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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This project has 3 aims: 1) Determine if different BCG produce variable amounts of AM and DG. 2) Determine if variations in AM and DG correlate with differential BCG activation of host innate and adaptive immunity. 3) Determine if modifications to culture media affects BCG AM and DG levels. In this reporting period, CP analysis of additional BCG strains (including the six strains initially proposed) led to the identification of strains with significant defects in AM and DG production. The variations are more pronounced when BCG are cultured in Sauton's medium. Culturing in 7H9 medium results in increased AM production consistently for all BCG strains, while DG production is variably increased among different BCG strains. Although variations in CP among BCG exist when cultured in 7H9, infection of macrophage cells with BCG cultured under the same condition induces pro-inflammatory responses that do not correlate with CP composition. As such, the relationship between CP composition and induction of innate immune responses may be more complex than initially perceived. We have also discovered that culturing under low glycerol conditions uniformly increases AM production but variably increases DG production among the twelve BCG strains. Inclusion of additional exogenous cues to low glycerol, specifically lowered phosphate and SHX enhances AM and DG production in only some BCG strains. Collectively, the results of our investigation indicates that CP production is far more variable in BCG than previously appreciated and could certainly impact their effectiveness as vaccines. We have also discovered that CP production is negatively regulated by glycerol as well as by low phosphate and SHX in some but not all BCG strains.

15. SUBJECT TERMS

BCG sub-strains, variations in capsular polysaccharides, BCG-macrophage interactions, innate immune response, cell-mediated immune response

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1. Introduction

Tuberculosis (TB) is an airborne bacterial infection caused by members of the *M. tuberculosis* complex (1). Live attenuated *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) is the only vaccine available for TB. However, the efficacy of BCG is not 100% and is in fact quite poor at consistently protecting against the disease (2). This is likely due to the genetic heterogeneity seen in extant BCG vaccine strains that are used in different parts of the world (2). Indeed this genetic heterogeneity may well impact the synthesis and cell surface localization of the major capsular polysaccharides (CP) arabinomannan (AM) and α-D-glucan (DG), both of which are implicated in mycobacteria-host interactions and immune activation (3). Whether AM and DG vary among different BCG strains is not known. It is also not known if variations in CP among BCG strains correlate with their abilities to activate innate and adaptive immunity. Finally, recent studies show that growth conditions can influence multiple aspects of BCG immunobiology including their protective efficacy. Whether this is mediated through changes in CP is also not known. As such, this project has 3 main objectives:

1) Determine if genotypically different BCG strains produce different amounts of AM and DG.
2) Determine if variations in AM and DG correlate with differential BCG activation of host innate and adaptive immunity.
3) Determine if modifications to culture media affects BCG AM and DG levels.

The results of this study could explain why some BCG sub-strains are more immunogenic and protective than others. On a practical level, changes to growth media used to culture BCG may be a simple way to enhance the vaccine strain's immunogenicity and enable the production of vaccines that are more effective in the fight against TB.

2. Keywords

BCG, capsular polysaccharides, BCG-macrophage interactions, innate immune response, cell-mediated immune response

3. Accomplishments

3.1. Major goals of the project.

Aim 1. Examine CP (AM and DG) of BCG strains.
Major task 1 - Optimize CP extraction and analyses of CP.
Milestone: BCG CP samples prepared and analyzed; Local AREB and ACURO approval – 100% complete.
Major task 2 - LC-MS analyses of CP.
Milestone: BCG CP samples analyzed by LC-MS – rest of proposed work in revised NCE SOW on hold due to COVID-19 pandemic.

Aim 2. Assess CP-mediated macrophage-BCG interactions.
Milestone: Assessment of CP-mediated macrophage-BCG interactions – 100% complete.

Aim 3. Assess CP-mediated immune-stimulation in mice by different BCG.
Major task 1 - Prep 7H9-grown BCG^{+CP} and BCG^{-CP}, inoculate mice and assess cell-mediated immunity.
Major task 2 - Prep Sautons-grown BCG^{+CP} and BCG^{-CP}, inoculate mice and assess cell-mediated immunity.
Milestone: Assessment of CP-mediated immune-stimulation in mice – rest of proposed work in revised NCE SOW on hold due to COVID-19 pandemic.

Aim 4. Identify exogenous factors that enhance CP (AM and DG) production.
Major task 1 - Assess growth in modified Sautons, prep CP and analyze.
Major task 2 - Assess growth in Sautons with serine hydroxamate on BCG CP and cell-mediated immunity in mice.
Milestone: Assessment of effects of exogenous factors on BCG CP and immunogenicity – 100% complete.

3.2. Accomplishments to date.

Aim 1. Examine CP of BCG strains.

Major task 1 – We had previously reported the optimization of CP extraction and dot-blot analyses. Initially, six BCG strains – BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur - grown in 7H9 and Sautons media (without detergent typically added to prevent clumping) were analyzed. We showed that the amounts of AM did not vary among these six BCG strains when they were cultured in the same type of growth media. However, AM levels produced by BCG grown in 7H9 medium was on average by 2 to 3-fold higher than that produced by BCG strains grown in Sauton's medium. In contrast, BCG-Moreau was found to produce more DG than the other 5 sub-strains when they were cultured in either 7H9 or Sauton's medium. Morever, the DG produced by BCG-Russia, Japan, Moreau and Tice was increased when they were cultured in 7H9 medium compared to Sauton's medium. Noting these glaring differences, we wanted to determine if additional BCG strains in our collection also exhibited variations in AM and DG production. Accordingly, BCG-Sweden, Birkhaug, Prague, Glaxo, Frappier and Phipps were also included in the analysis **(Figure 1).** In working with the twelve extant BCG strains in our collection, we were able to replicate our previous results with BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur. We were also able to identify BCG strains with noticeable defects in CP composition. For example, BCG-Prague cultured in Sauton's medium was found to produce little to no AM compared to the other BCG strains grown under the same culture conditions. Consistently however, culturing all BCG strains including BCG-Prague, in 7H9 medium resulted in noticeable increases in AM production. With respect to DG, BCG-Prague, Birkhaug and Glaxo cultured in Sauton's medium were found to be poor producers of this CP. With the exception of BCG-Danish and Pasteur, most BCG strains cultured in 7H9 medium appeared to increase production of DG albeit to varying degrees.

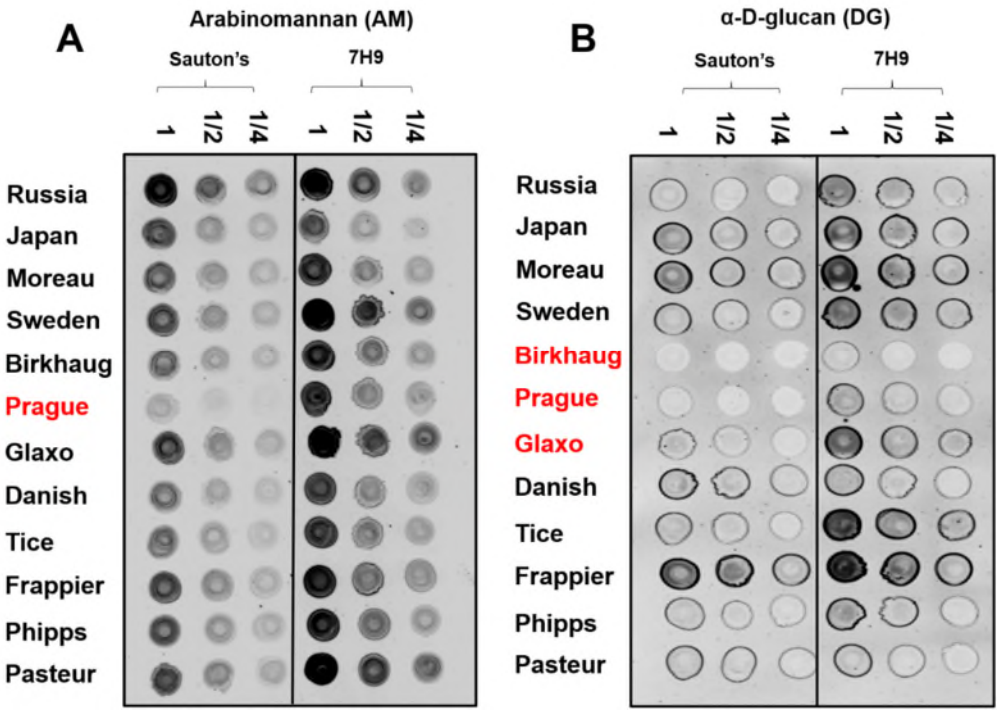

Figure 1. Dot-blots of AM (A) and DG (B) produced by 12 different BCG strains grown in Sauton's and 7H9 medium without detergent. The first undiluted spot is 3 µL PBS with 0.5% tyloxapol extracts of the freeze-dried BCG^{+CP} cells at 20 mg/mL. Subsequent spots are 3 µL of 2-fold serial dilutions. Representative of 4 independent experiments.

These results enable us to conclude the following: 1) Both AM and DG production is highly variable among extant BCG strains but this is dependent on the type of growth medium that they are cultured in. 2) Culturing in 7H9 medium leads to **consistent** increases in AM production in **all** BCG strains and **variable** increases in DG production in most but not all BCG strains when compared to growth in Sauton's medium. 3) Regulation of AM and DG production in BCG due to exogenous cues is variable and more complex than previously appreciated.

Our attempts to assess AM and DG production by different BCG strains using ELISAs has been unsuccessful and challenging. Due to the COVID-19 pandemic, consequent shut-down of our lab from March to July 2020, and limited operational capacity since then, we have opted to place these efforts on hold.

Major task 2 - LC-MS analyses of CP. As reported last, BCG CP extracts were analyzed by LC-MS but the results proved to be unclear, likely due to the biological complexity of the extract. Again, due to the COVID-19 related shutdowns and limited operational capacity of our lab since then, we have opted to place this work on hold.

Aim 2. Assess CP-mediated macrophage-BCG interactions.

We previously reported that attempts to generate BCG-Russia, Japan, Moreau, Danish, Tice and Pasteur lacking CP (BCG^{-CP}) by vigorous mixing and sonication for use in macrophage infections were unsuccessful. However, incubation of these six BCG strains in PBS supplemented with 0.5% tyloxapol removed both AM and DG from the BCG cell surface without affecting its viability. We observed that BCG^{-CP} is phagocytosed 2 to 3-fold more by human THP-1 macrophage cells than BCG^{+CP}. We also observed that BCG^{+CP} cultured in Sauton's medium tended to be better phagocytosed than BCG^{+CP} cultured in 7H11 medium. However, once the BCG strains had entered THP-1 cells no differences in intracellular replication could be observed. Further interrogation also revealed that BCG^{+CP} induced higher IL-1β production by THP-1 cells than BCG^{-CP}, regardless of growth in Sauton's or 7H9 medium. Unfortunately, no correlation between AM or DG levels and IL-1β induction by the six BCG^{+CP} was seen. This may be due to the fact that we only assessed six BCG strains and that IL-1β is poorly induced by BCG due to their lack of the type-7 protein secretion system ESX-1. As such, in more recent work we assessed the induction of TNF-α in THP-1 cells by the twelve extant BCG strains cultured in 7H9 medium in the absence and presence of detergent to yield BCG^{+CP} and BCG^{-CP} respectively **(Figure 2).** As observed previously, BCG cultured without detergent (BCG^{+CP}) tended to induce significantly more inflammation than BCG cultured in the presence of detergent (BCG^{-CP}). This result strongly suggests that the CP is required to induce macrophage inflammation by BCG although we cannot rule out the involvement of other cell surface antigens that may also be passively removed by detergents. While BCG strain differences in the induction of inflammation were observed, the correlation with CP variation is weak. This suggests that the pro-inflammatory effects of CP in BCG is far more complex than has been appreciated. Moreover, CP-mediated inflammation may be masked by other pro-inflammatory as well as anti-inflammatory cell surface antigens whose expression might be just as variable among different BCG strains.

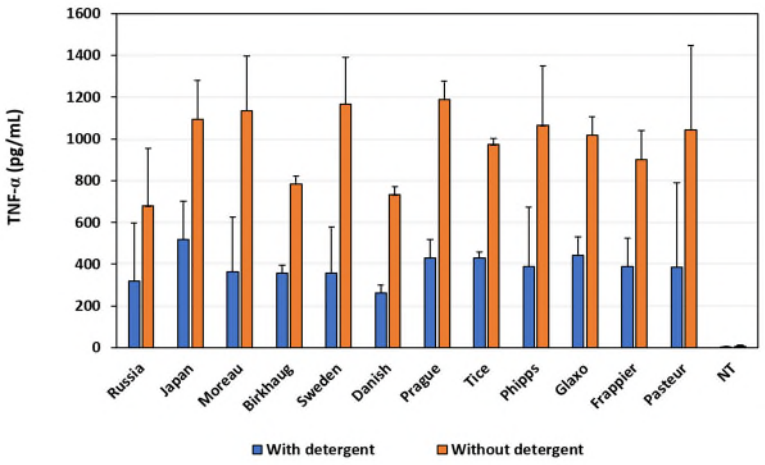

Figure 2. Induction of TNF-α production by THP-1 macrophages treated with BCG^{-CP} and BCG^{+CP}. Values are means of two independent experiments with each biological replicate consisting of two technical replicates. Error bars show the standard error of means.

Aim 3. Assess CP-mediated immune-stimulation in mice by select BCG^{-CP} vs BCG^{+CP}.

Although a clear correlation between AM or DG abundance in BCG strains and their induction of pro-inflammatory responses in macrophages has not been observed, variations in AM and/or DG abundance may still impact cell-mediated immunity. The animal use protocol enabling us to investigate this has been approved by both the institutional animal research ethics board and ACURO. BCG^{-CP} or BCG^{+CP} were prepared and immunization trials

in C57BL/6 mice had been initiated but due to COVID-19 related shutdowns and limited operational capacity of our lab, the trial had to be prematurely terminated. As such, this work has been delayed and is presently on hold.

Aim 4. Identify exogenous factors that enhance CP production.

Our results thus far indicate that BCG grown in Sauton's medium tend to produce lower amounts of both AM and DG compared to growth in 7H9 medium. A major difference between Sauton's and 7H9 medium is the presence of significantly higher concentrations of glycerol in the former (4). Sauton's medium contains 60mL/L glycerol while 7H9 medium contains 2mL/L glycerol. We therefore hypothesized glycerol negatively regulates CP synthesis. To test this, all twelve BCG strains were cultured in standard Sauton's medium and modified Sauton's medium with 2mL/L glycerol (hereafter referred to as Sauton LG). CP extracts were prepared and analyzed as described previously **(Figures 3 & 4).** All twelve BCG strains when cultured in Sauton's LG produced more AM, albeit to varying degrees than in regular Sauton's medium **(Figure 3).** The up-regulatory effect of reduced glycerol on the production of DG in BCG was less consistent **(Figure 4).** With the exception of BCG-Russia, Danish, Phipps and Pasteur, most BCG strains produced increased amounts of DG albeit to varying degrees when cultured in Sauton's LG. These results suggest that increased DG production seen in BCG-Russia and Phipps cultured in 7H9 relative to Sauton's medium **(Figure 1)** may be due to factors other than glycerol. Moreover, the ability of BCG-Russia to respond specifically to glycerol levels in the growth medium may be impaired as its production of AM is unaffected. In contrast, the lack of changes in DG production by BCG-Danish and Pasteur, regardless of their growth in regular Sauton's, Sauton's LG or 7H9 media suggests their ability to respond to exogenous cues in the growth medium may be compromised.

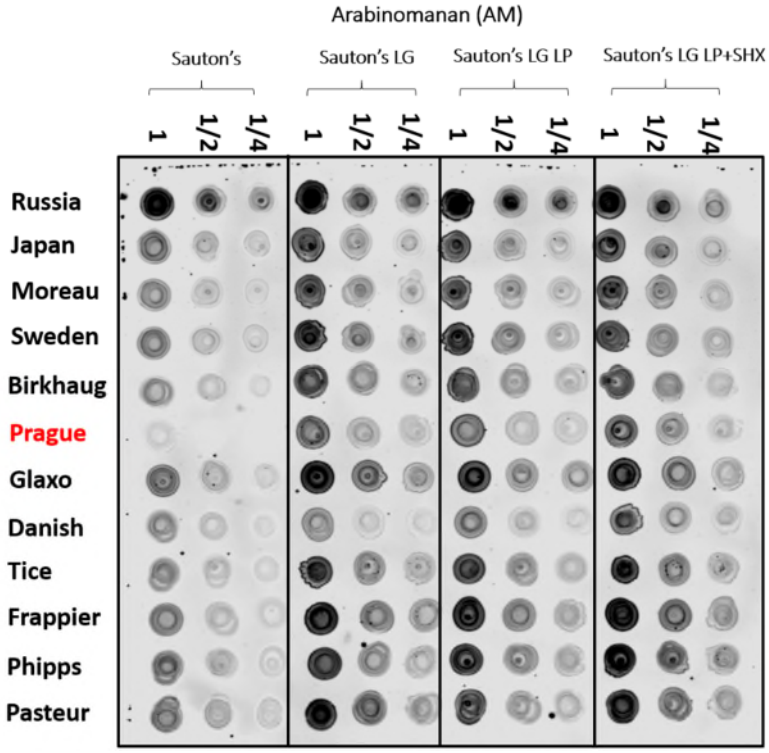

Figure 3. Dot-blots of AM produced by 12 different BCG strains grown in Sauton's medium and modified versions (without detergent). The first undiluted spot is 3 µL PBS with 0.5% tyloxapol extracts of the freeze-dried BCG^{+CP} cells at 20 mg/mL. Subsequent spots are 3 µL of 2-fold serial dilutions. Representative of 3 independent experiments.

It has been reported that reduction of phosphates in culture media can trigger the bacterial stringent response and upregulate CP production in mycobacteria (5). Moreover, serine hydroxamate (SHX) has also been shown to trigger the stringent response (5). As such, we wanted to efficiently determine if the presence of these two additional conditions might synergize with the lowered glycerol in Sauton's LG medium to substantially boost AM and DG production by BCG **(Figures 3 & 4).** Thus, Sauton's LG was modified by reducing the phosphate content to yield Sauton's LG LP. This was modified further with the addition of SHX to yield Sauton's LG LP+SHX. These media types were then inoculated with BCG and CP extracts analyzed. Lowered phosphate and SHX addition either induced no change or marginally increased production of AM by all BCG strains cultured in Sauton's LG **(Figure**

3). This suggests that under pre-existing conditions of low glycerol, the up-regulatory effects of reduced phosphate and SHX on AM production is either negligible or non-existent. In contrast, lowered phosphate and SHX addition appeared to increase DG production in BCG-Japan, Moreau, Prague and Tice only. This suggests that under pre-existing conditions of low glycerol, the up-regulatory effects of reduced phosphate and SHX on DG production is inactive inn some BCG strains but not in others.

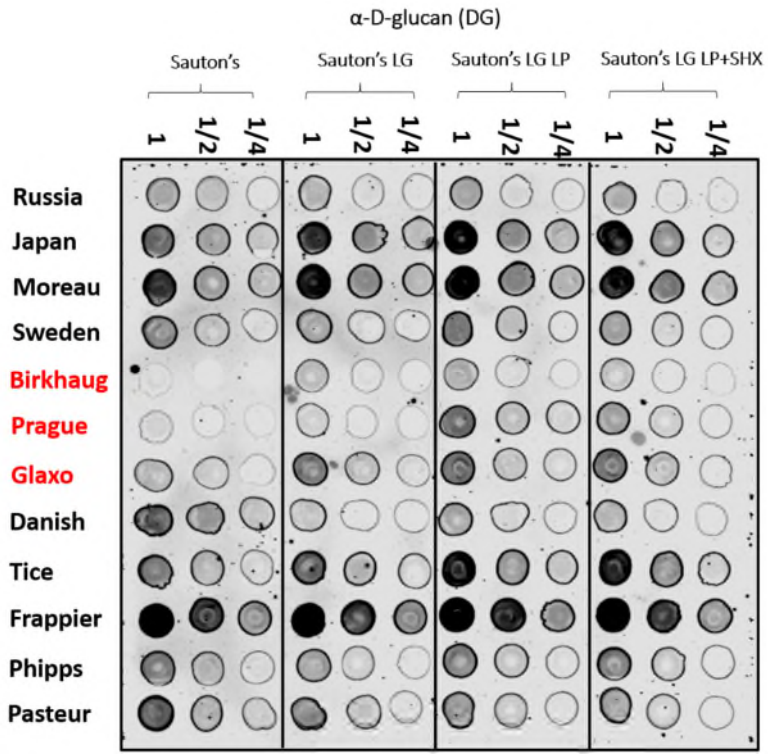

Figure 4. Dot-blots of DG produced by 12 different BCG strains grown in Sauton's medium and modified versions (without detergent). The first undiluted spot is 3 µL PBS with 0.5% tyloxapol extracts of the freeze-dried BCG^{+CP} cells at 20 mg/mL. Subsequent spots are 3 µL of 2-fold serial dilutions. Representative of 3 independent experiments.

Main Findings and Conclusions for this Reporting Period: CP analysis of additional BCG strains (including the six strains initially proposed) has led to the identification of several strains with significant defects in AM and DG production when they are cultured in both Sauton's or 7H9 medium. The variations are more pronounced especially when BCG are cultured in Sauton's medium. Culturing in 7H9 medium results in increased AM production consistently for all BCG strains. Under the same culture conditions however, DG production is variably increased among different BCG strains.

Although variations in CP among BCG exist when they are cultured in 7H9, infection of macrophage cells with BCG cultured under this condition induces pro-inflammatory responses that do not correlate strongly with CP composition. As such, the relationship between CP composition and induction of innate immune responses may be more complex than initially perceived.

We have also discovered that culturing under low glycerol conditions uniformly increases AM production but variably increases DG production among the twelve BCG strains. Inclusion of additional exogenous cues to low glycerol, specifically lowered phosphate and SHX enhances AM and DG production in only some BCG strains.

Collectively, the results of our investigation indicates that CP production is far more variable in BCG than previously appreciated and could certainly impact their effectiveness as vaccines. We have also discovered that CP production is negatively regulated by glycerol as well as by low phosphate and SHX in some but not all BCG strains.

3.3. Opportunities for training.

Nothing to report.

3.4. Dissemination of results to communities of interest.
Nothing to report.

3.5. Plans during next reporting period to accomplish goals.
As outlined in the proposal and statement of work, experiments to assess cell-mediated immunity in BCG^{-CP} and BCG^{+CP}-immunized mice will be performed. In parallel, the effects of culture media-dependent changes to BCG CP on cell-mediated adaptive immunity in mice will also examined.

4. Impact

4.1. Impact on the development of the principal discipline of the project.
Our results to date show for the first time that CP varies significantly among different BCG strains, many of which are used in different parts of the world in national TB vaccination programs. Given that CP impacts BCG protective efficacy, the variations in CP has clinical implications with respect to global TB control. We have also discovered that rational changes to culture media can enhance production of CP components. This means BCG efficacy can be boosted by simply culturing vaccine preparations in the necessary medium prior to their administration. This information will be of significance to commercial TB vaccine producers.

4.2. Impact on other disciplines.
Nothing to report.

4.3. Impact on technology transfer.
Nothing to report.

4.4. Impact on society beyond science and technology.
Nothing to report.

5. Changes/Problems

5.1. Changes in approach and reasons for change.
Our initial plan to focus on only six BCG strains has been revised to include additional strains – bringing the total analyzed to twelve. The reason for this change was the observation that the six initial BCG strains assessed did not exhibit significant variations in CP. Going forward, additional experiments to be performed will involve all twelve BCG strains.

5.2. Actual or anticipated problems or delays and actions or plans to resolve them.
The COVID-19 pandemic, particularly the shutdown of our laboratory from March to July 2020 significantly hampered progress. Partial resumption of operations since August 2020 with strict public health restrictions in place, has allowed us to conduct some of the proposed experiments indicated in the NCE SOW but at a much slower speed. We intend to complete *in vivo* mouse work to assess the impact of CP on activation of adaptive immunity but we will be streamlining the treatment groups. As such, funding from another source has been secured to perform this work that may go beyond the end of the US DoD-funding period of Jan 31, 2021.

5.3. Changes that had a significant impact on expenditures.
Nothing to report.

5.4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Not applicable and nothing to report.

5.5. Significant changes in use or care of human subjects.
Not applicable

5.6. Significant changes in use or care of vertebrate animals.
Nothing to report.

5.7. Significant changes in use biohazards and/or select agents.
Nothing to report.

6. Products

6.1. Publications, conference papers, and presentations.
Nothing to report.

6.2. Website(s) or other Internet site(s).
Nothing to report.

6.3. Technologies or techniques.
Nothing to report.

6.4. Inventions, patent applications, and/or licenses.
Nothing to report.

6.5. Other Products.
Nothing to report.

7. Participants & Other Collaborating Organizations

7.1. What individuals have worked on the project?

Name:	*Jeffrey Chen Ph.D.*
Project Role:	*Principal investigator*
Researcher Identifier:	***http://orcid.org/0000-0001-8431-3802***
Nearest person month worked:	*10*
Contribution to Project:	*Conceptualized, planned, helped execute experiments, collected and analyzed data*
Funding Support:	*VIDO-InterVac, University of Saskatchewan*

Name:	*Ze Lim M.Sc.*
Project Role:	*Research technician*
Researcher Identifier:	*None*
Nearest person month worked:	*12*
Contribution to Project:	*Mr. Lim executed experiments, collected and analyzed data*
Funding Support:	

7.2. 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.

7.3. What other organizations were involved as partners?
Nothing to report.

8. Special Reporting Requirements

8.1. Collaborative Awards.
Not applicable.

8.2. Quad Charts.

Not applicable.

9. Appendices

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