Prevalence of serotypes, production of Apx toxins, and antibiotic resistance in strains of *Actinobacillus pleuropneumoniae* isolated in the Czech Republic

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ABSTRACT: Results of serotyping of 122 strains of *Actinobacillus pleuropneumoniae* isolated on 21 swine farms in the Czech Republic from January 1999 to December 2000 are presented. The highest prevalence was found for serotype 9 (n = 81, 66.4%), followed by serotype 2 (n = 19, 15.7%). Serotypes 1, 3, 5, 7, 8, 11, and 12 were identified only sporadically. Twenty-six of the isolates were tested for gene-toxic profiles and haemolytic and cytotoxic activities. The investigations confirmed the association between serotype on the one hand and Apx genotype and Apx phenotype on the other hand. Results of tests of resistance to antibiotic by the disc diffusion method have justified the apprehension of development of resistance due to long-term treatment with antibiotics. Strains with full or intermediary resistance against all the commonly used penicillin- and tetracycline-type antibiotics were found. None of the strains was resistant to florfenicol, norfloxacin, or cefalotine.

Keywords: porcine pleuropneumonia; apx toxin; PCR; pneumonia; antibiotics; pig; Czech Republic

In the last 20 years, porcine pleuropneumonia has been recognised world-wide as one of diseases causing the most serious economic losses in the pig husbandry. The causative agent *Actinobacillus pleuropneumoniae* induces a highly contagious disease characterised by acute or chronic fibrinohaemorrhagic necrotising pneumonia (Sebunya and Saunders, 1983). Two biotypes of *A. pleuropneumoniae* are recognised. Biotype 1 is NAD dependent and biotype 2 is NAD independent. So far, 14 serotypes have been recognised, 12 within biotype 1 and 2 within biotype 2 (Nicolet, 1992). Although the virulence of *A. pleuropneumoniae* is multifactorial, the major factor in the induction and development of the disease is the production of exotoxins. *A. pleuropneumoniae* produces three different exotoxins that belong the RTX family poreforming toxins (Frey *et al.*, 1993). RTX toxins are widely distributed among Gram-negative bacteria in which they are now recognised as being relevant virulence factors. Members of this group share structural and functional properties, including the presence of glycinerich repeats, a particular mode of secretion with a signal sequence at the C-terminus, post-translational activation, and cell toxicity via poreforming mechanism. RTX toxins are encoded by operons that consist of four contiguous genes arranged in the order, *C*, *A*, *B*, and *D*. The *C* gene encodes a product that directs the cytoplasmic conversion by an acylation reaction of the structural toxin encoded by the *A* gene to the active form, exported by the products of the *B* and *D* transporter genes (Welch, 1991). RTX toxins in *A. pleuropneumoniae* are called Apx toxins (for *A. pleuropneumoniae* RTX toxins): two of these toxins, ApxI and ApxII, are haemolytic and cytotoxic, whereas ApxIII possesses cytotoxic but not haemolytic activity. This finding encouraged the development of a simple and rapid molecular biological method allowing the classification by PCR of *pleuropneumoniae* in terms of Apx toxin production into the following five groups (Frey *et al.*, 1993, 1995).

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Group 1 – strongly pathogenic and epizootologically most relevant serotypes 1, 5, 9, and 11 producing the ApxI and ApxII toxins
Group 2 – less pathogenic, but epizootologically relevant serotypes 2, 4, 6, and 8 producing the ApxII and ApxIII toxins
Group 3 – the least pathogenic serotype 3 producing only the extracellular ApxIII toxin
Group 4 – serotypes 7 and 12 producing only the ApxII toxin
Group 5 – serotype 10 producing only the ApxI toxin

The first massive outbreaks of porcine pleuropneumonia were diagnosed in the Czech Republic towards the end of 1982 and increasing incidence was observed already in 1983 at a time of establishment of farms specialising in piglet production and pig finishing. This change in pig husbandry probably contributed to the spreading of the disease in the Czech Republic. Serological surveys carried out in 1989 demonstrated a high prevalence of antibodies to *A. pleuropneumoniae* (especially against serotypes 9 and 2), even in swine herds that were free of clinical disease. Another increase in the incidence of porcine pleuropneumonia, observed in the early nineties, was probably due to liberalisation of trade, increase in among-herd transfers of animals and purchase of breeding animals from abroad. All these factors contributed to a widening of the array of serotypes identified in local herds and to an increase in the number of herds affected by clinically manifested porcine pleuropneumonia. To cope with the emerging danger, extensive immunoprophylactic measures were implemented including using of antibiotic for treatment and prophylaxis of the bacterial respiratory syndrome. This situation raises the issue of development of antibiotic resistance in bacterial strains.

MATERIAL AND METHODS

Strains

The material under study included 122 strains of *A. pleuropneumoniae* isolated from dying animals showing clinical manifestations or post-mortem lesions typical of porcine pleuropneumonia and from lesions detected on post-mortem meat inspection. The tested isolates originated from 21 farms with open or closed herd turnover located in various regions of the Czech Republic. The strains were grown on blood agar plates using a strip of a *St. aureus* culture as the source of NAD. Twenty six strains were selected for the detection of haemolytic and cytotoxic activity and subsequent subtyping using gene specific PCRs.

Coagglutination test

For the preparation of antisera against capsular antigens, reference strains of *A. pleuropneumoniae* representing all twelve serotypes were used. The following strains were used to prepare immune rabbit blood sera: ATCC 27088 as serotype 1; ATCC 27089 as serotype 2; ATCC 27090 as serotype 3; NCTC 11384 as serotype 4; NCTC 11383 as serotype 5; ATCC 33590 as serotype 6; CAPM 3800 as serotype 7; CAPM 3803 as serotype 8; CAMP 3888 as serotype 9; CAMP 6279 as serotype 10; CAMP 6325 as serotype 11; CAMP 8329 as serotype 12. Antigens for immunization of rabbits were prepared from 18 hour-old mucoid growth on *Haemophilus* test medium base (HTM) with HTM supplement (OXOID) enriched with Vitox (OXOID) as recommended by the manufacturer. The growth from each plate was harvested gently in 3 ml physiologic saline solution containing 0.3% formalin and kept at room temperature for 2 days. This antigen is referred to as formalinized whole-cell suspension. A cell suspension of an optical density (OD) of 1 at 550 nm was used for the immunization of rabbits. Two young adult rabbits were injected *i.v.* 2 times a week with increasing doses of formalinized whole-cell suspension of each serotype. The doses were 0.5, 1, 2, and 3 ml followed by 2 *i.v.* injections of 3 ml each of 6-hour-old live culture at weekly intervals. Blood samples were obtained from the rabbits 7 days after the last injection was given. Preimmunization sera of rabbits were tested for their antibody contents to different type strains of *A. pleuropneumoniae*. Only rabbits that were negative for antibodies were used. The details of the preparation of coagglutination reagents and the procedure of the coagglutination test have been described by Mittal *et al.* (1983).

Haemolytic activity test

The haemolytic activities were tested by modification Haemolytic activity test (Tarigan *et al.*, 1996). The *A. pleuropneumoniae* isolates were inoculated onto chocolate blood agar (CBA) and incubated 18 h at 37°C. Colonies were rinsed with 5 ml of RPMI-1640 (SIGMA) supplemented with 10 mM calcium chloride per plate. The resulting suspensions were incubated at 37°C for 2 h and centrifuged at 10 000 × g for 10 min

and supernatants were kept for haemolytic activity tests. The activity was tested in serial geometrical dilutions in a final volume of 1 ml by adding 1 ml of 1% suspension of sheep erythrocytes in physiological saline using RPMI-1640 as the negative control and addition of 0.5% of TRITON X-100 (SIGMA) as the positive 100% haemolysis control. Unlysed erythrocytes were separated by centrifugation at 700 × g for 5 min after 2 hours of incubation at 37°C. Some of the supernatant (125 µl) was transferred to a 96-well, flat-bottomed microplate, and the optical density (A_{540nm}) of the supernatant was measured in a microplate reader. The degree of haemolysis (% haemolysis) was calculated as follows:

$$100 \times (A_{540nm} \text{ sample} - A_{540nm} \text{ RPMI-1640}) / (A_{540nm} \text{ Triton X-100} - A_{540nm} \text{ RPMI-1640})$$

A_{540nm} RPMI-1640 and A_{540nm} Triton-100 represent the absorbance of 0% and 100% lysis, respectively. One haemolytic unit (HU) was defined as the lowest concentration of the Apx preparation causing 50% haemolysis, determined by plotting the sample % haemolysis against dilution on semilog graph paper.

Cytotoxicity test

Lung macrophages were collected from piglets aged 8 to 10 weeks (Leengoed *et al.*, 1989) reared conventionally in the premises of the Institute. Tested strains were inoculated into a liquid medium (BHI) supplemented with NAD at 10 µg/ml and incubated overnight at 37°C. Supernatant was separated by centrifugation and used in the test in geometrical dilution series (PBS) in a final volume of 50 µl. Each well was completed with 50 µl of a suspension of alveolar macrophages containing 5×10^6 cells per 1 ml. The mixture was incubated at 37°C for 5 heurs. Thereafter the medium was removed and killed macrophages were stained by addition of 50 µl of 0.2% nigrosin into each well. Nigrosin was removed after 5 min at room temperature and remaining nigrosin droplets were sucked up with blotting paper. Then 50 µl of PBS was pipetted into each well and stained macrophages were enumerated in an inverse microscope. The cytotoxicity titre was defined as the highest dilution showing less than 50% of stained macrophages.

DNA purification

A. pleuropneumoniae strains were first grown in BHI supplemented with NAD at 10 µg/ml at 37°C 18 h. Then 200 µl of the BHI grown culture was inoculated onto CBA and incubated at 37°C 18 hours. The culture was rinsed with 2 ml of PBS and 100 µl of the resulting suspension was used for DNA purification using QIAamp Tissue Kit (QIAGEN) according to the instructions of the manufacturer. The resulting DNA was used in PCR.

PCR

PCR were run in 20 µl of reaction mixture containing 2 µl of tenfold concentrated reaction buffer, 200 µM of dNTPs, 10 pmol of each primer, 0.5 U of *Taq* Polymerase (PROMEGA), and 2 µl of sample DNA purified with the QIAamp Tissue Kit (QIAGEN). The volume was completed to 20 µl with deionised (MILIPORE) water. Positive and negative controls were run with each PCR. DNA of reference strains

Table 1. Primers used for amplification of Apx genes of *A. pleuropneumoniae* (Frey *et al.*, 1995)

Target genes	Sequence (sense, antisense)	Primer location	Amplified product size (bp)
ApxICA	5'-TTGCCTCGCTAGTTGCGGAT-3' 5'-TCCCAAGTTCGAATGGGCTT-3'	440–459 2 860–2 841	2 420
ApxIICA	5'-CCATACGATATTGGAAGGGCAAAT-3' 5'-TCCCCGCCATCAATAACGGT-3'	599–621 2 687–2 658	2 088
ApxIIICA	5'-CCTGGTTCTACAGAAGCGAAAATC -3' 5'-TTTCGCCCTTAGTTGGATCGA-3'	595–619 2 351–2 331	1 755
ApxIBD	5'-GTATCGGCGGGATTCCGT-3' 5'-ATCCGCATCGGCTCCCAA-3'	4 986–5 003 6 433–6 416	1 447
ApxIIIBD	5'-TCCAAGCATGTCTATGGAACG-3' 5'-AACAGAATCAAAATCAGCTTGGTT-3'	5 655–5 675 6 623– 6 600	968

serotypes 2 (ATCC 27089) and 9 (CAMP 3888) were used as positive control and negative control was deionised (MILIPORE) water. Five PCRs were run with each sample. The initial 3-min step at 95°C was followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min and reaction was completed by the final step at 72°C for 3 minutes. The amplification product was detected by electrophoresis in agarose gel containing 0.5 μg of ethidium bromide per 1 ml and visualised in UV light in a transilluminator. Primers used in this study are presented in Table 1.

Antibiotic resistance tests

The resistance was tested by the disc diffusion method in petri dishes containing the *Haemophilus* test medium base (HTM) with HTM supplement (OXOID) enriched with Vitox (OXOID) as recommended by the manufacturer. The strains to be tested were first incubated at 37°C overnight and then the cultures were resuspended in PBS. The density of the suspension was adjusted to 0.5 McFarland standard (1 to 4 × 10^8 CFU/ml). The plates were inoculated by spilling the suspension over the surface. Inhibition zones of the individual drugs were read after 24 h of incubation at 37°C and strains were classified as sensitive, intermediary and resistant according to international standards (NCCLS, 1997).

The following antibiotic discs were used:

amoxicillin 25 μg; amoxicillin/clav. acid 30 μg; cephalotin 30 μg; norfloxacin 10 μg; oxytetracycline 30 μg; doxycycline 30 IU; erythromycine 15 μg, sulphonamides 300 mg; streptomycin 10 μg (Sanofi Diagnostics Pasteur), Cotrimoxazol 25 μg; tetracycline 30 μg; nalidixic acid 30 mg (Mast Diagnostics), ciprofloxacin (Lachema) 5 μg; florfenicol (Becton Dickinson) 30 μg.

RESULTS

All the isolated strains were classified as biotype 1 with growth dependence on β-NAD. Classification by the coagglutination test demonstrated the highest prevalence of serotype 9 (Figure 1) identified in 81 (66.4%) isolates, followed by serotype 2 (19 isolates; 15.7%). The serotypes 12 (9 isolates; 7.4%) and 11 (5 isolates; 4.1%) were identified less frequently. The serotypes were isolated from animals reared on large, or small farms. Further serotypes were identified rather sporadically.

Tests done with 26 selected field strains of *A. pleuropneumoniae* have confirmed relationships between serotype on the one hand, and haemolytic and cytotoxic activities on the other hand (Table 2). Both cytotoxic and haemolytic activities were demonstrated in the strains of serotypes 9 and 11 and cytotoxic activ-

Table 2. Genetic profiles of and toxin production

Serotype	Total strains	Haemolysis*	Cytotoxicity**	Results of PCR for Apx genes				
				ApxICA	ApxIICA	ApxIIICA	ApxIBD	ApxIIIBD
2	6	$X_{0.5}$ = 0 σ = 0.51	32 (83.3%) 16 (16.7%)	–	+	+	+	+
7	1	0	16	–	+	–	+	–
9	15	$X_{0.5}$ = 24 σ = 1.786	32 (73.3%) 16 (26.7%)	+	+	–	+	–
11	2	$X_{0.5}$ = 25 σ = 1	32	+	+	–	+	–
12	2	0	16	–	+	–	+	–

+ = positive PCR
– = negative PCR
* = haemolysis titre (highest dilution inducing haemolysis of 50% of erytrocytes)
** = cytotoxicity titre (highest dilution yielding less than 50% of stained macrophages)
$X_{0.5}$ = median of haemolysis titre
σ = standard deviation

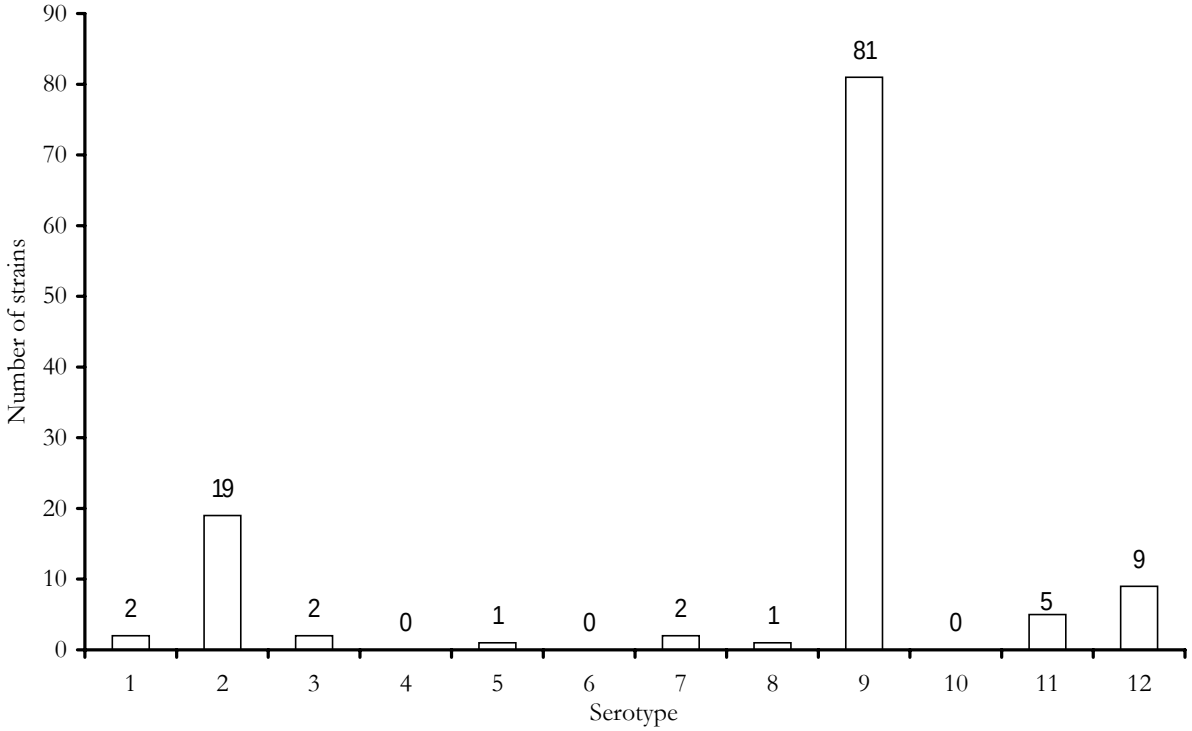

Figure 1. Serotypes of strains of *A. pleuropneumoniae* isolated in the Czech Republic from Jan. 1999 to Dec. 2000

ity alone in the strains of serotypes 2, 7, and 12. No significant within-serotype differences in toxin production were found.

The tests confirmed resistance to antibiotics in field strains of *A. pleuropneumoniae*. Strains with full or intermediary resistance against all the commonly used penicillin- and tetracycline-type antibiotics were found. No strain was resistant to florfenicol, norfloxacin, or cephalotine. Numbers and percentages of resistant strains are given in Table 3.

Resistance to erythromycine, streptomycin, or sulphonamides was demonstrated most frequently. No relationship between resistance and serotype was observed. Most of the strains were resistant to a specific

Table 3. Assessment of field strains of drug resistance

	Full resistance		Intermediary resistance		Sensitivity		Total strains
Erythromycine	10	(22.7%)	32	(72.7%)	2	(4.6%)	44
Streptomycin	13	(33.3%)	14	(35.9%)	12	(30.8%)	39
Sulfonamides	19	(40.4%)	3	(6.4%)	25	(53.2%)	47
Amoxicillin	11	(9.8%)	15	(13.4%)	86	(76.8%)	112
Oxytetracycline	8	(10.8%)	4	(5.4%)	62	(83.8%)	74
Tetracycline	11	(9.0%)	6	(4.9%)	105	(86.1%)	122
Doxycycline	3	(3.1%)	10	(10.2%)	85	(86.7%)	98
Ciprofloxacin	1	(1.2%)	10	(11.6%)	75	(87.2%)	86
Amox./clav.acid	1	(1.7%)	5	(8.5%)	53	(89.8%)	59
Cotrimoxazol	2	(1.7%)	4	(3.4%)	113	(94.9%)	119
Nalidixic acid	1	(2.4%)	1	(2.4%)	39	(95.2%)	41
Norfloxacin	0		3	(4.2%)	69	(95.8%)	72
Cephalotine	0		0		43	(100%)	43
Florfenicol	0		0		28	(100%)	28

group of antibiotics that had been used in the herd for a long period. However, three of our isolates were resistant against antibiotics of the penicillin and tetracycline types and also against erythromycine, streptomycin and sulphonamides.

DISCUSSION

The prevalence of different serotypes varies in different continents. Serotype 2 is predominant in Europe. Ninety per cent of the strains isolated in Denmark (Nielsen, 1984) and Germany (Schimmel and Hass, 1983) were serotype 2. The predominant serotypes in The Netherlands were 9 and 2 (Kamp *et al.*, 1987). Isolates of *A. pleuropneumoniae* belonging to serotypes 2, 3, 6, 7 and 8 were reported in England (McDowel and Ball, 1994). Serotypes 1 and 5 are most frequently identified in North America (Fales *et al.*, 1989; Mittal *et al.*, 1992) and serotypes 2, 5, and 6 in Korea (Min and Chae, 1999). In the present study, serotypes 9 and 2 were predominant and serotypes 1, 3, 5, 7, 8, 11 and 12 were present only in small numbers.

Interestingly, the occurrence of the individual serotypes is associated with geographical position of the finding. Serotype 1 ranking with the most frequently identified in North America is found in European countries only exceptionally. On the other hand, serotype 9 occurring very frequently in Europe has never been isolated in North America (Gottschalk, 1999). Similarly, the second most frequently identified serotype 2 is often isolated in Europe, but only sporadically in North America. These differences can be explained by results of toxigenicity tests. Unlike European serotype 2 strains which produce ApxII and ApxIII toxins, Canadian serotype 2 strains were found to produce only ApxII toxin. This characteristic is associated with a weaker virulence and hence was responsible for rare isolation of this serotype from clinical cases of porcine pleuropneumonia (Gottschalk, 1999).

PCR demonstrated that genetic profiles for the production of Apx toxin of all the tested 26 strains were identical with that of reference strains (Frey *et al.*, 1993). Then, all our isolates shared apx genotype and Apx phenotype with the reference strains. It has also been confirmed that there exist among-serotype, but not within-serotype, differences in toxigenicity (Beck *et al.*, 1994; Kamp *et al.*, 1994). Apx toxins rank with the major virulence factors of *A. pleuropneumoniae* (Inzana, 1991) and ApxI toxin-producing strains were isolated from outbreaks of acute porcine pleuropneumonia characterised by high death rate (Fales *et al.*, 1989). This finding can explain the predominance of serotype 9 among our strains most of which were isolated from acute and often lethal cases of porcine pleuropneumonia. Diseases caused by the less pathogenic serotypes 2, 3, 7, and 12 had mostly the character of chronic infections with a low or zero death rates. Most of these strains were isolated from lung samples collected from animals dying of another disease, or from slaughtered pigs.

The occurrence of antibiotic resistant strains, resulting often from inconsiderate drug use, poses a serious hazard for the development of the epizootological situation and the current state in the Czech Republic does not differ from that in other countries. Drug resistance of *A. pleuropneumoniae* is a world-wide problem which veterinary practitioners face when deciding on the treatment of acute porcine pleuropneumonia and/or preparing control programmes for large swine herds. The prevalence of drug resistant strains depends on geographical position, time of isolation, and up to now drug use. Nevertheless, more or less resistant strains occur world-wide (Wasteson *et al.*, 1996).

It can be concluded that, like in the neighbouring countries, the strongly pathogenic serotype 9 and the less pathogenic serotype 2 are the most frequently identified causative agents of porcine pleuropneumonia in the Czech Republic. Sporadic findings of other serotypes of *A. pleuropneumoniae*, apparently introduced by imported animals, emphasise the necessity of exact correct diagnostics including serotype classification apx gene profile determination for correct assessment of the epizootological situation which along with the knowledge of antibiograms of recently isolated strains, provide the basis for decisions on therapeutic and preventive measures.

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