# REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad.

1. Name and designation of ICMR- IF : Dr Mithilesh Kumar Singh
   Senior Scientist

2. Address : Immunology Section
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3. Frontline area of research in which training/research was carried out : Vaccinology

4. Name & address of Professor and host institute : Dr. Gourapura J. Renukaradhya
   Professor
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   OH, USA

5. Duration of fellowship with exact date : 1 year (28.03.2023 to 27.03.2024)

6. Highlights of work conducted :

   i) Technique/expertise acquired :

- Antibody dependent cell cytotoxicity (ADCC) assay for assessing the functional antibody response as one of the correlates of protective immunity in pigs immunized with inactivated influenza nanovaccine candidate.
- Complement dependent cell cytotoxicity (CDC) assay for assessing the functional antibody response as one of the correlates of protective immunity in pigs immunized with inactivated influenza nanovaccine candidates.
- Preparation/synthesis of chitosan-mannose nanoparticles and antigen encapsulation techniques for targeted delivery of antigen/vaccine to professional antigen presenting cells (dendritic cells, macrophages) located in mucosal tissues.
- Preparation/synthesis of trimethyl chitosan (TMC)-mannose nanoparticles and antigen encapsulation techniques for targeted delivery of antigen/vaccine to professional antigen presenting cells (dendritic cells, macrophages) located in mucosal tissues.
- Nanoparticle characterization using zeta sizer and scanning electron microscopy.
- Multi-color flow cytometry and data analysis using Flowjo software to assess the immune dynamics of T and or B cells and their subsets in vaccinated experimental animals.
- Cell culture, virus titration and virus neutralization.

- Avidity ELISA to determine the overall strength and stability of antibodies interaction to its corresponding antigen.
- Magnetic activated cell sorting (MACS) for NK cells purification.
- Characterization of monoclonal antibodies with flow cytometry (intracellular staining).

ii) Research results, including any papers, prepared/submitted for publication

**Introduction:**

Biosecurity measures to control Salmonellosis like using high-quality feed, and maintaining hygiene, are often costly and insufficient. Vaccination remains a critical strategy for controlling salmonellosis, with the ideal vaccine being cost-effective, broad-spectrum, and long-lasting. Live vaccines, while easy to administer orally, pose environmental risks, whereas killed vaccines require impractical parenteral administration for poultry. Subunit vaccines using conserved antigens like outer membrane proteins (OMPs) and flagellin (FLA) show promise by eliciting robust immune responses (1). Previously it has been demonstrated that a subunit *Salmonella* oral vaccine based on OMPs and flagellin encapsulated with mannose-chitosan nanoparticle and surface coated with flagellin has effectively reduced *Salmonella* load in cecum of broiler birds and also has elicited good innate and adaptive immune responses (2). This vaccine's distinctive feature is the use of chitosan known for its mucoadhesive and adjuvant properties along with mannose for targeting dendritic cells, enhancing antigen presentation. It is proposed that incorporation of a suitable adjuvant may be beneficial in order to further improve the efficacy of this vaccine candidate, Hence, present work was aimed at exploring the benefits of using adjuvants such as *Mycobacterium smegmatis* whole cell lysate (WCL) and cyclic di-GMP (c-di-GMP) with *Salmonella* oral nanovaccine candidate.

**Methodology and Results:**

**Preparation and characterization of mannose chitosan formulations**

The OMPs and flagella of *Salmonella* enteritidis (SE) was extracted and purity was verified as per the previously described method (2). The mannose-modified chitosan vaccine was prepared using the ionic gelation method as previous described but with few modifications(2). Briefly, 72 mg of lyophilized mannose-conjugated chitosan was dissolved in 72 ml of Milli-Q water under magnetic stirring, and the pH was adjusted to 4.3. To this solution, 7.2 mg of OMPs (3.6 mg) and flagella (3.6 mg) dissolved in 7.2 ml of 10 mM MOPS (pH 7.4) was added. Subsequently, 6 mg of TPP dissolved in 36 ml of Milli-Q water was added. Finally, 1.8 mg of flagellar protein dissolved in 1 ml of 10 mM MOPS buffer was added in drop-wise manner. The resulting nanoparticles were harvested as a pellet by centrifugation at 10,976g for 30 minutes. The formulation of adjuvant was prepared in similar manner using the ionic gelation method with either WCL or c-di-GMP. The two formulations were divided into different groups based on requirements, reconstituted in Milli-Q water, and used for vaccination. The entrapment efficiency was calculated using the supernatant of the formulation by the Bicinchoninic acid (BCA) method. The results of entrapment efficiency and physical nature of mannose chitosan nanoparticle entrapped with OMP and flagella or adjuvants are presented in Table 1.

**Table 1:** The entrapment efficiency and physical nature of mChitosan-NP entrapped with *Salmonella* antigens or adjuvants determined using the BCA method and Malvern Zetasizer respectively

| Nanoparticle formulation | Entrapment Efficiency | Size | Polydispersity Index | Zeta Potential (mV) |
|---|---|---|---|---|
| mChitosan-(OMPs+FLA)-NP | 70.11% | 239.6 | 0.357 | +21.0 |
| mChitosan-(GMP)-NP | 68.4% | 267.2 | 0.519 | +25.62 |
| mChitosan-(WCL)-NP | 64.42% | 254.0 | 0.462 | +20.01 |

**Immunizations and challenge studies in Chicken**

Day old Cornish Cross broiler chicks were randomly checked for the presence of *Salmonella* on arrival. The primary immunization was carried out on day 3 and the booster was given on day 21. Each vaccine dose contained 10 µg each of OMP and FLA encapsulated in mannose chitosan nanoparticles, along with varying doses of the adjuvants (Table 2), and a surface coating of flagella. The commercial vaccine Poulvac ST, a live vaccine from Zoetis was administered on day 1 and at the second week of age as per the manufacturer's instructions to the commercial vaccine group. At the 5$^{th}$ week, all birds except for the mock control group were challenged orally with SE @ 5 $\times 10^7$ colony forming units (CFU) /bird. The necropsy was conducted on day four post-challenge (DPC4).

**Table 2:** Immunization trial

| Group | Group names | Number of birds |
|---|---|---|
| 1 | Mock | 12 |
| 2 | Mock+ Challen | 11 |
| 3 | Commercial vaccine+ Challen | 12 |
| 4 | mChitosan(OMP+Fla)/F- NP+ Challen | 12 |
| 5 | mChitosan(OMP+Fla)/F-NP+mChitosan(WCL)/F-NP2.5µg+ Challen | 11 |
| 6 | mChitosan(OMP+Fla)/F-NP+mChitosan(WCL)/F-NP10µg+ Challen | 12 |
| 7 | mChitosan(OMP+Fla)/F-NP+mChitosan(WCL)/F-NP50µg+Challen | 12 |
| 8 | mChitosan(OMP+Fla)/F-NP+mChitosan(GMP)/F-NP2.5µg+ Challen | 12 |
| 9 | mChitosan(OMP+Fla)/F-NP+mChitosan(GMP)/F-NP10µg+Challen | 10 |
| 10 | mChitosan(OMP+Fla)/F-NP+mChitosan(GMP)/F-NP50µg+Challen | 12 |

**Determination of bacterial load**

To determine the SE loads in challenged birds, the caecal contents were processed and dilutions were plated in duplicate on Xylose Lysine Tergitol 4 (XLT4) agar plates with antibiotics Nalidixic acid and Novobiocin both at 20ug/ml. The plates were incubated at 37°C for 24 hours and the black colonies were counted. The *Salmonella* counts were recorded as CFU /ml of cecal content and then converted to log10 CFU/ml for statistical analysis. Marked reduction in cecal bacterial load was observed in high dose vaccinates. Specifically, cyclic-di-GMP at a concentration of 50µg greatly reduced the caecal bacterial load (1.2 $log_{10}$) as compared to commercial vaccinate group (0.51 $log_{10}$) in relation to mock challenge on day four of the challenge infection (Fig. 1).

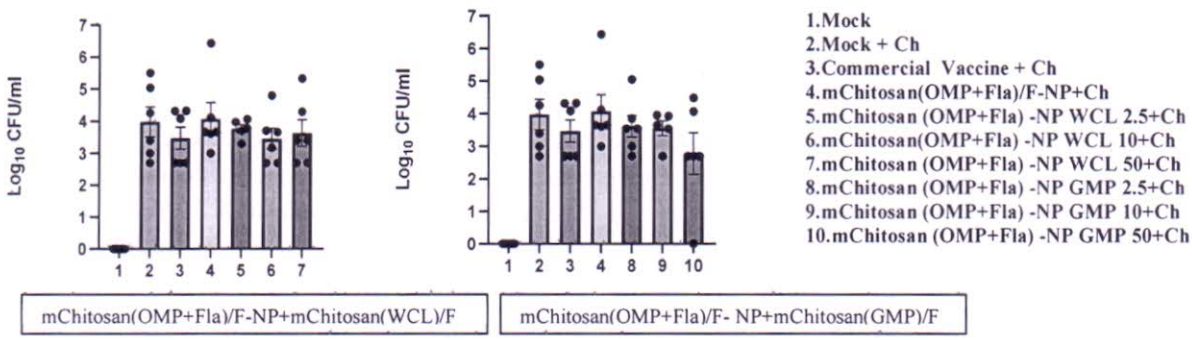



Fig 1: Bacterial clearance in cecum of birds immunized with different nanovaccine formulations at day 4 post-challenge. The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

## Flow Cytometry

Flow cytometry was used to assess key markers of immune response. Five million splenocytes in 1 ml of complete RPMI medium were seeded per well in a 48-well flat bottom plate. The cells were stimulated with OMP and flagella at 5ug/ml each for 48 hrs. The cells (1*10^6) were transferred to a non-sterile 96-well round bottom FACS plate, washed once in 200 µl FACS buffer/well and subjected to blocking for Fc receptors by using blocking buffer (1% normal mouse serum in FACS buffer) for 30 min at 4 C. This step was followed by two washes with 1X PBS and live/dead staining using the Fixable Dead Cell Stain Kit (Invitrogen, ThermoFisher Scientific) at a 1:1000 dilution in PBS, with 50 µl per well for 30 minutes at 4°C. The cells were then washed once in FACS buffer by centrifugation at 2,000 rpm for 2 minutes and proceeded to surface staining using the specified anti-chicken lymphocyte-specific antibodies or their corresponding isotype control antibodies at previously optimized concentrations in a final volume of 50 µl for 30 minutes at 4°C (Table 3). Samples were acquired using a live gate on a BD FACS Aria II machine and analyzed with FlowJo software (FlowJo™ v10.8, Becton, Dickinson & Company; BD). The frequency of CD3-BU1+ cells, indicative of B cells, increased in all vaccinated groups on day 4 of the challenge, except for the mChitosan (OMP+FLA)/F -NP GMP 10 and mChitosan (OMP+FLA)/F -NP GMP 50 groups, compared to the mock group (Fig. 2 A, B).

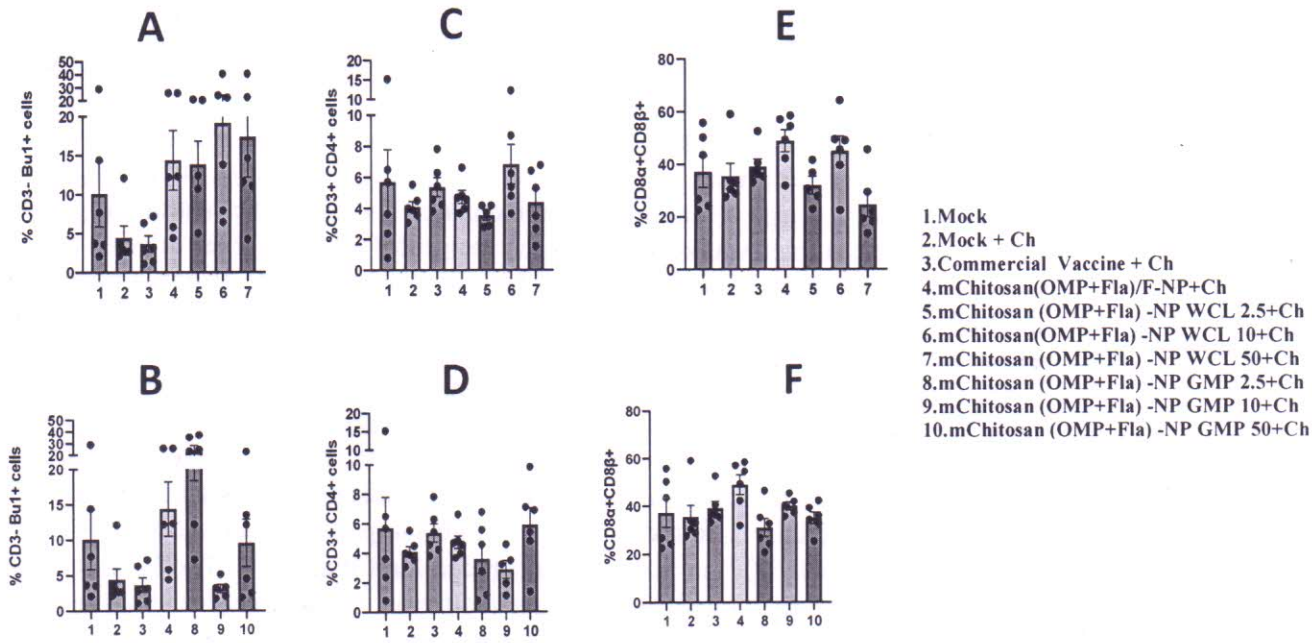



Fig 2: Flow cytometery analysis of immune cells on day 4 post-challenge; percentage of A) Bu1+cells with WCL adjuvant; B) Bu1+cells with GMP adjuvant; C) CD3+ CD4+ T-cells with WCL adjuvant; D) CD3+ CD4+ T-cells with GMP adjuvant; E) CD3+ CD8+ T-cells with WCL adjuvant; F) CD3+ CD8+ T-cells with GMP adjuvant. The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

In addition to the oral salmonella nanovaccination study, I have also got opportunity to work on archived vaccinated pig serum samples originated from previous two independent experiments (3,4). In Exp#1, mannose conjugated and unconjugated chitosan based whole inactivated H1N2 SwIAV (Chit-SwIAV-NPs and mChit-SwIAV-NPs) candidate vaccine was used (3) while in Exp#2 similar mChit- SwIAV-NPs plus STING agonist (S100) as an adjuvant either encapsulated (mChit-SwIAV+S100-eNPs) or surface adsorbed (mChit-SwIAV+S100-sNPs) was used (4). My study was aimed to understand whether functional differences in the humoral responses (neutralizing versus non-neutralizing antibodies) to inactivated swine influenza A virus (SwIAV) mannose chitosan vaccine candidates might contribute to differences in their efficacy. Results of both the experiments were analyzed with regards to different types of functional antibodies specific to SwIAV. Lactate dehydrogenase (LDH) release assay was employed for the detection of HA-specific antibodies with ADCC and CDC function in archived vaccinated pig serum samples in the laboratory, and virus neutralization (VN), hemagglutination inhibition (HAI) and avidity ELISA data obtained from two separate animal trials was compiled to get a comprehensive overview of the functional antibody responses following influenza vaccination and infection.

***Viruses:*** Vaccine virus used in both the experiments was SwIAV H1N2-OH10 [A/swine/Ohio/FAH10-1/2010 (H1N2)]. The challenge viruses used in both the experiments were heterologous to vaccine virus with Exp#1 pigs received SwIAV H1N1-OH7 (A/Swine/OH/24366/2007) and Exp#2 pigs received influenza A virus CA09-H1N1 [A/California/04/2009 (H1N1)].

**Virus neutralization and hemagglutination inhibition assay**

Briefly, a two-fold serial dilution (starting from 1:5) of respective heat inactivated serum samples were mixed with an equal volume of SwIAV H1N1/OH/2007 (100 $TCID_{50}$/well) in a 96-well round-bottom plate and incubated at 37 $^0$C in 5% CO2 for 1.5 h. One hundred microliter of sample and virus mix was transferred onto pre-adhered MDCK cells monolayer of over 90% confluency and incubated at 37 °C in 5% CO2 for 1.5 h before adding additional 100 µl/well 1x DMEM containing TPCK-treated trypsin (2.0 µg/ml) and plate was incubated for 44 h. The neutralizing activity of each serum samples was recorded as the reciprocal of the highest dilution showing complete inhibition of virus infection in MDCK cells. Finally, the virus neutralizing antibody titers were transformed to $log_{10}$ values for comparison between different experimental vaccine groups. The virus neutralizing antibody titers data of Exp #2 (DPC6) is already reported (4) and it is mentioned in Table 1 for comparison purpose. Data for hemagglutination inhibition (HAI) titer specific to SwIAV H1N1/OH/2007 and 2009 pandemic CA09-H1N1 IAV in sera and BAL fluid collected from both the experiments at DPC6 (Table 1) was determined as previously described. Briefly, heat inactivated and serially 2-fold diluted BAL fluid samples were mixed with 8 HAU of SwIAV (H1N1/OH/2007) in 50 µl and incubated for 1 h at 37 °C. The HAI titers were determined as the reciprocal of the highest dilution of the samples that completely prevented the turkey red blood cells hemagglutination. The HAI titers were transformed to $log_2$ values for comparison between different experimental groups. The HAI titers in serum samples from Exp #1 (DPC6) and in serum and BAL fluid from Exp #2 (DPC6) were reported earlier (3, 4) are mentioned in Table 1 for comparison purpose. The virus neutralizing (VN) and hemagglutinin inhibition (HAI) antibody titers in serum samples against the heterologous challenge SwIAV H1N1-OH7 were analyzed in all the vaccine groups at 6-day post-challenge (DPC6) (Fig. 3 A, B) belongs to experiment #1.

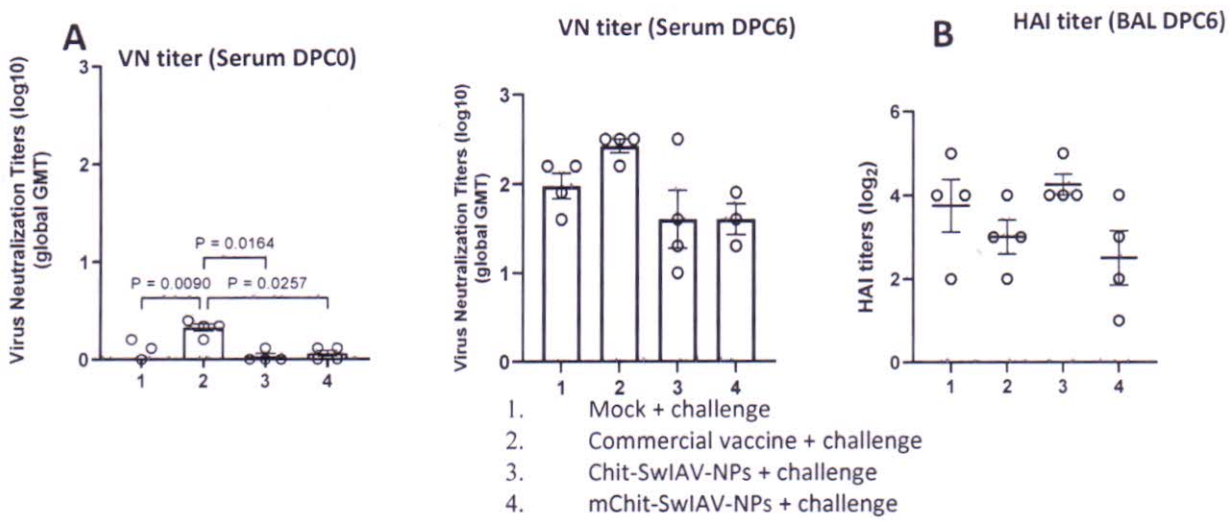
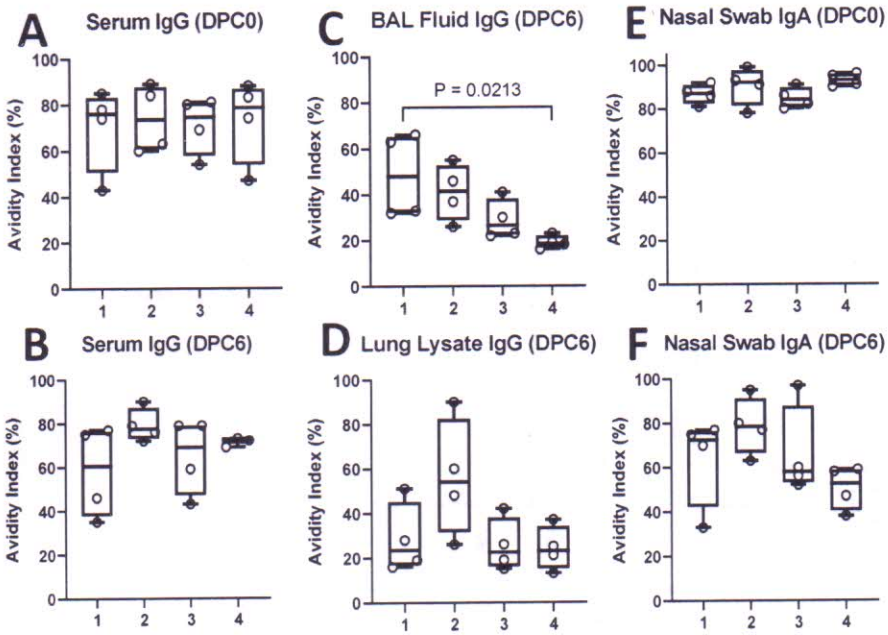



Fig. 3 Influenza A virus specific virus neutralization (VN) and hemagglutination inhibition (HAI) antibody titers in the serum and BAL fluid of experimental pigs, respectively (Exp#1). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

**Avidity of virus-specific antibodies**

Determination of SwIAV-specific antibody avidity in serum, BAL fluid, lung lysate and nasal specimens collected from experimental animals was performed as previously described. Briefly, avidity ELISA was performed using the dilution of samples: serum (1:100), BAL fluid (1:50), lung lysate (1:50) and nasal specimens (1:8). Briefly, the 96-well ELISA plates were adsorbed with pre-titrated inactivated SwIAV antigen (H1N1/OH/2007) and the procedure followed the same steps of ELISA as described earlier with an additional step of adding a chaotropic agent, ammonium thiocyanate (NH4SCN) at different indicated molar concentrations following serum incubation. Antibody avidity index was calculated as mean $OD_{450}$ (NH4SCN-treated samples)/ mean $OD_{450}$ (PBST-treated samples) and multiplied by 100. The antibody avidity data using serum, BAL fluid, lung lysate and nasal specimens from Exp#2 was already reported (4) and it is mentioned in Table 1 for comparison purpose. In order to determine the ideal concentration of chaotropic agent to induce dissociation in immune complexes, a nonlinear regression model was plotted using the $OD_{450}$ values obtained at 2-fold dilution of NH4SCN (5.0M, 2.5M, 1.25M, 0.625M and 0.31M) against control (0M). In experiment #1, with 2.5M of NH4SCN, serum antibodies were still bound to the antigen, at 1.25M concentration for BAL fluid and lung lysate antibodies, and 0.625M for nasal swabs IgA antibodies. Avidity of specific IgG and IgA in serum, lung lysate and nasal swab samples of both nanovaccine administered groups in experiment #1 were comparable. Avidity index values in serum samples in all the experimental groups were comparable (Fig 4, A, B). In contrary, mock challenge group demonstrated a significantly higher avidity index of virus specific IgG in BAL fluid compared to mChit-SwIAV-NPs vaccinates (Fig. 4 C), and relatively higher avidity index than nanovaccines groups with respect to virus specific IgG in lung lysate and virus specific IgA in nasal swab samples (Fig. 4 D, F).




1. Mock + challenge
2. Commercial vaccine + challenge
3. Chit-SwIAV-NPs + challenge
4. mChit-SwIAV-NPs + challenge

Fig. 4 Influenza A virus specific IgG and IgA antibody avidity index in the serum, BAL fluid, lung lysate and nasal swab samples of experimental pigs (Exp#1). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

**Antibody dependent cell cytotoxicity (ADCC)**

Stably transfected MDCK cells (provided by Dr Diego, Department of Population Medicine and Diagnostic Sciences, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA) with the lentiviral vector pScalps engineered to express the full-length open-reading frame of HA (HA1) from SwIAV A/H1N1/OH/2007 were used as target cells for the ADCC assay. The ADCC assay was performed as described previously with slight modifications [36]. Briefly, 8,000 MDCK-HA cells per well were seeded in round bottom 96-well plates overnight at 37 $^0$C with 5% $CO_2$. Cells were washed thrice with 1x phosphate buffered saline (PBS) and incubated with heat-inactivated serum samples (1:25 dilution) for 30 min at 37 $^0$C. Following three washings with 1x PBS, freshly isolated pig PBMCs (source of effector cells) from healthy slaughtered adult pigs were added in an 80 to 1 E:T ratio to the round bottom 96-well plates and incubated for 4 h at 37°C with 5% $CO_2$. Following incubation, 50 µl of cell-free supernatant was collected and transferred into a 96-well flat bottom plate and the lactate dehydrogenase (LDH) release is measured using the Cytotox 96 non-radioactive cytotoxicity assay kit (Promega, Leiden, Netherlands) according to the manufacturer's instructions. Serum samples collected at DPC6 but not at DPC0 from all the vaccinated pigs showed virus specific ADCC activity in experiment # 1 (Fig. 5 A, B), but the data was not statistically different among the groups. Specific cell lysis was comparable across all the vaccinated groups but profoundly higher than mock + challenge group at DPC6 in Exp# 1 (Fig. 5 B). While both at DPC0 and DPC6 serum samples of pigs had virus specific ADCC activity belongs to Exp# 2 (Fig. 6 A, B).

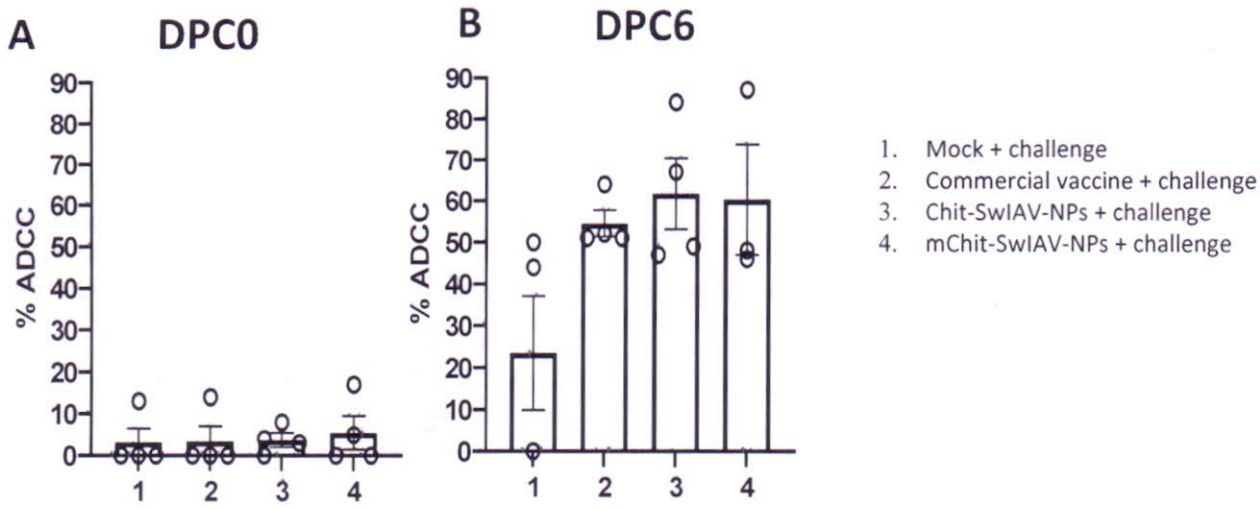



Fig. 5 Influenza A virus specific antibody-dependent cell-mediated cytotoxicity (ADCC) activity in the serum of experimental pigs (Exp # 1). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

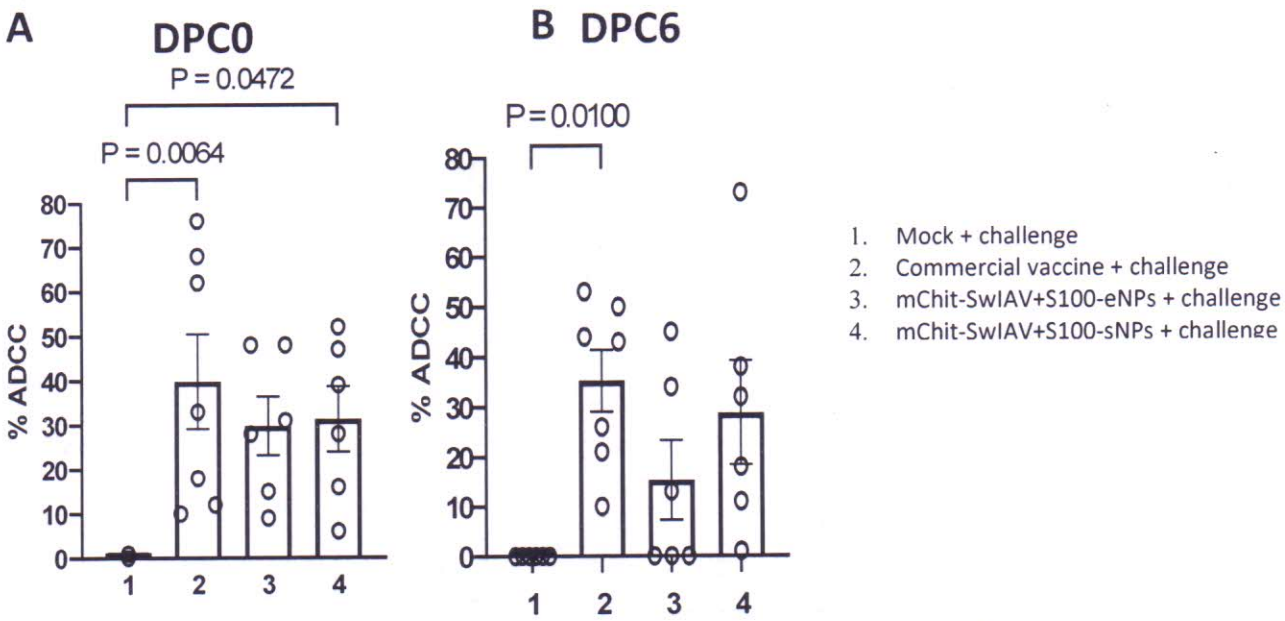



Fig. 6 Influenza A virus specific antibody-dependent cell-mediated cytotoxicity (ADCC) activity in the serum of experimental pigs (Exp # 2). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

**Complement dependent cell cytotoxicity (CDC)**

Briefly, 30,000 target cells (MDCK-HA) per well were seeded in round bottom 96-well plates overnight at 37 $^0$C with 5% CO2. Subsequently, cells were washed thrice with 1x PBS and incubated with heat-inactivated serum samples (1:25 dilution) diluted in serum-free AIM-V medium (Life Technologies, UK) containing 100 IU/ml penicillin and 100 µg/ml streptomycin for 30 min at 37 $^0$C. Following three washing with 1x PBS, 1:10-diluted Low-Tox rabbit complement (Cedarlane, Burlington, Ontario, Canada) in serum-free AIM-V medium was added, and the plate was incubated for 2 h at 37°C with 5% $CO_2$. Subsequently, 50 µl of cell-free supernatant was transferred into a 96-well flat bottom plate and complement-dependent lysis of target cells was determined by measuring the LDH release using the Cytotox 96 nonradioactive cytotoxicity assay kit (Promega, Leiden, Netherlands) according to the manufacturer's recommendations. Besides commercial vaccine group, none of the nanovaccine groups showed significant specific lysis of target cells in comparison to mock + challenge group both at DPC0 and DPC6 in Exp # 1 (Fig. 7 A, B). Specific CDC activity by commercial vaccine group serum samples was significantly higher than other three groups (Fig. 5, A and B). The group which received mChit-SwIAV+S100-eNPs vaccine showed relatively higher percentage of CDC activity than any other three groups both at DPC0 and DPC6 in Exp # 2 (Fig. 8 A and B).

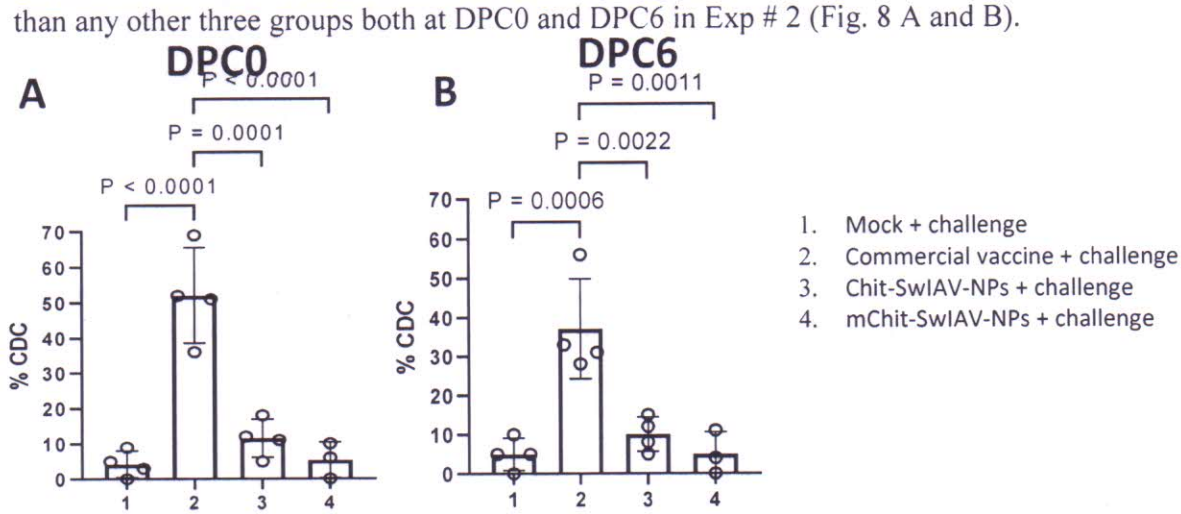


Fig. 7 Influenza A virus antibody specific complement dependent cytotoxicity (CDC) activity in the serum of experimental pigs (Exp #1). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

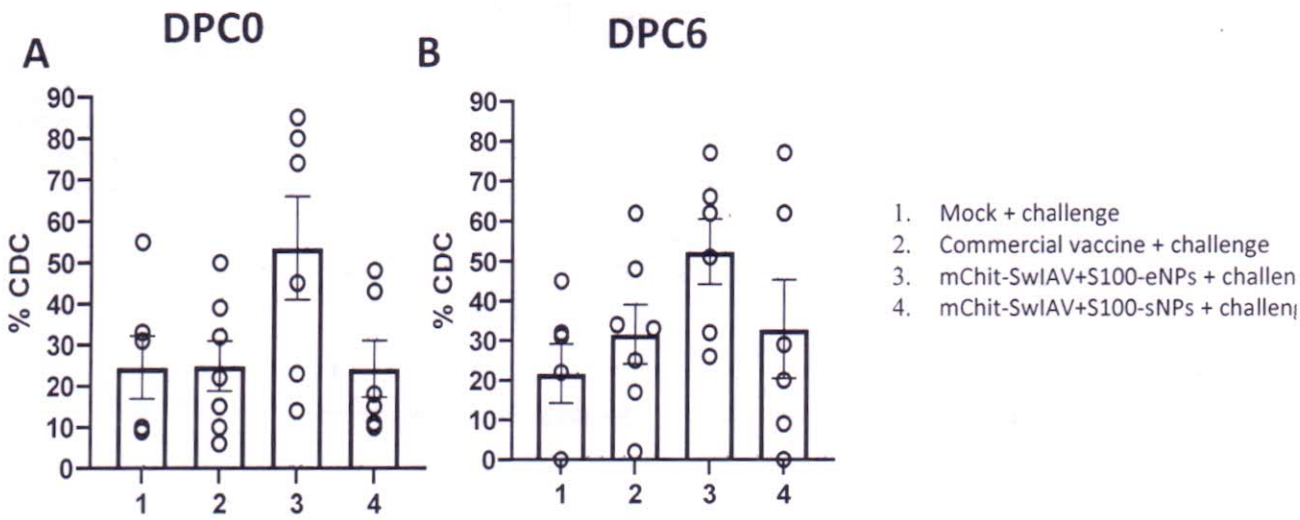



Fig. 8 Influenza A virus antibody specific complement dependent cytotoxicity (CDC) activity in the serum of experimental pigs (Exp #2). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

**References:**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Paper prepared/submitted/publication: 03

1. **Singh, M.**, do Nascimento G. M., Renu, S., Bugybayeva, D., Shekoni, O. C., Suresh, R., Schock., J., Dolatyabi, S., Diel, D. G., Renukaradhya, G, J. (2024).

Influenza specific functional antibodies in mannose conjugated chitosan-based influenza nanovaccine intranasal vaccinated pigs **(manuscript under preparation)**.

2. Bugybayeva, E. D., Patil, V., Yadagiri, G., Suresh, R., Singh, M., Schrock, J., Dolatyabi, S., Shekoni, O.C., Yassine, H.M., Opanasopit, P., HogenEsch, H. and Renukaradhya, G.J. (2024). Evaluation of efficacy of surface coated versus encapsulated influenza antigens in mannose-chitosan nanoparticle based intranasal vaccine in swine. Vaccines (**Under Reveiw**).
3. Hernandez-Franco, J. F., Yadagiri, G., Patil, V., Bugybayeva, D., Dolatyabi, S., Dumkliang, E., Singh, M., Suresh, R., Akter, F., Schrock, J., Renukaradhya, G. J. and HogenEsch, H. (2023). Intradermal Vaccination against Influenza with a STING-Targeted Nanoparticle Combination Adjuvant Induces Superior Cross-Protective Humoral Immunity in Swine Compared with Intranasal and Intramuscular Immunization. Vaccines, **11**: 1699.

Participation in conference/seminar/workshop: 03 (Page 23-25)

Abstract: 05 (Page 26-30)

iii) Proposed utilization of the experience in India:

During the last one-year training period, I had an opportunity and exposure to the research methodologies for the development of subunit mucosal vaccine, and understanding about the immune correlates of protection by employing the various modern state-of-the-art immunological techniques. During the training period, I also got opportunity to acquainted with the data analysis and interpretation tools for understanding the immune correlates of protection. I will apply all these gained knowledge and experience for development of novel oral and intranasal vaccines against other important pathogens (bacterial/viral) which primarily affect mucosal sites (respiratory and gastrointestinal tracts) of livestock species. Importantly, the techniques learnt could be applied to conduct vaccinology research to fight against various diseases of even large animals such as cattle, pigs, sheep, and goats. Some of the animal species such as pig is considered potential biomedical model for translation of novel rodent research findings.

27-05-24

Signature of ICMR-IFICMR

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