INTRADERMAL VACCINATION AND EXPERIMENTAL INFECTION OF ACTINOBACILLUS PLEUROPNEUMONIAE IN PIGS

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Abstract

Level of immunity introduced by single or multiple intradermal administrations of various concentrations of antigen in vaccination dose was studied in the paper. Intradermally administered vaccine against *Actinobacillus pleuropneumoniae* developed comparable immunity to vaccines administered intramuscularly. In spite of intramuscularly induced immunity, there is little known about immunity developed after intradermal administration of different concentrations of antigen in vaccination dose. Intradermal commercial vaccine, containing lipopolysacharid, outer membrane protein and toxins was used, diluted and/or concentrated. The challenge infection was used three weeks after revaccination and protection developed after administration of different concentrations was evaluated and compared to intramuscular administration. Intradermal route was found as comparable to intramuscular administration in protection of lungs against infection, three-time diluted dose was found unless equally effective as standard i.d. or i.m. dose, comparable to 4 times concentrated and 9 times diluted i.d. vaccine.

Introduction

Intradermal administration of vaccines is exciting, and advantageous route of administering antigens.
The animal and human beings have evolved skin systems for protection, using and storing information from environmental microbial threats and developed recognising and defence strategies. Strategies, based on deactivation and presenting of antigen are most frequent in the skin. The skin have been natural place for recognising environmental antigens[1][2][3].
Reasonable assumption of immune response after intradermal (i.d.) administration, lays in physiology of the skin[4]. The skin contains protective systems, as dendritic cells, in compare to muscles or subcutaneous tissue in multiple number [5], responsible for presenting of antigen and triggering immune reactions.
All mechanisms of immunity developed after either i.d. or i.m. administration of vaccines, is still not fully researched; important role plays momentary state of immune system, number of lymphocyte before vaccination[6], in dependence on age [7] and other aspects.
Intradermal administration of vaccines boosted not only specific antibody response, but induced cell mediated immunity as well [8], {Steinman R.M., 2002 121 /id;Faldyna, 2005 3 /id}. Protection after i.d. administration developed also mucosal immunity, as researched Nachvátalová [11].
This is a reason for development of new vaccines for i.d. administration, but there is little known about dosage of antigen in the vaccines.
One of the first products, developed for i.d. administration in pigs, was vaccine against porcine pleuropneumonia. Porcine pleuropneumonia is a contagious febrile disease of the respiratory tract of pigs, worldwide considered as a disease causing great direct and indirect economic losses within swine herds, mostly in the recent 20-25 years [12;13].
The aetiological agent of the infection, *Actinobacillus pleuropneumoniae* (APP), causes severe and fatal fibrinous hemorrhagic necrotizing pneumonia in pigs [13]. There are 14 different pathogenic strains of APP. The pathogenic impact depends on virulence of the strains (Nechvatalova [11]) .
There are many vaccines researched against the disease, the composition vary from stable vaccines, containing whole killed homologous bacteria, to subunit vaccines, containing purified inactivated exotoxins (APX), lipopolysacharides (LPS) and outer membrane protein (OMP) .
The already registered subunit vaccine Suivac APP (Dyntec, Czech Republic) against *Actinobacillus pleuropneumoniae* was used in the experiment. APP was used as a model of airborne infection and challenge infection was used to validate different protection developed after i.d. administration. The aims of the paper was compare i.d. and i.m. route of administration and declare dependence of intensity of immune response and protective efficacy on different concentrations of antigen in vaccination dose, administered intradermally.

Material and Methods

Animals and Experimental Design

The animal care protocol for the experiment followed the Czech guidelines for animal experimentation and was approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic. The piglets were purchased from *Actinobacillus pleuropneumoniae* (APP) seronegative farm, without APP history. Forty two conventional piglets Large White breed., 5 weeks old, average body weight of 10,25 kg (s=1,74), were randomly divided into 7 groups in the trial (n=6). Groups differed in dose and way of administration.-intradermal (ID) and intramuscular (IM) administrations were compared (Table 1).

Piglets were vaccinated and booster vaccinated under experimental protocol one week after housing, in day 0 and 21 respectively (age of 6 and 9 weeks).
Piglets were exposed to an experimental infection of the pathogenic strain of APP (challenge) 42 days post vaccination (PV). Experiment was finished one week later, 49 days PV; euthanized pigs were submitted to gross pathology and lungs were evaluated by scoring system. Blood samples for serologic analysis were taken weekly during the whole experiment.

Antigens and Injection Technique Used for Immunisation

Vaccine

Two modifications of commercial vaccine were used in the trial, Suivac APP ID and Suivac IM (Dyntec s.r.o., Czech Rep.) The vaccine contained proteins and lipopolysacharids (LPS) and equal mixture 6,0 ug of APX I,II,III inactivated toxins (1,8 ug of APX toxin I, 2,2 ug of APX toxin II and 2,0 ug of APX toxin III) in quantity corresponding to amount of bacteria at initial cultivation 10^9 CFU before inactivation at each standard vaccination dose.
Experimental vaccination doses were prepared using the same antigen and adjuvans, in different volume proportion.
Intradermal vaccine consisted of 0,2 ml; intramuscular vaccine consisted of 2 ml volume of oil adjuvanted vaccine.
Intradermal needle free injection automat Dermojet (producer AKRA DERMO JET, France), adjusted at dose of 0,2ml was used for vaccination. Retroauricular site was used for IM, the suprascapular region was used for ID administration of antigen.

Challenge Strain and Technique

Challenge Strain

Field strain of APP biotype 1, serotype 9 (APP 9) in the fourth passage (field isolate KL2-2000, VRI Brno) was used for the experimental infection. The strain has been classified as a highly pathogenic serotype with an acute course of pleuropneumonia and frequent deaths because of the production of RTX toxins in combination with APX I and APX II. The strain was used in our previously described infections [10;11;14] and belonged to the most frequently occurring serotype 9 in the Czech Republic and neighbouring countries [12].
The strain was cultured at 37°C in Brain–Heart Infusion Broth (HiMedia, India) supplemented with 10 ug/ml of nicotinamide adenine dinucleotide trihydrate (AppliChem GmbH, Germany) for 6 h. Concentration of the grown culture was measured by photometer and adjusted with sterile phosphate-buffered saline (PBS) to optic density OD_{550} = 1, which corresponded to the concentration 5×10^9CFU/ml APP.

Challenge Technique

Pigs were infected by intranasal way; total volume of each infection dose was 4 ml (2 ml sprayed inside each nostril). The final concentration of infection dose was $2,5\times10^7$CFU/ml.
Animals were euthanized by injection of embutramide, mebezonile, tetracain (T61, Intervet).

Clinical Monitoring

Health status was monitored during whole experiment and clinical signs of respiratory disorders were recorded (increased respiratory rate, dyspnoea, coughing, anorexia and lethargy). The pigs were regularly clinical observed, and temperature was measured daily after each vaccination and experimental infection for following seven days.
Pigs were weighed weekly.

Gross Pathology, Lung Scoring

Animals were submitted to gross pathology and lungs were examined. Lung lesions were scored by percentage of damaged surface by method described by Goodwin and Hannan [15;16] and modified by Christensen et al. [17]. Surface areas, showing pneumonia, were observed and measured. Total score of lung surface by per cent was 100. The surface division consists from left apical lobe 5%, right apical lobe 10%, left cardiac lobe 5%, and right cardiac lobe 10%, cranial edge of left diaphragmatic lobe 5%, and cranial edge of right diaphragmatic lobe 5%, intermediate lobe 5%, left diaphragmatic lobe 25% and right diaphragmatic lobe 30%. No another ratings or evaluating of severity damages were taken in consideration, to make the results simple and clear.
The extent of lung lesions was recorded into a lung scoring diagram sheet, the percentage of damaged surface was summarized for each tested group and statistically evaluated.

ELISA test

Serum samples were stored at -20˚C prior bulk serological analyse by ELISA.
Specific antibodies to APP LPS were determined in blood serum using indirect isotype-specific ELISA procedure. The antigen - crude LPS, extracted by the method of Darveau and Hancock (1983), in carbonate–bicarbonate buffer, pH 8,6 was pipetted into the wells (0,3 lg in 100 ul/well) of 96-well polystyrene microplates (Gama Dalečín, Czech Rep.). The plates were incubated in a refrigerator (4°C) for 18 h and subsequently washed three times with PBS, pH 7,2 containing 0,05% Tween 20. Then 100 ul of diluting solution (PBS,; pH 7,2 containing 0,05% Tween 20 and 0,5% casein hydrolysate) was pipetted into each well. The wells of the first row were completed with 50 ul of blood serum samples pre-diluted 1 : 33, and threefold dilution series were prepared transferring 50 ul of the mixture into 100 ul of diluting solution. After 60 min of incubation in a wet chamber at 37°C, the wells were washed three times, and 100 ul of goat anti-pig IgG, IgM horse-radish peroxidase conjugate (Bethyl, TX, USA) diluted 1 : 30 000; 1 : 10 000; 1 : 5000 respectively, was added into each well. After 1-h incubation at 37°C, the plates were washed four times and 100 ul of tetramethylbenzidine substrate (Test-line, Czech Republic) was added into each well. The reaction was stopped after 15-min incubation by adding 50 ul of 2M sulphuric acid and absorbance were read at 450 nm using the multichannel spectrometer iEMS Reader (Labsystems, Finland). The result of titration is an optical density red at dilutions 1 : 1000 for IgA, IgM and 1 : 3000 for IgG in serum samples.

Data Analysis

The results of serological examinations were expressed as mean values of optical densities (absorbance) obtained by ELISA test.

The values of lung score were obtained under protocol described above and were expressed in per cent of damaged surface of lungs and finally presented as average value of each group.

Significance of between-group changes, detected by ELISA, gross pathology findings, weighing of piglets and measuring temperature, was assessed with ANOVA non-parametric Mann-Whitney test; where $p<0,05$ was taken as the basic level of statistical significance. Each result of M-W test, i.e. comparisons of two independent samples, were multiplied by number of groups in the trial (x=7), to obtain statistically correct figures.

Graphs and statistical tables were performed by software Statistica v 7.1 (StatSoft,Inc.).

Results

Clinical Symptoms

Animals in experiment were regularly examined and none clinical signs of diseases were observed at any group, prior exposure to challenge infection. The value of average daily gain (ADG), important indicator of health was measured weekly, and matched to age and status.

Average Daily Gain

The general value of ADG was 0,46 kg (s= 0,13), during the whole trial, which is appropriate to age, status and conditions. There were not significant differences in weight and ADG between groups, until the time of challenge infection. Statistical differences were observed in AGD after challenge (days from 42PV to 49PV) between control group C, ID2/1, ID4/1, IM at one side and groups ID, ID1/3 at the other side, i.e. groups with severe lung inflammatory alterations (C, ID2/1, ID4/1, IM) became poorest ADG in last week and groups with little or none changes in lungs (ID, ID1/3) continued growth steadily (see Table 2).

Temperatures

Temperature was measured after each vaccination and challenge infection under trial design. There were seen none alterations of temperature at any group after vaccinations.

Challenge infection provoked all groups to clinical response in temperature increase, culminated at days 44PV and 45PV (2-3 days after challenge). Groups ID1/3,ID,ID2/1 and ID4/1 culminated shortly in range 39,9-40,1°C one day and dropped down to normal line in two days. Group ID1/9 aroused early (days 43-44PV) after challenge to 40,5°C and than decreased coincidently to other intradermally vaccinated groups. IM group reached 40,2°C in day 44PV and slowly decreased under level of 39,9°C in 3 days (day 47PV). Control group C showed early clinic response and reached temperature peak 40,8°C in two days after challenge (day 44PV) and didn't decreased under 40°C until end of experiment (day 49PV). The only significant result showed group ID1/3 in day 44PV and groups ID and ID1/3 in day 46PV, which temperatures were statistically lower in compare to control group C.

Isotype-specific humoral antibody response

IgG specific Ab against APX

Antigen, administered under protocol to groups IM, ID and ID1/3 produced highest level of specific Ab against APX (Figure 1) after first and booster vaccination (measured at day 14, 35 and 42PV, results without significant difference). Statistically significant lower level produced groups ID2/1, ID4/1 and ID1/9 in compare to groups ID,ID1/3, and IM. Control group C didn't produce any growth since day 0 until challenge (measured at day 49PV).

IgG specific Ab against LPS

Relatively low level of specific IgG Ab against LPS was measured after first vaccination of different concentrations of antigen, administered ID; until booster vaccination markedly increased level of specific IgG antibody isotope (Figure 2).

Markedly higher and significant volume of specific Ab, in compare to all groups, produced group IM, neither descent nor growth before and after booster vaccination, without registered response to challenge infection.

Other groups showed characteristic two fazed reaction after vaccination and booster vaccination, however didn't produce statistically significant diversity of specific Ab between groups ID1/3,ID,ID2/1. Both marginal groups ID1/9 and ID4/1 (most and less concentrated antigen) developed smaller volume of Ab (ID1/9) and dropped faster (ID4/1) after booster vaccination, thereby level of specific Ab at day 42PV (challenge) was significantly lower in compare to other groups. Challenge infection also provoked significant growth of Ab production in these two groups.

Results of control group statistically significant confirmed differences between vaccinated and not vaccinated groups.

IgM specific Ab against LPS

Dynamics of specific immune response, expressed by IgM antibody and figured out in Figure 3, showed rapid response – maximal level was reached in day 7 PV, regardless concentration and route of vaccination dose. Booster vaccination intensified level of specific IgM antibodies gradually in dependence of concentration of antigen in a dose; highest increase showed group ID4/1 (mostly concentrated antigen), to weak booster impact, observed in group vaccinated by highly diluted antigen ID1/9; though there were not statistically significant differences between groups.

All groups significantly had differed from control group C until day 42PV.

Gross Pathology and Lung Scoring

Gross pathology of lungs confirmed in general the presumptions, indicated by isotype-specific humoral Ab results.

Protection of lungs after challenge (49PV) reciprocally matched level of APX specific Ab, measured before challenge (42PV). Most complex protection showed groups ID, ID1/3 and ID2/1, in contrary to groups, where marginal concentrations of antigen were administered, i.e. groups ID1/9, ID4/1 produced markedly worse protection and peculiar but statistically significant was drop of immunity in IM group, expressed by extensive damages of lungs.

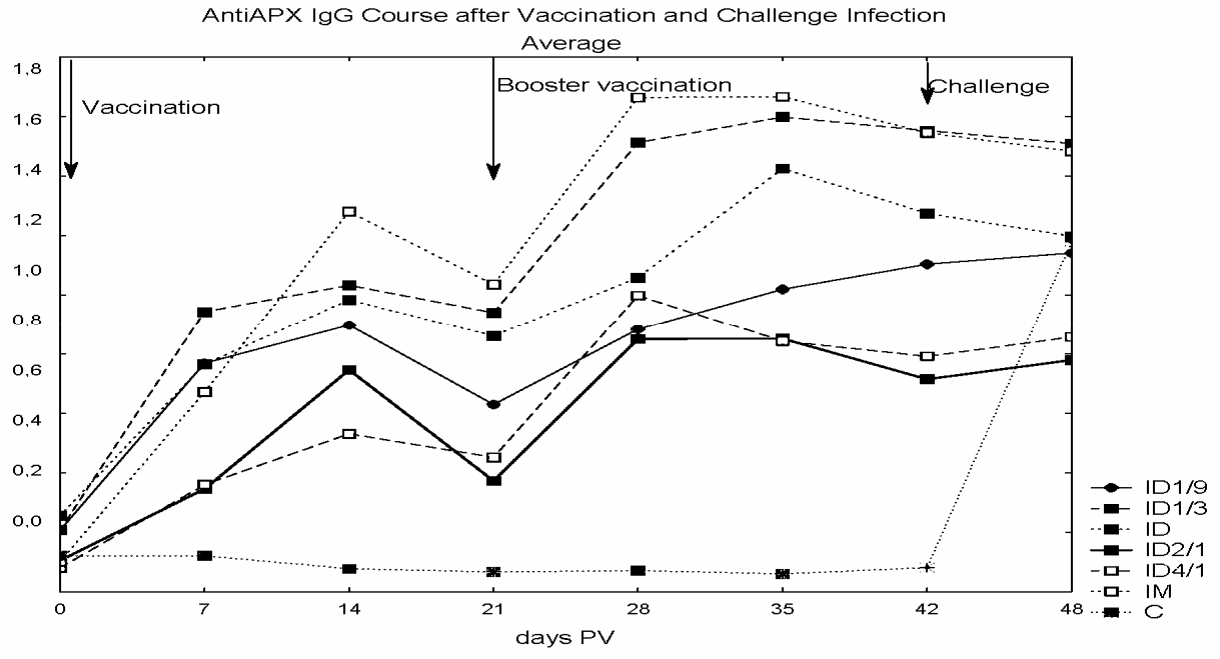
Least involved was group ID1/3 (*ID1/3: 7,5%*), significantly different to marginal groups ID1/9,ID4/1, and also IM.
Groups ID, ID2/1 shown mild inflammatory changes of parenchyma lung tissue (*ID: 15,8%; ID2/1: 20,8%*) and groups IM, ID1/9, ID4/1 shown more extent inflammatory changes (*IM: 22,5%; ID1/9: 25,0; ID4/1: 37,5%*).
Statistically significant were differences between group ID1/3 and marginal concentrations ID1/9, ID4/1 and also between ID1/3 and IM group.
There significantly similar ($p<0,05$) were results of APX specific Ab at groups ID1/9, ID2/1, ID4/1 and IM. None significant differences between groups ID1/3, ID and ID2/1 were recorded.
Group C significantly differs from all groups, and showed most extensive damages of lungs (*53,3%*).

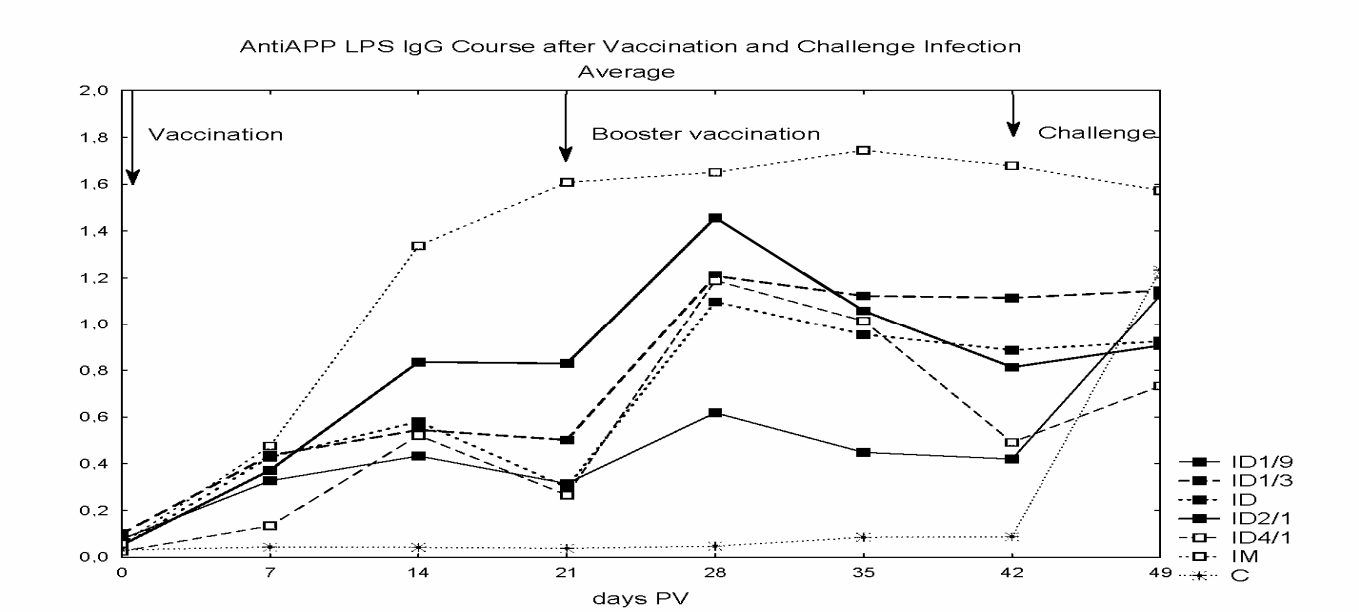
Table 1.: Groups of animals, routes of administration, dosage and vaccine volume, used in trials

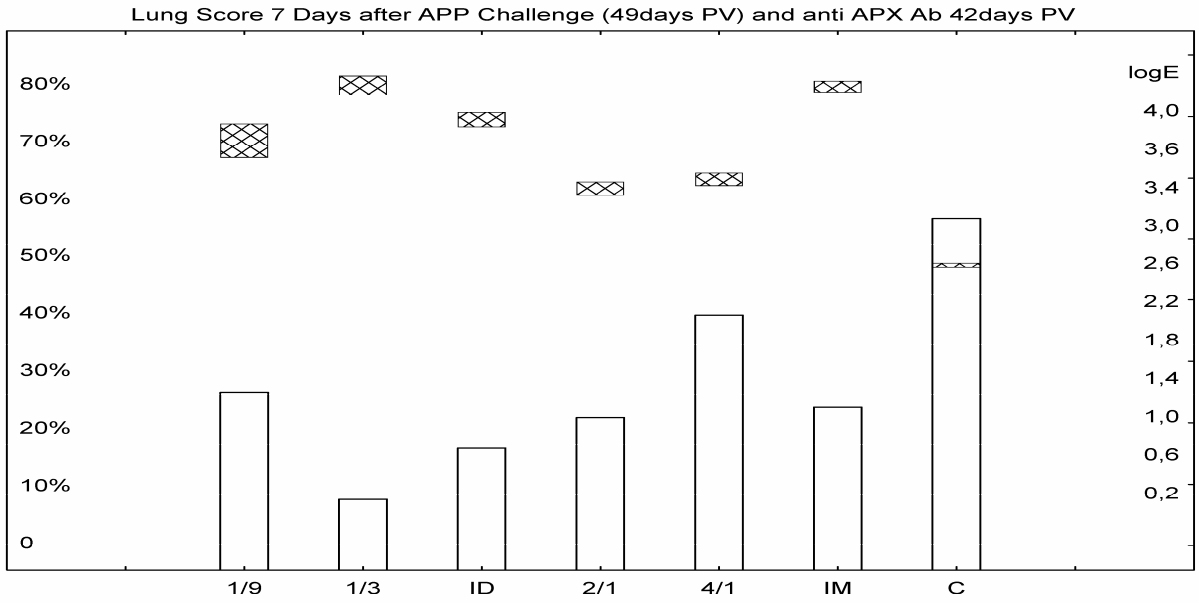
Group Label	Route of Admin.	Concentration/dilution	Remarks
ID	i.d.	Standard dose 0,2 ml	Vaccination dose, recommended by procedure
IM	i.m.	Standard dose 2 ml	Vaccination dose, recommended by producer
ID2/1	i.d	2x concentrated dose	Twice injected standard ID dose, i. e. double original dose
ID4/1	i.d	4x concentrated dose	Four-times injected standard ID dose, i. e. fourfold original dose
ID1/3	i.d	3x diluted dose	Diluted vaccine 2:1, 2 parts of adjutant : 1 part of vaccine; i. e. one third of original dose
ID1/9	i.d	9x diluted dose	Diluted vaccine 8:1, 8 parts of adjutant : 1 part of vaccine; i. e. one ninth of original dose
C	none	None	Not-vaccinated controls

Table 2.: Lung Score-results of challenge infection, expressed in percentage of damaged lung tissue 7 days

Lung Score	ID1/9	ID1/3	ID	ID2/1	ID4/1	IM	C
Average %	25,0	7,5	15,8	20,8	37,5	22,5	53,3
St.Deviation	9,57	5,59	10,96	13,36	9,46	6,29	7,45







Discussion

Clinical Status

Clinical data about temperatures, respiratory disease symptoms, behaviour and weight gain showed, that either different concentration of vaccination dose or administration route didn't altered clinical status and potency of piglets to grow.

Temperature growth and general status of piglets, confirmed by daily gain values showed, that vaccination against APP didn't completely protect against strong infection impact, however appropriate immunisation can augment and speed up immunity reaction so, that after short period of hours are animals able to manage infection and recover to normality.

Groups of piglets, where the lung affections were most severe (C, IM) showed temperature course higher and longer lasting in compare to other groups. Group with severe lung affections ID1/9, ID4/1 showed tendency to high febrile progression, lastly dropped down because of clinical failure of seriously diseased animals.

Groups ID1/3, ID and ID2/1 expressed better immunity in all observed clinical symptoms, confirmed by post-mortem lung status

Isotype-specific Humoral Anti-APP Antibody Response

Dynamics of specific antibody response of both antigens APX and LPS and both isotypes IgG and IgM, showed typical features of standard immune reaction after ID administered vaccines with different dosage of APP antigen; earlier formation of IgM followed by IgG antibody production [18].

Anti-APX IgG Antibodies
Prime and secondary responses of anti-APX IgG Ab showed typical tendency of dynamics, characterized by markedly higher uniform response to booster vaccination. Groups IM, ID and ID1/3 produced higher volume of specific Ab, after prime and booster vaccination as well, without difference of statistical significance, in compare to other groups; and without notable grow after challenge.
Groups ID1/9, ID2/1 and ID4/1 failed in production protecting level of specific IgG Ab and moderately responded to challenge infection. All groups statistically differed from control group C until challenge, moreover groups ID1/3 and IM preserved high level enough to differ continuously statistically significant after challenge one week later.
Anti-LPS IgG Antibodies
The course of IgG anti-LPS Ab response against different concentrations of antigen was characterised by strong reply of IM group to prime and booster vaccination, without gap and/or evident boost reaction to challenge infection.
ID1/9 showed low to mild production of specific Ab, group ID4/1 declined after booster vaccination rapidly to low level group ID1/9 of as much, as came near to level of control group C and reached statistically not significant difference to group C in day 42PV.
Anti-LPS IgM Antibodies
Two-phase curve figured out specific IgM antibody response to both routes of administration, whereas response to booster vaccination was in general less marked; in lower doses of antigen (groups ID1/3 and ID1/9) was response to booster vaccination almost unmarked, groups ID, ID2/1 and ID4/1 concentrations responded by gradual production of Ab, accordingly to concentration of antigen in a dose. Group IM responded markedly to booster vaccination; however production of specific IgG Ab didn't exceed other groups.

Gross Pathology and Lung Score
Clinical course showed tendency to higher protection of i.d. vaccinated animals in compare to i.m. administration in symptoms, developed after challenge infection at all monitored indicators. The tendency was observed most expressively in groups, vaccinated i.d. route by standard volume. Clinical status and lung score accordingly showed, that groups, vaccinated by i.d. administered antigen in standard dosage resulted in better protection, improved in ID1/3 group, vaccinated by 3times diluted antigen.
The most markedly and important result of artificial infection, mass of affected lung tissue, measured by lung scoring, didn't correspond to obtained specific Ab level(IgG and IgM), measured by ELISA in sera, i.e high level of antibody didn't correspond to low lung score and vice versa high lung score (i.e. more damaged lungs) showed not necessarily lower antibody titre.
Intradermal administration of antigen may involve specific cellular and mucosal immunity, as declared Nechvatalová 2005 in APP intradermally vaccinated piglets [11].
It could partially explain not corresponding levels of measured immunoglobulin and affection of lung tissue.
The lung scoring shown best results in the group ID1/3, where 3times diluted antigen was used; the antibody level in this group, measured in sera, did not confirm entirely results of lungs scoring.

Antigen Concentrations on Vaccination Doses
Quantity of antigen in vaccination dose, administered i.m. route, played a key role in ability of vaccine to produce better protective response of an individual or higher number of immune animals in vaccinated herd. An immunological principle of antigen volume in a dose after i.m. administration of antigen may be different in i.d. administration route, where different factors may be of high importance.
Results and level of protective immunity, measured either serologically either by necropsy after challenge, was different in equal volume of antigen administered i.m. and i.d.
The best results were obtained by 3 times lower volume of antigen, administered ID route; accordingly to findings in ID rabies vaccination [19] in human.
Comparable level of protection between i.d. and i.m. administering route, measured by surface damages after challenge and level of antibody titre in sera after vaccination and revaccination was found still after i.d. administration of 9 times diluted APP antigen (ID1/9).
Secondary immunological response after challenge was found also more effective in the range of evaluated parameters in animals, vaccinated by i.d. route [20], as resulted from i.d. and i.m. administration vaccine trial, provided by other researchers.
Van Rooij et al. declares, that intradermal inoculation induced significantly stronger antibody and cell-mediated immune responses and better protection against challenge infection than intramuscular inoculation.[21]
Barfoed et. al., accordingly to our findings, didn't find differences in administration parameters on antibody response after i.d. administering of model antigen at various sites (ear, thorax, inguinal area, tongue mucosa)[22].

Conclusion

The sufficient protective immunity was created via intradermal route of administration, confirmed by ELISA testing antibody response after vaccination, revaccination and challenge in blood and measured in damaged surface of lungs after autopsy of challenged animals.
Nevertheless, the comparisons, obtained from clinical monitoring, gross pathology and ELISA figures, showed discrepancy of results.
The different quantities of antigen in ID vaccination dose resulted in different protection of vaccinated animals. The level of protection was not proportional to the concentration of vaccination dose.
Highest levels of specific IgG antibody didn't response to better protection of the animal and humoral immune failure didn't match the results of clinical course and gross pathology.

The comparison of obtained ELISA response to APX and LPS specific antigens in sera and results of clinical status and lung score imply that humoral response of specific-Ab probably didn't interpret the most important components of protection against infection.
The experiment demonstrated efficacy of i.d. administration of vaccine in compare to i.m. administration route.
The most efficacious concentrations of i.d. administered vaccines, measured by clinical status and lung score, were seen in groups ID1/3, ID and ID2/1, i.e. standard concentrations. The tendency showed, that 3times diluted dose for i.d. administration was more effective than more concentrated ones.

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