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Comparative Genomic Analysis of *Avibacterium paragallinarum* using Next-gen sequencing to characterize Infectious Coryza infections in Domestic Poultry

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ABSTRACT

Avibacterium paragallinarum is a gram-negative bacterium that has significant impacts on poultry health and economics associated with the presence of reduced egg production when present. The disease caused by the bacterial pathogen is called Infectious Coryza (IC) and prevention of IC rely on the use of biosecurity measures, surveillance, and vaccination. The absence of cross protection among serovars makes rapid and efficient characterization of *A. paragallinarum* critical in reducing the risk of entry into poultry flocks. Current diagnostic tools such as conventional microbiology and serotyping are effective but are not as rapid and are labor-intensive causing for there to be a delay in the diagnostic process. Due to this, the goal of our study was to use comparative genome analysis to evaluate if next generation sequencing is a viable tool in diagnosing outbreaks and identifying the associated serogroups rapidly. To accomplish this, we performed comparative genome analysis of 40 clinical isolates collected from 2019 Pennsylvania (PA) IC outbreaks. The microbial genomes were assembled using Tychus and annotated using Prokka. Roary was then use to identify core genes and unique genes to evaluate how Page Serovars varied genetically. The outputs of Roary were then used to construct a phylogenetic tree to evaluate genetic relatedness of the clinical isolates to previously reported reference isolates obtained from NCBI GenBank. The study revealed genetic diversity among the AP serovars with an in-depth understanding of similarities and differences using reference strains and *A. paragallinarum* isolates from PA. Further an increased level of genetic heterogeneity among isolates compared to the reference genomes shed light to the open nature of the pan-genome.

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Chapter 1 : Introduction

The re-emergence of an acute and sporadically chronic respiratory disease in poultry caused by *Avibacterium paragallinarum* incurred severe losses to several states in Northeastern United States including Pennsylvania (PA) in 2019. The disease is called infectious coryza (IC) that has severe impacts on susceptible poultry populations when the etiologic agent, *Avibacterium paragallinarum*, is introduced into domesticated poultry flocks. Infectious coryza is characterized by its high morbidity and low mortality rates, having different manifestations of disease based on the susceptibility of the population and the presence or absence of comorbidities. The establishment of IC into susceptible populations can result in the production of acute inflammation of the upper respiratory tract, facial edema, and conjunctivitis. Subclinical signs such as reduction in egg production up 40% can also be present (Blackall & Soriano-Vargas, 2019) . The presence of a positive sample in a flock often requires flock termination, having severe economic impacts on poultry farmers. Spread within a flock is rapid via contact with infected birds, through ingestion of contaminated feed or water, and via airborne route. Once the susceptible birds get exposed, the signs of the disease are manifested within 24-72 hours (Rimler et al., 1977). These components paired with the significant economic losses experienced by the poultry industry make early detection of infectious coryza vital in controlling the spread of disease.

Avibacterium paragallinarum was classified into three serogroups (A, B and C) based on plate agglutination tests by Page in 1962 (Page, 1962). Subsequently, using the hemagglutination and hemagglutination inhibition tests a new classification system was proposed and divided the

isolates into three serogroups (I, II and III) and seven serovars (HA-1, HA-2, HA-3, HA-4, HA-5, HA-6 and HA-7) by Kume (Kume, 1983). Rapid, efficient, and effective diagnostics are critical in characterizing outbreaks of IC due to the lack of cross-protection amongst serogroups of *A. paragallinarum*. This pushes the importance of serogroup identification for the proper selection of vaccines for surrounding susceptible flocks that might not have been exposed to infectious coryza.

Bacterial isolation of *A. paragallinarum* has been used in the definitive detection and characterization of infectious coryza (Blackall, 1999). Although effective, this method has been limited due to the slow-growing fastidious nature of the bacteria and the necessity of specialized growth media. Serological detection of IC has also been used in detecting the disease in experimentally challenged animals (Blackall, 1999), however, serology lacks practical use in outbreak investigation due to the inability to characterize or serotype the isolates. As alternative to traditional serotyping methods, there exist a few PCR based efforts on molecular serotyping of AP isolates. For instance, the one targeting a specific hypervariable region on gene *hmtp210* encoding a 210-kDa outer-membrane protein (Sakamoto et al., 2012). Nevertheless, these methods have not been extensively validated and the genetic correlation to the serovars have not been fully established.

Next-generation sequencing stands as an uprising field in the genetic characterization of infectious diseases via genotyping. Such a method could provide a more rapid, cost-efficient method of characterizing outbreaks and selecting the appropriate autogenous vaccines for poultry facilities surrounding the outbreak area. Furthermore, genotyping can be a valuable tool in defining the epidemiology of infectious coryza to create vaccine programs based on predicted strains prevalence in the specified geographic area. The objective of this paper is to evaluate the genetic

basis for the serological differences in *A. paragallinarum* clinical isolates by whole genome sequence analysis and perform a comparative genomic analysis using reference genomes from three Page serogroups of *A. paragallinarum* to identify differences in core and accessory genes. Furthermore, this paper aims to evaluate if genotyping using next-gen sequencing can be used in the identification and characterization of *A. paragallinarum* serovars when outbreaks occur in domestic poultry.

Chapter 2 : Review of Literature

General Overview

Infectious Coryza (IC) is an acute respiratory disease caused by the *agent Avibacterium Paragallinarum*, also previously known as *Haemophilus Paragallinarum*. This disease affects poultry, and has major implications to the poultry industry due to the presence of growth retardation and marked decrease in egg production (10-40%) associated with infection (Blackall & Soriano-Vargas, 2019). Additionally, increased number of culls when disease is present have severe economic effects on the poultry industry. Vaccines are available as a preventative measure against infectious coryza in chicken flocks(Soriano-Vargas & Edgardo Horacio, 2004).

Etiology

Avibacterium Paragallinarum, previously referred to as "*Haemophilius Paragallinarum*", is a member of the family Pasteurellaceae. The length of this *A. paragallinarum* ranges between 1-3µm and its width ranges between 0.4µm-0.8µ(Blackall & Soriano-Vargasl,2019). This bacterium is a gram-negative, non-spore forming, non-motile organism that is pleomorphic. When plated onto an agar medium, the bacteria exhibit a coccobacilli morphology with a propensity to form filaments with short chains of bacteria. Pleomorphic rods and faded bacteria-like residues are observed in broth cultures. Longer bacilli form with metachromatic granules inside bacterial cells are often present in direct smears from nasal mucus. When colonies are exposed to obliquely transmitted light, smooth colonies are iridescent, while rough colonies do not exhibit iridescence (Kume et. al, 1983).

Growth requirements and culture medium

Within the genus *Avibacterium Paragallinarum*, Nicotinamide adenine dinucleotide (NAD) dependent and NAD independent strains have been identified, affecting the in vitro growth requirements. Most strains are NAD-dependent, requiring a reduced form of NAD (1.56-25 µg/ml medium) or an oxidized for (20-100 µg/ml) for effective in-vitro growth. NAD can also be supplied on agar cultures through simple addition or production by a feeder bacterium that is cultured as a cross streaked colony with *Avibacterium Paragallinarum*. NAD-independent isolates found in South America and Mexico are excluded. Additionally, 1-1.5% sodium chloride (NaCl) is required for optimal growth(Rimler et al., 1977).

A. paragallinarum strains are often grown in microaerobic or anaerobic conditions with higher levels of carbon dioxide (5-10%)(Rimler et al., 1976). Similar conditions have been accomplished through using the traditional candle-jar method or by using Campylobacter genus generated by commercial kits or gaseous mixtures. To successfully propagate *A. paragallinarum* the following basic mediums are used together with additional supplements: brain-heart infusion (BHI), chicken-meat infusion, and Haemophilus maintenance medium (HMM) (Blackall, 1998), Casman agar, gonococcal agar(Soriano-Vargas, 2004), test medium (TMA)(Rimler, 1979), or Colombia agar(Soriano-Vargas, 2004), Agar plates in 7%-10% bovine or sheep defibrinated blood agar plates are incubated for 16-24hrs at 37°C.

Biochemical Properties

Avi. Paragallinarum shows symbiotic growth with a requirement for 5-10% CO_2 in vitro passages (Blackall et al., 2005). Hemolysis and Catalase reaction were negative. Phosphate

reactions where variable, while ortho-Nitrophenyl- ß- galactoside (ONPG) was negative. Acid was produced from D-mannitol and D-sorbital, and lacked production for L-arabinose, D-arabinose, meso-inositol, D-galactose, glycerol, lactose, trehalose, and raffinose (Blackall et al., 2005). D-ribose, D-xylose, L-fructose, maltose, and dextrin has variable acid production.

Classification

The Page Scheme was the first methodology used for the classification of *A. paragallinarum* through identifying three serovars, Serotypes A, B, and C using slide agglutination tests(Soriano-Vargas & Edgardo-Horacio, 2004). Hemagglutination inhibition (HI) tests are now recommended for the isolate identification using the Page Scheme due to the decreased number of non-typeable isolates than compared to original agglutination technology(Eaves et al., 1989). Serotypes A, B, and C have been distributed in Argentina(Soriano-Vargas,2004), Brazil (Blackall et al., 1994), Egypt(Soriano-Vargas, 2004), Spain(Soriano-Vargas, 2004), United States(Soriano-Vargas, 2004), Philippines(Nagaoka et al., 1994), Indonesia(Poernomo et al., 2000), Mexico(Fernández et al., 2000), and South Africa(Blackall & Eaves, 1988) when characterized under the Page Scheme. Additionally, isolates Serotypes A and B have been located in Germany and China(Chen et al., 1993),(Zhang et al., 2003); Serovars A and C in Japan (Kume et al., 1978), Malaysia(Soriano-Vargas, 2004), Australia, and India(Soriano-Vargas, 2004); and Serotype C in Taiwan(Lin et al., 1996).

The Kume scheme stands as an additional serological classification method dependent on HI test using potassium thiocyanate-treated and -sonicated cells, rabbit hyperimmune serums, and glutaraldehyde fixed chicken erythrocytes (Kume et al., 1983). This method encompasses

Pages three serovars A, B, and C, and nine serogroups known as “Kume serovars”. Kume’s serovars include A-1, A-2, A-3, A-4, B-1, C-1, C-2, C-3, C-2 and C4(Blackall et al., 1990). Kume Scheme has the advantage providing a more detailed identification of serovars that were classified as non-typable strains under the Page scheme (Eaves et al., 1989). The following serovars have been identified using Kume’s scheme: A-1, A-2, B-1 and C-2 in Jalisco; A-1 and A-2 in the state of Mexico; B-1 and C-2 in Puebla; A-2 in Michoacan; C-2 in Morelos and B-1 in Sonora and Yucatan(Soriano-Vargas et al., 2001).

Blackall’s scheme is often used as a universal criterion for serovar nomenclature and serological typing due to confusion present when distinguishing Kume’s scheme with Page’s scheme. The following serovars have been classified based on Blackall’s scheme: A-1, B-1, C-2 in the United States (Kume et al., 1983) (Eaves et al., 1989), A-4, C-2, and C-3 in Australia(Eaves et al., 1989) (Blackall et al., 1990); A-1, B-1, C-2, and C-2 in South Africa(Eaves et al., 1989). A-1 and C-1 in Japan (Eaves et al., 1989); A-3 in Brazil (Kume et al., 1983); C-3 in Zimbabwe (R. Bragg, 2002); A-1, A-2, B-1, and C-2 in Germany(Kume et al., 1983) (Eaves et al., 1989). Kume’s scheme is not often used due to the tedious technical nature of classifying serovars.

Virulence Factors

Multiple factors have been associated with the pathogenicity *of A. paragallinarum* such as its capsule structure, Hemagglutinin (HA) antigens, sequestration of iron, and the RTX-toxin. The encapsulated structure of *A. paragallinarum* plays a significant role in epithelial adhesion to mucosal surfaces. A study analyzing the relationship between the amount of capsule present and

the virulence of the isolate found that there was a correlation, and that encapsulated organisms were able to effectively cause infection in chickens while unencapsulated chickens could not. Furthermore, the study found that chickens who were given the encapsulated variant exhibited increased loss of cilia, microvilli, increased leukocyte infiltration, and the production of mucopurulent substances on the nasal mucosa. In comparison, there was increased bacterial adhesion to mucosal surfaces with a reduction in virulence, and a lack of morphological changes in the nasal mucosa with chickens given the unencapsulated variant (Sawata & Kume, 1983). Additional studies found that the capsule also protective role in inhibiting bactericidal activity of the normal chicken serum (Sawata et. al, 1985). Such findings confirmed the importance of the capsule in cellular adhesion to effectively cause disease in chickens, as well as, their role in the production of severe lesions.

Hemagglutinin (HA) antigens have also been noted as a possible virulence factor for *A. paragallinarum.* The HA antigen is a 201kDA protein (HMTP210) that is a trimeric autotransporter that has been associated with biofilm formation, cell adherence, and hemagglutination. Mutants that are deficient of the HMTp210 gene for HA lacked HA activity and failed to elicit and immune response in immunized chickens. Additionally, such a mutation decreased adherence capacity and the ability to form biofilms on abiotic surfaces(Wang et al., 2014). Iron sequestration from chicken and turkey transferrin has also been noted as an importance factor in inducing disease due to the presence of 62 and 66-kDA proteins associated with transferrin binding proteins (TBP) 1 and TBP2(Ogunnariwo & Schryvers, 1992).

The final virulence method identified for *A. paragallinarum* is the production an RTX-toxin (Repeats in the Structural Toxin), a pore-forming toxin secreted by a range of gram-negative bacteria (Pan et al., 2012). RTX toxin operons are categorized using the CABD pattern,

where C encodes for the activation protein, A encodes for the structural protein, and B and D encode proteins that are involved in the type I secretion system(Pan et al., 2012). The secretion of the RT-toxin in gram-negative species is often associated with hemolytic or cytotoxic activities. *A. paragallinarum* contains an RTX-pore exotoxin where AvxA is encoded on a classical operon structure with the activator gene avxC, the serine-protease RTX toxin gene avxxA, and a type I secretion system avxBD(Küng & Frey, 2013). Proteases found in AvxA structural protein play a significant role in bacterial nutrition and invasion of the host, thus contributing to its virulence(Pan et al., 2012).

Transmission

Poultry are natural hosts of *A. paragallinarum* with birds of all ages being highly susceptible to infection. Isolation of *A. paragallinarum* has also been recorded in quails and psittacine. Additionally, clinical signs of sinusitis and infectious coryza were present in experimentally infected turkeys, with similar lesions being observed in chickens infected with the same bacterial culture. Infection in other poultry species has not been concluded, thus it is inferred that infection in avian species has not been effective. Rabbits, guinea pigs, mice, sparrows, ducks, and pigeons are resistant to experimental infection. Chickens who have chronic or healthy carriers of infection acts as the main reservoirs for infectious coryza. Molecular fingerprinting techniques have been used to monitor the role of reservoirs in the spread of infectious coryza, revealing that infection most frequently occurs in the fall and winter.

Infectious coryza is transmitted horizontally via direct contact, airborne droplets, or consumption of contaminated drinking water with nasal discharge present (Page, 1962). The

primary route of transmission within a flock is through waterborne transmission, in which infected and susceptible birds contaminated water sources. Nasal discharge from infected birds contains over 100 million *A. paragallinarum* organisms per ml. It is recorded that brief exposure of thirsty animals to *A. paragallinarum* in contaminated water containing less than 156 organism per ml can effectively transfer disease to susceptible birds(Page, 1962). Studies further confirmed durable nature of *A. paragallinarum* in water by observing its presence in nasal discharge in tap water (pH= 7.6) for 3 hours. A secondary route of intra-flock transmission of infectious coryza is via airborne infection in which infected respiratory discharge droplets or duct are inhaled. An experimental infection confirmed the viability of aerosol transmission for infectious coryza by exposing susceptible birds to infected birds in a single pen without food or water for 3 hours daily for four days (Page, 1962). The results of the study revealed airborne transmission was effective at inducing infection (55%), but at lower levels when compared to the 85% clinical sings rate when susceptible chickens are exposed to waters, common feed, and are in close quarters. Additionally, the study noted the slow transmission rate among chickens. Fomite transmission via contact with infected machinery is an additional source of transmission.

Mechanical transmission of infectious coryza cannot occur through transfer by hand, husbandry men, or flies(Page, 1962). It was recorded that flies could transfer organisms from their legs between the drinking water containers, however, effective transmission is unlikely due to the rapid nature required. One study observed that *A. paragallinarum* on the leges of common house flied that had been exposed to infectious nasal discharge only remained viable for 15-30 minutes, and that water contaminated by the same fly did not cause infection in poultry that had drank the water (Page, 1962).

Clinical Signs

Infectious coryza has a short incubation period that develops within 24-48 hours post-exposure whether it be through experimental infection or contact with infectious fluids (Blackall & Soriano-Vargas, 2019). Susceptible birds exposed to infected birds exhibit clinical sings within 24-72 hours. Infection with infectious coryza usually terminated in 2-3 weeks in the absence of concurrent infection. Infectious coryza causes acute inflammation of the upper respiratory system. This includes the transformation of nasal passage and sinuses from a serous nasal discharge to a mucosal discharge accompanies by facial edema and conjunctivitis(Blackall & Soriano-Vargas, 2019). Swelling of wattles occurs in males. Additionally, tracheal rales may be present if a lower respiratory tract infection is present. Co-infection with other infectious microorganisms may cause severe clinical respiratory outcomes. *Mycoplasma sinoviae, M. galliseptum, Ornithobacterium rhinotracheale, Escherichia coli, Salmonella spps, Pasteurella spps,* and infectious bronchitis are common in agents in co-infection with *Avi. Paragallinarum* (Vargas & Terzolo, 2004). A swollen head syndrome has been associated with *A. paragallinarum* co-infections in the absence of avian pneumoviruses and the presence of absence of other bacterial pathogens such as *M. synoviae* and *M. gallisepticum* in broiler chickens(Blackall & Soriano-Vargas, 2019). Additionally, arthritis and septicemia have been reported in broiler and layer flocks. Such clinical signs were associated with the presence of disease complex (Blackall & Soriano-Vargas, 2019).

Chickens may have diarrhea and decreased feed and water consumption. Additionally subclinical signs ranging from increased culls of growing animals and reduced egg production in laying flocks (10-40%) may be present (Blackall & Soriano-Vargas, 2019). Decreases in egg

production up to 58.7% have been recorded in flocks with severe disease(Vargas & Terzolo, 2004).

Morbidity & Mortality

Infectious Coryza is characterized by having a low mortality and high morbidity. Variations in age and breed influence the manifestation of disease. Factors such as parasitism, poor housing, and an inadequate nutrition may contribute to severity and duration of disease(Blackall & Soriano-Vargas, 2019). Older birds can suffer higher mortality rates noted by an outbreak in California that had mortality reach 48%. Co-morbidities with disease such as infectious bronchitis, laryngotracheitis, M*ycoplasma gallisepticum* infectious and pasteurollosis may increase the severity and duration of infectious coryza, resulting in increased mortality.

Diagnostics

Traditional method

The traditional definitive method for characterization of infectious coryza outbreaks requires the isolation of the organism and biochemical characterization of to confirm the identity(Blackall, 1999). This diagnostic method is challenging due to the fastidious and slow-growing nature of *A. paragallinarum*. Limitations such as outgrowth by surrounding commensal bacteria and specialized availability of resources such special growth media for NAD-dependent bacteria, cause for this methodology to be extremely tedious. Additionally, some diagnostic

laboratories cannot support the needs of specialized equipment due to accessibility issues(Blackall, 1999).

Molecular Identification

Molecular characterization via the use of PCR has provided a rapid and effective method of identifying infectious coryza outbreaks and characterizing them. A study comparing the use of PCR and the traditional culturing method by infecting 64 experimentally challenged chickens found that that PCR provided the same efficiency with more rapid timing, causing a shift to molecular characterization of outbreaks(Chen et al., 1998). Another advantaged of using PCR is the ability to identify over 40 *A. paragallinarum* isolates including NAD-independent strains (Blackall, 1999). Additionally, PCR has given negative reactions with many closely related bacteria such as NAD dependent forms of *P. volatium, P. avium*, and Pasteurella species, increasing ease of identification of *A. paragallinarum*. Newer access to whole genome sequences of *A. paragallinarum* isolates have allowed for the development of a real-time PCR that targets the recN gene(Kuchipudi et al., 2021). The recN gene is a DNA-dependent repair protein that has high divergence between different strains of *A. paragallinarum* and additional isolates, but remains conserved among the Page Serovars. In a study examining RT-PCR using the recN target sequence, they found 419 samples from suspected flocks yielded 100% agreement with bacterial-based detection(Kuchipudi et al., 2021). This represents a novel diagnostic method in the identification and characterization of outbreaks. Lastly, ribotyping has been used as an additional method of classification of *A. paragallinarum* into different serovars (Miflin et al., 1997).

Additional forms of PCR have been developed such as multi-plex PCR (mPCR) and ERIC-PCR ("Enterobacterial repetitive intergenic-PCR). The multiplex PCR targeted the HMTp210 gene, a major outer membrane protein associated with protection against infection(Sakamoto et al., 2012). The HMTP210 gene can be divided into three regions where regions 1 and 3 are highly conserved in serovars A and C, and region 2 which is 50% conserved. A study evaluating the effectiveness of mPCR in serovar identification found the sequence diversity in region 2 and Page serovars only holds true for a small set of strains examined(Morales-Erasto et al., 2014). This finding paired with the low sensitivity (Serovar C:46.3% and Serovar B:85.7%); illustrate the inability of mPCR to act as an effective diagnostic tool. ERIC-PCR was an additional method proposed for molecular identification through the use of ERIC intergenic consensus sequences, however, it is not ideal for serovar characterization justified by absence of a correlation between comparison of banding patterns of field strains and reference strains(Hellmuth et al., 2017).

Serology

Hemagglutinin inhibition tests (HI) are the primary diagnostic tool used for detection of antibodies in infected poultry. There are three main forms of HI tests: simple, extracted, and treated HI tests (Blackall, 1999). Simple HI tests are based on whole bacterial cells of Page Serovar A in fresh chicken erythrocytes, and are limited to detecting antibodies to serovars A. Simple HI test are often used for antibody detection in vaccination studies and with outbreaks. Extracted tests are based on KSCN-extracted and sonicated cells of *A. paragallinarum* and glutaraldehyde fixed chicken RBC, and are primarily used to detect antibodies to serovar C.

(Blackall, 1999) Extracted tests are often used with identification of antibodies against Serovar C in vaccination studies. HI tests are based on hyaluronidase-treated whole bacterial cells of *A. paragallinarum* fixed with formaldehyde. Treated HI tests have been used in the detection of antibodies of Page serovars A.B, and C, however it is not commonly used due to the lack of information to draw conclusions on correlation between high titer and protection using treated tests (Blackall, 1999). In contrast, vaccinated chickens with titers greater than 1 to 5 in simple or extracted HI tests are protected against experimental challenge.

The major limitation of serological assays is that their use is dependent on intended use and serovars, causing them to have lack practical use when serovars are unknown(Blackall, 1999). Due to this, additional robust assays are requiring for characterization of serovars. The production of the ELISA-assay is promising in detecting various antibodies to Page Serovars. A study illustrated the success of an ELISA in antibody detection by creating an ELISA that targeted the HMTp210 region of *A. paragallinarum*, and using the ELISA to test for antibodies in vaccinated chickens. The findings of this experiment found that the prevalence rates on ELISA were 80% (Serovar A) and 60% (Serovar C), and that the sensitivity for antibody detection was greater than that of HI tests(Sakamoto et al., 2012). Such findings illustrate an additional tool that can be used in the identification of antibodies in response *to A. paragallinarum.*

Treatment

Antibiotic are often use in the treatment of infectious coryza. This includes: streptomycin, spectiomycin, streptomyscin-spectino-mycin, sulphachloropiridazine-trimetroprim,

sulphadimetoxin-trimetropim, and other variations(Vargas & Terzolo, 2004). Quinolones such as Norfloxacin nicotinate and enrofloxacin have been observed to be excellent in treating of infectious coryza. A study evaluating the efficacy of norfloxacin in 26-week-old broiler chickens found that clinical signs disappeared rapidly 2-weeks post treatment, and that reisolated of *Avi. Paragallinarum* was not present when chickens were given 40mg norfloxacin/kg body weight(Lublin et al., 2019).

The rise of antibiotic resistance has increased the importance in sensitivity testing before administering antibiotics to infected chickens. A study done in Dutch commercial farms found that approximately 75% (32/44 isolates) of their isolates treated with tetracycline had an elevated minimum inhibitory concentration (MIC) (16 ≥g/ml) μ (Heuvelink et al., 2018). Such findings infer that commonly prescribed drugs such as doxycycline and oxytetracycline may no longer be effective at treating infectious coryza. These findings confirm the importance of preventative measures such as vaccination, disinfection, and proper biosafety procedures in effectively controlling the spread of infectious coryza.

Prevention

Prevention of the introduction of infectious coryza into domesticated poultry flocks depends on proper management, isolation, human traffic flow, disinfection, and the implementation of vaccine programs. Biosecurity practices such as monitoring human-traffic flow prevent *A. paragallinarum* from entering into the farming environment through contaminated material, supplies, or personnel. Furthermore, the use proper disinfection and sanitation aid in the elimination of exposure of infectious coryza to flocks via killing the agent. Although disinfection

and controlling traffic-flow reduce the probability of transmitting infectious coryza, vaccination is the most successful form of preventing infectious coryza.

Disinfection

Disinfection, proper management procedures and vaccination are key strategies in limiting the spread of infectious coryza. To evaluate the effect of a disinfection on the reduction of disease Braggs challenged vaccinated and unvaccinated birds with *A. paragallinarum* and implemented a continuous disinfection program using dideciIdimetylammonium chloride for one group of challenged chickens(Bragg, 2004). The findings of this study demonstrated a reduction in the severity of clinical signs and a less severe drop in egg production. An additional study found similar results when N-alkyl dimethyl benzyl ammonium chloride (TIMSEN) was administered in water to prevent horizontal transfer of *A. paragallinarum*(Huberman et al., 2005). The study also found that only one daily environmental spraying was needed to prevent clinical signs. The combination of these studying emphasizes the effect that disinfection can have on the reduction of transmission of infectious coryza.

Vaccination

Vaccination is an essential tool in preventing the introduction of infectious coryza into domesticated poultry flocks. Unvaccinated birds who recover from infectious coryza develop varying degrees of homologous natural immunity as early as two weeks post exposure. However, the absence of cross protection between serovars leaves birds with natural immunity susceptible

to infections with other serovars. Vaccines for infectious coryza are often in an inactivated bacterin form with the presence of adjuvants ranging from Aluminum Hydroxide gel, Single emulsion mineral oil, and a double emulsion mineral oil. The minimum antigenic level needed to establish protection in poultry is 10^8 colony forming units per vaccine dose. Protective antigens for infectious coryza are still being identified, however, purified HA antigen has proven to induce a protective response. Takagi further confirmed the role of the HA antigen in protection by illustrating the passive protection present when purified alone and when the HA antigen is purified with its protective antibody (Takagi, 1993). The HMTp210 outer membrane protein also plays a role in protection due to its ability to induce HI antibodies, however, it is usually expressed at lower levels (Sakamoto, 2013). One study aims to develop a recombinant vaccine that used region 2 of the HMTp210 protein of Serovars A and C as a fusion peptide. The findings of such study illustrated there was an absence of clinical signs and side effects at the injection site after being challenged with either Serovar A or C, indicating the possible use of the HMTp210 region as a protective antigen via recombination peptide fusion.

Cross Protection

Cross protection analysis within studies depends on their definition of “protection”. Some note protection as the presence or absence of clinical signs, while others define protection and the absence of mucus in sinuses, the absence of clinical signs, and the absence of the organism in the sinuses (Blackall, 1995). Due to such definitions, there are varying interpretations in the analysis of cross-protection. There is a general consensus that there is an absence of cross-protection between Page Serovars, with the exception of some cross-protection being found

between B-1 and C-4 (Soriano-Vargas, 2004) . These findings were exhibited in a study that found an absence of cross protection between Kume serogroup A-1 and C-1, A-1 and C-2, A-4 and C-2, and A-4 and C-4. In contrast, there is cross protection between Kume serovars, with some reduced levels of cross protection across the four C serovars. This cross-protection across Kume serovars was confirmed in an experiment where Serogroups A-1, A-2, and A-3 were strongly cross-protective, with A-4 having reduced cross-protection for serovar A. Furthermore, good cross protection between C-1, C-2, and C-3 was present with some limitations (Soriano-Vargas, 2004). Serogroup C-1 was noted to have lower protection against C-2 challenge, and C-2 had reduced protection for serogroups C-1 and C-3. An additional study observed similar results where only one out of four vaccines were cross-protective against C-1 strains found in Mexico and Ecuador (Morales-Erasto, 2015). Such findings illustrate the dependency of cross protection and HI antibody titer of inactivated Aluminum hydroxide vaccines on serovars present in the vaccine administered. Furthermore, these findings indicate the importance of the characterization and inclusion of serovars for effective protection.

Current Vaccines

Trivalent and Tetravalent forms of the infectious coryza vaccine are commonly used as a tool to increase the protection amongst serovars due the lack of cross protection amongst Page Serogroups. The efficacy and safety of trivalent vaccines containing one isolate from each Page Serovar was performed in specific pathogen free chickens (SPF) and conventional chickens (Gong, 2014). The protection rate in SPF chickens vaccinated on day 42 illustrated 80% protection 30 days post vaccination. Conventional chickens challenged 9 months post second

vaccination had a protection rate of at least 80% for all serovars. Such results indicate the production of protection against infectious coryza when all three serovars are included, however many commercial vaccines omit Serovar B, limiting protection in geographic areas where serovar B is present. This limitation was present in Indonesia where serovars A, B, and C were identified, while the vaccine only contained serovars A and C (Poernomo, 2000). Similarly, a study comparing the efficacy of monovalent vaccines with Argentinean serovar B strain bivalent (serovar A and C only), and trivalent strains (serovars A, B, C) found all commercial strains to be ineffective at protecting against a severe challenge of H8. In comparison, the monovalent strains with the Hp8 strain were more protective in chickens (Terzolo et. al, 1997). These findings indicated the need for the formation of a tetravalent vaccine that could contain all Page Serovars.

Chapter 3 : Materials and Methods

Sample Collection and bacterial DNA extraction

Clinical samples submitted to the Pennsylvania Animal Diagnostic Laboratory System (PADLS) from Infectious Coryza suspected chickens were streaked onto chocolate agar and incubated for 24 h at 37°C with 5% CO2 (Hsu, 2007). Forty-one isolates obtained were grown overnight in brain heart infusion broth supplemented with chicken serum and nicotinamide adenine dinucleotide (NAD). The clinical AP isolates were confirmed using biochemical reactions, growth conditions, a matrix- assisted laser desorption/ionization time-of-flight identification system (MALDI-TOF MS, Bruker Daltonics, Bremen, Germany) and a positive result in a real-time PCR specific for AP (Kuchipudi, 2021). Bacterial DNA was extracted using the Qiagen Genomic-tip 100/G following the manufacturer's instructions (Byukusenge, 2020). The concentration and purification of extracted DNA were quantified using Maestro Nano spectrophotometer® which was analyzed using fluorometric measurements. Illumina (San Diego, CA, USA) paired-end sequencing libraries were generated with the Nextera DNA Flex library prep kit (Illumina) and sequenced with the MiniSeq platform and midoutput reagent kit (2 × 150 bp). Base calling was performed with MiniSeq Local Run Manager software.

Reference isolates

Reference sequences were selected based on their Page serogroup, completeness of genome and global prevalence of infection in poultry. Genomic information of the reference strain genomes from all three serogroups A, B, C were obtained from the National Center of

Biotechnology Information (NCBI)-GenBank. The list of reference samples and associated Genbank metadata is provided in Table 2.

Table 1. Strain and Serovar Identification of *A. paragallinarum*

Strain	RefSeq assembly accession	Page Serogroup
JF4211	GCF_000442905.1	Serovar A
A221	GCF_000348525.1	Serovar A
NCTC 11296	GCF_900450705.1	Serovar A
Hp8	GCF_008633115.1	Serovar A
2671	GCF_004212345.1	Serovar B
FARPER-174	GCF_003864155.1	Serovar C
SA-3	GCF_004212355.1	Serovar C
ESV-135	GCF_011765605.1	Serovar C
Modesto (Japan)	GCF_004212375.1	Serovar C
Modesto (South Africa)	GCF_004931865.1	Serovar C

Genome Sequencing, Assembly and Annotation

Raw reads were assessed for their base quality using FASTQC and then Tychus (Dean et al., 2018) was used for genomic assembly and the construction of the consensus assembly. Briefly, Illumina paired-end reads were trimmed using Trimmomatic (Bolger, 2014) and for *de-novo* assembly the optimal k-mer length was estimated using KmerGenie (Chikhi and Medvedev, 2014). Preprocessed reads were then assembled using four assemblers were used, SPAdes (Bankevich, 2012), Velvet (Zerbino, 2010), IDBA-UD (Peng, 2012), and Abyss (Simpson, 2009). The contigs

of all assemblies were integrated using CISA (Contig Integrator for Sequence Assembly) (Lin and Liao, 2013) to create a higher quality consensus assembly for the isolates. Quality of assemblies was assessed by evaluating total length, the number of contigs, GC%, N50/75, L50/75, and the number of uncalled base pairs per 100,00 assembly bases. The master integral sequence was then used in the annotation of the sequences using Prokka (Prokka version 1.12; Seemann, 2014).

Pan-genome analysis

Characterizing the association between *A. paragallinarum* serotypes and their genetic attributes were accomplished via a pan-genome-wide association study (Pan-GWAS). The GFF3 files produced by Prokka from the isolate assemblies along with the annotation files of nine reference genomes that were deposited in Genbank were used for pan-genome analysis using Roary v. 3.11.2 (Page et al.2015). The core genome was defined and aligned using Roary, with the BLAST threshold set to 90-100%. The Roary uses an all-against-all Blast of sequences produced by an initial CD-Hit clustering. A pan-genome gene presence-absence matrix was visualized with Phandango (Hadfield, et al. 2017) using the gene presence/absence output file from Roary. Newick-formatted phylogenies of the core gene alignment were also created using Roary.

Phylogenetic analysis

Phylogeny was constructed using RAxML version 8.0.0 resulting phylogeny was tested against 100 bootstrap replications and the number of replications was determined by implementing

the autoMR convergence criteria in the RaxML. The phylogeny was subsequently visualized, annotated and rendered in circular phylogenetic tree using iTOL (Letunic and Bork, 2006).

Protein sequence-based genome comparison for reference genomes

The Proteome Comparison Service of PATRIC (https://www.patricbrc.org/) interface performs protein sequence-based genome comparison using bidirectional BLASTP. The unique gene of each serovar was performed via proteogenomic analysis of the Page serovars using PATRIC. An approximate list was then formed that compromised all of the Page Serovars and unique genes associated with each serogroup. BLAST was then used to confirm the alignment to the references and to confirm the presence/absence of a unique gene for each serovar. Additional analysis was performed using the proteogenomic analysis software on PATRIC for heat map formation depicting the distribution of unique protein families among reference genomes.

Chapter 4 : Results

Genome Annotation Analysis & Defining Core genes

Genomic features of 40 *A. paragallinarum* isolates were obtained by combining Tychus integrated genome assembly and Prokka annotation analysis. Previous studies reported that the average genomic size of *Avibacterium* is 2.4Mb with 41% GC content (Horta-Valerdi, 2017). The genomic size varied among isolates from Pennsylvania ranging between 2.11Mbp and 2.79Mbp. The total GC content is consistent across isolates, ranging between 40.71% and 41.55%.

Table 2 Comparison of 40 *A. paragallinarum* isolates based on key genome assembly and annotation parameters

ID	BPs#	N50	GC%	Largest Contig	# Contigs
AP01	2390963	195346	40.83	260696	36
AP02	2,790,793	257472	41.55	671256	31
AP03	2546519	116051	40.88	260558	47
AP04	2372742	133008	40.96	333343	26
AP05	2427704	213340	40.82	459119	36
AP06	2378369	128499	40.81	260564	49
AP07	2559121	193592	40.75	458964	39
AP08	2405609	193696	40.83	458568	40
AP09	2708891	193718	40.76	260795	42
AP10	2403862	146190	40.83	458944	37
AP11	2108833	102878	40.84	260686	53

AP12	2436695	135335	40.83	250237	40
AP13	2410605	193583	40.82	317992	45
AP14	2396959	198260	40.84	260837	34
AP15	2405901	111240	40.84	258023	47
AP16	2401024	193838	40.84	458883	34
AP17	2590462	193606	40.72	260923	41
AP18	2418510	193601	40.83	475183	35
AP19	2428282	146190	40.78	260754	42
AP20	2569699	172450	41.39	335918	30
AP21	2411167	193608	40.83	260727	39
AP22	2244066	102559	41.43	575929	43
AP23	2113767	104285	41.51	361358	38
AP24	2458097	101456	40.87	260557	49
AP25	2378317	193718	40.87	260698	23
AP26	2413493	193731	40.83	260570	42
AP27	2533935	198111	40.71	260563	26
AP28	2396931	146254	40.82	260721	39
AP29	2413484	145842	40.81	260559	45
AP30	2463570	227841	40.76	458999	19
AP31	2471378	168621	40.71	260567	28
AP32	2483645	220348	40.74	458606	20
AP33	2407737	193718	40.83	458973	39
AP34	2597917	193599	40.72	459176	39

AP35	2603288	203026	40.71	459253	36
AP36	2593701	116378	40.72	260693	45
AP37	2370112	193718	40.82	260698	42
AP38	2408398	146444	40.84	458967	40
AP39	2397192	193717	40.82	260569	47
AP40	2396789	146270	40.82	260714	39

Pangenome Analysis

Using the Roary program, the pan-genome containing the entire gene repertoire of 40 strains and core genes shared by all strains and nine references were obtained. Roary was used to define core genes and unique genes with the following parameters: core genes (99%<x<100%), softcore genes(95%<x<99%), shell genes(15%<x<95%), and cloud genes (<15%). The findings indicated that 3 core genes, 316 softcore genes, 2016 shell genes, and 5939 cloud genes were present. As the references JF4211_A, 2671_B_2, Modesto-JP_C, Modesto-SA_C, 221_A, SA3_C observed to be very distinct in the alignment resulting in a minimal number of core genes (**Figure 1),** we re-analyzed the dataset without those references. Roary re-analysis of *A. paragallinarum* pangenome identified 35 core, 1363 soft core, 859 shell and 2306 cloud genes out of 4563 total genes. The increased number of cloud genes indicate a higher-level heterogeneity exists among the isolates and references considered, highlighting the ‘open’ nature of the pan- genome. Single nucleotide polymorphisms were extracted from the core gene

alignment using SNP-sites version 2.3.316. In the set of 40 isolates, 32 were identical with 100 similarity and 0 SNPs identified.

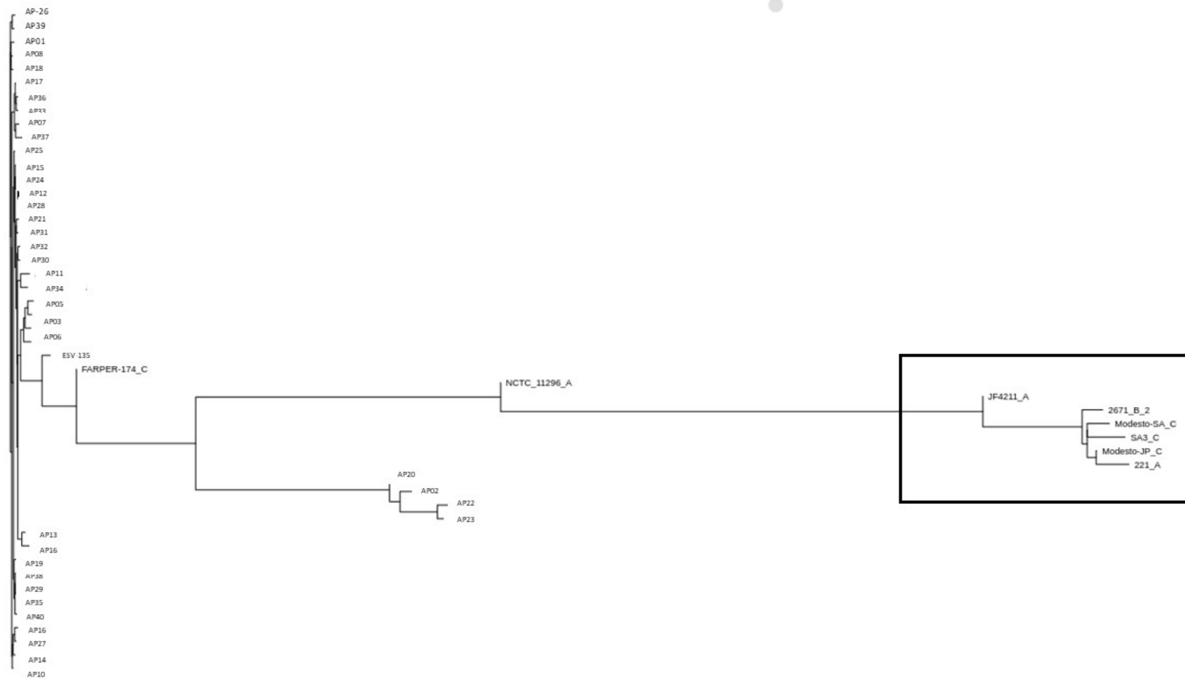

Figure 1. Phylogenetic relationship between isolates and reference strains: The phylogenetic tree contains all 40 isolates in addition to the nine reference genomes. The boxed area illustrates six reference genomes that do not share the same clade as any of the isolates, indicating an absence of relationship between six reference sequences and the isolates. Furthermore, a small clade is present containing AP2, AP20, AP22, AP23, illustrating that these isolates are related and share the serovar. The presence of a majority of the reference genomes does not depict a clear relationship.

Phylogenetic Analysis

Using RAxML core and accessory genome gene sequences were aligned and maximum likelihood trees were produced. All the three, core-genome, accessory-binary and SNP based

phylogenetic trees defined five main clades that showed a differential isolate distribution. **Figure 2** depicts the core-genome based phylogenetic tree edited and visualized using ITOL. The isolates AP22, AP23, AP02and AP20 observed to be belonging to a divergent A Serovar clade. AP06, AP05, AP03 and AP32 formed the third clade related to, but not identical to the Serovar C reference genomes. Remaining isolates were observed to be 100% identical, however, our analysis did not give any insights to their closest known serovar.

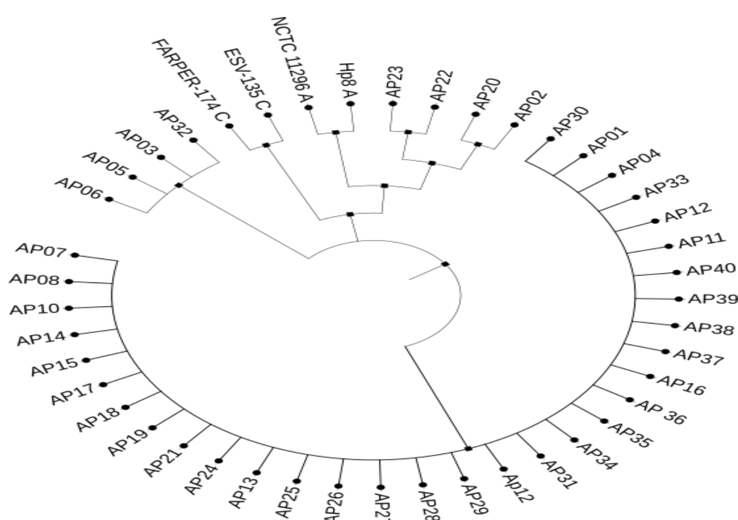

Figure 2. Phylogenetic Relationship among *Av. Paragallinarum* isolates and four reference strains. The phylogenetic tree contains 40 isolates and reference strains Hp8(A), NCTC11296(A), ESV-135(C), and FARPER-174(C). Three distinct clades are present. Isolates AP22, AP23, AP02 and AP20 observed to be belonging to a divergent A Serovar clade. Isolates AP06, AP05, AP03 and AP32 formed the third clade related to, but not identical to the Serovar C reference genomes. The remaining isolates were 100% identical, however, evaluation of proximity to the three serovars was unknown.

Identification of Serovar-Specific Candidate Gene Markers

Representative complete genomes for each serovar containing gene features were compared using the Proteome comparison service in PATRIC. However, being the isolate sample set observed to be highly diverged, the presence or absence of one or more serovar-specific gene markers was not predicted with sufficient statistical confidence. Heat map formation was also used in qualitatively analyzing unique genes in reference strains. **Figure 3** further confirms the highly divergent nature of *A. paragallinarum*, indicated by the low prevalence of conserved genes and high presence of unique genes among even the reference isolates. Further *in silico* analysis are required to understand the phylogenetic diversity among *A. paragallinarum* outbreak isolates to predict the serovar specific unique genes.

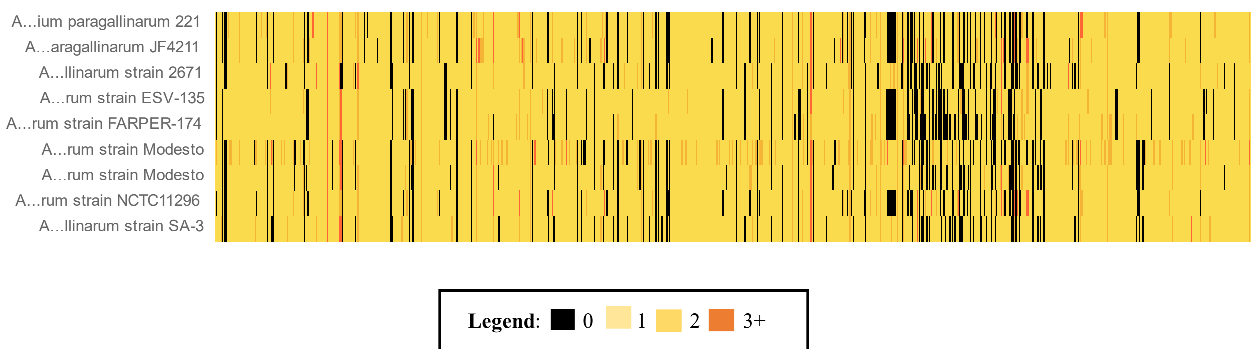

Figure 3. Heat Maps depicting protein family distribution for Reference strains of *Av. Paragallinarum:* Figure three depicts a heat map containing nine reference strains. Black lines indicate the absence of a specified gene, tan indicates the presence of at least one gene within the strain's genome, yellow indicates the presence of at least two genes within the strain's genome, and orange indicates the presence of at least three genes within the bacteria's genome. The following chart illustrates the absence of a conserved genome within *A. Paragallinarum*, exemplifying genetic diversity among reference strain independent of Serogroup.

Chapter 5 : Discussion

Avibacterium paragallinarum has significant impacts on poultry economics attributed to the large decrease in egg production when the infection is present. Recent outbreaks of Infectious coryza in Pennsylvania have pressured scientists to develop a more rapid and efficient diagnostic method for better epidemiological surveillance of IC for the characterization of prevalent local strains for vaccine selection. Such diagnostics are extremely important in the vaccine selection process due to the absence of cross-protection between most Page Serovars. Earlier characterization of common local strains of IC combined with proper biosecurity measures allows farmers to mitigate flock infections before they occur.

Forty isolates of *A. paragallinarum* strains assembled, annotated, and analyzed defined to determine core genomes and unique genes. Pan-genome analysis identified a minimum number of shared core genes, indicating that there is severe heterogeneity between isolates. PATRIC and BLAST were used to understand the distribution of the unique genes and their general function (antibiotic resistance, virulence, transport protein). Major proportion of the unique gene set from isolates analyzed were unannotated hypothetical proteins. The rareness of unique core genes and absence of annotated functions of the hypothetical protein limits the application of genotyping as a surveillance tool for diagnosing outbreaks due to the homogeneity.

Phylogenetic analysis was performed to establish the genetic relationship between the reference genomes and the 40 *A. paragallinarum* isolates. As the reference genome JF4211, 2671, Modesto-JP, Modesto-SA, 221, SA3 observed to be very distinct in the alignment in the preliminary analysis, phylogenetic relatedness of the clinical isolates was analyzed in comparison to HP8, NCTC 11296, FARPER-174 and ESV-135. Previously, Byukusenge et al. reported that

earlier clinical isolates of *A. paragallinarum* from PA observed to be genetically closer to genomes from Peru, strains 72, FARPER- 174, and strain AVPG2015 from Mexico (Byukusenge,2020). The isolates S13_S8, S14_S17, S01_S2 and S12_S9 observed to be belonging to a divergent A Serovar clade. S06_S10, S05_S3, S04_S11 and S03_S12 formed the third clade related to, but not identical to the Serovar C reference genomes. Remaining isolates were observed to be 100% identical, however, our analysis did not give any insights to their closest known serovar. Notably, the clinical presentation of the IC cases from which the *A. paragallinarum isolates* were obtained during the 2019 outbreaks in PA was very different from the typical IC clinical picture.

Chapter 6 : Conclusions

In conclusion, the study revealed genetic diversity among the *A. paragallinarum* serovars with an in-depth understanding of similarities and differences using reference strains and *A. paragallinarum* isolates from PA. Additionally, our study confirmed the highly divergent nature of *A. paragallinarum*, and their ability to constantly acquire various feature from serovars. Future studies should focus on annotating the unique hypothetical proteins with each serovar to evaluate their function and feasibility in acting as a diagnostic tool for IC Outbreaks. Furthermore, future studies can better characterize the methods of divergence of *A. paragallinarum*, and how divergence affects disease presentation, persistence, and resistance in domestic poultry flocks.

Table 1. Contact List for Experiment

First Name	*Last Name*	*Phone Number*
Jasmine	Morgan	703-919-7562
Meera	Surendran Nair	+1 814-867-2464
Manoj	Kumar	+1 814-863-9639
Lingling	Li	+1 814-863-7912

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Chapter 8 : Academic Vitae

EDUCATION: Pennsylvania State University, Spring 2022

Major: Veterinary and Biomedical Sciences;

Honors: Veterinary and Biomedical Sciences

RESEARCH

- **Lab Assistant | Kuchipudi Lab | Dr. Meera Surendran**
  Used command lines to analyze genomic sequences for Avibacterium paragallinarum. Used and pPCR and ELISA assays to analyze the population of wild and domestic animals infected with SAR-COV-2. Created diagnostic lateral flows to test for SARS-COV-2 to test large populations of wild animals.

- **Lab Assistant |Animal Diagnostic Lab, Dr. Justin Brown**
  Assessed the temporal dynamics of Avian Paramyxoviruses in waterfowl from Pennsylvania using the inoculation and PCR to identify the disease and serotype. Data entry and categorization for R to code was used to assess trends in season, gender, species, and age. Engages in two scientific presentations on the epidemiology of avian influenza.

- **Lab Assistant |Animal Diagnostic Lab, Dr. Justin Brown**
  Wrote a grant to receive funding for the project. Processed and Analyzed sample for studies on Avian Influenza using ELISA testing to test the sensitivity of samples. Data entry and categorization for R to code for trends in Avian Influenza temporal dynamics. Presented poster the Undergraduate Research Exhibition.

- **Student Lab Participant |Food Science Lab Dr. Darrel Cockburn**
  Selected to participate in study regarding the isolation and characterization of resistant starch degrading bacteria from the human gut microbiome. Prepared Biological samples and used DNA extraction and PCR testing to evaluate the Gut Microbiome. Presented Poster at the PA Space Grant Consortium Exhibition.

WORK EXPERIENCE: *Welcome Desk Attendant,* Pattee and Paterno Library, Penn State– 08/2019-Present. Veterinary Assistant/Lead, ShotVet, Alexandria, 06/2020-08/2020. *Kennel Assistant*, Hayfield Animal Hospital, 05/2019-08/2019

ACTIVITIES & AWARDS: Member of Gamma Sigma Delta Honors Society, Member of Phi Eta Sigma 2019-Present, National Success and Leadership Society (2020-Present), President (2021)/Secretary (2020) of Minorities in Agriculture, Natural Resources, and Related Sciences. Learning Assistant for Organic Chemistry (2021). Minority Mentor and STEM tutor (2020-2022).