



Published in final edited form as:

J Infect Dis. 2009 June 1; 199(11): 1671–1679. doi:10.1086/598966.

Experimental Infection of Human Volunteers with *Haemophilus ducreyi*: 15 Years of Clinical Data and Experience

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Abstract

Haemophilus ducreyi causes chancroid, which facilitates transmission of HIV-1. To better understand the biology of *H. ducreyi*, we developed a human inoculation model. Here, we describe the clinical outcomes of 267 volunteers who were infected with *H. ducreyi*. There was a relationship between papule formation and dose. The outcome (pustule formation or resolution) of infected sites within a subject was not independent; the most important determinants of pustule formation were gender and host. When infected a second time, subjects (n = 41) segregated towards their initial outcome, confirming the host effect. Subjects with pustules developed local symptoms, requiring termination from the study after a mean of 8.6 days. Hypertrophic scars developed in 16.2% of volunteers who were biopsied, but the model was otherwise safe. Mutant-parent trials confirmed key features in *H. ducreyi* pathogenesis, and the model has provided an opportunity to study differential human susceptibility to a bacterial infection.

Keywords

H. ducreyi; GUD; chancroid; human; human challenge model; host effect; pathogenesis

Haemophilus ducreyi causes chancroid, a sexually transmitted genital ulcer disease that is endemic in regions of Africa and Asia. Although the World Health Organization estimated the annual global prevalence of chancroid to be 4-6 million cases in the late 1990s, the epidemiology of the disease is not well characterized due to syndromic management and a lack of diagnostic testing [1,2]. Chancroid facilitates the transmission and acquisition of HIV-1 and is a public health concern [3].

H. ducreyi is a strict human pathogen that naturally infects genital and nongenital skin [4]. The bacteria presumably enter the skin by superficial abrasions that occur during intercourse [4]. Clinical disease is characterized initially by a painless papule that develops into a pustule at the site(s) of entry. Pustules erode into painful ulcers that prompt patients to seek medical attention 1-3 weeks after symptoms begin [2]. Purulent ulcers with ragged edges and suppurative lymphadenopathy typify the ulcerative stage [4].

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During the 1980s and 1990s, chancroid outbreaks occurred in New York City and New Orleans [5]. At the time of these outbreaks, data regarding the pathogenesis of *H. ducreyi* infection in humans were scarce [4,5] and findings from in vitro or animal models could not be correlated with human disease. Given the initial clinical course of natural disease, we reasoned that experimental infection up to the pustular stage would pose minimal risk to volunteers. Thus, a model of experimental infection with *H. ducreyi* in humans was developed to better define the pathogenesis of and host responses to the organism [6,7].

In this model, healthy adult volunteers are inoculated with *H. ducreyi* at multiple sites on the skin overlying the upper deltoid. Based on the delivery characteristics of the allergy testing device used for inoculation, the estimated delivered dose (EDD) is calculated by dividing the CFU loaded on the device by 1000 [8]. Papules develop within 24 hours, and either spontaneously resolve or evolve into pustules over the next 2-5 days. Volunteers remain infected until they reach clinical endpoint, which is defined as resolution of disease at all sites, development of a pustule that is either painful or > 4 mm in diameter, or 14 days after inoculation. At endpoint, all volunteers are treated with one dose of oral ciprofloxacin.

Within 24 hours of inoculation, fibrin and collagen are deposited in the wounds. PMN and macrophages traffic on the collagen and fibrin scaffolds, forming micropustules in the epidermis and dermis [9,10]. By 48 hours, PMN form an abscess that ulcerates through the epidermis [9,10]. Below the abscess is a dermal infiltrate of myeloid dendritic cells (DC), macrophages, memory and effector memory subsets of CD4 and CD8 T cells and activated NK cells [11-15] (unpublished data). The DC likely phagocytose *H. ducreyi* [14], migrate to regional nodes and sensitize naïve T cells to , as *H. ducreyi*-specific T cell lines can be propagated from pustules [16].

The histology of experimental pustules is nearly identical to natural ulcers [1]. In pustules, *H. ducreyi* are found in the abscess and dermis where they associate with collagen, fibrin, PMN and macrophages, which fail to ingest the organism [10]. *H. ducreyi* also colocalizes with fibrin and PMNs in natural ulcers [17]. Thus, evasion of phagocytosis and the bactericidal activity of serum that transudates into lesions are the major mechanisms of bacterial survival in experimental pustules and in natural disease.

Here, we report our cumulative 15-year experience with the experimental human infection model of *H. ducreyi*. We describe the clinical outcomes of the 267 volunteers who were infected with strain 35000 or its human passaged derivative, 35000HP. We describe the results obtained from 41 volunteers who were reinfected with 35000HP and of 20 mutant-parent comparison trials in the context of what is known about pathogenesis.

METHODS

Study Population

Between February 25, 1993 and December 31, 2007, we infected 267 volunteers (162 females, 105 males; 212 whites, 49 blacks, 6 Asians; age range 18 to 68 years; mean age \pm standard deviation, 33.7 \pm 10.3 years) at least once with *H. ducreyi*. Safety data, such as hypertrophic scar formation, are reported for the entire cohort. For analysis of initial infections, we included 220 subjects who were infected at 2 or 3 sites with the parent strain and who achieved clinical endpoint. If subjects participated in mutant-parent comparison trials, only sites inoculated with the parent strain were included in the analyses. Subjects (n=36) who participated in either dose-response [6] or chemoprophylaxis trials [18], or other time course studies [7,10,12], were excluded because of a short duration of infection (1-4 days). Subjects (n=11) who were HIV seropositive [15] were also excluded from the analysis.

Analysis of site independence

A total of 186 subjects, who received identical doses of the parent strain at both of two (n=106) or all of three (n=80) sites, were included in the analysis of site independence. Cumulative data for 90 of these subjects inoculated at two sites was reported previously [19]. The observed outcomes were compared to expected numbers under the assumption of site independence using chi square tests.

Papule and pustule formation rates

We estimated the odds of papule and pustule formation based on the EDD of 35000 and/or 35000HP, gender, race and age. For this analysis, we used logistic regression with generalized estimating equations (GEE) to predict papule and pustule formation rates. The GEE sandwich estimator for standard errors was used to calculate 95% confidence intervals for these rates.

Second Infections

Forty-one of the 220 subjects included in the analyses of initial infections were infected a second time [19,22] (unpublished data). For these 41 subjects, we estimated the odds of pustule formation based on their previous outcome using logistic regression as described above.

Mutant versus Parent Comparison Trials

To test the role of putative bacterial virulence factors in disease, we performed isogenic mutant-parent comparison trials [8,23-30]. These trials are double-blinded, multi-stage, dose ranging studies with at least 2 stages. The primary endpoint is the pustule formation rate. The first group of volunteers is inoculated at three sites with a fixed dose (x) of 35000HP on one arm and at three sites with varying doses (0.5 x, x, 2x) of an isogenic mutant on the other arm [23,24]. If the mutant and parent sites form pustules at similar rates, we inoculate a second group with similar doses. If pustules do not develop at sites inoculated with the mutant during the first iteration, the doses of the mutant are increased for each subsequent group until the EDD of the mutant is approximately 10-fold higher than that of the parent. Mutants are categorized as attenuated (unable to form pustules even at doses 10-fold that of the parent), partially attenuated (form pustules at doses 2- or 3-fold that of the parent, but not at doses equivalent to the parent) or virulent (form pustules at doses similar to the parent).

RESULTS

Outcomes of initial challenges

A total of 220 subjects (88 men, 132 women) contributed 538 sites (219 from men, 319 from women) to the analysis of papule formation. Of the 220, 192 subjects (76 men, 116 women) contributed 470 sites (192 from men, 278 from women) to the analysis of pustule formation.

Of the 192 subjects, 186 were infected with identical doses of 35000 or 35000HP at 2 or 3 sites, which were analyzed for independence. The expected number of people with 0, 1, 2 and 3 pustules (figure 1) was calculated based on the observed pustule formation rate and assumption of independence. A greater number of subjects than expected formed pustules at 0 or 2 sites for those inoculated at 2 sites ($P=0.001$) (table 1). Similarly, a greater number of subjects than expected formed pustules at 0 or 3 sites for those inoculated at a total of 3 sites ($P<0.001$) (table 1). Thus, the outcome of infected sites within a subject inoculated with identical doses of the parent strain was not independent, suggesting a host effect on outcome, as reported previously [19].

We calculated overall papule and pustule formation rates using logistic models and GEE to account for the within host effect on sites. Papules formed at 479 (89% [95% confidence

interval (CI, 85.7%-91.9%]) of 538 sites (figure 2). There was a significant effect of dose on papule formation ($P=0.0044$); the odds of papule formation increased by 2% per unit increase in EDD (figure 2). After adjusting for dose, we found no association between the likelihood of papule formation and either gender (figure 3) or race (data not shown).

A total of 470 sites were included in the calculation of pustule formation rates. The pustule formation rates for 85 sites inoculated with 35000 and 385 sites inoculated with 35000HP were not significantly different. Consequently, we analyzed results for the two strains together. Pustules formed at 256 of 470 sites for an overall pustule formation rate of 54.5% [95% CI, 48.7%-60.1%] (figure 2). There was no significant change in the odds of forming a pustule as the dose increased. When controlled for dose, men were significantly more likely than women to form pustules (OR 1.94; [95% CI, 1.23-3.09]; $P=0.0048$) (figure 3), consistent with previous reports [21]. There was no significant association between pustule formation and either race or age (data not shown). Thus, the most important determinants of pustule formation were host and gender.

Papules that progressed into pustules ($n=256$) did so within a mean \pm SD of 3.8 ± 2.2 days (range, 1-14 days). Papules that resolved ($n=156$) did so within 5.3 ± 2.6 days (range, 2-14 days). Only 7 pustules (2.7% [95% CI, 1.2%-6.2%]) reverted into papules and subsequently resolved without treatment. Thus, spontaneous resolution of papules is common, while resolution of pustules is uncommon in the model.

Of the 140 subjects who formed at least 1 pustule, 122 (87.1% [95% CI, 81.6%-92.7%]) developed pain or pruritis at the pustule site. Infection in these subjects was terminated due to symptoms after 8.6 ± 2.7 days. Only 18 subjects who developed pustules did not develop local symptoms and remained infected for 14 days.

Of the 192 volunteers, 139 (72.4% [95% CI, 66.1%-78.7%]) had at least 1 pustule at endpoint and were defined as a pustule former, while 51 (26.6% [95% CI, 20.3%-32.8%]) had all sites of infection resolve and were defined as resolvers. Two subjects had papules at 14 days and were not included in either category.

Outcomes of second challenges

Of the subjects who were initially classified as pustule formers or resolvers, 41 volunteered to be infected a second time [19,22] (unpublished observations). Of 15 (13 women, 2 men) resolvers challenged a second time, 7 (46.7%; 95% CI, 21.4% -71.9%) resolved all sites, 6 (40%; 95% CI, 15.2%-64.8%) formed at least 1 pustule, and 2 had papules at endpoint. Of 26 (16 women, 10 men) pustule formers challenged a second time, 23 (88.5% [95% CI, 76.2%-100%]) formed at least 1 pustule, 2 (7.7% [95% CI, 1.0%-25.1%]) resolved at all sites, and 1 had papules at endpoint. Adjusted for gender, a subject was more likely to be a resolver in the second infection if he/she was a resolver in the first infection ($P=0.017$). Similarly, a pustule former was more likely to form at least one pustule during a second infection if he/she was a pustule former during the first infection ($P=0.012$). The odds ratio of forming a pustule during the second infection for an initial pustule former was 3.42 [95% CI, 1.32-8.87] times that of an initial resolver (figure 4). Thus, subjects segregate towards their initial outcome when rechallenged.

Outcomes of sites inoculated with mutants

In isogenic mutant-parent comparison trials, subjects serve as their own controls for the host and gender effects. Two important themes emerge from the 20 trials published to date (table 2). First, the trials have validated the importance of the association of *H. ducreyi* with fibrin and collagen and evasion of phagocytosis [9,10]. Mutants that lack expression of OMPs

involved in serum resistance (DsrA and DltA), adherence to collagen (NcaA), fibrinogen binding (FgbA) or secreted proteins that are antiphagocytic (LspA1 and LspA2) are fully or partially attenuated in vivo [8,24-26,30]. In contrast, mutants whose gene products target host cells or compartments that do not have major roles in pathogenesis in vivo are dispensable for virulence [8]. For example, mutants that do not make paragloboside-like lipooligosaccharide (LOS), hemolysin and cytolethal distending toxin, which primarily target adherence to and invasion of keratinocytes or killing of keratinocytes and fibroblasts, are virulent [31-33]. A mutant in superoxide dismutase C, which promotes survival in phagolysosomes, a compartment that *H. ducreyi* evades in vivo, is virulent [34].

Second, the trials have helped identify vaccine candidates. Although some gram negative organisms contain as many as 40 TonB-dependent receptors, *H. ducreyi* contains only the hemoglobin receptor (HgbA), the heme receptor (TdhA), and TdX, whose function is not known [28]. A HgbA mutant is attenuated for pustule formation in the model, while a TdhA and TdX mutant is virulent [28,35]. Thus, HgbA is both necessary and sufficient for heme/iron acquisition by *H. ducreyi* in humans. HgbA is conserved in *H. ducreyi* strains and does not undergo phase or antigenic variation. Immunization with purified HgbA elicits protective antibody-mediated immunity in the swine model [28,36]. The results of these trials strengthen the idea that HgbA may be an excellent vaccine candidate in humans.

Risks and Adverse Events

We typically infect groups of subjects with a common inoculum. In 128 iterations, two groups (6 subjects) were infected with inocula that contained low levels of fungi or bacteria other than *H. ducreyi*. The subjects developed papules, were treated with ciprofloxacin once the contaminants were detected, and had no adverse outcomes. No subject developed fever or systemic symptoms due to experimental infection. Secondary transmission of *H. ducreyi* from a volunteer to another person has not occurred in 2,123 subject-days of infection.

Although we exclude persons who form keloids and hypertrophic scars, some subjects developed hypertrophic scars at sites of infection that were biopsied. Hypertrophic scars have not formed at infected sites that were not biopsied or uninfected sites that were biopsied. Subjects were followed for six months post-inoculation. Of 191 volunteers who were biopsied, 18 were lost to follow-up. Of the 173 remaining subjects, 21 women and 7 men (16.2% [95% CI, 10.7%-21.7%]) developed hypertrophic scars. More women (21.7% [95% CI 13.4-29.8%]) than men (9.2% [95% CI, 2.7%-15.7%]) developed hypertrophic scars ($P=0.028$). Of 31 African Americans, 12 (38.7% [95% CI, 21.6%-55.9%]) developed scars, while 14 of 140 Whites (10% [95% CI, 5%-15%]) developed scars ($P\leq 0.0001$). Both Asians who participated in the study developed hypertrophic scars. All subjects who developed hypertrophic scars received an intradermal injection of triamcinolone, and all scars became flat within 2 months.

DISCUSSION

If done under carefully regulated conditions, experimental infection of humans with *H. ducreyi* is safe, with minimal risk. No subject developed fever, lymphadenopathy or disseminated lesions. Most volunteers achieved clinical endpoint prior to 14 days because of local symptoms. Hypertrophic scars developed at infected sites that were biopsied in 16% of subjects. Although we did not assess lesional TGF- β levels in volunteers who formed hypertrophic scars, TGF- β transcripts are readily amplified from pustules (unpublished data), and expression of TGF- β may contribute to hypertrophic scar formation by enhancing localized production of collagen and fibronectin [37].

In the limited dose range employed in the model, the odds of forming a papule was dose dependent, while the odds of forming a pustule was not dose-dependent. The latter result differs

from analyses reported previously, in which site outcomes within a subject were assumed to be independent [8,20,21]. However, the outcome of multiple infected sites within a subject was not independent, indicating a host effect [19]. When reinfected, subjects segregated towards their initial outcome (pustule formation or resolution). The mechanism of disease resolution is unknown, but is likely due to enhanced phagocytic clearance in those subjects who resolve. Some naturally infected patients have spontaneous clearance of ulcers [2], while others have multiple recurrences of ulcers [38]. Taken together, the data suggest that some persons are prone to clearing infection while others are prone to disease progression, and that protective immunity does not develop in those who progress to pustules or ulcers [19,22].

The mechanisms of disease progression versus resolution may be determined in part by the microenvironment at the infected site. Gene expression profiles of infected sites from volunteers who were infected a third time after resolving infection twice are consistent with an effective immune response [39]. In contrast, profiles indicative of a hyperinflammatory, dysregulated immune response are present in persons infected a third time after developing pustules twice [39]. Monocyte-derived myeloid DC obtained from the volunteers who resolved twice expressed transcripts that should promote Th1 and Th17 responses in response to *H. ducreyi*, while DC from the volunteers who formed pustules twice express transcripts consistent with Th1 and regulatory T cell responses [39]. Th1 and Th17 responses may lead to a cytokine environment that promotes phagocytosis, while a combined Th1 and regulatory response may inhibit phagocytosis [39]. Alternatively, DC from the resolver group might direct NK cells to make an appropriate amount of IFN γ during early stages of infection, which augments phagocytosis.

Men and women formed papules, or became infected, at equal rates; however, men were two times more likely to form pustules. The male to female ratio of natural chancroid in endemic areas is 3:1, which likely reflects gender differences in susceptibility to disease progression [4]. In vitro, *H. ducreyi* dies at temperatures above 35°C. We were unable to assess whether gender differences in susceptibility were associated with elevated body temperatures at the time of ovulation in women because menstrual cycles of female participants were not assessed in detail [21]. Alternatively, the gender effect may reflect the fact that women are more prone to proinflammatory than tolerizing responses compared to men [40]. If women are less prone to tolerizing responses than men, and a tolerizing response promotes phagocytic failure, women should be less susceptible to pustule formation than men.

H. ducreyi is more closely related to the animal pathogens *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae* than human pathogens within the *Pasteurellaceae* [41]. Rabbit, primate, swine and murine models of chancroid were developed; the infectious doses in these models range from 10⁴ to 10⁷ CFU [2,8]. Murine lesions are due to the content of LOS in the inoculum, not viable bacteria. Bacteria are cleared over time in swine [34], and lesions in rabbits also spontaneously clear. In rabbits and swine, 1 to 2 weeks of infection evoke serum antibody responses and provide protection against repeated exposure; bactericidal activity develops in repeatedly infected swine [42,43]. The animal models do not simulate human disease sufficiently to study pathogenesis or host responses, reflecting the fact that *H. ducreyi* diverged from other pathogens to its unique niche in the human host [41].

In summary, experimental infection with *H. ducreyi* clinically and histologically mirrors natural infection and provides an excellent model for studying the pathogenesis of the infection. Since *H. ducreyi* naturally infects nongenital skin and causes a chronic limb ulceration syndrome [44,45], infection of the arm is not a major limitation of the model. Although our model is limited to the pustular stage of disease, the relationships between *H. ducreyi* and human cells in the model are maintained in natural ulcers [17], suggesting that the duration of infection is also a minor limitation. The model has significantly contributed to the

understanding of *H. ducreyi* pathogenesis and led to the discovery of immune mechanisms that may underlie differential human susceptibility to *H. ducreyi* infection.

Acknowledgments

This work was supported by grants AI31494 and AI27863 to S.M.S. and AI074657 to D.M.J. from the National Institutes of Allergy and Infectious Diseases (NIAID). The human challenge trials were supported by grant MO1RR00750 to the GCRC at Indiana University. All authors have no relevant financial relationships to disclose. We thank Kate Fortney and Stan Taylor for their assistance with the database as well as Byron Batteiger and Barbara Van Der Pol for their thoughtful critiques of the manuscript. We thank our many colleagues and collaborators and the volunteers who have participated in human challenge trials.

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Figure 1.
Outcomes of volunteers who were infected at three sites with 35000HP and developed 0, 1, 2 or 3 pustules.

Rates and 95% Confidence Limits

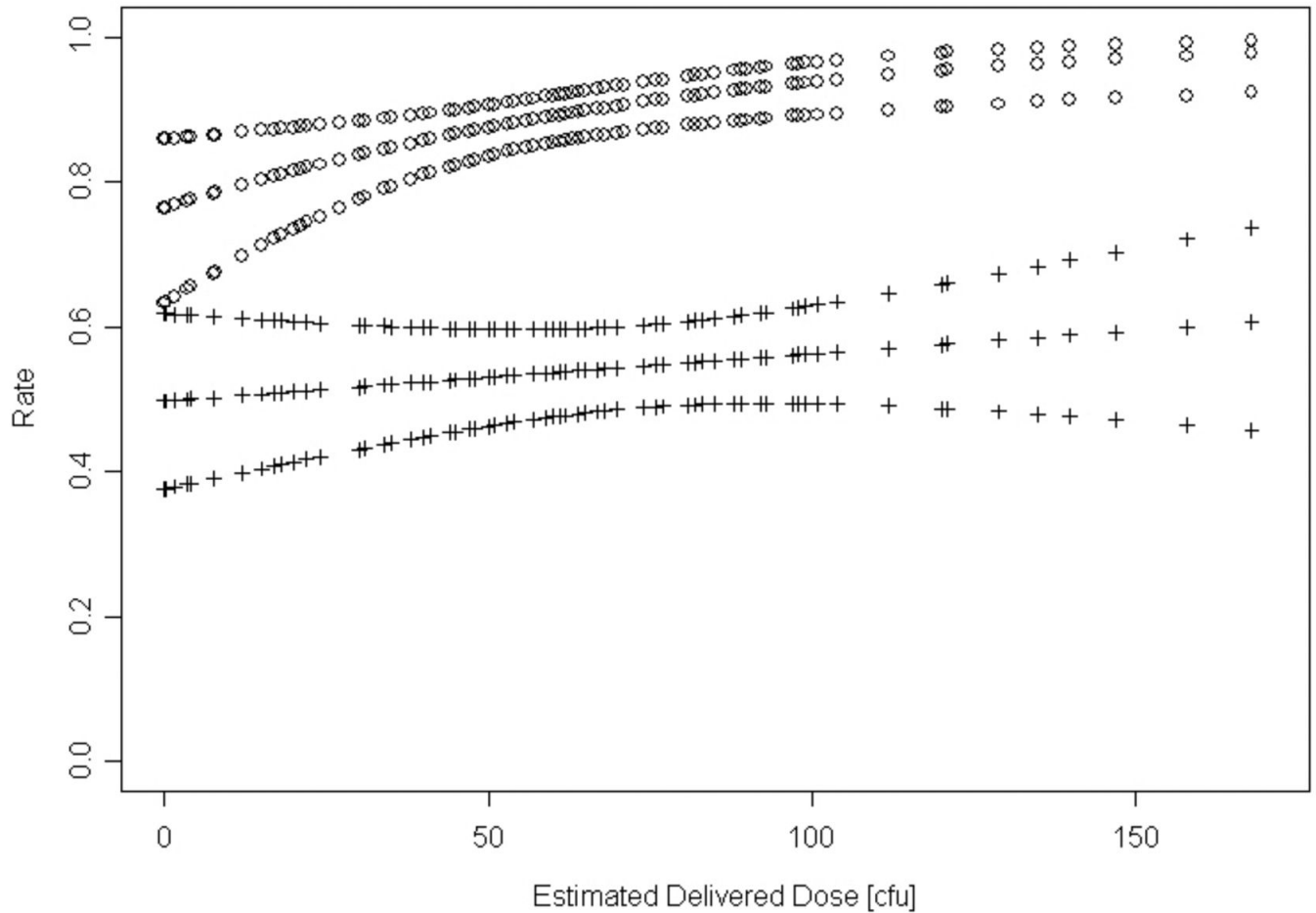


Figure 2. The relationship of estimated delivered dose and overall probabilities (with 95% CI) of papule (open circles) and pustule (+) formation, as predicted by logistic regression.

Papule and Pustule Formation Rates

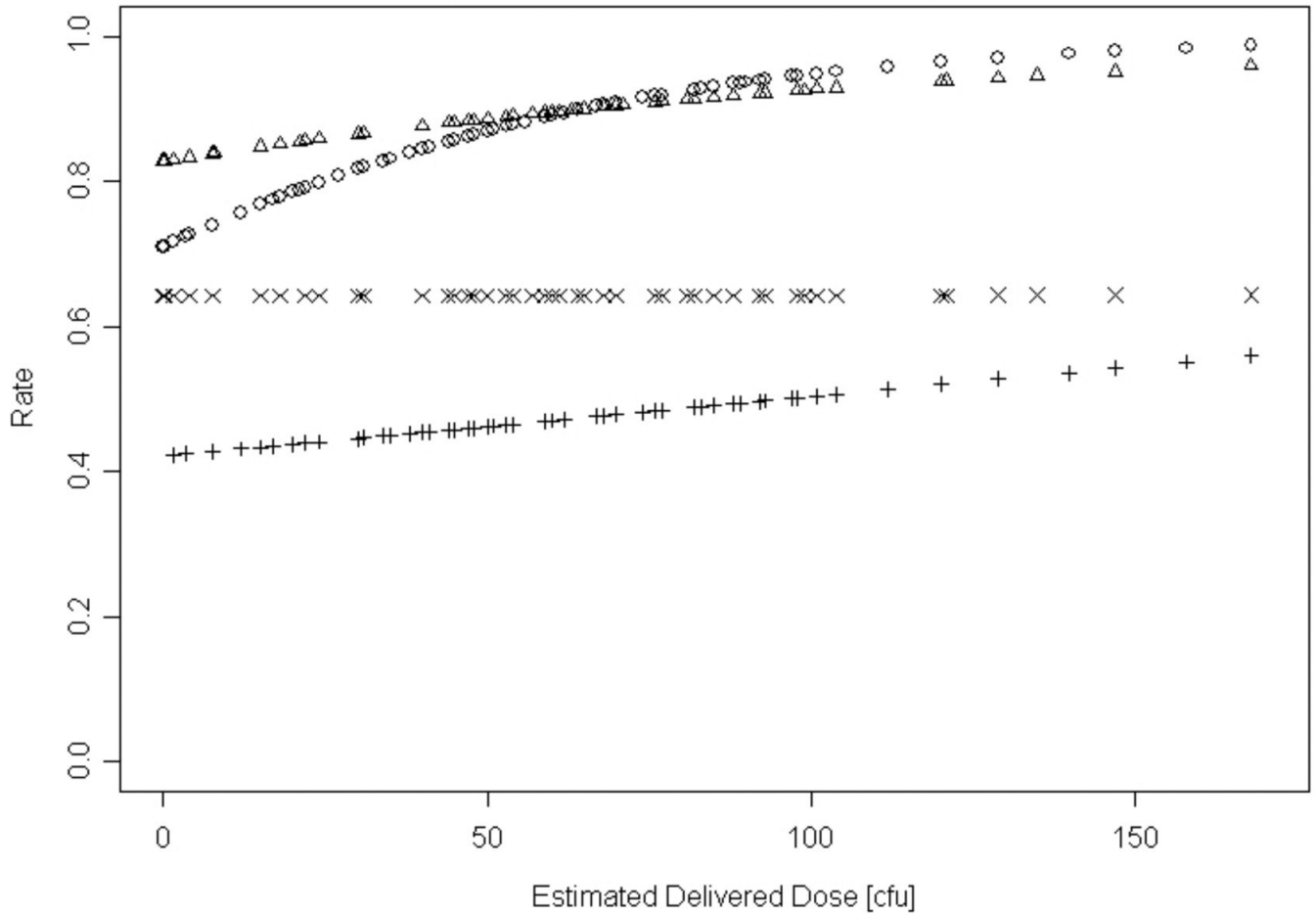


Figure 3. Papule formation rates for females (circle) and males (triangle) and pustule formation rates for females (+) and males (x) related to estimated delivered doses as predicted by logistic regression. For simplicity, the 95% CI are not shown.

Pustule Formation Rates of Second Infection

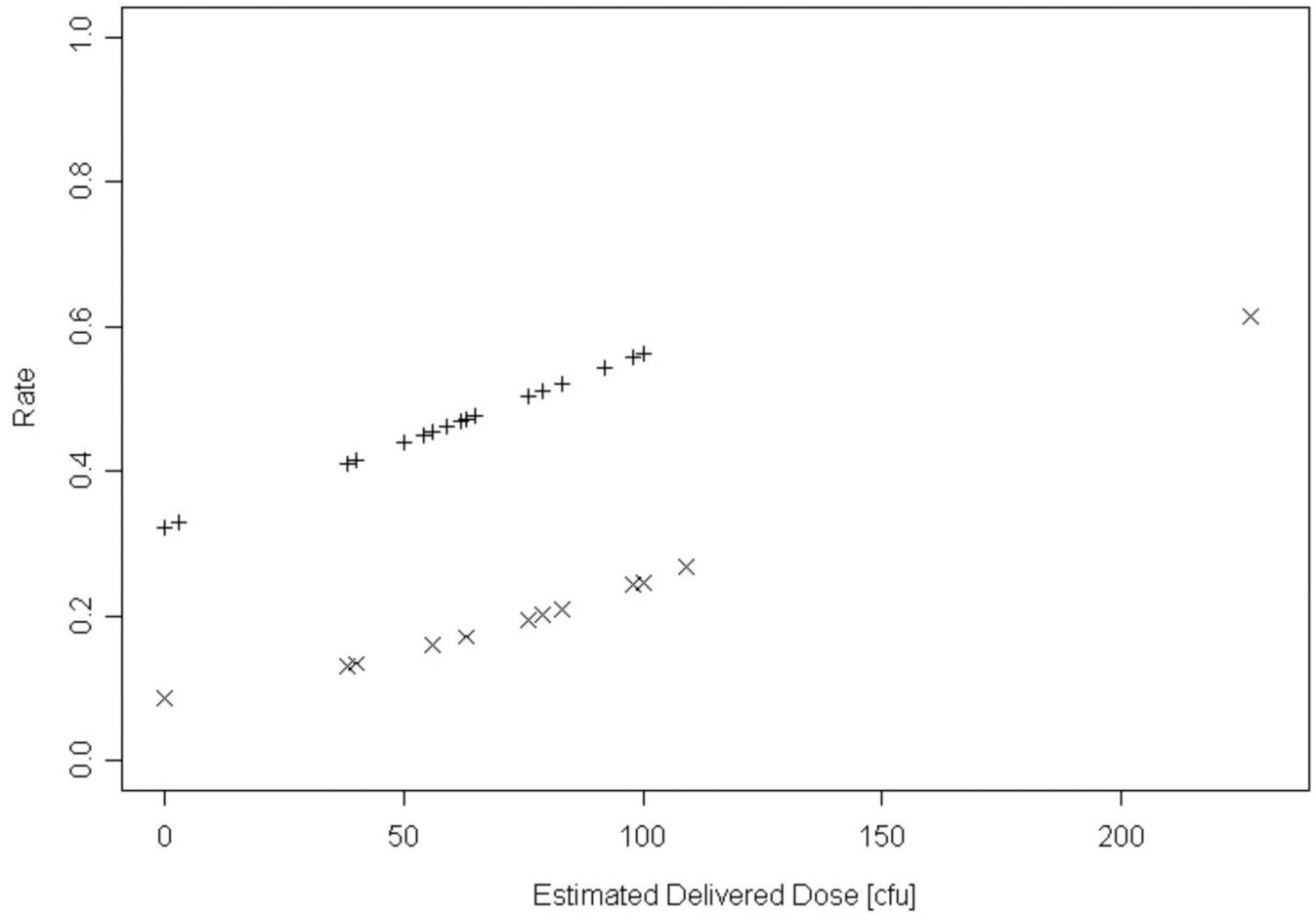


Figure 4. The likelihood of forming a pustule on the second infection for volunteers who formed pustules (+) or who resolved (x) during their first infections.

Table 1
Observed vs. expected number of people forming 0, 1, 2, or 3 pustules.

No. of pustules	Subjects Inoculated at 2 sites			Subjects Inoculated at 3 sites		
	No. of subjects observed	No. of subjects expected	No. of pustules	No. of subjects observed	No. of subjects expected	No. of subjects expected
0	33	22	0	18	7	7
1	31	53	1	19	27	27
2	42	31	2	16	33	33
3			3	27	13	13
Total	106	106	Total	80		80

NOTE: Expected numbers based on the observed overall pustule formation rate and the assumption of site independence.

Table 2

H. ducreyi mutants tested in the human challenge model.

Source	Gene	Definition	Function	No. of Subjects	Results [ref.]
Elkins	<i>dstrA</i>	ducreyi serum resistance A	OMP; major role in serum resistance	6	Attenuated [46]
Kawula	<i>ncaA</i>	necessary for collagen adherence	OMP; confers collagen binding	10	Attenuated [26]
Hansen	<i>lspA1</i> <i>lspA2</i>	double mutant, large supernatant proteins	secreted proteins; prevent Fc-g mediated phagocytosis	6	Attenuated [24]
Spinola	<i>pal</i>	peptidoglycan associated lipoprotein	lipoprotein; OM stability	9	Attenuated [47]
Hansen	<i>tada</i>	tight adherence protein A	type IV secretion; secretion of Flp proteins	15	Attenuated [23]
Bauer Janowicz	<i>fgbA</i>	putative fibrinogen binding adhesin	OMP that binds to fibrinogen in ligand blot	6	Partially Attenuated [30]
Elkins	<i>dlta</i>	ducreyi lectin A	OMP; partial serum resistance	7	Partially Attenuated [25]
Munson	<i>hhdB</i>	secretion/activation of hemolysin	lyses fibroblasts	6	Virulent [32]
Munson	<i>losB</i>	D-glycero-D-manno-heptosyltransferase	extends LOS beyond KDO-triheptose-glucose	5	Virulent [31]
Munson	<i>lst</i>	lipooligosaccharide sialyltransferase	adds sialic acid to major glycoform of LOS	5	Virulent [31]
Campagnari Munson	<i>glu</i>	glucosyltransferase gene	adds glucose to KDO-triheptose LOS core	5	Virulent [48]
Hansen	<i>cdtC</i>	cytolethal distending toxin	toxic for T cells, epithelial cells, fibroblasts	6	Virulent [33]
Hansen Munson	<i>cdtC</i> <i>hhdB</i>	double mutant, cytolethal distending toxin, hemolysin	see above	5	Virulent [33]
Kawula	<i>sodC</i>	superoxide dismutase C	detoxifies ROS for bacteria in phagolysosomes	6	Virulent [34]
Spinola Hansen	<i>momp</i>	major outer membrane protein	OmpA homologue; minor role in fibronectin binding	6	Virulent [49]
Campagnari	<i>ompP2A</i> <i>ompP2B</i>	double mutant, porin proteins	encode known classical trimeric porins	8	Virulent [27]
Spinola	<i>ftpA</i>	fine tangled pilus major subunit	unknown	7	Virulent [50]
Elkins	<i>hgbA</i>	hemoglobin binding outer membrane protein	heme/iron acquisition and transport from hemoglobin	9	Attenuated [35]

Source	Gene	Definition	Function	No. of Subjects	Results [ref.]
Elkins	<i>tdx/tdhA</i>	double mutant; ton B dependent receptors	heme uptake	6	Virulent [28]
Munson	<i>wecA</i>	first enzyme in the ECA biosynthetic pathway	initiates synthesis of putative ECA glycoconjugate	5	Partially Attenuated [29]