Laboratory Visit to Carlton University, Canada to work with Dr. Maria DeRosa

Dr. Valeria A. Torok

AUSTRALIAN SEAFOOD COOPERATIVE RESEARCH CENTRE

Project No. 2014/703

This project was conducted by
SARDI, Food Safety and Innovation, Adelaide, Australia
and
Carlton University, DeRosa Laboratory, Ottawa, Canada

The Australian Seafood CRC is established and supported under the Australian Government's Cooperative Research Centres Program. Other investors in the CRC are the Fisheries Research and Development Corporation, Seafood CRC company members, and supporting participants.

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Australian Government
Fisheries Research and Development Corporation

An Australian Government Initiative
CRC AUSTRALIA

ISBN: 978-0-9874218-5-2

NON-TECHNICAL SUMMARY

PROJECT NO 2014/703: LABORATORY VISIT TO CARLTON UNIVERSITY, CANADA TO WORK WITH DR. MARIA DEROSA

PRINCIPAL INVESTIGATOR: **Dr Valeria A Torok**

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OBJECTIVES OF RESEARCH TRAVEL GRANT

1) Assist in the development of improved methods of analysis for viruses in seafood, currently being undertaken in Seafood CRC project 2011/726
2) Increased knowledge in the process of generation of aptamers for food safety diagnostic tests, specifically in the area of pathogen, toxin, chemical or adulterant detection.

NON TECHNICAL SUMMARY:

Dr Torok spent two weeks (8^{th}-19^{th} September 2014) in the laboratory of Dr Maria DeRosa undertaking experiments to generate specific bioreceptors (aptamers) for human norovirus (NoV). The De Rosa Laboratory is actively involved in the development of biosensors and 'smart' materials based on DNA aptamers. Aptamers are single-stranded oligonucleotides that fold into distinct three-dimensional conformations, capable of binding strongly and selectively to a target molecule and have several advantages over antibody based approaches. The DeRosa group is developing aptamers to various targets including neurotransmitters, viruses, disease biomarkers and toxins, as well as utilising these in the development of electrochemical and optical biosensors.

Non-infectious norovirus like particles (NoV GII.4 VLP) generated within the SARDI Food Safety Laboratory to a recent pandemic strain were sent to the DeRosa laboratory to enable selection of aptamers specific to our target of interest. VLP are antigenically and structurally similar to the infectious virus, although they lack the virus genome and hence are non-infectious. An aptamer developed in the De Rosa laboratory to a related virus (murine norovirus; MNV) showed some specificity to human norovirus (NoV). The nucleic acid pool from which this aptamer was isolated was used to undertake further rounds of selection (Systematic evolution of ligands by exponential enrichment; SELEX) to identify aptamers highly specific to NoV. It was possible to recover nucleic acid from the pool during selection which appeared specific to NoV. By sequencing these resulting nucleic acid pools we will able to identify candidate aptamers specific to NoV. These will then be able to be used as bioreceptors in the development of novel biosensors to NoV.

OUTCOMES ACHIEVED TO DATE

- Selection for aptamers specific to human norovirus completed;
- Future plans to complete the work collectively agreed and in progress;
- The knowledge of systematic evolution of ligands by exponential enrichment (SELEX) established in the SARDI Food Safety and Innovation group;
- International collaboration with Carlton University established

(PROJECT) OUTPUTS DEVELOPED AS RESULT OF TRAVEL GRANT/ INDUSTRY BURSARY:

- Aptamer bioreceptors for human norovirus are being developed
- International collaborative relationship established between SARDI Food Safety and Innovation and Carlton University
- Professional development of the principal investigator

ABOUT THE PROJECT/ACTIVITY

BACKGROUND AND NEED

A major focus of our Seafood CRC funded project (2011/726: Wanted Dead or Alive - Novel Technologies for Measuring Infectious Norovirus Particles) is the development of sensitive biosensors for the detection and discrimination of infective human norovirus (NoV). A critical step in achieving this is the biological functionalisation of the sensor platform. We identified the De Rosa Laboratory as having specialised knowledge in bioreceptor production (aptamers) which would significantly assist in realising our goal. Aptamer based technology has shown potential for use as a diagnostics in many food safety areas, including pathogens, biotoxins and chemicals (pesticides, antibiotics and adulterants). Aptamers are single-stranded oligonucleotides that fold into distinct three-dimensional conformations, capable of binding strongly and selectively to a target molecule and have several advantages over antibody based approaches.

The DeRosa Laboratory also has knowledge in utilising aptamers for the development of electrochemical and optical biosensors. The majority (90%) of the work being undertaken in the DeRosa laboratory involves the production of aptamers to various targets. Some of the work currently being done includes SELEX selection to mycotoxins (fumonisin B1, ochratoxin, deoxynivalenol) and other toxins, plant exudates, biomarkers, food additives, viruses and flocculants. They also engage in research in the area of DNA nanotechnology and biosensor development. Within Australia there is only a narrow group of experts within this related field of research, hence limited opportunity for collaboration.

The DeRosa Laboratory had recently published a paper on the detection of murine norovirus (MNV) using an aptamer (AG3) developed to this target (Giamberardino *et al.,* 2013). When AG3 was incorporated into a simple electrochemical sensor using a gold nanoparticle-modified screen-printed carbon electrode it was reported to have a limit of detection of approximately 180 virus particles. This makes it one of the most sensitive biosensors reported to date for a member of the genus *Norovirus*. This aptamer was reported to have some cross reactivity to human norovirus (NoV GII.3). SARDI, Food Safety and Innovation used the published AG3 aptamer to try and capture another genotype of human norovirus (NoV GII.4). Using an ELISA based assay we found that AG3 cross-reacted with NoV GII.4. This finding led to Dr Torok making contact with Assoc Prof Maria DeRosa and discussions on collaborative work to develop a specific aptamer for NoV. Such an aptamer would be advantageous in developing a novel biosensor to NoV. Furthermore, the skills learned in aptamer production could be applied to other seafood related safety organisms and toxins, assisting in the development of rapid, sensitive, cheap and point of care diagnostic devices.

RESULTS

The international laboratory visit enabled detailed discussions and interaction with peers in the field for biosensor production. During the visit Dr Torok was invited to give a presentation at Carlton University on her Australian research (Norovirus & Hepatitis A virus: unlabelled ingredients you don’t want!). She was also invited to

give the same presentation at Health Canada, Bureau of Microbial Hazards in Ottawa. Following this presentation she was shown their diagnostic laboratories involved in foodborne virus research and the development of “laboratory on a chip” technology.

The exchange has also enabled the technical skill base within Australia to grow. This should support further research in the expanding area of novel diagnostics for food and seafood safety.

The results of experimental work done during the visit are detailed in Appendix A. In brief, NoV GII.4 virus like particles (VLP) were sent from SARDI to the DeRosa laboratory prior to the laboratory visit. These VLP are non-infectious as they lack the viral genome, but they are structurally and immunogenetically similar to an infectious virus. The integrity of the structure of the VLP had been reconfirmed by transmission electron microscopy (TEM) shortly before the visit. The MNV aptamer pool which had been developed to MNV following nine rounds of SELEX (DeRosa laboratory) were used as the starting point for screening for more specific aptamer to NoV GII.4. Four round of SELEX were undertaken and NoV GII.4 ssDNA pools identified. These will be further characterised collaboratively.

INDUSTRY IMPACT

Industry needs for rapid, reliable point of care diagnostics.

The presence of unsafe levels of chemical compounds, toxins, and pathogens in food constitutes a growing public health problem that necessitates new technology for the detection of these contaminants along the food chain from production to consumption. Many current food safety diagnostic methods have limitations in sensitivity, specificity, the ability to discriminate viable (infectious) from non-viable organisms, and the requirement for expert personnel and laboratories to undertake testing. Current methodologies are also often time consuming and expensive. The time to assess the risk of seafood products using available detection technologies is a significant issue for industry, as delays in analysis can impact harvest and transport strategies. Furthermore, limitation in both the sensitivity and specificity of some available detection methodologies may result in high risk products being present within the marketplace. Rapid, sensitive, cheap and on-site detection methodologies would allow industry to be proactive in testing, making increased screening for pathogens and contaminants financially viable and reducing testing turnaround times. This would allow industry to make timely decisions, either about product quality or safety, hence improving further decisions making (e.g. suitability for harvest, requirement/suitability for additional processing, potential risk management options). Examples, where rapid, reliable point of care diagnostics would benefit the industry include norovirus in oysters, ciguatoxins in finfish and marine biotoxins in seafood. Short term research needs to focus on building the processes to develop new technologies, including increasing national and international collaborations.

Internationally there is much research being undertaken into developing rapid diagnostics based on biosensors. The main components of a biosensor are the “bioreceptor”, which directly interacts with the target (pathogen, toxin or chemical),

and the “transducer”, which transforms this interaction into a physical or chemical response. The transducer determines the effectiveness of the signal processing and the output of the biosensor, while the bioreceptor determines the selectivity of the biosensor.

Aptamer based technology has shown potential for use as a diagnostic in many food safety areas, including pathogens, biotoxins and chemicals (pesticides, antibiotics and adulterants). Aptamers are single-stranded oligonucleotides that fold into distinct three-dimensional conformations, capable of binding strongly and selectively to a target molecule and have several advantages over antibody based approaches. Unlike the preparation of antibodies which relies on induction of an animal immune system, aptamer fabrication can be achieved for non-immunogenic and toxic targets. Moreover, it is possible to produce aptamers to specific regions of targets. Using aptamers as bioreceptors has the following advantages: high specificity and affinity to target; ability to target small molecules, large proteins and cells; once selected they can be synthesized with high reproducibility and purity from commercial sources (cost effective to produce); unlike antibodies or enzymes aptamers are highly chemically stable; and aptamers often undergo significant conformational changes upon binding the target. Together these offer great flexibility in the design of novel biosensors with high detection sensitivity and selectivity.

COMMUNICATION OF PROJECT/EXTENSION ACTIVITIES

Communication of the work will be via a collaborative scientific publication. Aptamers identified will be used to developed biosensors for NoV in oysters with collaborators possessing required skill sets.

KEY CONTACTS

Assoc Prof Maria DeRosa (Group Leader, Carlton University)

Ms Eman Hassan (PhD Student, Carlton University)

Ms Erin McConnell (PhD Student, Carlton University)

Mr Enrico Buenaventura (Section Head, Risk Assessment, Bureau of Microbial Hazards, Health Canada)

Dr Sabah Bidawid (Chief, Microbiology Research Division, Health Canada)

Ms Oksana Mykytczuk (Technician, Health Canada, Food Virology Laboratory)

ACKNOWLEDGEMENTS

Australian Seafood CRC

South Australian Research and Development Institute

APPENDIX A

Experimental work undertaken at Carlton University, Ottawa, Canada

Appendix A:

Experimental work undertaken at Carlton University, Ottawa, Canada

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Background

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodology

Systematic evolution of ligands by exponential enrichment (SELEX) is an *in vitro* selection for producing oligonucleotides of single-stranded nucleic acid that specifically bind to a target. The process begins with the synthesis of a very large oligonucleotide library consisting of randomly generated sequences of fixed length flanked by constant ends which enables the selected pool to be amplified by polymerase chain reaction (PCR). The sequences in the library are exposed to the target and those that do not bind the target are removed (positive selection). The bound sequences are eluted and amplified by PCR to prepare for subsequent rounds of selection in which the stringency of the elution conditions is increased to identify the tightest-binding sequences. The technique has been used to evolve aptamers of extremely high binding affinity to a variety of target ligands. Figure 1 outlines the process of selection.

One of the most critical steps in the SELEX procedure is obtaining single stranded DNA (ssDNA) after the PCR amplification step which the serves as input for the next cycle of selection. It is important that all the DNA is single stranded and as little as possible is lost. This can be achieved by a variety of methods. In our work this was achieved by adding a fluorescence tag to the forward primer and hexaethyleneglycol (HEGL) spacer followed several adenine nucleotides (polyA tail) to the reverse primer. The HEGL spacer acts as a terminator for the Taq polymerase during PCR. Hence, the DNA strand amplified with the forward primer is shorter than the DNA strand amplified by the reverse primer. The two strands can then be separated by size using electrophoresis under denaturing condition.

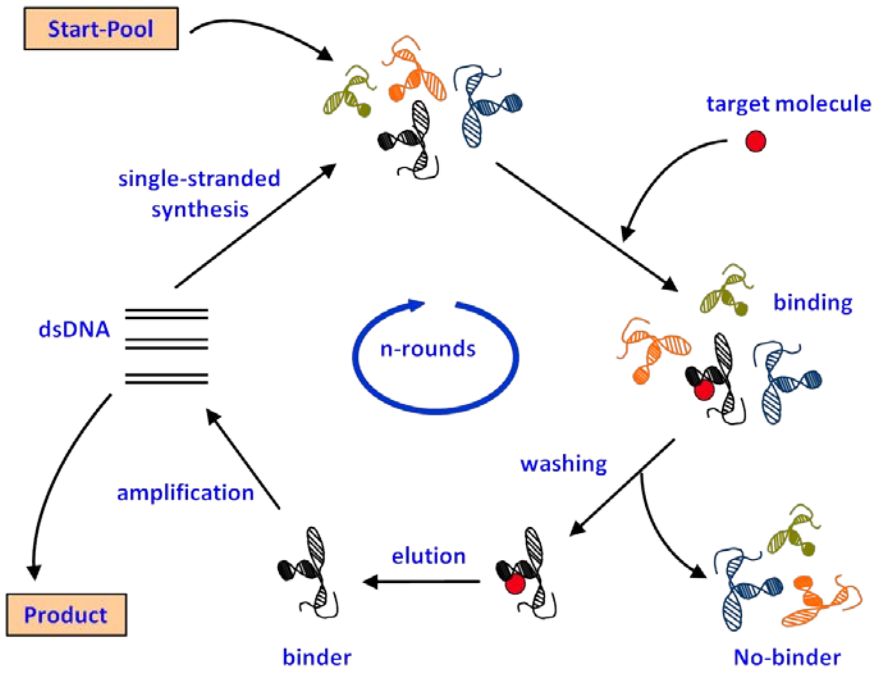

Figure 1: SELEX process.

Background work

Giamberardino *et al*., (2013) had developed an aptamer (AG3) specific to murine norovirus (MNV) capable of detecting 180 virus particles when used at the bioreceptor on an electrochemical biosensor. This aptamer also detected human norovirus (NoV) GII.3, although the level of sensitivity/specificity was much lower. We were interested to see if this aptamer also showed cross-reactivity with another genogroup of NoV (GII.4). Using enzyme linked immunesorbent assay (ELISA) I was able to show that AG3 bound NoV GII.4 virus like particles (VLP). This suggested that the SELEX pool used to generate the MNV aptamer may contain other DNA populations more specific to NoV. As part of the DeRosa laboratory visit, NoV GII.4 VLP generated in the laboratory of SARDI Food Safety and Innovation were sent to the DeRosa Laboratory to undertake further rounds of SELEX and hopefully identify a more specific NoV aptamer.

Aims:

1. To learn the SELEX methodology for aptamer generation to a target of interest.
2. To select aptamers specific to human norovirus like particles (NoV GII.4 VLP) from a SELEX library previously developed to murine norovirus (MNV) in the DeRosa laboratory.

Methodology:

SELEX on human NoV GII.4 VLP

Round 9 of the SELEX library generated to MNV by Giamberardino *et al*., (2013) was used as the initial DNA pool for selection against NoV GII.4 VLP. Four rounds of SELEX were performed, two during the laboratory visit and a further two post the laboratory visit by Eman Hassan. Negative selection was done to nitrocellulose. Positive selection was done with NoV VLP either in the absence of oyster digestive tissue (no Matrix) or supernatant of homogenized oyster digestive tissue in PBS (1:1) w/v (Matrix). Prior to each round of SELEX, approximately 100-500 pmol of DNA pool in 100 µL of general sensing buffer (GSB; 50 mM NaCl, 20 mM Tris-HCl pH 7.4, 3 mM MgCl2, 5 mM KCl) was denatured by heating at 95^{o}C for 5 minutes and allowed to cool for 10 min at 4^{o}C followed by 10 min at room temperature. A negative selection was first carried out to reduce non-specific interactions of the pool with the partitioning medium, a 0.45 µm nitrocellulose filter (Millipore). Following negative selection DNA was re-quantified to determine how much was lost. In the first rounds of SELEX, the pool was incubated with either no oyster matrix (400 µL GBS) or oyster matrix (400 µL oyster gut supernatant) for 30 min while shaking at room temperature and then filtered through partitioning media (nitrocellulose) to remove sequences binding non-specifically to oyster gut (in the case of the Matrix sample). The flow through was then incubated with 0.3 mL of 10^{7} VLP/mL (approximately 1,000,000 VLP) of NoV GII.4 for 60 minutes while shaking at room temperature and then filtered through a new nitrocellulose membrane to remove non-interacting sequences. The filters were washed with GSB to facilitate removal of non-binding sequences. The binding complex was eluted by suspending the filter in 300 µL elution buffer (7M urea, 50 mM HEPES-NaOH, pH 7.5, 10 mM EDTA) and heating at 95^{o}C for 5 minutes and removing buffer. This was repeated five times in total. The binding sequences were purified using phenol/chloroform extraction

followed by ethanol precipitation and re-suspension. The DNA pool was desalted with Amicon Y-30K ultrafiltration tubes, as per the manufacturer's instructions. The binding pool was checked by fluorescence spectroscopy and quantified by UV absorption (A_{260}).

The DNA binding pool was then amplified by conventional PCR. Primers used for amplification of ssDNA DNA library were PrFor (5'-6-FAM-CGT ACG GAA TTC GCT AGC-3') and PrRev (5'- A_{20}-HEGL-CAC GTG GAG CTC GGA TCC-3'). PCR was done in a reaction volume of 107 µL containing 50 µL of PCR buffer (100 mM KCl, 200 mM Tris, 2% Triton X-100, pH 9), 8 µL of 25 mM $MgCl_2$, 2 µL of 10 mM dNTP, 0.5 µL each of 0.2 mM PrFor and PrRev, 1 µL of 500U Taq polymerase, 40 µL sterile milliQ water and 5 µL of ssDNA pool template. PCR amplification cycling conditions were as follows: initial denaturation at 94^{o}C at 5 min followed by 20 cycles of denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C for 30 sec and extension at 72^{o}C for 30 sec and then a final extension at 72^{o}C for 10 min. PCR products were resolved on an 8% denaturing PAGE. Bands were excised, homogenized in 7 mL H_2O and incubated with shaking overnight at 37^{o}C. This was filtered through a 0.22 µm PES syringe filter and the then desalted as above. The ss DNA pool was quantified by spectrophotometry.

The stringency of SELEX was increased by lowering the level of target (VLP) and decreasing incubation time for binding of ssDNA pool with target. Second round SELEX was done with and without matrix and 100,000 VLP and incubated at room temperature for 30 min. Third round SELEX was done with and without matrix and 100 VLP incubated at room temperature for 30 min. Another round SELEX was done with ssDNA pool from round two with matrix and 1,000 VLP incubated at room temperature for 30 min.

Results and Discussion:

By incubating the ssDNA pool with oyster gut matrix it would be anticipated that ssDNA which would non-specifically bind oyster gut would be eliminated. Both matrix and no matrix selections were incubated with decreasing numbers of VLP to increase the stringency and select for the most specific aptamers to NoV VLP. In the first round of selection using 1,000,000 VLP resulted in 12-13% of the original ssDNA pool binding to NoV VLP, while 0.9-1.7% of the negative selection pool bound non-specifically to the nitrocellulose filter (Figure 2).

The second round of selection using 100,000 VLP resulted in a higher percentage of the DNA pool binding to the matrix sample than to the no matrix sample, although for this sample the negative selection binding was also higher than for the no matrix sample. It was decided to do a further more stringent round of SELEX before sequencing of the pool to optimise for the selection of more specific NoV aptamers. 100 VLP were used for the third round of selection, however this may have been too stringent as positive selection resulted in lower or similar percentage binding as the negative selections. For this reason a further selection was done using the round two ssDNA pool and a higher number of VLP (1000). Although only 2% of the ssDNA library bound for this positive selection it is not unexpected that a lower percentage binding would occur as fewer targets available for capture are present. It is promising that the percentage binding of the negative selection is lower than the positive selection.

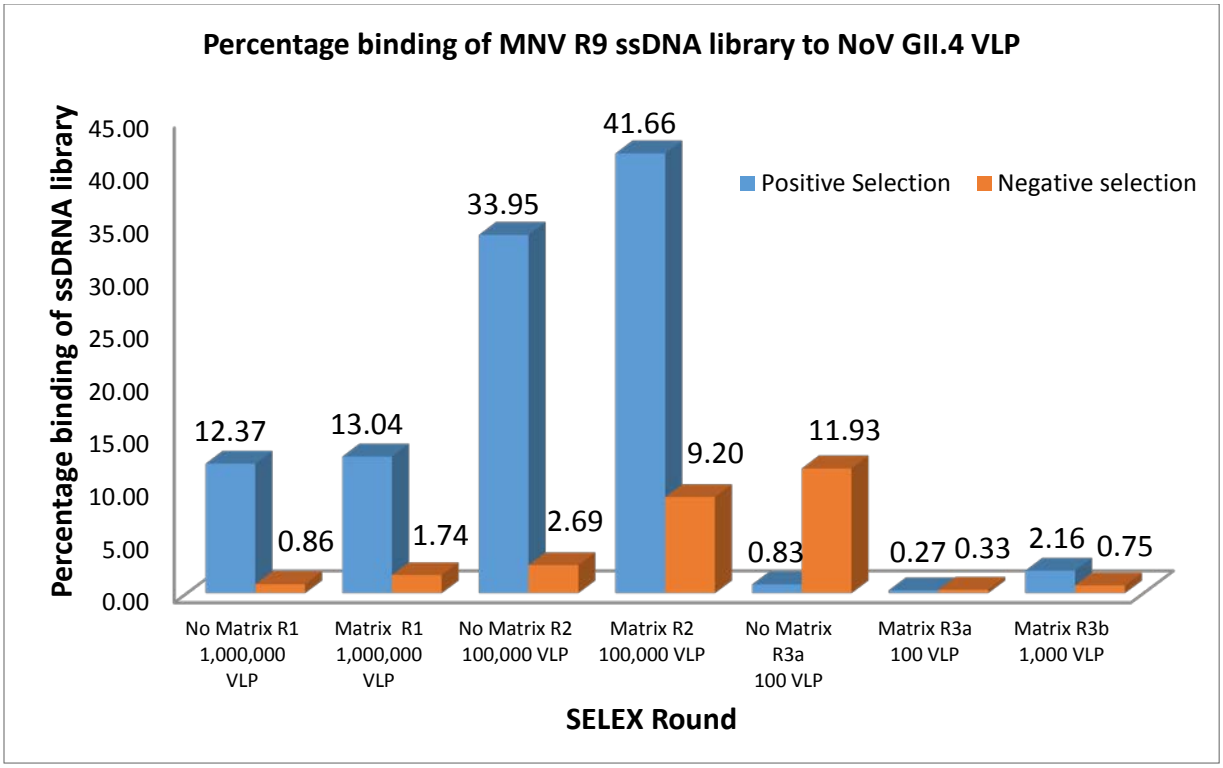

Figure 2: Percentage binding of MNV R9 ssDNA library to NoV GII.4 VLP over several rounds of SELEX each varying in stringency.

Future work:

DNA from the SELEX rounds 1, 2 and 3b will be sent to SARDI for high throughput sequencing (illumina). This will allow identification of sequences enriched and specific for NoV GII.4. The binding kinetics of identified aptamers to NoV will be determined by anisotropy of commercial surface plasmon resonance (Biacore or ProteOn). Characterised aptamers will be incorporated into a biosensor platform for NoV detection.

References

Giamberardino, A., M. Labib, E. M. Hassan, J. A. Tetro, S. Springthorpe, S. A. Sattar, M. V. Berezovski and M. C. DeRosa (2013). "Ultrasensitive norovirus detection using DNA aptasensor technology." PLoS ONE **8**(11): e79087.

Acknowledgement

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