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# Humoral and cellular immune response to *Histophilus somni* recombinant heat shock protein 60 kDa in farm animals

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**ABSTRACT**: The aim of this study was to evaluate the effects of immunising farm animals with the *Histophilus somni* recombinant heat shock protein 60kDa (*H. somni* rHsp60) in field conditions. Fifty piglets, 10 calves and 30 hens were immunised twice, and the same number of each species was used as the control. The humoral immune response was evaluated using ELISA in piglets (IgG, IgA and IgM) and calves ($IgG_1$, $IgG_2$ and IgM) sera and in hen egg yolks (IgY). Cell-mediated immune responses were evaluated using the skin test. Concentrations of serum haptoglobin in calves and piglets and plasma fibrinogen in calves, daily weight gain in piglets, as well as the inner body temperature and clinical signs in calves were measured to evaluate the clinical effects of vaccination. In animals immunised twice with *H. somni* rHsp60, a statistically significant increase in IgY antibodies in egg yolk as well as serum $IgG_1$ and $IgG_2$ antibodies in calves ($P < 0.05$) was found. In piglets, the antibody reaction against *H. somni* rHsp60 was higher in the experimental than in the control group, but the difference was significant only for the IgG class ($P < 0.05$). A moderate cell-mediated immune response to *H. somni* rHsp60 measured using the skin test was observed in piglets after 24 h ($P < 0.05$), but not in calves and hens. The daily weight gain was significantly higher in the experimental than in the control piglets ($P < 0.05$). The fibrinogen and haptoglobin levels in calves, as well as the inner body temperature, indicated a reduced risk of pathology in the experimental group of calves. The preliminary results confirmed the immunogenicity of *H. somni* rHsp60. A beneficial effect on piglet weight gain was observed. The obtained results warrant further studies of the protective effects of *H. somni* rHsp60 as an ingredient of subunit vaccines in farm animals.

**Keywords**: rHsp60; *Histophilus somni;* subunit vaccine; calves; piglets; hens

Bacterins developed against Gram-negative bacteria may cause side effects including endotoxic shock and can show unsatisfactory clinical efficiency (Meeusen et al. 2007). This limits their usefulness in field conditions, to prevent facultative pathogenic infections involving genera of *Pasteurella*, *Mannheimia*, *Histophilus*, *Escherichia* and *Salmonella*, which cause significant economic losses in livestock animals (Morin et al. 1976; Sivula et al. 1996; Gagea et al. 2006; Snowder et al. 2006; Blanchard 2012). Therefore, subunit vaccines, which use selected protective antigens, are introduced to improve the efficiency of immunisation and to avoid undesirable side effects, which happens frequently when whole-cell bacterins are used (Mann and Ark 2009; Hickey et al. 2011). The production of recombinant proteins circumvents the costs of purifying native antigens from whole bacteria.

Heat shock proteins (HSPs) play a protective role in stress conditions (Urban-Chmiel et al. 2013). Protist HSP60s are known as highly conserved, dominant antigens that induce the protective host immune response to various pathogens. They are recognised as immunodominant antigens in bacterial, fungal and parasitic infections, and are

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therefore capable of inducing strong humoral and cellular immune responses in mammals (Udvarnoki et al. 2007). Recombinant Hsp proteins such as *Salmonella* Typhi rHsp60 (Bansal et al. 2010), *Histoplasma capsulatum* rHsp60 (Gomez et al. 1995; Mannicki et al. 1995) and *Paracoccidioides brasiliensis* rHsp60 (Soares et al. 2008) are highly immunogenic and induce a protective immunity against lethal challenge by homologous pathogens. Dot blot hybridization revealed a close relation of Hsp60 from *Escherichia coli*, *Salmonella* and *Shigella* due to the close genetic relationship of these pathogens. However, *Campylobacter coli*, *C. fetus* and *C. jejuni* show only species-specific reactions. Similarly, *Vibrio vulnificus* and *V. parahaemolyticus* do not cross-hybridized with *Aeromonas* and *Plesiomonas* (Wong and Chow 2002). It has been confirmed that antibodies developed against bacterial Hsp60 show broad cross-reactivity. Polyclonal Hsp60 *Pseudomonas aeruginosa* antibodies and *Legionella micdadei* Hsp60 antibodies, as well as monoclonal *Mycobacterium leprae* Hsp65 and *Neisseria gonorrhoeae* Hsp65 antibodies, cross reacted with *Streptoccocus suis* Hsp60 (Benkirane et al.1997). Hsp60 isolated from *Giardia lamblia* cross reacted with polyclonal *Mycobacterium bovis* Hsp65 antibodies (Reiner et al. 1992). Polyclonal *S. suis* Hsp60 antiserum cross reacted with lysates of *Enterococcus faecalis*, *Streptoccocus equisimilis*, *S. agalactiae*, *S. bovis*, *S. pneumoniae*, *Escherichia coli*, *Shigella sonnei*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, and *Bordetella bronchiseptica* (Benkirane et al. 1997). Monoclonal *Pseudomonas aeruginosa* Hsp60 antibodies cross reacted with those of *P. stutzeri*, *P. alcaligenes*, *P. mendocina*, and *P. pseudoalcaligenes* (Luneberg et al. 1997).

*Histophilus somni* as a member of the family *Pasteurellaceae* is a Gram-negative bacterium and opportunistic pathogen associated with multisystemic diseases in cattle (Siddaramppa and Inzana 2004). *Histophilus somni* possesses a wide array of virulence attributes that are important in host-parasite relationships (Stefaniak 1999; Corbeil 2008).

Western blot analysis of goat, bovine and rabbit sera of animals hyperimmunised with whole cell or outer membrane protein (OMP) *H. somni* antigens, showed cross reactivity with some antigens of 20 Gram-negative bacterial strains (Stefaniak et al. 1998; Stefaniak et al. 1999; Galli 2009). A reaction with a 60 kDa antigen was identified in all examined strains of *Pasteurella*, *Mannheimia*, *Histophilus*, *Escherichia*, *Salmonella*, *Klebsiella*, and *Actinobacillus* genera. An Hsp60 antibody, affinity isolated from goat hyperimmune serum against *H. somni* OMPs cross reacted with 60 kDa antigens in all examined strains. Moreover, this antibody reacted with human rHsp60 (hrGroEL). It was concluded that the examined 60 kDa antigen belongs to the HSP60 family (Galli et al. 2009).

*Histophilus somni* recombinant Hsp60 (rHsp60) produced in our laboratory is highly immunogenic and induced broad cross-reactivity. Mouse and goat antibodies against *H. somni* rHsp60 reacted in immunoblots with 60 kDa antigens of other pathogenic strains of the genera mentioned above (Galli 2009). The murine *H. somni* rHsp60 antibodies inhibited *in vitro* biofilm production by *H. somni* (Zarankiewicz et al. 2012).

We previously showed that *H. somni* rHsp60 induces a humoral immune response in goats and mice. Therefore, the aim of this study was to evaluate the effects of immunising hens, piglets and calves with the recombinant *H. somni* heat shock protein 60kDa in field conditions.

## MATERIAL AND METHODS

**Antigen preparation**. Recombinant Hsp60 from *H. somni* was obtained using standard genetic engineering methods. The gene encoding Hsp60 was amplified from the genome of *H. somni* using PCR, and then the PCR products were inserted (using NcoI and BamHI restriction enzymes, Fermentas cat. no. ER0691 and ER0572, respectively) in frame into the expression vector pET22b(+) (Novagen, cat. no. 69337-3) with a histidine tag at the N-terminal end. The resulting plasmids were transformed into *Escherichia coli* Turner (DE3) expression cells (Novagen, cat. no. 70726-3).

**Protein expression**. Expression of the recombinant proteins was induced using 1mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Fermentas, cat. no. R0392). The transformed bacteria were cultured overnight at 37 °C in 100 ml of LB medium containing ampicillin (100 µg/ml) (PolfaTarchmin S.A.) and chloramphenicol (35 µg/ml) (Sigma Aldrich, cat. no. C0378). This pre-inoculum was added to 2 l of fresh LB medium with 2 ml of each antibiotic at the same concentrations. The bacteria were grown to an $OD_{600}$ = 0.6, were then induced using IPTG and were subsequently incubated for 4 h at 37 °C. The

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culture was centrifuged and the pellet was stored at –20 °C.

**Chromatography**. Proteins were purified using Ni-NTA columns. The pellet was resuspended in an 8M urea buffer (pH = 7.8) and sonicated (15 s, three times, at 80% amplitude of the ultrasonic homogeniser SonoPlus HD 2070 Bandelin). The denatured material was centrifuged at 10 000 × g, and the obtained supernatant was incubated with Ni-Sepharose (Invitrogen, cat. no. R901-15) for 90 min. The recombinant protein was eluted with buffer containing 20, 50, 200 and 500mM imidazole (Sigma Aldrich, cat. no. I0125) successively. To enhance protein solubility 10% glycerol (Sigma Aldrich, cat. no. G7893) was added. The eluates were dialysed against PBS containing 10% glycerol. The protein concentration was estimated using the Bradford method (Bradford 1976). The samples were stored at –20 °C until use.

**Immunisation of farm animals with rHsp60**. The experimental animals were as follows: 50 Polish White Landrase (pbz) weaners kept in one pen at the nursery sector of a commercial farm, 10 crossbred Polish h-f × Polish red and white calves kept at a dairy farm for 170 heads, and 30 laying hens (Lohmann Brown) kept in cages in a commercial laying hen farm. Animals were vaccinated twice. The first injection was given two weeks after weaning (piglets), two to four weeks after moving from the calving barn to the calf shed (calves) and two weeks after the beginning of egg laying (hens). The second injection was administered three weeks later. The experimental vaccine was composed of antigen (10 mg of *H. somni* rHsp60) dissolved in sterile saline solution containing 15% Emulsigen-D (MVP Laboratories). Animals were injected subcutaneously on the neck, calves and piglets with 1 ml, while hens received 0.4 ml of vaccine. Control groups included 50 weaners of a neighbouring pen, 10 calves kept together with experimental animals in four pens (seven heads per pen) and eggs from hens of neighbouring cages, respectively. Management and feeding conditions were the same in experimental and control groups. Weaners of both groups were immunised with *Actinobacillus pleuropneumoniae* vaccine two weeks before the experiment started according to the farm health programme.

**Sampling**. Three different blood samples were collected from 10 randomly chosen experimental and control piglets marked with ear tags and from all examined calves: before each vaccine injection and two weeks after the second injection. Eggs were collected from the hens once a week, until the 13$^{th}$ week after the first immunisation. The serum samples and eggs obtained from non-immunised animals were used as negative controls. Serum and yolk samples were stored at –20 °C until use.

**Skin test**. A skin test was performed in seven piglets, calves and hens of all experimental animals and in seven control animals representing each species. Animals were injected intradermally with 0.2 ml of rHsp60 (10 µl/ml) 14 days after the second immunisation. The skinfold thickness was measured immediately before the antigen injection and 24, 48 and 72 h after.

**Detection of serum antibodies by ELISA**. Sera obtained from piglets, calves and yolk samples from hens were examined for reactivity with *H. somni* rHsp60 in IgG, IgA, IgM classes of antibodies (piglets); $IgG_1$, $IgG_2$, IgM antibodies (calves) and IgY antibodies (hens). Microplates (NuncMaxisorp, cat. no. 439454) were coated with 100 µl per well of *H. somni* rHsp60 (3 µg/ml of 0.1M carbonate buffer, pH = 9.6) and 2% Tween 20 as a negative control. Serum samples were diluted 1 : 100, yolk samples 1 : 5000 with PBS containing 0.05% Tween 20 (PBS-T), and 100 µl of solution was added per well in duplicate. Plates were incubated at room temperature for 2 h. Goat anti-chicken IgG (Sigma Aldrich, cat. no. A-9046) diluted 1 : 20 000, anti-pig IgG (1 : 50 000), IgM (1 : 25 000), IgA (1 : 25 000) (Bethyl Laboratories, cat. no. E100-104, E100-100, E100-102, respectively) and sheep anti-bovine $IgG_1$ (1 : 40 000), $IgG_2$ (1 : 20 000), IgM (1 : 20 000, AbD Serotec, cat. no. AAI21P, AAI22P, AAI19P, respectively) HRPO conjugates in PBS-T were used. Plates were incubated for 90 min at room temperature. A substrate ortho-phenylenediamine (Sigma Aldrich, cat. no. P23938) in 0.01M phosphate-citrate buffer pH = 5.0 was used (100 µl per well followed by incubation in the dark at room temperature for 30 min (chicken IgG, pig IgG and IgA), 20 min (bovine $IgG_1$, $IgG_2$, IgM) and 15 min (pig IgM). The reaction was stopped by adding 2M $H_2SO_4$ (50 µl per well) The optical density was measured at a wavelength of 492 nm with an ELISA-Microplate reader µQuant (BioTek Instruments).

**Immunoblotting**. Recombinant *H. somni* rHsp60 was separated on SDS-PAGE (12%), and then transferred onto nitrocellulose membranes (using a von Keutz semi dry chamber). The membrane was blocked with 3% skim milk and later incubated with

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6xHistag MoAb (Clontech, cat. no. AKR-003) or goat-anti-*H. somni* rHsp60 antibodies obtained from goat hyperimmune serum against *H. somni* OMP. The reaction was developed with goat anti-mouse IgG HRPO conjugate and rabbit anti-goat IgG HRPO conjugate, respectively. 4-chloro-1-naphthol (Sigma Aldrich, cat. no. C8890) was used as a substrate. *H. somni* rHsp60 was also separated on SDS-PAGE (12%), and the gel was stained using Coomassie Brilliant Blue R-250 (Fluka, cat. no. 27816).

**Estimation of acute phase proteins**. Haptoglobin was measured according to Jones and Mould (1984) using human Hp 2-2 (Sigma Aldrich, cat. no. H9762) as the standard. Fibrinogen was measured according to Millar et al. (1971).

**Production and clinical parameters**. Piglets were weighed two weeks after the second injection and again three weeks later during regrouping. The lungs of calves were auscultated and the inner body temperature was measured between 10:00 and 12:00 h on the days of the first and second injections and 14 days later according to Baumgartner et al. (2009). Animals were supervised visually by a farm veterinarian every day, and in the case of illness the appropriate treatment was performed.

Animal tests were approved by the II Local Ethical Committee for Experiments on Animals in Wroclaw (resolution No. 179/2010).

**Statistical analysis**. Treatment comparisons were carried out using the analysis of variance procedures for a completely randomised design in the Statistica 10 statistical package (Stat Soft. Inc. (2013). Levene's test was used to assess variance homogeneity. The statistical differences between analysed means were determined using Tukey's test. The differences were considered statistically significant at $P \leq 0.05$. The obtained results are presented in tables and figures as the mean and standard deviation (mean ± SD).

## RESULTS

### Production of purified *H. somni* rHsp60

A protein of approximately 60 kDa was identified in 12% Coomassie-stained polyacrylamide gels (Figure 1.1). Its identity was confirmed as purified recombinant *H. somni* Hsp60 by reaction with an anti-His-tagged monoclonal Ab (Figure 1.2) on Western blot.

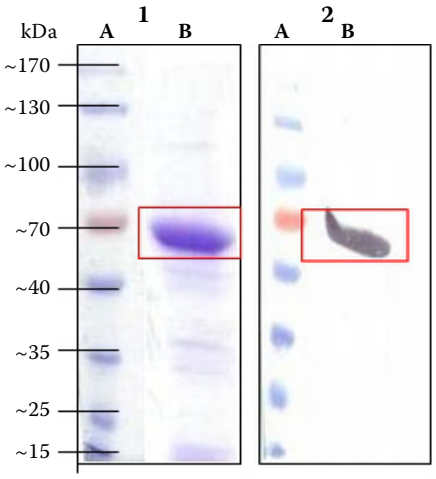


Figure 1. SDS-PAGE and immunoblotting of rHsp60 kDa *H. somni* protein with 6xHistag MoAb (1 : 2000) and goat anti-mouse IgG (1 : 2000) HRPO conjugate. **A** = marker page ruler prestained protein ladder (5 µl), **B** = purified rHsp60 protein (20 µl), **1** = SDS-PAGE, **2** = immunoblotting with 6xHistag monoclonal antibody

### Immunisation

Limited local swelling at the site of injection disappeared after a few days. No signs of pain were observed.

### Immune response

**Hens**. The intensity of antibody reaction (Figure 2) was similar in both groups before immunisation and persisted at a level of OD ≤ 1.0 in the control group up to the 12$^{th}$ week of the experiment. The activity of egg yolk antibody from the immunised group exceeded the OD of the control group from the second week to the end of the experiment. The differences between groups were statistically significant ($P < 0.05$) on the 2$^{nd}$, 4$^{th}$, 5$^{th}$, 8$^{th}$ and 11$^{th}$ week of observations. Two weeks after a booster dose, the average intensity of egg yolk antibody of the experimental group reached a peak (OD = 2.85).

**Piglets**. There were statistically significant differences in the activity of serum IgG and IgA *H. somni* rHsp60 antibodies between immunised and control animals on the "0" day (before immunisation) and on the last control day (two weeks after the 2$^{nd}$ immunisation) in the IgG class ($P < 0.05$) (Figure 3). Two weeks after the second immunisation the intensity of the antibody reaction was higher in the experimental than in the control group in all ex-

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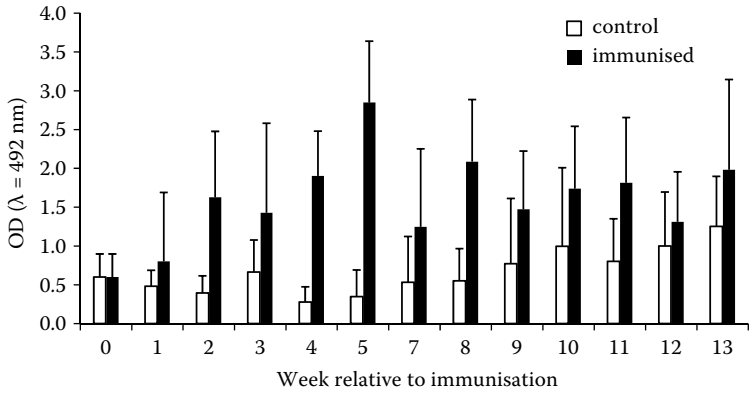


Figure 2. ELISA homologous IgY antibody activity in egg yolk of hens immunised with *H. somni* rHsp60 (OD at λ = 492 nm, egg yolks diluted 1 : 5000). Different letters shows differences between control and immunised in particular weeks ($P < 0.05$)

amined classes. The intensity of IgG class antibody activity increased statistically significantly (P < 0.05) by two-fold in the control and four-fold in the experimental group in comparison to pre-immunisation intensities. IgM antibody activity approximately doubled in both groups. IgA antibody activity was at a low level at the beginning of the experiment but also doubled in both groups of animals.

**Calves**. From the beginning of the experiment until two weeks after the second antigen injection, the OD of serum $IgG_1$ class antibodies increased by approximately 6.5-fold, and $IgG_2$ showed a 16-fold increase (Figure 4). At the same time, antibody activity in the control group remained stable at a low level in both classes. There were statistically significant differences ($P < 0.05$) in activities between the experimental and control groups in both IgG subclasses at the second antigen injection and two weeks later. No statistically significant differences occurred for IgM activity (Figure 4).

## Skin test

The skinfold thickness did not change in hens (differences were below 0.3 mm) for 72 h after intradermal antigen injection (data not shown). The largest increase in skinfold thickness was observed in experimental groups of piglets (Figure 5) 24 h after antigen injection ($P < 0.05$). In calves the skinfold thickness increased in both groups and there were no significant differences.

## Fibrinogen (Fb)

The Fb concentration in calves decreased stepwise in both groups from more than 5 g/l to approximately 3 and 4 g/l, respectively, in experimental and control calves at the last blood sampling (Table 1). The difference of nearly 1 g/l was statistically significant at $P < 0.05$.

## Haptoglobin (Hp)

No significant differences were observed for haptoglobin in piglets and calves. The serum Hp concentration in piglets (Table 2) increased from very low levels at the beginning of immunisation to exceed the normal range during the third week of the experiment. The serum Hp concentration de-

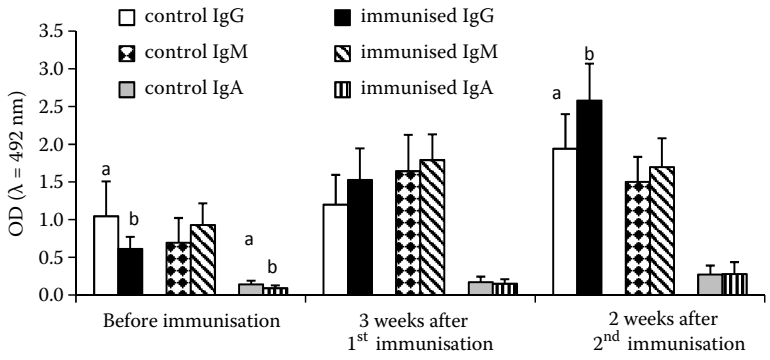


Figure 3. ELISA homologous serum IgG, IgM and IgA antibody activity in piglets immunised with *H. somni* rHsp60 (OD at λ = 492 nm, serum samples diluted 1 : 100). Different letters shows differences between control and immunised in particular weeks ($P < 0.05$)

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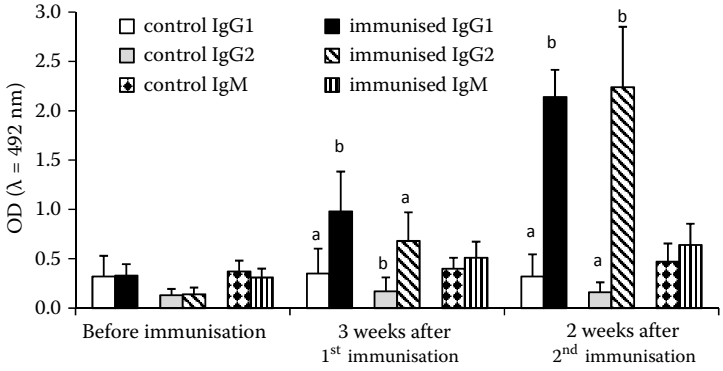


Figure 4. ELISA homologous serum $IgG_1$, $IgG_2$and IgM antibody activity in calves immunised with *H. somni* rHsp60 (OD at λ = 492 nm, serum samples diluted 1:100). Different letters shows differences between control and immunised in particular weeks ($P < 0.05$)

creased two weeks later in the experimental group, but increased in the control group. The mean Hp concentration in both groups of calves exceeded the normal range at the time of the first antigen injection. In the subsequent serum samples, it decreased to the normal range in the experimental group, but remained higher in the control group (Table 2).

## Daily weight gain in piglets

Immunised piglets showed a statistically significant daily gain of 210 g ($P < 0.05$) more than those in the control group in the last three weeks of rearing at the nursery sector.

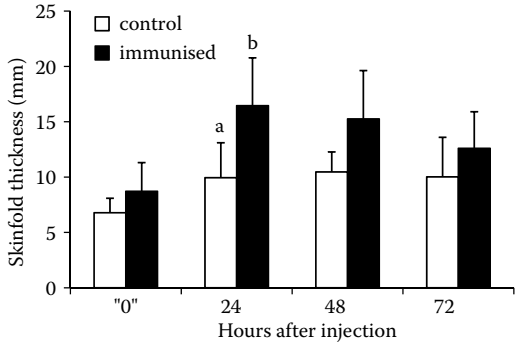


Figure 5. Skinfold thickness after intradermal injection of 0.2 ml *H.somni* rHsp60 (10 mg/ml) in piglets. Different letters shows differences between control and immunised ($P < 0.05$)

## Clinical examination of calves

Because of clinical signs of respiratory tract infection, four calves in each group were treated during the study with antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs). Despite this treatment, one calf in the control group died a few days after the end of observation with signs of bronchopneumonia. The inner body temperature two weeks after the second vaccination was elevated (over 39.2 °C) in one experimental, and in five control calves. Therefore, the mean inner body temperature was 0.5 °C lower in the experimental than in the control group (38.7 °C, and 39.2 °C, respectively), but no statistically significant differences were found between the experimental and control groups.

## DISCUSSION

Heat shock proteins can induce strong humoral and cellular immune responses in mammals, due to their role as immunodominant antigens in bacterial, fungal and parasitic infections (Udvarnoki et al. 2007). Therefore, they are frequently used to produce subunit vaccines. Numerous studies have shown the efficiency of immunisation with HSP family proteins in the induction of a protective immune response against pathogens (Kaufman et al. 1990; Mannicki 1995; Noll and Autenrieth 1996; Lee et al. 2006; Bansal et al. 2010). It has been demonstrated that *H. somni* rHsp60 induces an immune response in SPF mice

Table1. Mean and SD fibrinogen concentrations in calves (g/l)

| | Before the immunisation | 3 weeks after 1st immunisation | 2 weeks after 2nd immunisation |
|---|---|---|---|
| Control | 5.4 (2.0) | 4.3 (1.2) | 4.1 (1.2)[a] |
| Immunised | 5.1 (1.7) | 3.8 (1.2) | 3.1 (0.9)[b] |

Different letters shows differences between control and immunised (P < 0.05)

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Table 2. Mean and SD of haptoglobin concentrations in piglets and calves (g/l)

| | Before the immunisation | 3 weeks after 1st immunisation | 2 weeks after 2nd immunisation |
|---|---|---|---|
| Control piglets | 0.05 (0.05) | 1.07 (1.22) | 1.31 (0.76) |
| Immunised piglets | 0.12 (0.23) | 1.44 (0.79) | 0.95 (0.76) |
| Control calves | 0.50 (0.95) | 0.15 (0.24) | 0.25 (0.44) |
| Immunised calves | 0.15 (0.35) | 0.01 (0.02) | 0.06 (0.18) |

(Galli 2009; Bajzert 2013). The results of the current study confirm that vaccination of laying hens and calves in field conditions with recombinant *H. somni* Hsp60 induces an intense humoral immune response. There was no increase in anti-rHsp60 antibody activity in control hens and calves. In control piglets, the *H. somni* rHsp60 antibody activity may have increased due to an additional vaccination with whole-cell *Actinobacillus pleuropneumoniae* that was carried out two weeks before the beginning of the experiment in all weaned piglets (Figure 3). This vaccine contained *A. pleuropneumoniae* toxin and outer membrane protein (OMP), possibly including Hsp60 (Gardno and Faulkner 1998). Moreover, this farm was threatened by *A. pleuropneumoniae* and the animals may have survived repeated contact with this and other pathogens.

It has been confirmed that immunisation with homologous Hsp60 protects laboratory animals against experimental challenge with *Salmonella enteric* serovar Typhi (Paliwal et al. 2008; Bansal et al. 2010), *Helicobacter pylori* (Yamaguchi et al. 2000; Yamaguchi et al. 2003), *Histoplasma capsulatum* (Gomez et al. 1995; Deepe and Gibbons 2002), *Legionella pneumophila* (Blander and Horwitz 1993; Weeratna et al. 1994), *Yersinia enterocolitica* (Noll and Autenrieth 1996), *Chlamydia trachomatis* (Rank et al. 1995), *Paracoccidioides brasiliensis* (Soares et al. 2008), *Piscirickettsia salmonis* (Wilhelm et al. 2005) and *Francisella tularensis* (Hartley et al. 2004). Because of the broad cross-reactivity of *H. somni* Hsp60 antibodies (Galli 2009; Bajzert 2013) with Hsp60 of *Pasteurella multocida*, *Escherichia coli* and *Salmonella* Enteritidis, it seems to be an promising candidate to vaccinate farm animals and to induce cross-protection.

In the present study, a significant increase in the reaction of IgY *H. somni* rHsp60 antibodies in the egg yolks from experimental hens was maintained until the end of the 11th week of observation. The ability of *H. somni* Hsp60 to elicit an immune response and induce broad cross-reactivity to Gram-negative bacteria (Galli 2009; Bajzert 2013) suggests its possible use as an ingredient of subunit vaccines against salmonellosis in poultry. The increase in Hsp60 antibody activity in eggs from control hens that occurred during the second part of the experiment could be the consequence of repeated contacts with bacteria present in the farm environment that induced an immune response against the Hsp60 of those bacteria. Dera-Tomaszewska et al. (2003) found that in egg yolks of hens infected with *Salmonella* Enteritidis, IgY antibody reaction against homologous Hsp60 reached significantly higher levels than in *Salmonella*-free birds. We previously observed an effect of oral immunisation with whole, live *H. somni* cells, on the stimulation of cross-protection against poultry salmonellosis. An increase of the IgA anti-*H. somni*, *Salmonella* Enteritidis and *S.* Typhimurium antibody reactions was observed. The oral immunisation of chickens with whole *H. somni* cells increased protection against *S.* Enteritidis oral challenge and induced protection at a level comparable with commercial vaccines (Stefaniak et al. 1998; Wieliczko et al. 2000).

In the control group of piglets, symptoms of respiratory tract infections (sneezing) were observed, but there were no statistically significant differences in the haptoglobin and fibrinogen concentrations. Moreover, haptoglobin and fibrinogen concentrations were close to the normal range in the blood of experimental animals, indicating a low risk of inflammation. However, the lack of statistically significant differences in haptoglobin concentrations may be due to the considerable deviations between the results of individual animals.

Despite the difference in antibody response between the control and experimental hens, there were no significant differences in the response to intradermal injection of antigen in the cellular response test. This result was unexpected, because Hsp60 protein was described to stimulate a cellular response (Kaufmann et al. 1990; Galdiero et al. 1997; Bulut et al. 2002). The comparison of the skinfold thickness in piglets after intradermal injection of the antigen in both groups indicates a stronger response in the experimental group than in controls. In this study, the response was strong-

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est 24 h after injection, but in our earlier study (order-not yet published) the maximum increase of skinfold thickness at the site of injection of *H. somni* 270 kDa protein or supernatant of sonicated whole *H. somni* cells occurred in calves immunised with whole-cell *H. somni* vaccine (Somnuvac) after 72 h or later. The extent of the cellular response against *H. somni* antigens measured using the skin test correlated with the protection against respiratory tract diseases. In contrast, in mice sensitised with 2,4-dinitro-1-fluorobenzene the maximum skin reaction occurred at 24 h after intradermal injection of the antigen (Dhabhar et al. 2000).

*Histophilus somni* infections cause important losses in calves and young feedlot cattle, because of respiratory tract infections and meningoencephalitis (Kennedy et al. 1960; Balbierz et al. 1985), and are considered to be one of the major bacterial causes of losses in the course of respiratory organ diseases (BRDC) (Corbeil 2008). To avoid losses, many immunoprophylactic procedures have been developed and applied in beef and dairy cattle. In the present study, we demonstrated that vaccination with *H. somni* rHsp60 triggers an immune response in $IgG_1$ and $IgG_2$ classes. Stefaniak (1999) found that subcutaneous or intramuscular immunisation with a vaccine containing bovine *H. somni* whole cells leads to an increase in the intensity of antibody response especially in $IgG_2$ and IgA classes, but that a $IgG_1$, IgA, and IgM class response was typical of natural infection of the mucous membranes. Animals showing a specific immune response in the $IgG_2$ class were protected against experimental infection, whereas animals that reacted in the IgM and $IgG_1$ classes developed disease (Widders et al. 1989). Vaccination of piglets resulted in a beneficial production effect in the experimental group, which exhibited increased daily weight gains after the immunisation cycle. The observed weight gains in this study were very high in the experimental group compared to the reports of other authors (Falkowski et al. 2004; Ramesh et al. 2009). Two weeks before experimental immunisation piglets were treated with *Actinobacillus pleuropneumoniae* vaccine. Therefore, the observed higher gains in the experimental group may indicate a combined effect of administering both biopreparates.

Estimations of haptoglobin concentrations in piglets showed that no important changes occurred during the study. In calves, the average concentrations of both acute proteins were lower in the experimental than in control animals. Nevertheless, the effects of vaccination on the occurrence and intensity of inflammation was not confirmed.

The calves in this study were situated on a dairy farm with a persistent occurrence of respiratory tract infections. Calves selected for the study were in close contact with some ill individuals present in the same pen. Due to the lack of a specific diagnosis, it is difficult to unambiguously state that the vaccination had no protective effect on the calves.

In summary, this study shows that *H.somni* rHsp60 protein induces an immune response in hens, calves and piglets and may be a good candidate for a subunit vaccine component against infections caused by Gram-negative bacteria. However, it is necessary to conduct field experiments on larger groups of animals to assess its protective effect.

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