Bacteriological and Serological Studies on *Pasteurella multocida* Infection in Rabbits

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Bacteriological and serological examinations were made on *Pasteurella multocida* infection in two rabbit-breeding colonies. The organism was localized in the paranasal sinuses of 53 of 54 infected rabbits, excreting to the external nares of 49 rabbits. It was also isolated from the trachea and middle and inner ear of half of the infected animals and occasionally from the conjunctiva, lung and heart. The infection rate of the organism was very low (4.3%) in sucklings younger than a month old, but increased with advancing age, reaching nearly 100% in adults more than five months of age. A rapid increase of the infection rate was observed at two to three months of age. Serum antibody against somatic antigen of *P. multocida* was demonstrated in infected rabbits by use of the tube agglutination test, showing a close correlation with isolation of the organism in adults. Sucklings younger than one month of age from infected dams were significantly resistant to experimental nasal infection of *P. multocida*.

It is well known that *Pasteurella multocida* is an important causal agent of snuffles, pneumonia, otitis media, genital infection, abscess formation, conjunctivitis and septicemia in rabbits [7, 8, 13-16, 27-34], and these diseases are prevalent in rabbit colonies in this country. The significance of *P. multocida* in these disease and the clinical appearances and pathological changes of these diseases have been studied intensively by previous investigators [10-13, 15, 16, 28-30], but information on bacteriological and serological changes according to the age of the rabbits, which is indispensable for microbiological control of animal colonies, is not yet sufficient.

Recently, we found two rabbit-breeding colonies contaminated with this pathogen and performed epizootiological studies of the infection to obtain fundamental knowledge for bacteriological and serological monitoring and

control of the infection.

Materials and Methods

Rabbits: Two rabbit-breeding colonies were the subjects of the observations in this study. One of them consisted of about 200 breeding females and 20 males of the Japanese white rabbit strain and their offspring younger than three months of age, and the other of 150 female and 20 male breeders of the New Zealand white rabbit strain and their offspring of almost the same age as in the foregoing colony. Hereafter, they will be referred to as colony A and colony B, respectively. In both colonies, breeding rabbits were housed individually in cages, except for the time of mating, when a female was coupled with a male for less than an hour. Mating intervals for females were usually three months in colony A and two

months in colony B.

Their offspring were weaned at one month of age, after which several weanlings were housed together in a cage for about a month and then separated. All rabbits were given commercial pelleted feed and tap water. These colonies were contaminated with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Eimeria* spp. as well as *P. multocida*, but not with other detectable pathogens, such as *Salmonella* spp., *Mycoplasma* spp., Sendai virus and rabbit pox virus. In both colonies, room temperature was kept between 18°C and 28°C with air conditioners in the summer and heaters in the winter, but other climatic conditions were not controlled, except for exhaust fans for ventilation.

The epizootiological observations of *P. multocida* infection were mainly made on colony A and occasionally on colony B from November 1982 to July 1983.

Examination and identification of *P. multocida*: *P. multocida* was examined on the external nares, paranasal sinuses, trachea, middle and inner ear, conjunctiva, lung, heart, liver, spleen and kidney of rabbits killed by bleeding from the heart under chloroform anesthesia. Samples from the former five organs were obtained by swabbing the mucus membrane with cotton swabs moistened with saline and cultured directly onto 5% horse blood agar. From the latter five organs, pieces were taken aseptically, minced thoroughly with scissors and then stamped on 5% horse blood agar. From live rabbits, nasal swab samples were only taken for cultivation. After incubation at 37°C for 24 and 48 hours, bacterial colonies appearing on the blood agar were examined for *P. multocida*.

P. multocida was identified by colony appearance and the following biological and biochemical properties: gram-negative rod, bipolar staining, negative motility, negative hemolysis, positive reduction of nitrate to nitrite, positive production of indole, negative production of H₂S in SIM medium, negative growth on Simmons citrate medium, negative split of urea, positive fermentation of glucose and sensitivity to penicillin [22].

Examination of antibody: Rabbit sera were examined for *P. multocida* antibody by the tube

agglutination test according to Namioka's method [22, 23] with minor modifications. An antigen was prepared from a fresh isolate from a rabbit of colony A by the following method. About 50 mg of 10-hour culture on the 5% horse blood agar was suspended in 10 ml of 1 N HCl saline and incubated in a plugged tube at 37°C overnight. After centrifugation, sedimented organisms were washed repeatedly with saline of pH 2.2, 2.8, 3.5, 4.5 and 6.0 to avoid a sudden shift in pH from acid to base and finally suspended in phosphate-buffered saline, pH 7.0, adjusting the density to no. 3 of MacFarland's nephelometric series. Serum samples were inactivated at 56°C for 30 minutes before use. A mixture of 0.5 ml each of serum dilutions and the antigen in a tube was incubated at 37°C for two hours and then left at room temperature overnight for reading.

Antibody titer was recorded by the reciprocal of the highest serum dilution showing positive agglutination.

Experimental infection: Young rabbits of two age groups, 23 to 28 days and 36 to 40 days of age, were obtained from colony A and another colony that showed almost the same pathogen status as colony A, but was free from *P. multocida* infection. They were intranasally inoculated with 1–3 × 10², 10⁴ and 10⁵ organisms of a *P. multocida* strain that had been isolated from a rabbit of colony A. The inoculum was prepared by culturing the organism in nutrient broth at 37°C for 24 hours, and one drop (about 0.1 ml) each of appropriate dilutions of the culture was inoculated into the external nares of a rabbit with a micropipette. The inoculated animals were housed individually in metal cages with a wire mesh floor and given commercial pelleted feed (GB-1 made by Funabashi Farm) and tap water *ad libitum*. Two weeks after inoculation, they were killed and subjected to bacteriological examination by the same method described above for the naturally infected rabbits.

Results

1. Localization of *P. multocida* in rabbits: *P. multocida* was examined in various organs of 65 rabbits older than three months of age

Table 1. Localization of *Pasteurella multocida* in rabbits.

Table 1. Localization of <i>Chlamydia</i>										No. of rabbits		
Ext. nares	Paranasal sinuses	Trachea	Middle and inner ear	Conjunctiva	Lung	Heart	Spleen	Liver	Kidney	Colony	Colony	Total
										A	B	
+	+	+	+	+	+					0	2	2
+	+	+	+	+						2	1	3
+	+	+	+		+	+				5	0	5
+	+	+	+							9	1	10
+	+	+		+						1	0	1
+	+	+								3	3	6
+	+		+	+			+			1	0	1
+	+			+						0	2	2
+	+		+							3	1	4
+	+				+	+				2	0	2
+	+									13	0	13
	+	+								0	1	1
										3	0	3
			+							0	1	1
49	53	28	26	9	9	7	1	0	0	54		
(90.7)	(98.1)	(51.9)	(48.1)	(16.7)	(16.7)	(13.0)	(1.9)					
No. of rabbit positive for the organism (%)												

derived from both colonies. The results are summarized in Table 1. The upper respiratory tract was found to be the main site of the fsom organism, in that it was isolated from the paranasalsinuses in 53(98.1%) of the 54 infected rabbits. In addition, it was noteworthy that 49 (90.7%) of the 54 infected animals harbored the organism in their external nares, which were only sampling sites from live animals. White mucopurulent exudate was observed significantly often in the nasal cavity and paranasal sinuses of these infected animals. The organism was also frequently isolated from the trachea and middle and inner ear, being positive in 28 (51.9%) and 26 (48.1%) animals, respectively. Mucopurulent exudate was observed in the tympanic cavities of 12 of 26 animals harboring the organism in the ear. The organism was occasionally isolated from the conjunctiva, lung and heart of nine (16.7%) and seven (13.0%) animals, respectively, but gross pathological changes were not found in these organs. The spleen, liver and kidney were all sterile, except in one rabbit whose spleen harbored a small number of organisms without showing any

lesions.

2. The incidence of *P. multocida* in various age groups of rabbits: Nasal swab samples were taken from live rabbits of various age groups and examined for *P. multocida*. The results are shown in Table 2. The incidence of the organism was as low as 4.3% in sucklings younger than one month of age, but increased steadily with advancing age, reaching nearly 100% in adults older than five months. A rapid increase in the infection rate was observed at two to three months of age.

Table 2. Incidence of *Pasteurella multocida* in nasal cavities of rabbits of various age groups.

Age in months	No. of infected/No. of examined	
	Colony A	Colony B
< 1	6/140(4.3%)	
1 - 2	60/311(19.3%)	38/340(11.2%)
2 - 3	110/222(49.5%)	
3 - 4	139/172(80.8%)	108/160(67.5%)
4 - 5	148/166(89.2%)	
> 5	206/220(93.6%)	10/ 10(100.0%)

Table 3. Relationship between isolation of *Pasteurella multocida* from nasal cavities and agglutinating antibody titers in different age groups.

Age in months	Isolation of the organism	No. of rabbits examined	Agglutination titer								
			<10	10	20	40	80	160	320	640	1280
1-2	+	47	10	5	12	6	6	5	3		
	-	36	25	2	3	3	2	1			
2-3	+	13	1		1	3	1	4	3		
	-	14	7	5	2						
> 3	+	44			1	5	2	10	20	5	1
	-	10	7	2	1						

3. Serological examination: A close correlation between isolation of the organism and detection of serum antibody was observed in rabbits older than two months as summarized in Table 3. In rabbits aged one to two months, the antibody was not detected in 10 (21.3%) out of 47 infected animals; on the contrary, titers of 1:10 or higher were demonstrated in 11 (30.6%) of 36 noninfected ones. In animals older than two months of age, however, almost all the infected animals had antibody titers of 1:40 to 1:1,280, while all of the noninfected animals showed agglutination titers of 1:20 or lower.

4. Experimental infection: In the foregoing two experiments, it was suggested that rabbits younger than two months of age had maternal antibody and were resistant to *P. multocida* infection. To confirm this, experimental infections of *P. multocida* were performed in rabbits of these ages which were obtained from infected dams in colony A and also from a Japanese white rabbit colony free from *P. multocida* infection as control animals. The results are shown in Table 4. In 23- to 28-day-old rabbits, all animals from the *P. multocida*-free colony were infected with the organism, except for two that received 3×10^2 organisms, but in the animals from colony A, the infection was established in only one out of six, three out of nine and three out of six animals that received 3×10^2 , 3×10^4 and 3×10^5 organisms, respectively. However, no significant difference of susceptibility was observed in 36- to 40-day-old rabbits from the two colonies. Regardless of the sources and ages, infected animals usually

Table 4. Experimental infection in young rabbits derived from *Pasteurella multocida* infected and noninfected colonies.

Age	Inoculum size	Rabbits from noninfected colony	Rabbits from infected colony
23-28 days old	3×10^2	3/5*	1/6
	3×10^4	6/6	3/9
	3×10^5	5/5	3/6
36-40 days old	1×10^2	1/3	3/5
	1×10^4	3/3	3/5
	1×10^5	3/3	5/5

* No. of infected/No. of inoculated.

harbored the organism in their nasal cavity and paranasal sinuses and sometimes trachea and middle and inner ear, but remarkable lesions were not demonstrated in these organs, except for accelerated secretion of mucus in the nasal cavity and paranasal sinuses.

Discussion

It is generally considered that *P. multocida* infects rabbits via the respiratory route first, and then spreads to the middle and inner ear through the eustachian tube, to the conjunctiva through the nasolacrimal ducts, to the lung through the trachea or via the blood vascular route and to various organs, including the heart, genitalia and subcutaneous tissues via the blood stream [11, 26, 34]. Similar to findings by previous investigators, in the present study in which localization of *P. multocida* in rabbits was investigated, the organism was

isolated abundantly from the paranasal sinuses, a part of the nasal cavity, of almost all infected animals and less frequently from the trachea, middle and inner ear, conjunctiva and lung, which are anatomically connected to the nasal cavity. These results suggested that the organism spread from the nasal cavity to these organs. Although isolation rates were very low, the organism was also isolated from the heart and spleen, probably indicating translocation of the organism via the blood vascular route. On the other hand, it was noteworthy that more than 90% of the infected animals harbored the organism in their external nares, which were the only sampling sites in live animals.

Hagen [15] indicated that *P. multocida* might be spread from dams to offspring via the respiratory route shortly after birth. In our observations, however, sucklings younger than one month of age were rarely infected with the organism, in spite of the fact that almost all of their dams older than five months of age harbored the organism in the external nares. This phenomenon was also confirmed by an experimental infection in which young, 23- to 28-day-old, rabbits derived from a *P. multocida*-infected colony were significantly resistant to the infection. Such resistance was considered to be due to maternal immunity, because a considerable number of noninfected rabbits younger than one month of age had serum antibody, probably transferred from their dams.

The isolation rates of the organism increased with advancing age of the rabbits, reaching nearly 100% in adults as shown in Table 2. These findings indicated the rare occurrence of natural recovery from the infection and, at the same time, the usefulness of older animals as monitors for detection of *P. multocida* infection in a colony.

Recently, a few reports concerning antibody production in rabbit pasteurellosis [6, 17, 19, 21, 35] have been published. These investigators employed the tube agglutination test using formalinized whole cell antigen [35], the agar gel precipitation test using somatic antigen [19] and the enzyme-linked immunosorbent assay (ELISA) using boiled-cell extract, and appraisable results were obtained by the latter two

methods. In the present study, we also examined serum antibody in infected and noninfected rabbits with the tube agglutination test, in which somatic antigen of *P. multocida* treated with 1 N HCl [22, 23] was used, and found a close correlation between isolation of the organism and detection of antibody, especially in young adults to adults, when antibody titers of 1:40 or higher were regarded as positive. Antibody titers of 1:20 or lower seemed not always to indicate infection, because a considerable number of noninfected animals had serum antibody of these titers. Although *P. multocida* has many serotypes in combinations of capsular and somatic antigens [2-4, 18, 22, 23, 25], predominant serotypes of rabbit strains are not so varied [1, 5, 9, 19, 20, 24]. In particular, major somatic antigens are limited to two to four types. Therefore, serological diagnosis of rabbit pasteurellosis may be useful if the predominant strains are prepared as antigens.

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ウサギにおける *Pasteurella multocida* 感染の細菌学的 および血清学的研究

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日本白色種とニュージーランドホワイト種のウサギ生産コロニーで *Pasteurella multocida* 感染の細菌学的および血清学的観察を行い，以下の所見を得た。本菌は感染ウサギ54匹中53匹の副鼻腔に生息し，鼻孔では49匹が陽性であった。気管および中・内耳からの検出率は50%前後で，時には結膜，肺，心からも分離された。感染率は1カ月齢以下の哺乳仔ではきわめて低かった（4.3%）

が，月齢が進むにつれて上昇し，5カ月齢以上ではほぼ100%に達した。とくに，2～3カ月齢で感染率の急激な上昇を認めた。感染ウサギでは血清中に *P. multocida* のO抗原に対する凝集抗体が検出され，成熟動物では菌分離と抗体産生とがよく一致した。感染母体から生まれた1カ月齢以下の哺乳仔は本菌の経鼻感染に対して抵抗性を示した。