 genes (*hya*DC, *oma*87, *omp*A, *omp*H, *ptf*A-*hof*B) encoding surface localized proteins. The gene region of *hya*DC and *oma*87 showed the strains uniform but *omp*A and *ptf*A-*hof*B supported separation of strains isolated from Muskovy duck from other strains from poultry. PCR-RFLP of *omp*H gene region showed considerable diversity that harmonized with the presence of certain serotypes. Five PCR-RFLP patterns were differentiated (I., II., III., IV., VI.). They did not fit to all serotypes presented among our strains but serotype A:1 strains and strains with unique serotypes isolated from turkey could be separated by their uniform PCR-RFLP patterns.

The sequence analysis of *ptf*A gene encoding type 4 fimbrial subunit identified consensus nucleotide changes in the 3'-end region. These differences on the sequence defined two alleles of *ptf*A gene (allele type A and B). For rapid identification of these alleles we developed an allele-specific PCR assay (Sellyei et al. 2010) that demonstrated the dominancy of allele type A in serotype A:1 strains while allele type B was typical for the other serotypes.

Main new scientific results

In the course of the study:

- Connections between certain phenotypic features, like indole production, lacking of ornithine decarboxilase or lactose fermentation, and host origin of *P. multocida* strains were proved.

- Groups of strains separated according to host (Muscovy duck, galliformes, anseriformes) and geographic origin were delineated with ERIC-PCR.

- PCR-RFLP of *omp*H gene region was established that suitable for characterisation of strains thus supplementing traditional serological typing.

- Separation of strain isolated from Muskovy duck from other strains from poultry was confirmed by ERIC-PCR and PCR-RFLP on *omp*A and *ptf*A-*hof*B gene region.

- Two allele variants (allele A and B) of *ptf*A gene were characterised among strains isolated from poultry in Hungary.

- Allele specific PCR was developed for simple and rapid identification of allele variants using nucleotide sequence differences in the 3' end of the *ptf*A gene.

- A virulent group of strains, including primarily serotype A:1 strains, was separated and characterised successfully with the above mentioned methods.

Publication

Scientific articles:

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