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Prior exposure to *Bordetella* species as an exclusion criterion in the baboon model of pertussis

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ABSTRACT. The baboon model of *Bordetella pertussis* infection is the newest and most clinically accurate model of the human disease to date. However, among the 15 experimentally infected baboons in this study, a subset of baboons did not exhibit the expected high bacterial colonization levels or increase in white blood cell count. Moreover, cultures of nasopharyngeal wash samples from several baboons suggested *B. bronchiseptica* coinfection. Analysis of serum antibodies recognizing filamentous hemagglutinin, pertussis toxin and *B. pertussis* lipo-oligosaccharide indicated that several baboons had likely been previously exposed to *Bordetella* species and that prior exposure correlated with partial protection from *B. pertussis* infection. Notably, all animals with a baseline Fha titer of 5 IU/ml or below exhibited symptoms typical of the model, suggesting this value can be used as inclusion criteria for animals prior to study enrollment. While *B. pertussis* infection is endemic to human populations and *B. bronchiseptica* is common in wild small mammals, this study illustrates that baboons can readily harbor both organisms. Awareness of *Bordetella* species that share antigens capable of generating protective immune responses and tracking of prior exposure to those species is required for successful use of the baboon model of pertussis.

KEY WORDS: baboon, *Bordetella bronchiseptica, Bordetella pertussis*, filamentous hemagglutinin, whooping cough

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The resurgence of whooping cough infections in countries with high vaccination coverage has spurred renewed interest in pertussis pathogenesis, vaccination strategies and therapies. A major advance is the recent development of a baboon model that captures many clinical aspects of human pertussis [13]. Prior to this development, researchers relied on the mouse model of pertussis, which was used during development of the current acellular vaccines. Mice exhibit leukocytosis, the symptom most closely associated with disease severity, but do not cough, produce mucous or spread the disease via aerosols as in humans [4]. The baboon model appears to address many of these shortcomings: the animals cough, produce mucous, transmit disease through aerosols, are protected by commercial vaccines and exhibit leukocytosis [16]. Thus far, this model has helped to elucidate immune responses generated through infection versus vaccination [15], demonstrated that vaccinated baboons are able to asymptomatically spread disease [14], confirmed the effectiveness of maternal vaccination [17] and established that monoclonal antibody therapy effectively alleviates disease symptoms [9].

While *Bordetella pertussis* primarily infects humans, the related strains *B. parapertussis* and *B. bronchiseptica* also infect a variety of smaller mammals [8]. Boarded cats and dogs are subject to *B. bronchiseptica* infection, causing a disease commonly known as kennel cough. Laboratory animals, such as rabbits, guinea pigs and ferrets, have also been known to harbor the highly infectious pathogen [8]. All three strains share several key virulence factors, namely filamentous hemagglutinin (Fha), pertactin, adenylate cyclase toxin and dermonecrotic toxin [8]. Only *B. pertussis* expresses the pertussis toxin and generates a lipoligosaccharide (LOS) lacking the O-antigen repeats present in other strains while sharing core carbohydrates with *B. bronchiseptica* but not *B. parapertussis* [10].

Cross-protection resulting from infection with *Bordetella* species has been studied extensively in mice. In this model, immune

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responses against *B. pertussis* do not appear to protect against subsequent *B. parapertussis* infection, although *B. parapertussis* infection is able to protect against subsequent *B. pertussis* infection [19]. Co-infection with both organisms exacerbates the extent of *B. parapertussis* colonization [20]. Conversely, prior *B. pertussis* infection or vaccination protects against subsequent *B. bronchiseptica* infection [5]. Accordingly, researchers typically screen rodent and pig sera for reactivity to *B. bronchiseptica* antigens prior to use in pertussis experiments due to interference between antibody responses to the two organisms [3, 18].

As part of our on-going efforts to develop therapies to treat whooping cough, we performed a series of passive immunization experiments using the baboon model. Several animals exhibited atypical responses and *B. bronchiseptica* was definitively identified from the nasopharyngeal wash of one animal, while this organism was strongly suspected in these samples from three other animals. This is the first comprehensive analysis of spontaneous *B. bronchiseptica* infection of baboons and the corresponding partial protection against pertussis.

MATERIALS AND METHODS

Ethics statement

All animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with protocols approved by UT Austin (#2012-00084, #13080701) and the University of Oklahoma Health Sciences Center (#14-072-I) Animal Care and Use Committee and the principles outlined in the Guide for the Care and Use of Laboratory Animals.

Baboon challenge study

A total of 15 baboons between six and nine months old were infected intra-nasally and intra-tracheally with 10^9–10^{10} cfu *B. pertussis* strain D420 on day 0. Of these, eight animals were treated with human anti-PTx antibodies on day 2/3 (denoted T1-T8), while the remaining seven were untreated controls (denoted C1-C7). Serum and nasopharyngeal washes were collected from all animals every 3–4 days. Serial dilutions of nasopharyngeal washes were plated on selective Regan-Lowe agar plates supplemented with cephalexin (40 μg/m*l*). The specific details of procedures performed at the Oklahoma Baboon Research Resource at the University of Oklahoma Health Sciences Center have been described previously [9]. Microbial identification was performed by IDEXX (Westbrook, ME, U.S.A.) using MALDI-TOF for a preliminary identification based largely on 16S ribosomal RNA followed by a panel of standard microbiological tests, including Gram stain, lactose fermentation on MacConkey agar, urease and oxidase activity and the triple sugar iron test.

Detection of serum antibodies

ELISA was used to detect and quantify antibodies binding Fha, LOS and PTx antigens from *B. pertussis* (List Labs, Campbell, CA, U.S.A.) as described [9]. Curves were fit using a 4-parameter logistic model and compared to a reference monoclonal human antibody or high titer baboon serum run on each plate. The reference was standardized by comparison to the WHO Reference Reagent Pertussis Antiserum 06/142 (NIBSC, Potters Bar, Hertfordshire, U.K.), and serum antibody concentrations converted to IU/m*l*. Day 0 serum anti-Fha titers were log-transformed, and the values for baboons in the "typical" and "atypical" groups were found to be statistically different with a *P*-value of <0.001 in a T-test. For LOS ELISAs, plates were coated with 5 μg/m*l* LOS, with results reported as raw absorbance values in the dose-response regime at a serum dilution of 1:625. All ELISAs were performed in duplicate with standard deviations of the measurements reported as error.

RESULTS

During a series of studies designed to assess the potential for passive immunization to treat pertussis, several baboons had unexpectedly variable WBC and bacterial colonization levels 2-to-3 days after inoculation with *B. pertussis* (Fig. 1A). Six of 15 animals exhibited typical responses for this model: namely, a rapidly rising WBC and high bacterial colonization level on day 2/3. However, six animals had low WBC and colonization, one was heavily colonized but had a low WBC count, and two were not heavily colonized but had a two-to-four-fold WBC rise. Additionally, nasopharyngeal wash samples from four baboons (C2, C3, C4 and T5) yielded rapidly growing colonies apparent one day after plating on Regan-Lowe media selective for *Bordetella* species and overgrew *Bordetella pertussis* colonies appearing one to three days later.

Cultures of the rapidly growing bacteria from animal C4 were positively identified as *B. bronchiseptica*. MALDI-TOF was used to make a preliminary species identification, followed by a panel of biochemical tests to confirm the Bordetella assignment and discriminate between *B. bronchiseptica* and *B. pertussis*. The results showed that the cultured organism was Gram negative, did not ferment lactose on MacConkey agar, did not ferment lactose, sucrose or dextrose but catabolized peptone in the triple sugar iron test (K/K result) and was positive for oxidase and urease. Notably, *B. bronchiseptica* but not *B. pertussis* is urease-positive, and only *B. bronchiseptica* has flagella and is motile [6]. While not definitively confirmed, the other three animals were strongly suspected of *B. bronchiseptica* coinfection by virtue of similar culture characteristics. In all, nine of 15 baboons behaved aberrantly in this model (Table 1, Supplementary Table 1).

With compelling evidence of an active *B. bronchiseptica* infection in several animals, we sought to determine whether any other baboons in this study had been previously exposed to the organism. This question was addressed by examining antibody titers to Fha and LOS prior to infection and the kinetics by which those titers rose after infection. Specifically, high titers

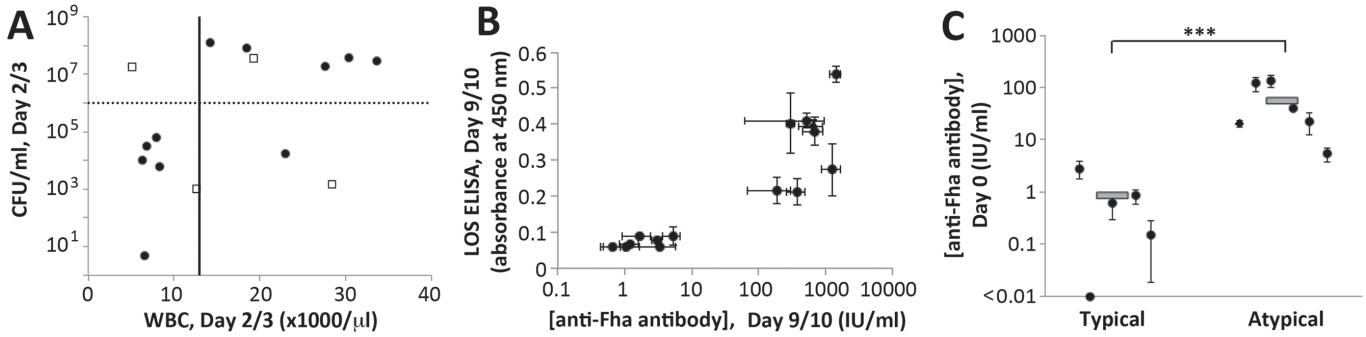

Fig. 1. Serum antibody levels indicate susceptibility to pertussis infection. A: WBC and *B. pertussis* colonization levels were measured on day 2 or 3 after experimental infection. Levels typical of the baboon model on day 2/3 are shown for WBC (>13,000/μl; solid line) and colonization (>10^6/m*l*, dashed line). Baboons exhibiting expected symptoms fell into the upper right quadrant, while baboons that appeared to be protected from disease fell into the lower left quadrant. Open squares indicate baboons with confirmed or suspected *B. bronchiseptica* co-infection. B: The day 9/10 LOS ELISA absorbance values correlated with the log-transformed day 9/10 Fha antibody responses with a Pearson correlation coefficient of 0.90. Animals exhibiting low values in both assays were classified as naïve, while animals exhibiting high values in both assays were classified as previously exposed. C: Anti-Fha antibody sera concentrations collected on Day 0 correlated with typical and atypical model responses; *** P<0.001 in a T-test of the log-transformed concentration values. Typical responses include high levels of colonization, a marked WBC rise and lack of a secondary antibody response. Shown are responses for animals without *B. bronchiseptica* co-infection; bars indicate geometric means.

before pertussis infection and a rapid rise after infection indicate a secondary immune response and strongly suggest prior *B. bronchiseptica* exposure. Prior experiments indicated that naïve baboons do not exhibit primary responses to Fha until after day 12, while secondary responses are detectable on day five [9, 13]. Thus, a high antibody titer on day 9/10 to Fha indicates prior exposure to any *Bordetella* species, while a similar response against LOS indicates prior exposure to either *B. bronchiseptica* or *B. pertussis* [1]. Detection of antibodies against PTx at day 9/10 was used to identify prior exposure to *B. pertussis* as opposed to *B. bronchiseptica,* which does not express this antigen due to mutations in the promoter.

Accordingly, serum titers against Fha and LOS on day 9/10 were determined for each baboon serum sample. Comparison of the resulting anti-Fha and anti-LOS serum titers revealed two distinct groups: seven baboons with low anti-Fha and low anti-LOS titers and eight baboons with high titers for both *Bordetella* antigens (Pearson correlation 0.90; Fig. 1B). Next, anti-Fha titers were determined for all time points, as these responses were stronger than those recognizing LOS. Animals with high anti-Fha titers on day 9/10 also exhibited high titers early in the experiment, and these remained high for the ~21 day experiment (Supplementary Fig. 1). Six of the seven baboons with no apparent secondary response had rising anti-Fha titers starting around day 16 or later, indicative of a primary response to the experimental infection. One baboon, C2, had no response at day 9/10, but a very strong response starting at day 13. Rapidly growing *Bordetella* colonies were recovered from this animal on day 9, suggestive of *B. bronchiseptica* co-infection.

Only the seven untreated baboons could be analyzed for the presence of anti-PTx IgG on day 9/10 due to interference from humanized anti-PTx antibodies administered to the treated baboons. One baboon, C5, was positive for anti-PTx antibodies as well as anti-Fha and anti-Bp LOS antibodies on day 9/10 (Supplementary Table 1, Supplementary Fig. 2). Interestingly, C5 had a colony count of 5 CFU/m*l* on day 2/3 and no WBC rise, indicating near complete protection from *B. pertussis* colonization. While we cannot exclude the possibility that this baboon was exposed to *B. bronchiseptica* as well, this result indicates that C5 had likely been previously exposed to *B. pertussis*.

We next aimed to develop a quantitative description of "typical" versus "atypical" baboon responses to pertussis infection. Published data [14, 16] indicate typical responses on day 2/3 include bacterial colonization >10^6 cfu/m*l*, an increase in WBC count to >13,000/μl and the absence of secondary immune responses to *Bordetella* antigens. Atypical responses do not meet one of more of these metrics (Table 1). Baboons with confirmed or suspected active *B. bronchiseptica* infection (C2, C3, C4 and T5) were excluded from this analysis to avoid potential confounding factors, such as competition with *B. pertussis*, unpredictable WBC rise and unpredictable timing of the secondary antibody response.

For future studies, a predictive test to exclude baboons likely to exhibit atypical responses to *B. pertussis* infection would be useful. Thus, the presence of serum anti-Fha antibodies on day 0 was evaluated as a potential screening tool. When baboons with active *B. bronchiseptica* infections were removed from analysis, anti-Fha antibody presence on day 0 was significantly predictive of the baboon's subsequent responses (P<0.001; Fig. 1C).

DISCUSSION

The baboon model of pertussis brings substantial improvements over previous models and has generated considerable enthusiasm due to its similarity to human disease. Unfortunately, the majority of baboons (nine of 15) in this study did not develop

Table 1. Baboon responses to experimental pertussis infection

Baboon	Active *B. br* infection [a]	Anti-Fha titer >100 IU/m*l* [b]	Anti-LOS absorbance >0.1 [b]	WBC <13,000/μl [c]	CFU <10^6/m*l* [c]	Response to Pertussis Infection [d]
C1	-	-	-	-	-	typical
C2	yes	-	-	-	-	excluded
C3	yes	-	-	yes	-	excluded
C4	yes*	yes	yes	yes	yes	excluded
C5	-	yes	yes	yes	yes	atypical
C6	-	-	-	-	-	typical
C7	-	-	-	-	-	typical
T1	-	yes	yes	yes	yes	atypical
T2	-	yes	yes	yes	yes	atypical
T3	-	yes	yes	yes	yes	atypical
T4	-	yes	yes	yes	yes	atypical
T5	yes	yes	yes	-	yes	excluded
T6	-	yes	yes	-	yes	atypical
T7	-	-	-	-	-	typical
T8	-	-	-	-	-	typical

a) *B. br*=*B. bronchiseptica*; -animal did not exhibit this behavior ; *=B. br infection identified by standard microbiological techniques. b) Anti-Fha and anti-LOS titers were measured in serum samples collected on day 9/10 after experimental pertussis infection. c) WBC and CFU values were collected from day 2/3 serum samples. d) Typical baboon responses to experimental pertussis infection are defined by WBC >13,000 /μl, CFU >106/m*l* on day 2/3 and low Fha and LOS serum titers; animals with known or suspected *B. bronchiseptica* infection were excluded from this classification.

symptoms as expected (Fig. 1A). In an effort to improve the utility of this model, we aimed to understand why some baboons varied in their response to *B. pertussis* infection.

Three baboons (C3, C4 and T5) were likely infected with *B. bronchiseptica* when the study began and were partially protected against *B. pertussis* infection. Prior studies in mice have shown that exposure to an engineered *B. pertussis* strain can protect against subsequent *B. bronchiseptica* infection [7]; conversely, an engineered *B. bronchiseptica* strain can protect against *B. pertussis* infection [12]. There are numerous overlapping antigens between these subspecies which are likely responsible for cross-protection. Specifically, anti-LOS and anti-pertactin antibodies are often bactericidal and can be generated through exposure to both *B. bronchiseptica* and *B. pertussis.* Importantly, they would be expected to have the substantial impact on subsequent *B. pertussis* infection levels and symptoms caused by infection observed in this study [18]. Additionally, baboon C2 was cohoused with *B. bronchiseptica* infected baboon C3 and appears to have been infected during the study, as this organism was not observed on plates until day 9.

An additional six animals were suspected of prior *B. bronchiseptica* or *B. pertussis* exposure, based on antibody profiles indicative of secondary responses after experimental *B. pertussis* infection (Fig. 1B). These animals all exhibited lower than expected colonization levels either with or without a suppressed WBC rise. While informative retrospectively, these data cannot be used to screen baboons prior to initiating an experiment. To this end, day 0 anti-Fha antibody concentrations were predictive of the baboons' subsequent response to infection, although these exhibited greater variability than day 9/10 titers (Fig. 1C). Because Fha is a shared antigen present in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, it is particularly useful as a simple screen to rule out confounding prior exposures for studies involving *B. pertussis* infection.

While prior *Bordetella* exposure complicates scientific studies, it further supports the baboon model's relevance to human disease. In humans, antibody levels against *Bordetella* antigens have been shown to inversely correlate with pertussis incidence [11]. Whooping cough in humans can be difficult to diagnose, because of widely varying infection severity and clinical manifestations which depend on the patient's prior exposures to *B. pertussis* and other microbes with overlapping antigens and timing of that exposure, exact age and level of general health [2]. These factors may play a similar role in the diversity of the baboon responses seen here and must be carefully controlled.

In comparison to other model animals used for pertussis, the environment baboons live in poses additional challenges. Baboons are typically housed with access to the outdoors where small rodents, such as rabbits or mice, for whom *B. bronchiseptica* is endemic could pass disease to the research animals. In our hands, commercial baboon serum (Sigma) had a relatively high anti-Fha antibody concentration of ~90 IU/m*l* (Supplementary Table 1), indicating that *Bordetella* infections are likely common in baboon colonies. The well-documented, highly communicable nature of both *B. pertussis* infection in baboons [14] and *B. bronchiseptica* infection in other mammals [6] requires extremely careful handling, containment and observation of baboons involved in pertussis studies. The University of Oklahoma Health Science Center is home to the only "Specific Pathogen Free" indoor baboon colony that was the source of baboons C7, T7 and T8. These three Specific Pathogen Free baboons responded to infection with *B. pertussis* as expected and were typical of the model. In our ongoing work, neonatal baboons from the Specific Pathogen Free colony are pre-screened for anti-FHA titer and consistently fall below 5 IU/m*l* at the time of infection and exhibit high colonization as expected (data not shown).

To maximize the impact of pertussis experiments in baboons, we recommend the following baboon exclusion criteria: (1) clinical

evidence of illness, (2) *B. bronchiseptica*-culture positive nasopharyngeal wash and (3) anti-Fha titers >5 IU/m*l* indicating prior exposure to *Bordetella sp.* With close attention to these factors, the baboon model of pertussis is poised to be an important new tool in development of next-generation pertussis vaccines and therapeutics.

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