Studies on Protective Immunity Against

*Bordetella bronchiseptica* Infection

Byong-Kyu Kang, D.V.M., M.S., Ph.D.

*Department of Veterinary Medicine, College of Agriculture
  Jeonnam National University*

**Introduction**

Results of research on swine respiratory disease since 1956 have proved *B. bronchiseptica* to be major etiologic factor in the disease commonly called the infectious atrophic rhinitis of swine (AR). In their previous paper, the authors observed that the detection of *B. bronchiseptica* by the isolation culture was gradually difficult when the age of animals was increased, and that agglutinating-antibodies became detectable in naturally infected piglets about 3 months of age. These results suggested that the organism present in the nasal cavities decreased gradually in numbers and that it was one of a model of infection in relation to establishment of infection and the immune response of the host. However, many problems still remain to be solved, especially on the specific relationship between the establishment of the agent in the nasal cavity and apperition of symptoms, lesions and immune response.

The purpose of the present experiments is to evaluate the prophylactic value of *B. bronchiseptica* vaccine prepared by several different method including assessment of the biological characters of used strain originated from infected swine, and is to obtain further information concerning the host-parasite relationship in experimentally infected mice.

**Materials and Methods**

**Bacterial Strain:** A strain of W-1029 of *B. bronchiseptica* which had been isolated from the nasal cavity of a naturally infected pigs with turbinate atrophy was used (designated virulent W-1029 strain, Phase I). An isolate of *B. bronchiseptica* which was originally isolated from the above source and sub cultured on the pepton water for several times was also used (designated low-virulent strain H-969, Phase III). The phase variation of the organisms was determined as described by Nakase. Culture Media: MacConkey agar medium (Eiken) containing 1% of glucose was used for the selective isolation of *B. bronchiseptica*. Trypto-soy agar medium (Eiken) containing 5% horse blood was also used for vaccine preparation and the detection of the flora of various organs from mice. Trypticase soy broth (Eiken) was used to obtain the challenge living organism for intranasal inoculation.

**Experimental Animal:** White ddS-line, male mice weighing 15 g of body weight, were distributed into cages as a group. Two mice selected at random from each cage were employed to confirm Bordetella-free state by cultivating the materials from the respiratory organs as well as by serological agglutination test.

**Experimental Procedure:**

*Experiment 1.-*

Virulence and mouse protection tests were conducted by the method described by Nakase with slight modification. Each organisms of Phase I and III cultured on the Trypto-soy agar plate medium for 48 hours at 37°C were suspended in the pepton water in the concentration of 1 mg of harvested organism per ml and 0.5 ml of serial ten-fold dilution was inoculated intraperitoneally and intracerebrally. Death or survive was recorded for 14 days and LD₅₀ was calculated by the Behrens-Kärber and/or probit method.
Comparisons of the protective potency between the *B. bronchiseptica*, Phase I and III was also done with the challenge of virulent W-1029, Phase I organism in the dose of 1 mg per ml inoculated intraperitoneally. Death or survive was also recorded for 14 days.

To confirm the toxicity of virulent W-1029 strain, Phase I organism, mouse toxicity test was done with the sonic extracted and the fraction obtained by the DEAE-cellulose column chromatography as described previously.

*Experiment 2.*

Vaccine Preparation: The organism (virulent W-1029 strain, Phase I) for preparing a whole culture bacterin was grown on the tryptose blood agar base medium, incubated at 37°C for 20-24 hours and harvested with 0.85% saline solution (1 mg per ml). This bacterin containing 10^7 colony forming units (CFU) per ml was kept at 37°C for one week or 0°C for 10 to 14 days until it was inactivated in the presence of formalin (0.5%) or merthiolate (1:10,000). The sterility was checked by inoculating 1 ml of bacterin into 10 ml of the trypticase soy broth or on the tryptose blood agar plate medium and after inactivation, all bacterins were kept at 4°C until used. From the above preparation of bacterins, the following four different killed vaccines were designated as merthiolate-killed at 4°C, merthiolate-killed at 37°C, formalin-killed at 4°C and formalin-killed at 37°C vaccine respectively.

Inoculation of Vaccine: A group of 30 mice received a single intraperitoneal injection of 0.3 ml vaccine. This volume of vaccine was calculated to contain either 0.3 mg of organism harvested from grown cells or 3×10^6 CFU.

Challenge Inoculation: Prophylactic values of the vaccines were assessed by challenge inoculation with the virulent strain W-1029, Phase I. After two weeks, the immunized mice were challenged with the dose of 10, 200 and 250 LD₅₀. This volume of challenge organisms was calculated as 10^6 CFU in 1 LD₅₀. Procedures for these examinations were reported in detail elsewhere.

*Experiment 3.*

<table>
<thead>
<tr>
<th>Immunized group</th>
<th>Non-immunized group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td><strong>Group B</strong></td>
</tr>
<tr>
<td>Merthiolate</td>
<td>Formalin</td>
</tr>
<tr>
<td>0 or 37°C</td>
<td>0 or 37°C</td>
</tr>
<tr>
<td>vaccinated</td>
<td>vaccinated</td>
</tr>
</tbody>
</table>

Challenged with living organisms (10, 200 and 250 LD₅₀)
Survived mice

Challenge inoculation, intranasally (0.03 ml of virulent strain W-1029, Phase I)
Observed for one month and examined by

<table>
<thead>
<tr>
<th>Bacteriological</th>
<th>Serological</th>
<th>Pathological</th>
</tr>
</thead>
<tbody>
<tr>
<td>nasal cavity</td>
<td>A.G. test</td>
<td>macroscopically</td>
</tr>
<tr>
<td>trachea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lung</td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver</td>
<td></td>
<td></td>
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<tr>
<td>spleen</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Experimental procedure to determine the effects of vaccines on mice after intranasal challenge.
Table 1. Comparisons of Virulence between *B. bronchiseptica*, Phase I and Phase III Organisms on Mice

<table>
<thead>
<tr>
<th>Strain Used</th>
<th>Phase Variation</th>
<th>Inoculation Route</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-1029, Virulent</td>
<td>I</td>
<td>Intracerebral Intraperitoneal</td>
<td>10^{-3.8*} 10^{-4.5**}</td>
</tr>
<tr>
<td>H-969, Low-virulent</td>
<td>II</td>
<td>Intracerebral Intraperitoneal</td>
<td>10^{-1.0k} 2×10^{11.8**}</td>
</tr>
</tbody>
</table>

* Calculated by the method of Behren’s—Kärber method.
** Calculated by the method of probit method.
LD<sub>50</sub> was shown as mg per ml in 0.03ml intracerebral and in 0.5ml intraperitoneal inoculation, respectively.

Table 2. Comparisons of Protective Potency with *B. bronchiseptica*, Phase I and Phase III Organisms to Mice

<table>
<thead>
<tr>
<th>Strain Used</th>
<th>Dose (0.5ml)</th>
<th>Mouse Survived/Immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immunized (mg/ml)</td>
<td>Challenged (mg/ml)</td>
</tr>
<tr>
<td>W-1029, Virulent</td>
<td>10^{-4}</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>1.0</td>
</tr>
<tr>
<td>H-969, Low-virulent</td>
<td>10^{-4}</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* Challenge was done with virulent strain W-1029, Phase I inoculated intraperitoneally.

Schematic diagram of experimental procedure to clarify the prophylactic value of the vaccines and the host-parasite relationship in relation to the immunological response of mice after challenged intranasally was shown in Fig. 1.

The immunized mice which were included Experiment 2 were challenged with 0.03ml of virulent strain W-1029, Phase I. And the non-immunized mice were also challenged as control. The external nares of unanesthetized mice were smeared with 10<sup>4</sup> or 10<sup>6</sup> CFU of 18 hour cultures in the trypticase soy broth and one or two mice were autopsied at 5-day intervals for a month after inoculation for cultivation of the challenged organisms in the respiratory system and internal organs and for serum agglutination test. Gross examinations were performed especially on the lung and the methods of cultivation and agglutination test were reported in detail previously.<sup>11,25</sup>

Results

Experiment 1: The virulence of strains used in this experiment was tested and a desirable LD<sub>50</sub> calculated from the results. The virulence of strains used are shown in Table 1.

In intracerebral inoculation, the LD<sub>50</sub> of Phase I organism was approximately 10^{-3.5} mg per ml and that of Phase III was 10^{-1.0} mg per ml. The LD<sub>50</sub> of Phase I was 10^{-4.5} mg per ml when inoculated intraperitoneally and the viable cell counts were 1×10<sup>7</sup> CFU per ml and that of Phase III was 2×10^{11.8} mg per ml.

To confirm the protective potency of the organisms used by the phase variation, mouse protection test was carried out. As shown in Table 2, the mice immunized with virulent W-1029, Phase I organism survived completely. On the contrary, none of mice immunized with the low-virulent H-969, Phase III organism survived when they were challenged with a virulent strain of Phase I organism.

Toxicity of obtained fractions from the sonic extracted crude or starting material by diethylaminoethyl (DEAE)-cellulose column chromatography was
tested on mice by intraperitoneal inoculation with a dose of 0.5ml of each fraction, and obtained results which included the various characters such as K-agglutinin, skin necrotic toxicity and hemagglutinability described in a previous paper. The results are shown in Table 2.

With the dosage employed, 80 to 100% of the mice died one to 6 days after inoculated and no differences between the fractions were observed although the K-agglutinin, skin necrotic toxicity and hemagglutinability showed difference in each fractions.

No different lethal activities of the fractions and it was presumed that the toxic fraction could not been separated by the DEAE-cellulose column chromatography and further rechromatography will be necessary to separate the toxic fraction.

Obtained results from the above, the active, biological and immunological characters of virulent W-J029 strain, Phase I organism were confirmed and this strain was used in a further experiment.

Experiment 2: Protective effect of vaccines prepared by four different procedures was evaluated and results are shown in Table 3. There was a marked difference between the non-immunized, control and the active immunized groups; all mice were dead in the non-immunized, control group, whereas all survived in the immunized group when they were challenged with the 10, 200 and 250 LD_{50} doses of virulent organism. However, no difference was obtained in protecting potency by the four vaccines and it was apparent from these findings that the vaccines prepared by the merthiolate or formalin treatment showed protective effect to B. bronchiseptica infection and harmless to mice, and therefore, subsequent

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### Table 2

<table>
<thead>
<tr>
<th>Tube number</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction number</td>
<td>F1</td>
<td>F2</td>
<td>F3</td>
<td>F4</td>
<td>F5</td>
</tr>
<tr>
<td>K-agglutinin*</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Skin necrotic toxicity</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hemagglutinin*</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse toxicity**</td>
<td>10/10</td>
<td>7/10</td>
<td>10/10</td>
<td>10/10</td>
<td>9/10</td>
</tr>
</tbody>
</table>

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Fig. 2. Chromatography of B. bronchiseptica, Phase I cells on a DEAE-cellulose column. Sonic extracted crude antigen (50mg/ml in 0.005M PBS at pH 7.4) was chromatographed on a column (1.3×23cm). The flow rate was 10 ml per hr. Total recovery of protein was 89% as measured by the Lowry-Folin reaction.

* Methods were described in a previous paper.

** Mouse died/mouse inoculated
Table 3. Protective Effects of Vaccines with Whole Cell Killed Organisms of
B. bronchiseptica, Phase I

<table>
<thead>
<tr>
<th>Strain Used</th>
<th>Treatment of Vaccine</th>
<th>Dose of Vaccine Inoculated*</th>
<th>Challenge Dose (LD&lt;sub&gt;50&lt;/sub&gt;)**</th>
<th>Survival Rate***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merthiolate</td>
<td>at 0 C</td>
<td>3×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>250</td>
<td>9/10</td>
</tr>
<tr>
<td>W-1029 Virulent</td>
<td>at 37 C</td>
<td>3×10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>250</td>
<td>10/10</td>
</tr>
<tr>
<td>Control (Non-immunized)</td>
<td>-</td>
<td>-</td>
<td>250</td>
<td>0/10</td>
</tr>
</tbody>
</table>

* Viable organisms in colony counts.
** 1 LD<sub>50</sub>=1×10<sup>7</sup> CFU.
*** Numbers of mice survived/challenged.

Fig. 3. Sequential detection of B. bronchiseptica in Experiment 3 (non-immunized, control).

- Nos. of colony from which B. bronchiseptica was isolated (### = more than 100).
- : B. bronchiseptica was not isolated.
- D: Die out. P: Lesions of pneumonia was recognized. K: Klebsiella sp. isolated.

Experiment 3: To determine whether or not there exists a relationship between the antibody response and protection against B. bronchiseptica infection, further intranasal infection was conducted on the survived mice in Experiment 2 and results are shown in Figs 3, 4 and 5.

In the non-immunized group of mice, a definite respiratory wheeze persisted for several hours after the inoculation. During the next 24 hours, the mice became less active, refused food, lost body weight, and showed rough coat. But in the immunized group of mice, clinical symptoms were not recognized.

Necropsy findings of the non-immunized group of mice are as follows. There was often a small amount of free bloody fluid in the serous cavities and pectechial hemorrhages were recognized in the peritoneum and pleura. The liver, spleen and kidneys were enlarged. The lungs were distended and diffuse hemorrhagic areas were noted, particularly on the apical regions. Bronchi contained frothy exudate. On the contrary, typical signs were not observed in the immunized, control group of mice.

The recovery of the organisms from the respiratory system in the immunized group of mice decreased remarkably in number by the isolation culture in comparison with the non-immunized group of mice.
but a long-term harbouring of the organism was recognized until the experiment ended in some cases of the immunized group of mice. Summary of incidence of *B. bronchiseptica* recovered from the various organs of the immunized and non-immunized, control groups of mice are presented in Table 4.

Results of the serum agglutinin response detected against *B. bronchiseptica* antigen after the intranasal challenge inoculation in Experiment 3 are shown in Fig. 6. The average serum agglutinin titers produced by the immunized groups of mice (group A & B) ranged from 1:40 to 1:2,560 and persisted continuously during the experimental period, while the titers of the non-immunized, control group of mice were negative or from 1:10 to 1:80, which appeared from 4 days after the intranasal inoculation.

**Discussion**

Antigenic fractions of bacterial cells concerning the protective action were not particularly demonstrated in the present study, even though they were separated from the sonicated extracted materials to the fractions of agglutinogen, necrotizing factor and hemagglutinin by DEAE-cellulose column chromatography. However, it was suggested that antigenic fractions were located in capsule antigens of cells, because a vaccine heated at 100°C for 1 hour lost the protective ability. These considerations may be supported by the fact that no protective ability was demonstrated in an avirulent or Phase III organism.

On the other hand, Fetter et al. reported that *B. bronchiseptica*, in producing atrophic rhinitis, must release substance which diffuses into the tissue and elicits changes in the osseous core without inciting an inflammatory reaction and that the endotoxin of *B. bronchiseptica* possibly was the initiating agent. Harris et al. also reported that Boivin extracts of *B. bronchiseptica* inhibited or uncoupled the energized process of bovine heart and porcine heart mito-

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Table 4. Summary of Incidence of *B. bronchiseptica* Recovered from Organs of Immunized and Non-immunized, Control Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Nasal Cavity</th>
<th>Trachea</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive %</td>
<td>Positive %</td>
<td>Positive %</td>
</tr>
<tr>
<td>A&amp;B (Immunized)</td>
<td>47/97*</td>
<td>23/97</td>
<td>14/97</td>
</tr>
<tr>
<td>C(Non-immunized)</td>
<td>30/30</td>
<td>20/30</td>
<td>20/30</td>
</tr>
</tbody>
</table>

* Numbers of mice from which Bordetella recovered/numbers of mice tested.
chondria, and elaboration of endotoxin or other factor(s) could have resulted in a direct cytotoxic effect on bone cells.

As shown in Table 1 and 2, low-virulent H-969, Phase III obtained by subculturing showed lower virulence compared with the virulent strain of W-1029. Phase I and completely lost the protective ability. Nakase\textsuperscript{14} reported that, although the organism originally obtained from pneumatic lesions of infected mice was Phase I, it easily varied to Phase II. Phase III or rough phase within 3 passages on blood agar. On the other hand, Nakagawa et al.\textsuperscript{16} reported that the 20th subcultures of strain 64 L of *B. bronchiseptica* originated in guinea pig had no remarkable effects of subculturing on the infectivity and ability to form pneumatic lesions of the organism. Further investigation will be needed to clarify the difference of virulence and infectivity among the strain originated from various sources, such as the mouse, rabbit, guinea pig, dog and pig.

Harris and Switzer\textsuperscript{10} demonstrated accelerated nasal clearance of *B. bronchiseptica* in pigs by injecting subcutaneously sonicated cells of *B. bronchiseptica* strain or pertussis vaccine for the prevention of whooping cough in man. Ganaway et al.\textsuperscript{55} reported that, in guinea pigs, the administration of formalized bacterin emulsified in Freund’s incomplete adjuvant prevented *B. bronchiseptica* infection completely. Nakagawa et al.\textsuperscript{16} reported that formalin and merthiolate killed vaccines were effective to prevent *B. bronchiseptica* infection in guinea pigs and merthiolate killed vaccine was most effective compared with heat or formalin treatment and same results were obtained in pertussis vaccine for human use\textsuperscript{16}. In the present study in which prophylactic effects of four different vaccines were compared, it was demonstrated that no difference between the treatments of formalin or merthiolate and 0 C or 37 C inactivation methods, and that almost all mice survived when challenged with large dose (250 LD\textsubscript{50}) of virulent strain.

Although it is commonly demonstrated that most naturally or artificially infected AR pigs showed the agglutinin in the sera by the agglutination test, the nature of the immunity especially in relation to prevention of *B. bronchiseptica* infection has never been clearly defined. Harris and Switzer\textsuperscript{17} reported that the serum of four swine intramuscularly inoculated with whole culture *B. bronchiseptica* formalized bacterin contained high titers of agglutinating antibody but resistance against nasal infection did not occur and the serum of swine intranasally inoculated with low-virulent strain did not contain detectable titers of agglutinating antibody. They also described that the simultaneous intranasal inoculation of both virulent and low-virulent strains did not accelerate the clearance of the infection\textsuperscript{55}. Recently, however, Harris and Switzer\textsuperscript{10} demonstrated accelerated nasal clearance of *B. bronchiseptica* in pigs by injecting subcutaneously sonicated cells of *B. bronchiseptica* strains or pertussis vaccine for human use and finally they concluded that this differs from the resistance against reinfection produced in pigs given a live low-virulent strain of *B. bronchiseptica*.

On the other hand, Yoda et al.\textsuperscript{25} reported that agglutinins were demonstrable in a few recovered guinea pigs submitted to experimental infection and that these animals were highly resistant to the challenge infection; most of the recovered guinea pigs had acquired nasal and tracheal resistance against the reinfection of the organism. Nakagawa et al.\textsuperscript{18} reported that there was a general correlation between protective potencies and serum agglutinin
titers, although discrepancies were observed in some guinea pigs and agglutinin production was not always accepted as evidence of protective potency.

It is particularly interesting to note that the age of animals was related to resistance. It is difficult to accept that the resistance was heightened with age, because no differences in susceptibility had been found among guinea pigs\(^2\) or swine\(^3\).

Recently, Koshimizu et al.\(^{13}\) reported that *B. bronchiseptica* could be established and persisted in the nasal cavities of piglets having some level of the maternal antibodies and suggested that the maternal antibody may have blocked the production of antibody.

As shown in Fig. 4, 5, 6 and Table 4, agglutinin was demonstrable in the immunized group of mice and these animals were highly resistant to the challenge infection. However, the organisms which were inoculated into the nasal cavities or respiratory organs of the immunized mice decreased gradually in numbers and became undetectable at 30 days after inoculation. The authors\(^{23}\) reported that agglutinating antibodies became detectable in naturally infected piglets about 3 months of age when the causative organism were gradually decreased in number and undetectable by the isolation culture. Consequently, it will be reasonable to conclude that the present experiment may be regarded as a model of natural infection, especially in relation to the establishment of infection and the immune response. Similar observations were also made by Koshimizu et al.\(^{13,14}\) in conventional piglets. And it is assumed that the favorite localization sites of the organism are the mucous membrane of the respiratory tract as was already stated by Ganaway et al.\(^5\) Harris et al.\(^9\) and Fetter et al.\(^4\). Further studies are needed to find any factor of the organism responsible for the resistance of the respiratory system in case of either natural infection or vaccination.

**Conclusion**

An experimental *Bordetella bronchiseptica* infection was carried out by using the ddS-line mice to evaluate the immunogenicity of the organisms originated from the naturally infected atrophic rhinitis of swine(AR) and to clarify the host-parasite relationship.

Results obtained are summarized as follows:

In the virulence and protective potency test, the phase I strain (virulent, W–1029) showed strong lethal toxicity and high protective potency, while the phase III strain (low-virulent, H–969) lacked it. This indicated that the virulent, phase I organism is the best and perhaps the only antigen for the preparation of active immunity in mice.

Whole cell vaccines (virulent, Phase I, W–1029) killed by formalin and merthiolate produced much effective protection potency and no differences were found by the inactivation methods.

There was a general correlation between the post-challenged bacterial recovery of the reinjected organisms from the respiratory organs or protective potency and serum agglutinin levels, although discrepancies were observed in some mice during a long-term observation. Therefore, agglutinin production was not always accepted as evidence of protective potency.

**References**

Animal Care (1965) 15 : 156.


Bordetella bronchiseptica의 감염세균에 관한 연구

康炳奎

全南大學校 農科大學 獸醫學科

抄録

바이러스 중 풍자성 합병증이 주요한 가역적 암균으로 생각되는 Bordetella bronchiseptica(以下B菌)에 자연감염을 분리한 나라에서 사용하여 mouse 내성 면역에 대한 B균의 감염의 효과를 검토하고자, 먼저 페티피아에 따르면 B균의 독자력과 감염성에 따른 실험 결과를 비교하였다. I相菌을 혼합하여, 치료는 구강내에 접촉에 따르는 B균의 독자력과 관련하여, 실험 및 비교는 진동실의 결과를 검토하였다. 이에 실험을 요약하면 다음과 같다.

1. B菌 I相菌(W-1029株)의 독자력은 III相菌(H-969株)에 비하여 독자력이 강하였으며, I相菌은 감염성에 보유하고 있었으나 III相菌은 감염성에 거의 없었다.

2. B菌 I相菌은 코막에 있어서 formalin 및 merthiolate 처리 및 불활성화 도수(0℃ 및 37℃)에서는 독자력이 보유한 독자력의 차이가 인정할 수 없었다.

3. 전염을 증가한 mouse 종에 B균의 통합내에 있는 독자력과 관련하여, 실험 및 비교는 전한 양적 인자, 그리고 실험적 구성물에 의하여 독자력이 적응적으로 검토되었으며 또한 혈청 정체 계상에 따르면 내성상의 완전적인 전제에 대한 B균의 결과를 인정하였고, 이로 인한 폐쇄율은 다소 큰율이 인정되었다.

이상의 내용으로, B菌의 감염을 사용하여 독자력의 영향을 시험적으로, H-969株에 있어서의 B菌의 독자력은 독자력에 대한 가능성이 제시되며, 이를 사용하여, B菌의 독자력은 내성상의 결과를 보고하였다.