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Antibody-Based Diagnostics and Production for High Consequence Animal Pathogens II

Final Report for PLA 6/ CRADA PNNL/176- with New Horizons Diagnostic Corporation

Larry Loomis

January, 2012

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Pacific Northwest National Laboratory
Richland, Washington 99352

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1.0 Background

New Horizons Diagnostic Corporation (NHD) develops, manufactures, and tests products for the detection of bacteria and toxins in human, environmental, surface, food, and water samples for government, municipal and corporate customers. It offers diverse medical products, such as products for testing cholera, gonorrhea, syphilis, and drug abuse screening products. The major product line for NHD is the development, marketing and sales of rapid antibody based diagnostic kits. Infectious diseases spread through food or water is a common, distressing, and sometimes life-threatening problem for millions of people in the United States and around the world. World Health Organization (WHO) studies have shown that in 1996, infectious and parasitic diseases accounted for 43% of the 40 million deaths in developing countries. The U.S. Centers for Disease Control and Prevention (CDC) estimates 76 million people suffer foodborne illnesses each year in the United States, accounting for 325,000 hospitalizations and more than 5,000 deaths.

There are more than 250 known food borne diseases. Bacteria cause most cases, followed by viruses and parasites. Some diseases are caused by toxins (poisons) from the disease-causing organism, others by bodily reactions to the organism itself. People infected with food borne germs may have no symptoms or develop symptoms ranging from mild intestinal discomfort to severe dehydration and bloody diarrhea. To reduce the severity as well as the numbers, a proactive farm to table approach may be useful. The first level of safety begins with the animal safety and health. Addressing this area will not only improve safety but also improve the economic position of the agro market. In addition, pesticides in food give rise to an estimated 6,000 cancer cases annually in the US. The Environmental Protection Agency (EPA) estimated, in the early 1990s, that approximately 43% of the water systems serving 120 million people had EPA violations, and nearly one million people became ill due to contaminated water.

Recently, national authorities in countries around the world have expressed growing concern over keeping a nation's food and water supply safe from deliberate acts of adulteration or contamination. The bioterrorism threat is being studied by a number of U.S. agencies, including the Food and Drug Administration, Department of Agriculture, Centers for Disease Control and Prevention, Environmental Protection Agency, Department of Homeland Security

and National Institutes of Health. The company also provides bio threat detection products, including diagnostic kits, collection kits, and environmental kits.

The Scientific Research Agricultural Institute of Kazakhstan (NISKhI) has, since 1958, identified specific diagnostic antigens and produced specific antibodies to a wide range of critically important animal and crop diseases of interest. The institute has broad expertise in highly pathogenic and exotic diseases of livestock and crops caused by viruses and other agents. Among these are Rinderpest virus, Newcastle disease virus, African swine fever virus, sheep pox virus, goat pox virus, fowl pox virus, blue-tongue virus, herpes virus and cereal rust fungi.

This collaborative effort, if successful, will permit the identification and development of new and improved diagnostic kits for the targeted diseases. New Horizon Diagnostics matched DOE's funding through a Cooperative Research and Development Agreement (CRADA). That CRADA is the subject of this report. The proposal was ultimately funded and a CRADA between NHD and PNNL was established. PNNL managed the activities at the Scientific Research Agricultural Institute of Kazakhstan (NISKhI) to support the efforts between NHD and (NISKhI) in developing commercially viable diagnostics and therapeutics.

2.0 Progress

The goal of this project was to use existing data and archived inventories for a series of antigens and antibodies specific Rinderpest and Newcastle Disease virus to develop new and improved rapid diagnostic kits (3-5 min.) for animal, environmental, and food-borne disease detection and therapeutic reagents for treating livestock diseases. NHD has global distribution and sales networks for both low-end and high-end diagnostic kits and treatments. Based on its prior experience with former-BW facilities in the FSU, NHD strongly believes that the antibody systems have high commercial value. The Scientific Research Agricultural Institute of Kazakhstan (NISKhI) was responsible for assuring the production of all reagents called out in this project by standard, conventional methods (hybridomas, polyclonal production, tissue culture, or recombinant DNA). All antigens (Ag) were certified inactivated by standard laboratory practices, and used to determine assay sensitivity, product release, and quality assurance. NISKhI screened existing collections and libraries to select candidate reagents that targeted the prioritized viruses. This was a one-way disclosure of technology and capabilities. New Horizon Diagnostics (NHD), the commercial partner for this project, established its priorities based on the maturity and availability of antigens and antibodies for specific pathogens, the need to further reduce any

reagents to practice, and market drivers. The project is now complete. The research activities were divided into two tasks that were completed during two separate phases. As noted below Task 1 was focused on the Rinderpest virus and Task 2 addressed the development of reagents for the detection of Newcastle Disease virus.

Task 1. NISKhI to produce and supply antigen and antibody for Rinderpest virus specific diagnostic kits.

During the first year of research, activities were conducted to develop reagents (antigens and antibodies) for the detection of the Rinderpest virus including the selection, propagation, concentration and purification of the selected virus strain ("K_{37}70"). The virus biomass was obtained from cultured tissue cells exhibiting significant morphological changes associated with viral infections. Density gradients and differential centrifugation methodologies were used to concentrate and purify virus particle (Figure 1) from virus-containing suspensions. Using a starting suspension of 2 liters yielded 4 ml (~10mg) of 99.8% pure viral suspension with an infectivity titer logarithms of 5.5 lg TCE50/ml in tissue culture (Table 1).

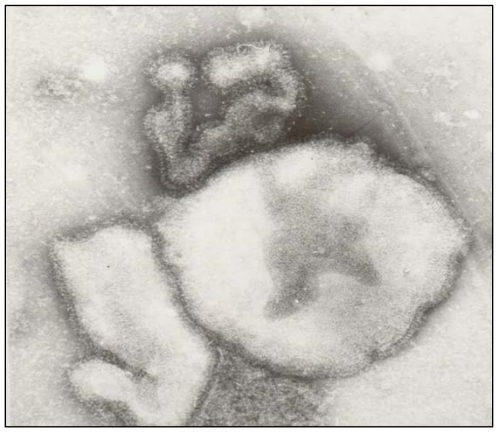

Figure 1 – The purified preparation of the Rinderpest virus, strain "K_{37}70". Negative contrasting with 2% PTA. Magnification ×100000

Purification Step	Volume, ml	Protein, mg/ml	Protein, mg	Purity, %
Original suspension	5000	3.33	16650.00	-
Clarification at 2000g	4990	3.03	15119.7	12.0
Precipitation with PEG6000	500	2.98	1490.00	90.3
Stepped gradient	20	0.97	19.4	99.1
Linear gradient	10	0.04	0.40	99.8

Table 1 – Rinderpest virus purification and concentration parameters

The next step was the isolation and purification of Rinderpest viral antigen to determine specificity and activity in selected serological assays (ELISA, DPT and CAE). During the 3rd quarter, the activities

were directed at the characterization of the isolated Rinderpest viral antigens using SDS-PAGE electrophoresis and immunization studies for the induction of antibody formation in challenged mice. Results from the electrophoresis studies indicated the presents of six basic polypeptides with molecular weights 212.0, 92.3, 68.0, 65.0,58.9 and 38.3 kD amounting to ~ 90 % of the isolated viral protein (Figure 2).

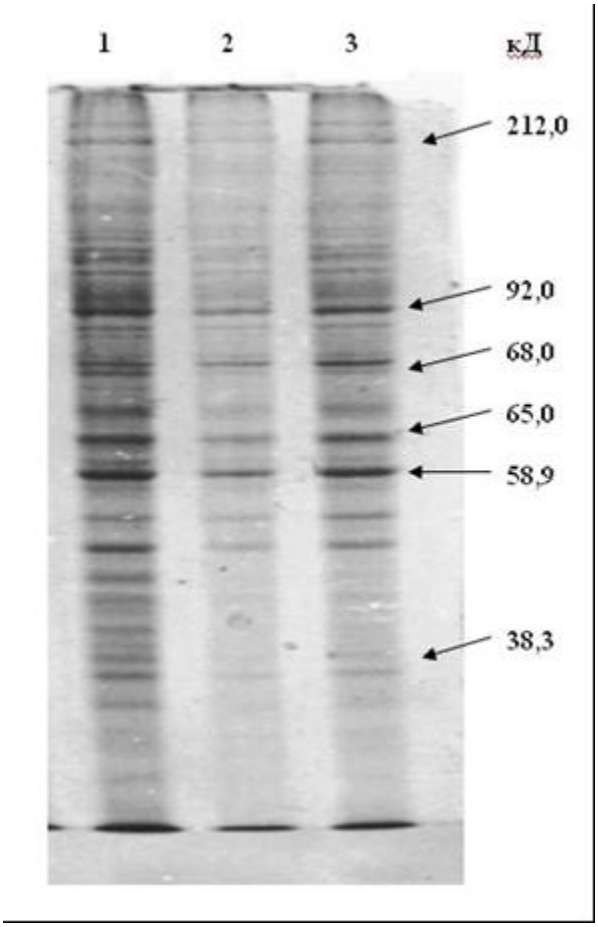

Figure 2 – Electrophoresis of the Rinderpest virus polypeptides in 5-20% PAAG gradient. 1, 2 & 3 – Three independent protein preparations of the Rinderpest virus, strain "$K_{37}70$"

For the preparation of immune spleen cells, mice were challenged using a mixture of Freund's adjuvant and 100 ug of viral protein. These immune spleen cells were fused with myeloma cells to produce hybridoma for the production of monoclonal antibodies with specificity for Rinderpest virus. Initial screening and characterization of hybridoma cells producing monoclonal antibodies were completed during the first year.

During the second year, efforts were focused on the production, purification and screening for specificity and binding activity of antibodies of selected clones As a result, 13 clones were identified producing specific antibodies to the Rinderpest virus. To expand the yield of antibodies, mice were

immunized intraperitoneally to induce ascites fluid formation. Antibodies were purified from ascites fluid using ammonium sulphate fractionation. Monoclonal antibodies were produced from six clones with two clones exhibiting high titer activity based on immune fluorescent methodology. These two clones were shown to not cross-react with antigens from related viruses (canine distemper, and peste des petit ruminants). During the last two quarter of year 2 the investigators screened clones for specificity to Rinderpest virus and cross-reactivity to canine distemper (CD) and peste des petit ruminants (PPR) using ELISA assays. Two antibodies (4G2D4 and 4G2G4) were shown to bind to all three viral antigen preparations and are thus group specific. One clone (2C1F7) was species-specific and only exhibited binding activity with the Rinderpest virus. Additional characterization experiments were conducting using immunoblotting methodologies. This permitted the visualization of activities to specific antigen protein fractions. The results from this investigation indicated that the two antibodies (4G2D4 and 4G2G4) reacted with the F-protein (molecular weight of 46kDa). There was significant activity observed with several protein bands by the 2C1F7 antibody. It was recloned. This process yield 7 new clones. The specificity of these new clones were tested against homologous and heterologous antigens. All 7 clones were positive for binding activity to Rinderpest virus and negative for activity against CD and PPR, as well as, avian influenza and Newcastle virus. Immunoblotting analysis against Rinderpest virus proteins was conducted using monoclonal antibodies from all 7 clones (Figure 3). Only activity was seen for the protein migrating at a molecular weight of 46kDa. All clones were isotyped and determined to be of the IgM class.

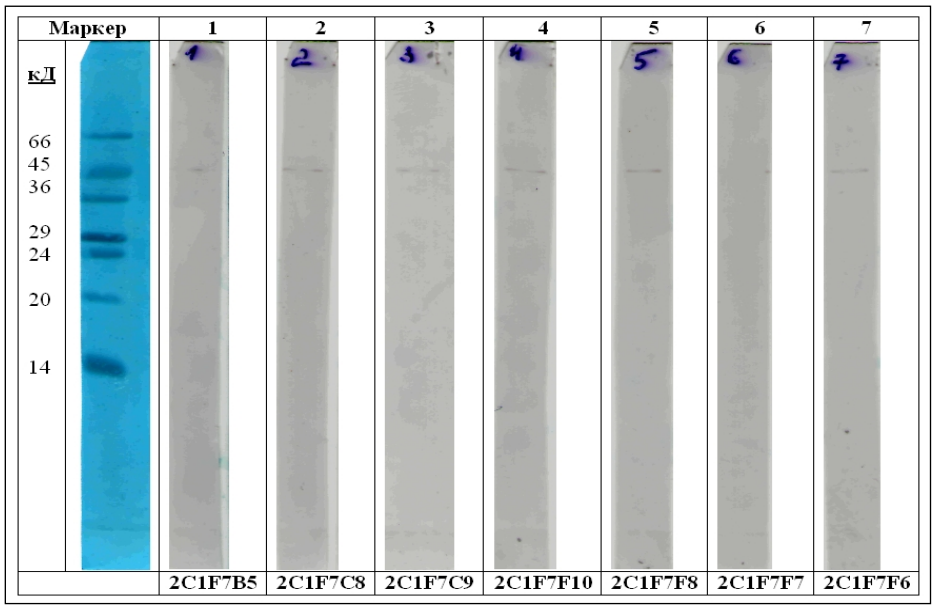

Figure 3 – Results of immunoblotting of seven Mabs to the Rinderpest virus antigens (a reactive band can be seen at the 46 kDa molecular binding position).

Task 2. NISKhI produces and supplies antigen and antibody for Newcastle disease virus (NDV) specific diagnostic kits.

Research activities focused on the Newcastle disease virus were initiated during the second quarter of the first year. This included the cultivation, purification, and concentration of the selected "La-Soto" Newcastle disease virus stain (Table 2). Following the inoculation of chicken embryos with virus, eggs were incubated at 37^0C for 3 days. The inoculated fertile eggs were chilled to 4^0C and allantoic fluid collected. From this exercise, 2 liters of allantoic fluid was collected with a infectivity activity of 8.5 lg EID_{50}/ ml. Virus purification and concentrated was accomplished using low and high speed centrifugation with viral preparations assessed using electron microscopy (Figure 3). The yield of material was ~ 5 ml (12mg).

Purification Step	Amount, cm^3	Protein, mg/cm^3	% of purification
Original AF	2000	103.61	-
Clearing 2000g	1950	94.43	9.41
Sucrose pad	5	2.33	99.98

Table 2 – Parameters of the Newcastle disease virus purification and concentration

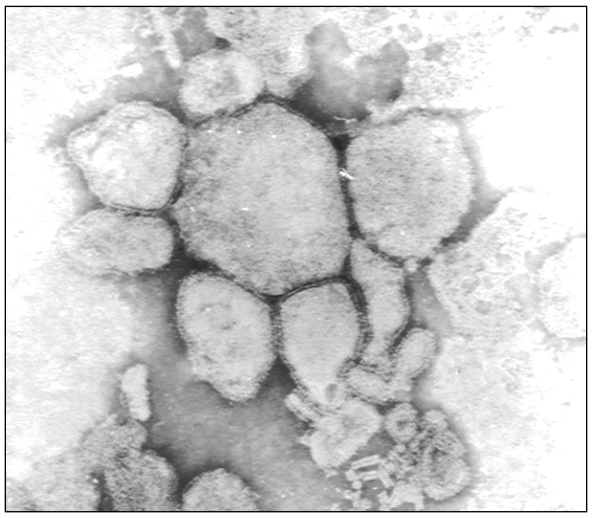

Figure 4 – Purified preparation of the Newcastle disease virus, "La-Sota" strain. Negative contrasting with 2% PTA × 100000.

During the third quarter of year 1, Newcastle disease virus proteins were isolated, purified and characterized. SDS-PAGE electrophoresis was employed to determine the molecular weight of identified polypeptide molecules. Four separate proteins were identified exhibiting bands at 202.0, 83.3, 56.0 and 43.6 kDa (Figure 5). This antigen preparation was used to immunize mice for monoclonal antibody preparation. Balb-C mice were challenged with a mixture of Freund's complete adjuvant and 100 ug of

the Newcastle disease virus preparation to generate immune lymphocytes for the fusion studies to generate hybridoma cells.

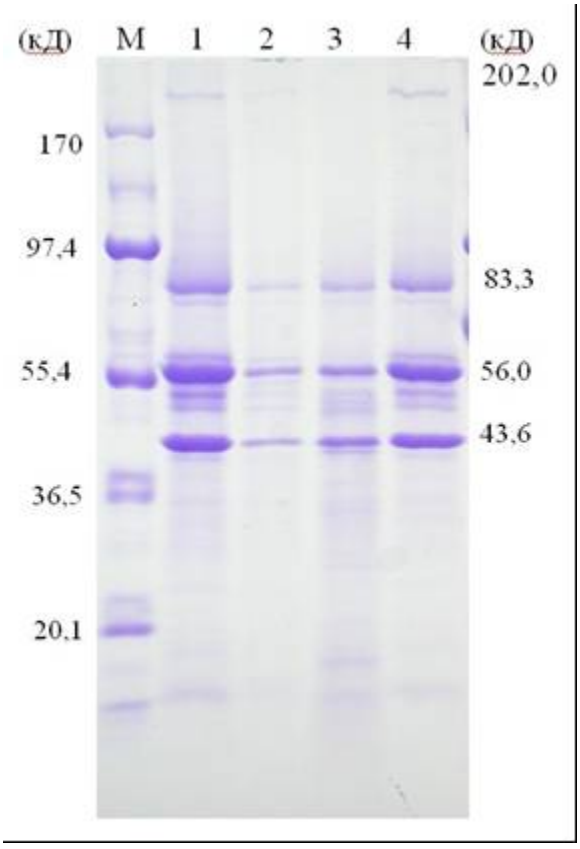

Figure 5 – Electrophoresis of the Newcastle disease virus polypeptides in 5-20% SDS – PAGE gel system.

During year 2 activities, hybridoma cells were generated using lymphocytes from immunized mice and myeloma cells. Following immunization of mice, ELISA assays were performed to determine the serum titer of induced antibodies approximately 30 days post primary challenge with several booster injections. Based on observed antibody response to the Newcastle disease virus antigen preparations, mice with very high titers (1:51200) were used for the fusion study. Two hybridoma cell experiments were conducted and generated 34 and 37 cell clones, respectfully. The next series of experiments were the identification of hybridoma clones producing monoclonal antibodies of acceptable affinity and specificity (Task 8 and 9). These experiments required the cloning of identified clones using the dilution method. As a result of these experiments, one clone 3D9 was identified with the appropriate activity for the Newcastle disease virus. This clone was subcloned and 5 subclones were identified for further study. These clones were 1D9B5, 1D9B6, 1D9E3, 1D9D3, and 1D9D8. These clones were tested against homologous and heterologous antigens. These antigens included the prepared Newcastle disease virus

antigen prepared for this study and CD, PPR, AI and Rinderpest preparations. Only activity was observed versus the Newcastle disease virus antigen preparation. No binding activity was observed for the other antigens. When these same monoclonal antibody subclones were employed in immunoblotting assay systems to identified specific activity to the various proteins based on molecular binding, activity was observed with a protein migrating with a before molecular weight of 43.6 kDa (Figure 6). All of the identified monoclonal antibodies produced and studied were of the IgG1 subtype.

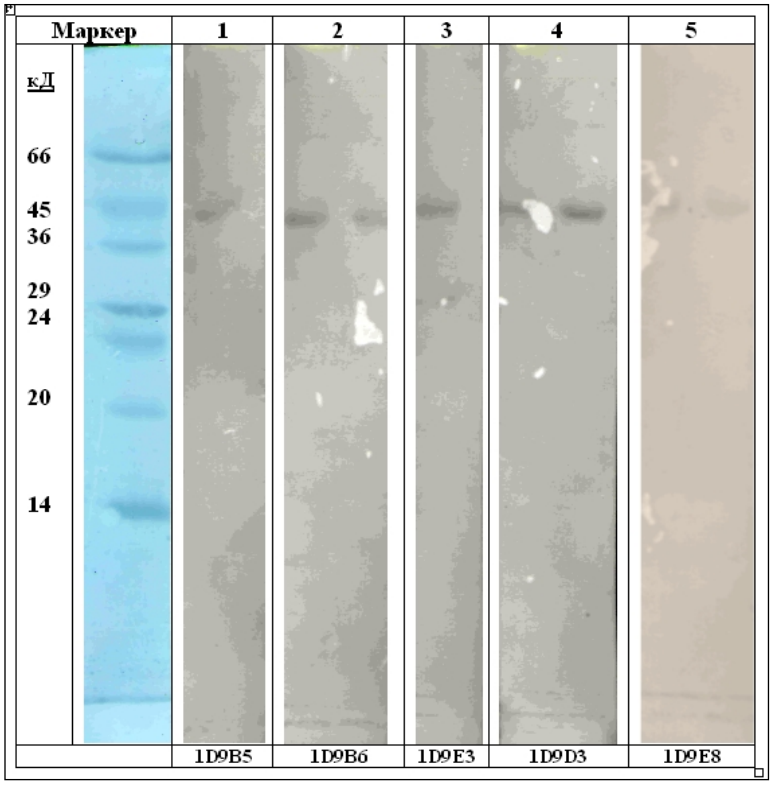

Figure 6 – Results of immunoblotting of MAb to the Newcastle disease virus antigens

3.0 Partners Evaluation of Success

New Horizon Diagnostics has been encouraged by the results of the project and projected considerable promise for commercialization of products that are developed as a result of work conducted under this CRADA, noting that "From a commercial – technical perspective, the testing will be further expanded to include other countries in the Far East as well as Europe." To provide additional perspective the commercial partner added this comment "Given the global threat to food and animal supply chains, this project is of extreme importance to all, both government and industry." This communication from NHD conveys its considerable appreciation and expectation of success for this GIPP project in product/technology development as well as supporting the global importance for collaborative efforts among all participants.

4.0 Future Plans and Commercialization Activities

The plans for commercialization are being pursued on several fronts. This includes the continued demonstration, evaluation and validation of the antibody-based diagnostic systems developed under this CRADA. NHD has continued to market these systems. The relationship of these systems to food safety and animal health and safety is spelled out clearly in a statement received recently from Dr. Larry Loomis who is the CEO of NHD, "New Horizons Diagnostics has a subsidiary called Safe Food Technology which has great interest in this work."

5.0 Inventions as a Result of this CRADA

No new inventions were derived or disclosed under this project.

6.0 Conclusion

Based on the data provided via interim and the final report, it is clear that the investigators have identified appropriate antigen and monoclonal antibodies for Task 1 directed at the Rinderpest virus and Task 2 directed at Newcastle disease Virus. It should be noted that Task 3 was not completed because the commercial partner was not able to supply the appropriate test kits to conduct the validation of tests kits for FMD virus developed under a related project. The CRADA project between NHD and PNNL was successful and is generating commercial opportunities in the U.S. and among partner institutes to use the results of this project to develop, manufacture and market diagnostic systems for animal diseases of considerable economic importance globally. As can be seen from the efforts generated by NHD, the global value of these detection systems for animal diseases could be considerable in terms of disease detection, prevention, treatment and return on investment and play a key role in developing significant procedures and protocols to detect, monitor and ultimately control disease outbreaks.

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