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## Isolation of cilia-associated respiratory (CAR) bacillus from pigs and calves and experimental infection of gnotobiotic pigs and rodents

Jerome C. Nietfeld, Barry L. Fickbohm, Douglas G. Rogers, Craig L. Franklin, Lela K. Riley

**Abstract.** Filamentous, gram-negative bacteria morphologically similar to cilia-associated respiratory (CAR) bacillus of rodents and rabbits were isolated from the tracheas of 5 pigs and 4 calves. All pigs but none of the calves had histologic lesions of chronic tracheitis. In silver-stained histologic sections, CAR bacilli were adhered to the tracheal epithelium of each pig but were not found in the calves. Like CAR bacillus of rats, the bacteria displayed gliding motility and grew only in cell culture or cell culture medium supplemented with fetal serum. Initially, all isolates were contaminated by *Mycoplasma* spp. This contamination was eliminated from 4 pig isolates by limiting dilutions, and mycoplasma-free isolates were used to intranasally inoculate gnotobiotic pigs and CAR bacillus-free mice and rats and to immunize guinea pigs. The gnotobiotic pigs remained healthy, and when they were necropsied 4 and 7 weeks after infection no macroscopic or microscopic lesions were found in the respiratory tract. However, CAR bacillus was isolated at both times from the nasal cavities and tracheas of inoculated pigs, and the ciliated tracheal epithelium of infected pigs necropsied 7 weeks after infection was colonized by low numbers of CAR bacillus-like bacteria. The rats and mice remained healthy through week 12 postinoculation, and evidence of short- or long-term colonization was not detected by histologic examination or culture. When used as primary antibody for immunohistochemical staining, sera from guinea pigs immunized with pig CAR bacillus specifically stained CAR bacilli colonizing the respiratory epithelium of naturally infected pigs, whereas sera collected prior to immunization failed to react with the bacteria. These results indicate that CAR bacilli are unlikely to be primary pathogens of pigs or cattle and that rodents do not act as reservoirs.

Cilia-associated respiratory (CAR) bacillus is a descriptive name for filamentous, gram-negative, gliding bacteria that colonize the ciliated respiratory epithelium of rats, mice, rabbits, white tailed hamsters, cattle, goats, and pigs.<sup>1,6–9,11,12,17,18,25</sup> Natural and experimental CAR bacillus infections in mice and rats cause chronic respiratory disease that clinically and pathologically resembles disease caused by *Mycoplasma pulmonis*.<sup>1,2,7,8,12–15,21,25</sup> Lesions consist of lymphoplasmacytic tracheitis and bronchitis with loss of cilia in areas of CAR bacillus colonization. Naturally infected rabbits reportedly are clinically normal, and macroscopic and microscopic lesions are absent.<sup>11</sup> However, after experimental inoculation with rabbit-origin CAR bacillus, rabbits developed a mucopurulent nasal discharge and lymphoplasmacytic bronchitis.<sup>4</sup> Most naturally infected pigs, cattle, and goats have chronic tracheitis and bronchitis, and the numbers of cilia are decreased in areas colonized by CAR bacillus.<sup>6,9,17</sup> However, it is

not known if lesions in livestock are caused by CAR bacillus.

In vitro cultivation of CAR bacillus is very difficult, as indicated by the 5-year gap between identification of CAR bacillus in histologic sections from rats with chronic respiratory disease<sup>25</sup> and isolation of the organism in embryonated chicken eggs.<sup>7</sup> Subsequently, CAR bacillus has been isolated from rats and rabbits using cell culture and cell culture medium supplemented with fetal bovine serum,<sup>2,4,22</sup> but growth of CAR bacillus on solid medium has not been achieved. Isolation of CAR bacillus from livestock has not been reported.

In this study, filamentous, gram-negative, gliding CAR bacillus-like bacteria were isolated from the tracheas of 5 pigs and 4 calves. All isolates were initially contaminated with *Mycoplasma* spp. After elimination of mycoplasmal contamination, pig isolates were used to intranasally inoculate gnotobiotic pigs and CAR bacillus-free rats and mice and to immunize guinea pigs whose sera were used for immunohistochemical staining of tissues from pigs naturally infected with CAR bacillus.

### Materials and methods

*Isolation of CAR bacillus.* Isolates of CAR bacillus were obtained from tracheas of pigs submitted to the Kansas State University Veterinary Diagnostic Laboratory, pigs at an ab-

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attoir, and 4 healthy calves necropsied as part of a trial to evaluate the safety of a cell line for production of vaccine virus. Each isolate was cultivated in cell culture using the rat intestinal epithelium cell line IEC-18.<sup>a</sup> Cell culture medium consisted of Dulbecco's modified Eagle's medium with 4.5 g glucose/liter<sup>b</sup> supplemented with 5% heat-inactivated fetal bovine serum<sup>c</sup> or 5% fetal porcine serum<sup>b</sup> and 0.1 unit insulin<sup>d</sup>/ml.

Samples were collected by incising the tracheas transversely, vigorously rubbing the mucosa with cotton-tipped swabs, and placing the swabs individually into 15-ml plastic centrifuge tubes containing 3 ml of cell culture medium. After transport on ice to the laboratory, the centrifuge tubes were vortexed vigorously, and 100–500  $\mu$ l of cell culture medium from each sample was placed into a well of a 6-well cell culture dish or a 25-cm<sup>2</sup> cell culture flask containing a monolayer of IEC-18 cells. The IEC-18 cells were incubated at 37 C in a CO<sub>2</sub> incubator (7%), and the medium was changed every 3–5 days. Flasks that became visibly cloudy were considered to be contaminated by bacteria other than CAR bacillus and were discarded.

Three to 5 days after inoculation and every 1–3 days thereafter, the flasks were examined for growth of cell-associated filamentous bacteria at 400 $\times$  with an inverted phase-contrast microscope. The cell culture medium also was examined for filamentous bacteria by placing a drop of medium on a microscope slide, covering the medium with a glass coverslip, and examining the drop at 1,000 $\times$  with a phase contrast microscope. Growth of CAR bacillus-like bacteria was maintained by transferring 100–500  $\mu$ l of medium from cell cultures with bacterial growth to fresh monolayers of IEC-18 cells. The CAR bacillus isolates were stored by freezing in cell culture medium at –80 C. After cultivation of the first isolate, it was found that the CAR bacillus could pass through a filter with a 0.45- $\mu$ m pore diameter. Thereafter, samples were filtered with a 0.45- $\mu$ m syringe filter<sup>e</sup> before inoculation onto IEC-18 cells.

**Testing CAR bacillus isolates for contamination.** For *Mycoplasma* isolation, 50  $\mu$ l of cell culture medium from flasks with CAR bacillus growth were spread over the surface of agar plates prepared as previously described.<sup>10</sup> The plates were incubated in a candle jar (~2–3% CO<sub>2</sub>) at 37 C and examined for colony formation at 2–3-day intervals for 3 wk. Colonies were mixed with a drop of sterile cell culture medium on a microscope slide and covered with a coverslip. Bacterial morphologic characteristics were determined by examination at 1,000 $\times$  with a phase contrast microscope. In addition, medium from cell cultures with CAR bacillus growth were tested for *Mycoplasma* spp. by polymerase chain reaction (PCR) using genus-specific primers as previously described.<sup>24</sup> To detect bacterial growth other than *Mycoplasma* spp., medium from CAR bacillus isolates was plated onto blood and chocolate agar plates and incubated for 96 hr at 37 C in air and in a candle jar (~2–3% CO<sub>2</sub>).

**Elimination of *Mycoplasma* spp. from CAR bacillus isolates.** Mycoplasmal contamination was eliminated from 4 pig CAR bacillus isolates by limiting dilutions. To harvest CAR bacilli, a 25 cm<sup>2</sup> flask of IEC-18 cells with CAR bacillus growth was scraped with a rubber-tipped scraper and the contents were transferred to a centrifuge tube. The IEC-

18 cells were pelleted by centrifugation for 15 min at 150  $\times$  g, and the supernatant containing the CAR bacilli was saved. Serial 10-fold dilutions of the supernatant were made using cell culture medium as diluent. Ten microliters of each bacterial dilution from 10<sup>-4</sup> through 10<sup>-9</sup> were inoculated into 18 wells of 48-well culture dishes containing IEC cells. The IEC-18 cells were incubated in 7% CO<sub>2</sub> at 37 C, and the medium was changed at 3–4-day intervals. Beginning 7 days after inoculation and daily thereafter, culture medium from each well was examined at 1,000 $\times$  with a phase contrast microscope for CAR bacillus growth. Medium from the wells with CAR bacillus growth that were inoculated originally with the most dilute CAR bacillus suspension were used to inoculate fresh flasks of IEC-18 cells. After 3–7 days of incubation, medium from these flasks was used to inoculate agar plates for mycoplasmal growth. If no growth occurred after 7 days, CAR bacilli from the flasks were transferred serially to fresh IEC cells. To be considered mycoplasma-free, culture medium from 3 consecutive passages had to be negative for *Mycoplasma* spp. as determined by culture and PCR.

**Experimental inoculation of gnotobiotic pigs, rats, and mice.** Eleven gnotobiotic piglets obtained by closed hysterotomy<sup>16</sup> were housed and maintained as previously described.<sup>20</sup> At 3 days of age, 9 pigs were inoculated intranasally with CAR bacillus suspended in cell culture medium as follows: 3 pigs with 0.5 ml of isolate 95-15405/nostril; 3 pigs with 1.0 ml of isolate 95-15405/nostril; and 3 pigs with 0.5 ml of isolate 96-1590/nostril. Two control pigs received 2 ml of sterile culture medium/nostril. Each group was housed in a separate isolation unit. The pigs were observed twice daily for signs of respiratory disease, lethargy, or anorexia and for other clinical signs of illness. On days 9 and 15 after inoculation, 1 treated pig was euthanized because of lethargy and poor growth. Twenty-nine days after inoculation, 5 treated and 1 control pig were euthanized and necropsied. The remaining 2 treated and 1 control pig were euthanized and necropsied on day 47 after inoculation. The pigs were anesthetized with tiletamine HCl and zolazepam HCl<sup>f</sup> (0.09 ml/kg) by the intramuscular route and then euthanized by electrocution. Portions of nasal turbinates, trachea, and lung were preserved in 10% neutral buffered formalin and routinely processed for histologic examination. Sections of each tissue were stained with hematoxylin and eosin and by the Warthin-Starry method and examined for histologic lesions and for bacilli colonizing the ciliated epithelium. Swabs were obtained from the tracheas and nasal cavities and cultured for CAR bacillus as described above.

To determine if rodents are a potential reservoir for pig CAR bacillus, rats and mice were infected intranasally with the PigCAR-1 isolate from an abattoir pig. Three breeding pairs each of Sprague-Dawley rats and BALB/c mice were obtained from colonies known by histologic and serologic screening to be free of rodent CAR bacillus.<sup>2,3</sup> The breeding pairs were designated A, B, and C, and the litters from each pair were inoculated intranasally according to the following schedule. In litter A, each pup received 10<sup>8</sup> CAR bacillus in 50  $\mu$ l of phosphate-buffered saline (PBS), in litter B, half of the pups were inoculated with 10<sup>8</sup> CAR bacillus in 50  $\mu$ l PBS, and in litter C, each pup was inoculated with 50  $\mu$ l

**Table 1.** Source of CAR bacillus isolates.

Isolate	Host	Host age	Tracheitis	CAR bacillus histology	Other findings
PigCAR-1	pig	6 mo	yes	yes	abattoir pig
243-54	calf	10-12 mo	no	no	none
244-55	calf	10-12 mo	no	no	none
245-56	calf	10-12 mo	no	no	none
246-57	calf	10-12 mo	no	no	none
95-15405	pig	5-6 wk	yes	yes	<i>Streptococcus suis</i> septicemia
96-1590	pig	2-3 mo	yes	yes	prolapsed bladder and peritonitis
96-4763	pig	3-4 mo	yes	yes	multisystemic abscesses
96-20304	pig	1-4 mo	yes	yes	PRRS,* bronchopneumonia, polyserositis

\* PRRS = porcine reproductive and respiratory syndrome.

PBS. Pig CAR bacillus was grown on mouse 3T3 fibroblast cells, and the bacterial inoculum was prepared as previously described.<sup>2,3</sup> Blood was collected from each rodent 10 wk after inoculation, and the sera were tested for antibodies to rat CAR bacillus.<sup>2</sup> All animals were euthanized and necropsied 12 wk after inoculation.<sup>2,3</sup> Portions of trachea and lung were preserved in 10% neutral buffered formalin, processed for histologic examination, stained, and examined in a manner similar to that described for the pigs. A second rodent experiment was done to determine if transient colonization occurred. This experiment was identical to the first, except that the rodents were examined for evidence of bacterial colonization at 2 and 4 wk after intranasal inoculation.

*Preparation of inocula for gnotobiotic pigs.* Eight flasks of IEC-18 cells with CAR bacillus isolate 95-15405 and 8 flasks with isolate 96-1590 were used to provide bacteria for challenge of gnotobiotic pigs. Both isolates were culture and PCR negative for *Mycoplasma* spp. and had been passed in vitro fewer than 10 times. For each isolate, the flasks were scraped to suspend the IEC cells and associated bacteria. The culture medium was placed in 15-ml conical centrifuge tubes and vortexed vigorously to disassociate bacteria from the IEC cells. The IEC-18 cells were pelleted by centrifugation at 150 × g for 15 min at room temperature. The supernatant was transferred to a second centrifuge tube, and the bacteria were pelleted by centrifugation at 27,000 × g for 30 min at 4 C. The supernatant was discarded, and the pellet was resuspended in 10 ml of cell culture medium. Centrifugation and resuspension of the bacterial pellet in 10 ml of medium then were repeated. The bacterial suspensions were placed in sterile rubber-stoppered glass tubes and transported on ice approximately 226 km for inoculation of the gnotobiotic pigs. The unused portion of each inoculum was brought back to the lab and cultured on IEC-18 cells to test for viability.

*Immunization of guinea pigs and immunohistochemical staining.* Pig CAR bacillus isolates 95-15405 and 96-1590 were used to prepare antigen for immunization of 2 guinea pigs because natural CAR bacillus infection has not been reported in guinea pigs. The bacteria were harvested in a manner identical to that used for inoculation of gnotobiotic pigs, except that PBS was substituted for cell culture medium for washing and resuspension of CAR bacillus. Each guinea pig was vaccinated 3 times with 1 isolate at 4-wk intervals. At each vaccination, 0.5 ml of bacterial suspension was given subcutaneously in 2 equally divided doses, and

0.25 ml was given intramuscularly. Immediately before the first vaccination and 3 wk after the last vaccination, the guinea pigs were anesthetized with halothane, and blood was collected by cardiac puncture. Sera from the 2 guinea pigs were used at a dilution of 1:200 as the primary antisera for immunoperoxidase staining of formalin-fixed, paraffin-embedded tracheas and nasal turbinates from pigs naturally colonized by CAR bacillus, as determined by Warthin-Starry staining. Because both isolates of CAR bacillus were grown in medium containing fetal pig serum, the diluted guinea pig sera were incubated for 1 hr with 1.5% fetal pig serum before use. Otherwise, the staining was performed as previously described.<sup>17</sup>

## Results

*Isolation of CAR bacillus.* Filamentous, gram-negative bacteria were isolated in cell culture from tracheas of 5 pigs and 4 calves (Table 1). Each pig was from a different herd, whereas the calves were from the same group. After 3-7 days of incubation, a few filamentous bacteria were seen by examination of a drop of culture medium at 1,000× with a phase contrast microscope. Most bacteria floated in the medium, but a few were attached to the slide by one pole. Many attached bacteria appeared to flex and glide across the slide. After an additional 2-5 days, small colonies of filamentous bacteria were attached to scattered IEC cells. Many of the bacteria appeared to lie flat on the cells, but numerous groups of bacteria also were attached by one pole forming pin-cushion-like aggregates, similar to those described for rat CAR bacillus.<sup>2,22</sup> Flexing or waving motion was evident, and some bacteria glided across the monolayer by utilizing this flexing motion. With time, bacteria covered the cells, after which the medium needed to be changed every 2-3 days or it became acidic (yellow), which was followed rapidly by loss of the monolayer and loss of bacterial viability. If the medium was changed at 2-day intervals, the monolayer and attached bacteria remained viable for 2-3 weeks, but eventually the IEC-18 monolayer detached completely.

The first isolate, PigCAR-1, was from a tracheal



**Figure 1.** CAR bacillus isolate PigCAR-1 grown on the cell-free surface of a tissue culture dish. Crystal violet stain.

swab collected from a pig at an abattoir. Approximately 100 samples were cultured, 77 from the abattoir, before successfully cultivating PigCAR-1. All abattoir pigs were from a single herd, and examination of histologic slides from the abattoir pigs demonstrated CAR bacillus-like bacteria in 40% of the tracheas, but contamination and overgrowth of the cell cultures by other bacteria was a serious problem. When it was discovered that PigCAR-1 could pass through a filter with a 0.45- $\mu\text{m}$  pore diameter, subsequent samples were filtered before inoculation of IEC-18 cells. When the samples originated from the trachea, 80–90% of the filtered samples were free of bacterial growth other than CAR bacillus and *Mycoplasma* spp. However, filtered samples originating from the nasal cavities had a high incidence of contamination by *Streptococcus* spp. and *Haemophilus parasuis*. For that reason, sampling of the nasal cavities was discontinued.

As the IEC-18 cells detached, pig PigCAR-1 grew in areas previously occupied by cells, and after the entire monolayer detached, this isolate continued to grow and soon covered the flask's surface (Fig. 1). PigCAR-1 could be maintained by scraping the flasks, aspirating the bacteria and medium, adding new medium, and incubating. If the flasks were not scraped and the medium was not changed at approximately 3–7-day intervals, the bacteria rounded up, fragmented, and became nonviable. PigCAR-1 also could be maintained in new cell culture dishes not conditioned by previous cell growth. However, isolates from the other 4 pigs and 4 calves could not be maintained in culture flasks without living cells. If transferred to flasks devoid of cells, within 1–3 days the bacteria could not be propagated when transferred onto monolayers of IEC-18 cells. Even in flasks with large numbers of CAR bacilli attached to IEC-18 cells, isolates other than PigCAR-1 did not attach to the flask's surface in spaces from which the epithelial cells detached, and

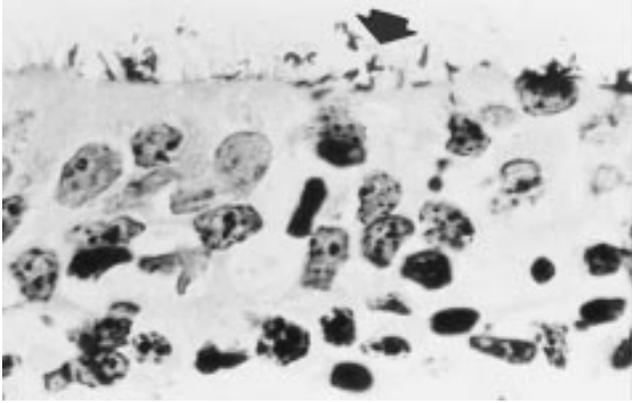
once the monolayer detached, the bacteria rapidly became unculturable.

All isolates were cultivated initially on IEC-18 cells, which were in use and available in the laboratory when the project began. Subsequently, 4 pig and 2 calf isolates have been grown on Madin Darby bovine kidney cells, Madin Darby canine kidney cells, and 3T3 mouse fibroblast cells. Each of the cell lines supported CAR bacillus growth equally well, so the cell line used for isolation is probably not critical. Insulin was added to the culture medium because it is necessary for growth of the IEC-18 cells. When other cell lines were used for maintenance of CAR bacillus, insulin was not added to the culture medium and bacterial growth was not affected. PigCAR-1 and the 4 calf isolates initially were cultivated in medium supplemented with 5% fetal bovine serum. When examined 3–5 days after inoculation, an additional 4–6 pig samples cultured in medium with fetal bovine serum contained filamentous bacteria that were morphologically indistinguishable from PigCAR-1 and the calf isolates. However, after approximately 1 week, the bacteria had died out. For that reason, supplementation with 5% fetal pig serum was initiated, and the remaining 4 pig isolates were cultivated using fetal pig serum. After initial isolation, each of the pig isolates was passed serially in medium supplemented with fetal bovine serum, so whether fetal pig serum was necessary for isolation is not known.

Two to 4 days after plating, culture medium from each CAR bacillus isolate yielded pinpoint clear colonies on the agar used for isolation of *Mycoplasma* spp., but no growth occurred on blood or chocolate agars. The bacteria in the colonies were small and pleomorphic and were assumed to be *Mycoplasma* spp. In addition, culture medium from each isolate was PCR positive using *Mycoplasma* genus-specific primers. After the limiting dilutions procedure, culture medium from 4 pig isolates was consistently culture and PCR negative for *Mycoplasma* spp.

The pig isolates were from pigs with chronic tracheitis, and CAR bacillus-like bacteria were identified in silver-stained sections of trachea from each pig. Lesions consisted of loss of cilia, squamous metaplasia of the epithelium, and lymphocytic infiltration of subepithelial tissue. The CAR bacillus-positive calves were healthy and free of macroscopic and microscopic lesions of the respiratory tract, and CAR bacillus-like bacteria were not seen in silver-stained sections of their tracheas.

*Infection of gnotobiotic pigs, rats, and mice.* CAR bacillus isolates 95-15405 and 96-1590 were isolated from the respective inoculums after inoculation of the gnotobiotic pigs. Except for the pigs euthanized 9 and 15 days after inoculation, all gnotobiotic pigs remained healthy. At necropsy, all pigs, including those eutha-



**Figure 2.** Sparsely scattered, black, argentophilic bacilli (arrow) interspersed among the tracheal cilia of a gnotobiotic pig euthanized 47 days after intranasal inoculation with CAR bacillus isolate 95-15405. Warthin-Starry stain.

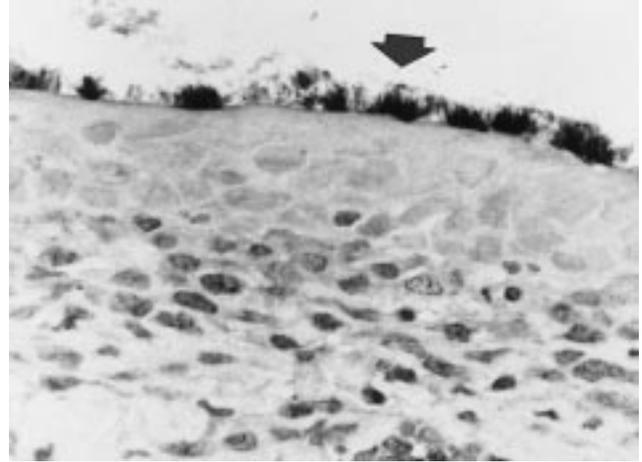
nized 9 and 15 days after inoculation, were free of macroscopic lesions. No histologic lesions were observed in the nasal turbinates, tracheas, or lungs of the treated or control pigs. Silver-stained sections of trachea from the 2 treated pigs necropsied 47 days after inoculation contained widely scattered small groups of black, filamentous bacteria-like structures consistent with CAR bacillus lying among and parallel to the cilia (Fig. 2). CAR bacillus-like bacteria were not seen in tissues from the control pigs or from treated pigs necropsied at 9, 15, and 29 days following inoculation. CAR bacillus was isolated from the nasal cavities and trachea of 1 treated pig necropsied 29 days after inoculation and from both treated pigs necropsied 47 days after inoculation. It was not isolated from the other pigs in the study.

All rodents infected with PigCAR-1 remained healthy and did not display clinical symptoms of respiratory disease. CAR bacillus was not found in silver-stained sections of rodent tracheas and lungs, and the rodents did not seroconvert to rat CAR bacillus.

**Immunohistochemical staining.** Specific staining was absent when sera collected prior to immunization were used as the primary antibodies for immunoperoxidase staining of tracheas and nasal turbinates from pigs naturally infected with CAR bacillus. Sera collected after immunization gave specific staining of the CAR bacillus-like bacteria colonizing the luminal surface of the ciliated respiratory epithelium (Fig. 3).

### Discussion

The bacteria described in this study have not been isolated previously from swine or cattle, and they possess several characteristics that make them likely candidates to be porcine- and bovine-origin CAR bacillus isolate. Like rat and rabbit CAR bacillus isolates,<sup>2,7,22</sup> the pig and calf isolates are gram-negative, filamentous



**Figure 3.** Immunohistochemically stained trachea from a pig naturally infected with CAR bacillus. The primary antibody was serum from a guinea pig immunized against pig CAR bacillus isolate 96-1590. The positive-staining CAR bacilli (arrow) form a dense black lawn and outnumber the interspersed lightly staining cilia. Immunoperoxidase stain, hematoxylin counterstain.

bacteria that display gliding motility, form pin-cushion-like colonies, and do not grow on solid agar. Two pig isolates colonized the ciliated epithelium of the nasal cavities and trachea of gnotobiotic pigs for at least 47 days. In addition, serum from guinea pigs immunized with pig CAR bacillus was used to detect CAR bacillus in immunoperoxidase-stained sections of tissues from naturally infected pigs, which indicates that the bacteria are related immunologically to pig CAR bacillus.

In the gnotobiotic pig study, tracheal culture samples probably contained small numbers of CAR bacilli. Medium from cultures of CAR bacillus was examined by phase contrast microscopy before and after filtration, and only a small proportion of the bacteria passed through the filter (J. Nietfeld, personal observation). Transport medium from samples collected 29 days after inoculation was filtered with a 0.45- $\mu$ m pore diameter filter prior to inoculation of cell cultures. Samples collected 47 days after infection were inoculated onto IEC-18 cells prior to and after filtration. CAR bacillus was isolated from the day 47 postinoculation samples only when cell cultures were inoculated with unfiltered transport medium. If the culture samples collected at 29 days after inoculation had not been filtered, probably more than 1 of the 5 treated pigs would have been culture positive.

Elimination of mycoplasmal contamination from isolate PigCAR-1 was difficult, and PicCAR-1 was passed in vitro over 100 times before *Mycoplasma* spp. could no longer be detected. Because PigCAR-1 did not colonize rats or mice and, in a preliminary study, did not colonize 4 colostrum-deprived pigs, it was as-

sumed that in vitro passage had decreased virulence. Isolates 95-15405 and 96-1590, which had been passed less than 10 times, were therefore used for inoculation of gnotobiotic pigs.

Although the gnotobiotic pigs were culture positive for CAR bacillus, no histologic evidence of cilia loss or inflammation in the nasal cavities, tracheas, or lungs was observed. CAR bacillus-like bacteria were also difficult to identify in silver-stained histologic sections of trachea and nasal turbinates. Similarly, the 4 calves from which CAR bacillus was isolated were healthy and had no histologic evidence of inflammation or cilia loss in their tracheas, and CAR bacillus could not be identified positively in silver-stained tracheal sections. The sparse colonization, lack of clinical disease, and absence of pathologic changes in experimentally infected pigs and naturally infected healthy calves suggest that CAR bacillus is probably not a primary pathogen of these animals.

However, untested strains of pig CAR bacillus may be virulent. CAR bacillus is recognized as a cause of chronic respiratory disease of rats,<sup>7,13,14,21</sup> but isolates from rats differ considerably in virulence.<sup>21</sup> Two rat isolates induced chronic inflammation of the respiratory tract of 3 strains of rats, but 2 other rat isolates were avirulent.<sup>21</sup> One of the avirulent isolates was contaminated with *Mycoplasma pulmonis* and caused chronic respiratory disease in an earlier study.<sup>7</sup> Isolates of rabbit CAR bacillus also vary in virulence.<sup>4,11</sup> Rabbits intranasally infected with rabbit CAR bacillus developed a mucopurulent nasal discharge and lymphocytic tracheitis<sup>4</sup>; however, no clinical symptoms or histologic lesions of respiratory disease were observed in rabbits naturally infected with CAR bacillus.<sup>11</sup> Thus, pig and calf CAR bacillus isolates may also differ in their pathogenicity.

It is also possible that the number of viable bacteria given to the gnotobiotic pigs was sufficient to cause infection but not clinical disease. With rats and mice, approximately  $10^5$  CAR bacilli are required to establish progressive infection that results in lymphocytic rhinitis, tracheitis, and bronchitis (C. L. Franklin, personal observation). The dosage of CAR bacilli given to the gnotobiotic pigs was not determined, but based on the limiting dilution studies there were probably  $10^8$ – $10^9$  viable bacteria/ml of inoculum. Harvesting of the CAR bacilli was begun at 9:30 AM, the pigs were inoculated at 2:00 PM, and the bacterial inocula were cultured to test for viability at 7:00 PM. Normally, when CAR bacillus isolates are serially passed in cell culture, bacteria can be detected by examination of the culture flasks at 400 $\times$  after 24 hours of incubation. Bacterial growth in cells inoculated with the CAR bacillus used to inoculate the gnotobiotic pigs was not detectable until after 48 and 72 hours of incubation,

which indicates that the number of viable bacteria decreased between harvest and reinoculation of cell cultures. The number of viable bacteria may have decreased between harvest and inoculation of the pigs, but the extent of the decrease is not known.

Coinfection with *Mycoplasma* spp. may predispose the colonization of CAR bacillus in calves and pigs. All CAR bacillus isolates in the present study were contaminated with *Mycoplasma* spp., and *M. hyopneumoniae* is known to exacerbate pneumonia caused by other bacteria, such as *Pasteurella multocida*.<sup>19</sup> Mycoplasmal infections are common in pigs and calves, and simultaneous infection with the 2 organisms could easily occur. Recently, infection with *M. pulmonis* did not exacerbate clinical signs or pathologic changes in rats inoculated with virulent CAR bacillus; however, the *M. pulmonis* isolate used in that study was avirulent.<sup>21</sup> In the current study, mycoplasmal infection did not appear to exacerbate CAR bacillus infection of naturally infected calves, but it is unknown if the *Mycoplasma* sp. that infected the calves was virulent. Thus, the effects of virulent *Mycoplasma* spp. on CAR bacillus infection remain unknown.

Prior damage to the ciliated respiratory epithelium may be necessary for extensive colonization by pig CAR bacillus because all pigs from which CAR bacillus was isolated and all CAR bacillus-infected pigs in an earlier study<sup>17</sup> had tracheitis with loss of cilia, squamous metaplasia, and chronic inflammation. However, most pigs from which isolation of CAR bacillus was attempted and the pigs in the earlier study<sup>17</sup> that were not infected with CAR bacillus also had chronic inflammatory lesions that were indistinguishable from those of pigs naturally infected with CAR bacillus. Therefore, the effect of tracheal damage on CAR bacillus colonization is unknown.

The lack of colonization of mice and rats by pig CAR bacillus indicates that rodents are probably not reservoirs for this organism. This finding is consistent with results from other studies. Isolates from rats are avirulent in rabbits and guinea pigs and have reduced virulence in hamsters.<sup>2,4,15,23</sup> Similarly, rabbit isolates are avirulent for rodents.<sup>2,4</sup> Transmission is most likely by direct contact between infected and uninfected pigs. Rodent studies indicate that airborne transmission probably does not occur.<sup>15</sup> Rats housed on bedding previously used by CAR bacillus-infected rats remained free of CAR bacillus,<sup>5</sup> most likely because CAR bacillus is very susceptible to drying and dies out quickly if not kept moist (J. Nietfeld, personal observation).

Although they are morphologically indistinguishable in vivo and in vitro, CAR bacilli from different animal species may be distinct organisms. Sequence analyses of the 16S ribosomal RNA (rRNA) gene demonstrated that rat isolates are related most closely

to members of the genera *Flavobacterium* and *Flexibacter*<sup>3,22</sup> and that rabbit isolates are related most closely to members of the genus *Helicobacter*.<sup>4</sup> Preliminary results from a more recent study also indicated that rabbit CAR bacillus isolates are distinct from rat isolates, but based on 16S rRNA sequences, rabbit isolates were more closely related to *Flavobacterium* than to *Helicobacter*.<sup>21</sup> Immunohistochemical staining of CAR bacillus-infected tissues from pigs and goats using antisera to rat CAR bacillus and PCR testing of CAR bacillus-infected pig tracheas using primers specific for rat CAR bacillus gave negative results,<sup>6,17</sup> which indicates that pig and goat CAR bacilli are probably different bacteria than rat CAR bacillus. Studies to sequence the 16S rRNA genes of 4 pig and 2 calf isolates are in progress to help determine the phylogeny of these CAR bacilli. Preliminary results indicate that they are distinct from known species of bacteria, with their closest relative being rat CAR bacillus (C. L. Franklin, personal communication). These findings further support the conclusion that the bacterial isolates described in the present study are CAR bacillus of pigs and calves. Additional work is required to fully characterize CAR bacillus of different animal species and to determine the role of CAR bacillus as a respiratory pathogen.

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### Sources and manufacturers

- a. CRL 1589, American Tissue Culture Collection, Rockville, MD.
- b. Gibco BRL, Grand Island, NY.
- c. Atlanta Biologicals, Norcross, GA.
- d. Sigma Chemical Co., St. Louis, MO.
- e. Acrodisc, Low Protein Binding, Gelman Sciences, Ann Arbor, MI.
- f. Telazol, Ft. Dodge Laboratories, Ft. Dodge, IA.

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