IMMUNOLOGICAL RESPONSE
TO OUTER MEMBRANE PROTEINS OF PASTEURELLA MULTOCIDA SEROTYPE A:3 IN CALVES

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The studies focused on the evaluation of the immunogenicity of the vaccines prepared from outer membrane proteins (OMPs) or iron-regulated outer membrane proteins (IROMPs) of Pasteurella multocida serotype 3 with aluminium hydroxide gel as adjuvant. The vaccines were given twice within a 14-day period. The sera were sampled before vaccination and weekly for 6 weeks thereafter. The immunogenicity of the vaccine was evaluated by the immunoblotting and ELISA. It was found that the immunization with the complexes of OMPs i IROMPs antigens obtained from the strain of serotype A:3 stimulated the production of specific antibodies.

Key words: calf, Pasteurella multocida, outer membrane protein, vaccine, immunization.

Pasteurella multocida causes pasteurellosis, so-called Bollinger’s disease (serotype B:2), in older cattle, and it enters for polyethiological diseases of calf’s respiratory tract (serotype A:3) (4, 9). Numerous infection factors such as viruses, mycoplasms, bacteria, and first of all Pasteurella multocida, Pasteurella haemolytica and Haemophilus somnus take part in enzootical pathogenesis of bronchopneumonia in calves.

The immunological methods along with stress reduction play a key role in the program of bronchopneumonia prophylaxis. An active specific immunostimulation is the most effective method to protect calves against the infection and clinical development of the disease.

The aim of the present studies was to prepare subunit vaccines comprising outer membrane proteins (OMPs) or iron-regulated outer membrane proteins (IROMPs) of P. multocida of serotype A:3 and to evaluate the immune response in calves vaccinated with the antigens.

Material and Methods

Bacterial strains. P. multocida strain P94 (serotype A:3) isolated from calf affected with bronchopneumonia was used (3).
Preparation of OMPs and IROMPs for SDS-PAGE. The examined strain was cultured on BHI medium (swine organ extract, IDG) and on BHI medium supplemented with 150µM 2,2'-dipyridyl. The OMPs were obtained according to the modified method of Morton et al. (13) as described in the previous publication (3). The amount of proteins was evaluated using the Protein Assay Kit (Sigma).

Immunization of calves. Experiments were carried out on 15 mixed breed calves weighing 100 kg. The animals were kept in a traditional bedding cow-shed. The experimental protocol involved vaccination with the following antigen complexes:
- OMPs from P94 strain amounting 2 mg – group 1,
- IROMPs from P94 strain amounting 2 mg – group 2.

Each dose of the vaccine (2 mL) contained suspension of proteins in PBS with a 10% addition of aluminium hydroxide gel as adjuvant. The animals were immunized sc twice at a 14-day interval, in the neck fold. The controls were given PBS with the addition of adjuvant. The blood was taken before the first immunization, and 7, 14, 21, 28, 35 and 42 d thereafter. The sera were stored at –20°C till tested.

The immunogenicity of the administered antigens was evaluated by the immunoblotting and ELISA.

Separation in SDS-PAGE. Electrophoretical separation of the proteins was performed in 10% polyacrylamide gels according to Laemmli (11) as described in the previous study (3).

Immunoblotting. The immunoblotting was performed using the calf sera obtained after OMPs or IROMPs immunization (19).

ELISA. Microplates were coated with antigen complexes identical to OMPs or IROMPs used in immunization of calves, at a concentration of 1 µg/mL. This test was conducted similarly to that reported earlier (1).

Statistical estimation of results. Obtained results were analysed statistically using Student’s t-test (Statistica 5.0 application).

Results

The experiment was carried out to evaluate the immunogenicity of the antigenic complexes of OMPs from P. multocida P94 (serotype A:3) cultivated on BHI medium and BHI medium supplemented with iron chelate (IROMPs) with immunoblotting and ELISA.

Fig. 1 shows the picture of electrophoretic separation of OMPs and IROMPs. The electrophoregrams of serotype A:3 strains demonstrate the presence of proteins with molecular weights of 22, 28, 34, 37, 42, 46, 56, 64, 75 and 84 kDa. The highest value of OD was found in the fractions of 34 and 84 kDa. The strain cultured on a medium supplemented with 2,2’-dipyridyl produced additional proteins with molecular weights of 98 and 104 kDa or an increased OD expression in band corresponding to molecular mass of 84 kDa (3).

Figs 2 and 3 show the results of the detection of specific antibodies against outer membrane proteins in the sera of calves immunized with the complexes of OMPs and IROMPs antigens. As can be seen in the pictures, the sera of two immunized groups of calves revealed the presence of the specific antibodies to the protein fractions found in the examined strain. The densitometric analysis of the detectability results of the anti-IROMPs antibodies demonstrated the presence of additional protein fractions and bands with enhanced expression.
Fig. 1. Electrophoretic profiles of the OMPs of *P. multocida* strain of serotype A:3: P94 (line 2) and P94+d (line 3). Lines 1 and 4 comprise the weight standards.

Fig. 2. Detection of the immunogenic OMPs of *P. multocida* after vaccination of a calf (No 4) with OMPs from strain P94 (serotype A:3): before immunization (line 2) and 7 d, 14 d, 21 d, 28 d, 35 d and 42 d after immunization (lines 3 to 8). Lines 1 and 9 comprise the weight standards.
Fig. 3. Detection of the immunogenic OMPs of *P. multocida* after vaccination of a calf (No 9) with OMPs from strain P94+d (serotype A:3): before immunization (line 2) and 7 d, 14 d, 21 d, 28 d, 35 d and 42 d after immunization (lines 3 to 8). Lines 1 and 9 comprise the weight standards.

Fig. 4. Detection of the immunogenic OMPs of *P. multocida* strain P94+d (serotype A:3) with the use of control calf serum (No 22) within the experimental period. Lines 1 and 9 comprise the weight standards.

In contrast to the sera of vaccinated calves, the sera of non-immunized ones can hardly recognise the OMPs of *P. multocida* strains. Fig. 4 illustrates immunoblotting pictures of the control calf serum with the OMPs of the examined *P. multocida* strain. A normal calf serum very hardly recognised the OMPs and IROMPs of *P. multocida* strains within the whole experimental period.
Fig. 5. ELISA. Anti-OMPs antibodies of *P. multocida* in the serum of calves vaccinated with OMPs and IROMPs from strain P94 (serotype A:3): I – before vaccination, II – 7 d after the first vaccination, III – 14 d after the first vaccination,
IV – 21 d after the first vaccination, V – 28 d after the first vaccination, VI – 35 d after the first vaccination.

The ELISA permitted us to detect the specific antibodies against OMPs and IROMPs of *P. multocida* strains both in the control and vaccinated animals. The mean absorbance values before and after vaccination and those in the controls are illustrated graphically in Fig. 5.

Fig. 5 depicts an increase in the level of anti-OMPs antibodies specific to the serotype in the groups immunized with the above antigens in comparison to that found before immunization; this increase could be seen as early as 7 d after the first vaccination. The level of anti-OMPs antibodies continued to increase up to 14 d after the first vaccination. An additional increase in absorbance was found after the second vaccination. The highest OD value was noted at the fifth blood sampling (2 weeks after the second vaccination) and since then mean absorbance values of the sera from the immunized animals retained at similar levels till the end of the experiment (6 weeks after the first vaccination).

The two immunized groups of animals revealed a statistically significant increase of absorbance at each time interval following day 7 after the first vaccination in comparison to the mean absorbance value found in the sera before immunization.

Absorbance values noted in the sera of the control calves treated with a diluted adjuvant did not differ in a statistically significant manner within the whole experimental period.

**Discussion**

*P. multocida* is the pathogen commonly present in enzootic bronchopneumonia of calves. Kielstein and Schimmel (10) isolated the microorganism from 50.4% of enzootic bronchopneumonia cases. Bovine pneumatic pasteurellosis (8) has been recognised as a major economic problem for European and North American cattle industries. Preliminary attempts to prevent the disease were complicated by incomplete knowledge of the causative organisms. Bacterins of *Pasteurella multocida* and *Pasteurella haemolytica* (2, 17), bacterin-virus combinations (8), live vaccines (5), and antigen extracts (17) were used for immunization.

The outer membrane proteins are potential candidates as immunogens to prepare subunit vaccines. The immunogenicity of selected outer membrane proteins of *P. multocida* was found in experiments involving rabbits (12) and chickens (21). Lu et al. (12) reported that the protective efficacy of a vaccine from the *P. multocida* A:3 outer membrane proteins was evaluated in rabbits. The results indicate that this vaccine provides a significant protection in rabbits against homologous challenge. Confer et al. (7) found that an intranasal vaccination of rabbits with *P. multocida* A:3 outer membrane proteins containing IROMPs stimulated immunity against experimental pneumatic pasteurellosis. On the other hand, Zhang et al. (21) documented protection of chickens vaccinated with a 35.5 kDa *P. multocida* cell-membrane antigen.

Few works on the immunogenic role of the OMPs of *Pasteurella* strains in calves were reported. Successful efforts concerning the immune response to the vaccination with outer membrane protein fractions of *Mannheimia* (*Pasteurella*) *haemolytica* were described in cattle by Morton et al. (14), Sreevatsan et al. (18), Puchalski et al. (16) and Wernicki et al. (20). Morton et al. (14) observed that administration of an N-lauroylsarcosine-derived outer membrane protein fraction of *P. haemolytica* A1 induced a protective response in calves against intrathoracic challenge
exposure with the homologous serovar. Sreevatsan et al. (18) investigated P. haemolytica subunit vaccines against pneumonic pasteurellosis in cattle. The results of their study demonstrate the usefulness of IROMPs in vaccine preparations to the development of a protective immune response in experimental pneumonic pasteurellosis. In Poland, Wernicki et al. (20) immunized calves with a subunit vaccine containing outer membrane proteins of M. haemolytica serotype 1. This vaccination stimulated the induction of a high level of specific antibodies anti-OMPs detected by ELISA. The immunogenic properties of these proteins were supported by immunoblotting reactions with the sera of immunized calves. The analysis of the pictures observed on a nitrocellulose film provided evidence of the presence of specific reactions for OMPs antigens.

Serum antibodies to OMPs are necessary for protection of cattle against P. multocida serotype A:3 which takes part in the pneumonia evoking. In Confer et al. study (6) cattle with experimentally induced resistance to a transthoracic challenge with live P. multocida had high antibody responses to P. multocida OMPs detected by immunoblotting and ELISA. Moreover, a protective action of the OMPs of P. multocida serotype B:2 against haemorrhagic septicaemia was found (15).

In our study, calves treated with OMPs and IROMPs vaccines containing antigens of the Polish isolate of serotype A:3 stimulated antibodies against specific outer membrane proteins detected by immunoblotting. ELISA test made on microplates coating with OMPs and IROMPs antigens also allowed us to find the increase of antibodies against specific outer membrane proteins in the sera of the immunized animals.

Summing up, OMPs and IROMPs P. multocida serotype A:3 vaccines possess satisfactory immunogenic properties.

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References


