ANTI-*ACTINOBACILLUS PLEUROPNEUMONIAE* HEMOLYSINS ANTIBODIES DETECTION BY ELISA IN PIG SERA

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Summary

An ELISA Apx test was proposed as a diagnostic test for infectious pleuropneumonia produced by *Actinobacillus pleuropneumoniae* in pigs. The antigen extracted from bacteria was characterised using serum from a vaccinated rabbit. Optimal antigen concentration of 1 μg/ml was found and used for evaluating two pig sera with known diagnostic. Relative good sensibility was observed but specificity not tested.

Key words: *Actinobacillus pleuropneumoniae*, Apx, ELISA.

Of the virulent factors of *Actinobacillus pleuropneumoniae (A.pp.)*, respiratory pathogen bacteria for pigs , Apx hemolysins have been proven to be of particular importance for the induction of protective immunity. Evaluating such an immunity was considered important for diagnosis of infectious pleuropneumonia in pigs and the recently most used method for this is ELISA in different variants.

For creating an ELISA test for evaluation pig serum antibodies against Apx it was necesary a coating antigen. This Apx antigen was extracted from culture supernatant and was characterized by a rabbit serum. The rabbit serum was obtained after vaccination with all three Apx hemolysins.

The prepared antigen was a mixture of two hemolysins and antibodies reactivity was considered cross-overed because of the structural and genetic relatedness of the all three Apx hemolysins.

Materials and methods

Two rabbits were vaccinated against pleuropneumonia using a recombinant vaccine, Actinoporc / Porcilis APP (Intervet Romania). In this vaccine are present three types of detoxified hemolysins (ApxI, ApxII, ApxIII) and an outer membrane 42 kDa protein (OMP). It was created to induce protective immunity against infection with any *A.pp*. serotype.

Before and after vaccination rabbits sera were tested by indirect ELISA for the presence of antibodies against Apx using some of the components of kit APP (VS035) produced by Cypress Diagnostics. These kit components were used as manufacturer mentioned: coated strips, washing solution, sample diluent solution, substrate solution and stop solution. Also, the serum dilution was 1/200 as indicated. Only the anti-rabbit peroxidase conjugate was from Sigma and was used at 1/20000 dilution and strips were incubated for 1 hour at 37°C. The repeated

administrations of the vaccine were at two weeks intervals, concomitantly with the bleedings.

An Apx antigen was obtained with a simplifier method insspired by **Devenish (1990a**). Briefly, after obtaining an 18 h culture on agar chocolate the bacterial lawn was removed by washing with RPMI 1640 medium. Following incubation at $37^{O}C$ for 90 minutes, the culture was centrifuged and the supernatant was precipitated by addition of ammonium sulfate to 80% saturation. The precipitate was redissolved in phosphate-buffered saline, pH 7.4 corresponding to approximately 1/30 the original volume of RPMI 1640 culture and dialysed against the same buffer.

Two antigens were prepared. The first Apx antigen was prepared from *A.pp*. reference serotype 5A (strain K17) which secrete ApxI and ApxII. The other antigen was obtained from *A.pp*. reference serotype 2 (strain S1536) which produce ApxII and ApxIII.

The obtained Apx antigens were tested by ELISA in a checkerboard titration using Actinoporc vaccinated rabbit serum. Also, the antigens were tested for hemolytic activity (**Devenish, 1990 a)** defined as the dilution of hemolysin which caused 50% hemolysis of a 1% washed rabbit erythrocyte suspension (units UH_{50}/ml).

Also, the antigens were used in testing two pig sera already diagnosed by commercial kit APP (VS035) from Cypress Diagnostics.

Results and discussions

Immunising rabbits with a vaccine for pigs was not very successful. Rabbit 1 was considered adequately immunised (with serum optical density O.D.x1000 = 737) and further used for Apx antigens characterisations. In Table 1 are presented the O.D.x1000 values by testing with kit VS035 at each bleeding of the two rabbits.

Table 1

ELISA results (O.D.x1000) for Actinoporc vaccinated rabbits

	bleeding I* (initially)	bleeding II	bleeding III	bleeding IV	bleeding V
rabbit 1	273	649	737 (finally)	-	-
rabbit 2	252	349	457	383	480 (finally)

- - before vaccination

The Apx antigen from *A. pp*. 5A had a total proteic concentration of 0.9 mg/ml and hemolytic activity of 16 UH50/ml, and the antigen from *A. pp*. 2 had a concentration of 0.8 mg/ml and 2 UH50/ml.

After checkerboard titration of the antigens using rabbit's sera (positive from vaccinated rabbit 1, and negative from normal rabbit) was concluded that a concentration of 1.25-0.6 μg/ml and a 1/200 serum dilution were the proper conditions for an ELISA method with Apx antigen. In Tables 2 and 3 are presented O.D.x1000 values for the titrations and in graphics 1 and 2 are, also presented the

arithmetic diferences and rapports between positive serum O.D and negative serum O.D. The results showed the antigenic relatedness between Apx hemolysins.

Table 2

ELISA checkerboard titration (O.D.x1000) of Apx antigen from *A. pp*. serotype 5A using rabbit serum 1 (positive +) and normal rabbit serum (negative -)

proteic antigen concentration (μg/ml)	20	10	5	2.5	1.25	0.6	0.3	0.15
serum + dilution 1/100	3077	2910	2835	2276	1645	1191	1210	862
serum – dilution 1/100	2345	1938	1559	1214	1013	883	1194	922
serum + dilution 1/200	3036	2609	2236	1622	1106	781	807	542
serum – dilution 1/200	1756	1375	1097	814	628	584	788	554
serum + dilution 1/400	2401	1967	1497	1096	758	543	591	369
serum – dilution 1/400	1186	910	729	556	407	392	576	371

Table 3

ELISA checkerboard titration (O.D.x1000) of Apx antigen from *A. pp*. serotype 2 using rabbit serum 1 (positive +) and normal rabbit serum (negative -)

proteic antigen concentration (μg/ml)	20	10	5	2.5	1.25	0.6	0.3	0.15
serum + dilution 1/100	2931	3015	3069	2971	2608	2071	1428	892
serum – dilution 1/100	2715	2482	2245	1734	1179	925	772	697
serum + dilution 1/200	3202	2960	3069	2846	2261	1573	1022	576
serum – dilution 1/200	2229	2023	1698	1251	804	603	527	418
serum + dilution 1/400	2998	2866	2779	2343	1606	1079	689	354
serum – dilution 1/400	1763	1575	1353	961	625	431	378	322

Two pig sera had been diagnosed by kit VS035: one was positive and the other one negative. These sera were also, tested with the prepared Apx antigens from *A.pp*. serotype 5A and 2. The comparing results from the both ELISA methods are presented in **Table 4**. The conditions for ELISA Apx proposed by this paper were: coating antigen 1 μg/ml, serum dilution 1/100 for 1 hour at 37°C, anti-pig peroxidase conjugate 1/20000 for 1 hour and 37°C, ABTS chromogen incubation for 1hour. The serum dilution was established as the same as in commercial kit for an adequate comparison. It can be observed in both ELISA that the negative serum had a high value for negative diagnostic. The positive serum, expressed like positive 1 by commercial kit had a low O.D. value in our ELISA test. So, the obtained antigens are not able to detect specific antibodies with great sensibility. But probably, more intense positive sera could be detected by the method presented. The accuracy of such type of ELISA in pig infectious pleuropneumonia would be best when used with acute- and convalescent- phase sera (Ma, 1990). And that because after infection with a virulent serotype antibodies against ApxI are present in pig serum. After several studies, Nielsen (2000) confirm the other authors findings that convalescent pigs produce strong humoral response to Apx and also, that all hemolysins are structurally and antigenically related. He suggested that ApxII might be the antigen of choice in diagnosing *A.pp*. infection by ELISA.

Table 4

Comparative ELISA results (O.D.x1000) for two pig sera

	positive pig serum	negative pig serum
kit VS035 (Cypress Diagnostics)		
diagnostic	positive 1	negative
IPRC=relative index for interpretation of kit results	58.9	18.5
O.D.x1000	930	384
proposed ELISA method in this paper		
Apx antigen from *A.pp*. 5A (O.D.x1000)	622	317
Apx antigen from *A.pp*. 2 (O.D.x1000)	475	184

ELISA Apx method was studied by many authors. **Devenish** (**1990a**) tested pig sera after vaccination by ELISA and by neutralizing technique using a purified 104-kilodalton hemolysin from *A. pp*. serotype 1. The author suggested that a titer of at least 1:10,000 would protect against disease, in which titer was expressed as the reciprocal highest serum dilution with O.D. similar with that of a negative serum. It must be mentioned that serodiagnosis of pleuropneumonia by Apx ELISA could have very high sensitivity but poor specificity (**Devenish, 1990b**), probably as a result of the presence of *Actinobacillus suis* and possibly alpha-hemolysin-producing *Escherichia coli*, in many swine herds.

Conclusions

An ELISA Apx test was proposed using an antigen extracted from bacteria. The test had relative good sensibility which can be improved by further purification of the antigen and could be used as an initial screening test to be followed by a test with high specificity to confirm all positive results.

References

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