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Original article

Production and characterization of swine hyperimmune serum against recombinant, common antigens of Gram-negative outer membrane bacteria

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Abstract

The application of immune serum is one of the most efficient method used formerly in the protection of raised piglets/weaners' health. The objective of the study was to determine specific antibody response during hyperimmunization of fatteners with a self-prepared subunit vaccine, and to propose production method of immune serum against Gram-negative bacteria antigens. The vaccine was administered every two weeks, 4 times. Individual and pooled serum samples were assayed for IgM, IgG and IgA antibodies against *Histophilus somni* recombinant Hsp60, *H.somni* rOMP40 and *Pasteurella multocida* LPS. Additionally total serum IgG and haptoglobin concentrations were measured.

Two weeks after the first vaccination IgM antibody raised significantly against H.s. rOMP40 and LPS, whereas after 4 weeks it increased against rHsp60 antigens. Anti-LPS IgM antibody raised up stepwise till the end of the observation, but IgM antibody against H.s. rHsp60 and H.s. rOMP40 decreased in further samplings. A significant raise in IgG class H.s. rHsp60-antibody was found 4 weeks after the first immunization and a similar raise against two remaining antigens after 6 weeks. The intensity of the reaction increased till the end of the experiment. The raise in IgA antibody level was observed only for H.s. rHsp60 antigen. Clinically observed, proper animal health and welfare were confirmed by haptoglobin concentration, which remained in physiological range. At least 4 booster doses were necessary to obtain hyperimmune serum containing a high level of antibodies against examined antigens. The number of immunizations influenced response profiles for specific IgM, IgG, IgA antibodies.

Key words: swine, recombinant outer membrane proteins, antibody response, hyperimmune serum, subunit vaccine

Introduction

An increasing number of bacterial strains that show multidrug resistance is an unprecedented challenge on livestock farms today. Many groups are looking for antibiotic alternatives to prevent diseases and maintain animal health (Rzaśa et al. 2007, Bhandari et al. 2008, Sjolund et al. 2010, Thacker 2013, Cheng et al. 2014). Even if high efficacy of commercial vaccines in protection against various pathogens is well-known (Busque et al. 1997, Haesebrouck et al. 2004, Wilson et al. 2013, Rose and Andraud 2017), it is difficult to implement effectively a comprehensive vaccination programme in a time as short as the pig production cycle is. Before starting a course of vaccinations it is essential to choose those which will be the most effective against the most common pathogens on the farm. As a rule, monovalent vaccines induce a better protective immune response than polyvalent ones (Kollipara et al. 2013). Limited protection induced by multivalent vaccines and increased risk of undesirable side effects force the search for alternative solutions (Appel 1999). Raising the protective immunity needs at least two vaccinations and about two weeks to develop secondary immune response after booster injection. Due to this fact it is difficult to organize the vaccination schedule to include simultaneous temporary immunosuppression around weaning and the desire to stimulate the immune system by the booster at this time, especially if many vaccinations are needed. Therefore, majority of known immunization programmes may induce the insufficient protection or the protective immunity can occur too late.

One of efficient methods used formerly in the protection of raised piglets'/weaners' health is parenteral application of immune serum. Antibodies administered this way show a broad spectrum of activity, i.e. they can attach to microbial surface antigens, neutralize toxins, promote bacteriolysis or block virus entry into cells. Passive immunotherapy or prevention using plasma/serum from convalescent patients or hyperimmune polyclonal antibodies from hyperimmunized donors were effective in human (Luke et al. 2010) and animals (Gogolewski et al. 1987, Gawel et al. 2005, Rzaśa et al. 2006) infections. A cascade of events is activated by antigen-antibody complexes, resulting in a suppression and inhibition of infection (Rzaśa et al. 2006, Chan et al. 2009).

Significant role of passive immunization in the prevention of infections is played by IgG-antibodies through their complement activation and Fc-receptors binding on phagocytic cells' surface and therefore bridging the adaptive and innate immune systems (Kapur et al. 2014).

Pig production costs could be decreased as a result of a prophylactic application of immune serum, or through combined applications of immune serum and vaccines, compared to vaccination programmes used alone. Using purified immunoglobulin preparations could be worth consideration due to high specificity and efficacy. Similar, but a little bit lower, efficacy of whole serum is noted, while it is also less expensive (Duhem et al. 1994). A lack of grace period for serum is another important factor when animal treatment is necessary, especially at the end of the production cycle. Broad access to antibiotics in large farms caused replacement of seroprophylaxis with excessive metaphylactic application of chemotherapeutics. As the consequence, today the occurrence of multidrug resistant pathogenic strains causes the need to search for alternatives for antibiotics (Suwantarat and Carroll 2016). Nowadays it seems worth consideration to return to passive immunization with antibodies in both prevention and treatment of infectious diseases in farm animals, particularly when 'on-farm harvested serum' could be prepared. Serum preparations belong to the safest biological products available (Duhem et al. 1994).

The evaluation of humoral immune response in vaccinated/infected (naturally or experimentally) animals has been widely discussed in literature, what could be used to establish the efficiency of vaccination (Loemba et al. 1996, Vezina et al. 1996, Nodelijk et al. 2001, Mengeling 2004, Kitikoon et al. 2006, Anderson et al. 2014, Rose and Andraud 2017). Little information is available concerning fatteners' immune response to hyperimmunization to obtain serum for farm usage and the influence of this process on further fattening performance (Blouin et al., 1994, Nedbalcova et al. 2011). The protective effect of hyperimmune serum was confirmed under field and experimental conditions (Blouin et al. 2011). It is commonly known that double vaccination conducted in two-weeks intervals gives usually the most effective immunological response. Sometimes in routine practice increase of this response could be boosted by subsequent injection (Jones and Mould 1984).

Previous study of our group showed that sera of animals hyperimmunized with whole cells or outer membrane complex of *Histophilus somni* (formerly *Haemophilus somnus*) cross-react with outer membrane antigens of many Gram-negative bacteria (Stefaniak et al. 1998). Hyperimmune sera against *H.somni* showed excellent clinical efficiency in prevention and treatment of infections caused not only by this bacteria, but as well by other Gram-negative bacteria, including most common facultative pathogens, like *Pasteurella multocida*, *Mannheimia haemolytica* and *Escherichia*

coli. We assumed that this phenomenon indicates that there are strongly immunogenic antigens that present significant interspecies similarity, cross-react and induce protective immunity.

Among the immunogenic, cross-reactive antigens we selected and characterized partly three as vaccine candidates: *H.somni* recombinant Hsp60 (Zarankiewicz et al. 2012, Jankowska et al. 2015, Bajzert et al. 2018), *H.somni* rOMP40 (Szydłowska et al. 2011) and *Actinobacillus pleuropneumoniae* LPS core oligosaccharide conjugated with both proteins (Jennings et al. 1984). Basic assumption of the subunit vaccine utilized for hyperimmunization of porkers was: „few antigens = immunity against many bacteria”, what is fundamentally different from traditional practice, where: „many antigens = immunity against few strains”.

The aim of the study was to evaluate the immunogenicity of experimental vaccine containing three conservative outer membrane antigens characterized by broad cross-reactivity (recombinant Hsp60 *H.somni*, rOMP40 *H.somni* and core oligosaccharide from *Actinobacillus pleuropneumoniae* LPS conjugated with both overmentioned proteins) in course of hyperimmunization of fatteners-donors of immune serum designed for piglets' protection.

Materials and methods

Animals

All the procedures were approved by the II Local Ethics Committee at the University of Environmental and Life Sciences, Wrocław, Poland (permission 110/2014). The trial was performed on 10 crossbred fatteners (Polish Landrasse x Polish Large White) kept in one pen in a fattening sector of a commercial farm. They were fed ad libitum standard feed used on the farm. The animals were subject to routine care and veterinary treatment; no health disorders occurred during the study.

Vaccine composition

Self-prepared vaccine containing following antigens of broad cross interspecies reactivity was used: recombinant 60 kD heat shock protein from *Histophilus somni* (*H.somni* rHsp60; 10 µg/dose; produced by Pure-Biologics Ltd. Wrocław), recombinant 40kDa outer-membrane protein from *H. somni* (*H.somni* rOMP40; 20 µg/dose; produced by PureBiologics Ltd. Wrocław) as well as conjugated *Pasteurella multocida* LPS core oligosaccharide with *H.somni* rHsp60 (20 µg/dose) and conjugated *Pasteurella multocida* LPS core oligosaccharide with rOMP40 (40 µg/dose). Obtained antigens

dissolved in saline were emulsified with Emulsigen® (20%, MVP Technologies) immediately before injection. The composition of used vaccine was tested in previous study and the best proportion of existed components was chosen (Szydłowska et al. 2011, Jankowska et al. 2015, Bajzert et al. 2017).

Hyperimmunization

The vaccinations were started at 15th week of age (2 weeks after moving to the fattening sector), with the last dose given at 21st week of age. The injections of experimental subunit vaccine were given intramuscularly 4 times every two weeks (1 mL/pig).

Blood sampling

Blood samples (“0”, “2”, “4”, “6”) were collected from *vena cava cranialis* immediately before each vaccination, starting directly prior to the first one. The final blood sample (“8”) was collected two weeks after the fourth immunization at slaughter. The collected serum samples were stored at -80°C until tested.

Pooled serum derived from fatteners (“8”) was protected with phenol at the concentration of 0.5 % and stored for six months at 4°C. Finally, after filtration and sterility control approximately 30 litres of serum were poured into 500 mL glass bottles.

Laboratory analysis

Obtained sera were examined for the IgG, IgM and IgA antibody against *H.somni* rHsp60, *H.somni* rOMP40 and whole LPS from *P. multocida*. Pooled (collected from all hyperimmunized fatteners at slaughter: “8”) and individual serum samples (“0”, “2”, “4”, “6”) from 10 fatteners were examined.

Microplates (Nunc Maxisorp, F) were coated with the above-mentioned antigens (3 µg/mL in 0.05M carbonate buffer pH=9.6 for rHsp60 and LPS, and 3 µg/mL in PBS buffer pH=7.4 for rOMP40; 100 µL per well). The plates were blocked using 1 % Tween 20 (250 µL per well) for 90 min at 37°C. Serum samples were diluted 1:100 (to detect IgA antibody against rOMP40 and LPS); 1:500 (to detect IgA antibody against rHsp60 and IgM antibody against rOMP40 and LPS); 1:1,000 (to detect IgM antibody against rHsp60); 1:8,000 (to detect IgG antibody against rOMP40 and LPS) and 1:50,000 (to detect IgG antibody against rHsp60). As PBS diluent containing 0.05 % Tween 20- PBST was used, 100 µL of the solution was added per well. The plates were incubated at room temperature (22±2°C) for 90 min. Goat anti-pig FcIgG HRPO conjugate (diluted 1:60,000), goat anti-pig IgM HRPO conjugate (diluted 1: 50,000), goat anti-pig IgA HRPO

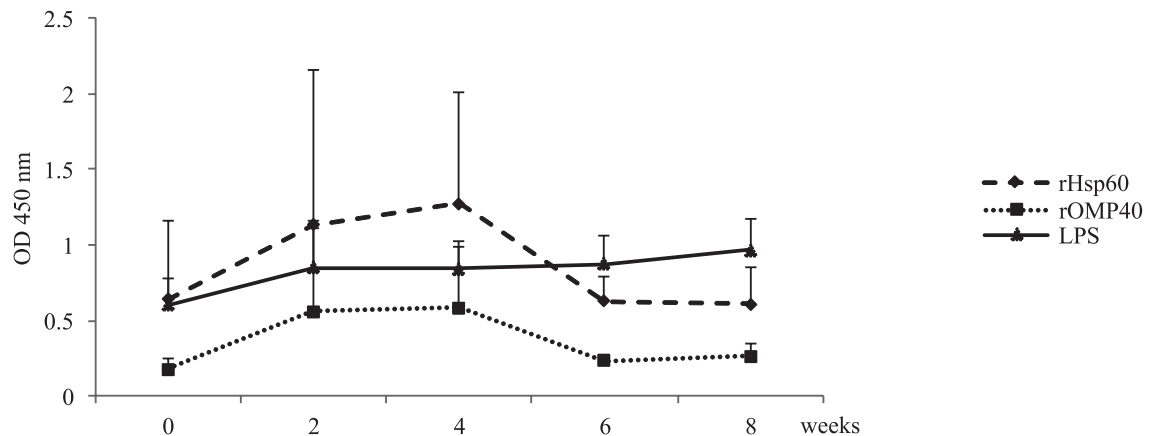


Fig. 1. Determination the reactivity of IgM antibody against rHsp60, rOMP40, and LPS.

Table 1. Statistical differences between IgM antibody levels in serum taken in course of hyperimmunization; a,b ≤ 0.05 ; A,B ≤ 0.01

	0 week	2 week	4 week	6 week	8 week
rHsp60	b	-	a	b	b
rOMP40	b	a	a	b	b
LPS	Bb	a	a	A	A

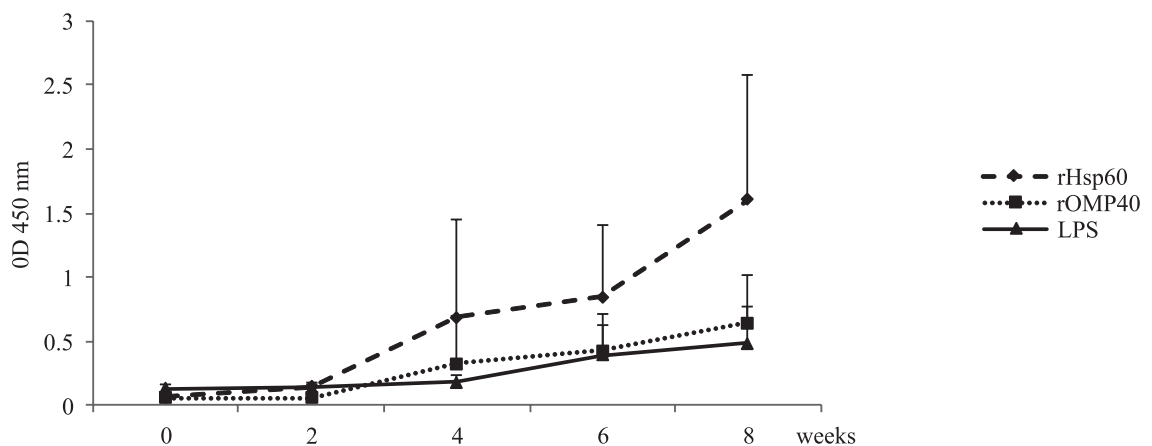


Fig. 2. IgG antibody response against rHsp60, rOMP40, and LPS in hyperimmunized porkers.

Table 2. Statistical differences between IgG antibody levels in serum taken in course of hyperimmunization; a,b ≤ 0.05 ; A,B ≤ 0.01

	0 week	2 week	4 week	6 week	8 week
rHsp60	Bb	Bb	Ba	Ba	A
rOMP40	B	B	B	A	Aa
LPS	B	B	B	A	A

conjugate (diluted 1:15,000) (Bethyl Laboratories) were used as the secondary antibody (100 μ L per well). The plates were incubated at room temperature ($22 \pm 2^\circ\text{C}$) for 90 min. TMB-supersensitive (Sigma Aldrich) was used as substrate (100 μ L per well). The plates were incubated in the dark at room temperature for 25 minutes. The reaction was stopped with 2M H_2SO_4 (50 μ L per well). Optical density was measured at the wavelength of 450 nm with ELISA-Microplate reader μ Quantum (BioTek Instruments).

The concentration of the total IgG in the blood serum was measured using commercial Pig IgG ELISA Kit (Cat No. E101-104 Bethyl Laboratories, Inc.).

The concentration of haptoglobin (Hp) was determined by guaiacol method according to Jones and Mould (1984).

Statistical analysis

Statistical analysis was performed using STATISTICA 12.5 statistical package (Stat.Soft.Inc.). The diffe-

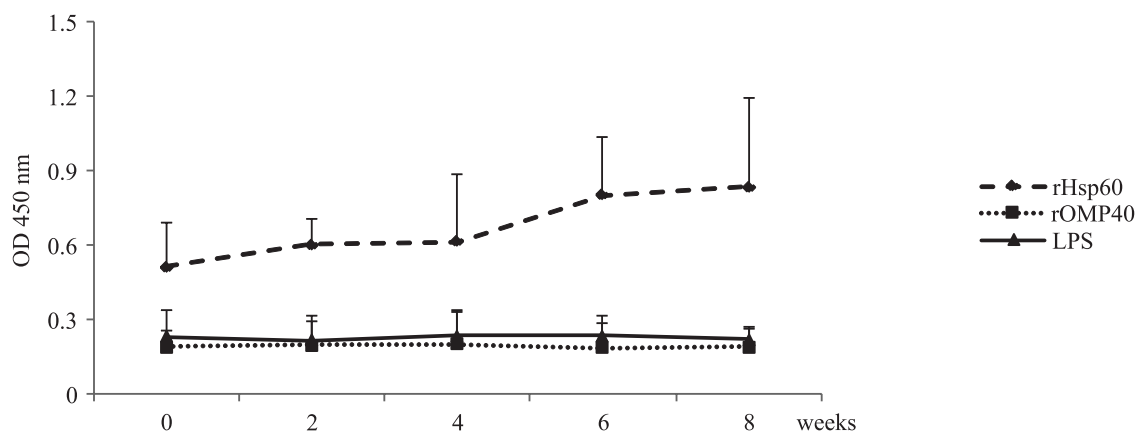


Fig. 3. Determination of reactivity of IgA antibody against rHsp60, rOMP40, and LPS.

Table 3. Statistical differences between *H. somni* rHsp60 IgA antibody levels in serum taken in course of hyperimmunization A, B ≤ 0.01

	0 week	2 week	4 week	6 week	8 week
rHsp60	B	-	-	-	A

rences between the means were determined by the post-hoc Duncan test at $p \leq 0.05$ and $p \leq 0.01$ levels. The obtained results are presented in figures as a mean and standard deviation (mean \pm SD); for clarity the statistical differences are presented in tables below adequate figures.

Results

The highest increase in *H. somni* rHsp60 IgM antibody after the first vaccine injection was observed (Fig. 1, Table 1). Two weeks after the second immunization ("4") the IgM antibody level differed statistically significantly from the samples taken in weeks "0", "6" and "8". The *H. somni* rOMP40 IgM antibody showed the highest level in weeks "2" and "4" and these results differed statistically from those obtained in observation weeks "0", "6" and "8". Reactivity to LPS showed only minor differences. In the last two samplings LPS IgM antibody level differed statistically significantly from the results from week "0" ($p \leq 0.01$). Moreover, the reactivity of the samples obtained at immunization weeks "2" and "4" was higher than those from "0" week ($p \leq 0.05$).

As shown in Fig. 2 and Table 2 the reaction of IgG antibody against particular antigens (*H. somni* rHsp60, *H. somni* rOMP40, LPS) increased during the experiment and reached its maximum two weeks after the fourth (last) vaccination, which result differs statistically from others ($p \leq 0.01$). Additionally, it was noted that the reactivity of IgG antibody against *H. somni* rHsp60 in the serum taken two weeks after the second and third immunization differed significantly ($p \leq 0.05$) from the

results obtained in weeks "0" and "2". A similar increasing tendency of IgG antibody against *H. somni* rOMP40 and LPS antigens was observed throughout the experiment. The results obtained on the first 3 samplings differed statistically from 2 last ones (Table 2).

The IgA antibody response to *H. somni* rOMP40 and LPS antigens showed no difference between respective sampling times (Fig. 3 and Table 3). However, it was observed that the mean intensity of IgA antibody reaction against rHsp60 at the last sampling differed statistically significantly from the sample taken before immunization.

The comparison of antibody responses in pooled (designed for use in piglets) and non-pooled sera (mean of the 10 individual reactivities) derived in the slaughterhouse revealed small differences between them (Fig. 4). The same or a slightly higher reactivity was observed for *H. somni* rHsp60 antigen in non-pooled serum, a reverse trend being observed for *H. somni* rOMP40 ones. For LPS antigen, a higher reactivity in non-pooled serum was noted for IgM antibodies, whereas for IgG and IgA higher results were observed in pooled serum.

The highest, about 60 %, increase in total IgG (Fig. 5), concentration was observed after the first immunization ("2nd week"). After the third vaccine injection ("6th week") the total of IgG was nearly the same as in week "0". On the last sampling day the mean concentration of IgG was lower by 1.24 g/L compared with the results from weeks "2" and "3". Moreover, it was noted that the concentration of IgG in pooled serum ("8") was lower by 1.41 g/L than the mean from the individual samples ("8").

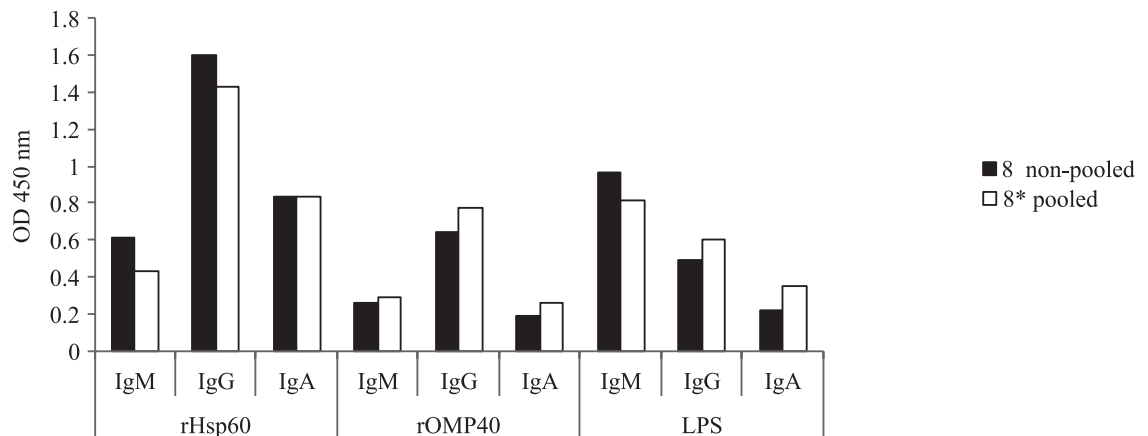


Fig. 4. Determination the reactivity of antibodies (IgM, IgG, IgA) in non-pooled and pooled samples on the last sampling day.

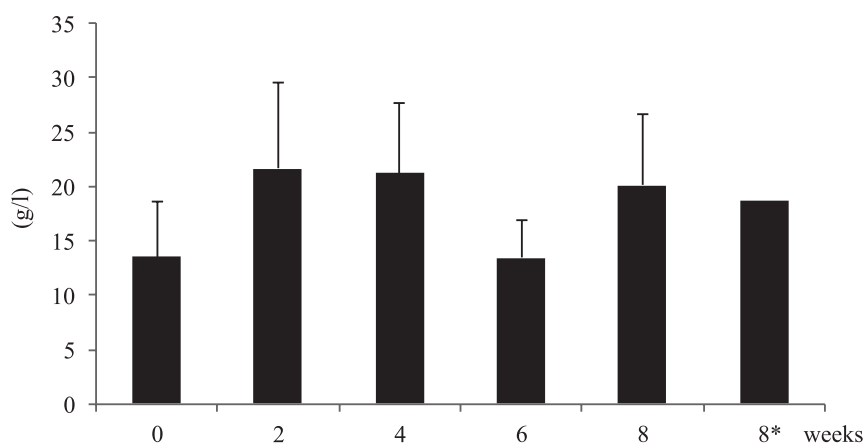


Fig. 5. Total IgG concentration in serum samples of hyperimmunized fatteners.
* pooled serum

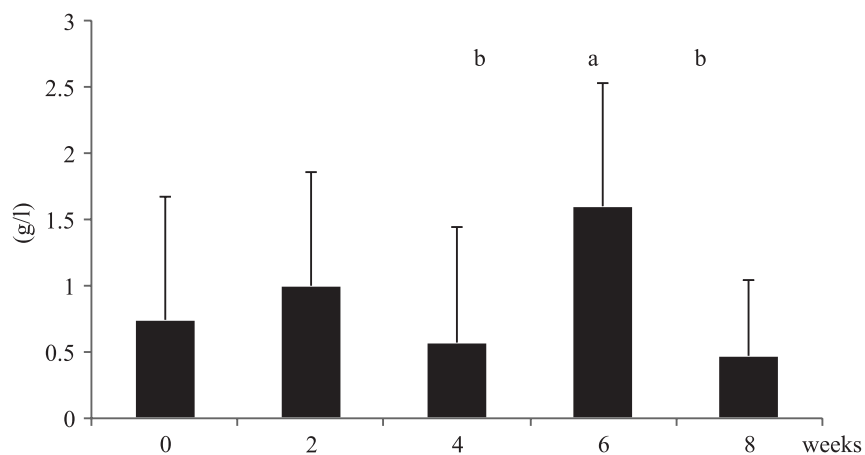


Fig. 6. Haptoglobin serum concentration during fatteners' hyperimmunization.
a,b ≤ 0.05 ; * pooled serum

Throughout the experiment the mean concentration of Hp was 0.83 g/L and varied from 0.47 (the last sampling) to 1.6 g/L on the fourth sampling day ("6", Fig. 6). The mean concentration noted on that day varied significantly ($p \leq 0.05$) from the results two weeks before and two weeks later.

Discussion

Evaluation of immune response after double or single-shot vaccination is the most common practice. However, there is insufficient information about a suitable vaccination schedule to obtain satisfactory hyper-immune serum under field conditions. Little is known

about the course of antibody response in pigs hyperimmunized under field conditions. Assessment of fatteners' immune status changes induced by hyperimmunization spanning a 10-week period, as in the experiment carried out by our team, is rarely found (Rzasa et al. 2006).

The decrease of anti-*H. somni* rHsp60 and anti-*H. somni* rOMP40 IgM antibodies after the fourth week of hyperimmunization and a concurrent further rise of IgG antibody in response to vaccination is associated with phases of immune response and switch of genes involved in production of heavy chain C_H domain of antibody molecule (Senger et al. 2015). The whole process from first vaccination to the appearance of IgG class antibodies takes at least three weeks and may be boosted to get a true anamnestic secondary response (Loemba et al. 1996, Wilson et al. 2013, Jankowska et al. 2015, Baxter et al. 2016).

IgM antibody against *H. somni* rHsp60 and LPS antigens were present in preimmune serum. Elevated IgM antibody activity may reflect natural exposure to infection (Kitikoon et al. 2006) and was found by Jankowska et al. (2015) in piglets vaccinated with *H. somni* rHsp60 two weeks after weaning. This proves the prevalence in the environment of antigens that cross react with vaccine antigens (Udvarnoki et al. 2007). The reactivity of those immunoglobulins raised after the first two immunizations (IgM specific to rHsp60 and rOMP40 antigens) and fell after reaching a plateau. Although one or two vaccine doses were sufficient to increase IgG-specific antibody response against each vaccine component (*H. somni* rHsp60, *H. somni* rOMP40, LPS core), it was noted that antibody reactivity rose after each subsequent immunization. It seems that additional booster doses may be recommended to increase antibody response in swine (disease outbreaks, poor health status). Preimmune serum contained low IgG level, what may confirm limited stimulation of humoral immunity by natural exposure to environmental pathogens.

In the present study the dynamics of specific antibody response in classes against three different antigens varied significantly. The highest levels of specific antibodies in all Ig classes were observed in response to *H. somni* rHsp60 antigen. This protein is an example of a highly conservative antigen (Siddaramppa and Inzana 2004).

Because of differences in blood volume harvested at slaughter (and indirectly the amounts of serum) from respective hyperimmunized individuals a limited differences in serum antibody reactivity and total IgG concentration between pooled and non-pooled serum samples occurred.

It is worth highlighting that the mean values of Hp

were the highest two weeks after the third immunization and exceeded the normal range, whereas the IgG concentration was the lowest at that time. Transient decrease of serum IgG concentration may cause the rise of risk of infections by farm-local pathogenic microflora and induce rise of acute phase proteins including Hp (Richter 1974, Frenyo et al. 1981, Petersen et al. 2004, Chmielowska-Korzeniowska et al. 2012). It seems that higher Hp and lower IgG concentrations can be related to intensive stimulation of the fatteners immune system during hyperimmunization or/and stress (Temple et al. 2012). Despite the fact that the mean values of Hp during the fourth blood collection were slightly above the physiological level, the remaining results varied from 0.4 to 1.1 g/L, which may confirm that hyperimmunization did not disturb significantly the health status of the fatteners. An additional confirmation that hyperimmunization had no negative effect on animal health was the weight gain of immunized fatteners, that did not differ significantly from non immunized animals kept at the same condition. Lack of negative impact on the health status and production parameters makes the production of farm-derived hyperimmune serum worth taking into account. Donors of hyperimmune serum used for seroprophylaxis/serotherapy should be obligatorily tested to avoid the risk of transmission of infectious diseases (Bier 1981).

The slaughter of 10 hyperimmune fatteners allowed to yield approximately 30 liters of immune serum with a satisfactory concentration of antibodies against vaccine antigens. This volume is sufficient for 10-millilitre single-shot subcutaneous administration to 3000 piglets. The protocol of serum injection should be tailored to the needs of the farm and could be modified (a few injections at various time intervals) because of a high risk of infection or to apply in animals exhibiting poor production parameters. A single serum injection can provide effective animal protection for a few weeks, which was observed in previous studies of IgG dynamics after single vaccinations (Rzasa et al. 2006). Preparation of farm-made serum may be recommended for farms with a well-known challenges and immune status of animals, where adequate data are regularly updated. Farm-made serum could be used not only for a quick improvement of animal health status. In the cited experiment serum inoculations were used successfully to eliminate PRRS from the farm, which confirms the value of such treatment in field conditions (Stukelj et al. 2015). Weaned piglets at age of 4-8 weeks are especially endangered to respiratory tract infections because of exhausted colostrum immunity and insufficient time to develop the protective immunity after vaccinations. Therefore, this age group seems to be a tar-

get number one where the passive immunization (with hyperimmune serum containing antibodies against common Gram-negative bacteria antigens) may minimize the risk of infection development. Such procedure is an alternative to antibiotics, while the increased number of multidrug-resistant bacterial strains is reported (Canton et al. 2008, Suwarant and Carroll 2016).

Conclusions

The present results indicate that hyperimmunization of fatteners with two or four doses of vaccine to obtain good quality immune serum would be better solution than three injections. Described above hyperimmunization schedule is an example of immune response development after vaccinations. Due to the fact that similar immune response with following rise of antibody titres is expected in case of using other antigens described study has an application potential in farm veterinary practice.

Acknowledgements

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