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United States Department of Agriculture
Project Initiation

Project Data for Accession Number 1034302

Project Title

PARTNERSHIP:Harnessing Bovine Organoids to Advance HPAI Influenza A Virus Research

Project Details

Sponsoring Institution National Institute of Food and Agriculture	**Project Status** ACTIVE
Funding Source NIFA Non Formula	**Grant Year** 2026
Grant No. 2026-67016-46036	**Cumulative Award Amt.** $796,000.00
Proposal No. 2024-08752	**Multistate No.** (N/A)
Project Start Date Mar 15, 2026	**Project End Date** Mar 14, 2029
Program Code [A1221] Animal Health and Production and Animal Products: Animal Health and Disease	

Project Director

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Recipient Organization

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Performing Department

Microbiology and Cell Biology

Non Technical Summary

The emergence of High Pathogenicity Avian Influenza (HPAI) H5N1 in U.S. dairy cattle poses significant challenges, and raises concerns about animal health, milk production, and economic stability within the dairy industry. This research aims to mitigate the impact of influenza virus infections in dairy cow populations by developing organoid models from bovine respiratory, intestinal, and mammary tissues. These organoids will facilitate essential studies on the virus-host interactions that underpin HPAI pathogenesis, an area critically understudied due to the constraints of agricultural BSL3+ facilities. Historical data indicate that HPAI predominantly affects poultry, yet recent spillover events have demonstrated its ability to infect mammals, including cattle, highlighting potential zoonotic risks. Notably, the viral tropism in bovine hosts remains poorly defined, with high viral loads observed in mammary tissues and milk, while the presence of the virus in the respiratory tract remains contentious. Utilizing organoid technology, this study will explore the variable pathogenesis of IAV across different bovine tissues and assess the impact of cellular

heterogeneity on viral infection and host responses. Additionally, the role of specific host pathways, such as the Rab11-mediated genome trafficking pathway, in the adaptation and virulence of HPAI will be investigated. Overall, this research addresses critical gaps in understanding HPAI in bovine hosts, aiming to inform future surveillance and control strategies to prevent further outbreaks and safeguard both public health and agricultural interests.

Animal Health Component

Animal Health Component: 100%

Research Effort Categories

Basic	80%
Applied	20%
Developmental	0%

Classification

Knowledge Area (KA)	Subject of Investigation (SOI)	Field of Science (FOS)	Percent
311	3440	1101	100%

Knowledge Area

[311] Animal Diseases

Subject Of Investigation

[3440] Meat, dairy cattle

Field Of Science

[1101] Virology

Keywords

Bovine, Dairy Cows, Influenza A Virus, Organoids, H5N1, Host-pathogen

Goals / Objectives

The emergence of High Pathogenicity Avian Influenza (HPAI) H5N1 in U.S. dairy cattle poses significant challenges and raises concerns about animal health, milk production, and economic stability within the dairy industry. The main goal of this research is to develop organoid models from bovine respiratory, intestinal, and mammary tissues to answer key questions about the virus-host relationship in cattle, which remains poorly defined.To accomplish this overarching goal we will focus on the following objectives and sub-objectives:Objective 1: A Toolkit of Bovine Organoid Systems: Compare and Contrast Diverse Tissues for Influenza A Virus Susceptibility.Sub-objective 1a: Establish and differentiate organoids from the bovine organoid toolkit.Sub-objective 1b: Sialic Acid Profiling of bovine organoid toolkit.Sub-objective 1c: Characterizing Tissue Resident Goblet Cells and Mucus Production in the Bovine Organoids Toolkit.Sub-objective 1d: Test organoid susceptibility to HPAI H5N1 and other low pathogenic avian IAV strains.Objective 2: HPAI Infection Dynamics and Innate Immune Responses in Bovine Hosts: From Organoid to Single Cell.Sub-objective 2a. Generation and infection of bovine single cell microgel cultures.Sub-objective 2b.

Analysis of viral productivity and extracellular viral diversity.Sub-objective 2c. Heterogeneity of innate immune responses of infected bovine organoids.Objective 3: Cattle as the new mixing vessel: Role of the Bovine Rab11 Pathway in IAV Infection of Bovine Organoids.Sub-objective 3a: Generate GFP-tagged Rab11 to visualize Rab11 localization in bovine organoids.Sub-objective 3b: Interaction of Rab11 with bovine IAV.Sub-objective 3c: Reliance of bovine and human seasonal IAV on bovine Rab11.

Project Methods

Objective 1a:Establish and differentiate organoids from the bovine organoid toolkit.We will build organoid models fromtissue from slaughterhouses located in Montana and Vermont. Post-mortem, lungs, intestines, and mammary glands will be extracted, and crypts will be isolated. Bovine fibroblasts will be isolated by digestion and subsequently grown in DMEM supplemented with 10% FBS.We will assess tight junction formation by electron microscopy and indirect immunofluorescence. Additionally, we will measure the transepithelial resistance of organoid-derived monolayers. Lastly, we will use commercial antibodies against intestinal epithelial cell markers, lung epithelial cell markers, and mammary gland markers to determine cell differentiation and the percentage of remaining crypt cells in the population.Objective 1b: Sialic Acid Profiling of bovine organoid toolkit. We will use specific lectins to determine the expression of 2,3- and 2,6-Gal in undifferentiated and differentiated bovine lung, intestine, and mammary gland organoids.Objective 1c: Characterizing Tissue Resident Goblet Cells and Mucus Production in the Bovine Organoids Toolkit.Total mucin production will be determined by collecting the supernatant, separating in polyacrylamide gel, and staining it with Periodic acid-Schiff. Both experiments will use undifferentiated organoids derived from the same tissue as controls.Objective 1d: Test organoid susceptibility to HPAI H5N1 and other low pathogenic avian IAV strains. The differentiated organoids will be infected with various HPAI H5N1 strainsat various MOIs to compare cell susceptibility. We will collect apical washes in pre-warmed, serum-free media to determine the amount of infectious virus produced using standard plaque assays.Readouts/Analysis: Organoid growth will be assessed daily by an inverted microscope, and WRN medium quality will be internally tested in our standard organoid cultures. All immunofluorescence assays will only contain positive and negative controls, including solely secondary antibodies or fluorophores. All experiments will be biological replicates, and appropriate statistical analysis will be employedExpected Outcomes/ Interpretation: The proposed research will yield a comprehensive toolkit of bovine organoid systems, highlighting the cellular composition, sialic acid content, and mucus production in bovines. We expect mammary bovine organoid lines to be more susceptible to IAV infections and present higher viral titers than other bovine organoids.Objective 2a. Generation and infection of bovine single cell microgel cultures. We will process the organoids to generate single cells that will be seeded onto microgels. We will optimize culture conditions and microgel seeding using primary bovine embryonic fibroblasts (BeEF). The seeded microgels will be infected using our co-flow inoculation method using a both the bovine and avian H5N1 IAV strains. Following optimization of assays with the BeEF cells, we will utilize primary cells isolated from bovine organoids.Objective 2b. Analysis of viral productivity and extracellular viral diversity. Viral supernatant will be collected by breaking the drops and separating the aqueous phase containing viral progeny from the oil and surfactant. The supernatant will be analyzed for virus production via plaque assays at multiple time points post infection. The viral RNA from progeny virions in the supernatant will be extracted to serve as a template for RT-PCR and for downstream analysis and Illumina based deep sequencing.Objective 2c. Heterogeneity of innate immune responses of infected bovine organoids. To examine the intracellular viral and host diversity from single infected cells, the cells will be collected and analyzed using a 10X genomics platform followed by Illumina sequencing. This will allow us to compare the cellular response from different cell types to IAV infection, complementing the analysis of viral diversity. This will enable comparison of the cellular response from different cell types to IAV infection, quantify differences in innate immune responses, and identify critical host factors associated with enhanced virulence or pathogenicity.Readouts/Analysis: IAV infection of cells can have differing extents of productive replication as measured by burst size and viral gene expression. Extracellular viral diversity will be analyzed from the individually infected cells using standard Illumina sequencing. The DeepSNV pipeline can be used to identify minor sequence variants with a 2% minimum frequency threshold set for variant calling. To analyze heterogeneity in innate immune responses cell samples will be processed for 10X single cell RNA sequencing as defined by the manufacturer. Expected Outcomes/ Interpretation: We expect infection of the BEFS and bovine organoid cells cultured on microgels

to be straightforward based on our preliminary data. Infection of the primary cells is expected to yield more diverse outcomes, with specific subsets of cells being susceptible to infection and having differential expression of viral genes, as previously observed. We will use scRNA-seq analysis to determine the specific cell types that become infected. We also expect to identify differences in innate immune responses in bovine organoids of different tissue origin in response to infection.Objective 3a: Generate GFP-tagged Rab11 to visualize Rab11 localization in bovine organoids. We will create lentiviral expression systems to transiently or stably express bovine Rab11a, Rab11b and Rab25 ('Rab11c) fused to GFP, mCherry or Halo tags to allow simultaneous visualization. We will also create constructs expressing well characterized dominant negative versions of each protein, to examine the progression of IAV infection in the absence of functional Rab11a, Rab11b or Rab25 pathways. Rab9 WT and DN lentiviruses will also be constructed as negative controls. Cells will be infected with recent seasonal isolates of H1N1 and H3N2 viruses of human origin as well as the HPAI H5 of bovine origin. This timing will be adapted for the length of a single replication cycle in each cell type as necessary.Objective 3b: Interaction of Rab11 with bovine IAV.Infected as well as mock infected cells expressing fluorescently tagged Rab proteins will be fixed and processed for fluorescent in situ hybridization with probes specific to each of the IAV gene segments, as we have previously published. Samples will be analyzed using automated pipelines developed in imageJ/FlowJo to quantify the number and size of IAV assembly intermediates, distance from the nucleus, as well as the relative incorporation of each genome segment. Halo-tagged Rab proteins from infected/mock infected cells will be affinity purified using magnetic Halo resin, and the relative binding affinities of components of the influenza RNP (NP, viral RNA), as well as structural proteins not expected to bind Rab11 (HA, M2) will be determined using western blot and RT-qPCR.Objective 3c: Reliance of bovine and human seasonal IAV on bovine Rab11. Cells expressing WT or DN versions of each Rab protein will be infected or mock infected for a single round of viral replication. Viral titer for each sample will be determined by plaque assay, as we have previously published.Expected Outcomes/ Interpretation:We expect IAV-infected cells of bovine origin to form larger Rab11+ puncta where IAV genome segments package together before release. In addition we expect to see colocalization between tagged forms of Rab11 and influenza viral gene segments. Ifthe Rab11 pathway is essential in bovine hosts we expect to see a >10-fold drop in viral titer in bovine cells that have been transduced with a dominant negative Rab11. Finally, we expect to see specific binding between bovine Rab11 and components of the viral RNP (viral RNA) but not structural proteins (HA, M2) or an unassociated Rab (Rab9).