Pneumonia in Horses Induced by Intrapulmonary Inoculation of \textit{Streptococcus equi} subsp. \textit{zooepidemicus}

Hiroyasu YOSHIKAWA\(^1\), Tomiyasu YASU\(^1\), Hideaki UEKI\(^1\), Toshifumi OYAMADA\(^1\), Hideo OISHI\(^1\), Toru ANZAI\(^2\), Masaaki OIKAWA\(^3\) and Takashi YOSHIKAWA\(^1\)

\(^1\)Department of Veterinary Pathology, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada 034–8628, \(^2\)Epizootic Research Station, Equine Research Institute, The Japan Racing Association, Kokubunji-machi, Shimotsuga-gun, Tochigi 329–0412 and \(^3\)Pathology Division, Equine Research Institute, The Japan Racing Association, 321–4 Tokami-cho, Utsunomiya-shi, Tochigi 320–0856, Japan

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ABSTRACT. To evaluate the possibility that \textit{Streptococcus equi} subsp. \textit{zooepidemicus} (\textit{S.z}) the causative bacterial agent of equine shipping fever pneumonia (ESFP), as well as to investigate its pathogenesis, 10 horses (seven Thoroughbreds and three Anglo-Arab species, ranging from 2–4 years in age) were experimentally inoculated, via an endoscope, into bronchus of the lung lobe with a dose of 30 ml of 1–7 \(\times\) 10\(^8\) CFU/ml of \textit{S.z}. After inoculation, autopsy and pathological examinations were sequentially conducted 30 min, 1, 2, 3, 4, 17, 20 hr and 2 weeks later. Pneumonia induced by the intrapulmonary inoculation of \textit{S.z} was characterized by small purulent pneumonia in the inoculated areas. With the lapse of time, these foci developed into serous hemorrhagic pneumonia, hemorrhagic purulent pneumonia, and then purulent, coagulation necrotic pneumonia. These pathomorphological characteristics of experimental pneumonia closely resemble those naturally occurring ESFP. There is strong evidence that \textit{S.z} is implicated as a causal factor in ESFP. \textit{S.z} grew in the mucus, exudate, and pulmonary effusions. Further, the bacteria showed resistance against phagocytosis by pulmonary alveolar macrophages (PAM) and neutrophils. Inhibition of PAM and neutrophil function is considered to be important in the development of pneumonia. With the progression of the disease, the neutrophils often adhered to the endothelial surface of the alveolar capillary lumen and played a role in generating coagulation necrosis of lung tissues.

KEY WORDS: equine, intrapulmonary inoculation, pneumonia, shipping fever, \textit{Streptococcus equi}.


Transportation stress has been implicated as a predisposing factor to respiratory diseases, such as so called “shipping fever pneumonia (ESFP)” and/or pleuritis/pleuropneumonia complex in horses [10, 12–14, 19]. Several hypotheses have been forwarded as to pathogenesis of the disease as follows. Pre-existing mild respiratory infections may develop into pneumonia when horses are transported [18]. Pulmonary defense mechanisms may be adversely affected by transit-associated stress [1, 22]. Elevation of the horse’s head, causing restriction in the range of neck movements, has been documented to compromise the immune system and increase the number of bacteria in transtracheal aspirates [15–17]. In recent years, there have been reports that \textit{Streptococcus equi} subsp. \textit{zooepidemicus} (\textit{S.z}) was frequently isolated as the predominant organism from the pulmonary foci of horses suffering from ESFP and \textit{S.z} may play a key role in the pathogenesis of ESFP [6, 12, 14]. As \textit{S.z} is a common commensal organism in the equine tonsil and nasopharynx and is not an airborne organism [9, 14, 23]. ESFP may be associated with an endogenous opportunistic infection by resident bacteria, \textit{i.e.}, \textit{S.z} [14]. However, there is little information on the effects of the \textit{S.z}’s invasion into the lungs of horses. In the present study \textit{S.z} was inoculated into the lungs of horses that have not yet transported for long distance, and the lungs were examined pathomorphologically to evaluate the possibility that \textit{S.z} is the causative bacterial agent of equine pneumonia associated with transport and to contribute to knowledge of the pathomorphogenesis of the lung lesions observed in ESFP [25].

MATERIALS AND METHODS

Horses: Ten horses (Nos. 1–10: 7 Thoroughbreds, 3 Anglo-Arabs) aged 19 months to 4 years were used (Table 1). These horses were with drawn from training because of locomotor disorders. Despite repeated treatment, the horses had failed to return to good health. Thus, the horses were clinically diagnosed for an absolute certainty that they have no chance of recovery. The animals were fed normally during the whole experiment period.

Bacteria: The bacterium used for inoculation was \textit{S.z} isolated from the pneumonia lesions of horse affected with naturally occurring ESFP [12].

Collection of bronchoalveolar lavage fluid (BALF): Seven days prior to \textit{S.z} inoculation into the lung each horse was secured in stocks and sedated with medetomidine hydrochloride (7.5 mg/kg body weight) administered intravenously. After applying a nose twitch and wiping off the horse’s nares with gauze, a 300 cm long, 8.7 mm diameter bronchoscope (Video-bronchoscope, Olympus, Tokyo, Japan) was passed through the nasal passage to the carina, and its distal end was then passed into a main-stem bronchus. The tracheal mucosa and bronchial mucosa were topically anesthetized with 2% lidocaine hydrochloride solution. The tip of the endoscope was wedged in the second branch of the principal right (chosen bronchus for inoc-
ulot) and left (no inoculation; control) bronchus located ventrally in the right and left caudal lung lobe (bronchus 1, 2) [20]. Then, air was extracted through the bronchoscope biopsy channel by syringe. One hundred ml of 37°C sterile isotonic saline solution was infused under pressure through the bronchoscope biopsy channel into the lung and was immediately aspirated manually. This procedure was performed within 10 min. The amount recovered lavage fluid was approximately 100 ml. The recovered BALF fluid was examined bacteriologically. The method for BALF was as described previously [7].

**Inoculation procedure:** Ten horses were inoculated endobronchially with a dose of 30 ml of the S.z suspension (1.7 × 10⁸ CFU/ml), via an endoscope by means of BALF described above, into the cranio-ventral region of the right caudal lung lobe, which is the site where pneumonic lesion associated with transport is most frequently found [14]. After an endobronchial inoculation was finished, the bronchoscope was soaked in 0.1% benzalkonium chloride solution for 10 min, and the bronchoscope was flushed, first with 0.02% benzalkonium chloride solution, then with 70% ethyl alcohol solution, and with sterile water. Finally air is sucked into dry the biopsy channel. Next, by using the sterile bronchoscope, the corresponding cranioventral region of the left caudal lung lobe was infused at a dose of 30 ml of 37°C sterile isotonic saline solution.

**Bacteriological examination:** BALF recovered prior to inoculation from 10 horses (Cases 1–10) and tissue specimens collected from the pulmonary foci in the inoculated areas of the 7 study cases (Cases 1, 2, 5, 7–10) were examined as described previously [12].

**Pathological examination:** After inoculation, necropsy was sequentially conducted 30 min (Case 1), 1 hr (Case 2), 2 hr (Case 3), 3 hr (Cases 4 and 5), 4 hr (Cases 6 and 7), 17 hr (Case 8), 20 hr (Case 9), and 2 weeks (Case 10) later. All horses were euthanized by intravenous injection of pentobarbital (30 mg/kg; Pitman-Moor Pharmaceuticals Ltd., U.S.A.), and immediately necropsied. Samples for histological, electron microscopical and bacteriological examination were collected within approximately 1 hr of euthanasia.

**Histopathology:** The lungs were resected by the method of Blunden and Mackintosh [4] so that all lobes could be obtained. The resected lungs were fixed in a 10% neutrally buffered formalin solution. The samples of lungs tissue were then embedded in paraffin by the routine method. Thin sections were cut and double-stained with hematoxylin-eosin (HE), and then observed with an optical microscope. For the study of S.z. within the pulmonary tissues, immunostaining was also conducted using anti-S.z serum of a rabbit as the primary serum (at a dilution ratio of 1 to 128) [12] and the simple stain, MAX PO (Nichirei Corp., Japan), as the secondary serum.

**Transmission electron microscopy:** Small sections of pneumonic tissues were doubly fixed with glutaraldehyde-OsO₄ and then were embedded in EPOK-812 (Okenshoji Corp., Japan) by the routine method. After ultramicrotomy, the ultrathin sections were double-stained with uranyl acetate and lead. The stained sections were observed on the electron microscope model H-7000 (Hitachi, Japan).

### RESULTS

**Clinical findings:** No obvious clinical symptom was observed at 1–4 hr after the inoculation (Cases 1–7), except for the minor change in rectal temperature. However, at 17 and 20 hr after the inoculation (Cases 8 and 9), transient increase in rectal temperature varying between 38.6°C and 39.2°C, inappetence and lethargy were observed. At 2 weeks (Case 10), the horse showed nasal discharge with purulent exudates, shallow frequent respirations (tachypnea) and pyrexia. These clinical signs of respiratory disease developed in the matter of 48 hr after the inoculation, and continued until 12 days later just before euthanasia.

**Microbiological findings:** No bacteria was recovered from any of BALF collected 7 days prior to inoculation from 10 horses. Results of re-isolation of S.z. from the lung tissues were shown in Table 1. S.z. was collected from pulmonary foci seen in the inoculated areas of the right caudal lung

### Table 1. Materials examine

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Breed a)</th>
<th>Age b)</th>
<th>Inoculation value c) of the S. zooepidemicus</th>
<th>Postinoculation times or weeks</th>
<th>Recovery value d) of the S. zooepidemicus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Th</td>
<td>2.5</td>
<td>1 × 10⁶</td>
<td>30 min</td>
<td>3 × 10⁸</td>
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<td>2</td>
<td>Th</td>
<td>2.6</td>
<td>1 × 10⁸</td>
<td>1 hr</td>
<td>4 × 10⁴</td>
</tr>
<tr>
<td>3</td>
<td>Th</td>
<td>2.11</td>
<td>7 × 10⁸</td>
<td>2 hr</td>
<td>NE</td>
</tr>
<tr>
<td>4</td>
<td>AA</td>
<td>2.1</td>
<td>7 × 10⁸</td>
<td>3 hr</td>
<td>NE</td>
</tr>
<tr>
<td>5</td>
<td>AA</td>
<td>2.0</td>
<td>7 × 10⁸</td>
<td>3 hr</td>
<td>6 × 10⁸</td>
</tr>
<tr>
<td>6</td>
<td>Th</td>
<td>2.9</td>
<td>7 × 10⁸</td>
<td>4 hr</td>
<td>NE</td>
</tr>
<tr>
<td>7</td>
<td>AA</td>
<td>2.1</td>
<td>7 × 10⁸</td>
<td>4 hr</td>
<td>3 × 10⁸</td>
</tr>
<tr>
<td>8</td>
<td>Th</td>
<td>1.7</td>
<td>7 × 10⁸</td>
<td>17 hr</td>
<td>3 × 10⁸</td>
</tr>
<tr>
<td>9</td>
<td>Th</td>
<td>2.6</td>
<td>1 × 10⁸</td>
<td>20 hr</td>
<td>2 × 10⁷</td>
</tr>
<tr>
<td>10</td>
<td>Th</td>
<td>4.0</td>
<td>1 × 10⁸</td>
<td>2 weeks</td>
<td>6 × 10²</td>
</tr>
</tbody>
</table>

a) Th: Thoroughbred, AA: Anglo-Arab, b) Years, c) CFU, d) CFU, e) Not examind.
lobe of the 7 study cases (Cases 1, 2, 5, 7–10). *S.z* was not isolated from the corresponding region of the left caudal lung lobe where sterile isotonic saline solution was infused (Cases 1, 2, 5, 7–10).

**Gross post-mortem findings:** There was no detectable pneumatic lesion in the cranioventral region of the left caudal lung lobe.

Pulmonary foci had developed around the inoculated areas. At 30 min, 1 and 2 hr after inoculation, only small, localized foci were found and they mainly comprised hyperemia and spotted hemorrhage in the inoculated areas. A yellow, bubbling fluid had exuded from bronchioles. At 3 to 4 hr after inoculation, there were pneumatic foci with the size of a goose egg accompanied by remarkable hemorrhage (Fig. 1). The interior of the bronchioles was filled with a purulent effusion. Interlobular and subpleural interstices were expanded and filled with a yellow edematous fluid. At 17 and 20 hr after inoculation, there were red hepatized foci about the size of a fist size, and a large amount of purulent effusion ran out through its cut surface (Fig. 2). At 2 weeks after inoculation, the pneumatic areas went into necrosis, and showed the purulent adhesive pleuritis. A large amount of purulent pleural fluid was accumulated in the thoracic cavity.

**Histological and electron microscopic findings:** Lesions started to develop at 30 min after inoculation, and with the progress of time, there were slight cell reactions mainly in the bronchiolar areas. Next, these reactions developed into serious purulent pneumonia, then purulent fibrous pneumonia or pleuritis.

**Thirty min to 1 hr after inoculation:** Alveoli in the inoculated areas were dilated with emphysema, and were accompanied by hyperemia of the alveolar walls. The bacterial associates showing a positive response to the *S.z* antibody were scattered inside the alveoli and in the alveolar septum (Fig. 3). Mucus secretion was accelerated in the bronchiolar epithelial cells, and at the same time, these cells were ablated. At 1 hr after inoculation, the alveolar walls were covered with a great deal of mucus. Macrophages that had phagocytosed the bacterium were observed in the alveoli, along with a large number of the bacteria. The alveolar walls were disintegrated from the infiltration by neutrophils...
neutrophils agglutinated to the alveolar walls and capillary vessels, causing obvious alveolitis and vasculitis (Fig. 6). Vasculitis was found in connection with coagulation necrotic microfoci inside the pneumonic foci. During this period, far more bacterium were found in the mucus and the macrophages than in the foci observed during the period up to 2 hr. Electron microscopic observations showed that, although bacterium had been phagocytosed by macrophages, the cell structure was retained relatively well and the mitosis of bacterium was often observed (Fig. 8). After macrophages had phagocytosed the bacterium, it was found that the phagosome was destroyed. Furthermore, these macrophages were surrounded by neutrophils, and many micro-abscesses had been formed.

Seventeen-twenty hr after inoculation: Pneumonic foci were characterized by remarkable infiltration by neutrophils, the many S.z and the formation of abscesses, with caseous necrotic foci. Observations by an electron microscope showed that the bacterium is incorporated into the

(alveolitis). In some cases, neutrophils were agglutinated in clumps to the capillary vascular walls of the alveoli (vasculitis). The electron microscopic observations confirmed that the macrophages engulfed the bacterium.

Two hr after inoculation: Interstitial edema and emphysema became conspicuous over the entire area of inoculation. The desquamation of bronchiolar epithelial cells, hemorrhage, the infiltration of numerous macrophages and neutrophils, and the precipitation of fibrin were observed in this area. Many bacterium, all showing a positive response to the anti-S.z serum, had been taken up into the cytoplasm of macrophages and neutrophils in the bronchiolar cavity.

Three to four hr after inoculation: Enhanced mucous secretion and catarrh were remarkable in the bronchiolar epithelial cells, and the bronchiolar cavity was filled with desquamated products and macrophages that had phagocytosed the anti-S.z serum positive bacterium (Fig. 4). The pulmonary lesions included various types, such as serous, hemorrhagic, or purulent lesions (Fig. 5). At the same time,
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phagosome of macrophages have survived, but that various organelles in the cytoplasm of macrophages had become vacuoles and degenerated. Meanwhile, immature granulation tissues were also formed around the pneumonic foci. They were accompanied by the regeneration of bronchiolar epithelial cells and the increases in lymphofollicles. Lobular interstitium dilated because of inflammatory edema accompanied by neutrophils and fibrin, and involvement of the fibrinous pleuritis (Fig. 7).

Two weeks after inoculation: Coagulation necrosis with a size as large as a lobule developed in the pneumonic foci in the inoculated areas. Abscesses included S.z were formed around the large necrosis. Encapsulation by the connective tissues was observed near these abscesses. Lobular interstices around the pneumonic foci expanded remarkably, and caused fibrous or purulent inflammation in the thoracic cavity.

DISCUSSION

Pneumonia cannot be easily formed by bacterial inoculation because of the defense mechanism of the respiratory tract mucosa [5]. In this inoculation study, therefore, horses were subjected to air expulsion treatment for the purpose of retaining bacterium inside the respiratory tract [24]. Horses were then infected with S.z via the respiratory tract, and pneumonia was observed over time. Important histological findings are summarized in the following points: (a) Well-defined pneumonia starts at 3 hr after inoculation, but initially develops in the bronchioles and the nearby alveoli. The S.z grows in mucus, exudate, and effusion, when the inflammation begins to change. (b) S.z shows resistance to the phagocytosis by macrophages and neutrophils. This bacterium grows in the phagosomes inside the macrophage, and causes phagocytes to be degenerated. (c) The pneumonia show the serous, hemorrhagic, fibrinous, and purulent inflammation. (d) No response from lymphocytic cells is observed during the progress of the inflammation. (e) The infiltration of bacterium by the macrophages and neutrophils within the alveoli (alveolitis) and the agglutinated of neutrophils to the capillary walls of the alveolus play important roles in the aggravation of the lesions. (f) The pleuritis is the result of exudative inflammation in the lobular interstitium.

ESFP is characterized by hemorrhagic, purulent, pleuritic pneumonia and the purulent pneumonia accompanied by pleuritis [6, 11, 18]. In a loading test based on the S.z infection, in which horses were transported for long hours, they developed a serous, hemorrhagic bronchial pneumonia of a highly purulent nature and multiple, coagulation necrotic pneumonia [14]. The basic pathological changes of this pneumonia, which were observed at various, experimentally approved times of inoculation, included the exudative inflammation of a serous, hemorrhagic, fibrous, or purulent type. It is obvious that these inflammatory lesions have been caused by S.z. Alveolitis caused by the infiltration of neutrophils, along with capillary vasculitis in the alveolar walls, is observed in the initial period of infection. They seem to play important roles in the aggravation of pneumonia. It is likely that the increase in mucus in the alveolar walls is involved in the colonization of bacterium. Both the neutrophils and macrophages are considerably degenerated and destroyed, and S.z survived within the macrophages. This indicates that neutrophils and macrophages have lost their normal functions and that S.z has resistance to macrophages and neutrophils. It is known that M-protein with anti-phagocytic effects exists in the capsules of S.z [21]. It has also been reported that as an anti-phagocytic effect of M-protein, this protein inhibits the bonding of C3b, a complementary component, to the cell surface of S.z and that this protein combines with fibrinogen [2, 3]. In pneumonia, remarkable infiltration by neutrophils and the subsequent destruction of neutrophils are observed, suggesting that some substances like the M-protein of S.z may have a chemotactic effect on neutrophils, or leukocytes may have some toxic effects [12]. These substances are deemed to play a role in the outbreak of coagulation necrosis [8, 26]. The profiles of pneumonia obtained from our study of inoculating horses with S.z by way of the respiratory tract closely resembled ESFP [14]. It has been shown that S.z is highly involved in the outbreak of this disease. In the future, it will be necessary to examine, from a local immunity aspect, the settling of S.z on the mucosa of the respiratory tract.

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REFERENCES


