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Human *Bordetella bronchiseptica* Infection Related to Contact with Infected Animals: Persistence of Bacteria in Host

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Within a period of 2 1/2 years, *Bordetella bronchiseptica* was isolated four times from a 79-year-old woman with bronchopneumonia. We have demonstrated by pulsed-field gel electrophoresis that this infection was related to contact with infected rabbits. The initial human *B. bronchiseptica* isolate had a phenotype characteristic of usual *B. bronchiseptica* clinical isolates; it produced toxin and adhesins, such as adenylate cyclase-hemolysin, filamentous hemagglutinin, and pertactin, and was able to induce lethality in a murine respiratory model. By contrast, although the three successive human isolates produced adhesins, they did not express adenylate cyclase-hemolysin and were unable to induce lethality. This implies that adenylate cyclase-hemolysin is required to induce lethality. We suggest that *B. bronchiseptica* may persist in the host, with expression of adenylate cyclase-hemolysin being essential for the initiation of infection and expression of adhesins being essential for persistence.

Bordetella bronchiseptica has been recognized as a respiratory tract pathogen of mammals since 1910 (16). It is primarily responsible for kennel cough in dogs, snuffles in rabbits, and atrophic rhinitis in piglets (6, 17, 18, 33). Evidence suggests that the agent may occasionally colonize the human respiratory tract and cause infection in compromised hosts (14, 18, 31, 34, 44). In some cases infected animals were found in the patient's environment (8, 26), but direct transmission was never demonstrated.

B. bronchiseptica is closely related to *Bordetella pertussis*, the agent of whooping cough, as shown by DNA hybridization (27), multilocus enzyme electrophoresis (35), and sequence analysis (2). The abilities of the two species to colonize and to establish upper respiratory tract infection depend on the production of a wide array of virulence factors. *B. bronchiseptica* synthesizes all of the factors implicated in *B. pertussis* virulence except for pertussis toxin (4). These factors include adhesins such as filamentous hemagglutinin (FHA), fimbriae, and pertactin (PRN) and toxins such as dermonecrotic toxin, tracheal cytotoxin, and adenylate cyclase-hemolysin (AC-Hly). AC-Hly is a member of the RTX family of bacterial toxins, which can enter mammalian host cells and disrupt their cellular functions by catalyzing cyclic AMP (cAMP) production and by inducing apoptosis of alveolar macrophages (11, 12, 25, 38). The expression of these factors, with the exception of tracheal cytotoxin, is subjected to a coordinated positive regulation mediated by the BvgAS proteins. These proteins belong to the large family of environment-sensing regulatory proteins which comprises two-component regulatory systems (3). In addition to positively regulating adhesins and toxins that are products of the *vag* genes (for virulence-activated genes), BvgAS proteins also negatively regulate motility in *B. bronchiseptica* and a collection of genes in *B. pertussis* called *vrg* genes (for virulence-repressed genes) (1, 5). Two regulatory phenomena, phase variation and phase modulation, have been characterized. Phase variation, which causes spontaneous *Bordetella* variants at a frequency of 10^{-3} to 10^{-6}, results from a specific DNA frameshift due to insertion of a single nucleotide into the *bvg* operon. In these variants, *vag* genes are not expressed and *vrg* genes are expressed. Phase variation is rarely reversible (41). Phase modulation results in bacteria phenotypically similar to the phase variants. Modulation, however, is a reversible event and occurs in response to environmental changes, such as a change in temperature, or the presence of modulators, such as magnesium sulfate or nicotinic acid, in the culture medium. Both phase variation and modulation are controlled by the BvgAS proteins.

In the present paper, we report the isolation of *B. bronchiseptica* from a woman with bronchopneumonia. Within the course of 2 years, the patient showed the same symptoms four times and *B. bronchiseptica* was isolated every time. Using pulsed-field gel electrophoresis (PFGE), we showed that the recurrent *B. bronchiseptica* infection was related to contact with infected rabbits. The abilities of the different isolates to express adhesins and toxins and to induce lethality in a murine respiratory model were also analyzed.

MATERIALS AND METHODS

Patient. We report the case of a 79-year-old woman admitted to our hospital in September 1989 for acute febrile bronchopneumonia. Her past medical history included brucellosis in 1952, pulmonary tuberculosis in 1954, pneumonia in 1983, and several episodes of bronchitis in 1984. She did not have a history of chronic respiratory disease (asthma, emphysema, or chronic bronchitis) or of exposure to tobacco smoke or known predisposing factors for infection (e.g., alcohol abuse or congenital or acquired immunodeficiency). She lived on a farm and had significant exposure to a cat and to rabbits.

In April 1989, the patient had a first episode of probable bronchitis, which was treated successfully at home with amoxicillin. A second episode occurred in July 1989 and was treated with mucolytics only. The third episode started in September of the same year with cough, dyspnea, and high-grade fever. She was initially treated at home with cefapirine (1 g/day intravenously) and was hospitalized after 6 days of treatment because of persistence of cough and dyspnea. Radiographs of the chest showed a bilateral pleural thickening of the lung apices consistent with past tuberculosis, bilateral perihilar infiltrates, and a right heterogenous paracardiac opacity. A bronchoscopy examination showed abundant mucopurulent secretions which grew only *B. bronchiseptica* (strain R1, $>10^7$ CFU/ml). The bronchial aspiration, two sputum samples, and two urine samples were cultured for mycobacteria and remained sterile, and the purified protein

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TABLE 1. Strains studied

Strain	Origin	Yr of isolation or reference
R1	Human (patient)	1989 (this study)
R2	Human (patient)	1990 (this study)
R3	Human (patient)	1991 (this study)
R4	Human (patient)	1992 (this study)
L2	Rabbit	1990 (this study)
9.73	Rabbit	19
5	Pig	19
Del	Human	19
Sei	Human	19

derivative skin test was positive. Serological tests for *Chlamydia* spp., *Coxiella burnetti*, *Mycoplasma pneumoniae*, *Rickettsia* spp., *Legionella pneumophila*, and human immunodeficiency virus were negative. The patient improved after a 2-week course of minocycline.

In September 1990, the patient was readmitted with similar complaints and pneumonia on chest radiography; she was treated with roxithromycin for 1 week before she was admitted. Mucopurulent secretions were seen by bronchoscopy, but no specific etiologic agent was cultured. The patient was treated with ofloxacin from day 10 to day 27 and with trimethoprim-sulfamethoxazole from day 10 to day 15 and was discharged on day 19. The patient returned 3 months later (December 1990) for recurrent pneumonia. Two cultures of purulent sputum showed only an abundant growth of oropharyngeal commensals, but a fibroscopic examination revealed abundant and mucopurulent secretions which when cultured grew only *B. bronchiseptica* (strain R2) in pure culture. An extensive evaluation for mycobacterial infection and for other causes of recurrent pneumonia was negative. The patient's immunological status was explored. Immunoglobulin G (IgG) (subclasses IgG1, IgG2, IgG3, and IgG4) and IgM levels were normal, but IgA levels were very high (6.7 g/liter). Concentrations of complement factors (CH50, C2, C3, C4, C5, and C9) were normal. Polymorphonuclear leukocyte chemotaxis, latex phagocytosis, and tetrazolium nitroblue reduction were normal. Exploration of the lymphocytic populations revealed a normal total count (1,890/mm^3), a slightly elevated number of B lymphocytes (23%; normal value, 8 to 12%), and normal T-cell counts (CD3, CD4, and CD8), except for CD57 cells, which were elevated (28%; normal value, 5 to 15%). Minocycline (200 mg/day orally) was started on day 5. The patient was discharged on day 19 with a 1-month prescription of minocycline (which she did not take regularly) and was told to avoid contact with animals. Five follow-up visits between January and October 1991 confirmed the absence of any pulmonary infection. However, in October an emphysematous respiratory insufficiency necessitated oxygen therapy 18 h/day.

In December 1991, the patient presented with cough and production of purulent sputum. Three days of treatment with amoxicillin (1.5 g/liter orally) did not improve the symptoms, and *B. bronchiseptica* (strain R3, 2×10^6 CFU/ml) was again isolated from sputum. The patient's condition slowly improved following 5 weeks of minocycline therapy (200 mg/day orally). In February 1992, a follow-up visit and X-ray examination revealed no sign of infection.

In July 1992 the patient was seen for bronchitis, and self-medication with minocycline was started. After 3 days, a culture of a purulent sputum showed, for the fourth time, 2×10^6 CFU of *B. bronchiseptica* (strain R4) per ml. A further evaluation for other infectious agents was again negative. Minocycline was continued for 1 month at home, with gradual improvement. The patient has remained free of infection for over 30 months of follow-up.

Epidemiological investigation. Epidemiological and bacteriological investigations were performed on the animals living in close proximity to the patient at the times that the first and the second *B. bronchiseptica* strains were isolated from her. She had very little contact with the farm animals. However, she lived with a young cat that slept with the 20 farm rabbits. At the time of the first documented infection of the patient, the cat and the rabbits had symptoms compatible with bordetellosis (cough associated with rhinorrhea, conjunctivitis, and diarrhea). Nasal and pharyngeal swabs were performed on the cat, but *B. bronchiseptica* was not isolated. During the second infection, the cat had no sign of bordetellosis, but the rabbits did. One rabbit was sacrificed, and the respiratory tract was removed. Cultures from the upper respiratory tract were sterile, but *B. bronchiseptica* (strain L2) was isolated in pure culture from the hilus and bronchi.

Bacterial identification and culture conditions. The strains studied are listed in Table 1. Bacteria were grown on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood (BGA) at 36°C for 48 h and again for 24 h. Subcultures in liquid medium (Stainer-Scholte medium [40]) were performed for 20 h at 36°C, until the optical density at 650 nm reached 1.0.

For Western blot (immunoblot) analysis, bacteria grown on BGA were resuspended in saline at a concentration of 2×10^{10} CFU/ml, diluted in Laemmli buffer (29), and boiled for 15 min.

Chromosomal DNA preparations. DNA was prepared as described by Khattack and Matthews (21) with some modifications. Briefly, bacteria were grown on BGA and resuspended in TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA [pH 8.0]) to an optical density of 0.7. One milliliter of cell suspension was mixed with an equal volume of low-melting-point agarose (1.0%). Each cell suspension-agarose mixture was dispensed in a 12-plug mold (Pharmacia). For each strain the plugs were incubated overnight with shaking at 37°C in 25 ml of EC buffer (6 mM Tris-HCl [pH 7.5], 1 M sodium chloride, 100 mM EDTA [pH 8.0], 0.5% [wt/vol] *N*-lauryl-sarcosine [Sigma], 1 mg of lysozyme [Sigma] per ml). Subsequently, this lysis buffer was replaced with 10 ml of ESP solution (0.5 M EDTA [pH 8.0], 1% [wt/vol] *N*-lauryl-sarcosine, 2 mg of proteinase K per ml) and incubated for 48 h at 50°C. The blocks were washed three times for at least 15 min in 30 ml of TE buffer and stored at 4°C in 0.5 M EDTA (pH 8) until further analysis.

Restriction digestion of chromosomal DNA. Restriction digestion with *Spe*I or *Xba*I was performed as described previously (21).

PFGE. PFGE was performed with a Pharmacia system. The gels (15 by 15 cm) were made up of 1% agarose in 0.5× TBE buffer (5.45 g of Tris-HCl per liter, 2.75 g of boric acid per liter, 0.038 g of EDTA per liter). The agarose blocks were loaded into wells along with DNA multimers from a 48.5-kbp bacteriophage lambda derivative (FMC Bioproducts) and *Saccharomyces cerevisiae* YNN295 chromosomes (Bio-Rad), which were used as molecular size markers. Electrophoresis was performed with pulse times of 10 s for 10 h, 20 s for 12 h, 30 s for 10 h, 60 s for 4 h, and 120 s for 4 h at a field strength of 10 V/cm.

Adenylate cyclase assay. Adenylate cyclase activity was measured as described previously (28). One unit corresponds to 1 nmol of cAMP formed per min at 30°C and pH 8.

Electrophoresis and immunoblotting methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with ready-to-use 8 to 25% polyacrylamide gels and the Pharmacia PhastSystem. Following electrophoresis, the proteins were transferred from polyacrylamide gels to Hybond C-Super membranes (Amersham). After blocking, membranes were incubated with a 5×10^{-4} dilution of mice polyclonal serum and human serum for the detection of anti-PRN antibodies and at a 2.5×10^{-5} dilution for the detection of anti-FHA and anti-AC-Hly antibodies at 4°C overnight. The immunochemical detection was performed with horseradish peroxidase-labelled sheep anti-mouse immunoglobulins and an enhanced chemiluminescence system (Amersham).

Immune sera. Groups of 10 4-week-old female BALB/c mice were immunized subcutaneously four times at 3-week intervals with 10 μg of either purified *B. pertussis* FHA, purified *B. pertussis* PRN, or *B. bronchiseptica* AC-Hly, adsorbed on aluminium hydroxide. Mice were bled 7 days after the last injection. The specificities of polyclonal antibodies were checked by Western blotting analysis with purified antigens and a whole *B. pertussis* bacterial suspension.

Intranasal infection of mice. *B. bronchiseptica* was grown on BGA as described above. Bacteria were resuspended and diluted in 1% Casamino Acids and serially diluted to provide challenge inoculum dilutions to determine the 50% lethal dose (LD_{50}). For the respiratory infection, 50 μl of bacterial suspension was injected intranasally into groups of 10 3- to 4-week-old female Swiss mice (CERJ, St. Berthevin, France). The LD_{50} for the challenge inocula were determined by recording the number of dead mice daily for 30 days.

RESULTS

Human *Bordetella* infection. *B. bronchiseptica* was isolated four times from the same patient. During the first two episodes, this patient had clinical respiratory infection. At this time, she was living with a cat which was often sleeping close to rabbits with signs of bordetellosis. During the second episode, *B. bronchiseptica* was also isolated from one of the rabbits, confirming the animal infection. *B. bronchiseptica* was isolated twice again from purulent bronchial secretions, 12 and 18 months after the second episode, when the patient was no longer in contact with infected animals.

Analysis of *B. bronchiseptica* clinical isolates. No difference between the four patient isolates, the rabbit isolate, and other human or animal isolates was detected by either culture, bacteriological characteristics, or antibiotic resistance. The patient and rabbit isolates were, as determined by the diffusion method (National Committee for Clinical Laboratory Standards), sensitive to amoxicillin plus clavulanic acid, ticarcillin, ticarcillin plus clavulanic acid, piperacillin, and piperacillin plus tazobactam; resistant to cefazolin, cefoxitin, cefotaxim, and aztreonam; sensitive to ceftazidime, moxalactam, imipenem, gentamicin, tobramycin, amikacin, chloramphenicol, minocycline, and erythromycin; resistant to josamycin; sensi-

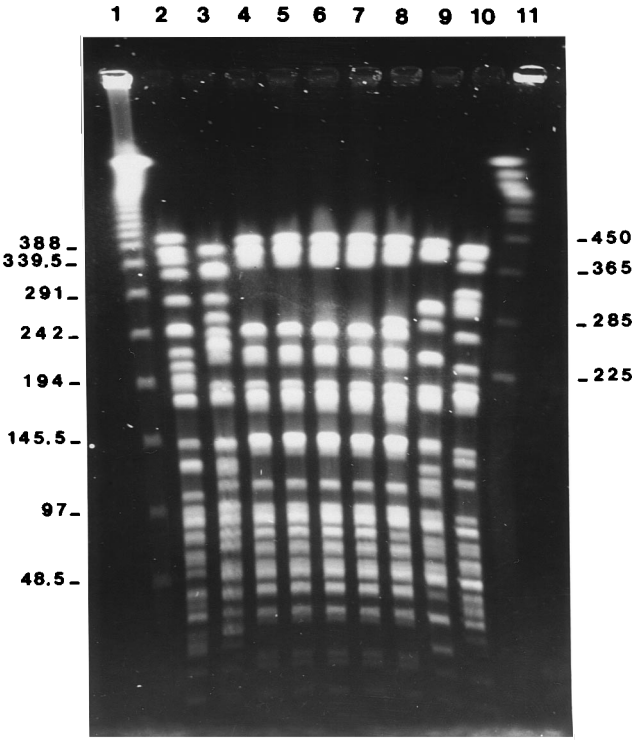

FIG. 1. Analysis of *Xba*I-digested DNA by PFGE. DNA was prepared from clinical isolates, digested with *Xba*I, and resolved by PFGE. The DNA profiles shown correspond to nine *B. bronchiseptica* clinical isolates, including the five analyzed in this study. Lane 1, ladder molecular size marker (multimers of DNA from lambda bacteriophage); lane 2, clinical isolate 5; lane 3, rabbit isolate 9.73; lane 4, rabbit isolate L2; lane 5, clinical isolate R1; lane 6, clinical isolate R2; lane 7, clinical isolate R3; lane 8, clinical isolate R4; lane 9, human isolate Del; lane 10, human isolate Sei; lane 11, molecular size marker DNA. Numbers on the left and right are sizes in kilobase pairs.

tive to ofloxacin and ciprofloxacin; and resistant to fosfomycin and bacitracin.

Analysis of *B. bronchiseptica* clinical isolates by PFGE. Recent studies (15, 21, 42) have shown PFGE to be a more effective technique for examining the epidemiology of *B. pertussis* than multilocus enzyme electrophoresis (35) or serology (21). Typing by PFGE was based on *Xba*I digestion of *B. pertussis* total DNA, which generated a small number of restriction fragments, and those authors suggested that PFGE typing could be used to trace the movement of *B. pertussis* strains within towns or intrafamilial epidemics (21, 22). Therefore, we used this technique to differentiate the *B. bronchiseptica* isolates from the patient from *B. bronchiseptica* isolates of different origins. We analyzed more than 60 isolates, and the results for 9 of them are presented in Fig. 1. We confirmed that 14 to 17 restriction fragments of 97 to 388 kb were generated by *Xba*I digestion and that fragments smaller than 97 kb were not sufficiently resolved to provide useful information. The restriction profiles of the 60 isolates were very heterogeneous. In Fig. 1, only nine DNA profiles are presented, and among them, there are six different profiles. However, the *Xba*I profiles for isolates L2, R1, R2, and R3 were identical (Fig. 1), suggesting that a single strain was responsible for the patient and the rabbit infections. The fourth patient isolate, R4, had a profile very similar to that of L2, R1, R2, and R3 except for an additional two bands of 160 and 288 kb. Consistent results were obtained when the DNA were digested with *Spe*I (data not shown).

TABLE 2. Expression of AC-Hly, FHA, and PRN by *B. bronchiseptica* clinical isolates

Clinical isolate	Growth conditions	Hemolysis[a]	AC[b]	Expression of[c]:			LD_{50}[d]
				AC-Hly	FHA	PRN	
L2	Normal[e]	+	40	+	+	+	2×10^6
	Modulator[f]	−	0	−	−	−	
R1	Normal	+	63	+	+	+	10^7
	Modulator	−	0	−	−	−	
R2	Normal	−	0	−	+	+	ND[g]
	Modulator	−	0	−	−	−	
R3	Normal	−	0	−	+	+	$\geq 10^9$
	Modulator	−	0	−	+	+	
R4	Normal	−	0	−	+	+	ND
	Modulator	−	0	−	−	−	

[a] Ability to produce hemolysis on BGA plates.
[b] Adenylate cyclase (AC) activity (milliunits per milliliter) assayed in bacterial suspensions.
[c] Detected by Western blot.
[d] Determined after intranasal infection of 4-week-old mice.
[e] Bacteria were grown in Stainer-Scholte medium at 36°C.
[f] Bacteria were grown in Stainer-Scholte medium in the presence of either 50 mM $MgSO_4$ or 5 mM nicotinic acid at 36°C or in the absence of a modulator at 25°C.
[g] ND, not determined.

Factors expressed by the different clinical isolates. The clinical isolates analyzed were not differentiated by PFGE. However, they were phenotypically different: no hemolysis was detectable when strains R2, R3, and R4 were grown on BGA plates, in contrast to the results for strains R1 and L2 (Table 2). Moreover, no adenylate cyclase activity could be detected in bacterial suspensions of R2, R3, and R4, in contrast to the results for R1 and L2 (Table 2). Since these three isolates were devoid of hemolytic and adenylate cyclase activities, we used specific polyclonal antibodies to determine if AC-Hly, FHA, and PRN were expressed. Since *B. pertussis* and *B. bronchiseptica* virulence factors have a high level of homology (4, 7, 19, 32), we used specific polyclonal sera against *B. pertussis* AC-Hly, FHA, and PRN. As shown in Table 2, whereas strains L2 and R1 expressed AC-Hly, FHA, and PRN, as described for previously studied *B. bronchiseptica* clinical isolates (19), isolates R2, R3, and R4 expressed only FHA and PRN. Moreover, isolates R1 and L2 could induce lethality in the murine respiratory model, but the R3 isolate could not, suggesting that as for *B. pertussis* (24), AC-Hly expression is required for lethality.

Regulation of factor expression by the different clinical isolates. The expression of *Bordetella* genes is positively or negatively regulated at the transcriptional level by the BvgS and BvgA proteins. The expression of FHA, PRN, and AC-Hly is positively regulated; i.e., these factors are produced at 36°C but not at 25°C or in the presence of $MgSO_4$ or nicotinic acid at 36°C. As shown in Table 2, for isolates L2, R1, R2, and R4 expression of FHA and PRN, and for L2 and R1 expression of AC-Hly, was observed at 36°C but not at 25°C or when the cultures were performed in the presence of modulators, as expected (similar results were obtained when cultures were performed in the presence of $MgSO_4$ or nicotinic acid). Surprisingly, the expression of FHA and PRN was constitutive in the R3 isolate, since expression was still observed at 25°C or in the presence of modulators at 36°C. However, the fact that R3 but not R4 was not sensitive to modulation suggests that both constitutive and nonconstitutive strains were present at the same time.

Analysis of antibodies in patient and rabbit sera. Sera were collected from the infected rabbit and from the patient after

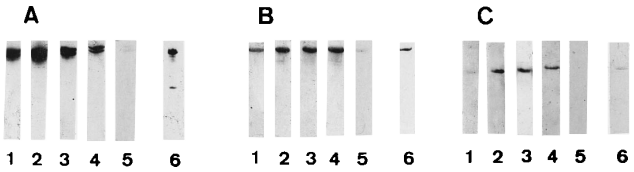

FIG. 2. Western blot analysis of purified AC-Hly, FHA, and PRN with patient and rabbit sera. Two hundred nanograms of purified AC-Hly (A), FHA (B), or PRN (C) was subjected to SDS-PAGE with 8 to 25% polyacrylamide gels, and proteins were transferred to Hybond C-Super membranes and incubated with sera from the patient collected after each isolation of *B. bronchiseptica* (1989 [lanes 1], 1990 [lanes 2], 1991 [lanes 3], 1992 [lanes 4], and 1993 [lanes 5]) and with rabbit serum collected after isolation of the *B. bronchiseptica* strain (lanes 6). The immunodetection was performed with peroxidase-labelled sheep anti-mouse immunoglobulins, using the enhanced chemiluminescence detection system from Amersham.

each *B. bronchiseptica* infection. Antibodies specific to AC-Hly, FHA, and PRN were detected in patient sera (Fig. 2, lanes 1, 2, 3, and 4) and in rabbit serum (Fig. 2, lanes 6) collected during infection. These results indicate that factors such as AC-Hly, FHA, and PRN were expressed during infection in vivo, since they induced the synthesis of the corresponding antibodies. Sixteen and 26 months after the fourth infection, the amounts of antibodies were significantly lower than after each infection (Fig. 2, lanes 5, and data not shown, respectively), suggesting that these factors were no longer produced.

DISCUSSION

In the present paper we report the case of a woman who had four successive *B. bronchiseptica* respiratory infections within a period of 2 years. Infections 1 and 2 occurred when the patient was living close to animals that were also infected by *B. bronchiseptica*. However, infections 3 and 4 happened when she was no longer in contact with infected animals, suggesting persistence of the bacteria. These infections occurred despite a 5-week course of treatment with minocycline. Minocycline was chosen because of its good in vitro activity and favorable pharmacokinetics. Despite this, three relapses occurred. The first can be explained by the short course of antibiotics, and the second can be explained because of poor patient compliance. However, the third treatment, 5 weeks long, was unable to prevent the fourth relapse. A fourth identical course was necessary to eradicate the bacteria. Treatment of *B. bronchiseptica* bronchopulmonary infections is difficult, and relapses have been often described (10, 20, 36). A long duration of antibiotic therapy, especially in compromised patients, seems to be important for a definitive cure.

Our aim was to compare the four human strains and one rabbit strain. It was not possible to differentiate these isolates by cultural or bacteriological characters or by resistance to antibiotics. Since recent results provide evidence that PFGE of *Xba*I-digested chromosomal DNA provides a sensitive means for discrimination between *Bordetella* isolates (21, 42), we used this technique to compare the five clinical isolates with various *B. bronchiseptica* isolates of animal or human origin. Analyses of 60 strains showed a much greater heterogeneity among *B. bronchiseptica* isolates than among *B. pertussis* isolates (43). This heterogeneity may indicate that *B. bronchiseptica* isolates are more adaptable than *B. pertussis* to a wide range of hosts and even to a saprophytic stage (37). However, as observed during an intrafamilial *B. pertussis* infection (15), the four strains isolated from the patient and that isolated from a rabbit have similar PFGE profiles. This result indicates that the human *B. bronchiseptica* infection was related to contact with infected rabbits. These data illustrate the potential of PFGE for studying the epidemiology of strains involved not only in *B. pertussis* infections but also in *B. bronchiseptica* infections or other bacterial infections.

Although not distinguishable by PFGE, the strains isolated had different phenotypes. The initial patient strain as well as that isolated from the infected rabbit expressed all *vag* genes and induced lethality in the murine model. These *vag* genes were positively regulated, as in all clinical isolates previously studied (19). However, the strains isolated during the last three patient infections produced FHA and PRN but not AC-Hly. These AC-Hly-deficient strains were not able to induce lethality in the murine model, confirming the fact that expression of AC-Hly is necessary to initiate infection (24). The strains isolated expressed FHA and PRN (one strain expressed them constitutively), confirming the importance of these adhesins for persistence of bacteria in the host, as previously suggested (9, 13, 23).

As already observed in a murine respiratory model (29), *B. bronchiseptica* persisted in the host despite the presence of specific antibodies. This suggests that *B. bronchiseptica* may persist intracellularly while expressing some of its *vag* genes or may stop *vag* gene expression and then express *vrg* genes. In fact, we have data showing that *B. bronchiseptica* strains expressing only *vrg* genes can be isolated several weeks after the onset of infection in humans or animals (30). *B. bronchiseptica* may persist in alveolar macrophages and must therefore repress AC-Hly expression, since this enzyme can induce apoptosis of alveolar macrophages (25). *B. bronchiseptica* may also persist in epithelial cells, as demonstrated recently in vitro (39). This property was shown to be independent of *vag* gene expression. The present observations allow us to propose a three-step model for *B. bronchiseptica* infection. First, the bacteria need to express all *vag* genes in order to initiate infection: adhesins such as FHA and PRN are necessary for the bacteria to adhere to ciliated cells, and toxins such as AC-Hly are necessary in order to alter host cell functions and to initiate infection. Second, in order to persist, the bacteria must stop producing AC-Hly, which is deleterious for extra- or intracellular survival, and continue to produce adhesins such as FHA and PRN. Third, the bacteria must cease all *vag* gene expression and turn on *vrg* gene expression in order to escape the host immune response.

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