EVALUATION OF IMMUNOGENICITY OF OUTER MEMBRANE PROTEINS OF \textit{PASTEURELLA MULTOCIDA} SEROTYPE B:2,5 IN CATTLE

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The studies involved \textit{P. multocida} of serotype B:2,5 isolated from animal affected with haemorrhagic septicaemia. Outer membrane proteins (OMPs) and iron-regulated outer membrane proteins (IROMPs) were prepared by extraction with 1% sarcosyl from the strain cultured on BHI medium and on a medium supplemented with 2,2’-dipyridyl. The antigen complexes were used to immunize calves. Each vaccine dose comprised 400 µg of OMPs suspension and 10% addition of aluminium hydroxide gel as adjuvant. The animals were injected twice at a 14-day interval. The blood was collected on the day of the first vaccination and then weekly for 6 weeks. The immunization of calves with the complexes of OMPs and IROMPs antigens caused occurrence of the specific antibodies detected with the immunoblotting and ELISA.

Key words: cattle, \textit{Pasteurella multocida}, outer membrane protein, vaccine, immunization.

\textit{Pasteurella multocida} is a dangerous pathogen for numerous mammal and bird species. This pathogen can cause in cattle the primary infection called haemorrhagic septicaemia (HS) or Bollinger’s disease in older animals. According to the OIE opinion HS is regarded as an acute disease with a high mortality rate in cattle and buffaloes (2, 7, 12). Bollinger’s disease is caused by strains with capsule B or E according to Carter (6) and somatic antigen 2 according to Heddleston (14).

Haemorrhagic septicaemia occurs in Africa, Asia, Central and South America. In Europe the cases of HS were found in Portugal, Greece and Romania. Enzootic focuses were reported in Spain, Belarus, Russia, Estonia, Latvia and Ukraine. In Poland HS cases were noted in the Masuria-Warmia Region and Masovia Region.

Limited information is available on antigens of \textit{P. multocida} that stimulate immunity in cattle. In haemorrhagic septicaemia, capsular antigen, LPS or LPS-protein complex, and outer membrane proteins, including iron-regulated outer membrane proteins, are effective immunogens for serogroups B and E (7).

The purpose of the present studies was the use of antigenic complexes of outer membrane proteins (OMPs) and iron-regulated outer membrane proteins (IROMPs), prepared from strain of serotype B:2,5 for the immunization of calves and evaluation of their immunogenic properties.
Material and Methods

**Bacterial strain.** *P. multocida* strain P260 (serotype B:2,5) isolated from animal affected with haemorrhagic septicaemia was used (5, 16).

**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 150 µM 2,2'-dipyridyl. The cultures were incubated for 18 h at 37°C. OMPs were obtained according to the modified method of Morton *et al.* (19) by extraction with 1% sarcosyl solution (sodium salt of N-Laurylo-Sarcosine) of bacterial cells previously exposed to ultrasounds. 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 antigen complexes of:
- OMPs from P260 strain amounting 400 µg – group 1,
- IROMPs from P260 strain amounting 400 µg – 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 an 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 evaluation of the immunogenicity of the antigens administered was performed with the immunoblotting and ELISA.

**Separation in SDS-PAGE.** Electrophoretical separation of the proteins was performed in 10% polyacrylamide gels according to Laemmli (17), as described in the previous study (5).

**Immunoblotting.** The immunoblotting was performed using the calf sera obtained after OMPs or IROMPS immunization and diluted at the 1 to 100 ratio. Anti-bovine IgG peroxidase conjugate (Sigma) was applied; 4-chloro-1-naphthol (Sigma) served as a substrate (25).

Gels and immunoblotting pictures were photographed using an ImageMaster VDS apparatus (Pharmacia Biotech) and subjected to the Analysis of One Dimensional Separation and Dot Blots program.

**ELISA.** Microplates were coated with the same antigen complexes (1µg/mL) as those of the OMPs or IROMPs used in immunization of calves. The test was conducted similarly to that described before (4).

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

**Results**

The experiments were carried out to evaluate, with the immunoblotting and ELISA, the immunogenicity of the antigen complexes of the OMPs of *P. multocida* strain P260 (serotype B:2,5) cultured on BHI medium and the same medium supplemented with the iron chelate (IROMPs).

The electrophoregrams of the separation of the OMPs and IROMPs are shown in Fig. 1.
The electrophoretical separation of OMPs revealed protein fractions with molecular weights amounting 22, 28, 32, 36, 46, 50, 58, 64 and 84 kDa. The highest OD value was demonstrated in the 32 kDa band. The strain harvested on the medium with iron chelate produced additional proteins about 102 and 110 kDa whereas the 84 kDa fraction revealed an increased OD expression in comparison with the iron-enriched medium (5).

Figs 2 and 3 show the results of the detection of the antibodies specific to outer membrane proteins in the sera of calves immunized with the antigen complexes of OMPs i IROMPs. The results showed that the sera of both immunized group of calves comprised the antibodies specific 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 an increased expression.

In contrast to the sera of vaccinated calves, the sera of non-immunized calves could hardly distinguish the OMPs of P. multocida. Fig. 4 shows the immunoblotting pictures of the serum of a control calf with the OMPs of the examined strain. A normal calf serum could hardly recognise the OMPs and IROMPs of P. multocida during the whole experimental period.
Fig. 2. Detection of the immunogenic OMPs of *P. multocida* after vaccination of a calf (No 13) with OMPs from strain P260 (serotype B:2,5): 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 19) with OMPs from strain P260+d (serotype B:2,5): 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.
The ELISA permitted us to detect the presence of the specific antibodies to the OMPs and IROMPs of *P. multocida* in the sera of the controls and immunized calves.

Table 1 shows the mean absorbance values of the sera before vaccination and after immunization; the table also comprises the absorbances of control sera.

The results presented in Table 1 indicate that in the groups immunized with the tested antigens an increase in the level of the anti-OMPs antibodies specific to the examined strain could be already seen at the second blood sampling (7 d after the first vaccination) as compared to that before immunization. The level of these antibodies continued to increase up to 14 d after the first immunization. An additional increase in the absorbance occurred after the second vaccination. The highest OD values were found at the fifth blood sampling (2 weeks after the second vaccination). Then, the mean absorbance value of the sera of all immunized calves attained nearly the same level till the end of the experiment (6 weeks after the first vaccination).

The two immunized groups of animals demonstrated a statistically significant increase in serum absorbance on day 7 after the first vaccination and continued until the end of the experiment in comparison to the mean absorbance value of the sera collected before immunization.

The absorbance values of the sera collected from the control calves treated with a diluted adjuvant failed to differ significantly and attained a similar level within the whole experimental period.
Table 1
ELISA. Anti-OMPs antibodies of *P. multocida* in the sera of calves vaccinated with OMPs and IROMPs from strain P260 (serotype B:2,5)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Examined sera*</th>
<th>Mean value of absorbance</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>P260</td>
<td>I</td>
<td>0.295 ± 0.0618</td>
<td>0.220 – 0.367</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.383 ± 0.0685</td>
<td>0.295 – 0.474</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.584 ± 0.0484</td>
<td>0.508 – 0.640</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>0.652 ± 0.0510</td>
<td>0.586 – 0.728</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.687 ± 0.0594</td>
<td>0.622 – 0.775</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>0.673 ± 0.0156</td>
<td>0.650 – 0.691</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>0.657 ± 0.0372</td>
<td>0.609 – 0.693</td>
</tr>
<tr>
<td>P260+d</td>
<td>I</td>
<td>0.225 ± 0.0434</td>
<td>0.185 – 0.288</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.358 ± 0.0806</td>
<td>0.292 – 0.467</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.656 ± 0.1117</td>
<td>0.539 – 0.788</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>0.759 ± 0.1438</td>
<td>0.586 – 0.940</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.801 ± 0.1062</td>
<td>0.652 – 0.944</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>0.780 ± 0.1023</td>
<td>0.672 – 0.910</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>0.787 ± 0.0547</td>
<td>0.734 – 0.879</td>
</tr>
<tr>
<td>Control group</td>
<td>I</td>
<td>0.202 ± 0.0331</td>
<td>0.152 – 0.240</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>0.206 ± 0.0201</td>
<td>0.188 – 0.238</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0.232 ± 0.0183</td>
<td>0.211 – 0.261</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>0.203 ± 0.0148</td>
<td>0.179 – 0.219</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>0.184 ± 0.0115</td>
<td>0.164 – 0.193</td>
</tr>
<tr>
<td></td>
<td>VI</td>
<td>0.191 ± 0.0101</td>
<td>0.180 – 0.207</td>
</tr>
<tr>
<td></td>
<td>VII</td>
<td>0.215 ± 0.0171</td>
<td>0.196 – 0.238</td>
</tr>
</tbody>
</table>

*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
VII – 42 d after the first vaccination

Discussion

In all countries where pasteurellosis occurs vaccinations are considered as an effective means of controlling this disease (2, 7, 12). Local isolates are usually used for vaccine preparation. Initially, simple bullion bacterins were used for immunization. Additionally, precipitated alum or aluminium hydroxide gel vaccines were manufactured (8, 9). The use of the vaccine with an oil adjuvant (water-oil emulsion) with a dense bacterin and mineral oil as water and oil phases, respectively, were advantageous in controlling this disease (2, 4, 20). Also several authors immunized cattle with live vaccines (10, 13). In order to improve the immunogenicity of the
vaccines, the causative organism has been fractionated and various cell surface components have been studied.

Outer membrane proteins of many Gram-negative bacteria are known to function as protective antigens. In *E. coli* the OMPs was first reported to serve as a receptor for high iron binding system responsible for providing protection in turkeys against *coli*septicaemia (3). Later, protective ability of OMPs was also demonstrated with *Pseudomonas aeruginosa* (22) and *Shigella* species (1). Among pastuerellae, OMPs from *P. haemolytica* have been found to be associated with immunity in cattle against pneumonic pasteurellosis (19). In recent years strains of *P. multocida* have been reported to possess membrane proteins which are protective to rabbits (11, 18) and poultry (26). Few reports considered the immunogenic properties of OMPs from *P. multocida* strain belonging to B:2,5 (5, 15, 21, 23, 24).

Srivastava (23) studied the role of OMPs from this serotype in providing protection of animals against *P. multocida* infection. Mice immunized with whole cell vaccine showed at challenge a survival rate of 84% whereas in mice given OMPs vaccine, it was 67%. Though the level of protection against OMPs was lower than the whole cell vaccine, still it was above the acceptable level, i.e. 67%. None of the non-immunized control mice resisted the challenge. This experiment gave the evidence that OMPs vaccine was protective to laboratory animals. This was further confirmed by employing the passive mouse protection assay. Eighty percent of the mice treated with antisera against whole cells or OMPs were protected against the challenge infection.

In the next study Srivastava (24) found that vaccines prepared from *P. multocida* serotype B:2 cells grown under iron-restricted and iron-sufficient conditions did not differ from each other in providing protection in mice and rabbits. However, the antibody titres detected in rabbits were significantly higher with the vaccine consisting of *P. multocida* cells grown under restricted than with those grown under iron-sufficient conditions. Similar results were also observed in cattle. It was concluded that a vaccine consisting of *P. multocida* cells grown under iron-restriction may be more effective than the vaccine prepared from cells grown conventionally.

Also Kennet *et al.* (15) observed protection of mice vaccinated with OMPs from *P. multocida* type B:2. They reported that OMPs prepared in iron-deficient medium afforded better protection against experimental challenge exposure than did OMPs prepared in iron-sufficient medium.

Subunit vaccines comprising OMPs from *P. multocida* serotype B:2 were used by Pati *et al.* (21) to immunize buffalo calves. Immunoblotting suggested that proteins of 44, 37 and 30 kDa were the major immunogens. Antibody production started from day 7 after vaccination and ELISA titres rose significantly at day 14. The titres further increased at day 21 but remained unchanged at day 26. All buffalo calves given OMPs vaccine survived challenge with virulent *P. multocida*. Results suggested that OMPs were protective and could be used in vaccines against haemorrhagic septicaemia.

In our experiment we used OMPs and IROMPs antigen complexes of *P. multocida* serotype B:2,5 to immunize calves. The occurrence of antibodies against specific outer membrane proteins detected by immunoblotting and ELISA in the sera of immunized cattle argued for a beneficial immunogenicity of the vaccines.
References


