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PRINCIPAL INVESTIGATOR: Jerrold Turner, M.D., Ph.D.

CONTRACTING ORGANIZATION: Brigham & Women's Hospital, Inc., Boston,,MA

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Jerrold Turner, M.D., Ph.D.

E-Mail: jrturner@bwh.harvard.edu

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Brigham & Women's Hospital, Inc.
75 Francis Street
Boston, MA 02115-6110

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

The research proposed here will explore this unanticipated interaction that has the intestinal barrier at its center. In retrospect, one might have anticipated that, as our first line of defense, the barrier would govern communication between intestinal contents and the immune system. Nevertheless, the underlying processes are almost entirely undefined. The studies described in this application will elucidate the mechanisms that regulate this communication, the means by which decreased channel activity either reduces or increases severity of IBD or infectious gastritis, respectively, and how excessive or insufficient dietary sodium impacts these processes. Further, the results will create a foundation that will help us to design a new class of therapies that modulate claudin-2 channel function. The data will also provide guidance as to whether interventions to reduce or increase channel activity are appropriate for an individual patient at a specific time, i.e. personalized medicine.

15. SUBJECT TERMS

None listed.

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TABLE OF CONTENTS

1. **INTRODUCTION:** This application in the topic area of inflammatory bowel disease (IBD) focuses on intestinal barrier loss, a highly significant but mechanistically underexplored topic in IBD pathogenesis. As a result of limited study, opportunities for therapeutic regulation of the intestinal barrier have been neglected. It is nevertheless imperative to develop deep understanding of the causes and effects of barrier regulation and means to correct mucosal permeability defects in IBD and other disorders. The work proposed is designed to generate molecular, pathophysiological, and translational data that will enable development of barrier-directed therapies. The studies will test the central hypothesis that claudin-2-dependent regulation of the intestinal epithelial barrier negotiates communication between dietary Na^+, mucosal immunity, and microbial pathogens. Because the barrier is compromised in many intestinal and systemic diseases, the knowledge and tools generated are also expected to provide insight into other immune-mediated and infectious diseases.

2. **KEYWORDS:** diarrhea, inflammatory bowel disease, enteric infection, mucosal immunity, tight junction, sodium, claudin-2, enteropathogenic *E. coli*, *C. rodentium*, host defense, microbiome

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.*
    - **What were the major goals of the project?**
        - SPECIFIC AIMS AND MILESTONES PROPOSED (months 1-12)
            - AIM 1: To elucidate the mechanisms by which claudin-2 and dietary Na^+ interact to regulate the mucosal environment and T cell differentiation.
                - Determine the effect of claudin-2 expression on lamina propria Na^+ concentration; months 0 – 12
                - Define the impact of claudin-2 and dietary Na^+ on epithelial function; months 2 - 22
                - Define the impact of claudin-2 and dietary Na^+ on microbial populations; months 4 - 22
                - Define the impact of claudin-2 and dietary Na^+ on basal T cell differentiation; months 4 - 22
                - Define the impact of claudin-2 and dietary Na^+ on stimulated T cell differentiation; months 6 - 26
            - AIM 2: To characterize the impact of claudin-2 expression and dietary Na^+ on immune-mediated, infectious, and damage-induced colitis.
                - Define the mechanisms of by which claudin-2 and dietary Na^+ modify immune-mediated colitis; months 6 - 36

- Define the impact of claudin-2 and dietary Na^+ modify immune responses in *C. rodentium* colitis; months 6 – 26
- Define the impact of claudin-2 and dietary Na^+ modify immune responses in DSS colitis 12 - 30

- AIM 3: To define how claudin-2 limits mucosal colonization and enhances pathogen clearance during enteric infection.
  - Define effects of claudin-2-mediated Na^+ and water flux on early colonization in vitro; months 0 – 12
  - Determine how claudin-2-mediated Na^+ and water flux disrupts adherence and persistent infection 12 - 34
  - Determine impact of claudin-2 and dietary Na+ on mucus structure and pathogen clearance 14 - 36

- **What was accomplished under these goals?**
  - All milestones have been affected by COVID-19 stay-at-home orders, which mandated cessation of all experimental activities. We were also ordered to reduce mouse colonies to the bare minimum needed to maintain each line. This changed June 1, when will entered Phase II of the research start-up progress. This allowed up to 50% of lab members to be present, with appropriate social distancing, and also permitted expansion of our colony. Reestablishment of the breeding colony was complete by November, considering 3 week gestation and 8 week postnatal development until sexual maturity. Larger scale breeding began at that time, and we began to have mice available for experimental use in mid-February. Availability continues to be suboptimal.
  - Due to COVID and these issues, the individual working to develop tools for measurement of lamina propria Na^+ concentrations in *Cldn2*

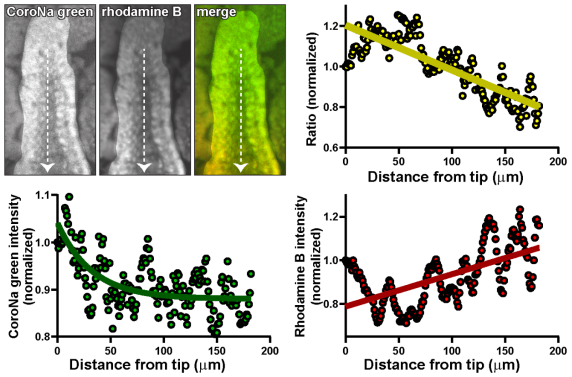

Figure 1. Preliminary measurements of lamina propria Na^+ concentration. The jejunum of WT mice was perfused with Kreb's saline containing glucose, CoroNa green (1 μg/ml), and rhodamine B (100 μg/ml) for 30 min. After snap-freezing, 20 μm thick frozen sections were image while frozen (using a microscope with a cooled stage). Because coroNa green fluorescent intensity varies as a function of both Na^+ concentration and dye concentration, rhodamine B, whose fluorescent intensity is unaffected by Na^+ concentration, was used to correct for differential coroNa green concentrations. Fluorescence of coroNa green and rhodamine B as well as a merged overlay image are shown. Intensity was measured along the line indicated in each image. The graphs below show that coroNa green intensity (green) is higher in the lamina propria of the upper villus. In contrast, rhodamine B intensity (red) is relatively constant and actually increases at the base of the villus. The ratio of these values (yellow) demonstrates greater Na^+ concentrations in the lamina propria of the upper villus, consistent with previous studies. Some further optimization of the method is needed, but these data indicate preliminary success.

knockout and *Cldn2* transgenic mice was required to leave the country. We have now made substantial progress (Fig. 1) and will complete development and begin to have measurements of Na^+ concentrations over the next reporting period.

- We have now collaboratively developed a bar-coded population of *C. rodentium*. Remarkably, we have found that there is a very narrow bottleneck for infection within the first few days of infection (Fig. 2). This was unexpected, as the bottleneck is more extreme than observed for any pathogen previously studied. As a result, the number of barcodes that remain within one week of infection is limited to ~10 (range 1 – 15). We are examining the effects of claudin-2 knockout and transgenic overexpression on these numbers. However, our previous data suggest that that the effect of claudin-2 expression on *C. rodentium* infection is, primarily, during the clearance stage of disease. We are, therefore, in parallel, working to develop ways to expand the early bottleneck.
- We have examined the effect of claudin-2 expression on epithelial function and, preliminarily, have found that claudin-2 regulates expression and activity of the epithelial Na^+ channel ENaC in the distal colon (Fig. 3). We are now in the process of characterizing Enac expression in claudin-2 knockout, wild type, and claudin-2 transgenic mice fed high or low Na^+ diets.
- In recent months (since experimental animals became available) we have begun using DSS colitis as a model to stimulate immune activity in

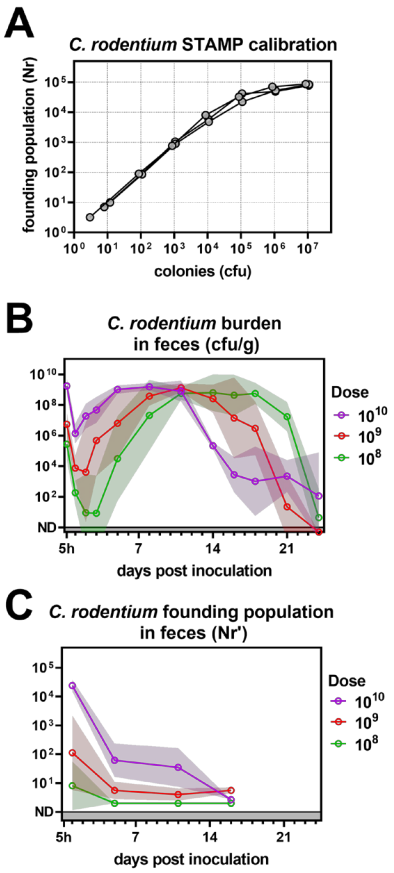

Figure 2. The bottleneck for *C. rodentium* colonization is extremely small. *A.* Calibration of barcoded *C. rodentium* library for STAMP studies. ***B.*** Peak fecal bacterial counts are independent of *C. rodentium* inoculum CFU. ***C.*** The number of founder *C. rodentium* at peak infection is <10 and is not affected by inoculum CFU.

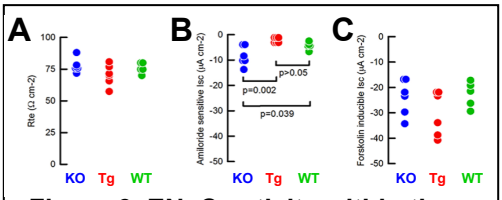

Figure 3. ENaC activity within the distal colon is upregulated in *Cldn2*$^{-/-}$ mice. *A.* Resistance of CLDN2 knockout (blue), claudin-2 transgenic (red), and wildtype (green) mice colon is similar. ***B.*** Amiloride-sensitive (ENaC) current is significantly greater in CLDN2 knockout, relative to transgenic and wildtype, mice. ***C.*** Forskolin-induced (cAMP) CFTR activation is similar in colons of CLDN2 knockout, claudin-2 transgenic, and wildtype mice.

claudin-2 knockout and claudin-2 transgenic mice. We found that claudin-2 altered severity of DSS colitis by affecting luminal water content, i.e., increased claudin-2 expression resulted in increased luminal water content. By simple dilution, this reduced the severity of DSS colitis. In addition, we found that manipulating dietary Na^+ affected the amount of water the mice drank. Since DSS is provided in the drinking water, this affected DSS doses. We have instead used TNBS, which induces a similar chemical colitis but is introduced intrathecally (Fig. 4). This allows us to standardize treatment. This has resulted in much more uniform outcomes and it is clear that treatment of claudin-2 transgenic mice results in much greater activation of Th1 and Th17 inflammatory pathways relative to claudin-2 knockout.

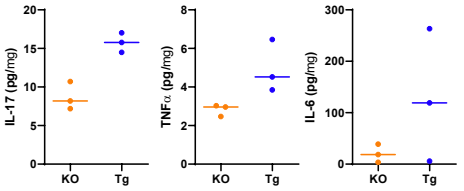

Figure 4. Claudin-2 overexpression primes the mucosal immune response. TNBS was administered rectally to CLDN2 knockout (orange) and claudin-2 transgenic (blue) mice. After 48 hours, colonic segments were harvested and homogenized. Data show cytokine content, as measured by ELISA, is significantly greater in claudin-2 transgenic mice.

- In collaboration with Prof. Gunnar Hansson we've done a careful functional analysis of intestinal mucin structure in claudin-2 transgenic and knockout mice. Unfortunately, our hypothesis that mucin structure would be affected by claudin-2 expression turned out to be incorrect. We are, however, continuing these studies after manipulating dietary Na^+.

- **What opportunities for training and professional development has the project provided?**
  - The project was not intended to provide training and professional development opportunities. Nevertheless, several postdoctoral fellows, including Preeti Raju, PhD, Nitesh Shashikanth, PhD, and Peter Steinhagen, MD (all authors on the JCI paper) have substantially advanced their professional skills through activities including experimental design, data analysis, data presentation, and manuscript preparation and submission. A new fellow, Dr. Yan Sweat, PhD, is being supported by the this DOD award. Dr. Sweat has replaced Dr. Steinhagen on the team and has been exceptionally productive thus far. Although not supported by this award, all other lab members have benefited intellectually from the activities supported by this project.

- **How were the results disseminated to communities of interest?**
  - Publications as below.
  - Lectures as below

- **What do you plan to do during the next reporting period to accomplish the goals?**
  - As mice continue to become available we are working to bring our program to full speed and continue the research project as originally proposed.

4. **IMPACT:**
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - The idea that increased intestinal paracellular cation permeability can be detrimental was reported. Previous to this, paracellular cation permeability increase were only reported to be detrimental.
        - The data demonstrate benefit of a drug that reduces intestinal paracellular cation permeability and provide a pathway for development of therapeutics.
        - New interest from pharma to develop small molecules that regulate claudin-2 function

    - **What was the impact on other disciplines?**
        - Nothing to Report.

    - **What was the impact on technology transfer?**
        - Nothing to Report.

    - **What was the impact on society beyond science and technology?**
        - *Nothing to Report.*

5. **CHANGES/PROBLEMS:**
    - **Changes in approach and reasons for change**
        - Nothing to Report.

    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - As described above, COVID19 caused major delays. We are now nearly back to 100% with some loss of efficiency due to continued COVID social distancing requirements.

    - **Changes that had a significant impact on expenditures**
        - Staff were paid while prevented from doing experimental work on site.
        - Some mouse care was required when no experimental work was permitted (see above).
        - These accommodations for COVID19 resulted in some fund depletion with limited productivity.

    - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
        - Nothing to Report.

6. **PRODUCTS:**
    - **Publications, conference papers, and presentations**
        - **Journal publications.**

- Raju P, Shashikanth N, Tsai PY, Pongkorpsakol P, Chanez-Paredes S, Steinhagen PR, Kuo WT, Singh G, Tsukita S, Turner JR. Inactivation of paracellular cation-selective claudin-2 channels attenuates immune-mediated experimental colitis in mice. J. Clin. Invest. 2020; 130:5197-5208. PMC7524482. 10.1172/JCI138697
- Shashikanth N, Rizzo HE, Pongkorpsakol P, Heneghan JF, Turner JR. Electrophysiologic analysis of tight junction size- and charge-selectivity Curr Protoc Cell Biol 2021; In press. PMCID in progress
- Chanez-Paredes SD, Abtahi S, Kuo WT, Turner JR. Differentiating Between Tight Junction-Dependent and Tight Junction-Independent Intestinal Barrier Loss In Vivo. Methods Mol. Biol 2021. PMCID in progress NIHMS1697601 10.1007/7651_2021_389
- Pongkorpsakol P, Turner JR, Zuo L. Culture of Intestinal Epithelial Cell Monolayers and Their Use in Multiplex Macromolecular Permeability Assays for In Vitro Analysis of Tight Junction Size Selectivity. Curr Protoc Immunol 2020; 131:e112. PMCID in progress. NIHMS1697612 10.1002/cpim.112
- Abtahi S, Gliksman NR, Heneghan JF, Nilsen SP, Muhlich JL, Copeland J, Rozbicki E, Allan C, Dudeja PK, Turner JR. A Simple Method for Creating a High-Content Microscope for Imaging Multiplexed Tissue Microarrays. Curr Protoc 2021; 1:e68. PMCID in progress. 10.1002/cpz1.68
- Zuo L, Kuo WT, Turner JR. Tight Junctions as Targets and Effectors of Mucosal Immune Homeostasis. Cell Mol Gastroenterol Hepatol 2020; 10:327-340. PMC7326733. 10.1016/j.jcmgh.2020.04.001
- Inczefi O, Bacquie V, Olier-Pierre M, Rincel M, Ringot-Destrez B, Ellero-Simatos S, Eutamene H, Betoulieres C, Thomas J, Laine J, Gros L, Leveque M, Leonard R, Harkat C, Robbe-Masselot C, Roka R, Mercier-Bonin M, Theodorou V, Darnaudery M, Turner JR, Ferrier L. Targeted Intestinal Tight Junction Hyperpermeability Alters the Microbiome, Behavior, and Visceromotor Responses. Cell Mol Gastroenterol Hepatol 2020; 10:206-208 e3. PMC7296230. 10.1016/j.jcmgh.2020.02.008
- Caron TJ, Scott KE, Sinha N, Muthupalani S, Baqai M, Ang LH, Li Y, Turner JR, Fox JG, Hagen SJ. Claudin-18 Loss Alters Transcellular Chloride Flux but not Tight Junction Ion Selectivity in Gastric Epithelial Cells. Cell Mol Gastroenterol Hepatol 2021; 11:783-801. PMC7847960. 10.1016/j.jcmgh.2020.10.005
- Yan J, Pandey SP, Barnes BJ, Turner JR, Abraham C. T Cell-Intrinsic IRF5 Regulates T Cell Signaling, Migration, and Differentiation and Promotes Intestinal Inflammation. Cell reports 2020; 31:107820. PMC7409536. 10.1016/j.celrep.2020.107820
- Abtahi S, Turner JR. Exploiting Alternative Brush Border Trafficking Routes to Treat Microvillous Inclusion Disease. Gastroenterol. 2020; 159:1233-1235. 10.1053/j.gastro.2020.08.006
- Xing T, Turner JR. New Pieces in the Puzzle That Leads to Spontaneous Bacteria Peritonitis. PracticeUpdate 2021.

https://www.practiceupdate.com/content/novel-pathomechanism-for-spontaneous-bacterial-peritonitis/115228/65/9/1

- Labarta-Bajo L, Nilsen SP, Humphrey G, Schwartz T, Sanders K, Swafford A, Knight R, Turner JR, Zuniga EI. Type I IFNs and CD8 T cells increase intestinal barrier permeability after chronic viral infection. J. Exp. Med. 2020; 217. PMC7953738
- Kang JW, Yan J, Ranjan K, Zhang X, Turner JR, Abraham C. Myeloid Cell Expression of LACC1 Is Required for Bacterial Clearance and Control of Intestinal Inflammation. Gastroenterol. 2020; 159:1051-1067. PMCID in progress. 10.1053/j.gastro.2020.07.024
- Donowitz M, Turner JR, Verkman AS, Zachos NC. Current and potential future applications of human stem cell models in drug development. J. Clin. Invest. 2020; 130:3342-3344. PMC7324189. 10.1172/JCI138645
- Yvellez OV, Rai V, Sossenheimer PH, Hart J, Turner JR, Weber C, El Jurdi K, Rubin DT. Cumulative Histologic Inflammation Predicts Colorectal Neoplasia in Ulcerative Colitis: A Validation Study. Inflamm Bowel Dis 2021; 27:203-206. PMC7813748. 10.1093/ibd/izaa047
- Ye S, Matthan NR, Lamon-Fava S, Solano-Aguilar G, Turner JR, Walker ME, Chai Z, Lakshman S, Chen C, Dawson H, Urban JF, Jr., Lichtenstein AH. Colon transcriptome is modified by a dietary pattern/atorvastatin interaction in the Ossabaw pig. J Nutr Biochem 2021; 90:108570. PMCID in progress. 10.1016/j.jnutbio.2020.108570
- Ye S, Matthan NR, Lamon-Fava S, Aguilar GS, Turner JR, Walker ME, Chai Z, Lakshman S, Urban JF, Jr., Lichtenstein AH. Western and heart healthy dietary patterns differentially affect the expression of genes associated with lipid metabolism, interferon signaling and inflammation in the jejunum of Ossabaw pigs. J Nutr Biochem 2021; 90:108577. PMCID in progress. 10.1016/j.jnutbio.2020.108577
- Stevens TW, Gecse K, Turner JR, de Hertogh G, Rubin DT, D'Haens GR. Diagnostic Accuracy of Fecal Calprotectin Concentration in Evaluating Therapeutic Outcomes of Patients With Ulcerative Colitis. Clin Gastroenterol Hepatol 2020; PMCID in progress. NIHMS1697617 10.1016/j.cgh.2020.08.019
- Pai YC, Weng LT, Wei SC, Wu LL, Shih DQ, Targan SR, Turner JR, Yu LC. Gut microbial transcytosis induced by tumor necrosis factor-like 1A-dependent activation of a myosin light chain kinase splice variant contributes to IBD. Journal of Crohn's & Colitis 2020; 15: 258–272. PMC7904084. 10.1093/ecco-jcc/jjaa165
- Christensen B, Hanauer SB, Gibson PR, Turner JR, Hart J, Rubin DT. Segmental Histological Normalisation Occurs in Ulcerative Colitis but Does Not Improve Clinical Outcomes. Journal of Crohn's & Colitis 2020; 14:1345-1353. PMC7533894. 10.1093/ecco-jcc/jjaa068
- Christensen B, Erlich J, Gibson PR, Turner JR, Hart J, Rubin DT. Histologic Healing Is More Strongly Associated with Clinical Outcomes in Ileal Crohn's Disease than Endoscopic Healing. Clin

Gastroenterol Hepatol 2020; 18:2518-2525 e1. PMC7586726. 10.1016/j.cgh.2019.11.056

- Cao F, Zhu L, Zhang J, Pongkorpsakol P, Kuo WT, Turner JR, Zhou Q, Wang Y, Chen F, Liu Y, Zuo L. Myosin light chain kinase is a potential target for hypopharyngeal cancer treatment. Biomed. Pharmacother. 2020; 131:110665. PMCID in progress.NIHMS1697616 10.1016/j.biopha.2020.110665

- **Books or other non-periodical, one-time publications.**
  Nothing to Report.

- **Other publications, conference papers, and presentations.**
  Tight Junction Channelopathy: A New Perspective on Mucosal Disease
  Institute of Clinical Physiology, Campus Benjamin Franklin, Charité - Universitätsmedizin Berlin, Berlin, Germany

  Therapeutic regulation of mucosal barriers
  Division of Gastroenterology. Johns Hopkins University School of Medicine, Baltimore, MD

  Paracellular channels: Fundamentals mechanisms and disease implications / Visiting Professor
  Division of Gastroenterology. Johns Hopkins University School of Medicine, Baltimore, MD

  Tight junction channelopathy: A new perspective on mucosal disease
  Division of Gastroenterology. Beth Israel Deaconess Medical Center, Boston, MA

  What is a mucosal barrier? Immunologic and pathologic implications
  Broad Institute Food Allergy Science Initiative (FASI) and Yale University School of Medicine

  Approaches to and impact of barrier restoration
  American Gastroenterological Association Annual Meeting (DDW)
  This presentation can be viewed at: https://jrturnerlab.com/2021-barrier-restoration-talk/

- **Website(s) or other Internet site(s)**
  - Nothing to Report.

- **Technologies or techniques**
  - 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: Jerrold Turner
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-0627-9455
Nearest person month worked: 1.8CM
Contribution to Project: oversight, data interpretation, and presentation
Funding Support:
Name: Yunuo Liu
Project Role: Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12 CM
Contribution to Project: mouse care and breeding and data management
Funding Support: None
Name: Heather Rizzo
Project Role: Technical Research Assistant
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 9 CM
Contribution to Project: Sample analysis
Funding Support: None
Name: Preeti Raju
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6 CM
Contribution to Project: All experimental work
Funding Support: None

Name: Yan Sweat
Project Role: Research Fellow
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6 CM
Contribution to Project: All experimental work
Funding Support: Salary
Name: John Heneghan
Project Role: Research Lab Manager
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2.2 CM
Contribution to Project: All experimental work
Funding Support: None

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to Report.

- **What other organizations were involved as partners?**
  - Nothing to Report.

8. **SPECIAL REPORTING REQUIREMENTS**
   - Nothing to Report.

9. **APPENDICES:**
   - None.