Characterisation of *Actinobacillus pleuropneumoniae* strains isolated from Hungarian swine herds

Actinobacillus pleuropneumoniae, the causative agent of porcine pleuropneumonia is an important pathogen of swine which can cause considerable losses in the swine industry all over the world. *A. pleuropneumoniae* has two biotypes; biotype 1 strains need nicotinamide adenine dinucleotide (NAD) to propagation, while biotype 2 strains do not. Both biotype 1 and biotype 2 strains cause haemorrhagic-necrotic pneumonia in swine, however their epidemiological impact is different. *A. pleuropneumoniae* biotype 1 strains are responsible for large outbreaks and great economic losses, while *A. pleuropneumoniae* biotype 2 strains are the aetiological agents of sporadic pleuropneumonia cases. For a long time 15 serovars of the agent were described, but as a result of the present project three further serovars became identified. Different virulence mechanisms of the agent are involved in the pathogenesis, adhesins, lipopolysaccharide (LPS) complex, iron uptake systems, surface polysaccharides, outer membrane proteins, fimbriae and different toxins are considered to be the main virulence factors. These toxins belong to the RTX (repeats in toxin) toxin family, which are widely prevalent in Pasteurellaceae, and are secreted by the type I secretion system. Four such toxins have been described up to now, ApxI-IV and the different toxins are produced by the *A. pleuropneumoniae* strains in different patterns. In order to reduce the losses caused by porcine pleuropneumonia, large amount of antibiotics is used for therapeutic or metaphylatic treatment of pigs and different kinds of vaccines, containing inactivated bacteria, inactivated toxins or different outer membrane proteins are used for specific prevention.

The project aimed to characterise *A. pleuropneumoniae* strains isolated from Hungarian swine herds:

1. *A. pleuropneumoniae* isolates were collected both from carcasses sent for diagnostic evaluation and slaughtered pigs having lesions in order to build a representative strain collection.
2. The isolates collected were serotyped using the indirect haemagglutination test, and some strains were also typed on the basis of toxin production.
3. Exotoxin production of the isolated *A. pleuropneumoniae* strains was detected
4. The metabolic phenotypical characteristics of the isolates were examined and their metabolic fingerprint was defined on the basis of assimilation of 95 carbon sources.
5. Genetic diversity of the *A. pleuropneumoniae* strains was analysed by comparing pulsed field gel electrophoresis fingerprints and a strain library was created.
6. A. *pleuropneumoniae* strains non-typable with indirect haemagglution test were analysed in order to find phylogenic connection with other strains.
7. Correlation between virulence, clinical form, severity of the clinical signs, post mortem lesions and characteristics of the *A. pleuropneumoniae* strains (biotype, serovar, metabolic type, genotype) was analysed.

A total of 255 *A. pleuropneumoniae* strains were isolated from 634 lung samples showing typical post mortem lesions of porcine pleuropneumonia. The samples were received from 70 swine herds located in different parts of Hungary. The isolates were identified as *A. pleuropneumoniae* strains on the basis of cultural, morphological and biochemical methods, and the identification was confirmed with PCR, too.

We aimed to examine the frequency of the different serovars of *A. pleuropneumoniae* in Hungary. The strains were serotyped using the indirect haemagglutination test but only 77 strains were involved in the evaluation after serotyping, since 178 strains were duplicates or multiple serovars from the same farm. In the case of 7 herds strains of two different serovars were identified. Fourteen *A. pleuropneumoniae* strains from the culture collection and isolated earlier were taken in the evaluation, one each strain from 12 farms and two each from two herds, where two serovars occurred. Out of the total 91 *A. pleuropneumoniae* strains 72 strains belonged to biotype 1 and 19 ones could be allocated into biotype 2. Serovar 2 (39.5%), 13 (15.4%), 8 (8.8%) and 16 (8.8%) were the most common ones, but several times serovar 9 (5.5%), 11 (3.3%) and 12 (3.3%) were isolated. Twelve strains (13.2%) were non-typable.

Non-typable *A. pleuropneumoniae* strains occur all over the world, and we also found some. Five *A. pleuropneumoniae* strains isolated from typical pathological lesions of porcine pleuropneumonia could not be assigned in any of the that time accepted 15 serovars. Using hyperimmune serum raised against these non-typable *A. pleuropneumoniae* strains in rabbits proved that they form a distinct group and there is no cross reaction between them and the type strains of *A. pleuropneumoniae*. All five strains harboured the genes for production (*apxIA*) and for secretion (*apxIB*) of ApxI, the gene for expression of ApxII and the largest size (2800 bp) of *apxIV* gene. The carbon source utilisation pattern and the sequence analysis of the 16S rRNA gene confirmed the species identification of them. A new serovar of *A. pleuropneumoniae* – serovar 16 – was proposed with *A. pleuropneumoniae* A-85/14 as reference strain. Whole-genome sequencing of the proposed reference strain was carried out and the presence of a unique capsular polysaccharide biosynthetic locus was confirmed. For molecular diagnostics, primers were designed from the capsule locus of *A. pleuropneumoniae* A-85/14, and a PCR was formulated that differentiated serovar 16 isolates from all that time known serovars and other common respiratory pathogenic and commensal bacteria of pigs. Analysis of the capsule locus of strain A-85/14 combined with the serological data confirmed the existence of a new serovar – serovar 16 – of *A. pleuropneumoniae.*

In the framework of international collaboration 9 *A. pleuropneumoniae* strains were received to serotyping. All strains proved to be non-typable in the indirect haemagglutination test when hyperimmune sera produced against the that time known 16 serovar specific type strains of *A. pleuropneumoniae* were used. Hyperimmune sera were raised against these non-typable strains in rabbits and these immune sera reacted well with the homologous antigens. Cross agglutination tests confirmed that these non-typable strains formed two types. Seven strains belonged to the same serovar and two strains to another one. The seves strains gave cross reaction of a low titre with serovar 8 of *A. pleuropneumoniae* in addition to their reaction with the homologous hyperimmune sera. The other type including two formerly non-typable strains gave only homologous reaction. On the basis of the results of the indirect haemagglutination test we supposed that these types represent two additional serovars, serovar 17 and 18. Strain 16287-1 was suggested to reference strain of serovar 17, while strain 7311555 was proposed to be the type strain of serovar 18. Whole genome sequencing to identify the capsule loci proved that serovar 17 strains have the same six-gene type I capsule locus and share common *cpsABC* genes with serovars 2, 3, 6, 7, 8, 9, 11 and 13. They share the same O-antigen genes, found between *erpA* and *rpsU*, as serovars 3, 6, 8 and 15. The shared genes common to capsule loci of serovars 8 and 17 explain the results of the serological examinations. The serovar 18 strains contain a unique three-gene type II capsule locus, having *cpsA* gene similar to that of serovars 1, 4, 12, 14 and 15. They had the same O-antigen genes as serovar 7. The *A. pleuropneumoniae* strains belonging to serovar 17 and 18 had only genes required for production of ApxII toxin. The above data confirmed the existence of serovar 17 and 18 of *A. pleuropneumoniae.*

We aimed to characterise *A. pleuropneumoniae* strains on the basis of assimilation of 95 carbon sources, and wanted to examine whether metabolic fingerprinting using the Biolog system (GN2 Microplate, MicroLog3 Version 4.20.05 software) can be used for the identification of *A. pleuropneumoniae* and can give correlation between metabolic pattern and biotypes, serovars or other characteristics of the strains. Sixty-eight *A. pleuropneumoniae* strains isolated from porcine acute pleuropneumonia cases and 6 reference strains were included in the metabolic examinations. Twentynine field strains were correctly identified by the Biolog system as *A. pleuropneumoniae*, 36 strains as *A. lignieresii*, two strains as *H. paraphrohaemolyticus* and one strain as *A. equuli* after 24 h of incubation. Among the six *A. pleuropneumoniae* reference strains the Biolog system identified one strain as *A. pleuropneumoniae*, four as *A. lignieresii* and one as *H. paraphrohaemolyticus*. There was no correlation between biotypes and serovars of *A. pleuropneumoniae* and the carbon source utilisation pattern and species identification by the Biolog system. These data show that Biolog system can be used for identification of *A. pleuropneumoniae* only if the database is extended and the diversity of the species is represented in it.

Another aim of the project was investigation of the genetic diversity of *A. pleuropneumoniae* in Hungary. *A. pleuropneumoniae* shows considerable geographic variation; differences in the dominant serovars in different regions have been reported. Outbreaks and sporadic cases are frequently caused by different serovars in a given region. In Europe, the dominant serovar is serovar 2, nevertheless, NAD-independent strains are more commonly isolated in Europe, e.g. in Hungary in Denmark or in Spain than in the US. High frequency of serovar 13 *A. pleuropneumoniae* isolates was reported earlier in Hungary. Seeking an explanation, the genetic diversity of serovar 13 compared with that of other serovars was especially focused. Connection between biotypes, serovars, antibiotic susceptibility, toxin production and genome structure was one of our aims. Pulsed field gel electrorophoresis was used to study the genetic diversity of the strains. Altogether 116 isolates from pathologic cases were biotyped and serotyped; susceptibility to beta-lactamics, tetracyclines, florfenicol, tiamulin and enrofloxacin was determined by broth microdilution. Toxin gene profiling was performed by PCR, genetic relatedness was assessed by pulsed-field gel electrophoresis.

Serovars differed markedly in their macrorestriction diversity. The dominant serovar 2 was highly diverse with nine distinguishable clusters (G1-3, H, I1-4, J), featuring a major important cluster I3 including 27 isolates (roughly half of all serovar 2 isolates) from at least 14 different herds. Serovar 13 biotype 2 was markedly less diverse, forming two potentially related subclusters with 14 and 11 isolates each from four herds (A1 and A2, respectively). All other major serovars (serovars 9 and 16) were represented by a single cluster (cluster C and D, respectively) showing high genetic relatedness. All major genetic clusters had a relatively long history of presence in Hungary, first isolates of clusters A1, A2, C, D and I3 dated to 1995, 1998, 2002, 2002 and 1998, respectively. All these major clusters has been shown to be present in the same herd for years.

Resistance against beta-lactam antibiotics was frequently found in cluster A2; 4/11 isolates representing two herds were resistant against penicillin G and amoxicillin, while 2/11 were resistant against cefoperazone. Isolates of cluster A2 was also frequently resistant against oxytetracycline (6/11 representing two herds) and against doxycycline (5/11), all doxycycline resistant isolates harboured the *tet*L gene. The single oxytetracycline resistant but doxycycline susceptible isolate exhibited a relatively low minimum inhibitory concentration of 16 mg/L, and harboured neither *tet*L nor any other tested tetracycline resistance gene. Resistance against tetracyclines was also frequent in cluster A1, 6/14 isolates were resistant against oxytetracycline and doxycycline. Five of these isolates (from the same herd) carried a

*tet*L gene, *tet*B was present in the remaining isolate. A single sporadic serovar 13 isolate harboured a *tet*B gene.

Regarding biotype 1 isolates, a single isolate in cluster C (serovar 9) carried a *tet*H gene, in cluster D (serovar 16) one isolate harboured a *tet*B, another one a *tet*L gene. One of the isolates of cluster F carried a *tet*B gene; two isolates from different herds in cluster I1 carried *tet*L genes. Of the isolates of the large I3 cluster one showed decreased susceptibility to penicillin G (MIC=4 mg/L), one had a *tet*B and another had one a *tet*L gene. In cluster K two of three isolates showed penicillin resistance, of which one was also intermediate to amoxicillin. Other isolates were susceptible to the drugs tested; beta-lactamase genes bla_{ROB} and bla_{BRO} were not detected.

All isolates carried the species-specific *apx*IV gene, confirming species identification, but major differences were detected in toxin gene carriage patterns among biotypes, serovars and clusters. All biovar 2 isolates (serovar 13 clusters A1 and A2, serovar 14 cluster B) lacked the gene *apx*III and *apx*IA, carrying the genes *apx*II and *apx*IB, indicating ability of production of ApxII toxin only. Biovar 1 serovar 2 isolates generally carried *apx*IB, *apx*II and *apx*III (clusters G-I), indicating ability of production and secretion of ApxII and ApxIII, but not ApxI associated with high virulence. Biovar 1 serovar 2 isolates of cluster J carried only the genes *apx*II and *apx*IB, they were capable of producing and secreting only ApxII, similarly to biotype 2 isolates. Sporadic biotype 1 isolates (serovar 12) behaved similarly, i.e. carried only *apx*II and *apx*IB. In contrast, clusters C (serovar 9), D1 (serovar 16), D2 (serovar 16) and E (non-typeable isolates) carried the genes *apx*IA, *apx*IB and *apx*II, thus these isolates are likely to produce and secrete the toxin ApxI associated with high virulence together with ApxII.

Antibiotic resistance was not uniform within clusters, however, tetracycline resistance was relatively frequent in the two clusters of serovar 13; one of them was also frequently resistant against beta-lactams. Resistance in other serovars was sporadic. All isolates carried *apx*IV. Toxin profiles of serovar 2 were characterized by ApxII and ApxIII toxin production excepting a small cluster of three isolates; serovar 9 and 16 isolates exhibited ApxI and ApxII. Serovar 13 carried *apx*II and *apx*IB indicating production of ApxII but not of ApxI or ApxIII. Unusually high frequency and low diversity of serovar 13 is not explained by its virulence properties, but high frequency of resistance against beta-lactamics and tetracyclines may have played a role in its spread. Emergence of serovar 16 may be facilitated by its high virulence, also explaining its high clonality

In a part of the present project correlation between virulence, clinical form, severity of the clinical signs, post mortem lesions and characteristics of *A. pleuropneumoniae* strains was also examined. Lungs of 28-31 day old piglets showing lesions of aute haemorrhagic-necrotic pneumonia and fibrinous pleuritis were sent from a large-scale farm. *A. pleuropneumoniae* serovar 11 strains, producing ApxI, ApxII and ApxIV toxins were isolated from the lungs. This serovar was not detected in this farm earlier. The data confirmed that *A. pleuropneumoniae* can cause disease not only in grower and feeder pigs, but a newly introduced serovar can also result severe clinical signs and lesions of the disease around weaning in the absence of maternal protection.

Since *A. pleuropneumoniae* serovar 16 is a recently described serovar, it was of special importance to collect data on its pathogenicity. In a large scla farm severe porcine pleuropneumonia and its high losses caused by *A. pleuropneumoniae* 16 were diagnosed, however the animals were vaccinated with a commercial vaccine containing inactivated *A. pleuropneumoniae.* In order to reduce the losses this vaccine was replaced with a registered vaccine containing inactivated toxins including that of produced by *A. pleuropneumoniae* serovar 16 and outer membrane proteins. As a result of the vaccination with the toxoid

vaccine the acute clinical signs disappeared proving that inactivated toxin vaccines can provide protection in the case of different serovars, too.

In order to evaluate the pathogenicity of *A. pleuropneumoniae* serovar 16 an animal infection trial was carried out. A total of 30 12-week-old piglets were divided in three groups and they were infected intra nasally with 10^8, 10^7 or 10^6 colony forming units of the bacterium. The clinical signs were scored for 6 days, their body mass was measured, the post mortem and the histological lesions were evaluated. Clinical signs could be seen in all three groups already 6 hours after the infection; however they were not very serious. Severity of the clinical signs and the number of febrile days was parallel with the infective dose and significant differences in the body mass were also evident. Typical post mortem and histological lesions could be observed in all groups, while piglets left at the place of origin remained healthy. The examination confirmed that the type strain of serovar 16 *A. pleuropneumoniae* A-85/14 was able to cause typical clinical signs and post mortem lesions in 12-week-old piglets in vivo confirming the high pathogenecity of this serovar.

The present project helped us in extending our knowledge on the importance and impact of porcine pleuropneumonia, the characteristics of the *A. pleuropneumoniae* strains in Hungary, the frequency of the different serovars, the genetic diversity of the strains and furthermore three new serovars were identified. These data help the better understanding of the characteristics of the agent and the pathogenesis of the disease; they can help the development of vaccines in order to prevent the disease. Our well characterised large culture collection of *A. pleuropneumoniae*, the complete set of hyperimmune sera produced against all serovars give a solid basis to continuing this research. Whole genome sequencing of *A. pleuropneumoniae* strains could provide data to understanding the connection between genome structure, virulence of the strains and epidemiology of the disease. The present research is planned to be extended into this direction.

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