Epidemiology of Invasive *Klebsiella pneumoniae* with Hypermucoviscosity Phenotype in a Research Colony of Nonhuman Primates

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Invasive *Klebsiella pneumoniae* with hypermucoviscosity phenotype (HMV *K. pneumoniae*) is an emerging human pathogen that, over the past 20 y, has resulted in a distinct clinical syndrome characterized by pyogenic liver abscesses sometimes complicated by bacteremia, meningitis, and endophthalmitis. Infections occur predominantly in Taiwan and other Asian countries, but HMV *K. pneumoniae* is considered an emerging infectious disease in the United States and other Western countries. In 2005, fatal multisystemic disease was attributed to HMV *K. pneumoniae* in African green monkeys (AGM) at our institution. After identification of a cluster of subclinically infected macaques in March and April 2008, screening of all colony nonhuman primates by oropharyngeal and rectal culture revealed 19 subclinically infected rhesus and cynomolgus macaques. PCR testing for 2 genes associated with HMV *K. pneumoniae*, *rmpA* and *magA*, suggested genetic variability in the samples. Random amplified polymorphic DNA analysis on a subset of clinical isolates confirmed a high degree of genetic diversity between the samples. Environmental testing did not reveal evidence of aerosol or droplet transmission of the organism in housing areas. Further research is needed to characterize HMV *K. pneumoniae*, particularly with regard to genetic differences among bacterial strains and their relationship to human disease and to the apparent susceptibility of AGM to this organism.

Abbreviations: AGM, African green monkey; HMV *K. pneumoniae,* invasive *Klebsiella pneumoniae* with hypermucoviscosity phenotype; NHP, nonhuman primate; RAPD, random amplification of polymorphic DNA.

Klebsiella pneumoniae is an enteric, gram-negative, lactose-fermenting bacillus with a prominent capsule. This bacterium has been associated with peritonitis, septicemia, pneumonia, and meningitis in both Old and New World primates,[10,13,29] although it also is reported to constitute normal fecal and oral flora in many nonhuman primates (NHP).[12] Pathogenic strains associated with the upper respiratory tract typically are heavily encapsulated.[12] Over the past several decades, human medical literature indicates the emergence of an invasive *K. pneumoniae* disease in Taiwan and other Asian countries, in which community-acquired pyogenic liver abscesses have been attributed to strains of invasive *K. pneumoniae* with a unique hypermucoviscous phenotype (HMV *K. pneumoniae*).[6,17-19,21,26,34] The hypermucoviscous phenotype has also been associated with other serious complications, including bacteremia, meningitis, and endophthalmitis. This strain of *Klebsiella* has become an emerging cause of pyogenic liver abscesses in some nonAsian countries, including the United States.[16,20,36,39] The majority of clinical cases of HMV *K. pneumoniae* are in the Asian population, particularly in patients with diabetes mellitus.[3,4,33] Determination of the HMV phenotype typically is based on a positive string test.[8,35,39]

Several virulence factors have been associated with HMV *K. pneumoniae*. *Klebsiella* spp. generally develop prominent polysaccharide capsules which increase virulence by protecting the bacteria from phagocytosis and preventing destruction by bactericidal serum factors. Capsular serotypes K1 or K2 have been reported as the major virulence determinants for human HMV *K. pneumoniae* liver abscesses.[5,8,37,38] In addition, the mucoviscosity-associated gene *magA*, which encodes a structural outer membrane protein of the K1 serotype, and *rmpA* (regulator of the mucoid phenotype gene; located on a plasmid) have been proposed as virulence factors.[9,27,31,40,41] Recently, it was suggested that 2 clones, CC23 [K1] and CC82[K1], are strongly associated with primary liver abscess and respiratory infection, respectively.[2]

Over a period of several months in 2005 to 2006, 7 African green monkeys (AGM; *Chlorocebus aethiops*) in the US Army Medical Research Institute of Infectious Diseases research colony developed abscesses in multiple locations and either died or were euthanized when the abscesses were determined to be nonresectable.[35] HMV *K. pneumoniae* of the K2 serotype and carrying *rmpA* was determined to be the cause of the infection in 1 case, and the 6 other cases had similar clinical and pathologic features. This report[35] is the only documentation, to our knowledge, of natural infection with HMV *K. pneumoniae* in NHP. As a result of these cases, the US Army Medical Research Institute of Infectious Diseases instituted policies to exclude HMV *K. pneumoniae* from the colony. The organism was included as a specific pathogen-free requirement for vendors, and *K. pneumoniae* culture results were reported during quarantine periods and on routine semiannual examination for all colony NHP.

Case Study

In March 2008, a 3.5-kg male rhesus macaque tested positive for HMV *K. pneumoniae* on an oropharyngeal sample obtained during routine semiannual physical examination. This animal was not assigned to an experimental protocol and had not been used in any previous experimental protocols. The macaque was serologically negative for cercopithecine herpesvirus 1, simian type D retrovirus, SIV, and simian T-cell leukemia virus 1 and tested negative for parasites by fecal flotation and direct smear. Although this animal showed no clinical signs of disease, concerns about transmission of HMV *K. pneumoniae*, which had previously caused fatal disease in AGM at the facility, led to the decision to euthanize the macaque.

Approximately 3 wk after the index case was identified and euthanized, the other 35 rhesus macaques that were housed in same room were cultured by using oropharyngeal and rectal swabs in an effort to increase sensitivity of testing. Culture results were positive for HMV *K. pneumoniae* in 3 male rhesus macaques in the room; 2 of the 3 animals were positive on both oropharyngeal and rectal culture, and the remaining macaque was positive on oropharyngeal culture only. At 5 wk after the index case, a 5.2-kg male cynomolgus macaque, located in a separate building and with no history of direct contact with the infected rhesus macaques, tested positive for HMV *K. pneumoniae* on a rectal culture during medical screening for an experimental protocol to which the animal had been assigned. This animal had cleared a 6-wk quarantine, during which he tested serologically negative for cercopithecine herpesvirus 1, simian type D retrovirus, SIV, and simian T-cell leukemia virus 1; tested culture negative by oral swab for *Klebsiella* spp., *Salmonella* spp., and *Shigella* spp.; and tested negative for parasites by fecal flotation and direct smear. Because of the severity of disease previously associated with this organism in AGM in our colony and because the pathogen had now been found in 2 separate buildings, we initiated screening of the entire NHP colony for HMV *K. pneumoniae*.

Here we describe an epidemiologic investigation of cynomolgus and rhesus macaques in our research colony that were infected with HMV *K. pneumoniae*. Our investigation included bacteriology, PCR, and random amplified polymorphic DNA (RAPD) typing of HMV *K. pneumoniae*,; we also tested the hypothesis that aerosols or droplets created during cage cleaning were responsible, in part, for transmission of the organism.

Materials and Methods

Animals. The animals described in this report were maintained in AAALAC-accredited facility. All research was conducted as part of protocols approved by our institutional animal care and use committee and adhered to the *Guide for the Care and Use of Laboratory Animals*.[28]

The NHP colony consisted of 307 animals (51 AGM, 125 cynomolgus macaques, and 131 rhesus macaques). Rooms housing NHP contained only 1 species at a time, but multiple species were housed within a single wing or corridor of the NHP hallways and there was no defined entry order for the rooms. Semiannual evaluations of NHP included physical examination; complete blood counts and blood chemistry; serologic testing for measles, cercopithecine herpesvirus 1, simian type D retrovirus, SIV, and simian T-cell leukemia virus 1 in macaques; serologic testing for measles virus, SIV, simian T-cell leukemia virus 1, African green α herpesvirus, and rotavirus (group A) in AGM; culture of the oropharynx; and screening for parasites via fecal flotation and direct smear. Until identification of the 1st infected macaque, rectal cultures were not performed routinely unless an NHP showed clinical signs of enteric disease. All NHP were housed in 4.5-ft^2 or 6.0-ft^2 cages with 4 cages per rack (Allentown Caging Equipment, Allentown, NJ). The environmental conditions were maintained as recommended in the *Guide for the Care and Use of Laboratory Animals*[28] (temperature, 16 to 29 °C; humidity, 30% to 70%; and 12:12-h light:dark cycle). Animals were fed a standard primate diet (diet 8714, Harlan Teklad, Madison, WI) supplemented with fruit and other food treats. Fresh water, provided ad libitum, was chlorinated at the municipal level and filtered (Edstrom Industries, Waterford, WI). Environmental enrichment (Challenge Ball, Kong, and Hercules Dental Device, Bio-Serv, Frenchtown, NJ) was provided, and cages were arranged so that the animals were facing each other across the room.

Bacteriology. Oropharyngeal and rectal swabs were taken from 307 cynomolgus macaques, rhesus macaques, and AGM. Any animal that tested positive was removed from the room and housed in a room that contained only NHP that cultured positive for HMV *K. pneumoniae*. The rooms from which these NHP originated then underwent 6 wk of quarantine, during which weekly oropharyngeal and rectal swabs were cultured in an effort to eliminate all infected NHP from general housing. A total of 2297 oropharyngeal and rectal samples were collected and cultured from midMarch to early September 2008.

All swabs were collected by using culture collection and transport systems (BBL Culture Swab, Becton Dickinson, Franklin Lakes, NJ). The swabs were delivered to the microbiology laboratory, used to inoculate MacConkey agar plates, and incubated at 35 °C without CO_2. The plates were examined the day after inoculation for the presence of hypermucoviscous, lactose-fermenting, gram-negative rods. All suspect colonies were analyzed by using an automated bacterial identification system (Vitek 2, bioMerieux, Hazelwood, MI) and subcultured on a 5% sheep blood agar plate to ensure purity. Any isolate yielding a probability of 90% or greater for *K. pneumoniae* was further analyzed through the string test, in which a bacterial loop was touched to a suspect colony on a 5% sheep blood agar plate and withdrawn slowly. Bacteria forming a 'string' of at least 5 mm were determined to be positive (Figure 1). Isolates determined to be HMV *K. pneumoniae* were boiled and the resulting DNA was frozen at –70 °C for molecular analysis.

Real-time PCR. Rapid real-time PCR assays (*Taq*Man and *Taq*-Man Minor Groove Binder assays, Applied Biosystems, Foster City, CA) were used to detect *rmpA* and *magA*, genes that are highly associated with the HMV phenotype; all HMV *K. pneumoniae* cultures from midMarch 2008 to September 2008 were tested as described previously.[15] A total of 78 HMV *K. pneumoniae* samples were tested via PCR for the *magA* and *rmpA* genes as part of the current epidemiologic study.

RAPD analysis. Bacterial genomic DNA was extracted (Illustra Genomic Prep Mini Spin Kit, GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. RAPD analysis (Ready-To-Go RAPD Beads, GE Healthcare) was performed by using a primer provided in the kit (primer 2, 5' GTT TCG CTC C 3'); we tested six different random 10mer primers provided and selected primer 2 because it yielded easily readable patterns and the most bands that were well separated. Ampli*Taq* polymerase, dNTPs (0.4 mM each), bovine serum albumin (2.5 µg), buffer (3
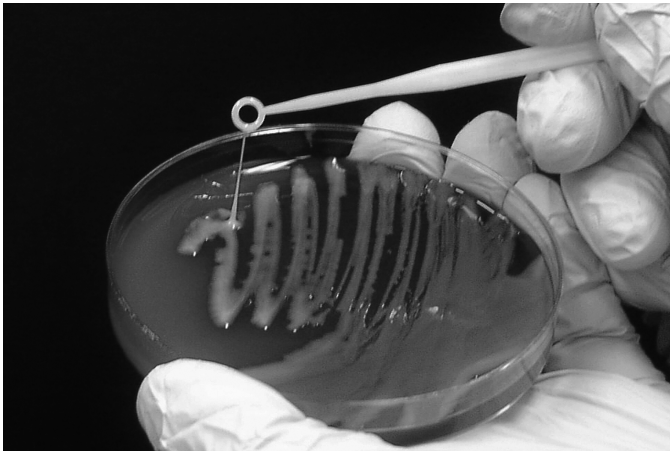
Figure 1. Positive string test. The HMV phenotype of *K. pneumoniae* is defined by a positive-string test. The test is performed by touching a colony with a bacterial loop and gently lifting. If a mucoid 'string' of 5 mm or more forms, the string test is considered positive.

Figure 2. Culture plates affixed in a grid pattern around and on NHP cages. Once the plates were in place, the animal caretaker performed routine spray-down of organic material from the cages. The plates then were removed and cultured to determine the extent of environmental spread of HMV *K. pneumoniae* during routine cage-cleaning procedures.

mM $MgCl_2$, 30 mM KCl, 10 mM Tris [pH 8.3]), and Stoffel fragment were provide as dried beads that were stable at room temperature. Primer 2 (10 µL; 5 pmol/µL), template DNA (10 µL; 5 ng/µL), and 5 µL sterile distilled water were added to the reaction beads for a total reaction volume of 25 µL. PCR amplification was performed in a thermocycler (Mastercycler, Eppendorf, Hamburg, Germany) by using 5 min at 95 °C followed by 45 cycles of 95 °C for 1 min, 36 °C for 1 min, and 72 °C for 2 min. PCR products were separated on a 1.5% agarose gel, which was stained with ethidium bromide and visualized by UV transillumination. DNA fragment patterns were compared by visual inspection.

Environmental testing. A total of 326 samples were collected from various NHP housing rooms in the facility and divided among 3 test methods. For the first test, 258 environmental swabs were collected from predetermined sites over 6 wk. Swabs were taken at midday, after rooms had been cleaned and sanitized by the animal caretaker. Each week, 42 swabs were taken from the door knob, room corner, drain cover, handle of hose spray nozzle, lixit, air handling vent, and rubber surface of floor squeegee in 6 NHP housing rooms (total, 252 swabs). These rooms contained either infected, quarantined (HMV *K. pneumoniae* status unknown), or culture-negative animals. During week 5, additional swabs were taken from cage fronts of 3 different NHP cages in the room housing infected animals, and during week 6, swabs were taken from feces in pans of 3 infected macaques (total, 6 swabs).

The second test involved affixing 14 MacConkey plates to the wall and on the surfaces of 2 banks of cages in a grid pattern (Figures 2 and 3), removing lids, allowing the caretaker to spray solid and liquid organic material out of 1 bank of cages, and then promptly removing the culture plates. This test was performed twice: once while the first bank of cages was sprayed out, and then again when the second bank of cages was sprayed. A total of 28 MacConkey plates were submitted for culture by using this test method.

The third test involved exposing MAC culture plates, placed on trays throughout the room immediately after the caretaker cleaned the cages by using a high-pressure spray. Culture plates remained in place for various periods of time. Tray stands were constructed by using polyvinyl chloride material and contained 2 trays each: 1 at the height of upper cages, and the other at the height of the lower cages. The tray stands were placed in the center of the room, at 5-ft intervals along the length of the room (Figure 4). Each tray held 2 culture plates. One set of 10 plates (position A in Figure 4) was left in place from the completion of spraying to 60 min afterward. The second set of 10 plates (position B in Figure 4) was in place from the completion of spraying until 5 min after spraying; these plates then were replaced with 10 additional plates that remained in place from 5 min to 10 min after spraying. The plates were exchanged again; the final set of plates was placed in position B from 10 min to 60 min after spraying. A total of 40 culture plates were submitted for culture by using this third test method.

Results

Bacteriology. From midMarch 2008 to early September 2008, a total of 2297 swabs from 307 rhesus macaques, cynomolgus macaques, and AGM were tested by oropharyngeal and rectal culture for HMV *K. pneumoniae*. Although 195 samples were positive for *K. pneumoniae*, only 81 of those samples were of the HMV phenotype. The 81 HMV *K. pneumoniae* samples represented 19
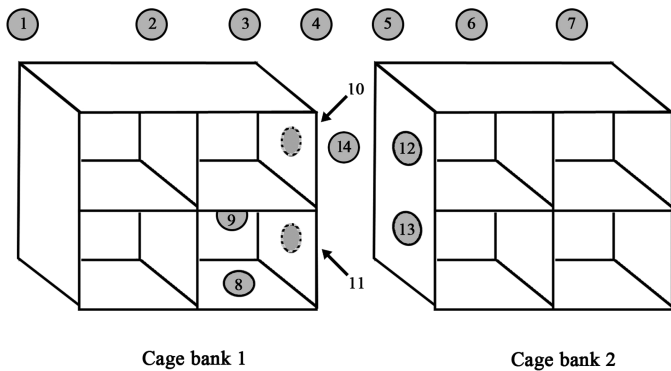
Figure 3. Grid pattern configuration of culture plates. MacConkey cultures plates were taped in positions 1 through 13, and the lids were removed. Plates 8 and 9 were placed in an empty cage, directly below and beside infected NHP. The animal caretaker then followed routine procedures to spray waste material out of cage bank 1, avoiding wetting of NHP in cages. Once the gross contamination was removed from cage bank 1, all culture plates were covered and removed. A second set of MacConkey plates were taped in positions 1 through 13 and the experiment repeated for cage bank 2.
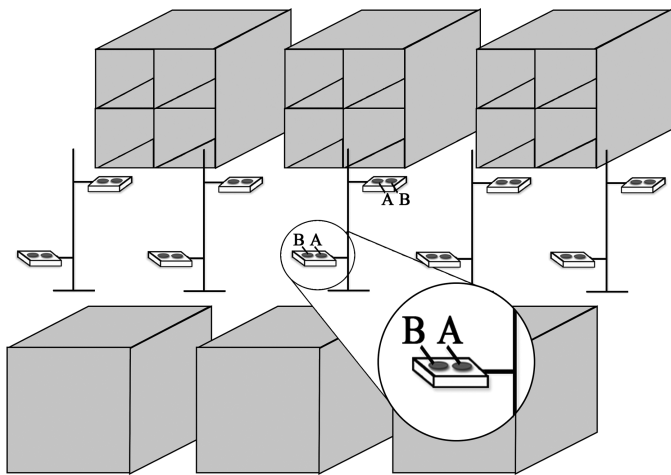

Figure 4. Room configuration for culturing for HMV *K. pneumoniae* after cage spray. In an effort to determine the environmental spread of HMV *K. pneumoniae*, covered MacConkey culture plates were placed on trays while an animal caretaker sprayed waste material from cages. As soon as the caretaker had completed the spray-down, plate covers were removed. Plates in position A (inset) remained in place from 0 to 60 min after spraying. In position B, 3 sets of plates were rotated at predetermined time points: set 1 remained in place from 0 to 5 min, set 2 from 5 to 10 min, and set 3 from 10 to 60 min after spraying.

infected animals, of which 14 had multiple positive cultures during the monthly physical examinations. The single index animal (a macaque) was euthanized promptly after yielding a single positive sample, and another NHP had a single positive culture and then tested negative on multiple subsequent cultures. Another 3 animals were swabbed and then moved into biocontainment as part of an experimental protocol; positive results were identified after the animals were in biocontainment, which made further evaluation inadvisable. As a result, 78 positive HMV *K. pneumoniae* isolates were evaluated as part of this study.

PCR gene analysis. Real-time PCR testing of the 78 HMV *K. pneumoniae* samples resulted in 49 *rmpA⁺/magA⁻* isolates, 17

rmpA⁻/magA⁺ isolates, and 12 *rmpA⁻/magA⁻* isolates. PCR results from 16 NHP (the index case and 15 NHP that were available for repeated cultures) are presented in Table 1. In some cases, an animal had PCR results that were variable between culture sites or over time.

RAPD analysis. A subset of 18 isolates, identified as HMV *K. pneumoniae* in light of bacteriology and phenotypic analysis, was characterized by RAPD typing in an attempt to confirm genetic variability suggested by the PCR results. Visual inspection readily distinguished multiple RAPD types among the HMV *K. pneumoniae* isolates (Figure 5). Patterns were considered to be distinct if 2 or more bands were present or absent between 2 isolates. According to this criterion, 4 clusters (A, B, C, D) were identified. There was no apparent association of RAPD patterns with animal species, given that rhesus macaques were infected with 3 different types (A, D, B), and cynomolgus macaques were infected with 2 different types (C and B). Likewise, RAPD pattern was not associated with *rmpA/magA* status. However, RAPD pattern related loosely to vendor or vendor shipment cohort. For example, cluster A was uniquely associated with vendor 1. However, these associations were not absolute: shipment cohort 2 from vendor 2 contained animals infected with HMV *K. pneumoniae* of both clusters B and D.

A single animal, Cy4 from research institute 1, yielded 2 different strains isolated from rectal swabs taken about 3 wk apart. The first isolate appeared similar to cluster C bacteria, but the second isolate was unique and did not correlate with any other identified cluster. The HMV *K. pneumoniae* isolated from an abscess in 2005 from an AGM with fatal multisystemic disease appeared to be a minor variant of cluster A from vendor 1 (Figure 5).

Environmental testing. In the first test method, 252 environmental swabs were taken from predetermined locations in NHP rooms over 6 wk, and none of those samples cultured positive for HMV *K. pneumoniae*. Six additional environmental swabs were added during weeks 5 and 6. HMV *K. pneumoniae* was cultured during week 5 from the cage front of an infected macaque in the room housing known infected animals, and HMV *K. pneumoniae* was cultured from feces in pans of 3 infected macaques during week 6.

The second test method, which used a grid system of MacConkey culture plates affixed to the walls and cages in the room housing known infected animals, yielded only 1 positive culture. The plate that was placed in position 8 (Figure 3) during spray-down of cage bank 1 yielded HMV *K. pneumoniae*.

The final test method, which involved uncovering culture plates for different times after completion of spray down of organic material from cages in the room housing known infected NHP, yielded no samples that were positive for *K. pneumoniae*.

Discussion

Although HMV *K. pneumoniae* has emerged as an important pathogen of humans, causing pyogenic liver abscesses and other metastatic lesions, this organism previously caused fatal abdominal abscesses in AGM at our facility.[35] To our knowledge, these cases reflect the only documented cases of clinical disease attributed specifically to HMV *K. pneumoniae* in NHP. After a subclinically infected rhesus macaque was identified in March 2008, subsequent screening and testing of 307 NHP resulted in the collection of 2297 oropharyngeal and rectal swabs and 326 environmental swabs over 7 mo. The HMV phenotype was found

Table 1. HMV *K. pneumoniae* and *rmpA/magA* status in macaques

Animal ID	Culture type	HMV *K. pneumoniae* status[a] by date									
		3/6/08	3/13/08	4/7/08	4/28/08	5/9/08	5/20/08	5/28/08	6/17/08	7/21/08	8/18/08
Rh1[b]	Oral	+/−									
	Rectal										
Rh2	Oral		Neg	+/−		Neg	+/−	Neg	Neg	+/−	Neg
	Rectal			Neg		Neg	+/−	+/−	+/−	+/−	Neg
Rh3	Oral		Neg	+/−		Neg	Neg	Neg	Neg	Neg	Neg
	Rectal			+/−		+/−	+/−	Neg	+/−	+/−	+/−
Rh4[c]	Oral		Neg	+/−		Neg	Neg	Neg	Neg	Neg	Neg
	Rectal			+/−		+/−	+/−	+/−	Neg	−/+	Neg
Rh5	Oral		Neg			+/−	+/−	+/−	Neg	+/−	+/−
	Rectal					+/−	+/−	+/−	+/−	Neg	+/−
Rh6	Oral	Neg				Neg	Neg	Neg	Neg	Neg	Neg
	Rectal					+/−	Neg	+/−	Neg	Neg	Neg
Rh7	Oral	Neg				+/−	+/−	Neg	Neg		Neg
	Rectal					+/−	+/−	+/−	+/−		Neg
Rh8	Oral	Neg						+/−	Neg	Neg	Neg
	Rectal							+/−	+/−		−/+
Rh9	Oral	Neg						Neg	−/−	Neg	Neg
	Rectal							Neg	Neg	Neg	Neg
Rh10	Oral	Neg						Neg	+/−	Neg	Neg
	Rectal							Neg	Neg	Neg	Neg
Cy1	Oral				Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Rectal				+/−	Neg	+/−	Neg	Neg	Neg	Neg
Cy2[c]	Oral				Neg	Neg	Neg	−/−	−/−	+/−	Neg
	Rectal				+/−	+/−	−/−	+/−	−/−	+/−	+/−
Cy3[c]	Oral	Neg			−/−	+/−	−/−	−/−	−/−	Neg	Neg
	Rectal				Neg	+/−	Neg	Neg	Neg	Neg	Neg
Cy4[c]	Oral		Neg		Neg	Neg	Neg	Neg	Neg	Neg	Neg
	Rectal				−/−	Neg	+/−	Neg	−/−	Neg	−/−
Cy5	Oral				Neg	Neg	+/−	Neg	Neg	+/−	Neg
	Rectal				+/−	+/−	+/−	+/−	+/−	Neg	Neg
Cy6	Oral							Neg	Neg	Neg	Neg
	Rectal							−/+	−/+	−/+	−/+

Rh, rhesus macaque; Cy, cynomolgus macaque. Blank cells indicate that culture was not performed.
[a]HMV *K. pneumoniae*-positive isolates indicated by *rmpA/magA* status
[b]This animal was the index case and was euthanized after initial positive culture.
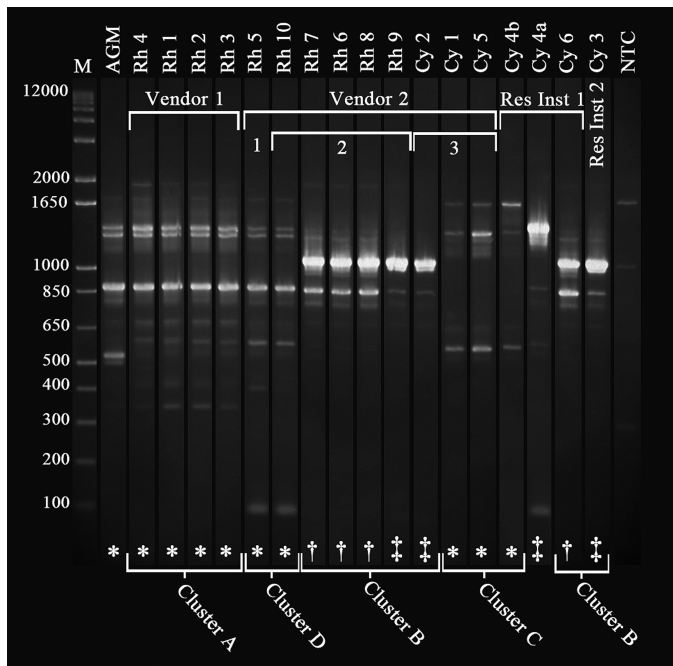[c]Animal had variable PCR results over time.

Figure 5. RAPD-PCR banding pattern of a subset of isolates from NHP infected with HMV *K. pneumoniae*. Isolates were identified as being HMV *K. pneumoniae* based on Vitek analysis and a positive string test. "M" indicates a 1 kb plus marker, and NTC indicates a negative control. The isolate labeled AGM was an HMV *K. pneumoniae* culture from a fatal abdominal abscess in an African green monkey in 2006. All other isolates were from oropharyngeal or rectal swabs from asymptomatic macaques. Animal identifications correlate with those in Tables 1 and 2. Isolates labeled Cy4$_a$ and Cy4$_b$ represent 2 rectal samples taken from NHP Cy4 about 3 wk apart (Cy 4$_a$, 2008 Apr 28; Cy4$_b$, 2008 May 20). Animals from Vendor 1 comprised a single shipment, whereas NHP that came from Vendor 2 arrived in 3 separate shipments, as indicated. The remaining 2 macaques were transferred from 2 different Department of Defense research centers, indicated as Research Institute 1 (Res Ins 1) and Research Institute 2 (Res Ins 2). PCR results were correlated with the RAPD-PCR banding patterns. Isolates were determined to be *rmpA+/magA−* (*), *rmpA−/magA+* (†), or *rmpA−/magA−* (‡). Clusters A through D indicate distinct RAPD types, which were determined based on the absence or presence of 2 or more bands.

in 81 samples, representing a total of 19 subclinically infected macaques. Three samples, obtained from animals that were moved into biocontainment, were unavailable for further analysis, leaving a total of 78 samples. In addition, 2 of the 326 environmental swabs tested positive for HMV *K. pneumoniae*.

Fifteen of the 19 infected animals were isolated and monitored clinically with monthly evaluations, which included physical exam, hematology and serum chemistry, and oropharyngeal and rectal culture. The infected macaques, regardless of species, remained asymptomatic from the time they were diagnosed through early September 2008, when they were used on a separate research protocol to further characterize HMV *K. pneumoniae*.

Asymptomatic carriers of *Klebsiella* are common in both animals and humans. In 1 study,[24] prevalence of fecal shedding in healthy adult dairy cows was over 80%. In humans, fecal carriage of *Klebsiella* is also common, ranging in prevalence from 5% to 38% in the general population to 77% in hospital settings.[30] Rates of *Klebsiella*-positive cultures from the human nasopharynx range from 1% to 6% in the general population to 19% in hospital set-

tings.[30] In our study, the index case was identified by positive results from an oropharyngeal swab, which was the standard sampling site before the detection of infection within the colony. Once colony screening began, both oropharyngeal and rectal swabs were submitted for culture. This procedural change likely altered the sensitivity of testing, perhaps resulting in improved detection of subclinical carriers of HMV *K. pneumoniae*.

Four of the 15 infected NHP had PCR results that varied from culture to culture. A study using RAPD typing to characterize fecal *Klebsiella* shedding in dairy cows showed marked variation in strains within and between fecal samples collected from individual cows on multiple consecutive days.[25] Two cows were found to harbor multiple strains of *Klebsiella* in fecal samples taken on a single day. In our study, a single cynomolgus macaque (Cy4) had 2 different strains, as determined by RAPD typing, isolated from rectal samples taken 3 wk apart. Although the study evaluating dairy cows[25] examined *Klebsiella* species in general and not HMV *K. pneumoniae* specifically, its findings of coinfection with multiple strains may still help to explain the variable PCR results in our current study. Here, pure culture isolates were generated by picking a single bacterial colony based on phenotype and performing a boil preparation for PCR. If various strains coinfect a single animal, several phenotypically similar colonies from those strains may grow on a plate from which only a single colony would have been selected. Depending on the genotype of the colony selected, PCR results would vary from culture to culture. An additional consideration is that, once diagnosed as positive for HMV *K. pneumoniae*, each animal was moved into a single room that housed all infected macaques, regardless of genotype. The potential for coinfection or superinfection with different strains was increased during that period.

A persistent carrier status did not appear to exist in dairy cows; instead a transient presence of *Klebsiella* was evidenced by the large variety of *Klebsiella* strains found within samples and between samples collected from individual animals on multiple consecutive days.[25] The suggestion that infection in dairy cows is transient differs from literature on *Klebsiella* spp. in other species, which indicates that the organism colonizes the mucosal surfaces of mammals including humans, horses, and swine.[30] HMV *K. pneumoniae* infections in macaques do not appear to be as transient as those in the dairy cows previously described.[25] Most macaques in the current study maintained infection with at least 1 strain of *K. pneumoniae* for weeks to months; however, some animals appeared to clear the organism, testing positive once or twice and then subsequently testing negative.

In our facility, animals from an individual quarantine group (shipment cohort) generally stay grouped together until they are assigned to an experimental protocol. There appear to be some broad (but not absolute) cohort associations with regard to *rmpA* and *magA* gene status (Table 2). However some building renovation projects in our facility in the months preceding identification of the index case necessitated moving NHP into different rooms. As a result, identifying all NHP that an individual animal might have been exposed to during this time was impossible. Consequently, an NHP may have had unknown exposure to an NHP with a subclinical infection of HMV *K. pneumoniae*.

The RAPD technique has been used frequently to study genetic variability in populations. Unlike PCR, which requires well-defined and specific DNA sequences as primers, the RAPD technique uses primers of arbitrary nucleotide sequence to access

Table 2. Cohort associations between macaques infected with HMV *K. pneumoniae*

| Animal | Initial PCR results | | Date of arrival | Source |
	rmpA	*magA*		
Rh1[a]	+	−	July 2007	Vendor 1
Rh2	+	—	July 2007	Vendor 1
Rh3	+	—	July 2007	Vendor 1
Rh4[b]	+	—	July 2007	Vendor 1
Cy1	+	—	January 2008	Vendor 2
Cy2[b]	+	—	January 2008	Vendor 2
Cy5	+	—	January 2008	Vendor 2
Cy4[b]	—	—	January 2008	Research institute 1
Cy6	—	+	May 2008	Research institute 1
Cy3[b]	—	—	June 2007	Research institute 2
Rh5	+	—	May 2007	Vendor 2
Rh6	—	+	August 2007	Vendor 2
Rh7	—	+	August 2007	Vendor 2
Rh8	—	+	August 2007	Vendor 2
Rh10	—	—	August 2007	Vendor 2
Rh 9	—	—	August 2007	Vendor 2

[a]This animal was the index case and was euthanized after initial positive culture.

[b]Animal had variable PCR results over time.

random segments of genomic DNA to reveal polymorphisms. In epidemiologic studies, although pulsed-field gel electrophoresis generally is considered to be the 'gold standard'[7], it is technically demanding, requires specialized equipment, and is time-consuming.[14] Both pulsed-field gel electrophoresis and RAPD typing have proved useful for differentiating between groups of epidemiologically related extended-spectrum–β-lactamase-producing *K. pneumoniae*.[14] Whereas pulsed-field gel electrophoresis was more discriminating, RAPD typing proved to be more efficient for screening of clonally related isolates.[14] In our current study, the RAPD technique was applied to a subset of HMV *K. pneumoniae*-positive cultures to further elucidate some of the variability seen in PCR results. Cohort associations identified on PCR appeared to hold relatively consistent with RAPD banding patterns. Two different isolates from the same cynomolgus macaque (Cy4) underwent RAPD testing. During PCR analysis, the 28 April 2008 positive rectal culture from Cy4 was *rmpA*−/*magA*−, whereas that on 20 May 2008 was *rmpA*+/*magA*− (Table 1). The RAPD genotypes of those 2 isolates showed banding patterns that differed from each other: 1 of the isolates had a banding pattern consistent with cluster C, but the other isolate appeared unique and did not fit into any other cluster grouping (Figure 5).

In the initial stages of this epidemiologic investigation, a great deal of attention was given to the potential for environmental spread of this organism within the NHP colony and to concerns about human health. *K. pneumoniae* is ubiquitous in nature, existing in surface water, sewage, and soil and on plants,[30] and it is well known to exist in biofilms.[22,23] In addition, *K. pneumoniae* is a common cause of nosocomial infections in the human healthcare systems.[30] It is an opportunistic pathogen that has a survival time of 4 to 27 d (median, 7.5 d) in common hospital materials.[11]

Because of these characteristics, requirements regarding personal protective equipment were enhanced at our facility, and investigators, caretakers, technicians, and veterinary staff working with NHPs were trained about the human health risks associated with HMV *K. pneumoniae*. Concerns about the zoonotic potential of this known human pathogen led to the decision to initiate environmental surveillance for *Klebsiella*. Because we were able to culture HMV *K. pneumoniae* from the feces of 3 infected macaques, a potential for fecal–oral transmission existed. Screening involved taking cultures from areas in 6 different NHP rooms that we considered most likely to support the growth of *K. pneumoniae*. The results of these screenings revealed that environmental contamination was not a major factor in transmission of this agent between NHP rooms. As a primary respiratory pathogen, *K. pneumoniae* is known for droplet or aerosol transmission.[1,32] Accordingly, 2 experiments were initiated to determine how readily *K. pneumoniae* was dispersed both during and after high-pressure spraying of cages within the room housing known infected NHP. The results of each experiment failed to support a theory of widespread droplet or aerosol transmission of the bacteria either during or immediately after high-pressure cage wash by animal caretakers.

Although HMV *K. pneumoniae* has been documented to cause abdominal abscesses in AGM[35] and pyogenic liver abscesses (sometimes with bacteremia, endophthalmitis, and meningitis) in humans in Taiwan and other Asian countries,[6,17-19,21,26,34] the organism was maintained as a subclinical infection in a colony of macaques at our facility. Despite initial concerns about clonal spread of the organism through environmental, aerosol, or droplet contamination within the colony, environmental testing did not suggest widespread contamination in the environment. In addition, PCR and RAPD testing verified the existence of multiple genotypes which could broadly be associated with vendor cohort groups. Infection with multiple strains appeared likely in cohoused, infected macaques through a fecal–oral transmission pattern. As a result of this study, our institute has initiated procedures to increase physical separation between AGM and macaque species, including designation of room entry order and changes of personal protective equipment between species.

Future research efforts should focus on immunologic changes that result in macaques from subclinical infection with HMV *K. pneumoniae* and the potential for use as a research model for human disease. In addition, further characterization of the organism in AGM, a species that appears particularly susceptible to clinical disease resulting from infection with HMV *K. pneumoniae*, may prove to be valuable by providing insight into virulence factors and potential immune-evading measures used by this pathogen in infected AGM and humans.

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