J. Rakuno Gakuen Univ., 13: 83~121 (1988)

Ecological Studies on *Klebsiella pneumoniae* in Horses and Virulence of the Organisms of Capsular Type 1 in Relation to the Size of Capsule*

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(April, 1988)

Contents

* This is a Ph. D thesis presented to the Faculty of Veterinary Medicine, Hokkaido University. The outline of this study was published in Veterinary Microbiology **15**: 219-228 (1987), Japanese Journal of Veterinary Research **35**: 263-273 (1987) and Japanese Journal of Veterinary Science **50**: 313-323 (1988).

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Preface

Klebsiella, a genus of the family Eneterobacteriaceae, is a Gram-negative, non-motile, non-sporebearing, encapsulated rod. The bacteria is widely distributed in the gasterointestinal tract of man and animals, and in soil, water, grain etc [23].

Klebsiella organisms are opportunistic pathogens that can give rise to bacteremia, pneumonia, infection of the urinary tract and several other types of human infection. Due to strains with multiple antibiotic resistance, an increase in *Klebsiella* infection has been reported in recent years particularly in hospitals [41]. In animals, *Klebsiella pneumoniae* can cause genital infection in horses, mastitis in cows, fatal pneumonia with septicaemia in calves, abcesses and cervical adenitis in laboratory mice, and pneumonia, fibrinous pleurisy and peritonitis in guinea-pigs [41].

Klebsiella possesses both O (lipopolysaccharide) and K (polysaccharide) antigens, but serological typing is based on examination of the K antigen. This is because the O antigen types are fewer in number than the K antigen types and because O antigen determination is hampered by the heat-stable K antigens. *Klebsiella* has been classified into 77 capsular types [23].

Klebsiella is divided into 4 species, *K. pneumoniae*, *K. oxytoca*, *K. terrigena* and *K. planticola* [23]. In mares the most troublesome of the *Klebsiella* species is considered to be *K. pneumoniae*. The organism is a causative agent in metritis, cervicitis, infertility and abortion in mares [1, 5, 7, 13, 14, 17, 20, 21, 24, 27, 37, 38]. Equine metritis due to *K. pneumoniae* has been on the rise in many countries, causing a considerable economic loss. Capsular types 1, 2 and 5, in particular, have been associated with epizootic equine metritis [7, 14, 24].

Little information has been available on the distribution of *K. pneumoniae* in the genital tract of stallions. Studies on the isolation of and on the capsular typing of *K. pneumoniae* from feces and nasal swabs of horses have not been extensive enough. The present study provides evidence that capsular type 1 is a predominant type in metritis cases due to *K. pneumoniae*

in mares. Moreover, the same type was also found in the genital tract of stallions. The less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from the genital tract of stallions and from cervical swabs of mares sufferining from metritis, although the less heavily encapsulated strains have not been reported in animals before. The less heavily encapsulated and the heavily encapsulated strains were compared on their virulence for mice and on their capability for causing metritis in mares. This study focuses on the ecology of *K. pneumoniae* in horses and virulence of the organisms of capsular type 1 in relation to the size of capsule.

Chapter I. Capsular types of *Klebsiella pneumoniae* isolated from the genital tract of mares with metritis, extragenital sites of healthy mares and the genital tract of stallions

Introduction

The incidence of equine metritis due to *Klebsiella pneumoniae* has increased in many countries, including Japan [1, 13, 17, 20, 24]. *Klebsiella* has been classified into many capsular types; at present, 77 capsular types (capsular types 1 to 72, 74, and 79 to 82) are known [23].

Some capsular types, such as capsular types 1, 2 and 5, have been associated with epizootic equine metritis [7, 14, 24]. Compared to these types, capsular type 7 was found to be less capable of causing metritis in mares [20, 24]. A shift of the predominant capsular type of *K. pneumoniae* from capsular type 5 to capsular type 1 was found around 1973 in cases of equine metritis in England [24].

It was also found in England and Germany that capsular types 5 and 7 were isolated from the genital tract of stallions [5, 24, 38]; the former was implicated in metritis [5, 24], and the latter, which was the most common capsular type and was probably representative of normal preputial flora, was less implicated in the disease [24]. Since these findings, little information has been available on the distribution of *K. pneumoniae* in the genital tract of stallions [5, 21, 24, 37, 38]. Capsular type 1, which has been predominantly isolated from cases of metritis in mares, is a rare finding in the genital tract of stallions [24, 38].

Studies on the isolation and capsular types of *K. pneumoniae* from feces and nasal swabs of horses have not been extensive enough and thus further investigations of the ecology of *K. pneumoniae* are desirable.

The present survey of *K. pneumoniae* in the horse-breeding areas of Hokkaido, Japan, focuses on: (1) the isolation and capsular types of *K. pneumoniae* from cervical swabs of mares of racing and draft breeds suffering

from metritis; (2) the presence of capsular type 1 in the genital tract stallions; (3) the isolation and capsular types of *K. pneumoniae* from feces and nasal swabs of healthy mares; (4) the presence of less heavily encapsulated strains of capsular type 1 in semen.

Materials and Methods

Animals

Eight hundred and ninety-eight mares of racing breeds with metritis reared on 51 breeding farms and 182 clinically healthy mares and 13 mares that had recovered from metritis due to *K. pneumoniae* reared on 9 breeding farms in Hokkaido, Japan, were examined. These mares ranged from 5 to 18 years of age. Ninety-four healthy stallions of racing breeds reared on 7 studs were examined for a bacteriological survey of semen and genital tracts. Forty-seven mares of draft breeds with metritis reared on 6 breeding farms in Hokkaido were also examined. The breeding farms for racing and draft breeds were located far apart and there was no exchange of horses between the farms.

Collection of specimens

Samples were obtained from cervical swabs, feces and nasal swabs in mares and from semen and swabs of the fossa glandis in stallions.

Cervical swabs were collected only from mares of racing and draft breeds suffering from metritis. Before sampling, the vulva was cleaned with chlorhexidine gluconate and wiped with 70% ethanol, after which a sterile swab was handled with cervical forceps and inserted through a vaginal speculum to scrape the cervix.

Feces and nasal swabs were collected from clinically healthy mares and from mares that had recovered from metritis (mares which had been infected with *K. pneumoniae* 2 to 6 months previously, but showed no clinical signs of infection at the time of sampling). Nasal specimens were collected from the nostrils; a sterile swab was introduced into the nostrils to a depth of about 20 cm.

The swabs from the fossa glandis of stallions of racing breeds were collected after washing the penis with tap water before the stallions were mated. The residual semen after mating was collected for culture.

Culture method

Cervical sawbs were cultured on both trypticase soy agar (BBL, Cockeysville, U. S. A.) supplemented with 5% sheep blood and deoxycholate hydrogen sulfide lactose (DHL) agar (Nissui, Tokyo, Japan) at 37°C for 18 to 24 hours. The number of colonies isolated was recorded.

Swabs of the fossa glandis of stallions were examined likewise. For semen, 0.1 ml of 10-fold serial dilutions of specimens were inoculated onto both media.

Feces and nasal swabs were cultured in Koser's citrate broth (BBL) at 37°C for 18 to 24 hours. After cultivation, one loopful of the broth was inoculated on solidified Koser's citrate medium and incubated at 37°C for 18 to 24 hours. The isolates were identified as *Klebsiella* as described by Cowan [4] and Ørskov [23].

*Preparation of anti-*Klebsiella *capsular sera*

Rabbit antisera against all of the *Klebsiella* reference strains, which were kindly supplied by Dr. Ørskov, International Escherichia and Klebsiella Centre. Statens Seruminstitut, Copenhagen, were prepared by the procedure descirbed by Edwards and Ewing [10]. The reference strains were cultured on Worfel-Ferguson agar [10] to select the strains which produced capsules of moderate size. The selected strains were inoculated into infusion broth (BBL) to which 0.2% of glucose had been added. After several hours of incubation, the culture was killed by addition of 0.5% formalin. The killed suspension was injected into rabbits in amounts of 0.5, 1.0, 2.0, 3.0, 4.0 and 4.0 ml at intervals of 4 days. The immunized rabbits were bled 5 to 7 days after the last injection. When some antisera showed cross-reactivity with other reference strains, these antisera were absorbed with the cross reacted strains. After complete absorption, the sera without cross-reactivity were used as anti-capsular monovalent sera. A total of 65 antisera against the reference strains (capsular types 1 to 56, 60, 64, 66, 68, 70 to 72, 79 and 80) was used in this study. The remaining 12 antisera, anti-capsular types 57 to 59, 61 to 63, 65, 67, 69, 74, 81 and 82, which were rare types, were not used because of the very low titers.

Method of capsular typing

Capsular typing was carried out as follows. The strains tested were cultured on Worfel-Ferguson agar at 37°C for 18 to 24 hours. After cultivation, a heavy suspension of the bacteria was tested first by slide agglutination by using 65 antisera gainst the reference strains of *Klebsiella*. When the isolates showed a positive reaction by the slide agglutination test, the positive reaction was confirmed by the capsular swelling reaction under the microscope.

Microscopic examination of capsules of K. pneumoniae

Capsules of *K. pneumoniae* were examined microscopically after negative staining using Indian ink [9]. Thickness of the capsule was measured with a micrometer eyepiece.

Less heavily encapsulated strains were regarded as those having a thickness of capsule that was approximately 1.2 μm. Heavily encapsulated strains were regarded as those possessing a capsule that was approximately 3.5 μm. These figures were based on the thickness of the capsule of 100 organisms of each representative strain, which was an average of 1.23 ± 0.43 and 3.51 ± 1.23 μm, respectively.

Statistical analysis

The results were analysed by the chi-square test.

Results

Isolation and capsular types of K. pneumoniae *from cervical swabs of metritis cases in mares of racinig and draft breeds*

Isolation of *K. pneumoniae* from 1980 to 1984 from cervical swabs of metritis cases in mares of racing and draft breeds is shown in Table 1. About 77% (on average) of the cervical swabs of mares of racing breeds suffering from metritis yielded bacterial growth. *K. pneumoniae, Streptococcus zooepidemicus, Escherichia coli, Staphylococcus* spp., *Pseudomonas aeruginosa* and fungi were isolated mainly from the cervical swabs of mares of racing breeds. Approximately 80% of the mares positive for bacteria

Table 1. Isolation of *K. pneumoniae* in Hokkaido from 1980 to 1984 from cervical swabs of metritis cases of mares

Year	No. of horses (A)	No. positive for bacteria (B)	Rate (B/A)	No. positive for *K. pneumoniae* (C)	rate (C/A)
Racing breeds					
1980	159	113	71.1	2	1.3
1981	172	123	71.5	10	5.8
1982	310	251	81.0	55	17.7
1983	118	97	82.2	15	12.7
1984	138	105	76.1	29	21.0
Total	897	689	76.8	111	12.4[a]
Draft breeds					
1981	11	8	72.2	3	27.3
1982	23	19	82.9	7	30.4
1983	13	11	84.6	3	23.1
Total	47	38	80.9	13	27.7[a]

a Significant difference between the isolation rates from mares of racing and draft breeds ($P<0.05$).

yielded a single predominant colony type. Of those, more than 90% of the cases yielded more than 50 colonies per plate to an innumerable number. The isolation rate of *K. pneumoniae* from the cervical swabs was only 1.3% in 1980, but increased thereafter; the highest isolation rate was 21.0%, in 1984. In total, 111 out of 897 (12.4%) of the cervical swabs examined were positive for *K. pneumoniae* in mares of racing breeds suffering from metritis.

About 80% of the cervical swabs from mares of draft breeds suffering from metritis yielded bacterial growth. *K. pneumoniae*, *S. zooepidemicus*, *E. coli* and *Candida* spp. were isolated mainly from the cervical swabs of mares of draft breeds. The isolation rate of *K. pneumoniae* from cervical swabs obtained from 1981 to 1983 ranged from 23 to 30%. In total, 13 out of 47 (27.7%) of the cervical swabs were positive for *K. pneumoniae* in mares of draft breeds.

Thus, the isolation rate of *K. pneumoniae* from metritis cases was higher in mares of draft breeds than in those of racing breeds ($P<0.05$).

The capsular types of the isolates from mares with metritis are shown in the upper half of Table 2. In mares of racing breeds suffering from

Table 2. Capsular types of *K. pneumoniae* isolated from mares with metritis and from stallions

Specimens	No. examined	Capsular types				
		1	7	22	39	Untypable[a]
Cervical swabs of mares with metritis						
Racing breeds	88[b]	79	1	1		7
Draft breeds	13				13	
Stallions of racing breeds						
Semen	19[c]	5	7			7
Swab of fossa glandis	1[d]	1				

a Untypable by the antisera against 65 capsular types of *K. pneumoniae* (shown in MATERIALS AND METHODS).
b 88 of 111 isolates were examined.
c 19 of 30 isolates were examined.
d 1 of 4 isolates was examined.

Isolation and capsular types of K. pneumoniae *from semen and swabs of the fossa glandis of healthy stallions of racing breeds*

Isolation of *K. pneumoniae* from semen and swabs of the fossa glandis of healthy stallions of racing breeds is shown in Table 3. *K. pneumoniae* was not isolated from semen or swabs of the fossa glandis in 1981 and 1982, but isolations were made in 1983 and 1984. In 1984, more than 40% of the stallions examined yielded *K. pneumoniae*. A lower percentage of *K.*

Table 3. Isolation of *K. pneumoniae* from 1981 to 1984 from semen and swabs of the fossa glandis of stallions of racing breeds

Year	Semen			Fossa glandis		
	No. positive	No. examined	Rate (%)	No. positive	No. examined	Rate (%)
1981	0	4		0	1	
1982	0	9		0	1	
1983	7	25	28.0	1	17	5.9
1984	23	56	41.1	3	26	11.5
Total	30	94	31.9	4	45	8.9

metritis, 79 out of 88 (about 90%) of the isolates from the cervical swabs were capsular type 1. In mares of draft breeds suffering from metritis, all of the 13 isolates from the cervical swabs belonged to capsular type 39. *pneumoniae* was isolated from swabs of the fossa glandis than from those of semen.

Of the 34 isolates (30 from semen and 4 from the fossa glandis, Table 3) of *K. pneumoniae*, 20 (19 from semen and 1 from the fossa glandis, Table 2) were typed. Five out of 19 (26.3%) isolates of *K. pneumoniae* from semen and 1 isolate from a swab of the fossa glandis were identified as capsular type 1 (lower half of Table 2). Almost the same number of capsular type 7 and untypable strains were isolated from the semen. It is possible that the untypable strains belonged to some of the 12 capsular types, 57 to 59, 61 to 63, 65, 67, 74, 81 and 82, for which immune sera were not available.

Isolation and capsular types of K. pneumoniae *from feces and nasal swabs of healthy mares*

Attempts were made to isolate *K. pneumoniae* from feces and nasal swabs in healthy mares of racing breeds and from mares that had recovered from metritis (Table 4). *K. pneumoniae* was isolated from 21 of 182 (11.5%) healthy mares and similarly from 4 of 13 (30.8%)mares that had recovered from 4 of 23 (17.4%) healthy mares and from 3 of 13 (23.1%) mares that had recovered from metritis due to *K. pneumoniae* capsular type 1. Isolation of *K. pneumoniae* from feces was lower in percentage in the healthy mares than in the mares that had recovered from metrities due to *K. pneumoniae* capsular type 1 ($P=0.05$), though there was no significant difference in isolation of *K. pneumoniae* from nasal swabs.

The capsular types of *K. pneumoniae* isolated from feces and nasal swabs are shown in Table 5. Untypable strains were most predominant in feces and nasal swabs of healthy mares and in the mares that had recovered

Table 4. Isolation of *K. pneumoniae* from 1983 to 1986 from feces and nasal swabs of mares of racing breeds

Mares	Feces			Nasal swabs		
	No. positive	No. examined	Rate (%)	No. positive	No. examined	Rate (%)
Healthy	21	182	11.5[a]	4	23	17.4
Recovered	4	13	30.8[a]	3	13	23.1

a Significant difference between the isolation rates from healthy mares and mares recovered from metritis ($P=0.0.5$).

Table 5. Capsular types of *K. pneumoniae* isolated from feces and nasal swabs of mares

Specimens	No. examined	Capsular types				
		1	30	31	32	Untypable[a]
Faces from mares						
Healthy	21			1	1	19
Recovered	4		1			3
Nasal swabs from mares						
Healthy	4		1			3
Recovered[b]	3			1	1	1

a Recovered 2 to 6 months ago from metritis due to *K. pneumoniae* capsular type 1.
b Untypable by the antisera against 65 capsular types of *K. pneumoniae* (shown in MATERIALS AND METHODS).

from metritis due to *K. pneumoniae* capsular type 1; capsular type 1 was not isolated. Although feces and nasal sawbs of the mares suffering from metritis were not surveyed as a group, one mare suffering from metritis revealed capsular type 1 in feces and in the nasal cavity.

Presence of less heavily encapsulated strains of capsular type 1 in semen

Two colonial types were found among the 6 strains of capsular type 1 isolated from semen. One type was heavily encapsulated and similar to the colonial morphology of capsular type 1 strain from mares of racing breeds with metritis; it was 3 to 5 mm in diameter, honey-colored, between opaque and translucent in density, dome-shaped, moist, nonhemolytic and very mucoid and tended to coalesce on blood agar. The other type was less heavily encapsulated, small (2 to 3 mm in diameter), milky and yellowish-white-colored opaque, dome-shaped, moist and nonhemolytic and less mucoid on blood agar. The heavily encapsulated strain was isolated from semen samples from 2 stallions. The remaining 4 stallions yielded only the less heavily encapsulated strains. Each isolate was either of the heavily or less heavily

encapsulated type, but did not contain a mixture of the two. Only mares bred to stallions carrying the heavily encapsulated type developed metritis, whereas mares bred to stallions carrying the less heavily encapsulated type did not.

Discussion

The rates of *K. pneumoniae* isolation from the cervical swabs of mares of racing and draft breeds from 1980 to 1984 averaged 12.4% and 27.7%, respectively. Capsular typing of the isolates revealed clearly that most of the isolates from metritis-infected mares of racing breeds belonged to capsular type 1, whereas those from mares of draft breeds belonged to capsular type 39. The fact that capsular type 1 was predominant in mares of racing breeds was similar to previous reports [17, 24]. Metritis due to *K. pneumoniae* capsular type 39 was reported only once in a horse whose breed was not specified [24].

Predominance of capsular type 39 as the causative agent of metritis in mares of draft breeds is interesting. In the present study, all the mares examined came from 6 closed populations, which were different in location and no exchanges had been made with racing breeds. Perhaps capsular types 1 and 39 were distributed among racing and draft breeds, respectively. The possibility that capsular type 39 causes metritis in mares of racing breeds and that capsular type 1 causes metritis in mares of draft breeds cannot be excluded and is the subject of future study.

Increase of the isolation rate of *K. pneumoniae* from the genital tract of stallions continued for two years in Hokkaido after the increase of the rate of isolation of *K. pneumoniae* from metritis cases. It was presumed that *K. pneumoniae* infection was spread among horses of racing breeds by mating; stallions were contaminated after mating with mares suffering from metritis, the infection was then transmitted to other mares.

Of 20 isolates from stallions, 6 were capsular type 1 and 7 were capsular type 7. In metritis cases, however, capsular type 1 was the predominant type (about 80%) and capsular type 7 was isolated from only one case. The findings that capsular type 1 was implicated in metritis and capsular type 7 had less potential to cause metritis and was representative of the normal flora agreed with the previous report [24]. To prevent the spread of metritis due to *K. pneumoniae*, it is necessary to make periodical examinations of semen and the genital tract of stallions.

Two types of capsular type 1 strains with a different colonial morphology (mucoid and heavily encapsulated, and less mucoid and less heavily encapsulated) were isolated from the semen of stallions. Each stallion possessed either a heavily encapsulated or a less heavily encapsulated strain. The

first type of colony, which was heavily encapsulated, large and mucoid, was similar to that of capsular type 1 strains from metritis cases and presumably caused metritis in the mares that were mated with stallions, whereas mares mated with stallions carrying the second, less heavily encapsulated type of colony, did not develop metritis. Difference in virulence between the two types of capsular type 1 strains is important and is described in the chapters 2 and 3.

The capsular types of *K. pneumoniae* from feces of horses has been reported only once and the presence of capsular types 7, 21 and 30 was noted [24]. The same report showed the isolation of capsular types 5 and 11 from the nasal cavity. In the present study, untypable *K. pneumoniae* was predominant (25 out of 32, 81.3%), but no capsular type 1 was present in feces or the nasal cavity of healthy mares of racing breeds, or in the mares that had recovered from metritis due to *K. pneumoniae* capsular type 1.

Contagious equine metritis broke out in 1980 for the first time in south-western Hokkaido, Japan [15, 19, 32], in the same areas surveyed in the present study of *K. pneumoniae*. To prevent the spread of contagious equine metritis, antibiotics such as penicillin, aminobenzyl-penicillin, chloramphenicol, streptomycin and kanamycin were used extensively in mares of racing breeds in these areas, particularly in 1980 and 1981. Since *K. pneumoniae* is resistance to these antibiotics [16, 30], the use of these durgs might have changed the bacterial flora in the mares of racing breeds and this may have been responsible for the increase in cases of metritis due to *K. pneumoniae*.

Summary

A survey of *K. pneumoniae* was performed on cervical swabs, feces and nasal swabs of mares and on samples from the genital tract of stallions from 1980 to 1986 in south-western Hokkaido, Japan. Capsular type 1 was the predominant type (79 of 88, 89.8%) in the metritis cases due to *K. pneumoniae* in mares of racing breeds. The same type was isolated from semen and swabs of the fossa glandis of 6 of 20 (30.0%) of the stallions of racing breeds. Heavily encapsulated and less heavily encapsulated strains of capsular type 1 were isolated from the stallions. Mares bred to stallions carrying heavily encapsulated strains developed metritis, whereas those bred to stallions carrying less heavily encapsulated strains did not. Capsular type 39 was isolated from cervical swabs solely from metritis-infected mares of draft breeds and not from any mares of the racing breeds examined. Untypable strains were isolated from cervical swabs in 7 of 88 (8.0%) metritis cases of mares of racing breeds and from semen in 7 of 19 (36.8%) stallions of

racing breeds and they were predominant in feces (19 of 21, 90.5%) and nasal swabs (3 of 4, 75.0%) of healthy mares of racing breeds.

Chapter II. Presence of less heavily encapsulated *Klebsiella pneumpniae* capsular type 1 in semen of healthy stallions and cervical swabs of mares suffering from metritis, and comparison of virulence between heavily and less heavily encapsulated strains

Introduction

Klebsiella pneumoniae is an important cause of metritis in mares. Epizootics of metritis are usually associated with *K. pneumoniae* capsular types 1, 2 and 5 [1, 5, 14, 24]. Predominance of *K. pneumoniae* capsular type 1 as the causal agent of metritis in mares has been reported [11, 17, 24]. The same capsular type was isolated also from the urogenital tract of stallions [11, 24, 38]. In the previous chapter, capsular type 1 was predominant type in cervical swabs of mares suffering from metritis due to *K. pneumoniae* and same type was isolated from semen and fossa glandis of stallions, likewise. Furthermore, less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from semen and swabs of fossa glandis of healthy stallions. Mares bred to stallions carrying less heavily encapsulated strains did not develop metritis, whereas those of bred to stallions carrying heavily encapsulated strains did.

Less heavily encapsulated strains of *K. pneumoniae* were isolated from clinical specimens of patients [33, 34, 35] and were less virulent for mice [35]. Variants of *K. pneumoniae* possessing a small capsule also arose from the culture of heavily encapsulated strains, while heavily encapsulated strains were subcultured repeatedly in soft agar [36]. Less heavily encapsulated variants of *K. pneumoniae* capsular types 1 and 2 isolated by Domenico et al. were comparatively non-virulent in experimental lobar pneumonia and burn wound sepsis in mice [6, 8].

Non-capsulated variants of *K. pneumoniae* capsular type 1, on the other hand, have been known to arise from heavily encapsulated strains with [25] or without mutagen [31] and are known to be weakly virulent for mice [31] and to be easily ingested by human polymorphonuclear leukocytes (PMNL) compared with encapsulated parent strains [31, 39, 40].

The present study focuses on equine *K. pneumoniae* capsular type 1 strains which were less heavily encapsulated. In this chapter, distribution of heavily and less heavily encapsulated strains of *K. pneumoniae* capsular type 1 in mares and stallions, virulence of those strains for mice and susceptibility to phagocytosis are described and compared.

Materials and Methods

Bacterial strains

Eighty-eight strains of *K. pneumoniae* capsular type 1 including the strains described in the previous chapter and newly isolated strains, were used in this study. These strains were isolated from horses raised in southwestern Hokkaido. Of these, 5 were isolated from semen of healthy stallions and 83 from cervical swabs of mares suffering from metritis. Five strains (RK93, RK26, RK31, RK43 and RK55) isolated from semen of stallions and 10 strains (F6, H9, E134, E135, RK72, RK73, RK78, RK79, RK80 and RK84) isolated from cervical swabs of mares were selected from among a total of 88 strains to observe the capsule and to examine the mean lethal dose (LD_{50}) value for mice and the degree of phagocytosis by PMNL. Strain A 5054, which was supplied by Dr. Ørskov, International Escherichia and Klebsiella Centre, Statens Seruminstitut, Copenhagen, was used as the reference strain of *K. pneumoniae* capsular type 1. All the strains were suspended in 10% skim milk and stored at −80°C until used.

These strains were cultured on trypticase soy agar at 37°C for 24 hours for observation of colonial morphology and for 48 hours for observation of the capsule.

Isolation of non-capsulated variants of K. pneumoniae

Non-capsulated variants (f6, h9, e132 and e135) were obtained by 120 to 130 *in vitro* passages on trypticase soy agar without mutagenic treatment from 4 strains (F6, H9, E134 and E135) of *K. pneumoniae* capsular type 1 isolated from cervical swabs of mares suffering from metritis. These variants were translucent and non-mucoid in colonial morphology, and the main characteristics were not different from those of the parent strains. Non-capsulated variants did not show any positive capsular sweiling reaction [3]. These were used as non-capsulated variants in this study.

Observations of capsule of K. pneumoniae *under a light and electron microscopy*

Observations of the capsule were carried out with a light microscope after negative staining with Indian ink by the method of Duguid [9], and with an electron microscope after negative staining with 0.1% phosphotungstic acid. Bacterial strains cultured on trypticase soy agar at 37°C for 48 hours were suspended in Phosphate-buffered saline solution (PBSS, pH 7.2) and examined.

The capsule was observed in very thin wet Indian ink film and micrographs were randomly taken. Thickness of capsule was expressed as the

transverse diameter, which was determined by measuring both the width of the bacillus and its surrounding capsule. One hundred bacteria cells were measured at random.

Observation of the capsule under an electron microscope was performed by using the following technique. One drop of the bacterial suspension was placed on a carbon-coated copper grid. The excess drop was removed with a pointed piece of filter paper and a drop of 2% ammonium acetate was added to the grid. This drop was also removed with filter paper and a drop of 0.1% phosphotungstic acid was placed on the grid for 10 minutes. The excess drop was removed with filter paper and the grid was allowed to dry overnight before examination by the electron microscope for the presence of capsules. The specimens were examined under a JEM-100S (Japan Electron Optics Laboratory, Tokyo, Japan) transmission electron microscope under an accelerating voltage of 80 kV.

Virulence for mice

Both sexes of ddY mice weighing 20 to 25 grams were used. Just before use, the organisms forming smooth colonies were selected and cultured at 37°C overnight in trypticase soy broth (BBL). The organisms were harvested and washed twice by centrifugation and resuspended in PBSS. The lethality of the organisms for mice was tested by the intraperitoneal inoculation of graded doses in 10-fold steps of test material in 0.2 ml of PBSS into groups of mice (five mice per group). Mice receiving injections were observed for 2 weeks. The LD_{50} of the organisms was calculated from the death rate of each group of mice by the method of Karber [18] and were expressed as colony forming unit (CFU).

Preparation of PMNL of horse

Heparinized venous blood was obtained from a healthy adult horse. Two ml of heparinized blood were mixed with 6 ml of 0.85% NaCl, layered over 3 ml Conray 400 (Daiich Pharmaceuticals, Tokyo, Japan) — Ficoll (Pharmacia Fine Chemicals, Sweden) mixture (containing 10 ml of 33.4% Conray 400 and 24 ml of 9% Ficoll in water), and centrifuged at 1,550 rpm for 30 minutes at room temperature. The PMNL-rich fraction was obtained from a thin layer just above the large pellet containing erythrocytes. To remove erythrocyte contamination, the PMNL-rich fraction was treated with 0.2% NaCl solution for about 20 seconds, made isotonic with 1.6% NaCl solution, and the PMNL were washed twice at 1,550 rpm for 10 minutes. The PMNL were resuspended in Hank's medium to 1×10^6 cells/ml for observation of phagocytosis.

Phagocytosis assay

Trypticase soy broth cultures of strains in the logarithmic phase of

growth were harvested and washed twice by centrifugation and resuspended in Hank's medium. For observation of phagocytosis, the bacterial suspension was diluted to 1×10^8 organisms/ml. Observation of phagocytosis by PMNL was performed both *in vitro* and *in vivo*. Phagocytosis experiments *in vitro* by healthy equine PMNL were performed as follows: 0.1 ml of bacterial suspension (1×10^8 bacteria/ml), 0.1 ml of PMNL (1×10^6 cells/ml) (bacteria : PMNL ratio of 100 : 1), 0.1 ml of pooled healthy horse serum and 0.2 ml of Hank's medium were incubated with shaking in a water bath at 37°C for 15 minutes. After incubation, the mixture was centrifuged in Hank's medium three times at 500 rpm for 5 minutes to wash out and remove any uningested bacteria. The resulting pellet was then smeared on slide glass.

In an experiment *in vivo*, PMNL was induced in the abdominal cavity of ddY mice by the intraperitoneal injection of 0.2% sodium caseinate, and mice were injected with the bacterial suspension by the intraperitoneal injection after 7 hours. After 30 minutes, the ascites were collected and smears were made. The smears were air-dried, fixed in methanol and stained with Giemsa staining solution. One hundred PMNL were examined microscopically to determined phagocytic index (the number of bacteria ingested per leukocyte).

Results

Presence of heavily encapsulated and less heavily encapsulated strains of K. pneumoniae *capsular type 1 in semen of healthy stallions and cervical swabs of mares suffering from metritis*

Capsules of heavily encapsulated and less heavily encapsulated strains of *K. pneumoniae* capsular type 1 isolated from semen of healthy stallions and cervical swabs of mares suffering from metritis are described in Table 6. The strains shown in the table include all the heavily and less heavily encapsulated strains (1 and 4 strains, respectively) from the semen of stallions and all the less heavily encapsulated strains (7 strains), but only some representative (3/76) strains of heavily encapsulated strains from cervical swabs of mares suffering from metritis. Non-capsulated variants isolated in the author's laboratory from equine heavily encapsulated strains were used as control, together with reference strain A 5054. All the strains of *K. pneumoniae* capsular type 1 isolated from semen and cervical swabs showed a positive capsular swelling reaction with homologous antiserum, whereas the 5 non-capsulated variants showed a negative reaction.

Strains possessing a capsule of more than 3.0 μm in transverse diameter were judged as heavily encapsulated, whereas those possessing a capsule diameter ranging from 1.3 to 2.2 μm were judged as less heavily encapsulated strains (Table 6). The non-capsulated variants isolated in the author's

Table 6. Capsules of heavily encapsulated and less heavily encapsulated strains of *K. pneumoniae* capsular type 1 isolated from semen of healthy stallions and cervical swabs of mares suffering from metritis

Strains	Capsule		
	Thickness measured microscopically (mean±μm)[a]	Sort of encapsulation	Colonial morphology
Semen			
RK 93	3.4±1.0	Heavily encapsulated	BOT[b], very mucoid
RK 26	1.3±0.2	Less heavily encapsulated	Opaque, less mucoid
RK 31	1.5±0.3	〃	〃
RK 43	1.4±0.3	〃	〃
RK 55	1.4±0.3	〃	〃
Cervical swabs			
F 6	3.5±1.1	Heavily encapsulated	BOT, very mucoid
H 9	3.0±1.0	〃	〃
E 134	3.1±0.9	〃	〃
E 135	1.7±0.2	Less heavily encapsulated	BOT, mucoid
RK 72	2.2±0.3	〃	〃
RK 73	2.0±0.3	〃	〃
RK 78	2.1±0.4	〃	〃
RK 79	2.0±0.4	〃	〃
RK 80	1.9±0.3	〃	〃
RK 84	2.0±0.3	〃	〃
Variants			
f 6	—[c]	Non-capsulated	Translucent, non-mucoid
h 9	—	〃	〃
e 134	—	〃	〃
e 135	—	〃	〃
Reference strain			
A 5054	3.2±0.1	Heavily encapsulated	BOT, mucoid

a Thickness of capsule was regarded as the transverse diameter, which was determined by measuring both the width of the bacillus and its surrounding capsule under a light microscope after negative straining with Indian ink. One hundred organisms of trypticase soy agar culture for 48 hours under a light microscope were measured, and the average of the thickness was calculated.

b BOT: between opaque and translucent in density.

c Capsule was not observed under a microscope after negative staining with Indian ink, and was negative in capsular swelling reaction.

laboratory did not possess any capsules under a light microscope after staining with Indian ink. The reference strain A 5054 was heavily encapsulated.

The colonies of the heavily encapsulated strains were between opaque and translucent in density and very mucoid, whereas those of the less heavily encapsulated strains were opaque and less mucoid (semen-origin strains) or between opaque and translucent and mucoid (cervical swab-origin strains). Non-capsulated strains were translucent and non-mucoid.

Light and electron micrographs of each of the heavily encapsulated, less heavily encapsulated and non-capsulated strains are shown in Figs. 1

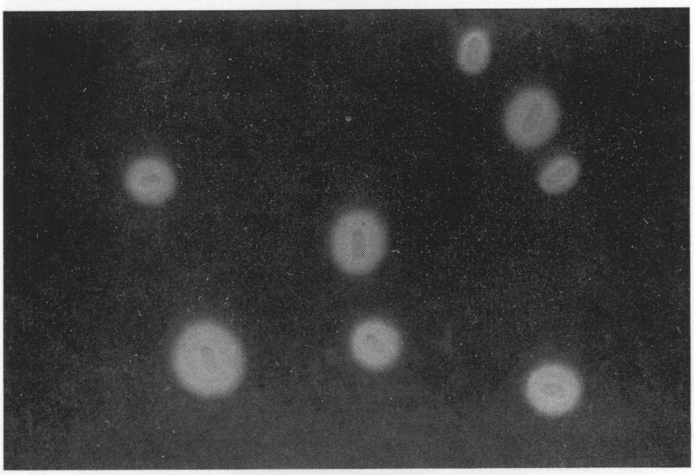

Fig. 1. Heavily encapsulated strain (F 6) under a light microscope after staining with Indian ink. × 1,200.

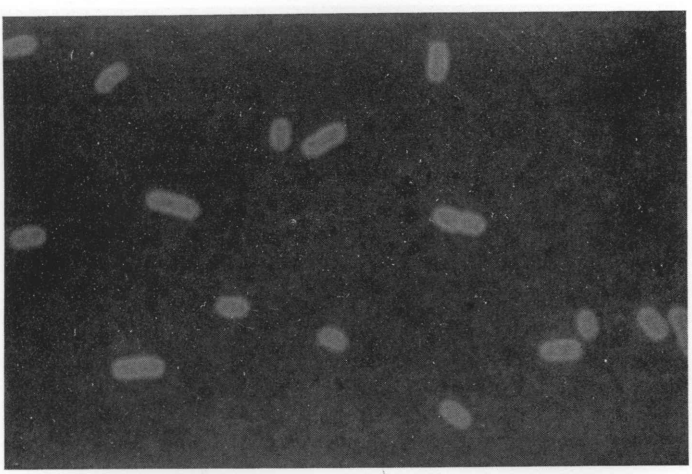

Fig. 2. Less heavily encapsulated strain (RK 26) under a light microscope after staining with Indian ink. × 1,200.

to 6. Heavily encapsulated strain F6 revealed as thick capsule which was almost 5 to 6 times the minor axis of the organisms (Fig. 1). Less heavily encapsulated strain RK26 possessed a thin capsule which was almost 2 times the minor axis of the organisms (Fig. 2). Non-capsulated variant f6 possessed no capsule (Fig. 3). These light micrographs were taken under the same exposure. Capsules looked bright, whereas bacilli looked dark. In addition, non-capsulated bacilli (Fig. 3) were usually out of focus due to

Fig. 3. Non-capsulated strain (f 6) under a light microscope after staining with Indian ink. × 1,200.
The micrograph was taken under the same exposure as Figs. 1 and 2.

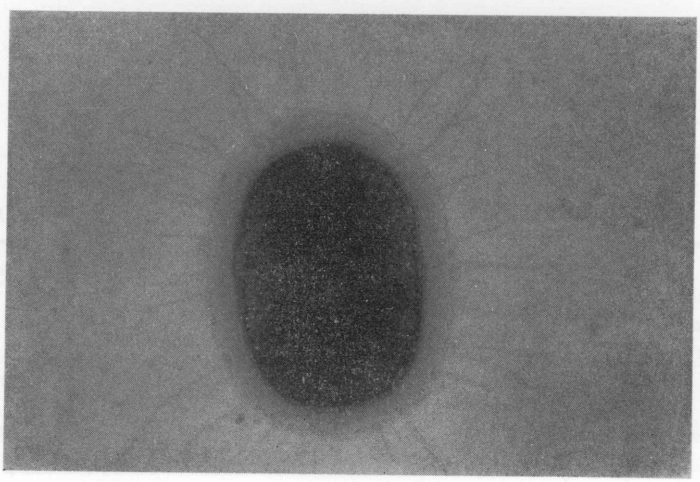

Fig. 4. Heavily encapsulated strain (F 6) under an electron microscope after negative staining with 0.1% phosphotungstic acid. × 18,000.

Brownian movement, which was more remarkable in non-capsulated bacilli than in heavily and less heavily encapsulated organisms. Observed under electron microscope, heavily encapsulated strain F6 was obviously surrounded by a layer of amorphous materials about 220 nm in thickness (Fig. 4). A thin capsule-like substance was found around the organisms of less heavily encapsulated strain RK26 (Fig. 5). Neither a capsule nor an amorphous layer was observed around the bacilli of non-capsulated variant f6 (Fig. 6).

Thus, the difference between the capsule of heavily encapsulated and

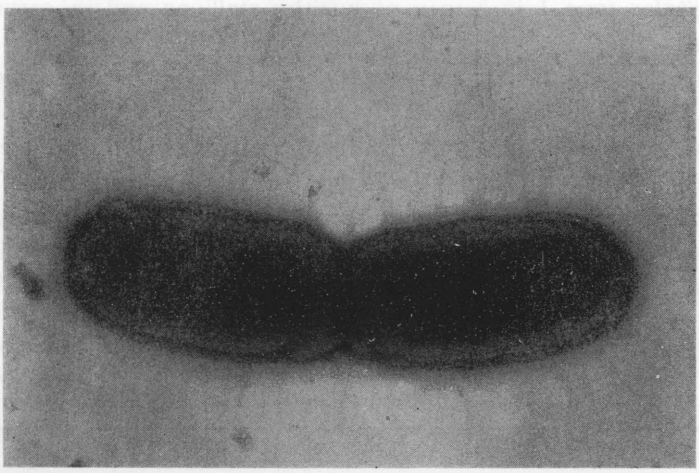

Fig. 5. Less heavily encapsulated strain (RK 26) under an electron microscope after negative staining with 0.1% phosphotungstic acid. × 18,000.

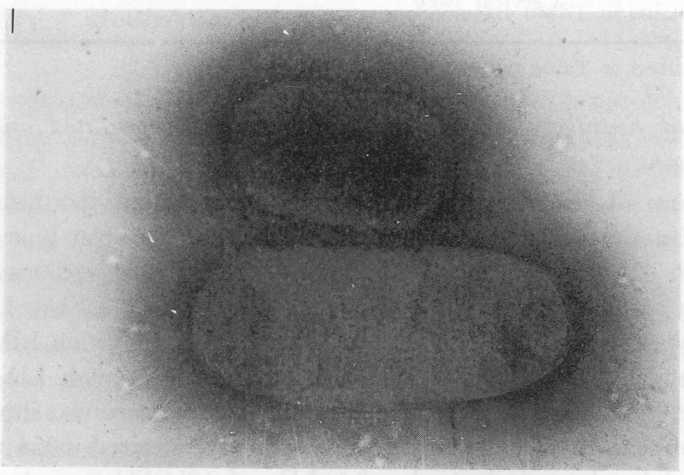

Fig. 6. Non-capsulated strain (f 6) under an electron microscope after negative staining with 0.1% phosphotungustic acid. × 18,000.

less heavily encapsulated strains was more clearly shown under a light microscope than under an electron microscope. The capsule was probably shrunk by unavoidable drying during preparation of the specimens for electron microscopy.

Distribution of heavily encapsulated and less heavily encapsulated strains of K. pneumoniae *capsular type 1 among healthy stallions and mares suffering from metritis.*

Distribution of heavily and less heavily encapsulated strains of *K. pneumoniae* capsular type 1 in semen of healthy stallions and in cervical swabs of mares suffering from metritis is shown in Table 7. Of the 5 isolates from the semen of stallions, one (20.0%) was heavily encapsulated and the remaining 4 (80.0%) were less heavily encapsulated. Of the 83 isolates from the cervical swabs of mares suffering from metritis, 76 (91.6%) were heavily encapsulated and only 7 (8.4%) were less heavily encapsulated. Thus, less heavily encapsulated strains were predominant in semen of healthy stallions, whereas heavily encapsulated strains were predominant in cervical swabs of mares suffering from metritis.

Table 7. Distribution of heavily encapsulated and less heavily encapsulated strains of *K. pneumoniae* capsular type 1 in semen of healthy stallions and in cervical swabs of mares suffering from metritis

Horses	Source	Encapsulation[a]	No. of isolate
Healthy stallions	Semen	Heavily encapsulated	1
		Less heavily encapsulated	4
Mares suffering from metritis	Cervical swab	Heavily encapsulated	76
		Less heavily encapsulated	7

a As described in Table 6.

Virulence of heavily encapsulated, less heavily encapsulated and non-capsulated strains

Virulence of randomly selected heavily encapsulated, less heavily encapsulated and non-capsulated strains of *K. pneumoniae* capsular type 1 is shown in Table 8. Heavily encapsulated strains, either from stallions or mares, were highly virulent for mice, showing LD_{50} of 10^2 to 10^4 CFU. Less heavily encapsulated strains from stallions were less virulent, showing LD_{50} of 10^7 to 10^8 CFU, which was similar to non-capsulated variants with LD_{50} of 10^6 to 10^8 CFU. A less heavily encapsulated strain from a mare was intermediately virulent, showing LD_{50} of 10^5 CFU. The reference strain A 5054 was heavily encapsulated but less virulent, showing LD_{50} of 10^7 CFU.

Susceptibility of heavily encapsulated, less heavily encapsulated and non-

Table 8. Virulence of heavily encapsulated and less heavily encapsulated strains of *K. pneumoniae* capsular type 1 isolated from semen of healthy stallions and cervical swabs of mares suffering from metritis

Strains	Encapsulation	LD_{50} for mice (CFU)	Phagocytic index[a]	
			PNML of horse[b]	PMNL of mice[c]
Semen				
RK 93	Heavy	1.4×10^{2}	0.19	0.36
RK 26	Less heavy	2.5×10^{8}	3.41	3.89
RK 55	"	1.7×10^{7}	1.82	2.26
Cervical swabs				
F 69	Heavy	1.5×10^{3}	0.16	0.40
H 1	"	1.2×10^{3}	0.15	0.30
E 34	"	1.9×10^{4}	0.09	0.38
E 135	Less heavy	1.3×10^{5}	1.49	2.01
Variants isolated in laboratory				
f 6	Non	6.3×10^{6}	3.77	NT[d]
h 9	"	1.9×10^{7}	3.59	NT
e 134	"	5.5×10^{7}	3.22	NT
e 135	"	5.2×10^{8}	3.88	NT
Reference strain				
A 5054	Heavy	3.8×10^{7}	0.02	NT

a Expressed as number of *K. pneumoniae* ingested per leukocyte.
b Susceptibility to phagocytosis *in vitro* by PMNL of horse.
c Susceptibility to phagocytosis *in vivo* by intra-abdominal PMNL of mice.
d NT, not tested.

capsulated strains to phagocytosis by PMNL of healthy horse *in vitro* and PMNL of mice *in vivo* is also shown in Table 8. Heavily encapsulated strains ingested were 0.09 to 0.19 bacteria per cell by PMNL of horse *in vitro* and 0.30 to 0.40 bacteria per cell by peritoneal PMNL of mice, which showed they were protected from phagocytosis. Of the less heavily encapsulated strains, one strain from a cervical swabs was ingested at 1.49 bacteria per cell by PMNL of horse and 2.01 bacteria per cell by PMNL of mice, and 2 strains from semen were ingested at 1.82 to 3.41 beacteria per cell by PMNL of horse and 2.26 to 3.89 bacteria per cell by PMNL of mice. Non-capsulated variants were ingested at 3.22 to 3.88 bacteria per cell by PMNL of horse. Phagocytic index for each strain was paralleled with LD_{50} for mice, except the reference strain A 5054.

Discussion

The present study showed that less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from semen of healthy stallions and cervical swabs of mares suffering from metritis. Distribution of the heavily encapsulated and less heavily encapsulated strains showed a distinct difference in that the less heavily encapsulated strains were predominant in semen of healthy stallions whereas the heavily encapsulated strains were predominant in cervical swabs of mares with metritis. Less heavily encapsulated strains were low or intermediate in virulence for mice and were easily ingested by equine and murine PMNL, whereas heavily encapsulated strains were highly virulent for mice and were protected from phagocytosis by the PMNL. The difference of virulence between heavily and less heavily excapsulated strains is considered to have some relation to the epidemiological findings (1) that mares bred to stallions carrying heavily encapsulated strains developed metritis whereas mares bred to stallions carrying less heavily encapsulated strains did not (chapter I), and (2) that less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated in the present chapter from the mares suffering from metritis at the rate of only 8.4% (7/83). Virulence of less heavily encapsulated strains of capsular type 1 derived from stallions and mares with metritis needs to be tested by experimental uterine infection in mares.

There was a slight difference in colonial morphology and thickness of the capsule between the less heavily encapsulated strains isolated from semen and those from cervical swabs. Semen-origin strains were less mucoid and opaque in colonial morphology and the thickness of the capsule was 1.3 to 1.5 μm in transverse diameter, whereas cervical swab-origin strains were between opque and translucent in density and mucoid in colonial morphology and the thickness of capsule was 1.7 to 2.2 μm in transverse diameter. It is unknown whether the less heavily encapsulated strains isolated from semen and cervical sawbs are originally different from each other, or whether the semen-origin less heavily encapsulated organisms might slightly increase the thickness of capsule and change to cervical swab-type less heavily encapsulated organisms in uterine cavity or the cervical swab-origin organisms might be changed to semen-type organisms in the urogenital tract of stallions. Experimental uterine infection of mares with less heavily encapsulated strains isolated from semen of stallions might partially answer this question.

Less heavily encapsulated strains were obtained by mutation from a population of heavily encapsulated strains *in vitro* [8, 12, 36]. Such mutation has not been evidenced *in vivo*. This is a subject of future study.

Equine PMNL were used in the present study to examine susceptibility

to phagocytosis of the heavily and less heavily encapsulated strains of *K. pneumoniae* of equine origin. Less heavily encapsulated strains were easily phagocytized whereas heavily encapsulated strains were protected from phagocytosis by equine PMNL. The difference between the heavily and less heavily encapsulated strains to phagocytosis was also shown by murine PMNL.

It is conceivable from the results of the present and previous chapters that the urogenital tract of stallions provides an advantageous environment for less heavily encapsulated organisms but a disadvantageous one for heavily encapsulated organisms. It is also conceivable that equine uterine cavity provides a beneficial environment for heavily encapsulated organisms (leading to metritis) but a disadvantageous one for less heavily encapsulated organisms. Experimental infection of mares with the heavily and less heavily encapsulated *K. pneumoniae* strains, which is stated in chapter III, seems to support the latter speculation.

Virulence of less heavily encapsulated strains was low and similar to virulence of non-capsulated strains. Virulence of heavily encapsulated *K. pneumoniae* capsular type 1 has been reported [6, 8, 12]. A non-capsulated strains did not develop metritis in mares (describe in chapter III). Studies are described in next chapter to determine whether less heavily encapsulated strains are able to cause metritis in mares, or whether full encapsulation is necessary for *K. pneumoniae* capsular type 1 to cause metritis in mares.

Summary

Less heavily encapsulated strains of *K. pneumoniae* capsular type 1, which were different from ordinary heavily encapsulated strains, were isolated from semen of healthy stallions and from cervical swabs of mares suffering from metritis. Less heavily encapsulated strains possessed capsules ranging from 1.3 to 2.2 μm in transverse diameter, which were obviously smaller than those of heavily encapsulated strains, which possessed capsules more than 3.0 μm in diameter. Of the 5 strains of *K. pneumoniae* capsular type 1 isolated from semen of healthy stallions, one (20.0%) was heavily encapsulated and the remaining 4 (80.0%) were less heavily encapsulated, whereas of the 83 strains of *K. pneumoniae* capsular type 1 from the cervical swabs of mares suffering from metritis, 76 (91.6%) were heavily encapsulated and only 7 (8.4%) were less heavily encapsulated. Less heavily encapsulated strains showed low (semen-origin strains, LD_{50} was 10^7 to 10^8 CFU) or intermediate (cervical swab-origin strain, LD_{50} was 10^5 CFU), virulence for mice and were easily phagocytized by equine and murine PMNL, whereas heavily encapsulated strains showed high virulence (LD_{50} was 10^2 to 10^4 CFU) for mice and were protected from phagocytosis by the equine and murine PMNL.

Chapter III. Difference of virulence in causing metritis in horses between heavily encapsulated, less heavily encapsulated and non-capsulated strains of *Klebsiella pneumoniae* capsular type 1

Introduction

Klebsiella pneumoniae is an important cause of metritis in mares. Predominance of *K. pneumoniae* capsular type 1 as the causal agent of metritis in mares has been reported [11, 17, 24].

In the chapter II, it was shown (1) that less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from semen of healthy stallions and cervical sawbs of mares suffering from metritis, and (2) that less heavily encapsulated strains were distributed predominantly in semen of healthy stallions whereas heavily encapsulated strains were distributed predominantly in cervical swabs of mares with metritis. Less heavily ecapsulated strains were low or intermediate in virulence for mice and were easily ingested by equine and murine PMNL, whereas heavily encapsulated strains were highly virulent for mice and protected from phagocytosis by the PMNL. The difference of virulence between heavily and less heavily encapsulated strains is considered to have some relation to the following epidemiological findings: (1) that mares bred to stallions carrying less heavily encapsulated strains did not develop metritis, whereas mares bred to stallions carrying heavily encapsulated strains did (chapter I); and (2) that less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from mares suffering from metritis at the rate of only 8.4% (chapter II). Virulence of less heavily encapsulated strains of capsular type 1 was thought to be tested in mares.

Experimental uterine infection of mares with *K. pneumoniae* capsular type 1 was reported only once [26]. A pony mare was inoculated with *K. pneumoniae* capsular type 1 strain (designated as *K. aerogenes* according to an old classification, encapsulation of the strain was not specified) with the inoculum size of 6×10^8 CFU. The strain was recovered from the cervix of the pony at 48 hours post ionculation only and from the clitoris/urethra at 24 hours, 48 hours and 20 days post inoculation. No sign of external vulval discharge and no real evidence of vaginal and cervical inflammation were shown. Experimental uterine infection of mares with *K. pneumoniae* capsular types 68 and 10 isolated from the genital tract stallions with a history of breeding problems showed that inoculated mares became infected and remained infected until the post-inoculation estrous cycle was initiated or completed, and that the numbers of *K. pneumoniae* decreased in the uterus of mares after completing the estruos cycle after inoculation [2].

In the present chapter, experimental uterine infection of mares with

heavily encapsulated, less heavily encapsulated and non-capsulated strains of *K. pneumoniae* capsular type 1 is made to determine if there is any relation between bacterial encapsulation and the ability of bacteria to cause metritis in mares.

Materials and Methods

Bacteria

F6, the heavily encapsulated strain, was isolated from cervical swab of a mare suffering from metritis (chapter II). RK26, the less heavily encapsulated strain, was isolated from semen of a healthy stallion (chapter II). Strain f6, the non-capsulated strain, was a variant obtained from strain F6 (chapter II). The organisms forming smooth colonies of these strains were suspended in 10% skim milk and stored at −80°C until used.

Horses

Seven horses (4 mares and 3 fillies) of thoroughbred breed, 1 to 20 years old, were allotted to 3 groups: group 1 consisted of 2 horses (a mare and a filly) inoculated with the heavily encapsulated strain; group 2 consisted of 3 horses (2 mares and a filly) inoculated with the less heavily encapsulated strain; group 3 consisted of 2 horses (a mare and a filly) inoculated with the non-capsulated variant. Mares were inoculated with 5 mg of dinoprost tromethamine (Prostin F2 Alpha, The Upjohn, Co., Kalamazoo, Mich.) to induce estrus, but the treatment was successful in only half of the mares, as described below.

Preparation of inoculum

The organisms forming smooth colonies were cultured at 37°C overnight in trypticase soy broth. Organisms were harvested and washed twice by centrifugation and resuspended in PBSS and stored overnight at 4°C. After viable count was determined by plating serial dilutions of the suspension on trypticase soy agar, the organisms were inoculated into the uterus. Viable count of residual bacterial suspension was finally determined.

Method of inoculation

Of the 7 horses, 4 mares were artificially induced into estrus with 5 mg of dinoprost tromethamine. Estrus was determined by rectal palpation of the ovaries. Only 2 (horse Nos. 3 and 4) of the 4 mares exhibited estrus. Three fillies were not treated with the hormone. The external genitalia were cleaned with benzalconium chloride solution. A vaginal speculum was passed into the vagina, after which a sterile swab was picked up with a cervical forceps and inserted carefully through the speculum and cervix into

the uterus. After the uterine swab was obtained, mares or fillies were inoculated with 1×10^8 to 6×10^9 CFU of bacteria into the uterus using a sterile insemination catheter fitted with a syringe.

Sampling and cultural methods

Uterine swabs for bacteriologic culture were collected on determined post-inoculation days (PIDs) with a sterile cottontipped swabs. Contamination of the uterine swabs by the cervix was unavoidable. Swabs were sampled every day in the 1st week and once every several days thereafter. Inoculated horses were sacrified on the 20th to 48th PIDs. Reproductive organs were removed and opened. Pieces of mucosa for bacterial recovery were removed from the right and left uterine horns, uterine body, cervix and vagina. The swab sample was streaked on trypticase soy agar supplemented with 5% horse blood and deoxycholate hydrogen sulfide lactose agar, and the mucosa specimens and a section of ovaries were stamped on the media, and incubated at 37°C for 24 hours. The number of colonies isolated was recorded.

Clinical and hematological observation

The inoculated mares were examined daily for signs of metritis (cervicitis, vaginitis and exudate), and the rectal temperature was taken. Hematological (white blood cell count, differential count of white blood cells) and rectal examinations were performed at the same time as the uterine swabs were sampled.

Pathological examination

Uterine spiecimens for histology were fixed in 10% neutral buffered formalin. The fixed tissue was embedded in paraffin, sectioned at 5 μm in thickness, and stained with hematoxylin and eosin.

Results

Clinical findings

Horse No. 1 (mare), inoculated in the anestrus with 1.0×10^9 CFU of heavily encapsulated strain F6, discharged yellowish-white colored fluid from the cervix from the 2nd PID for 19 days and exhibited severe hyperemia and edema of the cervical and vaginal mucosae from the 3rd PID for 23 days. No such clinical findings were found thereafter before sacrifice on the 48th PID. Horse No. 2 (filly) inoculated with 1.0×10^8 CFU of the same strain exhibited vaginal exudate from the 3rd PID for 10 days, as well as hyperemia and edema of the cervical and vaginal mucosae from the 3rd PID for 12 days. No clinical findings were found thereafter before sacrifice on the 23rd PID (Table 9, Fig. 7).

Horse No. 3 (mare) inoculated in the estrus stage with 6.1×10^9 CFU

of less heavily encapsulated strain RK26 exhibited discharge from the 3rd PID for 5 days, and hyperemia and edema of the cervical and vaginal mucosae from the 2nd PID for 3 days. No such clinical signs were observed thereafter before the day of sacrifice on the 27th PID. Two other horses (No. 4, mare in estrus and No. 5, filly) inoculated with 3.7×10^9 and 1.2×10^9 CFU of the strain, respectively, did not show any clinical signs during the period of 20 and 24 days after inoculation, respectively (Table 9, Fig. 8).

Horse No. 6 (mare in anestrus) and No. 7 (filly) inoculated with 1.3×10^9 and 1.0×10^9 CFU of non-capsulated variant f6, respectively, did not show any clinical signs (Table 9, Fig. 9).

No fibrile response was observed in any of the inoculated horses. No remarkable increase of leukocytes nor nuclear shift to the left of neutrophils was found in any of the horses.

Recovery of K. pneumoniae *from uterine swabs*

Recovery of *K. pneumoniae* from uterine swabs of the inoculated horses is shown in Table 9 and Figs. 7～9. Horse No. 1 inoculated with heavily encapsulated strain discharged numerous organisms from the 1st to the 25th PIDs. No recovery of the organisms was found on the 30th, 37th and 44th PIDs. Horse No. 2 inoculated with the same strain discharged the organisms from the 2nd to 21st PIDs, with varying numbers of organisms.

Horse No.3 inoculated with the less heavily encapsulated strain discharged the organisms from the 1st to 8th PIDs, with numerous organisms from the 3rd to 7th PIDs. No recovery of the organisms was found thereafter before the day of sacrifice on the 27th PID. Two other horses (Nos. 4 and 5) did not show any bacterial discharge (Table 9, Fig. 8).

Horse No. 6 and No. 7 ionculated with non-capsulated variant f6, did not discharge the organisms during the period of 21 and 20 PIDs, respectively. The single exception was discharge of organisms at only one day after inoculation in horse No. 7 (Table 9, Fig. 9).

Recovery of K. pneumoniae *from uterine and vaginal tissues at autopsy*

Recovery of heavily encapsulated strain from uterine and vaginal tissues at autopsy is shown in Table 9. Organisms were recovered in large numbers from the vagina, cervix, uterine body and uterine horns of horse No. 1, and in smaller numbers from the same tissue of No. 2 (Table 9).

Less heavily encapsulated organisms and non-capsulated organisms were never recovered from the vagina, cervix, uterine body, uterine horns and ovaries of the inoculated mares (Nos. 3 to 5 and Nos. 6 and 7, respectively) (Table 9).

Table 9. Clinical findings and recovery of *K. pneumoniae* organisms from horses inoculated with 3 differently encapsulated strains of *K. pneumoniae* capsular type 1

Horses				Inoculum			Clinical findings				Recovery of *K. pneumoniae* organisms[a]					
							Exudate		Cervicitis		Duration of bacterial isolation from uterine swabs (PID)	At autpsy				
No.	Age (years)	Estrous cycle	Termination (PID)	Strain	Encapsulation	size (CFU)	Day of appearance after inoculation	Duration (days)	Day of appearance after inoculation	Duration (days)		Vagina	Cervix	Uterine body	Uterine horn	Ovaries
1	14	Anestrus	48	F 6	Heavy	1.0×10^9	2nd	19	3rd	23	1st–25th	###	###	###	###	–
2	1		23	F 6	Heavy	1.0×10^8	3rd	10	2nd	12	2nd–21st	–	+	+	+	–
3	20	Estrus	27	RK 26	Less heavy	6.1×10^9	3rd	5	2nd	3	1st–8th	–	–	–	–	–
4	8	Estrus	20	RK 26	Less heavy	3.7×10^9	–	–	–	–	–	–	–	–	–	–
5	1		24	RK 26	Less heavy	1.2×10^9	–	–	–	–	–	–	–	–	–	–
6	9	Anetsrus	21	f 6	Non	1.3×10^9	–	–	–	–	–	–	–	–	–	–
7	1		20	f 6	Non	1.0×10^9	–	–	–	–	1st	–	–	–	–	–

a Colonies per plate grown from stamped specimens of pieces of mucosa from the vagina, cervix, uterine body and uterine horns, and of pieces from the ovaries were shown. ###, more than 401 colonies; +, 1 to 10 colonies; –, no growth.

Table 10. Pathological findings of uterus of horses inoculated with 3 differently encapsulated strains of *K. pneumoniae* capsular type 1

Macroscopic findings						Histological findings[a]							
Horse No.	Edema	Vesicle	Incrassation	Hyperemia	Pus	Edema	Hyperemia	Proliferation of luminal epithelium	Basal vacuolation of luminal epithelium	Degeneration or necrosis of luminal epithelium	Mononuclear cell infiltration	Neutrophilic leukocyte infiltration	Eosinophilic leukocyte infiltration
1	##	+	+	##	–	###	##	##	##	+	###	##	###
2	–	+	–	–	–	–	–	–	–	–	–	–	–
3	+	+	–	+	–	##	+	+	+	+	##	+	##
4	–	–	–	–	–	–	–	–	–	–	+	+	–
5	–	–	–	–	–	–	–	–	–	+	–	–	–
6	–	–	+	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	*b	*	*	*	*	*	*	*

a +, mild to ##, severe.
b Not done.

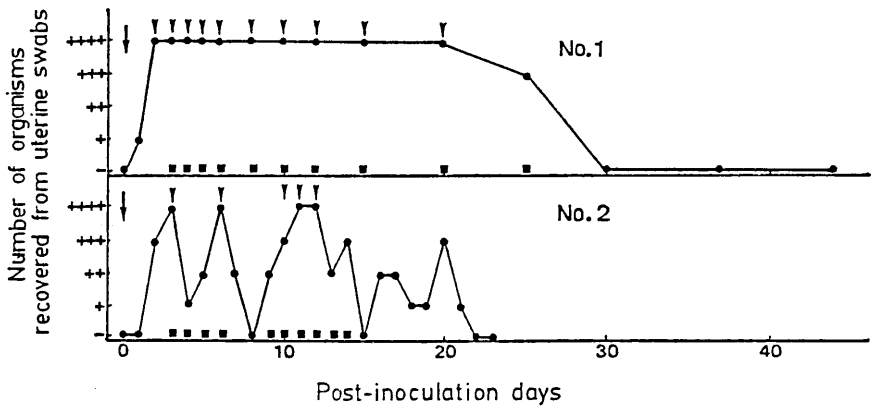

Fig. 7. Recovery of the organisms from uterine swabs and clinical signs of horses inoculated with heavily encapsulated strain F 6 of *K. pneumoniae* capsular type 1. ⧺, more than 401 colonies per plate were grown from streaked swabs; ⧺, 101 to 400 colonies per plate were grown from streaked swabs; ++, 11 to 100 colonies per plate were grown from streaked swabs; +, 1 to 10 colonies per plate were grown from streaked swabs; −, no growth; ↓, inoculation of organisms into the uterus; ▼, vaginal discharge; ■, cervicitis.

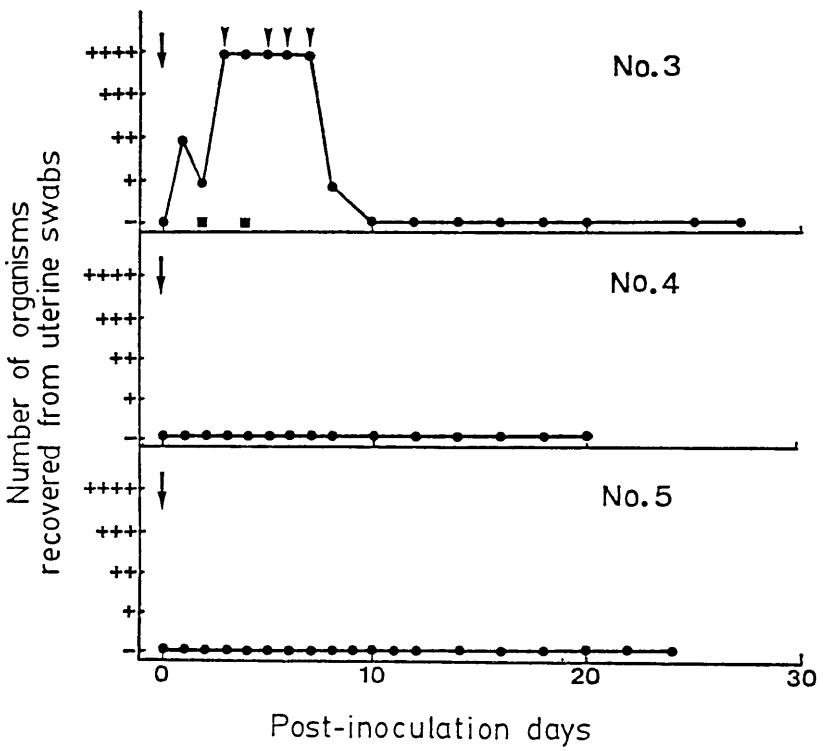

Fig. 8. Recovery of the organisms from uterine swabs and clinical signs of horse inoculated with less heavily encapsulated strain RK26 of *K. pneumoniae* capsular type 1. Symbols were described in Fig. 7.

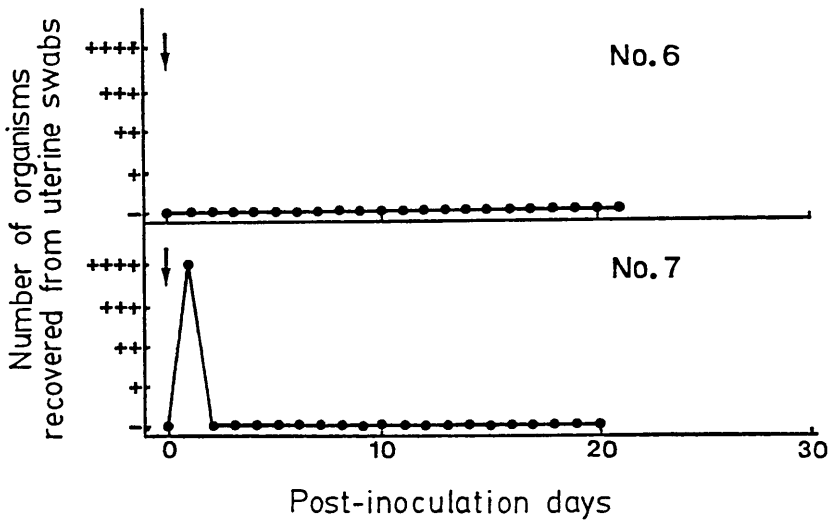

Fig. 9. Recovery of the organisms from uterine swabs and clinical signs of horse inoculated with non-capsulated strain f6 of *K. pneumoniae* capsular type 1. Symbols were described in Fig. 7.

Colonial morphology and encapsulation of organisms recovered from cervical swabs and reproductive tissues

Organisms recovered from samples of the mares and fillies inoculated with heavily encapsulated, less heavily encapsulated and non-capsulated strains were similar to each of the inoculated strains in colonial morphology and encapsulation (light microscopic examination after negative staining with Indian ink). Transverse diameter of the capsule of recovered beacteria of the less heavily encapsulated strain ranged from 1.2 to 1.3 μm, the same as the inoculated one.

Pathological findings

Results of pathological observation are shown in Table 10. Edema, vesicle and hyperemia of endometrium were observed macroscopically in horse No. 1 (mare) inoculated with heavily encapsulated strain. Edema and hyperemia in lamina propria, proliferation, basal vacuolation and degeneration or necrosis of luminal epithelium, and mononuclear cells, neutrophilic leukocytes and eosinophilic leukocytes infiltration in lamina propria were observed in the mare. No particular pathological change was observed in horse No. 2 (filly).

The almost same pathological changes werd observed in horse No. 3 (mare) inoculated with less heavily encapsulated strain. The degree of pathological change of the mare was milder than that of horse No. 1. Mild infiltration of mononuclear cells and neutrophilic leukocytes in lamina propria

was observed in horse No. 4 (mare). No particular pathological change was observed in horse No. 5 (filly).

No particular pathological change was observed in horse Nos. 6 (mare) and 7 (filly) inoculated with non-capsulated strain.

Discussion

Heavily encapsulated strain of *K. penumoniae* capsular type 1 ionculated into the uterus of mares caused severe metritis in 2 of 2 horses (mare and filly), whereas less heavily encapsulated strain caused very slight metritis in only 1 mare of 3 horses (2 mares and filly), and non-capsulated strain did not cause metritis in 2 of 2 horses (mare and filly). These results indicate that there is a difference in the ability to cause metritis in horses among the heavily encapsulated, less heavily encapsulated and non-capsulated strains of *K. penumoniae* capsular type 1.

It was shown in chapter II that heavily encapsulated strains were highly virulent for mice and were protected from phagocytosis by equine and murine PMNL, whereas less heavily encapsulated strains (semen-origin) and non-capsulated variants were low in virulence for mice and were easily ingested by the PMNL. The difference of virulence among 3 differently encapsulated strains in mice and *in vitro* was reflected in the present study in the difference in ability to cause metritis in mares.

In chapter I, it was epidemiologically shown that mares bred to stallions carrying the less heavily encapsulated strain did not develop metritis whereas mares bred to stallions carrying the heavily encapsulated strain did. In the present study, the less heavily encapsulated strain isolated from semen caused only very slight metritis in only 1 of 3 horses. The epidemiological findings in the previous chapter demonstrated were thus confirmed experimentally by the results of the present chapter.

Experimental uterine infection of a pony mare in winter anestrus with *K. pneumoniae* capsular type 1 was reported [26]. No sign of external vulval discharge and no real evidence of vaginal and cervical inflammation were shown, and the strain was recovered from the cervix of the pony at 48 hours post-inoculation only. Encapsulation of the strain inoculated into the pony was not clear. In the present study, a mare in anestrus and a filly, both inoculated with heavily encapsulates strain, exhibited metritis. Experimental production of metritis with *K. pneumoniae* capsular type 1 in mares in anestrus was thus shown. Of the 3 horses inoculated with the less heavily encapsulated strain, 2 mares were in estrus and were aged 20 years and 8 years. Of the 2, only 1 (20 years old) exhibited very slight metritis. The 8-year-old one, inoculated with organisms that were 6 times less than

those inoculated into the 2-year-old mare, did not show any signs of metritis. It is not clear whether the difference between the two was only due to the size of inoculum. It seems, however, that the less heavily encapsulated strain is less able to cause metritis in mares.

The mares used in the present study were not sufficient in number or uniform in conditions such as age and estrous cycle. It was supposed that during estrus, the uterus provides optimal conditions for growth of contagious equine metritis bacteria [28, 29]. Generally speaking, however, clearance of bacteria is accelerated in the uterus of horses in the estrous stage due to a large number of leukocytes accumulated in the mucosa of the uterus and in the uterine cavity, which resulted in increased phagocytosis [27]. Uterine IgG concentration in normal mares was higher in anestrus than in estrus [22]. In the present study, the heavily encapsulated strain of *K. pneumoniae* capsular type 1 caused severe and moderate metritis in a mare in anestrus and a filly, respectively, whereas the less heavily encapsulated strain produced only very slight metritis in only a mare of 2 mares and nothing in a filly. And non-capsulated variant did not cause any metritis in a mare in anestrus or in a filly. Although the number of horses was not sufficient and the conditions of the horses were not ideal, correlation between capability to cause metritis and encapsulation of *K. pneumoniae* capsular type 1 strains was demonstrated.

Pathological findings of uterus of the horses were paralleled in the present study with clinical findings of the horses. Only exception was a filly inoculated with heavily encapsulated strain which showed the signs of metritis for the first 12 days and bacterial recovery for 20 days, but did not show pathological change at autopsy on 23rd day. Pathological change might be revealed in the uterus of the filly if the filly was sacrificed during the time of clinical metritis.

Colonial morphology and encapsulation of the organisms recovered from samples from the mare inoculated with the less heavily encapsulated strain were similar to those of the inoculated strain, which was semen origin. The results indicated that the diamter of capsule and colonial morphology of the recovered organisms were similar to those of semen-type organisms (capsules were 1.3 to 1.5 μm in transverse diameter, colonies were opaque and less mucoid) and not cervical swab-type organisms (capsules were 1.7 to 2.2 μm in transverse diameter, colonies were between opaque and translucent in density and mucoid). The presumption that less heavily encapsulated organisms of semen-type might be changed to less heavily encapsulated organisms of cervical swab-type by increasing slightly the thickness of the capsule and changing the colonial morphology in the uterine cavity was thus negated in the present study.

Summary

Heavily encapsulated strain of *K. pneumoniae* capsular type 1 inoculated into the uterus of a mare and a filly caused severe and moderate metritis, respectively, whereas the less heavily encapsulated strain caused only very slight metritis in only 1 of 2 mares and nothing in a filly. Heavily encapsulated strain was recovered from the uterine swabs of the mare and filly until the 25th and 21st PIDs, respectively, and was recovered at autopsy on the 23rd and 48th PIDs from the uterus and vagina, respectively. Less heavily encapsulated strain was recovered from uterine swabs of only 1 mare until the 8th PID only, but not from the remaining mare and filly, and the recovery of the organisms from the uterus and vagina at autopsy on the 20th and 27th PIDs was negative. Non-capsulated strain did not cause any metritis in a mare and a filly and the organisms were not recovered from uterine swabs from the 2nd PID nor at autopsy on the 20th and 21st PIDs from the uterus and vagina.

Conclusion

Klebsiella pneumoniae is a causative agent in metritis, cervicitis, infertility and abortion in mares. Eqine metritis due to this bacterial organism has spread in many countries, including Japan. A considerable economic loss to the horse breeding farms can result from equine metritis traced to *K. pneumoniae*. A better understanding of both the epidemiology of the infection and the ecology of *K. pneumoniae* is necessary to improve the rate of equine reproduction.

In this study capsular type 1 was found to be the predominant type in metritis due to *K. penumoniae* in mares and also the predominant type in the genital tract of stallions. In addition, the less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from cervical swabs taken from mares suffering metritis and from the genital tract of stallions. Virulence of the less heavily encapsulated and the heavily encapsulated strains for mice, and the difference in the capability of each strain for causing metritis in mares are described and compared.

Capsular type 1 was the predominant type (79 of 88, 89.8%) in metritis due to *K. pneumoniae* in mares of racing breeds. The same type was isolated from the semen and from swabs of the fossa glandis in 6 of 20 (30.0%) stallions of racing breeds. Both the heavily encapsulated and the less heavily encapsulated strains of *K. pneumoniae* capsular type 1 were isolated from stallions. Mares bred to stallions carrying the heavily encapsulated strains develop metritis, whereas those bred to stallions carrying the less heavily encapsulated strains did not. Capsular type 39 was isolated from

cervical swabs taken solely from metritis infected mares of draft breeds but was not isolated from any mares of the racing breeds examined. Capsular type 1 strains of *K. pneumoniae* were not found in feces and nasal swabs of healthy mares of racing breeds.

The less heavily encapsulated strains of *K. pneumoniae* capsular type 1, different from the ordinary heavily encapsulated strains, were isolated from the semen of healthy stallions and from cervical swabs of mares suffering from metritis. The less heavily encapsulated strains had capsules ranging from 1.3 to 2.2 μm in transverse diameter, a size obviously smaller than those of the heavily encapsulated strains measuring more than 3.0 μm in diameter. The less heavily encapsulated strains were predominant in the semen of healthy stallions but the heavily encapsulated strains were predominant in the cervical swabs of mares with metritis. The less heavily encapsulated strains showed a low or intermediate virulence for mice and were easily phagocytized by equine and murine PMNL, whereas the heavily encapsulated strains showed a high virulence for mice and were protected from phagocytosis by equine and murine PMNL.

The heavily encapsulated strain of *K. pneumoniae* capsular type 1 inoculated into the uterus of the horses caused severe or moderate metritis in 2 of 2 horses; however, the less heavily encapsulated strain caused very slight metritis in only 1 of 3 horses, and the non-capsulated strain did not cause metritis in 2 of 2 horses. Recovery of the organisms and pathological findings on the uterus of the horses correlated with clinical findings on the horses. In conclusion, the virulence of strains of *K. pneumoniae* capsular type 1 for causing metritis in mares had a direct correlation to size of capsule.

Acknowledgements

I am deeply indebted to Professor Ryo Yanagawa, Faculty of Veterinary Medicine, Hokkaido University, for his kind guidance, encouragement and critical review of the manuscript of this thesis; and to Professor Chitoshi Itakura, Professor Hiroshi Kanagawa and Associate Professor Takeshi Mikami Faculty of Veterinary Medicine, Hokkaido University, for their helpful discussions and critical review of the manuscript.

I wish to express deep appreciation to Professor Takashi Hiramune, School of Veterinary Medicine, Rakuno Gakuen University, for his invaluable discussion, encouragement and critical reading of the manuscript.

I wish to thank former Professor Nobuo Murase, Professor Yoshimi Kawakami and Associate Professor Misao Onuma, School of Veterinary Medicine, Rakuno Gakuen University, for their invaluable advice and encouragement during the course of this study.

The assistance of all staff members of the Department of Epizootiology, School of Veterinary Medicine, Rakuno Gakuen University, has been greatly appreciated. I am also grateful to the veterinarians of Shadai Farm and Hidaka Agricultural Mutual Aid Association for their help in collecting samples for bacterial survey.

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要　　旨

Klebsiella pneumoniae は，馬の子宮内膜炎，子宮頚管炎，不妊症，流産の原因菌のひとつである。本菌による子宮内膜炎の発生は，日本をはじめ，多くの国で増加の傾向にあり，馬生産牧場にとって経済的損失は大きい。馬子宮内膜炎の疫学および本菌の生態を知ることは，本病の予防対策上有用と考えられる。

著者は，子宮内膜炎罹患馬子宮頚管ぬぐい液および種雄馬生殖道由来の *K. pneumoniae* 中で，莢膜型1が優勢であることを示し，さらに，それらの中に小莢膜保有株が存在することを明らかにした。そこで著者は，小莢膜保有株と大莢膜保有株のマウスに対する毒力を検討し，さらに，馬への子宮内接種試験を行い，子宮内膜炎発症に差が認められるか否

かを比較検討した。

子宮内膜炎罹患馬子宮頚管ぬぐい液，健康雌馬の糞便，鼻腔および健康種雄馬生殖道由来 *K. pneumoniae* の莢膜型：*K. pneumoniae* による子宮内膜炎の疫学及び本菌の生態を明らかにする目的で，1980年から1986年に，北海道の日高，胆振地方の軽種馬生産牧場およびスタリオンセンターに繋養されている，子宮内膜炎罹患馬の子宮頚管ぬぐい液，健康雌馬の糞便，鼻腔ぬぐい液および種雄馬生殖道について *K. pneumoniae* の検索を行った。また，石狩，空知地方の重種馬生産牧場において，子宮内膜炎罹患馬の子宮頚管ぬぐい液の菌検索も同様に実施した。軽種馬子宮内膜炎由来の *K. pneumoniae* は，莢膜型1が最も優勢(88株中79株，89.8%)であった。また，同じ莢膜型1が軽種種雄馬生殖道から分離 (20株中6株，30.0%) された。種雄馬由来の莢膜型1に属する株の中で，莢膜の厚さが異なる小莢膜保有株と大莢膜保有株の存在が明らかになった。大莢膜保有株を保菌している雄と交配した雌は子宮内膜炎に罹患し，小莢膜保有株を保菌している雄と交配した雌は子宮内膜炎に罹患しなかった。一方，重種馬子宮内膜炎由来 *K. pneumoniae* は，すべて莢膜型39に型別された。この莢膜型は，軽種馬由来株では認められなかった。軽種馬子宮内膜炎由来および種雄馬生殖道由来 *K. pnumoniae* のそれぞれ8および36.8%が，また，糞便，鼻腔由来株のほとんどが型別不能であった。

健康種雄馬精液および子宮内膜炎罹患馬子宮頚管における小莢膜保有株の存在ならびに小莢膜保有株および大莢膜保有株の毒力の比較：小莢膜保有株は，莢膜の厚さが1.3–2.2 μm で，大莢膜保有株のそれは3.0 μm 以上であった。種雄馬精液由来5株中4株 (80.0%) が小莢膜保有株であり，1株 (20.0%) が大莢膜保有株であった。一方，子宮頚管由来83株中7株 (8.4%) が小莢膜保有株であり，他の86株 (91.6%) が大莢膜保有株であった。このように，種雄馬精液と子宮内膜炎罹患馬子宮内における小莢膜保有株および大莢膜保有株の分離率に明らかな差が認められた。小莢膜保有株のマウスに対する毒力は弱 (精液由来株，LD_{50} が 10^7–10^8 CFU)，または中等度 (子宮頚管由来株，LD_{50} が 10^5 CFU) であり，馬，マウスの好中球に容易に食菌された。一方，精液および子宮頚管由来大莢膜保有株は強い毒力 (LD_{50} が 10^2–10^4 CFU) を示し，好中球の食菌作用に抵抗し，ほとんど菌食されなかった。

小莢膜保有株，大莢膜保有株および無莢膜変異株の馬子宮への接種試験：小莢膜保有株が，馬に子宮内膜炎を発症させることができるか否かについて，大莢膜保有株およびそれから作出した無莢膜変異株を用い，比較検討した。大莢膜保有株を接種された2頭の馬は，それぞれ重度および中等度の子宮内膜炎を起こし，長期間にわたり排菌し，剖検時にも子宮から本菌が回収された。小莢膜保有株を接種された3頭中1頭のみが軽度の子宮内膜炎

を起こしたにすぎず，その排菌は短期間であった。他の2頭は発症せず，子宮ぬぐい液および剖検時の子宮からも本菌は回収されなかった。無莢膜変異株を接種された2頭はともに子宮内膜炎の所見を示さず，1頭のみにおいて接種後1日目に子宮ぬぐい液から回収されたのみで，剖検時にも子宮から本菌は回収されなかった。

以上のように，馬子宮内膜炎発症における *K. pneumoniae* 莢膜型1の毒力は莢膜の厚さの程度と相関することが明らかになった。

以上，三章の結果から，今までに報告されていなかった小莢膜保有 *K. pneumoniae* 莢膜型1の存在と分布が明らかになり，子宮内膜炎発症と莢膜の厚さの程度が相関することが判明し，細菌の感染に莢膜の果たす役割の重要性を改めて指摘したものと考える。