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Development of Aptamer Rolling Circle Amplification Platform as a Vaccine Adjuvant to Control Salmonella in Broiler Chickens

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Development of Aptamer Rolling Circle Amplification Platform as a Vaccine Adjuvant to Control *Salmonella* in Broiler Chickens

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ABSTRACT

Salmonella has been a major source of concern in public health and food industry. It is the cause of gastrointestinal illness and fever called salmonellosis. Severe cases of salmonellosis have resulted in hospitalization and even death around the world. *Salmonella* can be found in the intestinal tract of animals including chickens and can be transmitted to human through contaminated food. Hence, it is highly important to find ways of reducing the transmission of *Salmonella* from chickens to humans. This work is focused on the control of *Salmonella* in broiler chicken through the development of novel vaccine adjuvant. Two different aptamer rolling circle amplification (RCA) products were developed and combined to produce the vaccine complex: CD40-specific and *Salmonella*-specific RCA. CD40-specific RCA (termed aptamer RCA II), presenting two CD40-specific aptamers, has the ability to boost immune responses in broiler chickens through activation of the macrophage, and *Salmonella*-specific RCA (termed 3Sal-aptamer RCA) presents 3 *Salmonella*-specific aptamers, displaying strong binding affinity against various *Salmonella* serotypes, including *S.* Typhimurium, *S.* Enteritis and *S.* Kentucky. These two RCA products (aptamer RCA II and 3Sal-aptamer RCA) were designed to contain the spacer regions complementary to each other to facilitate the binding of the two RCA products through hybridization of the spacer regions. The vaccine complex was formed in the following steps: (1) 3Sal-aptamer RCA was incubated with *S.* Typhimurium cells, and unbound *S.* Typhimurium cells were removed by extensive washing. (2) The 3Sal-aptamer RCA-*S.* Typhimurium complex was then incubated with aptamer RCA II to form the final *Salmonella* vaccine complex. We determined that on average 2.15×10^6 CFUs of *S.* Typhimurium cells were loaded in 1 ml of RCA complex. These results showed that the final complex that consists

of 3 components of *Salmonella*-specific RCA, *Salmonella* cells, and CD40-specific RCA can be a novel vaccine complex targeting CD40 for efficient control of *Salmonella* in broiler chickens.

ACKNOWLEDGEMENTS

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DEDICATION

I want to dedicate this to God almighty.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Chicken meat contaminated with *Salmonella* continues to be a critical food safety issue and salmonellosis is still a major source of concern when it comes to foodborne illness(Silva et al., 2014). According to Center for Disease and Control (CDC), an estimate of 1.35 million illnesses , 26,000 hospitalizations and 420 deaths per year in the United States is associated to salmonellosis in humans (Silva et al., 2014). The most common serovars associated with salmonellosis are *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) and Enteriditis (*S.* Enteritidis). However, a subset of outbreaks has been attributed to 30 other serovars (Silva et al., 2014). *S.* Typhimurium is linked with contamination in a variety of meat sources (beef, pork, and poultry) and causes more human cases of foodborne illness, while *S.* Enteriditis is primarily associated with poultry products, both poultry meat (Antunes et al., 2016; Chousalkar & Gole, 2016) and egg (Chousalkar et al., 2018). As this foodborne bacterial pathogen still afflicts a large portion of the human population, developing methods to decrease *Salmonella* contamination in poultry products would be beneficial for both the consumers and the poultry industry. This is particularly important for the United States as there is an extensive poultry industry that comprises multiple poultry integrators and growers, serving to provide a significant quantity of poultry within the country and for export.

Rolling Circle Amplification (RCA) and its applications

Rolling circle amplification (RCA) is an isothermal enzymatic reaction that uses DNA polymerase to produce a long single stranded DNA containing tandemly repeated sequences. A primer is annealed to a circular single stranded DNA template and the polymerase with strong strand-displacement activity continuously incorporate nucleotides to generate a long single stranded DNA (Ali et al., 2014). The major components needed to produce RCA are DNA

polymerase, primer, dNTPs and circular single stranded DNA template. The circular DNA template can be synthesized enzymatically by ligation of single stranded linear DNA. Although RCA is a simple isothermal process, it is becoming a powerful tool that is now used to produce nanomaterials with various functional properties (Ali et al., 2014). Particularly, when it is designed to contain DNA aptamer sequences, the resulting RCA products can display strong affinity against the target molecules via hundreds of DNA aptamers (Al-Ogaili et al., 2020)

Due to its ability to produce a single stranded DNA consisting of the same DNA sequences repeated thousands of times, and the simplicity of its reaction, RCA has been used in several applications such as protein detection, stem cell fishing, nanomaterials fabrication, and vaccine. In clinical practice, detection of protein plays an essential role and RCA has been used as a platform for electrochemical detection for protein (Zhou et al., 2007). In this method, immobilization of antibodies was done on electrode surface to capture protein target. The detection was done by the deposition of silver enzymatically which was enabled by highly efficient readout of the RCA. This readout by RCA is more efficient when compared with the redox-labeled probe platform with low readout efficacy. Because aptamers are widely available for many proteins, Zhou et al. (2007) concluded that this method is promising for ultrasensitive immunoassay. Stem cell is another important topic in biology that has been widely studied because of its importance in cell-based therapies (Yao et al., 2020). However, it is very difficult to capture stem cells from bone marrow because of its ultra-low concentration. RCA has been employed to effectively fish stem cell by using a double-RCA strategy that generates a physical cross-link DNA (Yao et al., 2020). In this strategy, two different RCAs were produced separately. RCA 1 contained aptamers that has high affinity for a specific membrane protein of bone marrow mesenchymal stem cells (BMSCs). RCA 2 was designed to be partially

complementary with RCA 1, thereby forming a crosslink with RCA 1. RCA 2 was hybridized with RCA 1 to form a cross-link of cell-anchored DNA. The stem cells once captured can be released using DNase I digestion of the DNA network. According to Yao et al. (2020), this method has been proven to be an effective way to capture stem cell amidst tens of thousands of other cells. The stem cell that is released enzymatically still maintains normal cell activity.

DNA is also an excellent tool for the fabrication of nanomaterial (Baker et al., 2021). There is limited chemistry to increase the functionality of this nanomaterial such as high-performance scaffold and DNA nanoflowers (DNFs). Baker et al. (2021) strategically used orthogonal method to produce RCA and DNFs. The DNA and protein cargo were modified to expand the chemical functionality of the system. This method has been used to produce DNFs with multiple aptamers surfaces capable of binding to cancer cell. This DNA-based nanomaterial is useful in the biomedical field for therapeutic and diagnostic purposed because of their high target affinity and specificity.

The use of aptamer that has high affinity for CD40 has also been used to develop RCA that was used as a vaccine adjuvant (Al-Ogaili et al., 2020). This was accomplished by first developing DNA aptamer with affinity for chicken CD40 extra domain (chCD40ED). These aptamers were tested and two were selected to make RCA containing aptamers with affinity for chCD40ED. The aptamer RCA was conjugated with M2e epitope peptide of influenza virus and the complex was injected into chicken. The result demonstrated that the RCA vaccine complex was able to stimulate anti-M2e IgG antibody that is significantly higher than the control group (Al-Ogaili et al., 2020).

Enhancing vaccination efficacy via CD40 targeting

A method to enhance vaccine efficacy is to purposely engage and activate immune cells, specifically the professional antigen-presenting cells which initiate the acquired immune

response. A common marker on these cells is the CD40 receptor, which is known for its ability to stimulate dendritic cells, increase the pro-inflammatory activity of macrophages, and provoke maturation of B-cells while inducing class-switching to more effective antibody isotypes. Previous work on targeting CD40 using monoclonal antibody (mAb) against the chicken CD40 receptor (Chen et al., 2010) has produced a significant result. When this anti-CD40 mAb is complexed with an antigen target (**Figure 1**) and used to immunize chickens, there was a significantly more rapid and extensive target-specific antibody response within a week as compared to immunization with the antigen alone (without the CD40 mAb) (Chen et al., 2012; Chou et al., 2016; Vuong et al., 2017). This was attributed to the ability of the mAb to both activate the antigen-presenting cell and also directly present the antigen to this cell to manipulate downstream immune responses. This complex has been proven to provide protection against disease when used as a vaccine, capable of protecting chickens against highly pathogenic avian influenza (100% survival after H5N1 challenge), ameliorating the immunosuppressive effects of infectious bursal disease, and decreasing enteric lesions associated with necrotic enteritis (Duff et al., 2019; Vuong et al., 2018). Although this system is particularly effective as an adjuvant (immunostimulator) and delivery vehicle for vaccination, the use of mAbs is very expensive for

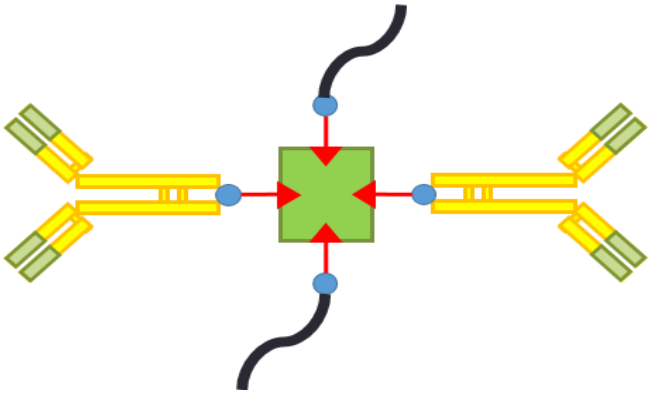


Figure 1. Original monoclonal antibody (mAb) based CD40-targeting adjuvant system. A central streptavidin is used as a central scaffold to complex biotinylated mAb and biotinylated antigen.

practical use in the poultry industry. Production of purified mAb is a very expensive and laborious process. The original mAb-based CD40-targeting complex also required the use of streptavidin protein as a central scaffold (able to bind 4 biotinylated proteins). This necessitated that both the CD40-targeting mAb and the vaccine antigen target need to be biotinylated for the separate vaccine components to be conjugated by the streptavidin to form a singular vaccine complex (**Figure 1**). This preparation process is expensive, laborious, and prone to handler error. To advance the CD40-targeting vaccine adjuvant system to a more accessible platform (poultry vaccine costs average less than 1 cent per dose), the mAb needed to be replaced with a cheaper and easier-to-produce component and the use of streptavidin as a scaffold needed to be eliminated from the design. A bivalent design, which would permit both CD40 receptor engagement/activation and single-step antigen loading (without the use of additional supplementary scaffolding proteins), would make this vaccination system more accessible.

Aptamer RCA as a novel adjuvant to enhance vaccination efficacy

To overcome these limitations in the mAb-based CD40-targeting immunization platform, aptamers were developed to replace the use of mAb. Aptamers are nucleotide-based (either DNA or RNA) and can be easily developed and produced at a low cost. Because aptamers are nucleotide-based, the final sequence can be documented, products easily screened for quality assurance, and can avoid master stock contamination issues. Aptamers have been successfully used as immunostimulators in various immunizations (Wengerter et al., 2014). The anti-CD40 aptamers screened and selected to replace the mAb were purposely constructed as a long chain of aptamers using RCA to ensure proper engagement with the CD40 receptor on antigen-presenting cells. When streptavidin was used as a central scaffold to complex the anti-CD40 aptamer RCA with a model peptide antigen target (M2e peptide from avian influenza virus), immunized chickens were

confirmed to be equally immunogenic responsive as compared to the original mAb-based system (Al-Ogaili et al., 2020). In this study, we aim to improve the adjuvant platform by eliminating the streptavidin scaffold system and use the improved adjuvant system to develop vaccines to control *Salmonella* in broiler chickens.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides used in this study shown in **Table S1** were synthesized through Integrated DNA Technology (IDT).

Aptamer binding assay

We obtained 3 *Salmonella* strains from the Poultry Health Laboratory (PHL) at the University of Arkansas, Fayetteville: *S.* Typhimurium, *S.* Kentucky, and *S.* Enteriditis. All strains were killed using formalin, when needed, and suspended in buffer. To quantify the binding affinity of each aptamer to different strains of *Salmonella*, the cells of each strain (live or killed) were washed three times, re-suspended in PBS with 10μM of FAM aptamer and incubated at room temperature for 1 hour. After incubation, the mixture was centrifuge at 12,000 rpm for 15 minutes and cells were washed 3 times to remove fluorescence-labeled aptamer (FAM labeled at 5' end; FAM aptamer) that was not bound to the cells. The washed cell-FAM aptamer pellet was transferred to black colored 96-welled plates to measure fluorescence (excitation 485 nm: emission, 528nm) using a microplate fluorescence reader (Park et al., 2014).

Design of the template for 3Sal-aptamer RCA

The sequences of 3 selected aptamers with a better binding affinity for *Salmonella* serotypes were used to design linear template for the production of 3Sal-aptamer RCA. Reverse complements of the 3 sequences were lined up side by side with spacer complementary to aptamer RCA II to give a sequence from the 3′ to 5′ end as shown in **Table 1**. The different colors show the sequences of the different aptamers used to design the template.

Table 1. DNA sequences of the aptamers

Oligonucleotide	Sequence
33	5′-TATGGCGGCGTCACCCGACGGGGACTTGACATTATGACAG-3′
S8-7	5′-CTGATGTGTGGGTAGGTGTCGTTGATTTCTTCTGGTGGGG-3′
C4	5′-ACGGGCGTGGGGGCAATGCCTGCTTGTAGGCTTCCCCTGTGCGCG-3′
3Sal-aptamer RCA template	5′ -Phospho-AAGACGTTCCCGCGCACAGGGGAAGCCTACAAGCAGGCATTGCCCCCACGCCCGT GTCACCCCACCAGAAGAAATCAACGACACCTACCCACACATCAGGTCA CTGTCATAATGTCAAGTCCCCGTCGGGTGACGCCGCCATATTCAGATGC -3′
3Sal-aptamer RCA	5′ -GCATCTGAATATGGCGGCGTCACCCGACGGGGACTTGACATTATGACAG TGACCTGATGTGTGGGTAGGTGTCGTTGATTTCTTCTGGTGGGGTGAC ACGGGCGTGGGGGCAATGCCTGCTTGTAGGCTTCCCCTGTGCGCGGGAACGTCTT -3′

Rolling Circle Amplification

The production of rolling circle amplification (RCA) started with annealing. Phosphorylated linear DNA template (25pmol = 10 µM, 2.5 µL) and biotinylated primer (250pmol=100 µM, 2.5 µL) dissolved in TE buffer (45 µL) were mixed, denatured at 95°C for 10 min, and annealed at 56 °C for 5 min. To synthesize the circular template needed for RC reaction, ligation of the naked template was done by mixing T4 DNA ligase (Cat# M0202S, NEB, USA; 400 units/µL, 1 µL), 10 × T4 ligase reaction buffer (6 µL), and Milli-Q water (3 µL) with the annealed product to make a total volume of 60 µL ligation system. The ligation reaction was carried out at 16°C for 8h, and the enzyme was heat inactivated at 65°C for 10 min. The next step was the RCA reaction which was done by hybridizing the ligated circular template with the

primer (volume of 60 µL) was mixed with phi29 DNA polymerase (Cat# M0269L, NEB, USA; 10 units/µL, 2 µl), 10 × phi29 DNA polymerase reaction buffer (8 µl), 100 × bovine serum albumin (BSA, 0.8 µl), and dNTPs (10 mM, 10 µl). RCA reaction was performed at 30ºC for 16 hrs. The size of the RCA products was characterized by 0.8% agarose gel electrophoresis which was stained with gel red, and image was viewed using ultraviolet light. RCA products were 10x diluted before running the electrophoreses.

Determination of *Salmonella*-loading capacity of aptamer DNA network complex.

Salmonella was cultured for 16 hours overnight in LB broth at 37˚C and washed 3 times in PBS. The washed cells were diluted 2,000 times. Eighty µL of the diluted cell suspension was transferred into two different 1.5ml microcentrifuge tubes (one for DNase I digestion and the other for no-digestion control) and 10 µL Sal-aptamer RCA was added to each of the tubes and incubated for 1 hour at room temperature with gentle shaking. An aliquot of the diluted *Salmonella* cell suspension was 10X-diluted and plated on LB agar plates to determine CFUs/ml. After an hour of incubation, 10 µL of aptamer RCA II was added to the mixture and incubated for another one hour. Biotinylated magnetic beads were added to the mixture in both tubes after incubation and the new mixture was incubated at room temperature for another one hour to allow binding of the magnetic beads with the *Salmonella*-RCA network. Magnetic rack was used to separate the *Salmonella*-RCA network that binds to the magnetic beads. The clear liquid was pipette out and the cell network was re-constituted again. One thousand µL of PBS was used to re-suspend the cell network and washed three times to ensure that unbound *Salmonella* was washed out. Final re-suspension of *Salmonella*-RCA network was done in 99 µL of DNase 1 buffer, and 1 µL (0.1U) of DNase 1 was added to one tube for DNase I digestion, while 1µL of sterile water was added to another tube (no-digestion control). The mixture was incubated for

1hour at room temperature to ensure release of the cell from the network by the enzymatic digestion of the DNA in tube one while tube two should remain undigested. Magnetic rack was used to separate the magnetic beads from the released cell. The supernatant was pipette out and 10x serial dilution of the supernatant was done, plated, and incubated at 37˚C for 24 hours. The colonies were enumerated to calculate the number of *Salmonella* cells loaded to *Salmonella*-RCA network.

Formation of RCA network complex through hybridization of two RCA products.

We performed hybridization between the following 3 pairs of RCA products and the progress in hybridization was monitored at 0 hour and 1 hour: (1) 3Sal-aptamer RCA and aptamer RCA II, (2) 3Sal-aptamer RCA and 3Sal-aptamer RCA, and (3) aptamer RCA II and aptamer RCA II. Briefly 3Sal-aptamer RCA was stained with Gel-red in a microcentrifuge tube and another 3Sal-aptamer RCA in a separate microcentrifuge tube with Gel-green. Aptamer RCA II was also stained with Gel-red and Gel-green separately. The reaction was set up as follows; 100µL of 3Sal-aptamer RCA stained with Gel-green was pipetted into a 1.5 ml tube and 100µL of aptamer RCA II stained with Gel-red was added. The second reaction was done by adding 3Sal-aptamer RCA stained with Gel-red and Gel-green together in a tube, while the third reaction was adding the aptamer RCA II that was stained with gel red and SYBR green together in a tube. The three reactions were viewed using ultraviolet light to see the physical interaction at 0 hour. The mixtures were incubated at room temperature with minimum shaking for 1 hour and monitored using ultraviolet light.

Preparation and evaluation of the aptamer RCA network complex

Killed *S.* Typhimurium was washed three times using PBS and finally re-suspended in PBS. It was then diluted 2000x to achieve a cell density of 2.15×10^6 CFUs. This cell

concentration was confirmed using the hemocytometer. 80µL of the diluted cell was pipette into a 1.5 ml microfuge tube each and 10µL of 3Sal-aptamer RCA was added to the tube. The mixture was incubated at room temperature for one hour with minimum shaking. After an hour, 10µL of aptamer-RCA II was added to the tube and incubated at room temperature for another one hour with minimum shaking to envelop the RCA-*Salmonella* network. Biotinylated streptavidin beads were added to the mixture to capture RCA-*Salmonella* complex. This was done by incubating the mixture for another one-hour after which a magnetic rack was used to remove the unbound *Salmonella* and the complex was washed three times and later resuspended in PBS.

RESULTS AND DISCUSSION

Comparison of the binding of *Salmonella*-specific aptamers against live vs dead cells

One question we wanted to ask was if aptamer binding to *Salmonella* cells would be the altered between the live and killed cells of *Salmonella*. It was due to the fact the use of live cells is more convenient and straightforward to perform the analyses related to binding of aptamers or RCA containing aptamers, while the final preparation of the finished *Salmonella*-RCA network complex for evaluation in chickens needed to be done using killed cells. Therefore, we performed the aptamer binding assay using 3 DNA aptamers (ST2P, SAL26, and ST1) against the live and killed cells for each of the 5 strains (*S.* Typhimurium, *S.* Enteritidis, *S.* Heidelberg, *S.* Kentucky, and *E. coli*). The results of the binding assay shown in **Figure 2** shows that aptamer binding is not different between the live and dead cells except for *S.* Kentucky.

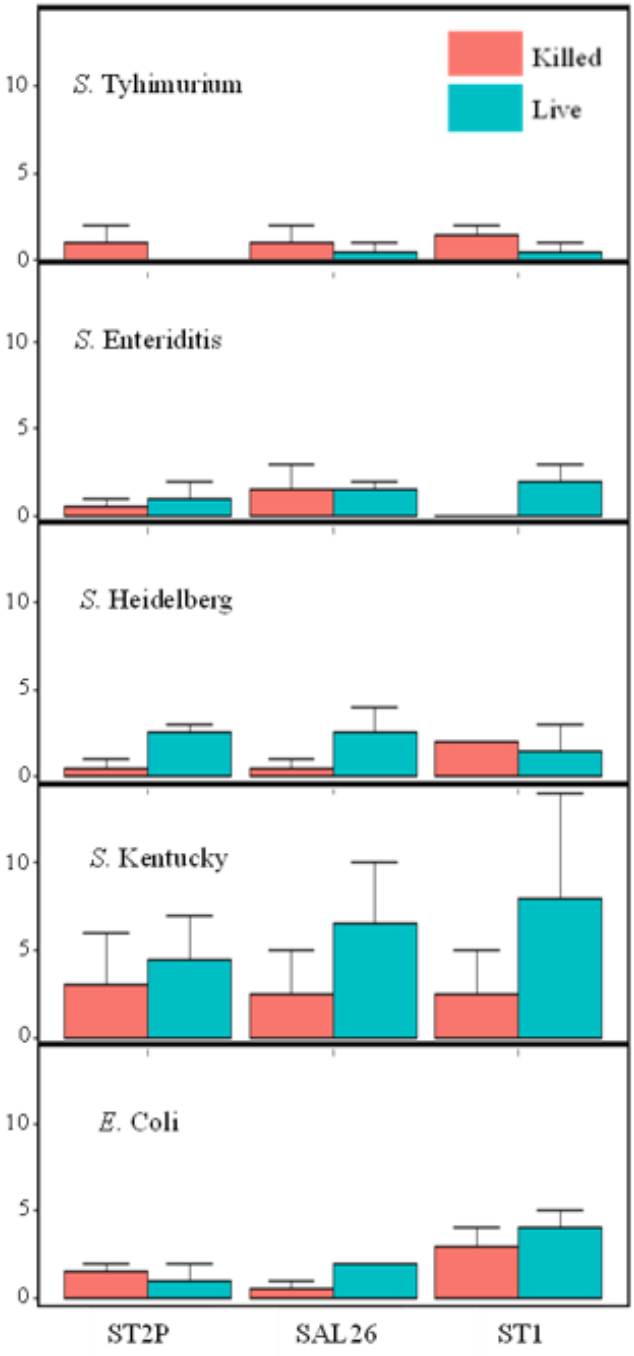


Figure 2. Binding affinity of 6-FAM aptamers for live and dead cells of different *Salmonella* serotypes and *E. coli*

Evaluation and selection of the *Salmonella*-specific aptamers

To produce RCA product with a good binding affinity to broad range of *Salmonella* serotypes, 6 DNA aptamers with binding capacity to different serovars of *Salmonella* were searched and selected from the published research (**Table S1**). We performed the assays to compare the binding affinity of these 6 aptamers against 3 serotypes of *Salmonella* strains (*S.* Typhimuirum, *S.* Enteritidis, and *S.* Kentucky). As shown in **Figure 3**, the result of the assay showed that the aptamer C4 has the highest binding affinity to all three *Salmonella* serotypes among all 6 aptamers evaluated. The aptamer ST2P on the other hand showed the least binding affinity of all aptamers evaluated. The 3 aptamers with higher binding affinity to *Salmonella* serotypes were selected (S8-7, 33 and C4) and used to design template for generating *Salmonella*-specific RCA product.

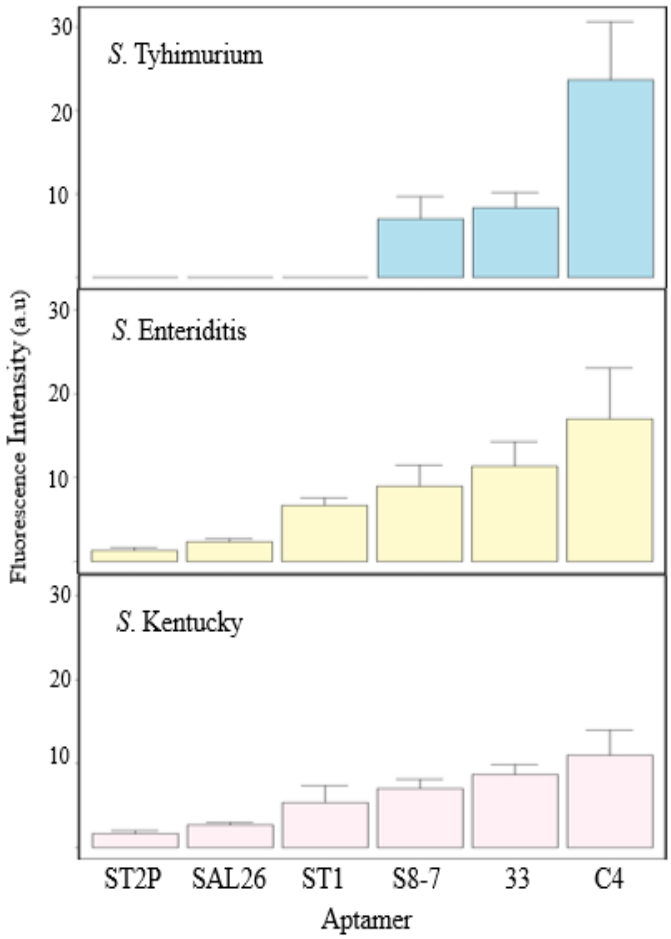
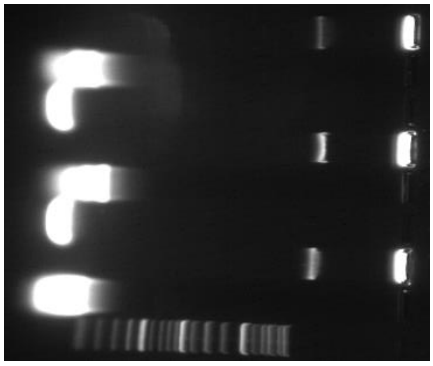


Figure 3. Binding affinity of 6 FAM-aptamers for *S.* Typhimurium, *S.* Enteriditis, *S.* Kentucky

Rolling Circle Amplification

The designed template for *Salmonella* RCA was used to produce the RCA and the RCA products were analyzed using 0.8% agarose gel electrophoreses. The RCA product was 10x diluted and loaded to run the agarose gel electrophorese to enable better visualization of the result. The result in **Figure 4** shows the RCA products of high molecular weight with strong intensity, which suggests successful production of RCA products.



1. 3Sal-aptamer RCA
2. 3Sal-aptamer RCA template
3. Primer for 3Sal-aptamer RCA
4. Aptamer RCA II
5. Aptamer RCA II template
6. Primer for aptamer RCA II
7. Aptamer NC-RCA
8. Aptamer NC-RCA template
9. 2-Log ladder

Figure 4. Analysis of the RCA products using agarose gel electrophoresis

Production and evaluation of the *Salmonella*-RCA complex network

The two RCA products (3Sal-aptamer RCA and aptamer RCA II) were designed to hybridize to each other partially through interaction between the two complementary spacer regions as illustrated in **Figure 5**. To confirm this cross-linking process, we used two different DNA staining dyes with different colors (Gel-Red and Gel-Green). The result in **Figure 6** showed that 3Sal-aptamer RCA and aptamer RCA II were able to bind well as evidenced by the colors changed from red and green to yellow upon mixing. On the other hand, the mixing of the same kind of RCA products (either 3Sal-aptamer RCA or aptamer RCA II) stained with red and green colors did not result in the change of the colors to yellow, suggesting separation of the two

RCA products even after 1 hour of mixing. Based on the result from this assay, we conclude that the design of our RCA products allowed cross-linking between the two RCA products through efficient hybridization between the two RCA products.

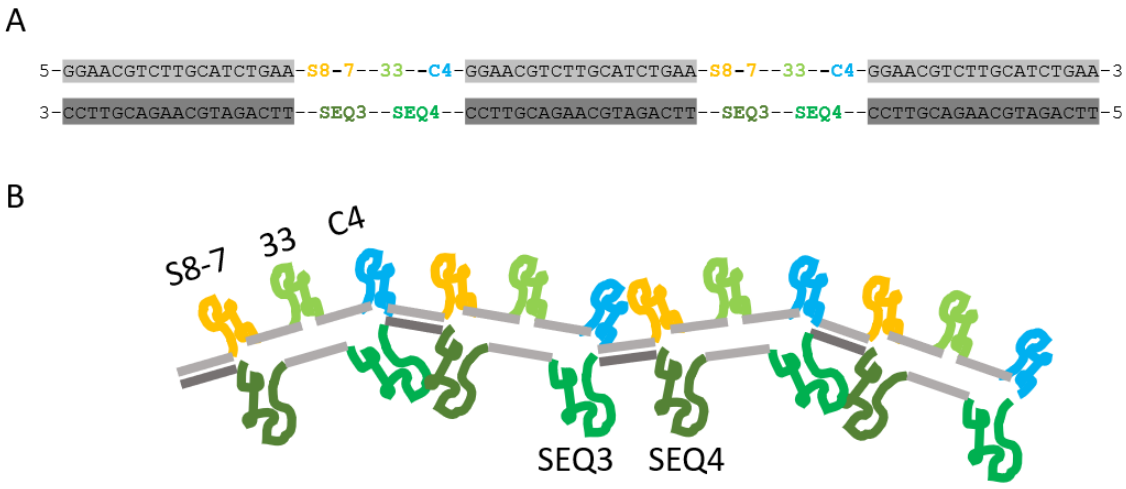


Figure 5. Design of the spacer regions of the two RCA products (3Sal-aptamer RCA and aptamer RCA II) to facilitate hybridization between the two spacer regions. (A) the 19nt sequences of the two spacer regions and (B) the diagram illustrating the cross-linking of the two RCA products through hybridization of the spacer regions.

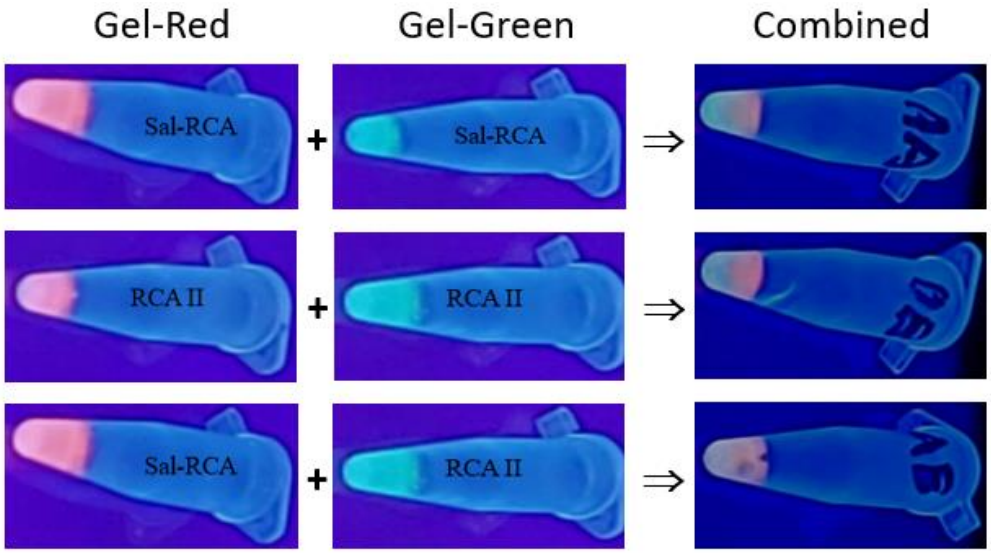


Figure 6. Interaction between the RCA products with the same or complementary spacer regions.

We also wanted to quantify the number of *S.* Typhimurium cells that can be loaded into the RCA network complex. For this purpose, we used DNA I enzyme to digest DNA network complex (3Sal-aptamer RCA + S. Typhimurium + aptamer RCA II), thereby releasing the captured *S.* Typhimurium cells as previous described by Yao et al. (2020). The same assay was performed, yet without DNase I digestion as a control group. The result in **Figure 7** shows that the strategy of using DNase I to release the captured *S.* Typhimurium cells was effective, and the count of *S.* Typhimurium cells were consistently and significantly higher with DNase I digestion as compared to the no-digestion control group. From this data, we were able to estimate the number of *S.* Typhimurium cells captured in the RCA network complex to be on average 2.15×10^6 CFUs of *S.* Typhimurium cells per 1 ml of RCA network complex by subtracting the count of the no-digestion control group from the number from DNase I digestion group.

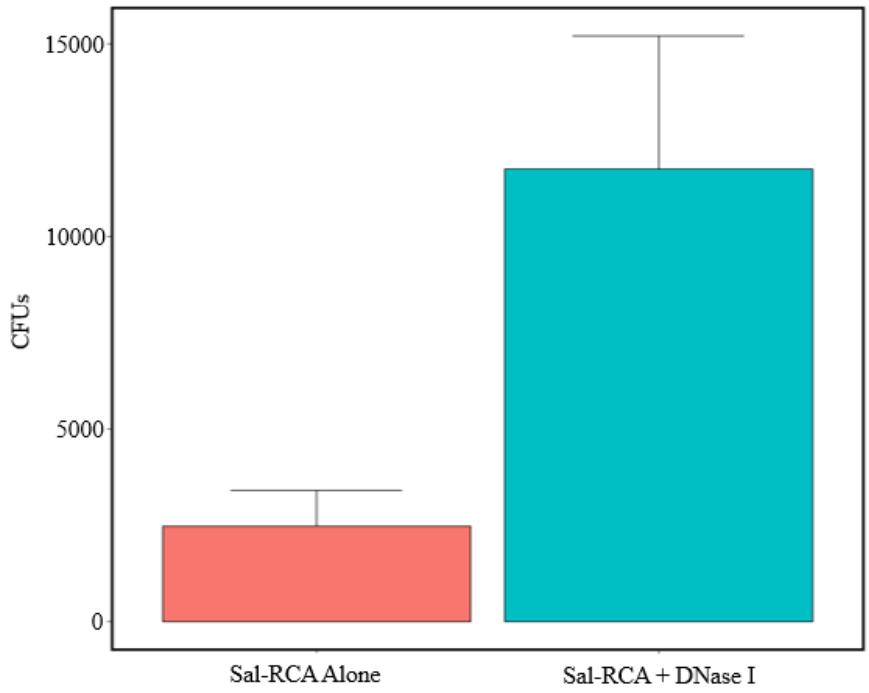


Figure 7. Estimation of the number of *S.* Typhimurium cells loaded to *Salmonella*-RCA network using DNase I digestion to release *S.* Typhimurium cells captured in RCA network

CONCLUSIONS

In the current study, we attempted to develop a novel RCA network complex as a vaccine candidate to control *Salmonella* serotypes in broiler chickens. This RCA network complex consists of two RCA products with specific binding affinity against various serotypes of *Salmonella* (3Sal-aptamer RCA) and CD40 receptor (aptamer RCA II). We produced this complex and demonstrated *S.* Typhimurium cells can be securely loaded within the complex. In the next step of this project, we will evaluate the efficacy of this novel RCA network complex loaded with *Salmonella* as a novel vaccine to control *Salmonella* in broiler chickens.

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SUPPLEMENTARY MATERIALS

Table S1. DNA sequences of the aptamers used in this study.

Name	DNA Sequence (5′ → 3′)	References
ST1	6FAM-CCGATGTCCGTTAGGGCTCCTCCATAGAT	Park et al., 2014; Silva et al.
SAL26	6FAM-TAGCTCACTCATTAGGCACATTTGTGGCACCAAATTTGAATTAATCAAGACAGTGTGGTGCATAGTTAAGCCAGCC	Lavu et al., 2016
ST2P	6FAM-ATAGGAGTCACGACGACCAGAAAGTAATGCCCGGTAGTTATTCAAAGATGAGTAGGAAAAGATATGTGCGTCTACCTCTTGACTAAT	Duan et al., 2013
33	6FAM-TATGGCGGCGTCACCCGACGGGGACTTGACATTATGACAG	Joshi et al., 2009
S8-7	6FAM-CTGATGTGTGGGTAGGTGTCGTTGATTTCTTCTGGTGGGG	Dwivedi et al., 2013
C4	6FAM-ACGGGCGTGGGGGCAATGCCTGCTTGTAGGCTTCCCCTGTGCGCG	Moon et al., 2013
SEQ3 (Illumina)	CCGAATTCGAAGGACAAGAGGTGGAATTGGTAATGGGGTGTAAATGGAGCAGTGAATTCGTCTTTTATGCTACGTCCCGC	
SEQ4 (Illumina)	CCGAATTCGAAGGACAAGAGTAGGGCTACATGGAATAGGGATCAGAAGAGCAGGGCTAGGTCTTTTATGCTACGTCCCGC	
Aptamer RCA II template	GCATCTGAAGCGGACGTAGCATAAAAGACGAATTCACTGCTCCATTTACACCCCATTACCAATTCCACCTCTTGTCCTTCGAATTCGGGATCCACCGTAGCAGCGGGACGTAGCATAAAGACCTAGCCCTGCTCTTCTGATCCCTATTCCATGTAGCCCTACTCTTGTCCTTCGAATTCGGGGAACGTCTT	
3Sal aptamer RCA template	Phospho-AAGACGTTCCCGCGCACAGGGGAAGCCTACAAGCAGGCATTGCCCCCACGCCCGTGTCACCCCACCAGAAGAAATCAACGACACCTACCCACACATCAGGTCACTGTCATAATGTCAAGTCCCCGTCGGGTGACGCCGCCCATATTCAGATGC	
B-primer	Biotin-GGGAACGTCTTGCATCTGAA	
Primer with no Biotin	GGGAACGTCTTGCATCTGAA	