AWARD NUMBER: W81XWH-21-1-0967

TITLE: Development of an Attenuated Vaccine for the Prevention of Lyme Disease

PRINCIPAL INVESTIGATOR: Ronald Mark Wooten

CONTRACTING ORGANIZATION: University of Toledo Health Science Campus

REPORT DATE: October 2022

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PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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4. TITLE AND SUBTITLE

Development of an Attenuated Vaccine for the Prevention of Lyme Disease

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5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Ronald Wooten

E-Mail:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Toledo Health Science Campus

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

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12. DISTRIBUTION / AVAILABILITY STATEMENT

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Borrelia burgdorferi (Bb) is the causative agent of Lyme disease, which is the leading vector-borne disease in North America and temperate regions across the northern hemisphere. Due to its rapid increase world-wide throughout the Northern hemisphere, there is great interest in developing preventative vaccines against Lyme disease. For many pathogenic microbes, the use of mutant strains that are attenuated for virulence have proved to be highly effective for generating protective immunity. This is because those strains can survive within a host long-enough to express virulence proteins that are critical for host survival, but not long enough to cause clinical disease while eliciting B and T cell responses against virulence-relevant antigens. Recently, our group has identified a Bb mutant was cleared from the host without developing Lyme disease, but the infection was sufficiently long to elicit significant levels of Bb-specific Abs, a substantial number of which were against antigens that are only expressed within a vertebrate host. Importantly, immunization of mice with this attenuated strain was able to protect 100% of the mice challenged with 10x the ID50 from this Bb strain, meaning that it possesses all the properties to act as an attenuated vaccine. The experiments outlined in this project intend to test the abilities of this attenuated vaccine to elicit antibodies capable of protecting mice against challenge with Bb via tick or syringe inoculation up to 1 year post-immunization, thus preventing the development of Lyme disease. The results of this study could lead to the development of a greatly needed vaccine to protect against Lyme disease.

15. SUBJECT TERMS

NONE LISTED

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Borrelia burgdorferi (Bb) is the causative agent of Lyme disease, which is the leading vector-borne disease in North America and temperate regions across the northern hemisphere. Due to its rapid increase world-wide throughout the Northern hemisphere, there is great interest in developing preventative vaccines against Lyme disease. For many pathogenic microbes, the use of mutant strains that are attenuated for virulence have proved to be highly effective for generating protective immunity. This is because those strains can survive within a host long-enough to express virulence proteins that are critical for host survival, but not long enough to cause clinical disease while eliciting B and T cell responses against virulence-relevant antigens. Recently, our group has identified a Bb mutant was cleared from the host without developing Lyme disease, but the infection was sufficiently long to elicit significant levels of Bb-specific Abs, a substantial number of which were against antigens that are only expressed within a vertebrate host. Importantly, immunization of mice with this attenuated strain was able to protect 100% of the mice challenged with 10x the ID_{50} from this Bb strain, meaning that it possesses all the properties to act as an attenuated vaccine. The experiments outlined in this project intend to test the abilities of this attenuated vaccine to elicit antibodies capable of protecting mice against challenge with Bb via tick or syringe inoculation up to 1 year post-immunization, thus preventing the development of Lyme disease. The results of this study could lead to the development of a greatly needed vaccine to protect against Lyme disease.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Borrelia burgdorferi, Lyme disease, tick-borne disease, vaccine, attenuated vaccine, immunization, chemotaxis, antibody, protective antigens, proteome array

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Based on discussions after the initial 1st annual technical report and our preliminary data, the Program Officers (Davy and McCarty) asked for a revised, tracked, unofficial copy of the SOW be submitted with this report, so we can reset our priorities based on the preliminary data while completing the major tasks of the initial SOW. This has been sent as a separate document

Specific Aim 1: Determine whether the attenuated Δ*cheY3* mutant can act as a vaccine to protect from challenge with WT *B. burgdorferi* (Bb) strains

Major Task 1: Determine whether a single dose of the attenuated Δ*cheY3* mutant can elicit antibody responses similar to the WT *B. burgdorferi* strain without the detrimental effects associated with persistent *Bb* infections.

Subtask 1 – Obtain ACURO approval (I already have IACUC approval for this work at UT, as I filed an amendment for all these techniques 2 months ago)
Subtask 2: Evaluate antibody responses to Δ*cheY3* immunization under different conditions
Subtask 2.1 Immunization dose analysis
C57BL/6 mice will be immunized i.d. with heat-killed Bb (B31-A3), live Bb (B31-A3), Δ*cheY3* Bb or vehicle control. Varying inoculum sizes will be used for Δ*cheY3* groups ranging from 10^6-10^9. Blood and serum will be harvested. n=10 C57BL/6 mice/group (7 groups = **73 C57BL/6 mice**) (source: CRL).
Subtask 2.2 Perform PCR analysis on ankle, heart, skin (local and distal to injection) and ear to detect Bb in those tissues
Subtask 2.3 Evaluate germinal center development via microscopy. If Δ*cheY3* Bb are seen to migrate outside of immunization site, then B and T cell populations in germinal centers will be assessed by flow cytometry.
Subtask 2.4 Evaluate antibody isotypes (IgG and IgM) and levels in immunized mice using ELISAs.
Subtask 2.5 Anti-sera proteome array analysis to identify which native Bb antigens generated an antibody response. Antisera from 3 best immunization groups as determined by ELISA will be used. Samples will be sent to Antigen Discovery (Irvine, CA) for processing and bioinformatics analysis.

Major Task 2: Evaluate how the different single-dose immunization protocols elicit protective immunity against challenge with two WT *B. burgdorferi* strains.
Subtask 1 - Evaluate the ability of Δ*cheY3* immunization to protect against syringe-challenge with two WT Bb strains up to one-year post- immunization.

Subtask 1.1 Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge. C57BL/6 mice will be i.d. immunized with BSK media only (control), heat-killed B31-A3 Bb (control) or live Δ*cheY3* (inoculum ranging from 10^6-10^9). Mice will be minimally bled 5 weeks after the immunization regimen to confirm the development of Bb-specific Abs by ELISA prior to challenge. Mouse numbers = 10 mice per group x 6 immunization groups x 1 challenge doses x 2 *B. burgdorferi* strains for syringe challenge x 3 challenge time points = **360 C57BL/6 mice**
Subtask 1.2 Challenge immunized mice with B31-A3 or N40 strain. Immunized C57BL/6 mice (Subtask 1.1) will be grouped and challenged with 5x the ID_{100} of the WT-GFP B31-A3 or N40 strain i.d. at 5 week, 6 months or 1 year post vaccination. Ears of WT-GFP-infected mice will be imaged over a 28-day period to assess bacterial load. Ears, skin, heart, and ankles will be harvested at 28d and Q-PCR will be performed to quantify Bb Subtask 1.3 Evaluate Ab quality and quantity using ELISAs and Proteomic Analyses. Antisera from different immunization schedules will be sent to Antigen Discovery for screening against the *B. burgdorferi* proteome array.
Subtask 2 - Evaluate the ability of Δ*cheY3* immunization to protect against tick-mediated challenge with 2 WT Bb strains up to one year after immunization.
Subtask 2.1 Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge These studies will be performed exactly as those outlined in **Subtask 1.1 above.** Mouse numbers = 10 mice per group x 6 immunization groups x 2 Bb strains for tick-challenge x 3 challenge time points = **360 C57BL/6 mice**
Subtask 2.2 Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by QPCR. Total mice needed will be **40 C57BL/6 mice**
Subtask 2.2 Challenge immunized mice from **Subtask 2.1**. Immunized mice will be split into groups and challenged at either 60 days, 6 months or 1 year post final immunization via tick bite with either B31 or N40 strains. Ticks will be evaluated by QPCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post challenge and Q-PCR will be performed to

quantify Bb levels in tissues.
Subtask 2.3 Evaluate Ab quality and quantity using ELISAs and Proteomic Analyses. Antisera from different immunization schedules will be sent to Antigen Discovery for screening against the *B. burgdorferi* proteome array.

Major Task 3: Determine whether booster doses of the ∆*cheY3*-vaccine can enhance protection against *B. burgdorferi* challenge

Subtask 3.1 - Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge. Groups of mice will be single, double, triple, or non- immunized intradermally (21 days between doses) with the optimal ∆*cheY3* dose observed in **Major Task 2.** The total number of mice needed for this study is 10 mice per group x 4 immunization schedules x 2 *B. burgdorferi* challenge strains x 3 challenge times = **240 C57BL/6 mice.**

Subtask 3.2 Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by Q-PCR. Total mice needed will be **30 C57BL/6 mice**

Subtask 3.3 Challenge immunized mice from **Task 3, Subtask 1.1**. Immunized mice will be split into groups and challenged at either 60 days, 6 months or 1 year post final immunization via tick bite with either B31 or N40 strains. Ticks will be evaluated by QPCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post challenge and Q-PCR will be performed to quantify Bb levels in tissues.

Subtask 3.4 Evaluate Ab quality and quantity using ELISAs and Proteomic Analyses. Antisera from different immunization schedules will be sent to Antigen Discovery for screening against the *B. burgdorferi* proteome array.

Major Task 4: Determine whether passive transfer of ∆*cheY3*-elicited antisera can protect against *B. burgdorferi* challenge

Subtask 4.1 - Generate antisera based on best immunization schedule from **Major Tasks 1-3.** Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge. The total number of mice needed are 10 mice per group x 4 antisera = **40 C57BL/6** mice total.

Subtask 4.2 Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by QPCR. Total mice needed will be **5 C57BL/6 mice**

Subtask 4.3 Passive transfer of sera to mice then challenge 1 day later via tick bite. Ticks will be evaluated by Q-PCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post challenge and Q-PCR will be performed to quantify Bb levels in tissues.

Specific Aim 2: Identify the *B. burgdorferi* antigens that are upregulated *in vivo* and correspond with antibodies present in protective antisera.

Major Task 5: Proteomic and bioinformatics analyses to identify *B. burgdorferi* proteins upregulated *in vivo* and determine immunogenicity

Subtask 1: Bioinformatic analyses will be performed on the antisera proteomic array data collected in Major Tasks 1-3.

Major Task 6: Develop and purify the identified Bb antigens from Major Task 5 as recombinant proteins

Subtask 1: Clone, express, and purify the 10-20 “best” vaccine candidates (as identified above) as recombinant fusion proteins in *E. coli.*

Major Task 7: Test the ability of antisera generated against the recombinant proteins to passively protect from challenge with Bb

Subtask 7:1. Generate antisera based on best immunization schedule from **Major Tasks 1-3.** Immunize mice and confirm development of Bb-specific Abs via ELISA. The total number of mice needed are (7 mice

per group for antisera x Bb antigens) + 10 mice per group for challenge x (5 Bb antigens + 1 control) x 2 *B. burgdorferi* strains for tick-challenge = **155 C57BL/6 mice total**.
Subtask 7.2. Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by QPCR. Total mice needed will be **20 C57BL/6 mice**
Subtask 7.3. Passive transfer of sera to mice then challenge 1 day later via tick bite. Ticks will be evaluated by Q-PCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post-challenge and QPCR will be performed to quantify Bb levels in tissues.
Subtask 7.4. Direct binding and killing assays will be performed using our established protocols to determine the relative effectiveness of the different antisera against Bb *in vitro*.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

I have attached a copy of the current SOP (SOW.2021-10.28b) that has an added column to show which tasks have been complete, in addition to the description below.

Approval for funding by the DoD was received by the University of Toledo on October 1, 2021. Correspondence was continued between the PI and the DoD to finalize the Statement of Work (SOW), which was finally approved on November 12, 2021. We then proceeded with paperwork to attain the approval for animal work, which is essential, as all work in the SOW involved immunization and/or challenge of mice *with B. burgdorferi.* We received final approval from ACURA on August 11, 2022 (see below) and were then able to start the vaccine work. *Note: all work on this proposal required mouse studies.

Major Task 1: Determine whether a single dose of the attenuated ∆*cheY3* mutant can elicit antibody responses similar to the WT *B. burgdorferi* strain without the detrimental effects associated with persistent *Bb* infections.

For overall issues related to Major Task 1, we have hired all personnel listed on our Budget Justification and trained them on all techniques needed for the studies. Using grant money that is NOT from the DOD and using our IACUC protocols separate from ACURA approval, we have performed ID_{50} studies to determine the infectious doses the infects 50% of mice (ID_{50}). This information is needed to allow for our challenge studies in Major Task 2. Earlier work in our lab found an ID_{50} of 215 B31 organisms, but this was over 5 years ago. This new study found an ID_{50} of 572 B31 organisms, we will use as the basis for our challenge studies.

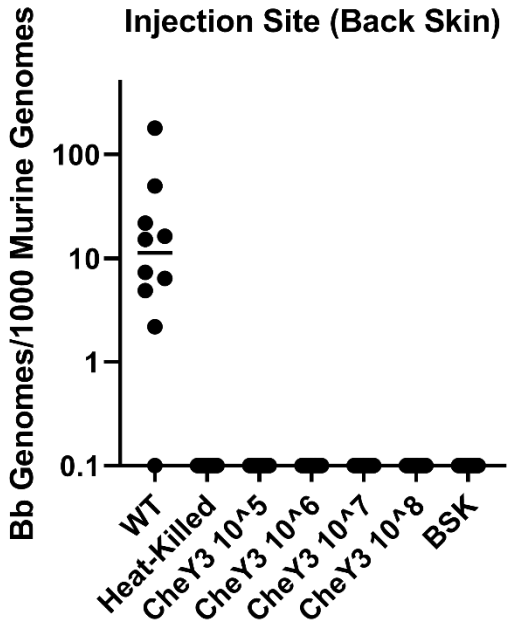
Subtask 1: Obtain ACURO approval. Our institutional IACUC protocol was amended and approved by our institutional committee on April 24, 2022. This protocol and the ACURA version was then submitted to USAMRIID for ACURA approval. After much correspondence, we received ACURA approval on August 11, 2022. With this approval, we moved forward with our studies.

Recently, our institutional IACUC protocol expired while going through repeated rounds of revisions. This new 3-year revised version of the IACUC protocol was finally approve on March 29, 2023.

Subtask 2: Evaluate antibody responses to Δ*cheY3* immunization at different conditions.

Subtask 2.1 Immunization dose analysis. Groups of 10 C57BL/6 mice (5 male and 5 female) were immunized i.d. with either 10^8 heat-killed Bb (B31-A3), 10^4 live Bb (B31-A3), Δ*cheY3* Bb at doses of 10^5-10^8, or BSK media control on September 6. All mice were sacrificed on October 10 (e.g. one month) and the following tissues harvested: blood, rear ankles (minus skin), ears, skin at injection site, heart, and lymph nodes (both axillary and inguinal = 4). Thus Subtask 2.1 is now **completed** and tissues are being processed for later subtasks.

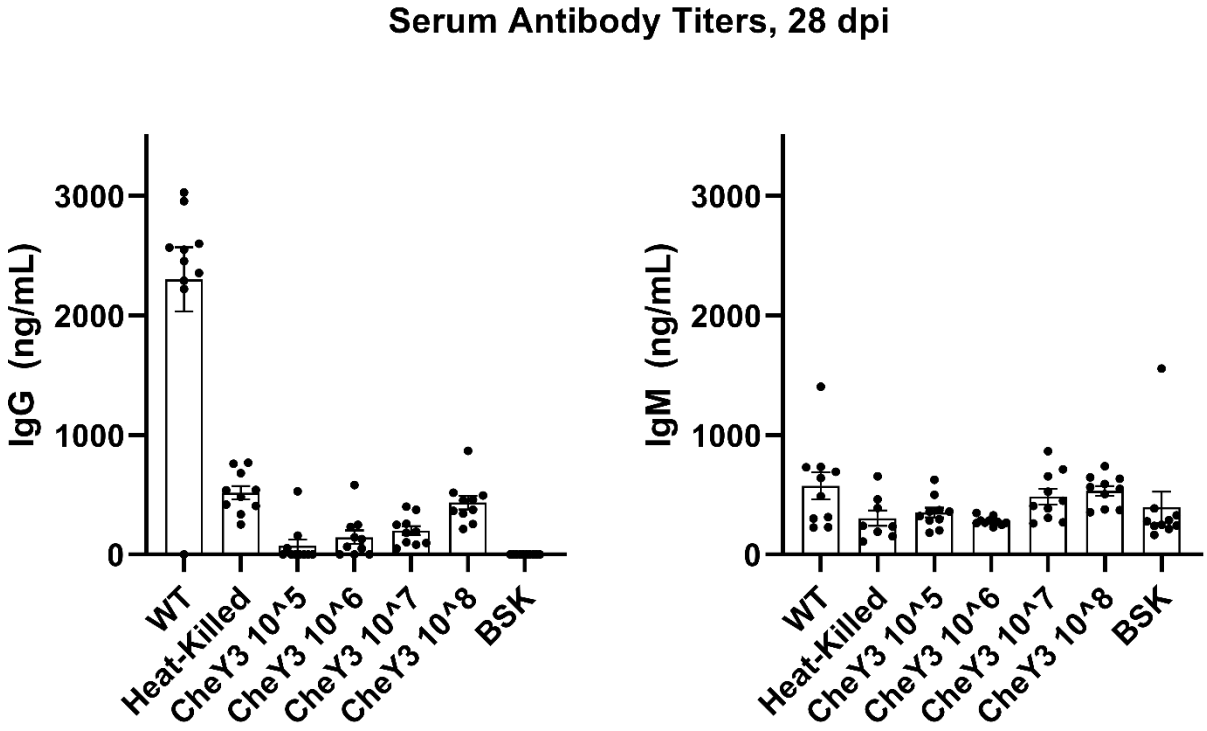
Subtask 2.2 Perform Q-PCR analysis on ankle, heart, skin (local and distal to injection) and ear to detect Bb in those tissues. These tissues (420 total) are processed for DNA using a very detailed and labor-intensive protocol that is required to get a final product clean enough to detect *B. burgdorferi* at the low numbers they normally persist at in different tissues. <u>These purifications are still ongoing</u>, but we have already purified and quantified the back skin (i.e. vaccine injection site), <u>which would be the most likely target to see persisting Δ*cheY3* or WT bacteria.</u> As shown below, injection with 10^3 WT Bb caused a persistent infection with significant numbers of Bb in the back skin whereas injection with 10^8 heat-killed Bb showed no persistent Bb genomes. Importantly, injection of the Δ*cheY3* Bb at doses of 10^5-10^8 showed no persistent Bb genomes at the infection site at 4 weeks post-infection, indicating that all Δ*cheY3* Bb were killed and did not persist at the infection site. This supports the non-persistent nature of the Δ*cheY3* Bb vaccine strain, even at doses as high as 10^8 Bb. We will continue to process the remaining tissues and should be able to finish quantification within the next 1-2 months.



Subtask 2.3 Evaluate germinal center development via microscopy. The lymph nodes harvested in **Subtask 2.1** have been fixed in formalin, sectioned, and H&E stained. We have now sent the slides to our collaborator (Dr. J.P. Lavik) at the Indiana University School of Medicine to perform a blinded assessment of germinal center integrity/dysregulation using standard Pathology scoring. We hope that Dr. Lavik will be able to score these samples relatively soon

Subtask 2.4 Evaluate antibody isotypes (IgG and IgM) and levels in immunized mice via ELISA. ELISA analyses were performed using our novel published Bb-specific methods, where temperature-shifted and killed Bb are adhered to all wells of an ELISA plate other than a standard lane that contains known amounts of a murine IgG or IgM standards, which allow for quantification of all lanes. For IgM, even the BSK-injected negative control possessed significant levels of Bb-specific antibodies at 4 weeks post-infection, which is expected since we have reported that murine hosts possess "natural" IgM antibodies that react with Bb. Interestingly, at 4 weeks post-infection all of the mice receiving the different live, dead, and attenuated Bb doses possessed

IgM levels similar to the BSK control, which was somewhat troubling since we and others have shown that Bb infection-elicited IgM levels above background can persist beyond 2 weeks due to Bb infections; more on that later. For IgG, the negative BSK control elicited no Bb-specific antibodies whereas the WT Bb elicited a significant and large IgG response even with a 10^3 inoculum. Immunization with the Δ*cheY3* Bb vaccine demonstrated a dose-dependent response, with the lowest 10^5 dose eliciting a minimal but significant IgG response, ranging up to the 10^8 dose which elicited levels similar to the 10^8 dose of heat-killed WT Bb. While the dose response was expected, the low levels even at the 10^8 dose is troubling, as we had seen higher levels in our preliminary studies. This has caused us to question whether we should stick to our current statement of work progressing with single dose inoculums and challenge, versus progress to the 3-dose regimen immediately before wasting time and resources on the single dose regimen, which now appears to be substandard. I hope to discuss this possibility with my program officer after reading this report, so that time and resources are not wasted by using an ineffective vaccine dose.



Subtask 2.5 Anti-sera proteome array analysis to identify which native Bb antigens generated an antibody response. The goal of this subtask was to take antisera from the 3 best immunization groups as determined by ELISA to be sent to Antigen Discovery (Irvine, CA) for processing and bioinformatics analysis, with the intention to identify Bb antigens associated with protective versus non-protective immunizations. However, because the antibody response appears to be substandard for the single dose immunization, we hesitate to send these sera for this very expensive (>$50,000) analysis when it may be more prudent to instead focus on antigens/antisera elicited by the 3-dose regimen. We have now discussed this with the Program Officer and have revised the SOP to continue to collect samples until Task 4, when we will send all of the samples at once to get the data.

These data are all for Major Task 1. We feel that we have enough data to begin Major Task 2, which has now been revised to involve performing a 3x dose of all vaccines, in the hopes

of gaining a stronger antibody response capable of protecting from long-term challenge with virulent *B. burgdorferi* strains.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Based on the data so far, it would appear that the single dose immunizations will provide sub-standard antibody responses for protective immunity, and that it would be prudent to subsequently change to a more standard 3-dose immunization schedule, then repeat the analyses from Subtask 2 to find the optimal procedures to proceed to Major Task 2. After discussions, the Program Officials are giving the go-ahead to proceed with the revised procedures.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Based on the data so far, it would appear that the single dose immunizations will provide sub-standard antibody responses for protective immunity, and that it would be prudent to subsequently change to a more standard 3-dose immunization schedule, then repeat the analyses from Subtask 2 to find the optimal procedures to proceed to Major Task 2. However, it was reassuring that even the highest dose of the attenuated vaccine did not result in any persistent *B. burgdorferi* in any tissues tested.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Based on the data so far, it would appear that the single dose immunizations will be provide sub-standard antibody responses, and that it would be prudent to change to a more standard 3-dose immunization schedule, then repeat the analyses from Subtask 2 to find the optimal procedures to proceed to Major Task 2. I have now recently had discussions with the Program Officer, and I am currently submitting a revised SOW that will allow us to rearrange the proposed studies to next focus on a 3x immunization dose, with the intent to produce a much stronger antibody response that will be able to protect against challenge with fully virulent *B. burg*dorferi strains.

Actual or anticipated problems or delays and actions or plans to resolve them

The major delay was in getting our IACUC and ACURO approval. Subsequently, we were able to pretty much complete Major Task 1 very quickly. We will be changing the order of experiments based on the Task 1 data, but will again be proceeding quickly onto the revised Major Task 2.

Changes that had a significant impact on expenditures

Since receiving the initial reward, pretty much all reagents and animal costs have increased dramatically. Thus, we will be watching our budget closely to see if studies can be consolidated or retooled to allow us to complete the studies under budget.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Not applicable

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

  Nothing to report.

  Books or other non-periodical, one-time publications.

  Nothing to report.

  Other publications, conference papers and presentations.

  Nothing to report.

- **Website(s) or other Internet site(s)**

  Nothing to report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *R. Mark Wooten*
No change

Name: *Jason Huntley*
No change

Name: *John Presloid*
Project Role: *Graduate Student*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*
Contribution to Project: *Preparation of experimental protocols and reagents needed for these studies. Performed mouse immunizations and processed all tissue collected post-mortum. Performed the ELISA and QPCR analyses reported in this report.*

Name: *Irum Syed*
Project Role: *Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*
Contribution to Project: *Helped perform mouse immunizations and processed all tissue collected post-mortum. Performed the ELISA and QPCR analyses reported in this report.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

STATEMENT OF WORK – Sept. 10, 2021
PROPOSED START DATE – Sept. 15, 2021

Site 1: University of Toledo Health Science Campus
3000 Arlington Ave. MS #1021
Toledo, OH 43614
PI: R. Mark Wooten

Site 2: Antigen Discovery, Inc.
1 Technology Drive
Irvine, CA 92618

Specific Aim 1: Determine whether the attenuated Δ*cheY3* mutant can act as a vaccine to protect from challenge with WT *B. burgdorferi* (Bb) strains	**Timeline**	**Site 1**	**Site 2**	**Task**
Major Task 1: Determine whether a single dose of the attenuated Δ*cheY3* mutant can elicit antibody responses similar to the WT *B. burgdorferi* strain without the detrimental effects associated with persistent *Bb* infections.	Months			
Subtask 1 – Obtain ACURO approval (I already have IACUC approval for this work at UT, as I filed an amendment for all these techniques 2 months ago)	1-2	X		Done
Subtask 2: Evaluate antibody responses to Δ*cheY3* immunization under different conditions	3-8	X		
Subtask 2.1 Immunization dose analysis C57BL/6 mice will be immunized i.d. with heat-killed Bb (B31-A3), live Bb (B31-A3), Δ*cheY3* Bb or vehicle control. Varying inoculum sizes will be used for Δ*cheY3* groups ranging from 10^6-10^9. Blood and serum will be harvested. n=10 C57BL/6 mice/group (7 groups = **73 C57BL/6 mice**) (source: CRL).	3-8	X		Done
Subtask 2.2 Perform PCR analysis on ankle, heart, skin (local and distal to injection) and ear to detect Bb in those tissues	3-8	X		Done
Subtask 2.3 Evaluate germinal center development via microscopy. If Δ*cheY3* Bb are seen to migrate outside of immunization site, then B and T cell populations in germinal centers will be assessed by flow cytometry.	3-8	X		Pending
Subtask 2.4 Evaluate antibody isotypes (IgG and IgM) and levels in immunized mice using ELISAs.	4-10	X		Done
Subtask 2.5 Anti-sera proteome array analysis to identify which native Bb antigens generated an antibody response. Antisera from 3 best immunization groups as determined by ELISA will be used. Samples will be sent to Antigen Discovery (Irvine, CA) for processing and bioinformatics analysis.	4-10	X	X	Delay
Milestone(s) Achieved: Determination of 1) whether any Δ*cheY3* immunization doses resulted in long-term persistence of the bacteria in any target tissues 2) the breadth of Bb antigens recognized by antisera generated by the different vaccination schedules 3) the amounts and isotypes of antibodies generated against *Bb* by each immunization schedule. 4) whether the immunization doses allowed development of normal germinal centers vs dysregulated germinal centers				
Major Task 2: Evaluate how the different single-dose immunization protocols elicit protective immunity against challenge with two WT *B. burgdorferi* strains.				Pending
Subtask 1 - Evaluate the ability of Δ*cheY3* immunization to protect against syringe-challenge with two WT Bb strains up to one-year post- immunization.	11-24			

Subtask 1.1 Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge. C57BL/6 mice will be i.d. immunized with BSK media only (control), heat- killed B31-A3 Bb (control) or live Δ*cheY3* (inoculum ranging from 10^6-10^9). Mice will be minimally bled 5 weeks after the immunization regimen to confirm the development of Bb-specific Abs by ELISA prior to challenge. Mouse numbers = 10 mice per group x 6 immunization groups x 1 challenge doses x 2 *B. burgdorferi* strains for syringe challenge x 3 challenge time points = **360 C57BL/6 mice**	11-24	X		
Subtask 1.2 Challenge immunized mice with B31-A3 or N40 strain. Immunized C57BL/6 mice (Subtask 1.1) will grouped and challenged with 5x the ID_{100} of the WT-GFP B31-A3 or N40 strain i.d. at 5 week, 6 months or 1 year post vaccination. Ears of WT-GFP- infected mice will be imaged over a 28-day period to assess bacterial load. Ears, skin, heart, and ankles will be harvested at 28d and Q-PCR will be performed to quantify Bb	11-24	X		
Subtask 1.3 Evaluate Ab quality and quantity using ELISAs and Proteomic Analyses. Antisera from different immunization schedules will be sent to Antigen Discovery for screening against the *B. burgdorferi* proteome array.	11-24	X	X	
Subtask 2 - Evaluate the ability of Δ*cheY3* immunization to protect against <u>tick-mediated challenge</u> with 2 WT Bb strains up to one year after immunization.				
Subtask 2.1 Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge These studies will be performed exactly as those outlined in **Subtask 1.1 above.** Mouse numbers = 10 mice per group x 6 immunization groups x 2 Bb strains for tick-challenge x 3 challenge time points = **360 C57BL/6 mice**	11-24	X		
Subtask 2.2 Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by Q- PCR. Total mice needed will be **40 C57BL/6 mice**	11-24	X		
Subtask 2.2 Challenge immunized mice from **Subtask 2.1**. Immunized mice will be split into groups and challenged at either 60 days, 6 months or 1 year post final immunization via tick bite with either B31 or N40 strains. Ticks will be evaluated by Q- PCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post challenge and Q-PCR will be performed to quantify Bb levels in tissues.	11-24	X		
Subtask 2.3 Evaluate Ab quality and quantity using ELISAs and Proteomic Analyses. Antisera from different immunization schedules will be sent to Antigen Discovery for screening against the *B. burgdorferi* proteome array.	11-24	X	X	
Milestone(s) Achieved: 1) Identify which immunization doses can protect against syringe and/or tick challenge with WT fully-virulent B31 (homogeneous challenge) and N40 (heterogeneous challenge) Bb strains 2) Compare the amounts and isotypes of antibodies generated by protective immunization doses vs. those that were not protective 3) Compare the Bb antigens recognized by antibodies generated by protective immunization doses vs. those that were not protective				
	Months			
Major Task 3: Determine whether booster doses of the Δ*cheY3*-vaccine can enhance protection against *B. burgdorferi* challenge				

Subtask 3.1 - Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge. Groups of mice will be single, double, triple , or non-immunized intradermally (21 days between doses) with the optimal Δ*cheY3* dose observed in **Major Task 2.** The total number of mice needed for this study is 10 mice per group x 4 immunization schedules x 2 *B. burgdorferi* challenge strains x 3 challenge times = **240 C57BL/6 mice.**	16-20	X	
Subtask 3.2 Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by Q-PCR. Total mice needed will be **30 C57BL/6 mice**	16-28	X	
Subtask 3.3 Challenge immunized mice from **Task 3, Subtask 1.1**. Immunized mice will be split into groups and challenged at either 60 days, 6 months or 1 year post final immunization via tick bite with either B31 or N40 strains. Ticks will be evaluated by Q- PCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post challenge and Q-PCR will be performed to quantify Bb levels in tissues.	16-28	X	
Subtask 3.4 Evaluate Ab quality and quantity using ELISAs and Proteomic Analyses. Antisera from different immunization schedules will be sent to Antigen Discovery for screening against the *B. burgdorferi* proteome array.	16-28	X	X
Milestone(s) Achieved: 1) Identify which immunization doses can protect against syringe and/or tick challenge with WT fully-virulent B31 (homogeneous challenge) and N40 (heterogeneous challenge) Bb strains 2) Compare the amounts and isotypes of antibodies generated by protective immunization doses vs. those that were not protective 3) Compare the Bb antigens recognized by antibodies generated by protective immunization doses vs. those that were not protective			
Major Task 4: Determine whether passive transfer of Δ*cheY3*-elicited antisera can protect against *B. burgdorferi* challenge			
Subtask 4.1 - Generate antisera based on best immunization schedule from **Major Tasks 1-3.** Immunize mice and confirm development of Bb-specific Abs via ELISA prior to challenge The total number of mice needed are 10 mice per group x 4 antisera = **40 C57BL/6** mice total.	27-32	X	

Subtask 4.2 Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by Q- PCR. Total mice needed will be **5 C57BL/6 mice**	27-32	X	
Subtask 4.3 Passive transfer of sera to mice then challenge 1 day later via tick bite. Ticks will be evaluated by Q-PCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post challenge and Q-PCR will be performed to quantify Bb levels in tissues.	27-32	X	
Milestone(s) Achieved: Identify whether antisera (i.e. antibodies) alone are sufficient to confer protection against tick-challenge with WT fully-virulent Bb (B31 (homogeneous challenge) and N40 (heterogeneous challenge) strains)			
Specific Aim 2: Identify the *B. burgdorferi* antigens that are upregulated *in vivo* and correspond with antibodies present in protective antisera.			
Major Task 5: Proteomic and bioinformatics analyses to identify *B. burgdorferi* proteins upregulated *in vivo* and determine immunogenicity			
Subtask 1: Bioinformatic analyses will be performed on the antisera proteomic array data collected in Major Tasks 1-3.	10-32	X	X
Milestone(s) Achieved: 1. Identify which Bb antigens are recognized by antibodies present in protective immune sera versus those present in non-protective immune sera. 2. Identify which of the protective antigens observed in #1 above have predicted functions associated with virulence in other pathogens. 3. Identify which of the protective antigens observed in #1 above have predicted properties that indicate they would be surface-expressed on Bb, and thus a putative vaccine target. 4. Identify which of the protective antigens observed in #1 above have predicted proteins that are common in multiple Bb strains 5. Identify which of the protective antigens observed in #1 above have predicted proteins that show little variability between multiple Bb strains 6. Utilize the information obtained from #1-5 above to decide the best Bb proteins to clone and purify in **Major Task 6** as potential recombinant vacciness.			
Major Task 6: Develop and purify the identified Bb antigens from Major Task 5 as recombinant proteins			
Subtask 1: Clone, express, and purify the 10-20 “best” vaccine candidates (as identified above) as recombinant fusion proteins in *E. coli.*	30-36	X	

Milestone(s) Achieved: 1. Will have generated between 10-20 of our "best" putative vaccine targets. 2. These will be tested for their immunogenic and protective potential in **Major Task 7**			
Major Task 7: Test the ability of antisera generated against the recombinant proteins to passively protect from challenge with Bb			
Subtask 7:1. Generate antisera based on best immunization schedule from **Major Tasks 1-3.** Immunize mice and confirm development of Bb-specific Abs via ELISA. The total number of mice needed are (7 mice per group for antisera x Bb antigens) + 10 mice per group for challenge x (5 Bb antigens + 1 control) x 2 *B. burgdorferi* strains for tick-challenge = **155 C57BL/6 mice total**.	30-36	X	
Subtask 7.2. Prepare Bb-infected *Ixodes scapularis* ticks. Naïve larval ticks will be infected by feeding on B31 or N40-infected mice. Tick infection will be evaluated by Q-PCR. Total mice needed will be **20 C57BL/6 mice**	30-36	X	
Subtask 7.3. Passive transfer of sera to mice then challenge 1 day later via tick bite. Ticks will be evaluated by Q-PCR to assess infection levels after feeding. Mouse ears, skin, ankles and urinary bladders will be harvested at 5 weeks post-challenge and Q- PCR will be performed to quantify Bb levels in tissues.	30-36	X	
Subtask 7.4. Direct binding and killing assays will be performed using our established protocols to determine the relative effectiveness of the different antisera against Bb *in vitro*.	30-36	X	
Milestone(s) Achieved: 1. Will have tested antisera generated against up to 10 different Bb antigens to protect against challenge with wild-type Bb. 2. Will have tested the abilities of these antibodies to directly bind and/or kill Bb *in vitro*			

Abbreviations List (if necessary)
Antibody – Ab
Borrelia burgdorferi - Bb C57BL/6
mice – B6
i.d. Intradermal