Experimental study of pathogenicity of serogroup F in rabbits

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Experimental study of pathogenicity of Pasteurella multocida serogroup F in rabbits

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Abstract

The role of *Pasteurella multocida* serogroup F in inducing disease in rabbits was investigated in this study. Three groups of 12 *Pasteurella*-free rabbits each were intranasally (i.n.), subcutaneously (s.c.), and perorally (p.o.) challenged, respectively. Six rabbits of each group were immunosuppressed using dexamethasone.

Eight rabbits (four of them immunosuppressed) inoculated i.n. showed symptoms of respiratory distress resulting in respiratory failure and died or were euthanized in the terminal stage of the disease 3–6 days post-infection (p.i.). The main pathological findings were fibrinopurulent pleuropneumonia (immunocompetent rabbits) or diffuse haemorrhagic pneumonia (immunosuppressed rabbits). Septicemic syndrome ending with shock occurred in 11 rabbits (six of them immunosuppressed) inoculated s.c., which died or were euthanized in the terminal stage of the disease 2–3 days p.i. The most significant pathological findings were extensive cutaneous and subcutaneous lesions. All of the p.o. inoculated rabbits survived the challenge showing no clinical signs of the disease and no macroscopic lesions.

The observations in this study indicate that in addition to serogroups A and D of *P. multocida*, serogroup F also can be highly pathogenic for rabbits and therefore might be a cause of considerable economic loss in commercial rabbit production.

Keywords: *Pasteurella multocida*; serogroup F; rabbit; pasteurellosis

Introduction

Pasteurellosis caused by *Pasteurella multocida* is one of the most significant bacterial diseases of rabbits and causes considerable economic losses in large production units
throughout the world (Takashima et al., 2001). The disease is characterised by various clinical
symptoms, including respiratory distress, genital infections, abscesses, otitis, and septicaemia,
but infection by *P. multocida* can also appear without manifesting any clinical signs (DeLong
and Manning, 1994). To date, only serogroup A and, to a lesser extent, serogroup D strains of
*P. multocida* have been considered as causative agents of rabbit pasteurellosis (Kawamoto et
al., 1990; Dabo et al., 1999).

*P. multocida* serogroup F was first described in turkeys in the USA (Rimler and Rhoades,
1987). Isolates of this serogroup originated mainly from birds in North America (Rhoades and
Rimler, 1987; Wilson et al., 1995) but they also have been found in birds in other parts of the
world (Jonas et al., 2001). Serogroup F has been predominantly described as a causative agent
of fowl cholera (Rimler, 1987; Jonas et al., 2001) and for a long time it was quite uncommon
in mammalian hosts. Nevertheless, findings of this serogroup in some mammalian species
(i.e., pigs, sheep and cattle) showing various clinical symptoms have been newly reported by
several authors in different parts of the world (Moreno et al., 2003; Davies et al., 2003; Catry
et al., 2005). Moreover, we previously reported on a relatively high incidence of serogroup F
among rabbit nests in the Czech Republic (Jaglic et al., 2004) and among a total of eight
serogroup F rabbit isolates we found seven distinct pulsed-field gel electrophoresis types
(Jaglic et al., 2006). This could indicate that the occurrence of serogroup F in rabbits is not the
consequence of a coincidental spreading of one clone to a rabbit host. Because of incomplete
data concerning the health and/or epizootiological status of the affected rabbit nests, the aim
of this study was to evaluate whether serogroup F can be a new potential agent of the rabbit
pasteurellosis or whether it is only a commensal bacterium within a rabbit host.

Materials and methods
Bacterial strain and culture media

*P. multocida* serogroup F rabbit isolate J-4103 (CAPM 6431) was selected for the experimental infection of rabbits. It was of ribotype 1, the predominant ribotype of the serogroup F rabbit isolates (Jaglic et al., 2004). Prior to the infection, the isolate was passaged three times in chicken embryos. The isolate was routinely grown at 37 °C on blood agar (Blood Agar Base No. 2, HiMedia, India) containing 5% sheep blood.

Experimental infection of rabbits

Rabbits and housing. Animal handling complied with the legal directives of the Czech Republic and with the institution’s policy. Specific pathogen-free New Zealand White rabbits of both sexes and weighing 1.8 to 2 kg were obtained from Charles River Laboratories, Germany GmbH. The rabbits were individually housed in stainless steel cages placed in isolated rooms with a barrier regime and independent ventilation. The rabbits were fed pellets (a complete food mixture for rabbits 4–12 weeks old, Bonagro, Czech Republic) *ad libitum* and had free access to water. The rooms were kept at a temperature of 21 °C, relative humidity of 40–60%, and ventilation of approximately 15 air changes per hour. Before infection, nasal, conjunctival and rectal swabs as well as whole blood were collected for bacteriological examination to ensure *Pasteurella*-free status of the rabbits. Sera were tested for the presence of *P. multocida* IgG and IgM antibodies by an ELISA described below.

Experimental design. A total of 57 rabbits were divided into three challenged groups of 12 rabbits each (according to the route of inoculation) and one negative control group of 21
rabbits. Each group was placed in a separate room. Six rabbits from each challenged group
and 12 rabbits from the control group were immunosuppressed by intramuscular
administration of dexamethasone (Dexameth, Medochemie LTD, Cyprus) at a dose of 2 mg/kg
of body weight. The dose had been established in our preliminary experiment. Dexamethasone was administered three times in 24-hour intervals, and the last application
was 24 hours post infection (p.i.).

Challenge. The rabbits were challenged with $6 \times 10^4$ CFU in 1 ml of phosphate saline buffer
(PBS) as follows: (i) intranasally (i.n.) by direct application of the bacterial suspension into
nasal cavities using an aerosol applicator, (ii) subcutaneously (s.c.) in the caudal region of the
left scapula, and (iii) perorally (p.o.) between the buccal wall and molars. To avoid aspiration
of the inoculum during the p.o. application, a neutral sterile puree (wheat flour and semolina)
was added to the bacterial suspension. The negative control rabbits were inoculated with 1 ml
of PBS alone as described above (seven rabbits per each route). The clinical status of the
challenged and control rabbits, including body weight, rectal temperature, attitude, and chest
murmur, was monitored twice daily. Challenged rabbits which were caught up in the terminal
stage of the disease and those which survived until 15 days p.i. were euthanized by bleeding
from v. jugularis under ketamine-xylazine narcosis. At the same time, three
immunosuppressed (IS), and two to three immunocompetent (IC) rabbits of the negative
control group were also sacrificed.

Pathology. All challenged and control rabbits were examined for the presence of gross and
histological lesions. Lungs, heart, liver, spleen and kidneys were histologically monitored in
all the rabbits. A part of the changed skin and subcutis of the subcutaneously inoculated
rabbits, as well as parts of the stomach, ileum (in the region of Peyer’s patches), caecum and
colon wall (including mesenteric lymph nodes) of the perorally inoculated rabbits were also histologically examined. The samples were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin-eosin and toluidine blue.

**Bacteriology.** For the bacteriological examination, nasal, conjunctival and rectal swabs as well as whole blood were sampled from the dead rabbits, from rabbits in the terminal stage of the disease prior to their euthanasia, and also 7 and 15 days p.i. from rabbits that survived the challenge (including the negative control rabbits). The blood samples (1 ml) were drawn from the *a. auricularis caudalis* except in the cases of the dead rabbits, from which the blood was obtained by sterile cardiac puncture. Also examined were 1 ml of whole urine (sterile puncture of the bladder) and impression smears of lung, myocardial, liver, spleen and kidney tissues that were obtained from all the challenged and control rabbits at necropsy. Impression smears of cut sections were made on agar plates and streaked for isolation. In the case of the peroral infection, a segment (wall and contents) of the stomach, ileum (the region of Peyer’s patches), caecum, and colon, as well as mesenteric lymph nodes, were also included. The samples were cultivated on non-selective and selective (396 U of neomycin and 30 U of bacitracin in 100 ml of the culture medium; Pamycon Biotika, Slovakia) blood agars (Blood Agar Base No. 2, HiMedia, India) containing 5% sheep blood. Suspected bacterial colonies were examined by species- and capsular-specific PCRs (Townsend et al., 1998; Townsend et al., 2001).

**ELISA.** Sera obtained from the challenged and control rabbits were examined for the presence of *P. multocida* IgG and IgM antibodies. They were collected with the whole blood at the intervals described above. Sera from the dead rabbits were not examined.
Bronchoalveolar lavages (BALs) obtained from all the challenged and control rabbits at necropsy were examined for the presence of *P. multocida* IgA antibodies. The lungs were washed with 15 ml of PBS and BALs (supernatants) were separated at 8000 x g for 10 min. The positive control serum and BAL were prepared by intramuscular and intranasal immunisation of New Zealand White rabbits with the serogroup F *P. multocida* isolate J-4103 inactivated by formalin. Serum and BAL obtained from a *Pasteurella*-free rabbit were used as negative controls.

The antigen used in the assay was prepared from the *P. multocida* J-4103 isolate as described by Kawamoto et al. (1994). An indirect ELISA was carried out as described earlier (Klaassen et al., 1985). HRP-conjugated goat anti-rabbit IgG-Fc antibodies (Bethyl Laboratories Inc., USA) diluted at 1:40,000, and HRP-conjugated goat anti-rabbit IgM-µ antibodies (Abcam Ltd., UK) diluted at 1:10,000 were used for determination of the *P. multocida* IgG and IgM antibodies, respectively. HRP-conjugated goat anti-rabbit IgA-λ antibodies (Abcam Ltd., UK) diluted at 1:40,000 were used for determination of the *P. multocida* IgA antibodies. The positive threshold (cut-off) was calculated as three standard deviations above the mean optical density for serum and BAL samples obtained from the *Pasteurella*-free rabbits (all rabbits prior to challenge and those belonging to the negative control group).

**Haematology.** Total leukocyte counts in the blood of the euthanized rabbits were determined using the Digicell 500 cell counter (Contraves AG, Switzerland). Differential leukocyte counts were calculated from blood smears stained with May-Grünwald and Giemsa-Romanowski.

**Lymphocyte subsets.** Lymphocyte subsets in the euthanized rabbits were quantified by
indirect staining and flow cytometry as described earlier (Jeklova et al., 2007). Briefly, the
blood was stained using a whole-blood lysis technique. Organ samples (spleen, as well as
mesenteric and tracheobronchial lymph nodes) were collected into RPMI 1640 medium and
cell suspensions were prepared by carefully teasing the lymphoid tissue using two forceps. All
cell suspensions were filtered through a fine nylon mesh. Commercially available monoclonal
antibodies (mAbs) were used for detecting lymphocyte subsets. For quantifying
B-lymphocytes, R-PE conjugated mouse anti-human CD79α (clone HM57, DakoCytomation,
Denmark) was used. Cell suspension was fixed and permeabilized with IntraStain kit
(DakoCytomation, Denmark) and labelled according to the manufacturer’s instructions. T
cells and their subsets were detected using the following anti-rabbit mAbs: anti-CD4
(RTH1A, IgG1), anti-CD8 (ISC27A, IgG2a), anti-panT2 (RTH21A, IgG1) and anti-CD45
(ISC18A, IgG2a) (VMRD Inc., USA). As a secondary immunoreagent, FITC (dilution 1:100)
or R-PE (dilution 1:500) labelled goat anti-mouse conjugates of appropriate subisotypes
(Southern Biotechnology Assoc., Inc., Birmingham, AL) were used. Propidium iodide was
used to stain DNA of dead and damaged cells to exclude these from analysis. Data were
acquired using a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, CA)
and CELLQuest™ software. Gating was based on forward and right angle scatter signals.

Lymphocyte activity. The activity of lymphocytes isolated from the blood, spleen, and
mesenteric and tracheobronchial lymph nodes of the euthanized rabbits was determined using
the mitogen-driven lymphocyte transformation test. The density of the mononuclear cell
suspensions obtained by gradient centrifugation (Histopaque® -1077, Sigma) was adjusted to
10^6 per ml of RPMI 1640 medium supplemented with 10% precolostral calf serum and
antibiotics. Cells were incubated in the presence of concanavalin A mitogen (Con A, Sigma,
St. Louis, USA) at a concentration of 10 µg/ml. Cells without added mitogen served as
control samples. The microplates were incubated at 37° C in an atmosphere of 5% CO₂ for 3 days. Twenty hours before harvesting (FilterMate Harvester, Packard Bioscience Instrument Company, Meriden, USA), 50 µl of medium with ^3^H-thymidine (5 µCi/ml) was added. The incorporation of ^3^H-thymidine was analysed by a microplate scintillation & luminescence counter (TopCount NXT™, Packard Bioscience Instrument Company, Meriden, USA). The results were expressed in stimulation indices, which were calculated as the ratio of counts per minute in stimulated samples versus non-stimulated controls.

**Results**

*Experimental infection of rabbits*

Before their use in the experiment, all the rabbits were confirmed as *Pasteurella*-free and were negative for IgG and IgM antibodies against *P. multocida*. During the experiment, all the rabbits belonging to the negative control group remained *Pasteurella* free and serologically negative, and no clinical or pathological signs of the disease were observed among them.

**Intranasal challenge.** Severe respiratory disease, with development of the first distinct clinical signs 2–3 days p.i., occurred in eight rabbits. All of them died or were euthanized in the terminal stage of the disease 3–6 days p.i. (Table 1). The main clinical signs were fever and respiratory distress resulting in respiratory failure (severe dyspnoea was observed in the terminal stage of the disease in two rabbits). Pathological findings from the clinically diseased rabbits are summarised in Table 2 and in figures 1 and 2. In the four remaining rabbits, which survived the challenge, only slight reddening of the conjunctivas and a weak nasal whistle
were sporadically detected. Three of them had weak pulmonary hyperemia accompanied by a slightly increased occurrence of alveolar macrophages.

*P. multocida* was re-isolated from the following sites or organs of the clinically diseased IC/IS rabbits: nose (n = 4/4), conjunctiva (n = 1/0), lungs (n = 4/4), blood (n = 1/3), myocardium (n = 4/4), liver (n = 2/3), spleen (n = 0/2), and kidneys (n = 1/3). In the cases of the rabbits surviving the challenge, the bacterium was isolated from the nose 7 days (all rabbits) and 15 days (only IS rabbits) p.i., and also from the myocardium of one IS rabbit.

Serological positivity for *P. multocida* was mainly detected in the surviving rabbits. All of them were positive for IgG 15 days p.i., whereas positivity for IgM was detected 7 days (one IC and one IS rabbit) and 15 days (two IC and one IS rabbit) p.i. Positivity for IgM was also confirmed in one IS rabbit prior to its euthanasia 6 days p.i. This challenge group was also the only one where IgA positive BALs were found. Clear IgA positivity was confirmed in all four rabbits that survived the challenge (15 days p.i.), whereas weakly IgA positive BALs were detected in two IS rabbits that died 6 days p.i. Lymphopenia (mainly due to a decrease of CD8+ lymphocytes) and depression of lymphocyte activity in the blood were mainly observed in the clinically diseased rabbits (Tables S1–S6; see the Supplemental Data).

**Subcutaneous challenge.** Eleven rabbits, showing the first clinical signs 1 day p.i., became seriously ill and died or were euthanized in the terminal stage of the disease 2–3 days p.i. (Table 1). All the diseased rabbits had an acute septicemic syndrome ending with respiratory symptoms and shock. One of the most significant signs was dark blue to violet or blackish skin rapidly spreading from the place of inoculation and affecting extensive areas of the lateral and ventral abdomen and thorax (including the left thoracic limb). Petechial and
ecchymotic haemorrhages, more numerous in the IS rabbits, were mainly located on the ear skin, parietal and visceral pleura, and pericardium. The main pathological findings observed in the clinically diseased rabbits are shown in Table 3 and Figure 3. The sole IC rabbit that survived the challenge showed no signs of the disease. This rabbit was also negative for *P. multocida* and the only one in this challenge group in which serological positivity was observed (for IgM 7 and 15 days p.i. and for IgG 15 days p.i.).

*P. multocida* was re-isolated from the following sites or organs of the clinically diseased IC/IS rabbits: nose (n = 4/2), urine (n = 0/1), blood, lungs, myocardium, liver, spleen, and kidneys (all rabbits). Among these rabbits, lymphopenia and decreased lymphocyte activity in the blood and monitored lymphoid organs were also observed (Tables S1–S6; see the Supplemental Data).

**Peroral challenge.** All the perorally inoculated rabbits survived the challenge showing no clinical signs of the disease and no macroscopic lesions. Only enlarged Peyer’s patches were found in five of them, the alteration of which was histologically confirmed as catarrhal nonpurulent ileitis. However, such changes may not have been solely induced by *P. multocida*. *P. multocida* was not recovered from any of the p.o. challenged rabbits but positivity for IgM antibodies 7 and 15 days p.i. was found in two of them (one IC and one IS rabbit). Even though all the perorally inoculated rabbits survived, decreased lymphocytes activity was found in the blood and monitored lymphoid organs in both the IC and IS animals. CD4+8+ double positive cells significantly increased in the blood, spleen and tracheobronchial lymph nodes of the IC rabbits (Tables S1–S6; see the Supplemental Data).

**Discussion**
The different routes of inoculation tested in this study resulted in different prevalences of the disease among the challenged rabbits. By the i.n. inoculation we aimed to reproduce a natural route of infection that is the most common in mammalian hosts (Adlam and Rutter, 1989). Subcutaneous inoculation bypasses the external immunological barriers and therefore it is not surprising that the highest morbidity occurred among the s.c. inoculated rabbits. However, after administering a serogroup A *P. multocida* strain at different challenge doses, Lu et al. (1982a) reported a higher mortality among i.n. inoculated rabbits (up to 75%) than in s.c. inoculated rabbits (17–50%). Finally, as serogroup F has been mainly described as a causative agent of fowl cholera and where transmission by aerosol has been reported to be less important (Rhoades and Rimler, 1989), we also tested the peroral route of infection that has been successfully used in an avian host (Pehlivanoglu et al., 1999). This route, however, failed to induce the disease in any of the challenged rabbits, indicating that peroral intake does not play an important role in transmitting the tested isolate.

Pasteurellosis of rabbits may break out under the influence of various stressors that suppress the animals’ immunity (Webster, 1924; DeLong and Manning, 1994). We therefore tried to simulate such immunosuppression by administering dexamethasone, which in our preliminary experiment resulted in lymphopenia associated with lymphocyte redistribution to lymph nodes and other lymphatic tissues (including bone marrow) and depression of lymphocyte activity (unpublished data). As a relatively high prevalence of the fatal disease was observed in both the IC and IS rabbits (except for those p.o. challenged), however, it could be assumed that the isolate tested in this study is highly virulent. Conversely, Lu et al. (1982b), using a serogroup A *P. multocida* strain, observed a significantly higher prevalence of pneumonia in hydrocortisone-treated rabbits (80%) than in non-treated rabbits (20%). The conclusion on
high virulence of the challenge isolate also can be supported by the finding that most of the
i.n. challenged rabbits became seriously ill even though they were inoculated with a relatively
low challenge dose. In general, many other authors have used quite higher challenge doses
($10^6$–$10^{10}$ CFU) in i.n. experimental infections of rabbits with *P. multocida* serogroup A
strains (DiGiacomo et al., 1987; Glavits and Magyar, 1990; Lu et al., 1991; Al Haddawi et al.,
2000). Failure to induce the disease in rabbits by i.n. application of *P. multocida* serogroup A
and D strains at a dose of $10^5$ CFU has been described (DeLong et al., 1992). Percy et al.
(1986) speculated that the upper respiratory tract serves as an effective barrier that protects
the animals from the disease and pulmonary lesions.

In the i.n. challenged rabbits, the most significant pathological changes were observed in the
lungs. In contrast to a study in which essentially the same pulmonary lesions were described
in both hydrocortisone-treated and non-treated rabbits (Lu et al., 1982b), the pathological
findings in lungs of IC and IS rabbits were generally different. In the IC rabbits, and as
already observed elsewhere (Al Haddawi et al., 2000), inflammatory events played an
important role in development of the pulmonary lesions. The fact that just evoking the
inflammatory reaction, which does not have to be induced by the microorganism itself, could
lead to pulmonary lesions was previously reported by Warner et al. (1988), who had used a
derived endotoxin alone. On the other hand, the diseased rabbits (with one exception) having
suppressed inflammatory response had diffusively spread pulmonary haemorrhages without
the characteristic inflammatory lesions. In this challenged group, bacteraemia or septicemic
disease also occurred, since *P. multocida* was found not only in the lungs but also in the blood
and parenchymatous organs. The fact that septicemic pasteurellosis in rabbits could be a fatal
sequel especially to rhinitis and pneumonia has been reported (Webster, 1924).
The most severe pathological changes found in the s.c. challenged rabbits occurred in the cutis and subcutis. To our knowledge, such diffuse and extensive lesions were quite unusual, since mainly localised (sub)cutaneous abscesses have been observed in both naturally infected and subcutaneously injected rabbits (Lu et al., 1982a; DiGiacomo et al., 1983; DeLong and Manning, 1994). Extensive subcutaneous haemorrhages and oedema have been primarily found in ruminants suffering from septicemic pasteurellosis (Carter and De Alwis, 1989; Carrigan et al., 1991). Furthermore, severe cellulitis and dermal lesions caused by \textit{P. multocida} have been mainly described in humans (Brue et al., 1992) and birds (Rhoades and Rimler, 1989; Jeffrey et al., 1993). The rapid expansion and extensiveness of the subcutaneous lesions observed in this study indicate that the challenge isolate, which was previously confirmed as dermonecrotin negative (Jaglic et al., 2005), is highly invasive. In the s.c. challenged rabbits, the lungs were also affected and positive for \textit{P. multocida}. Since the intravenous inoculation of \textit{P. multocida} was reported as a route that could produce acute pneumonic lesions in rabbits (Percy et al., 1986), it could be assumed the lungs may be a target organ without regard to the route of infection.

All of the diseased i.n. challenged rabbits and 54.5\% of those inoculated subcutaneously had \textit{P. multocida} positive nasal swabs. A frequent presence of \textit{P. multocida} in the nose and its general absence in other excretions indicate that nasal discharge could be one of the most important primary sources of the infection. Therefore, airborne spreading or direct contact could be assumed as the main routes of transmission of the disease, as mentioned previously (DeLong and Manning, 1994). \textit{P. multocida} was also recovered from the noses of rabbits that survived the i.n. challenge. While the positivity for \textit{P. multocida} disappeared in the IC rabbits after 7 days p.i., however, those rabbits with suppressed immunity were still positive 15 days p.i. A longer persistence of \textit{P. multocida} in the IS rabbits indicates that immunity-suppressing
conditions (e.g. stress) could contribute to the occurrence of bacilli carriers among the animals.

The serological examination showed that production of the antibodies against *P. multocida* was detectable by ELISA 6–7 days p.i. In general, no significant correlation was observed between the antibody production and immunosuppression. The finding of the *P. multocida* antibodies in those rabbits surviving the i.n. and s.c. challenges shows that the microorganism colonised these animals and interacted with them. On the other hand, an absence of the specific antibodies in most of the p.o. challenged rabbits (accompanied by an absence of *P. multocida* in all of the samples collected) could support speculation that the tested isolate is generally unable to colonise the rabbit host by this route.

Immunological examinations revealed that lymphocyte depression was characteristic for the clinically diseased i.n. and s.c. inoculated rabbits. Transient suppression of immune functions during the acute stage of inflammation prevents excessive damage of self tissues. Uncontrolled production of proinflammatory cytokines may lead to severe immunopathological changes and even be a threat to life. For this reason, it is essential that their production be accurately regulated by a feedback system. The same regulatory system, however, causes the suppression of a mitogen-stimulated lymphocyte transformation. Production of interleukins 10 and 4, together with transforming growth factor-beta, is suspected to be responsible for the anti-inflammatory effects (Oswald et al., 1992). Curiously, however, a decreased activity of lymphocytes was also detected in all p.o. inoculated rabbits even though they survived without observable signs of the disease.

**Conclusion**
Although it they cannot be applied for the *P. multocida* serogroup F in general, the observations in this study indicate that this serogroup can be highly pathogenic for rabbits and therefore might be a cause of considerable economic loss in commercial rabbit production. This speculation should especially be taken into account because of the fact that different clones of the serogroup F already have spread among rabbit nests in the Czech Republic.

Acknowledgements

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Supplemental Data

Supplemental Data showing comprehensive results of the immunological investigations are available at [http://www. ...](http://www. ...)

References


Table 1. Time-related numbers of euthanized or dead rabbits after challenge with *Pasteurella multocida* J-4103 and numbers of rabbits surviving the challenge.

<table>
<thead>
<tr>
<th></th>
<th>Intranasal challenge</th>
<th>Subcutaneous challenge</th>
<th>Peroral challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Euthanasia / Exitus</td>
<td>Survived</td>
<td>Survived</td>
</tr>
<tr>
<td></td>
<td>day 3</td>
<td>day 5</td>
<td>day 6</td>
</tr>
<tr>
<td>Immunocompetent rabbits</td>
<td>1 / 0</td>
<td>0 / 0</td>
<td>2 / 1</td>
</tr>
<tr>
<td>Immunosuppressed rabbits</td>
<td>1 / 0</td>
<td>0 / 1</td>
<td>1 / 1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2 / 0</strong></td>
<td><strong>0 / 1</strong></td>
<td><strong>3 / 2</strong></td>
</tr>
</tbody>
</table>
Table 2. Numbers of animals with occurrence of pathological lesions among the clinically diseased intranasally challenged rabbits (n = 8).

<table>
<thead>
<tr>
<th></th>
<th>Lungs 1)</th>
<th>Heart 2)</th>
<th>Others 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocompetent rabbits</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Immunosuppressed rabbits</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>8</strong></td>
<td><strong>5</strong></td>
<td><strong>5</strong></td>
</tr>
</tbody>
</table>

1) Severe fibrinopurulent and necrotizing pleuropneumonia in immunocompetent rabbits and diffuse haemorrhagic pneumonia in immunosuppressed rabbits (one immunosuppressed rabbit showed pulmonary changes similar to those observed in immunocompetent rabbits). 2) Dilatation of the right heart ventricle. 3) Congestions, haemorrhages, inflammatory cell infiltrations, and initial dystrophy (more obvious in kidneys).

Table 3. Numbers of animals with occurrence of pathological lesions among the clinically diseased subcutaneously challenged rabbits (n = 11).

<table>
<thead>
<tr>
<th></th>
<th>Cutis and subcutis 1)</th>
<th>Lungs 2)</th>
<th>Heart 3)</th>
<th>Others 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunocompetent rabbits</td>
<td>5</td>
<td>4</td>
<td>3</td>
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</tr>
<tr>
<td>Immunosuppressed rabbits</td>
<td>6</td>
<td>5</td>
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<td>5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11</strong></td>
<td><strong>9</strong></td>
<td><strong>8</strong></td>
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</table>

1) Significant and confluent haemorrhages with perivascular cell infiltrations rapidly spreading from the place of inoculation and affecting extensive areas mainly of the thorax and abdomen. 2) Pulmonary hyperemia with moderate infiltration of alveoli with erythrocytes and inflammatory cells. 3) Hyperemia and haemorrhages in myocardium, epicardium and endocardium (also hydropericardium in immunosuppressed rabbits and acute purulent myocarditis in one immunocompetent rabbit). 4) Congestions, haemorrhages, inflammatory cell infiltrations, and initial dystrophy.
Fig. 1. Severe fibrinopurulent pleuropneumonia with extensive fibrinous adhesions on parietal pleura and lung surfaces typical for the intranasally challenged IC rabbits. Serohaemorrhagic exudate in thorax also could be observed. Alveoli (and some bronchioli) were partly or completely filled with fibrin and mixed cellular infiltrate (erythrocytes, leukocytes, and desquamated epithelial cells).

Fig. 2. Pulmonary hyperemia and haemorrhagic pneumonia occurring in the intranasally challenged IS rabbits. Moderate to very extensive penetration of erythrocytes into the alveolar lumen was the only relevant histological finding.

Fig. 3. Very extensive haemorrhages in subcutis of the subcutaneously inoculated IC rabbit (oedematous and “dirty-coloured” subcutis was more characteristic for the IS rabbits). These changes penetrated the abdominal wall and affected the contiguous parietal peritoneum.