VACCINE IDENTIFIED BY IN VIVO GENOMIC SCREENING ENHANCES FERTILITY IN CATTLE DURING ENVIRONMENTAL CHALLENGE WITH *CHLAMYDIA*

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Introduction

Infection with *Chlamydia psittaci* is ubiquitous in cattle, causing metritis, infertility, and abortion, diseases that are of major economic importance to the dairy and beef cattle industries (1-5). Because of a lack of efficacious vaccines available for use in cattle and because conventional approaches to produce vaccines against *Chlamydia* have at best produced mixed results, we elected to develop a novel vaccine against *Chlamydia* using expression library immunization (ELI) (6,7). This approach was used to screen the entire genome of *C. psittaci* for protective antigens, using a mouse model of *C. psittaci* induced lung disease. This earlier study identified nine protective gene fragments (Stemke-Hale, et al., submitted for publication).

The five most protective gene fragments from this previous study, were selected for use in this study, as genetic and recombinant protein vaccines. This set of five genes included $dnaX_2$ (the *Chlamydia* DNA polymerase III subunit), gatA (Glu-tRNA Gln amido-transferase subunit A), gatC (Glu-tRNA Gln amido-transferase subunit C), pmp5 (*C. psittaci* outer membrane protein 90A), and pbp3 (transglycolase/ transpeptidase).

The purpose of this study was to extend the previous *in vivo* mouse work to cattle, the biologically and economically relevant natural host. To accomplish this, a natural infection model was used in which challenged cohort animals transmitted *Chlamydia* to, and induced infertility in, the experimental herd mates (5). The experimental herd mates were used to evaluate genetic and recombinant protein vaccines against *Chlamydia*, compared to mock vaccinated controls. We report here a 64% increase in fertility with vaccination against *Chlamydia*.

Materials and Methods

Animals – Normal Holstein heifers, 300-400 kg body weight, 13-17 months of age, n=51. Heifers were housed on pasture with access to hay and water. Heifers were fed a grain-based supplement two times per day.

Serology – Heifers were tested by ELISA for antibodies against an immuno-dominant peptide of the major outer membrane protein of *C. psittaci* strain B577, the ruminant abortion strain originally isolated from ovine abortion (4).

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Detection of *Chlamydia* – Vaginal cytobrush samples were collected from all heifers at weekly intervals. Cytobrushes were inserted 18 cm into the vagina, continuously rotated while sweeping a 5 cm line of mucosal surface. The brush was then placed into a vial containing 400µl stabilization reagent (RNA/DNA Stabilization Reagent for Blood/Bone Marrow, Roche Molecular Biochemicals, Indianapolis, IN, USA) and mixed by rotating rapidly for 5 seconds. Vials were stored at -85°C. DNA was extracted from samples by glass matrix binding and elution (High Pure PCR Template Preparation Kit, Roche Molecular Biochemicals, Indianapolis, IN, USA). *C. psittaci* B577 genomes were detected in extracted samples by *Chlamydia* 23S rRNA fluorescence resonance energy transfer (FRET), using real time quantitative PCR methods as described previously (8).

Vaccines – The Genetic Vaccine against *Chlamydia* was composed of a pool of plasmids that contained the mammalianized full ORFs of the five most protective *C. psittaci* strain B577 genes, previously identified in a mouse model of *C. psittaci* strain B577 induced lung disease (Stemke-Hale, et al., submitted for publication). The Protein Vaccine against *Chlamydia* was an Alum-Quil A based vaccine that contained 50 µg per 2 ml dose of each of the affinity-purified full-length proteins expressed in *E. coli*, using the same genes that were incorporated into the Genetic Vaccine. The Control Vaccine was a genetic vaccine that contained plasmids that expressed a bacterial gene unrelated to *Chlamydia*.

Administration of vaccines – Heifers (n=24) were randomly assigned to one of three groups, after blocking for body weight: Genetic Vaccine (n=6), Protein Vaccine (n=6), and Control Vaccine (n=12). Genetic Vaccine and Control Vaccine were delivered with a gene gun (6.2 µg per dose, five doses to the inner skin of one pinna and five doses to the vaginal mucosal surface). The Protein Vaccine was delivered as a 2 ml deep intramuscular injection in the semimembranosus muscle. The vaccination protocols were administered twice, six weeks apart.

Chlamydia challenge – 10^8 IFU *C. psittaci* strain B577 were loaded into 0.5ml insemination straws, slowly frozen to -85°C and stored in liquid nitrogen. Heifers were challenged 7-14 hours after the initiation of standing estrus, eight weeks after the second round of vaccinations. Challenge straws were thawed in a water bath at 35°C for 45 seconds and immediately inoculated 1 cm into the uterine body. Twenty-seven heifers were challenged with *Chlamydia*. The sole purpose of this group of heifers was to provide increased environmental contamination with *Chlamydia* throughout the environment of the remaining 24 heifers that had been vaccinated.

Detection of estrus – Monitoring for estrus was continuous, using a computerized estrus detection system (HeatWatch® system, DDx, Denver, CO, USA).

Breeding of heifers – Estrus was synchronized with two injections of dinoprost tromethamine (25 mg intramuscular, Lutalyse®, The Upjohn Co., Kalamazoo, MI, USA), 14 days apart. The second round of synchronization was scheduled two weeks after the inoculation of the challenge heifers with *Chlamydia*. This time point was selected from previous studies (DeGraves, et al., unpublished data) that determined this to be the point of maximum shedding, following inoculation with *Chlamydia*. The heifers were bred 7 to 14 hours after first standing heat and after standing for a

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minimum of ten mounts. All heifers were bred with semen from a single bull, which was listed as a superior settler (Marshall, Select Sires, Franklin, TN, USA). Semen was stored in insemination straws in liquid nitrogen, thawed in a water bath at 35°C for 45 seconds and immediately inoculated 1 cm into the uterine body.

Pregnancy testing – Pregnancy was determined by manual rectal palpation at 42 days post breeding.

This study was conducted with the approval of the Auburn University Institutional Animal Use and Care Committee.

Results

All fifty-one heifers had high titers for antibodies against *C. psittaci* strain B577, prior to challenge with *Chlamydia*, indicating relatively recent or active infection. In addition, low levels of *C. psittaci* strain B577 were detected in nine of fifty-one heifers (18%), prior to challenge.

Fertility results for this experiment are listed in Table 1. Six of the twelve heifers (50%) that received Control Vaccine were pregnant, while four of the five heifers (80%) that received Genetic Vaccine were pregnant, and five of the six heifers (83%) that received Protein Vaccine were pregnant. The Odds Ratio for improvement of fertility with vaccination against *Chlamydia* was 4.5 (0.67-30.2, 95% confidence interval). Only five heifers are listed in the Genetic Vaccine group, as one heifer was excluded due to a lack of estrus.

Table 1 – Number of pregnancies (%) by vaccine treatment, 42 days post breeding.

Vaccine Group	Pregnant	Not pregnant
Control Vaccine	6 (50%)	6 (50%)
Genetic Vaccine	4 (80%)	1 (20%)
Protein Vaccine	5 (83%)	1 (17%)

Table 2 – *Chlamydia* genomes detected by quantitative *Chlamydia* 23S rRNA FRET PCR. Values are total number of *Chlamydia* genomes detected per treatment group. Cytobrush samples were collected at weekly intervals, one cytobrush sample per heifer. Values in parenthesis are the number of heifers positive for *Chlamydia* genomes. Placebo Vaccine group, n=12; Genetic Vaccine group, n=6; Protein Vaccine group, n=6; Challenge group, n=27. Blank cells indicate sampling points for which *Chlamydia* genomes were not detected; there were no missing data points.

	week post *Chlamydia* challenge											
Group	-2.5	-1.5	-0.5	0.5	1.5	2.5	3.5	4.5	5.5	6.5	7.5	9.5
control vaccine	8 (1)		8 (1)	112 (1)		8 (1)		56 (5)		112 (6)		
genetic vaccine					168 (1)	8 (1)		8 (1)		32 (4)		
protein vaccine			8 (1)					112 (5)		72 (5)		
challenge heifers	24 (3)		32 (3)	$5x10^4$ (19)	10^5 (17)	$3x10^5$ (23)	$9x10^3$ (19)	$2x10^3$ (13)	$6x10^3$ (8)	312 (13)	400 (7)	

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Chlamydia genomes detected at weekly intervals by treatment group, are listed in Table 2. Peak shedding of *Chlamydia* in challenge heifers was detected 2.5 weeks after intrauterine *Chlamydia* inoculations were initiated. Peak shedding was detected in vaccinated heifers 4.5 and 6.5 weeks after challenge heifers were inoculated with *Chlamydia*. No *Chlamydia* was detected in any heifers, 9.5 weeks after challenge heifers were inoculated. Shedding of *Chlamydia* did not appear to be influenced by vaccine treatment.

Discussion

In this experiment, vaccination against *Chlamydia* reconstituted fertility back to what is naturally achievable for healthy cattle with proper nutrition. Similarly, for heifers that received mock vaccine, infertility was remarkably similar to what is frequently observed under natural conditions in cattle herds with poor fertility. In this study, *C. psittaci* genomes were detected in a high proportion of virgin heifers, prior to challenge with *Chlamydia*. This data suggests that *C. psittaci* strain B577 is likely to be ubiquitous in cattle, which is strongly supported by serological data of this and previous studies (4). When combined with the fact that *C. psittaci* is capable of causing infertility at relatively low doses (3), and that environmental contamination with *C. psittaci* may be associated with infertility in cattle (5), infection with *Chlamydia* is likely to be an economically important cause of infertility in cattle.

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