IDRC | CRDI

International Development Research Centre
Centre de recherches pour le développement international

**Project Title:** A non-replicative adenovirus vaccine platform for poultry diseases

**IDRC Project Number:** 108627-001

**Period covered by the report:** December 15, 2018 – December 14, 2019

## List of Annexes:

**Annex 1. Experimental design for the vaccination/challenge experiment in chickens**.

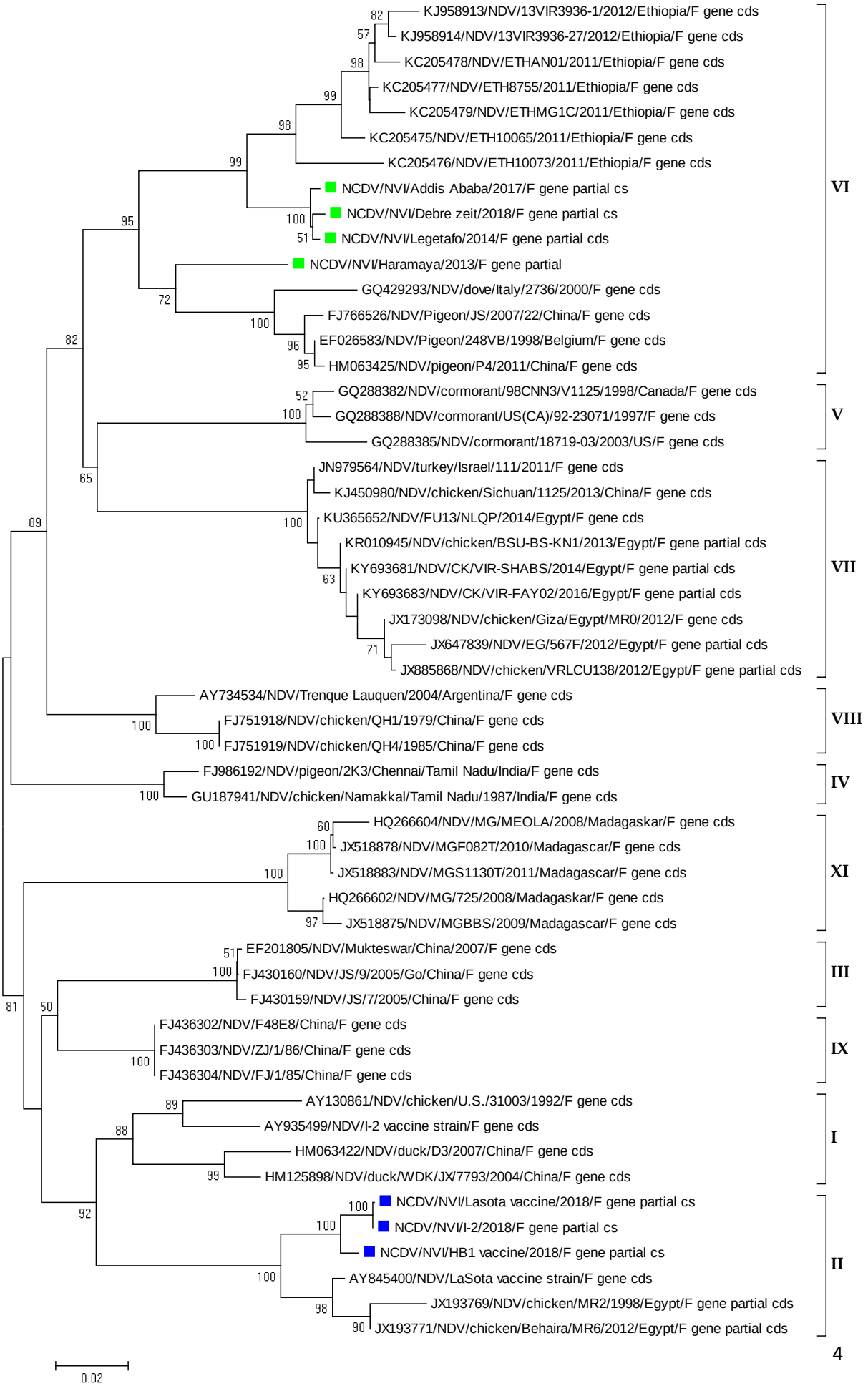
This trial will be finalized at the NVI, Ethiopia, by the beginning of March 2020. The animals will be immunized with the selected recombinant adenovirus-based vaccines prepared and their protective efficacy and possible superiority compared to the live vaccine produced in embryonated eggs will be assessed. The groups, experimental conditions, schedule for challenge and blood collection as well as description of *in vitro* analyses appear described below.

| **Group** | **Number of animals** | **Number of doses (Days)** | **Dose ($TCID_{50}$/ml)** | **Route of administration (via)** | **Estimated volume per dose** |
|---|---|---|---|---|---|
| Ad-F-CMV | 10 | 2 doses (Days 0, 21) | $1 \times 10^{10}$ | Parenteral, via intramuscular (i.m.) | 250-500ul |
| Ad-F-CMV | 10 | 2 doses (Days 0, 21) | $1 \times 10^{10}$ | Mucosal, eye drop instillation | 50-200 ul |
| Ad-F-CMV | 10 | 1 dose (Day 0) | $1 \times 10^{10}$ | Parenteral, i.m. injection | 250-500ul |
| Ad-F-CMV | 10 | 1 dose (Day 0) | $1 \times 10^{10}$ | Mucosal, eye drop instillation | 50-200 ul |
| Ad-F-βactin | 10 | 2 doses (Days 0, 21) | $1 \times 10^{10}$ | Parenteral, i.m. injection | 250-500ul |
| Ad-F-βactin | 10 | 2 doses (Days 0, 21) | $1 \times 10^{10}$ | Mucosal, eye drop instillation | 50-200 ul |
| Ad-F-HN-CMV | 10 | 2 doses (Days 0, 21) | $1 \times 10^{10}$ | Parenteral, i.m. injection | 250-500ul |
| Ad-F-HN-CMV | 10 | 2 doses (Days 0, 21) | $1 \times 10^{10}$ | Mucosal, eye drop instillation | 50-200 ul |
| NDV live vaccine | 10 | 2 doses (Days 0, 21) | As indicated by the manufacturer | Parenteral, i.m. injection | 250-500ul |
| NDV live vaccine | 10 | 2 doses (Days 0, 21) | As indicated by the manufacturer | Mucosal, eye drop instillation | 50-200 ul |
| Non vaccinated | 10 | - | - | - | - |

- Blood will be collected at day 0 previous to the first administration dose and at days 14 and 35 post-vaccination.
- Challenge with NDV will be practiced at day 42 and observation and monitoring of clinical signs will be recorded for one week. Total scheduled time = 50 days.
- Serum from samples taken will be separated from clotted blood, labeled and stored at -20ºC until *in vitro* assessment of specific responses to NDV by HIA, ELISA and *in vitro* neutralization assays.
- Surviving and non-surviving animals will be subjected to necropsy for the comparative analysis of clinical lesions.
- Part of the blood collected in each time point specified will be used for PBMC isolation for the assessment of cell-mediated immune responses (RNA extraction from lymphocytes and ddPCR for quantification of cytokines / intracellular staining of cytokines).

**Annex 2. Phylogenetic tree construction of NDV isolates based on the partial Fusion protein gene (F gene)**.

The F gene nucleotide sequences analyzed comprise a fragment of 749 bp. The analysis involved 53 nucleotide sequences. Four field isolates sequences from the present study, three vaccine strains and 46 sequences available in the GenBank database were used for the phylogenetic tree construction. The tree was constructed using the neighbor-joining algorithm and the software MEGA v7 (1000 bootstrap values). The evolutionary history was inferred using the Neighbor-Joining method. The current NDV isolates clustered under Genotype VI of the Class II of ND viruses. The bootstrap values are displayed above branches. The four field isolates (green) and vaccine strains (blue) sequenced in this study are marked with color rectangles. The isolates are named: NDV/Haramaya/2013, NDV/Legetafo/2014, NDV/Addis/Ababa/2017, and NDV/Debre/zeit/2018. The strains used for the preparation of current live vaccines are grouped under Genotype Class II.


KJ958913/NDV/13VIR3936-1/2012/Ethiopia/F gene cds
KJ958914/NDV/13VIR3936-27/2012/Ethiopia/F gene cds
KC205478/NDV/ETHAN01/2011/Ethiopia/F gene cds
KC205477/NDV/ETH8755/2011/Ethiopia/F gene cds
KC205479/NDV/ETHMG1C/2011/Ethiopia/F gene cds
KC205475/NDV/ETH10065/2011/Ethiopia/F gene cds
KC205476/NDV/ETH10073/2011/Ethiopia/F gene cds
NCDV/NVI/Addis Ababa/2017/F gene partial cs
NCDV/NVI/Debre zeit/2018/F gene partial cs
NCDV/NVI/Legetafo/2014/F gene partial cds
NCDV/NVI/Haramaya/2013/F gene partial
GQ429293/NDV/dove/Italy/2736/2000/F gene cds
FJ766526/NDV/Pigeon/JS/2007/22/China/F gene cds
EF026583/NDV/Pigeon/248VB/1998/Belgium/F gene cds
HM063425/NDV/pigeon/P4/2011/China/F gene cds
GQ288382/NDV/cormorant/98CNN3/V1125/1998/Canada/F gene cds
GQ288388/NDV/cormorant/US(CA)/92-23071/1997/F gene cds
GQ288385/NDV/cormorant/18719-03/2003/US/F gene cds
JN979564/NDV/turkey/Israel/111/2011/F gene cds
KJ450980/NDV/chicken/Sichuan/1125/2013/China/F gene cds
KU365652/NDV/FU13/NLQP/2014/Egypt/F gene cds
KR010945/NDV/chicken/BSU-BS-KN1/2013/Egypt/F gene partial cds
KY693681/NDV/CK/VIR-SHABS/2014/Egypt/F gene partial cds
KY693683/NDV/CK/VIR-FAY02/2016/Egypt/F gene partial cds
JX173098/NDV/chicken/Giza/Egypt/MR0/2012/F gene cds
JX647839/NDV/EG/567F/2012/Egypt/F gene partial cds
JX885868/NDV/chicken/VRLCU138/2012/Egypt/F gene partial cds
AY734534/NDV/Trenque Lauquen/2004/Argentina/F gene cds
FJ751918/NDV/chicken/QH1/1979/China/F gene cds
FJ751919/NDV/chicken/QH4/1985/China/F gene cds
FJ986192/NDV/pigeon/2K3/Chennai/Tamil Nadu/India/F gene cds
GU187941/NDV/chicken/Namakkal/Tamil Nadu/1987/India/F gene cds
HQ266604/NDV/MG/MEOLA/2008/Madagaskar/F gene cds
JX518878/NDV/MGF082T/2010/Madagascar/F gene cds
JX518883/NDV/MGS1130T/2011/Madagascar/F gene cds
HQ266602/NDV/MG/725/2008/Madagaskar/F gene cds
JX518875/NDV/MGBBS/2009/Madagascar/F gene cds
EF201805/NDV/Mukteswar/China/2007/F gene cds
FJ430160/NDV/JS/9/2005/Go/China/F gene cds
FJ430159/NDV/JS/7/2005/China/F gene cds
FJ436302/NDV/F48E8/China/F gene cds
FJ436303/NDV/ZJ/1/86/China/F gene cds
FJ436304/NDV/FJ/1/85/China/F gene cds
AY130861/NDV/chicken/U.S./31003/1992/F gene cds
AY935499/NDV/I-2 vaccine strain/F gene cds
HM063422/NDV/duck/D3/2007/China/F gene cds
HM125898/NDV/duck/WDK/JX/7793/2004/China/F gene cds
NCDV/NVI/Lasota vaccine/2018/F gene partial cs
NCDV/NVI/I-2/2018/F gene partial cs
NCDV/NVI/HB1 vaccine/2018/F gene partial cs
AY845400/NDV/LaSota vaccine strain/F gene cds
JX193769/NDV/chicken/MR2/1998/Egypt/F gene partial cds
JX193771/NDV/chicken/Behaira/MR6/2012/Egypt/F gene partial cds
VI
V
VII
VIII
IV
XI
III
IX
I
II
0.02

**Annex 3. Scale-up of adenoviral production to bioreactors.**

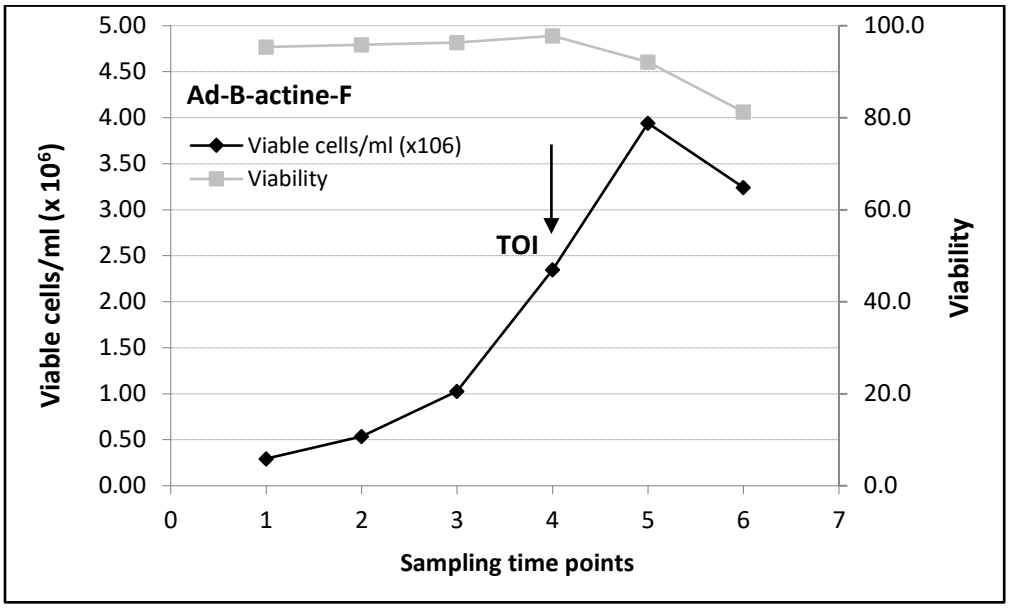
The production of the Ad-F-CMV, Ad-F-βactin and Ad-F-HN-CMV recombinant adenoviruses was undertaken by seeding at 0.35 x $10^6$ cells/mL the 1L (working volume 0.75L) or 3L (working volume 2.3L) bioreactors (Applikon Biotechnologies, USA), using the selected Xell HEK-GM medium and allowing cell growth until infection at approximately 2 x $10^6$ cells/mL in fed-batch mode, with initiation of the feeding supply at a viable cell count of approximately 1 x $10^6$ cells/mL. Viral production has been achieved in 3L bioreactors at cell densities over 4 x $10^6$ cells/mL.

Process parameters such as dissolved oxygen and pH have been strictly monitored, controlled (37°C; pH 7.00; DO 40%; 100 rpm) and documented as well as the on-line monitoring through the analysis of capacitance values (uF/cm) in the case of three liters cultures, as an indication of the total biovolume of the culture. The latter has been consistent with total cell counts and cell viability recorded at each time point before and after infection, providing a reliable production kinetics which is part of the documentation on the robustness of the process. Feeding supplement (Xell HEK-FS) have been supplied as 3% of the total bioreactor volume every 48h. Cultures have been infected at a MOI =1-3 and have been harvested when cell viability has started to decrease below 70%. Samples have been daily taken and determination of total viral particles (VP) per ml (by HPLC) and infectious viral particles per ml (IVP/ml) expressed as Median Tissue Culture Infectious Dose ($TCID_{50}$)/mL have been performed. Part of the present optimizations has been also the standardization of the $TCID_{50}$ working protocol for Ad titration, which has largely facilitated the evaluation on the functionality of the recombinant adenoviruses produced in a consistent and reliable manner. Titers at the time of harvest in the raw material (non-concentrated, non-purified rAds) have reached values over $10^8$ IVP/ml and around 5 x $10^9$ - $10^{10}$ VP/ml.

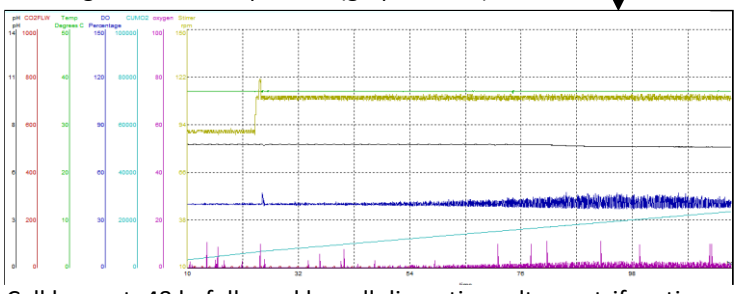
Purification after 10x concentrations by ultracentrifugation in a two-step CsCl gradient has been used for all the adenoviral productions intended for immunization experiments. For the rAd-ND variants produced, total viral particles have been quantified in the range of 1.3 to 5.3 x $10^{11}$ VP/ml after CsCl purification. After purification, IVP values per ml in the range of 1.7 to 7.5 x $10^{10}$ $TCID_{50}$/ml have been quantified. Alternative approaches for purification will be detailed further as part of one of the milestones included in this reporting period.

***In the following figures:*** A) Production of Ad-F-βactin in 1L bioreactor, in HEK293SF cells using Xell-HEK GM. A fed-batch regimen was used supplying Xell HEK-FS at 3 % every two days, before and after the time of infection. An MOI = 1 was used. The time point of infection is indicated with an arrow. B) Total viral particles/ml and infectious viral particles/ml for different viral productions (in shake flasks and 1 L bioreactors) appear tabulated. These productions were analyzed in C) and E), showing the HPLC chromatogram of raw disruption supernatant from a shake flask (700 ml) and 1L bioreactor productions, respectively. D) and F) show the HPLC chromatogram of the purified adenoviruses. Counts of viable cells were done manually using a hemacytometer and trypan blue dye and using a Vi-cell counter (Thermo Scientific). G) Data on capacitance values are shown as they were obtained from a 3L controlled bioreactor for production of Ad-F-HN-CMV in HEK Xell-GM. The bioreactor was operated in a fed-batch mode, infected as described above and harvested at 48h post infection. Infectious titers in all cases were calculated by $TCID_{50}$/ml in 96-well plates assays and total viral particles/ml were measured by HPLC using a UNO-Q polishing column. The red lines in the chromatograms correspond to UV detection at 260 nm and the arrows show the Ad5 peak.

**A)**




Profile of pH (black) and dissolved oxygen (blue) regulation during the whole bioprocess (graph below).



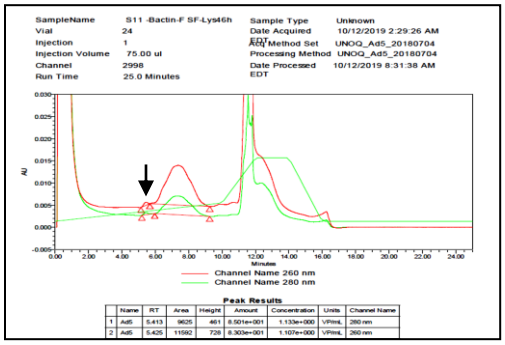
Cell harvest: 48 h, followed by cell disruption, ultracentrifugation, dialysis, and formulation.

| Time points in the graph | Days |
|---|---|
| 1 | Day 0 |
| 2 | Day 1 - S1 (24h) |
| 3 | Day 1 - S2 (36h) |
| 4 | Day 2 - Time of infection (TOI) |
| 5 | Day 1 p.i. (24 h.p.i.) |
| 6 | Day 2 p.i. (48 h.p.i.) |

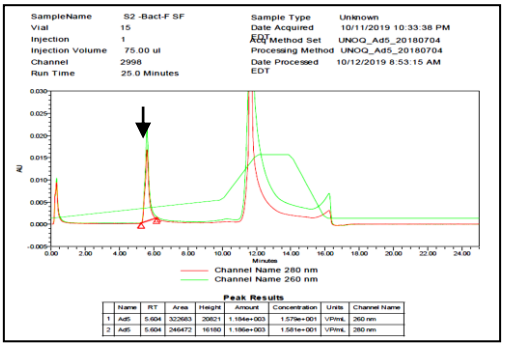
**B)**

| Adenovirus | VP/mL | TCID50/mL |
|---|---|---|
| Ad-βactin-F (Shake Flask 600 mL) (Lot No. 24/9/19) - Raw – 10x | 3.80E+09 | |
| Ad-βactin-F (Shake Flask 600 mL) (Lot No. 24/9/19) - Purified Oct/19 | 5.31E+10 | 1.95E09 |
| Ad-βactin-F (BioBatch-1L) (Lot 21/9/19) - Raw – 10x | 5.23+09 | |
| Ad-βactin-F (BioBatch-1L) (Lot 21/9/19) - Purified Oct/19 | 1.36E+11 | 3.2 E10 |

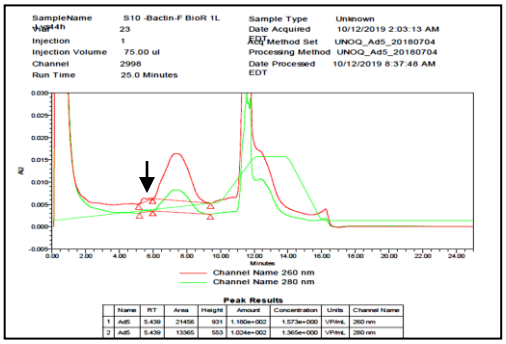
**C) Raw disruption supernatant concentrated**



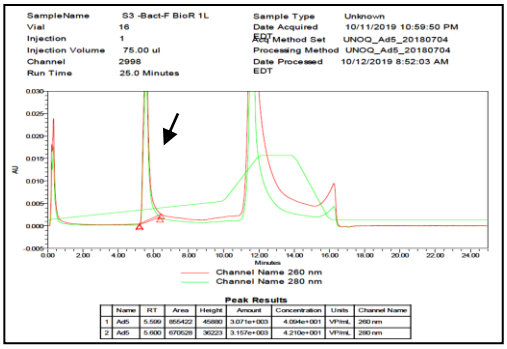

**D) Purified rAd**




**E) Raw disruption supernatant concentrated**




**F) Purified rAd**




**G)**

| **Ad-F-HN-CMV - 3L bioreactor HEK293SF** | | | |
|---|---|---|---|
| **Time point** | **Days** | **Capacitance (pF/cm)** | **IVP ($TCID_{50}$/mL)** |
| **1** | Day 0 | 1.073 | After purification<br><br>6.3E10 |
| **2** | Day 1 (16h) | 3.879 | |
| **3** | Day 1 (24h) | 7.236 | |
| **3** | Day 2 -Time of Infection | 14.505 | |
| **4** | Day 1 p.i. (24h) | 21.544 | |
| **5** | Day 1 p.i. (36h) | 18.506 | |
| **6** | Day 2 p.i. : (48h)<br>Viable cells 1.86E+06/ml<br>Viability 43.2% | 9.894 | |

**Annex 4. Analysis of cell lysis and clarification steps for the purification of the recombinant adenoviruses.**

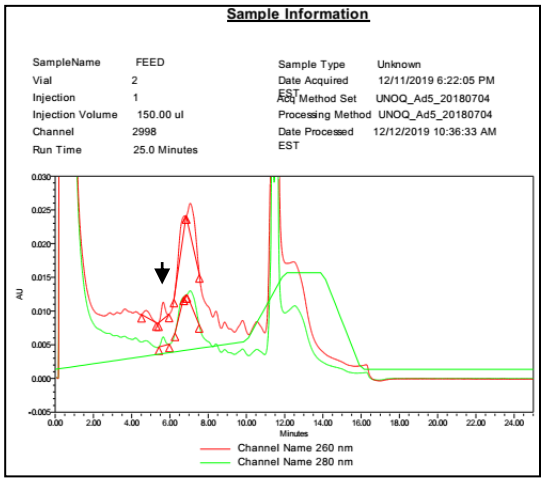
In the previous (18-months) report, various methods for the adenovirus extraction from the cell cultures were evaluated: infected cultures were treated by *i*) three cycles of freezing/thawing, *ii*) cell lysis initiated by addition of 1/9 culture volume of buffer containing 10% v/v polysorbate 20, 50% w/v sucrose, and incubation two hours at 37°C under agitation, *iii*) addition of 1/10 culture volume of a buffer containing 1% Triton X-100 and incubation 30 min at 37°C under agitation. The highest virus recovery, estimated by qPCR, was obtained when cells were treated with 1% Triton X-100. Presently, in experiments carried out using the Ad-HN-GFP-CMV construction, the cell disruption process (designed to be conducted directly in the bioreactor after its completion) have been adjusted to a lower concentration (0.1%) of Triton X-100, in 2mM $MgCl_2$, with treatment of 10 units/mL of Benzonase at 27.3 $^{o}$C and 120 rpm, for 1 hour of incubation. Both detergents, Triton X-100 and Tween 20, were used in this optimization for cell lysis and it was shown that both tend to stabilize the virion in a similar manner, with 100% recovery of a production having values of 1.8 x $10^{10}$ VP per mL. Subsequent clarifications through Sartoscale 25 Sartopure PP3 dead-end filters resulted in an elevated recovery, which was followed by depth filtration using using a Millistak Depth Filter, D0HC media filter that allowed a recovery of 100%.

| Description of disruption conditions assayed | Viral Particle/mL | Total VPs (in total volume) | Recovery |
|---|---|---|---|
| **48hrs - Harvest** | **$1.423x10^{10}$** | **$2.4x10^{9}$** | |
| **0.1%Triton+ 10Units Benzonase+2mM $MgCl_2$ -1 hour Incubation at 27.3$^{o}$C 120 RPM** | **$1.051x10^{11}$** | **$1.8x10^{10}$** | |
| **0.1%Triton+ 10Units Benzonase+2mM $MgCl_2$ -2 hour Incubation at 27.3$^{o}$C 120 RPM** | **$1.07 x10^{11}$** | **$1.8x10^{10}$** | **100%** |
| **0.1%Triton+ 10Units Benzonase+2mM $MgCl_2$, 5µm filtrate** | **$1.23 x10^{11}$** | **$2.1x10^{10}$** | **107%** |
| **0.1%Triton+ 10Units Benzonase+2mM $MgCl_2$, 3µm filtrate** | **$1.29 x10^{11}$** | **$2.2x10^{11}$** | **122%** |
| **S5- 0.1%Triton+ 10Units Benzonase+2mM $MgCl_2$, 1.2µm filtrate** | **$1.24 x10^{11}$** | **$2.2 x10^{10}$** | **103%** |
| **S6- 0.1%Triton+ 10Units Benzonase+2mM $MgCl_2$, 0.65µm filtrate** | **$1.22x10^{11}$** | **$2.0 x10^{10}$** | **85%** |

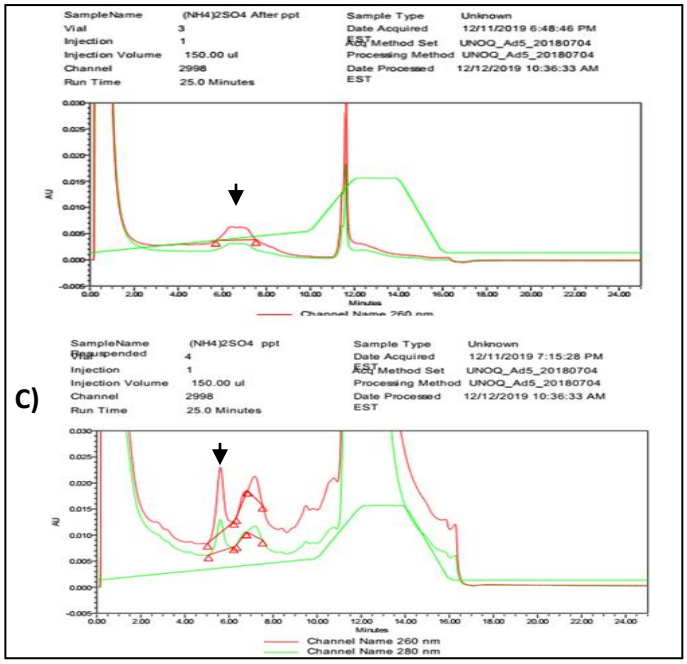
**Annex 5. Analysis of ammonium sulphate precipitation in the Ad5 purification steps**.

Chromatograms from HPLC show the analyses of HEK293 cell lysates after production of the Ad5-HN-GFP, clarified as described in Annex 4, and subjected to ammonium sulfate precipitation. The analysis corresponds to the initial (feed) material A); after ammonium sulphate precipitation B); and after resuspension in formulation buffer C). The red line corresponds to UV detection at 260 nm and the arrows show the Ad5 detection peak. The viral particles and the recovery achieved is summarized in D).

**A)**




**B)**




**C)**




**D)**

| Samples description | Viral Particle/mL | Total Viral Particles (in total volume) | Recovery |
|---|---|---|---|
| Post infection (48h) Harvested | $1.42 \times 10^{10}$ | $2.4 \times 10^{9}$ | |
| Ammonium sulfate precipitant | $9.15 \times 10^{10}$ | $1.5 \times 10^{12}$ | |
| Ammonium sulfate precipitant re-suspended with formulation buffer | $1.4 \times 10^{11}$ | $2.3 \times 10^{10}$ | 35% |

**Annex 6. Detection of specific anti-F antibodies by ELISA in chicken.**

The ELISA (ID Screen Newcastle Disease Indirect, IDVet) for detection of specific IgY antibodies against the F-antigen was used to assess the generation of the anti-NDV immune response elicited in chickens in the preliminary vaccination experiment. It showed specific Abs in animals receiving the recombinant adenoviruses Ad-F-CMV, Ad-F-HN-CMV and the live NDV vaccine. Positive specific immunity by ELISA was detected only in the animals shown in the table, with a sample to positive ratio (S/P) superior to 0.3, as indicated by the assay manufacturers. It has been previously described a good correlation between the anti-F titers measured using this assay and hemagglutination inhibition titers, which in turn correlate with protection against NDV (https://www.id-vet.com/wp-content/uploads/2016/01/encart-presse-IDvet.pdf). The table below also shows the *in vitro* hemagglutination inhibition titers obtained using sera from these animals against NDV.

| Group | ID | Detection of anti-F Abs expressed as S/P ratio (absorbance at 450 nm)* | HI titers |
|---|---|---|---|
| **Ad-F-CMV** | 302 | 0.32 | 1/32 |
| **Ad-F-CMV** | 306 | 0.56 | 1/8 |
| **Ad-F-CMV** | 307 | 0.33 | 1/16 |
| **Ad-F-HN-CMV** | 322 | 0.34 | 1/4 |
| **Ad-F-HN-CMV** | 330 | 0.31 | 1/32 |
| **NVI live vaccine** | 341 | 1.59 | 1:128 |
| **NVI live vaccine** | 342 | 0.54 | 1:32 |
| **NVI live vaccine** | 343 | 3.28 | 1:64 |
| **NVI live vaccine** | 344 | 3.48 | 1:256 |
| **NVI live vaccine** | 345 | 3.11 | 1:128 |
| **NVI live vaccine** | 346 | 2.82 | 1:128 |
| **NVI live vaccine** | 347 | 1.96 | 1:128 |
| **NVI live vaccine** | 348 | 2.72 | 1:128 |

* **Positive specific immunity at S/P values >0.3.**

**Annex 7. Experimental design for the safety evaluation of different formulations containing the Ad-F-CMV.**

Each formulation (being considered in the purification approaches in development) was administered to the groups of Balb/c mice as described below for a preliminary evaluation of their safety in the mice model. Experiments with repeated administrations and also 2- to 10-fold the selected dose will be conducted in the target species after the definition of the vaccine dosage. In this experiment, the parameters being monitored in mice are related to mice health (water and food consumption, general observation during 10 days, observation at the injection site). Importantly, biodistribution analysis by qPCR quantification of the Ad5 will be performed in liver, central nervous system, heart, lung, kidney and spleen.

| Group number | Mice number per group | Ad-F-CMV - $10^9$ VP/mouse |
|---|---|---|
| 1 | 3 | **Formulation 1: three cycles of freezing/thawing** |
| 2 | 3 | **Formulation 2: 1/9 culture volume of buffer (10% v/v Tween-20, 50% w/v sucrose, 20mM MgCl2, 500mM Tris pH 8.0)** |
| 3 | 3 | **Formulation 3: 1/10 culture volume of solution of 20 mM MgCl2, 1% Triton X-100 +37.3 mM MgSO4** |
| 4 | 3 | **Formulation 4: 0.1% Triton-X100** |
| 5 | 3 | **Formulation 5: 0.1% Tween-20** |
| 6 | 3 | **Non injected** |

**Annex 8. Standard Operating Procedures to support the technology transfer.**

These SOPs have been extensively advanced and their status appears summarized below.

| Protocol | Present status |
|---|---|
| Protocol for insertion of a defined gene into an universal vector for the generation of an adenovirus transfer vector | Prepared, revised |
| Protocol for the generation of the final adenoviral vector | Prepared, revised |
| Protocol for an enhanced transformation of HEK293 cells | Prepared, revised |
| Protocol for transformation of HEK293 serum free cells | Prepared, revised |
| Protocol for rescue and single-step amplification of an adenoviral vector | Prepared, revised |
| Protocol for the generation of a working cell bank of HEK293 and HEK293 serum free cells | Prepared, revised |
| Protocol for the thaw and freezing of HEK293 cells | Prepared, revised |
| Protocol for the generation of a master seed bank and working bank for the recombinant adenovirus | Prepared, revised |
| Protocol for production of the adenovirus in shake flask | Prepared, revised |
| Protocol for production of the adenovirus in 3L bioreactor | Prepared, revised |
| Protocol for cell harvest from bioreactor and lysis | Prepared, revised |
| Protocol for cell disruption in bioreactor and harvest of cell lysate | In preparation |
| Protocol for recovery of adenovirus from harvested cell lysate | In preparation |
| Protocol for purification of the adenovirus in a two step CsCl gradient | Prepared, revised |
| Protocol for the quantification of the recombinant adenovirus in HPLC | Prepared, revised |
| Protocol for the quantification of the recombinant adenovirus by ddPCR | Prepared, revised |
| Protocol for the quantification of a GFP-recombinant adenovirus by flow cytometry | Prepared, revised |
| $TCID_{50}$ protocol for the titration of the adenovirus | Prepared, revised |
| Protocol for formulation process of the recovered adenovirus | In preparation |
| Protocol for lyophilisation of the adenovirus vaccine preparation | In preparation |
| Protocol for potency test of the of the adenovirus-based vaccine | In preparation |

**Annex 9. Points of consideration for a potential extension period of the project.**

A funded extension of 18 months after the present funding period of the project will significantly advance two critical components of this product development project leading to **readiness** of the product for **commercial manufacturing**:

1) ***Improvement of product efficacy, safety and cost effectiveness*** *for enhanced protection of chicken against New Castle Disease Virus (NDV) infections.* Building on the data generated from the on-going target animal study:

a) Improvement of **Vaccine formulation, delivery and stability** will be re-assessed to achieve superior protection of the recombinant vaccine. The on-going animal experiments will provide key information on the formulation (*e.g.* adjuvantation) and delivery (dose, route) required for increased immunogenicity. Additional animal experiments will bring information on the minimum protective dose (dose-response), onset and duration of immunity and *in ovo* vaccination (effective against influenza using the Ad5 vector as vaccine). The challenge experiments would also benefit from the preparation of viruses from current circulating strains for the challenge (instead of phylogenetically distant reference strains such as La Sota. Lyophilisation, a technology well-established for adenovirus product will be considered for final product presentation and long-term storage. The selection of the final route of administration will determine the downstream process critical steps.

b) Improvement of **Vaccine Safety and Environmental compliance** as regulated within the field of application Additional development of analytical tools originally limited to support the process development will be extended to product safety and environmental monitoring. This includes the development of PCR protocols to monitor tissue distribution and environmental distribution of the Ad5 (as a genetically modified organism) used for vaccination. Also, the development of vaccine potency assays will be undertaken. Finally, analyses on the prevalence and transmission of NVDV after vaccination and challenge experiments (sterilizing immunity, shedding of NDV) will be also highly valuable for the final product final.

***An extensive cost-effectiveness study taking into consideration the societal and geographical environment will provide the fundamental bases for the selection of the most effective process to manufacture an effective and safe recombinant Adenovirus-based vaccine.***

2. A sustainable outcome of the original project is to enable NVI, Ethiopia to manufacturing the recombinant adenovirus vaccine against NDV locally through an **effective technology** transfer and to master the adenovirus technology as platform to design and develop new adenovirus-based vaccines against vaccines included in the IDRC eligible list of diseases and/or to major circulating poultry diseases like Gumboro or Marek's. The development of expertise in the area of cell culture and bioreactors operation at NVI with the creation of R&D capacity need to be intensified and further supported. Additional actions on the transfer of the technology platform, including on-site training of personnel would be needed. Specifically, for the recombinant NDV vaccine efforts toward the production of commercial lots at NVI and marketability of the vaccine product requires adoption the cell culture technology capacities for which the members of this project are fully committed to act over an extension of this project within an additional 18 month-extension period.

**Annex 10. Abstract for manuscript submission.**

This summary includes one part of the results obtained, which have been already included in the progress reports completed up to now.

**An adenovirus-based vaccine platform for poultry diseases: Establishing the technology for a Newcastle disease virus vaccine manufacturing process for sub-Saharan Africa**


**Abstract**

Developing novel vaccine technologies or improving existing processes to confront situations such as pandemic threats or zoonotic diseases is a worldwide increasing priority. Human health at a global scale can be influenced by the risk of transmission of infectious diseases from wildlife and domestic animals. Thus, veterinary vaccination and animal health monitoring are highly relevant for prevention purposes. In developing countries, also this contributes to increase food safety in animal production and aids to decrease poverty by providing economic autonomy to farmers and smallholder farmers. In regions such as Sub-Saharan Africa, farmers' activities are frequently affected by the impact of diseases in poultry such as avian influenza and Newcastle disease (ND). ND is one of the most critical, with several outbreaks per year and the need for vaccination with live vaccines produced in embryonated eggs. These vaccines lead to virus shedding by vaccinated poultry which may lead to disease in non-vaccinated birds. The purpose of this work was to develop an adenovirus (Ad) vectored vaccine platform technology suitable for the rapid adaptation to this or other avian viral threats. It is based on an efficient process operating production strategy using HEK293 suspension cells and serum-free medium, allowing high-cell density productive infection in bioreactors. The work comprised the phylogenetic analysis of local isolates of Newcastle disease virus (NDV) and the construction of adenoviral vectors using different regulatory elements, with and without GFP, bearing the F and HN antigens from NDV genotype VI, either as individual antigens or in bicistronic vectors. Their functionality was evaluated in vitro in HEK293 and chicken fibroblast (DF-1) cells by corroborating the expression of the encoded antigens. Remarkably, viral rescue and generation of primary stocks was simplified by developing one novel procedure for single step amplification in suspension cultures. A rapid production process was established in shake flask experiments by identifying and optimizing culture conditions and critical parameters. The production phase with the different Ad variants was initiated at cell densities of $2x10^6$ cells/ml in Xell AG HEK-GM medium. Scalability was further demonstrated in batch and fed-bath controlled bioreactor runs (1-3L) with production at cell densities over $4x10^6$ cells/ml and infectious titers above $5x10^8$ $TCID_{50}$/mL (>$10^{10}$VP/mL). Viruses were harvested at around 45hpi and subjected to clarification or purification steps. They were concentrated, formulated, and stocked at $10^{10}TCID_{50}$/mL for animal vaccination experiments. ELISA and hemagglutination inhibition assays on the humoral immune response elicited in mice and chicken showed the induction of specific, functional antibodies against NDV. These results consolidate the bases of a simple, scalable and reproducible vaccine manufacturing process for its deployment in these regions affected by ND.

**Annex 11. Technical work plan for the forthcoming months.**

1. Determine the level of protective capacity of the three vaccines in study (Ad-F-CMV, Ad-F-βactin, Ad-F-HN-CMV) by observation of clinical signs after administration of a lethal challenge of NDV consisting of $0.5 \times 10^{6.5}$ $ELD_{50}$. Develop ELISA assays, hemagglutination inhibition assays and neutralization *in vitro.* Conduct comparative analysis with live attenuated vaccine.
2. Prepare the Ad5-NDV required for an additional vaccination/challenge experiment in the next 6-months period, directed to identify the following: the optimal dose (dose-response study), minimum protective dose, onset and duration of protection, safety (once the dose has been established) and identification parameters that may remain pending.
3. Optimize the adenoviral production process in bioreactors at larger scale than 3L to master the technology. Final optimization of dynamic feeding strategies based on key process variables. Intensify and finalized the requirements on the monitoring of physiological changes, nutritional requirements and adjustments of feeding rates, and nutrient composition during growth or virus production phases.
4. Finalize the downstream processing step for rAd-ND recovery, achieving a cost-effective manufacturing process, based also in results from the challenge experiment.
5. Advance and finalize the elaboration and revision of the Standard Operating Procedures for the technology transfer to Ethiopia. Visit on site for this purpose.

**Table 1: Titration of the adenovirus vaccine preparations selected for the immunization/challenge experiment**. Summary of the main individual productions and values of infectious viral particles calculated as $TCID_{50}$/ml in the final preparations are described. These formulations comprise the recombinant adenoviruses Ad-F-CMV, Ad-F-HN-CMV, and Ad-β-CMV.

| rAd5 | Culture volume | TCID50/ml (IVP/ml) | Vials | Total | Harvest (H), Purification (P) |
|---|---|---|---|---|---|
| Ad-F-Beta-actin | Bioreactor 1 L | $3.2 \times 10^{10}$ | 14 vials 500ul each | 7 ml | H: 20/9/19 P: 1/10/19 |
| Ad-F-Beta-actin | Shake flask 700ml | $5.7 \times 10^{10}$ | 5 vials 1 ml each | 5 ml | H: 18/10/19 P: 6/11/19 |
| Ad-F-Beta-actin | Shake flask 700ml | $7.5 \times 10^{10}$ | 11 vials 1 ml each | 11 ml | H: 18/10/19 P: 24/10/19 |
| Ad-F-HN-CMV | Bioreactor 3 L | $6.3 \times 10^{10}$ | 10 vials 1 ml each | 10 ml | H:29 /9/19 P: 26/10/19 |
| Ad-F-HN-CMV | 700 ml Shake flask | $1.8 \times 10^{11}$ | 9 vials 1.5 ml each | 13.5 ml | H: 20/9/19 P: 30/10/19 |
| Ad-F-CMV | 700 ml Shake flask | $1.73 \times 10^{10}$ | 8 vials 500 ul each | 4 ml | H: 13/9/19 P: 20/9/19 |
| Ad-F-CMV | 700 ml Shake flask | $3.1 \times 10^{10}$ | 15 vials 500 ul each | 7.5 ml | H: 28/9/19 P: 30/9/19 |
| Ad-F-CMV | Bioreactor 1 L | $1.3 \times 10^{9}$ | 11 vials 500ul each | 5.5 ml | H: 29/9/19 P: 4/10/19 |