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Comparative Mutational Analysis and the Glycosylation Patterns of a Peruvian Isolated Avian Influenza a Virus H5N1: Exploring Possible Viral Spillover Events Within One Health Approach

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Comparative Mutational Analysis and the Glycosylation Patterns of a Peruvian Isolated Avian Influenza A Virus H5N1: Exploring Possible Viral Spillover Events Within One Health Approach

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Simple Summary: Influenza A virus is a pathogen of significant global concern for public health and wildlife conservation. A deeper understanding of its mutational/glycosylation profile is essential, given its broad host range capabilities. This study provides the comparative variations found in all segmented proteins of the H5N1 viruses analyzed, some of the findings must be highlighted within the One Health framework.

Abstract: (1) Background: The emergence of H5N1 Influenza A viruses clade 2.3.3.4b since 2020, have caused the mortality of thousands of birds/mammals worldwide, through evolutionary changes have been associated with acquired mutations and posttranslational modifications. (2) Methods: This study aimed to compare the mutational profile of H5N1 avian Influenza virus isolated from a Peruvian natural reserve, with recent data from other related international studies made in human and different species of domestic and wild birds and mammals. Briefly, the near complete protein sequences of Influenza virus coming from a *Calidris alba* were analyzed in a multisegmented level, altogether with 55 samples collected between 2022-2024 in different countries. Moreover, the glycosylation patterns were also predicted in silico. (3) Results: A total of 603 amino acid changes were found among H5N1 viruses analyzed, underscoring the detection of critical mutations HA:143T, HA:156A, HA:208K, NA: 71S, NP:52H, PA:336M, PA:36T, PA:85A/N, PB1-F2:66S, PB2:199S, PB2:292V, PB2:559T, as well as PA:86I, PA:432I, PA:558L, HA:492D, NA:70D, NS1-83P, PB1:515A, PA-X:57Q, PB1-F2:22E, NS1-21Q, NEP:67G, among others, considered of importance under One Health perspective. Similarly, changes in the N-linked glycosylation sites (NLGs) predicted in both HA and NA proteins were found, highlighting the loss/acquisition or changes in some NLGs sites such as 209NNTN, 100 NPTT, 302NSSM (HA) and 70NNTN, 68NISS, 50NGSV (NA). (4) Conclusions: This study provides our understanding about the evolution of current Influenza A viruses H5N1 HPAIV circulating globally. These findings outline the importance of surveillance updating mutational profiles and glycosylation patterns of these highly evolved virus.

Keywords: one health; Influenza A viruses; H5N1; mutational/glycosylation profile

1. Introduction

Influenza A virus (IAV), is an enveloped, negative-sense, single stranded and eight segmented RNA virus, belonging to the genus *Alphainfluenza,* family *Orthomyxoviridae*, which is a major treat within a One Health framework, of global concern for public health and wildlife conservation, due to highly contagious spread and the devastating impact it caused in breeding colonies of different species of domestic and wild birds and mammals globally [1–5]. The ultimate spread of these

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viruses across the Americas, left a record level of alarming mortality, with strong impacts on wild birds [6–10], and marine mammals with an increasing evidence of mammal-mammal transmission [1,11–13]. Likewise, the emerging infections of H5N1 avian origin virus reported in cattle [14,15], wild carnivores [16–20], domestic mammals [21–23] as well as reports of human infections [24] highlighting the characteristics of these exceptionally-evolved viruses and the risk of possible spillover events. Furthermore, the increase of unprecedent distribution, extensive circulation of Influenza H5N1 HPAIV clade 2.3.3.4b viruses worldwide must consequently be taken in consideration and monitored lastingly. Since the first detection, of avian Influenza virus of high pathogenicity (HPAIV) A/goose/Guandong/1/96 (Gs/Gd/96), the infectivity and transmissibility characteristics it has been gradually increasing [25], and adquiring mutational changes in a multisegmented level and showing adaptational features to new hosts [1,26–28]. Moreover, key amino acid changes constantly vary the biophysical properties of Influenza viruses facilitating immune evasion, introducing/removing an N-linked glycosylation motif or increasing the receptor binding avidity of the HA receptor [29]. Particularly, the variable degrees of glycosylation regulate the functional balance between receptor affinity to maintain viral fitness [30]. It is well-known that post-translational modifications confer capabilities to the globular head and stem of the HA ectodomain to bind host-derived glycans and hide or expose the functional region, a process required to initiate the cell viral entry [30]; moreover, changes in the glycosylation pattern of NA protein are also crucial for an appropriate protein folding, stability, solubility and budding, as well as influence the neurovirulence of Influenza viruses [31–34]. Besides, the glycosylation dynamics in the currently H5N1 Influenza viruses are not yet fully understood at all.

Given the critical role of mutations and glycosylation patterns in the dynamics of Influenza virus and its adaptative evolution, this study describes the comparative results obtained between several H5N1 Influenza virus genomes mammal and non-mammal within One Health approach.

2. Materials and Methods

2.1. Sample Collection

One duplicate sample oropharyngeal swab was collected from a *Calidris alba* with slowness fly and stagnation from Pantanos of Villa a Peruvian National Reserve during the period of time March-April 2023. The material was transported and preserved in a triple packaging system to perform molecular detection of Influenza A viruses following the standard procedures diagnostic by amplification [35].

2.2. Molecular Detection

Viral ARN was extracted using the Viral Nucleic acid extraction kit II (Geneaid, New Taipei, Taiwan) according to the manufacturer´s instructions in a class 2A biological safety cabinet (Biobase, China). The extracted RNA was subjected to purity measurement using a spectrophotometer DS11 instrument (Denovix, USA) and Influenza virus detection was performed using a high-resolution melting analysis (HRM) procedure to target the M and HA gene following the standard procedures of World Organization of Health (WHO, 2017).

2.3. Whole-Genome Sequencing

Whole-genome sequencing (WGS) of amplicons per each genome segment of the isolate H5N1 virus were obtained using Mytaq® Red DNA Polymerase kit (Meridian Bioscience, USA) and the sequencing were done using Illumina next generation sequencing (NGS) technology (Miseq system with a 250-cycle paired-end). The reads were analyzed according to the tools previously described [37].

 doi:10.20944/preprints202502.1501.v1

2.4. Data Sets

Full-length or near full-length protein sequences of 55 highly pathogenic avian influenza A viruses (HPAIV) H5N1 were downloaded from the National Center for Biotechnology Information (NCBI), altogether with data Peruvian isolate H5N1.A/Calidris alba/Lima/Villa01/2023, within One Health perspective. The sample list included 6 human isolates, 16 mammals isolates and 32 bird isolates reported in outbreaks from 2022 -2024 and considered by us of importance to be included into the analysis. The data sets obtained from GenBank are listed in Supplementary 1 (S1) Table 1. Data input were obtained from studies carried out in several species of mammals [12,21,38–41], and birds collected in South America and North America regions mainly [5,6,42–45], as well as from other representative areas outside the continent. Aditionally, quality reports, availability of complete/near complete protein sequences of H5N1 viruses and related studies published recently were considered to the data selection [1,16,46–48]. All Influenza virus proteins were included in the perusal: PB2, PB1, PA, HA, NP, NA, M y NEP (NS2), and the accessible information of Influenza virus accessory's proteins were taken into special consideration.

2.5. Mutational analysis and genotype identification

The data obtained in a multispecies level, were analyzed in the search of amino acid changes, segment by segment manually and in silico, using Clustal Omega [49], and the command-line tool FluMut [50]. All sequences available of PB2, PB1, PA, HA, M, NP, NA and NEP (NS2) were compared, including the accessory proteins PA-X, PB2-F1 and NS1.

Genotype was determined using GenoFLU (https://github.com/USDA-VS/GenoFLU) [51].

2.6. Prediction of Potential N-Glycosylation sites in HA and NA Influenza A H5N1 Viruses

Potential N-linked glycosylation sites (NLG) of the sequences HA and NA proteins of H5N1 viruses analyzed, were predicted and compared with the reference genome (A/goose/Guandong/1996(H5N1) using the high prediction accuracy NetN-Glyc 1.0 server [52], detecting the Asn-X-Ser/Thr sequons, (where X is any amino acid except proline)[53], and measures of prediction confidence >0.5 were taken into consideration as a threshold.

3. Results

3.1. Mutational Manual Analysis

Through mutational manual analysis carried out at 55 samples selected of H5N1 Influenza viruses, coming from both birds and mammals, a total of 603 amino acid changes were identified, distributed throughout the 8 protein segments PB1, PB2, PA, HA, M, NA, NP, NEP and the 4 accesories proteins M2, NS1, PB1-F2 and PA-X; where 76 mutations have been detected in the HA protein; 72 mutations in NA protein; 22 mutations in M1; 11 mutations in M2; 22 mutations in NP protein; 94 mutations in NS1 protein, 39 mutations in NEP (NS2) protein; 56 mutations of PB2 protein; 64 mutations in PB1 protein; 48 mutations PB1-F2; 30 mutations in PA-X and 75 mutations in PA protein. The complete mutational details can be observed in the Supplementary 1 (S1) y 2 (S2).

It is noteworthy, that the proportion of individual/grupal mutations detected per each protein segment, varied notoriously as follows: individual mutations (those found only in 1-3 of the samples evaluated) were observed more in PA, PB1, PB2, NP, NA and PA-X proteins; whilst grupal mutations (those identified in more than 4 samples), were detected in larger quantities in PB1-F2 and PB1 proteins. In addition, more mutations in "all" samples analyzed, were found in M1 and NS1 proteins (S1(Table 2) and Graphic 1).

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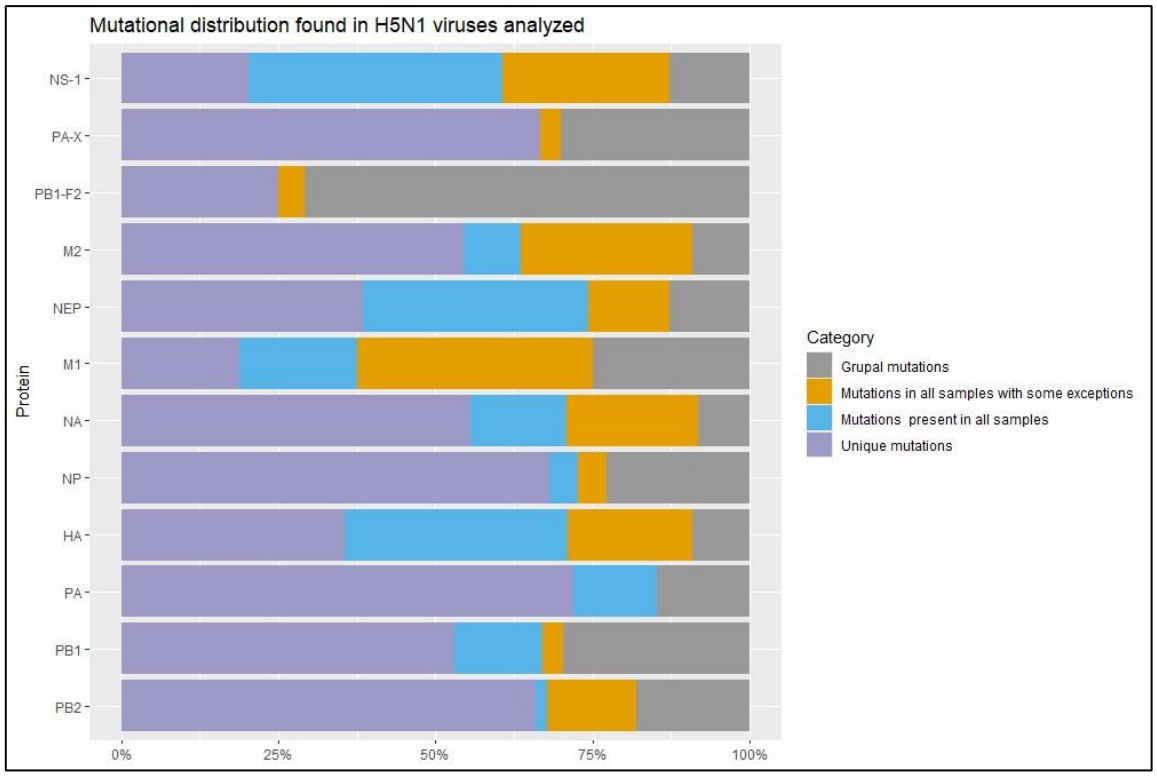

Graphic 1- Mutational distribution found in H5N1 viruses analyzed. The mutations were classified in 4 categories: unique mutations (mutations found only 1-3 samples), groupal mutations (mutations found in more of 4 samples), mutations present in all samples and mutations found in almost all samples with some exceptions.

Furthermore, considering the size of each virus protein segment, the detection of more quantity of amino variations presented in the accessories proteins PB1-F2, NS1 and NEP, particularly in the "PB1-F2 protein" was conspicuous . Particularly, certain virus, such as isolates coming from A/LOU/WT, polar bear/ALK, harbor-seal/ME and several birds species shared 23 variations in PB1-F2 protein; likewise, they also had in common 7 mutations in PB1, 4 mutations in HA, 2 mutations in NS1, 1 mutation in PA-X); as well as, grupal mutations PB1:375N, HA:492D, PB1-F2:22E, PB1-F2: 90N, NS1:83P, PA-X:215N. Also, a considerable number of individual mutations in other proteins were found, such as NA(19 variations found in A/LOU/WT, 3 in polar-bear/ALK) and PA(13 variations found in A/LOU/WT, 6 in polar-bear/ALK), like individual mutations NA:44N,45H,48T,53V,62I,81D,82P,84A,234I,286S,288V,399L and PA:201I/T,211I,322L,399V,626R among others detected, and keeping in mind that some of these specimens are the most recent isolates, it should be considered under One Health perspective (Tables 1 and 2).

Table 1. Unique mutations (only detected in 1-3 samples) in H5N1 viruses identified per each protein segment.

Mutation	Source
HA:9V	South America (Andean-guayata/ARG)
HA:10T	North America(harbor-seal/ME), continent outside
HA:87T	(duck/BD)
HA:99S	North America (vulture/FL)
HA:99D	South America (duck/UGY)
HA:102T	Continent outside (turkey/GER, duck/BD)
HA:104G	North America(vulture/FL), continent outside
HA:147M	(chicken/JPN)
HA:152S	North America (A/CA,emu/CA), South America
HA:201R	(Procellaria/BR)
HA:225M	North America (A/CA)

 doi:10.20944/preprints202502.1501.v1

HA:226T	North America (harbor-seal/ME), continent outside
HA:248L,259C, HA:277Y,285E,	(turkey/GER)
HA:316E,324T, HA:336R,493D	South America(Calidris-alba/LIM)
HA:304N	South America (Numida/BR)
HA:310V	North America (harbor-seal/ME)
HA:336N	South America (Numida/BR)
HA:473K	South America (Numida/BR)
HA:520R	South America (Numida/BR)
HA:520N	South America (Numida/BR)
HA:531L	South America (Sterna/BR, Humboldt-penguin/CHI)
	South America (panthera-leo)
	North America (A/CA, emu/CA)
	Continent outside (turkey/GER)
	North America (goat/MI)
	Continent outside (turkey/GER)
	South America (Fregata/BR)
NA:19V	South America (Numida/BR)
NA:20A	North America (goat/MI)
NA:23V	North America(A/LOU/WT)
NA:44N	North America(A/LOU/WT)
NA:45H	North America (A/LOU/WT)
NA:48T	North America (A/LOU/WT)
NA:53V	North America (A/LOU/WT)
NA:62I	North America (polar-bear/ALK), continent outside
NA:67I	(Eagle/JPN)
NA:74L	North America (A/LOU/WT)
NA:74C	North America (A/LOU/WT)
NA:75I	Continent outside (turkey/GER)
NA:81D	North America (A/LOU/WT)
NA:82P	North America (A/LOU/WT)
NA:84A	North America (A/LOU/WT)
NA:90P	North America (A/LOU/WT)
NA:221S	Continent outside (turkey/GER)
NA:216V	North America (A/LOU/WT)
NA:217R	South America (Belcher-gull/PER)
NA:223T	South America (Numida/BR)
NA:234I	North America (peregrine-falcon/NY)
NA:237F	North America (A/LOU/WT, Northern-pintail)
NA:241I	North America (peregrine-falcon/NY)
NA:254R	North America (A/LOU/WT)
NA:257R	North America (house-mouse)
NA:284N	North America (A/LOU/WT)
NA:286S	South America (black-necked-swam/UGY), continent
NA:288V	outside (chicken/JPN)
NA:308R	North America (A/LOU/WT)
NA:308K	North America (A/LOU/WT)
NA:329S	Continent outside (duck/BD)
NA:340Y	Continent outside (turkey/GER)
NA:340F	North America (A/LOU/WT)
NA:364N	South America (duck/UGY)
N:374V	Continent outside (duck/BD)
N:399L	South America (Belcher-gull/PER)
NA:432R	Continent outside (duck/BD)

 doi:10.20944/preprints202502.1501.v1

NA:436V	North America (polar-bear/ALK), continent outside (pintail/EGY)
NA:442I	North America (A/WT)
NA:460S	North America (polar-bear/ALK), continent outside (Eagle/JPN)
	South America (Calidris-alba/LIM)
	North America (grackle/TX)
M1:55M	Continent outside (turkey/GER, pintail/EGY)
M1:191H	South America (gallus/PER)
M1:218A	South America (Sterna/BR)
M2:12R	Continent outside (turkey/GER)
M2:19Y	South America (wild-duck/CO), North America (peregrine-falcon/NY)
M2:21G	Continent outside (turkey/GER)
M2:27A	North America (cattle/TX), continent outside (duck/BD)
M2:28T	North America (raccoon/IA)
M2:52S	South America (Calidris-alba/LIM)
PA:13V	North America (house-mouse/NM), continent outside (eagle/JPN)
PA:42V	South America (Numida/BR)
PA:36T	North America (Cattle/TX), South America (Numida/BR)
PA:45S	South America (Panthera-leo/PER)
PA:59K	Continent outside (duck/BD)
PA:59G	North America (vulture/FL)
PA:68S	North America (A/CA, emu/CA)
PA: 75Q	North America (polar-bear/ALK)
PA: 86I	South America (elephant-seal/ARG, tern/ARG)
PA:142E	North America (alpaca/ID)
PA:100I	North America (Racoon/IA), South America (Procellaria/BR)
PA:118U	North America (peregrine-falcon/NY)
PA:184S,207V	Continent outside (duck/BD)
PA:190F	South America (fregata/BR)
PA:201I	North America (A/LOU/WT), continent outside (duck/BD)
PA:201T	North America (polar-bear/ALK), continent outside (eagle/JPN)
PA:272N	North America (vulture/FL)
PA: 211I	North America (A/LOU)
PA:213K	North America (Northern-pintail)
PA:269K	North America (A/LOU/WT)
PA:322V	North America (A/LOU/WT)
PA:322L	North America (polar-bear/ALK), continent outside (Eagle/JPN)
PA:323I	North America (A/LOU/WT)
PA:330V	Continent outside (chicken/JPN)
PA:336M	South America (Chilean-dolphin)
PA:348L	North America (A/LOU/WT)
PA:351G	North America (A/WT)
PA: 354F	North America (polar-bear/ALK/ALK), continent outside (Eagle/JPN)
PA:382G	Continent outside (duck/BD)
PA:388G	North America (A/LOU/WT, polar-bear/ALK)
PA:399V	North America (A/LOU)
PA:404S	
PA:423T	
PA:425F	
PA:459V	
PA:465M	

 doi:10.20944/preprints202502.1501.v1

PA:465T	North America (cattle/TX)
PA:486M	Continent outside (chicken/JPN)
PA:486L	South America (black-necked-black-necked-swam/UGY)
PA:489S	South America (Chilean-dolphin)
PA:523L	South America (Procellaria/BR)
PA:538G	North America (harbor-seal/ME)
PA:545V	North America (A/CA)
PA:561V	North America (Northern-pintail)
PA:581I	North America (A/LOU)
PA:613Q	South America (Procellaria,Sterna/BR)
PA: 614D	North America (A/LOU)
PA:614S	North America (A/LOU/WT), continent outside
PA:621V	(Eagle/JPN)
PA:626R	Continent outside (chicken/JPN)
PA:655F	Continent outside (duck/BD)
PA:664R	South America (Procellaria/BR)
PA:688G	North America (polar-bear/ALK), continent outside
	(Eagle/JPN)
	North America (goat/MI)
	Continent outside (turkey/GER)
	North America (A/LOU)
	North America (A/CA)
	Continent outside (duck/BD)
	Continent outside (turkey/GER)
	South America (elephant-seal/ARG, tern/ARG)
PA-X: 20T	South America (Numida/BR)
PA-X:36D	North America (cattle-TX)
PA-X:36T	South America (Numida/BR)
PA-X:42V,52D	North America (vulture/FL), continent outside (duck/BD)
PA-X:59K	South America (Sterna/BR)
PA-X: 62T	North America (A/CA, emu/CA)
PA-X:68S	North America (harbor/seal/ME)
PA-X:70V	North America (polar-bear/ALK)
PA-X:75Q	South America (elephant-seal/ARG, tern/ARG)
PA-X: 86I	North America (peregrine-falcon/NY)
PA-X:118V	South America (turkey, duck/UY)
PA-X:122I	North America (alpaca/ID)
PA-X:142E	North America (polar-bear/ALK)
PA-X:160E	Continent outside (duck/BD)
PA-X:184N	South America (Fregata/BR)
PA-X:190F	North America (A/LOU/WT)
PA-X: 195K	South America (wild-duck/CO), North America
PA-X:207L	(peregrine-falcon/NY)
PA-X:211Y	North America (A/LOU)
PA-X:250P	North America (A/LOU/WT, peregrine-falcon/NY), South
	America (wild-duck/CO)
PB2:9N	North America (polar-bear/ALK), South America
PB2:79G	(Pelecanus/PER)
PB2:152V	North America (harbor-seal/ME)
PB2:190R	South American (elephant-seal/ARG)
PB2:191G	South America (Panthera-leo/PER)
PB2:199T	Continent outside (chicken/JPN)
PB2:251K	South America (black-necked-swam/UGY)

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PB2:255A	North America (goat/MI)
PB2:274V	North America (bovine/TX)
PB2:292V,339R	North America (goat/MI)
PB2:346A	Continent outside (turkey/GER)
PB2:353R	North America (goat/MI)
PB2:444G	North America (goat/MI)
PB2:453S	South America (Fregata/BR)
PB2:451V	North America (vulture/FL)
PB2:452V	Continent outside (duck/BD, pintail/EGY))
PB2:472D	South America (wild-duck/CO)
PB2:532L	South America (wild-duck/CO), North America
PB2:539V	(peregrine-falcon/NY)
PB2:560M	North America (polar-bear/ALK), continent outside
PB2:575V	(Eagle/JPN)
PB2:596A	North America (raccoon/IA), South America (wild-
PB2:639S	duck/CO)
PB2:660R	Continent outside (turkey/GER)
PB2:663R	Continent outside (turkey/GER)
PB2:666I	North America (red-fox)
PB2:667I	North America (Northern-pintail)
PB2:670R	South America (duck,turkey/UGY)
PB2:677K	North America (goat/MI)
PB2:679S	North America (polar-bear/ALK), continent
PB2:680G	outside(Eagle/JPN)
PB2:683A	North America (goat/MI)
PB2:684S,697M	North America (A/CA, emu/CA)
PB2:711S	North America (A/MO)
PB2:715S	South America (Calidris-alba/LIM)
	North America (polar-bear/ALK), continent outside(Eagle/JPN)
	Continent outside (turkey/GER)
	Continent outside(duck/BD)
	North America (peregrine/falcon/NY)
	North America (harbor-seal/ME)
PB1:11R	South America (Sterna/BR)
PB1:14V	South America (Sterna, Procellaria/BR)
PB1:40I	South America (Chilean-dolphin)
PB1:51E	South America (necked/UGY, Numida/BR)
PB1:53E	South America (Sterna/BR)
PB1:121N	South America (black-necked-swam/UGY)
PB1:147V	North America (Northern-pintail)
PB1: 171A	North America (A/CO)
PB1:176T	North American (harbor-seal/ME)
PB1:211K	North American (goat/MI, Northern-pintail)
PB1:291A	North American (polar-bear/ALK)
PB1: 321I	North America (peregrine-falcon/NY), South America
PB1:339V	(wild-duck/CO)
PB1: 348V	South America (Numida/BR)
PB1:371D	South America (duck,turkey/UGY)
PB1: 372I	South America (Numida/BR)
PB1:383G,455D	North American (harbor-seal/ME)
PB1:384P	Continent outside(duck/BD)
PB1:384T	North American (house-mouse/NM)

 doi:10.20944/preprints202502.1501.v1

PB1:384A	North American (cattle/TX)
PB1:390G	North American (turkey/GER)
PB1:394S	North American (A/WT)
PB1:431H	South America (Fregata, Procellaria/BR)
PB1:512L	North America (vulture/FL)
PB1:533S	Continent outside (turkey/GER)
PB1:576M	North America (polar-bear/ALK)
PB1:584H	Continent outside(duck/BD)
PB1:621K	North America (goose/ALK)
PB1:657H	South America (elephant-seal/ARG, tern/ARG)
PB1:660I	South America (Sterna/BR)
PB1:719I	North America (harbor-seal/ME)
PB1:738G	South America (Humboldt-penguin, cormorant/CHI)
PB1:739D	North America (harbor-seal/ME), South America (Procellaria/BR)
	South America (Procellaria,Sterna/BR)
	North America (Goat/MI)
NSI:36I	South America (royal-tern/ARG)
NS1:66D	North America (A/CA, emu/CA)
NS1: 67G	North America(harbor-seal/ME)
NS1:67Q	North America (A/LOU/WT)
NS1:75G	North America (A/LOU/WT)
NS1:76A	North America (House-mouse)
NS1:77R	Continent outside (duck/BD)
NS1:81V	South America (Belcher-gull)
NS1:88H	Continent outside (duck/BD,turkey/GER,chicken/JPN)
NS1:129T	North America (Goat/MI)
NS1:136M	North America (A/LOU/WT)
NS1:193Q	North America (Goat/MI)
NSI:201Y	Continent outside (duck/BD)
NSI:202T, 210R, NS1:217T	Continent outside (duck/BD)
NSI:213L	South America (Calidris-alba/LIM
NSI:219E	North America (Northern-pintail)
NS1:226T	South America (elephant-seal/ARG,tern/ARG, Chilean-dolphin)
	South America (Numida/BR), North America (Northern-pintail)
NEP:27G	North America (A/ LOU/WT)
NEP:36V	Outside continent (duck/BD)
NEP:52V	South America (Calidris/alba/LIM)
NEP: 56Y	North America (Cattle/TX)
NEP:60S	North America (grackle/TX)
NEP:61K	North America (A/LOU/WT)
NEP:63E	Continent outside (duck/BD)
NEP: 64T,76M, NEP:77K,85Q ,81G,	Continent outside (duck/BD)
NEP:82E	North America(peregrine-falcon/NY, vulture/FL), South (wild-duck/CO)
NEP:89T	North America (A/MO)
NEP: 89V	South America (Panthera-leo/PER)
PB1-F2:11R	North America (peregrine-falcon), South America (wild-duck/CO)
PB1-F2:11L	Continent outside (turkey/GER)
PB1-F2:29R	Continent outside (duck/BD, turkey/GER, chicken/JPN)
PB1-F2:35L	

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PB1-F2:39T	Continent outside (duck/BD)
PB1-F2:41L	North America (A/LOU)
PB1-F2:57Y	North America (vulture/FL)
PB1-F2:69L	Continent outside (duck/BD)
PB1-F2:73E	North America (vulture/FL)
PB1-F2:78R	North America (harbor-seal/ME)
PB1-F2:79Q	South America (royal-tern/ARG)
PB1-F2:90I	North America (vulture/FL), outside continent (duck/BD) South America (black-necked-swam/UGY)
NP:41V	South America (Procellaria/BR)
NP:48R	South America (Panthera-leo/PER)
NP:63T	North America (A/CO)
NP:119T	South America (Chilean-dolphin, elephant-seal/ARG, tern/ARG)
NP:119V	North America (A/CA, emu/CA)
NP:190A	South America (Calidris-alba/PER)
NP:221K	South America (Numida/BR)
NP:230L	South America (mammals/birds)
NP:234S	Continent outside (turkeyGER, pintail/EGY)
NP:253V	North America (peregrine-falcon/NY)
NP:318L	North America(A/LOU)
NP:323S	South America (Numida/BR, black-necked-swam/UGY)
NP:363I	South America (gallus/PER)
NP:411A	North America (A/CO)
NP:425V	South America (Panthera-leo/PER)

Likewise, several unique and/or grupal mutations stands out among other specimens coming from North/South American, highlighting many of them have been also found in both mammals and birds, such in case of individual variations shared found in North American isolates: A/CA, emu/CA (HA:104G, HA:336N, PA:68S, PA:486M, PA:655F, PA-X:68S, PB2:670R, NS1:67G, NP:119V); cattle/TX (M2:27A, PA:36T, PA:404S, PA-X:36T, PB1:384T, NEP:60S); South American Chilean-dolphin, elephant-seal/ARG and terns/ARG (NS1:26K, NSI:226T, NP:119T, PA:57Q, PA-X:57Q, PA:86I, PA-X:86I, PA:336M, PA-X:20T, PB2:152V, PB1:40I, PB1:548F, PB1:515A,PB1:621K. Similarly, other specimens had also their own particular mutations like in South America: Panthera-leo/PER (HA:310V, PA:45S, PB2:190R, NEP:89V, NP:425V), Calidris-alba/LIM (HA:201R,NA:442I, M2:52S, PB2:679S,NS1:213L, NEP:56Y, NP:190A); black-necked-swam/UGY (PA:425F, PB2:199T, NP:323S, PB1-F2:90I); Andean-guayata/ARG (HA:9V); and in North America: peregrine-falcon/NY (NA:223T, NA:237F,M2:19Y, PA-X:118V, PA-X:207L,PA-X:250P, PB2:472D), vulture/FL (HA:87T, HA:102T, PA:59G, PA:272N, PA-X:59K, PB2:453S, PB1:431H, NEP:82E, PB1-F2:41L, PB1-F2:69L); harbor-seal/ME (HA10T, HA:152S,HA:226T,PA:465T, PA-X:70V, PB2:79G, PB2:715S, PB1:176T, PB1:372I,PB1:660I, NS1:67Q, PB1-F2:73E), goat/MI (HA:520R, PA:614S, PB2:274V, PB2:346A, PB2:353R, PB2:663R, PB2:667I, PB1:211K, NS1:36I, NS1:136M, NS1:201Y); raccoon/IA (M2:28T, PA:100I, PB2:539V); house-mouse/NM (NA:254R, PA:13V,PB1:384P,NS1:77R); alpaca (PA:142E, PA-X:142E) and red-fox/MI (PB2:596A).

Additionally, it is underscored the presence of grupal mutations PB1:515A, NS1:26K, PA-X:86I and PA-X:57Q found shared between South America mammals and birds (that if are considered in conjunction with the unique mutations detected in these samples), maybe have significance trying to explain the never seen before, huge spread of Influenza H5N1 viruses in our region.

Further, certain samples accumulated more quantity of individual/grupal amino acid changes in different protein segments such in case of birds coming from Brasil (Numida/BR, Procellaria/BR mainly) and the specimens coming from Bangladesh, Japan, Egypt and Germany included within the present study. (Tables 2 and 3).

 doi:10.20944/preprints202502.1501.v1

Table 2. Mutations identified in H5N1 viruses per samples group (4 or more isolates).

Mutation	Source
HA:11I	North America(A/LOU/WT, polar-bear/ALK), continent outside (eagle/JPN)
HA:52A	North America(A/LOU/WT, polar-bear/ALK), continent outside (eagle/JPN)
HA:211I	North America (mammals/birds)
HA:242I	South America(Sterna,Numida,Fregata,Procellaria,thalasseus/BR)
HA:492D	North America (A/LOU/WT, polar-bear/ALK), continent outside (eagle/JPN)
HA:504Y	South America(Sterna,Fregata,Procellaria,thalasseus/BR), continent outside (duck/BD)
HA:527I	North America(A/LOU/WT,polar-bear/ALK), continente outside (chicken/eagle/JPN, pintail/EGY, duck/BD, eagleJPN)
NA:6R	North America(polar-bear/ALK), continent outside (duck/BD, turkey/GER, eagle/JPN, pintail/EGY)
NA:10T	North America(polar-bear/ALK), continent outside (duck/BD, turkey/GER, eagle/JPN, pintail/EGY)
NA:70N	North America(polar-bear/ALK), continent outside (duck/BD, eagle/JPN), South America (wild-duck/CO) North America (mammals, birds)
NA:71S	North America (mammals, birds)
NA:321I	North America (polar-bear/ALK), continent outside (duck/BD, turkey/GER, eagle/JPN, pintail/EGY)
NA:405T	
M1:82S	North America (mammals/birds)
M1:125A	South America (Sterna/BR)
M1:227T	North America (mammals,birds)
M1:236K	Continent outside (chicken/JPN)
M2:88N	North America (mammals,birds)
NP:52H	North America (mammals/birds), continent outside (Eagle/JPN)
NP:105M	North America (mammals/birds), continent outside (turkey/GER), South America (wild-duck/CO)
NP:293K	South America (Sterna,Procellaria,Fregata,thalaseus/BR), continent outside (turkey/GER)
NP:377N	North America (harbor-seal/ME,vulture/FL,peregrine-falcon/NY), South America (wild-duck/CO), continent outside (chicken/JPN)
NP:482N	North America (mammals,birds)
PB2:58A	North America (mammals/birds)
PB2:109I	North America (mammals/birds)
PB2:139I	North America (mammals/birds)
PB2:154F	South America (Procellaria,Sterna,Thalasseus,Fregata/BR, duck,turkey,black-necked-swam/UGY)
PB2:362G	North America (mammals/birds)
PB2:441N	North America (mammals/birds)
PB2:495I	North America (mammals/birds)
PB2:631L	North America (mammals/birds)
PB2:649I	North America (mammals/birds)
PB2:676A	North America (mammals,birds), raccoon (676V)
PB1:16D	North America (A/LOU/WT, polar-bear/ALK), continent outside (pintail/EGY, Eagle/JPN)
PB1:154S	North America (A/LOU/WT, polar-bear/ALK), continent outside (eagleJPN) North America (birds/mammals)
PB1: 171V	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK), continent outside (chicken/eagle/JPN, pintail/EGY, duck/BD, Eagle/JPN)

 doi:10.20944/preprints202502.1501.v1

PB1:171A	North America (A/CO)
PB1:172D	North America (A/LOU/WT, harbor-seal/ME,polar-bear/ALK), continent outside (eagle/chicken/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1:179I	North America (birds-mammals)
PB1:207R	North America (A/LOU/WT, polar-bear/ALK), continent outside (turkey/GER, eagle/JPN)
PB1: 215K	South American (birds/mammals), continent outside (duck/BD)
PB1:264D	South America (birds, mammals)
PB1:375N	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, peregrine-falcon/NY), continent outside (chicken/eagle/JPN, pintail/EGY, duck/BD,turkey/GER), South America (wild-duck/CO)
PB1:378M	South America (birds/mammals)
PB1:399D	South America (birds/mammals)
PB1:429R	South America (birds/mammals)
PB1:430K	North America (birds/mammals)
PB1:515A	South America (Chilean-dolphin, elephant-seal/ARG, tern/ARG, pelecanus, Humboldt-penguin,cormorant,chimango/CHI)
PB1:548F	South America ((Chilean-dolphin, elephant-seal/ARG, tern/ARG)
PB1:587P	North America (birds/mammals)
PB1:614D	North America (A/LOU/WT, polar-bear/ALK), continent outside (Eagle/JPN) South America (Procellaria,Numida,Fregata/BR, swam,duck,turkey/UGY)
PB1:646I PB1:694S	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK), continent outside (eagle/chickenJPN, turkey/GER, duck/BD)
PB1-F2:4G	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK), continent outside (pintail/EGY, eagleJPN)
PB1-F2:7I	North America/South America (birds and mammals)
PB1-F2:7T	North America (birds and mammals), South America/continent outside (birds) South America (Sterna/BR)
PB1-F2:7M PB1-F2:8Q	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, continent outside (Eagle/JPN) South America (birds/mammals)
PB1-F2:17S PB1-F2:18T	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, continent outside (eagle/chickenJPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:20R	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, continent outside (Eagle/JPN)
PB1-F2:21R	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, continent outside (chicken/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:22E	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, continent outside (Eagle/JPN), South America (Numida/BR,black-necked-swam/UGY)
PB1-F2:30L	South America (birds/mammals), North America (vulture/FL, peregrine-falcon/NY) North America/South America (birds and mammals)
PB1-F2:31E PB1:36T	North America (A/LOU/WT, harbor-seal/ME), polar-bear/ALK, continent outside (Eagle/JPN)
PB1-F2:40G	North America (A/LOU/WT, polar-bear/ALK), continent outside (Eagle/JPN, pintail/EGY), South America (Sterna/BR)
PB1-F2:42Y	North America (A/LOU/WT, harbor-seal/ME, polar-bear/ALK, continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:44R	South America (birds/mammals), North America (goose/ALK,vulture/FL, peregrine/falcon), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY) North America (A/LOU/WT, polar-bear/ALK,harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)

 doi:10.20944/preprints202502.1501.v1

PB1-F2:46T	North American (mammals/birds)
PB1-F2:47S	North America (polar-bear/ALK, harbor-seal/ME), continent outside (eagle/JPN, pintail/EGY)
PB1-F2:48R	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, pintail/EGY)
PB1-F2:49A	South America (birds,mammals), North America (goose/ALK,pintail)
PB1-F2:50G	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:54K	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:55I	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, pintail/EGY)
PB1-F2:56A	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:57C	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:58W	North America (A/LOU/WT, polar-bear/ALK), continent outside (eagleJPN)
PB1-F2:65R	North America (goose/ALK,pintail), South America (birds/mammals), continent outside (chicken/Eagle/JPN, duck/BD, pintail/EGY)
PB1-F2:66S	North America (birds/mammals), continent outside (pintail/EGY)
PB1-F2:68I	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:70G	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, duck/BD, pintail/EGY)
PB1-F2:75L	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, turkey/GER, pintail/EGY)
PB1-F2:82S	North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME), continent outside (chicken/Eagle/JPN, duck/BD, pintail/EGY)
PB1-F2:84S PB1-F2:90N	South America (wild-duck/CO), North America (A/LOU/WT, polar-bear/ALK, harbor-seal/ME, peregrine-falcon/NY), continent outside (chicken/JPN, turkey/GER, duck/BD, pintail/EGY)
NS1:7S	North/South America/outside of continent (birds and mammals)
NS1:7L	North America (3 birds, 14 mammals)
NSI:21R	North/South America/continente outside (birds and mammals)
NS1:21Q	North/South America (grackleTX, elephant-seal, A/CO, cattleTX)
NS1:26K	South America (Chilean-dolphin, elephant-seal/ARG, tern/ARG, Humboldt-penguin, chimango, cormorant/CHI)
NSI: 53G	North America (vulture/FL, harbor-seal/ME), South America (birds/mammals)
NS1:83P	South America (wild-duck/CO), North America (A/LOU/WT,harbor-seal/ME,polar-bear/ALK, peregrine-falcon, vulture/FL), continent outside (pintail/EGY, eagleJPN)
NS1: 87S	North America and continent outside (birds and mammals)
NS1: 88C	South America birds (Procellaria,Fregata,Sterna,thalasseus/BR)
NS1: 116C	North America/continent outside (birds and mammals)
NS1: 147I	North America (A/LOU/WT, polar bear), continente outside (pintail/EGY, eagle/JPN)
NS1:189N	South America, birds(chimango,cormorant/CHI, Humboldt-penguin/CHI),North America (peregrine-falcon/NY)
NEP:7V	North America (3 birds, 14 mammals), 2 continent outside
NEP:7S	North/South America/outside (birds and mammals)
NEP:31V	South America (Birds: chimango, Humboldt-penguin, cormorant)
NEP:67G	

 doi:10.20944/preprints202502.1501.v1

	North America (3 birds, 19 mammals), continent outside (Eagle/JPN, pintail/EGY)
	South America (mammals and birds)
PA: 57R	North America (mammals and birds)
PA:113R	North America (mammals and birds)
PA:219I	South America (tern/ARG, Calidris-alba/LIM, Chimango/Coquimbo/CHI)
PA:237K	South America (royal-tern/ARG)
PA:237A	North America (peregrine-falcon), South America (wild-duck/CO), continent
PA:272E	outside (duck-BD)
	North America (mammals and birds
PA:277P	North America (A/CA, emu/CA,peregrine-falcon/NY), South America
PA:432I	(cormorant, wild-duck/CO), continent outside (pintail/EGY, turkey/GER)
	South America, birds/BR/UGY
PA:479E	North America (birds/mammals), South America (Procellaria/BR)
PA:497R	South America (elephant-seal/ARG,tern/ARG, pelecanus/PER, Procellaria/BR,
PA:558L	turkey/duck/UY, Panthera-leo/PER,Calidris-alba/LIM), North America (goose/ALK, Northern-pintail)
PA-X: 57Q	South America (mammals,birds), continente outside (turkey/GER), North America (peregrine-falcon/NY, A/LOU/WT)
PA-X: 61I	South America (wild-duck/CO), North America (A/LOU/WT, peregrine-falcon/NY), continent outside (turkey/GER, duck/BD).
PA-X:85T	South America (wild-duck/CO), Continent outside (duck/BD,turkey/GER), North America (A/LOU/WT)
PA-X:113R	North America (mammals and birds)
PA-X:193S	South America (wild-duck/CO), North America (A/LOU/WT, peregrine-falcon/NY, polar-bear/ALK,vulture/FL, harbor-seal/ME), continent outside (duck/BD, pintail/EGY)
PA-X:215L	South America (Fregata/BR, Thalasseus/BR,Sterna/BR, Procellaria/BR, duck/UGY, turkey/UGY)
PA-X:245N	South America (mammals/birds)

Besides, a number of mutations were found in "all" specimens, (concerning to the reference genome) (Table 3), as well as, in other cases some specimens had mutations shared in almost all samples with little exceptions detected, (Table 4). Be aware, that several of these mutational changes could have been transmitted over time at a multispecies level, bringing them with/without a specific trait, effect, or significance; nonetheless, these were common findings even in specimens far away of our geographical region.

Table 3. Mutations detected in all H5N1 viruses analyzed.

Protein	Commentary
HA	3N,88R,100S,110S,111L,139P,142E,143T,154Q,156A,157P,171D,190I,197S,208K,228K,233S 234Q,239R,243D,256H,284G,326K,429K,499R,515K,539A
NA	46P,76A,78Q,99I,100Y,258I,289M,366S,382E,418M,434N
M1	140A,144L,165I,
M2	18N
NP	136L
PB2	699K,741S
PB1	177E, 478S, 490F, 535I,536N, 558T, 598P,609Y,610C
PB1-F2	No PB1-F2 sequence in the reference genome
NS1	6I,18V,22F,23S,24D,25Q,27L,28C,54I,60A,73S,84V,94T,95L,112A, 114G,117I,127R,137L,140Q,146L,153E, 158G,161S, 163L,170T,180V,191T, 194V, 197T, 198L,205S,206S,211R,221K,224R,225T,366S

 doi:10.20944/preprints202502.1501.v1

NEP (NS2)	6V, 14M,22G, 26E,37S, 40L, 48A, 49V,68Q,83V,86R,88K,100M,111Q
PA	63V,129I, 212C, 228N, 361K, 536K,544E, 585L, 586L,716R
PA-X	no PA-X sequence in the reference genome
PB2	355R,

In general, during the mutational scanning, certain mutations were acquired in all samples tested, whilst others were only detected in an individual or groupal presentation. Also, there was an existing different distribution in the mutations between the North American isolates versus the South America isolates, and even within the same group of samples of North American ones, where some of them showed more similarities in terms of mutations with Southern isolates. (Complete mutational scanning can be seen in Supplementary No.2 (S2).

Table 4. Mutations found in almost all H5N1 viruses with some exceptions.

NS1	**44R,55E,56T,59R,63Q,70E,71E,74D,90L,111V,118R,139D,145I,166L,171D,192V,204R,207N, 209D,213P,441V,553A, 608S,245S/N,252K/R**
PB1	59S,75D
PB2	334S,340R,463V,464M,471T,478I,590G, 616V
NP	450N
M2	28I,51V,61G
M1	85S,87T,101R,200V,230R,232D
HA	69K,98R,120M,131L,178I,185R,199N,201E,205N,226A,336S,341K,344R,527V,549M
NA	8T,20V,44Y,81T,155Y,188I,269M,287D,340S,336S,338M,339P,340S,395E,460G
NEP	63A, 64K,81E,85H,89I
PA-x	252R/K
PB1-F2	11Q,12L/S

3.2. Flumut Mutational Analysis

Our mutational analysis done, were compared with the results obtained with Flumut program, and a match in the detection of the next mutations/molecular markers was found as follows: HA:154N, HA:156A, HA:185A; M2: 27I; NA:155H, NA:223T, NA:364N; NS1:53D, NS1:55E, NS1:66E, NS1:74N, NS1:205S, NS1:210R; NS2:48A; NP:41V, NP:105V; PA:63I, PA:142E, PA:190S, PA:497R; PB1:207R, PB1:375S, PB1:598L; PB1-F2:56A, PB1-F2:66S; PB2: 9N, PB2:292V, PB2:339K, PB2:495I, PB2:590S, PB2:631L, PB2:676T, PB2:699R, PB2:715N; emphasizing that some of the mutations presented amino acid changes in the mutation designated, such in case of HA:154N (HA:154Q), HA:185A (HA:185R) or NA:53D (NA:53G), and taking into account, as previously mentioned, that certain mutations were found in all samples whilst others were only detected in individual samples. (Complete Flumut mutational analysis can be seen in Supplementary No.3 (S3).

3.3. Genotype Identification

Four genotypes were found in the H5N1 viruses analyzed: B3.13(30,5%), B3.2(37,3%), B1.3(3,4%), A3(3,4%), A2(1,7%), B1.1(1,7%); and in (22%) of specimens the genotype was not assigned, because not all the segments match found of total of segments in input file. (Complete Genoflu genotype determination can be seen in Supplementary No.4 (S4).

3.4. Glycosylation Patterns

3.3.1. N-linked Glycosylations in the HA of Influenza H5N1 Viruses

A total of 7 N-linked glycosylations (NLG) were found in HA protein of the reference genome (A/goose/Guandong/1996(H5N1) at positions 27 NSTE, 39 NVTV, 181 NNTN, 209 NPTT, 302 NSSM,

 doi:10.20944/preprints202502.1501.v1

500 NGTY and 559 NGSL respectively, taking in consideration that 209 NPTT position had included a warning (Pro-X1), due the presence of a proline in the sequon. The complete list of glycosylation predictions is Supplementary No.5 (S5).

Particularly, the 209 NPTT NLG, was absent in some samples analyzed, and it specimens carried a specific change into the HA protein sequence: a mutation in the amino acid position 211 from T to I; in other words, the samples that were absent in 209 NPTT site, all of them conserved this amino acid variation. In addition, the amino acid position 208 (previous to sequon 209 NPTT), had a variation from Q (reference genome) to K, in all samples tested.

For instance, the mammal isolates coming from North America were classified in two groups (according to the presence or absence of 209 NPTT NLG): the first group, included 16 samples (4 A/CA/MT/CO, 1 alpaca/ID, 3 bovine/TX/ID, 2 feline/TX/ID, 1 red-fox/MI, 2 goat/MI, 1 raccoon/USA, 1 house-mouse/NM, 1 domestic cat/TX), that only had 6 NLGs predicted and had lost 209 NPTT site; whilst the second group, included 4 samples (2 A/LA/WA, 1 harbor-seal/ME/ME, 1 polar bear/AK) showed 7 NLGs and it maintained invariable the 209 NPTT glycosylation position predicted.

In contrast, the mammal isolates coming from South America (elephant-seal/ARG, Panthera-leo/PE and dolphin/CHIL), had 7 NLG foreseen and maintained the 209 NPTT glycosylation predicted site. On the other hand, 19 bird isolates coming from South America (Andean-guayata/ARG, Sterna-hirundo/BR, Humboldt-penguin/CHIL, Procellaria-aequinoctialis/BR, Numida-meleagris/BR, cormorant/CHIL, belcher-gull/PE, wild-duck/CO, 2 pelecanus/PE, backyard-duck/UY, Fregata-magnificens/BR, chimango-caracara/CHIL, black-necked-swam/UY, royal-tern/AR, Calidris-alba/PE, gallus-gallus/PE, Thalasseus-acuflavidus/BR and South America-tern/ARG) presented 7 NLGs predicted and also preserved the position 209 NPTT invariable; but otherwise, 3 North America birds samples (Emu, chicken/Idaho and grackle/Texas), only had 6 NGLs and the 209 NPTT was absent, whilst others 6 specimens (peregrine-falcon/NY, goose/AK, Northern-pintail/USA, chicken/JPN, duck/BD, white-tailed-eagle/JPN, turkey/GER and pintail/EG) maintained a constant presence of the predicted 209 NPTT glycosylation site.

Likewise, the glycosylation predictor showed a substitution in the NLG site to 499 NGTY and 558 NGSL (from 500 NGTY and 559 NGSL of the reference genome). This ultimate characteristic has been seen in all analyzed genomes in this search, interestingly, it was also found a possible coincidence (there may or may not be) with the presence of a mutation in the amino acid sequence prior to NGTY sequon, from K to R in position 499, which was present also in all isolates analyzed (with exception of the reference genome). In addition, a slight increase in the glycosylation potential was seen of 0.5263 (reference genome) versus 0.58 finding present in all samples analyzed.

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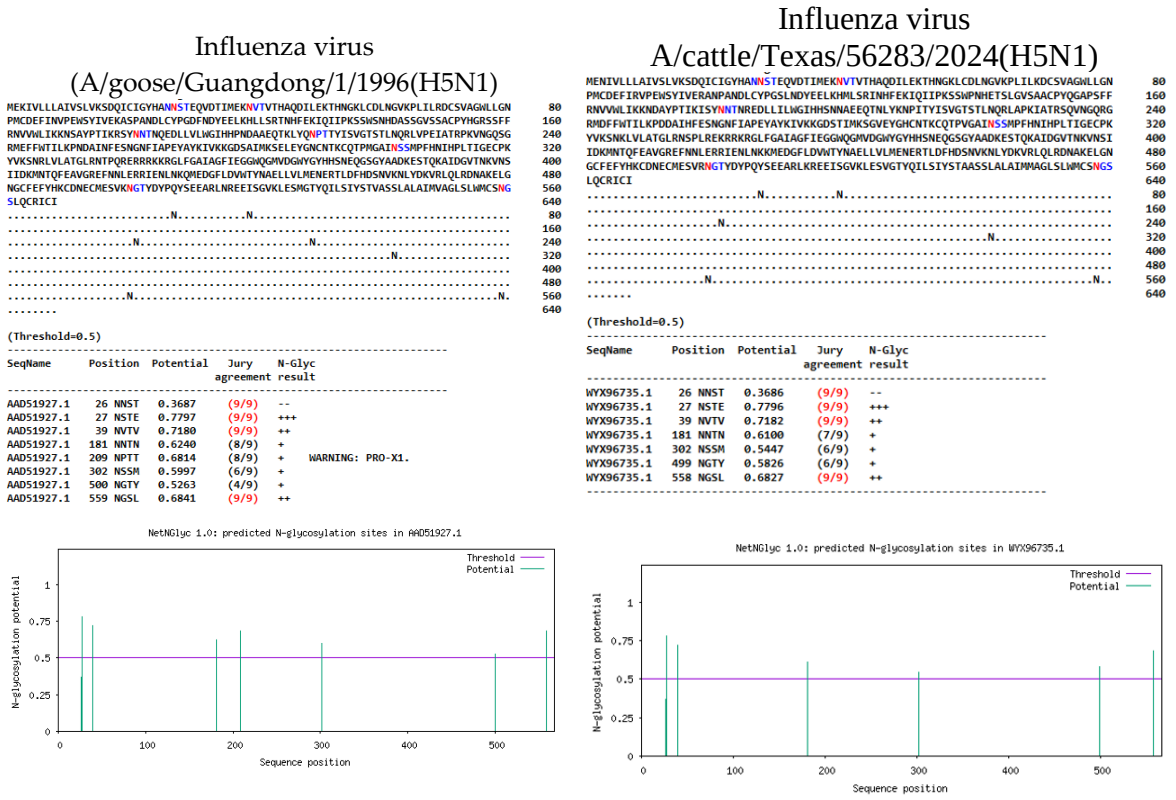

Figure 2. Comparison of glycosylation sites for HA protein of H5N1 viruses: (**A**). Left. Reference genome Influenza A virus (A/goose/Guangdong/1/1996(H5N1) had 7 NLGs predicted; (**B**). Right. Influenza A virus/cattle/Texas/56283/2024, had 6 predicted NLGs, with substitution of 2 NLGs sites (499 NGTV and 558 NGSL) and loss of the 209 NPTT site.

Furthermore, 2 North American avian isolates (chicken/JPN and vulture/FL) had 8 predicted NLGs, with an extra NLG site as 100 NPTN, that also concurred with a mutation in the HA protein, 102 site from A to T amino acid; it only was shown in these two isolates. It should be noted that a 100 NPTN glycosylation prediction was found in samples very far away geographically.

Besides, two South American avian samples (Sterna-hirundo/BR and Humboldt-penguin/CHL) showed 6 NLGs with a loss of glycosylation site 302 NSSM. A contrast was made with HA sequence protein where S amino acid was replaced by N at site 304 in both cases. This was only seen in these two isolates (Figure 3).

3.3.2. N-linked Glycosylations in the NA of Influenza H5N1 viruses

The predicted NLGs in NA protein of the reference genome (A/goose/Guandong/1996(H5N1) found 7 sites of glycosylation at 50 NQSI, 58 NNTW, 63 NQYT, 68 NISN, 88 NSSL, 146 NGTV and 253 NGSC positions. In the case of NA protein, no warming was issued for the presence of proline in any NLG. Differences in glycosylation patterns were found in some of the samples, showing changes in the major prediction NLGs sites predicted to NA protein. In comparison with the reference genome, these patterns were found in both mammals and bird's samples.

First, the 3 South American mammals' isolates (elephant-seal/ARG, Panthera-leo/PE and dolphin/CHIL), as well as 17 of the South America avian isolates (Andean-guayata/ARG, Sterna-hirundo/BR, Humboldt-penguin/CHIL (Figure 3), Procellaria-aequinoctialis/BR, Numida-meleagris/BR, cormorant/CHIL, belcher-gull/PE, pelecanus/PE, backyard-duck/UY, Fregata-magnificens/BR, chimango-caracara/CHIL, black-necked-swam/UY, royal-tern/AR, Calidris-alba/PE, gallus-gallus/PE, thalasseus-acuflavidus/BR and South America-tern/ARG), had the same 7 NLGs at 50 NQSI, 58 NNTW, 63 NQYT, 68 NISN, 88 NSSL, 146 NGTV and 253 NGSC positions, described for the reference genome. Nevertheless, the wild-duck/CO specimen substituted the 68 NISN

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glycosylation site predicted with 70 NNTN. This same feature was seen in other 2 avian isolates (duck/BD, white-tailed-eagle/JPN), and 1 sample of mammal origin (polar-bear/ALK).

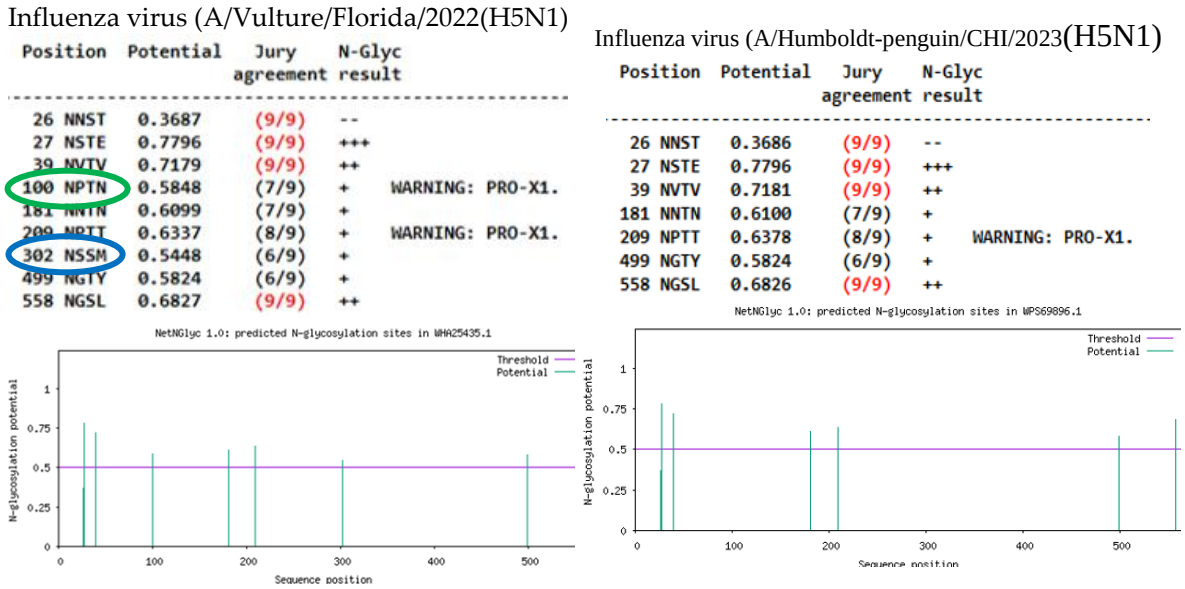
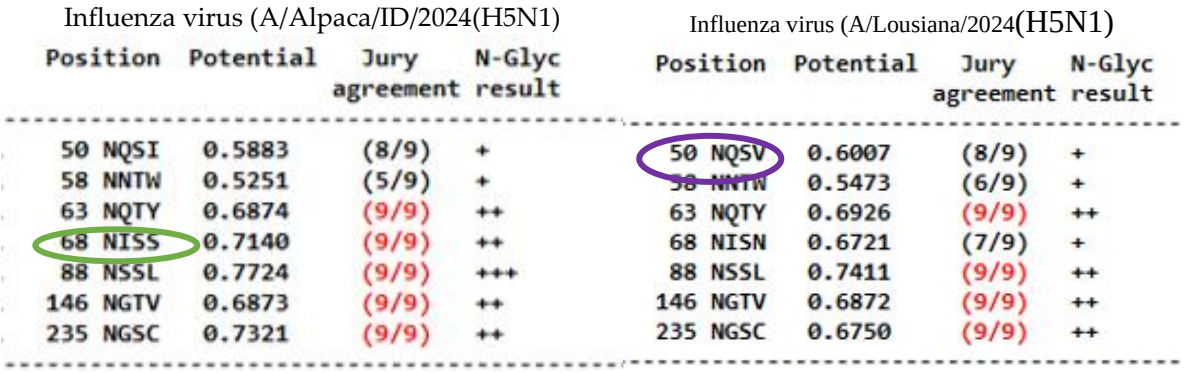


Figure 3. Comparison of glycosylation sites for HA protein of H5N1 viruses: (**A)**. Left. North American Influenza A virus (A/Vulture/Florida/2022(H5N1) had 8 predicted NLGs, with a gain of 100 NPTN site; (**B)**. Right. South American Influenza A virus/Humboldt-penguin/CHI/56283/2023(H5N1) had 6 NGLs predicted with loss of 302 NSSM site.

In the case of North American avian isolates, 2 samples (vulture/FL and Goose/AK, shared the same patron of glycosylation with the NA reference genome, while others 3 specimens (Emu/CA, grackle/TX, chicken/ID) had substituted the predicted NLG site at the 68 position from NISN to NISS. It is worth highlighting that this characteristic was also found in 11 North American mammal isolates (2 A/CA, and 1 A/CO, 1 domestic-cat/TX, 1 alpaca/ID, 3 bovine/TX/ID, 2 feline/TX and 1 racoon/USA). Moreover, 68 NISS feature, were also accompanied with a substitution in the amino acid of the 67 position from V to I, just prior to the sequon location. However, considering the potential of glycosylation predicted between the samples analyzed, was quite variable in this particular site. For example, the reference genome had a glycosylation potential of 0.73 being 68 NISN, whilst other samples being also 68 NISN had a potential of 0.67-0.68; this finding has been similar in other different species (3 A/LO/WT/MO, 1 red-fox/MI and 1 domestic cat/TX isolates). In addition, the 11 samples that are 68 NISS had a glycosylation potential predicted of 0.71. (Figure 4).

Furthermore, two recent North American mammal isolates (2 A/LO/WT), had the unique, characteristic of NLG 50 NQSV predicted; it was presented with a slight increase of predicted glycosylation potential from 0.50 to 0.60 compared with other samples (50 to NQSI), for instance the reference genome had a potential of 0.55 in this NLG predicted site.



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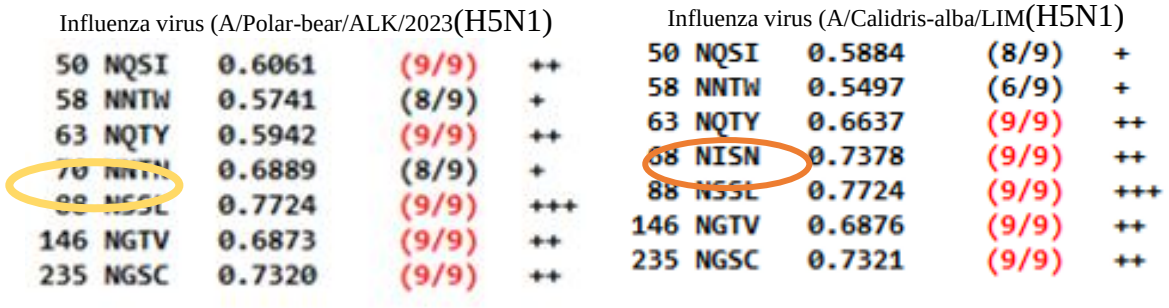

Figure 4. Comparison of glycosylation sites predicted for NA protein of H5N1 viruses: (**A**). Left/up: Influenza virus (A/Alpaca/ID/2024(H5N1) had 7 NLGs, with a 68 NISS site; Left/down: Influenza virus (A/polar-bear/ALK/2023) had 7 NLGs, with a 70 NNTN site (**B**). Right/up: Influenza virus (A/Lousiana/2024(H5N1) had 7 NGLs with the substitution 50 NQSV; Right/down: Influenza virus (A/Calidris-alba/LIM(H5N1) had 7 predicted NGLs with 68 NISN.

Conversely, 1 North American avian sample (peregrine-falcon/NY) had a variation in the NLG predicted from the position from 235 NGSC (present in the reference genome and other samples) to 221 NNTL, its difference, diminished notably the glycosylation potential from 0.73 to 0.47. In contrast, a decrease in the glycosylation potential was also seen in an avian sample (Northern-pintail/USA) that being 235 NGSC had a glycosylation potential of 0.67. This last feature was share with the two most recent human samples tested (A/LO/WT) that also had 0.67 of a glycosylation potential in this NLG. Coincidentally, these 3 samples (Northern-pintail/USA and 2 A/LO/WT), had the unique feature of a mutation detected in position 234 where V was replaced to I.

On the other hand, the sample (turkey/GER) had 6 NGLs, due to the loss of 88 NSSL glycosylation site predicted, and also it match with a respective substitution in the amino acid from S to P located in the 90 position of NA protein.

Besides, the isolate (chicken/JPN) showed a slight increase in the glycosylation potencial at the 146 NGTV site of 0.68 (presented in all other samples) to 0.7377, it may due to a substitution E in the amino acid position 150.

4. Discussion

Noteworthy, the treat of Influenza H5N1 viruses globally within One Health framework is undeniable, and one of the significant challenges of our era, focus on limiting exposure and preventing the spread of Influenza, considering now more carefully animal welfare, in order to maintain a sustainable connection between nature and people. Particularly, at this time, the protection of global biodiversity, is a growing concern for humanity, and current facts such as, the overexploit of wild and domestic animals, the unsustainable production systems [54], as well as the poorest water quality available to the creatures with whom we share our planet, leave us big stakes to maintain the balance in ecology, habitats, ecosystem services and conservation of threatened species. Hence, international collaborative strategies are required. Anthropogenic activities impact strongly aquatic habitats, altering water and sediment quality, affecting the organisms, wildlife and human [55,56], specially birds and marine mammals the principal reservoirs and victims of Influenza viruses; so it is crucial to consider ecology facts, and holistic perspectives, to mitigate the transmission risk (including zoonosis reversa), between mammals and humans.

Meanwhile, this study describes the mutational changes and glycosylation patterns found during the comparative analysis of H5N1 Influenza isolates, by exploring evolution of these highly variable viruses. In the search for critical mutations, an extended listing of amino acid variations, with potential impact on the biological characteristics of Influenza viruses were detected. Consisting with our findings, mutations detected HA:156A, HA:208T (HA:208K in our listing), NP:52H, PA:336M, PA:36T, PA:85I (PA:85A/N), PB1-F2:66S, PB2: 292V and PB2:559T (PB2:559N), have been associated with increased receptor specificity, enhanced haemagglutinin and

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neuraminidase functions, as well as increase of polymerase activity and inmune evasión [57]. Moreover, mutations found in PA:497R and PB2:631L, have also been connected with an enhance of polymerase activity, facilitating the replication in several mammalian cells [26]. Furthermore, amino acid changes observed HA:T143A (HA:143T in our listing) and NA:71S, have been recent, implicated in fostering virus infectivity and facilitating immune evasion, through resistance acquired to neutralizing antibodies, it is important to highlight that mutation NA:71S has not been previously reported in H5N1, and its functional implications are still unknow, but further research needs to be done since these mutations had been seen in isolates coming from domestic cats with marked neurotropism [22,58].

Now, considering that the largest mass mortality associated with H5N1 virus happened in the coast of Peru, South America [59], and the devastating impact on wild birds and mammals throughout the continent and globally has been happening to present, it is crucial to identify key mutations that could represent mammalian adaptation or that warrants further research. Some mutations recently reported in Argentina, PB1:548F, PB1:621K, PA:86I, PA:237E (PA:237K in our findings), NS1:21Q and NS1:226T(M. M. Uhart et al., 2024), have also been showed within our analysis, underscoring that these substitutions were found in others mammal/birds species of our region (including our own Peruvian specimen, as well as some North America isolates): PB1:548F/NS1:226T (in Chilean-dolphin), PA:237K/A (Calidris-alba/LIM, chimango-caracara/CHI), and NS1:21Q (A/CO, cattle/TX, grackle/TX). According to, evolution rates described to South America mammals and birds H5N1 viruses versus those circulating in cattle in the USA at present (6.2×10^{-3}; $5.3\text{-}7.2 \times 10^{-3}$ 95% HPD) [15], is concerning from One Health perspective, since a specific mutation shared in samples collecting with one year and a half of difference in South and North America and coming from different hosts was found; however is evident and well-know, that mutational changes should not be studied an individual level, this point should be analyzed together with individual mutations previously described (Tables 2–5), to achieve a better understanding of mutational evolution.

Besides, inside of the mutations marked as critical in FluMut are noteworthy the following: HA:154N (related to an increased virus binding to α2-6) [60]; M2:27A(found in cattle/TX and duck/BD samples, related to increased resistance to amantadine/rimantadine [60]; NA:223T, NA:364N(found in a peregrine-falcon), related to posible reduced inhibition to oseltamivir [61]; NP:105V, PA:63I(PA:63V in our listing), PB2:9N(polar-bear/ALK,pelecanus)[60], PB2:699R, related to increased virulence [62]; and PA:497R [63], PB2:631L [64], PB2:676T,PB2:495I,PB1:66S,NP:41V[60], related to an enhanced polymerase activity. Additionally, there are several spot mutations HA:185A, PA:142E(alpaca), PB1:375N, PB1:598L, PB1:56A, PB2:292V, PB2:590S that require further investigation to elucidate their biological functions. From our standpoint, the data displayed in the present study also include other new mutations that still had an unknown function, and bearing in mind that only one amino acid change or the combination of them, are vital in the evolution of Influenza viruses, is paramount to do the follow up. The last thing, to highlight at this point, which is based in our findings, is the importance of carrying out further analysis of new findings, especially in the accessory proteins PB1-F2 and PA-x, in view of striking mutational traits, such as PA:M86I, PA-X:M86I (South America), and PB1-F2:4G, PB1-F2:8Q, PB1-F2:17N, PB1-F2:39T, PB1-F2:47N, PA:142E, PA-X:142E (North America) coming from mammal/birds isolates.

On the other hand, talking about glycosylation facts, it is noted that glycoproteins are indispensable factors for the infectivity, survival and transmissibility of the Influenza virions. The acquisition or loss of active glycosylation sites, are crucial in the evolution of Influenza A viruses, and lots of variations exist in the glycosylation patterns between host-species and the virus strain. The present study showed some key NLGs found in both HA and NA proteins in multiples hosts of H5N1 Influenza viruses, where several of these differences were shared between some mammals and non-mammals' species, including certain amino acid mutations.

Amidst, the presence/absence of 209 NPTT in HA protein, as well as change in position of NGLs such as 499 NGTY, 558 NGSL, just as the finding of an extra NLG 100 NPTN or the loss of 302 NSSM

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are disparities that could require future analysis, considering that the addition of oligosaccharides in HA has been associated with changes in the ability to bind to cellular receptors, interaction with neutralizing antibodies (immune evasion), properly protein fold, fusion process, efficient transport, stability and a fit budding [31,65], so is essential to carry out the scanning, ir order to find out what kind of possible biological function are fulfilling these NLGs, and what specific area of HA is being strengthened, considering that HA NLGs in stem region are paramount for the membrane fusion, and correct protein folding; whilst NLGs in the head region are useful to receptor binding and to mislead the immune system (masking antigenic sites of the receptor binding domain)[53]. Specifically, in HA receptor-binding site (RBS), the occurrence of adaptative mutations and altered glycosylation in or near RBS are significant factors that influence viral receptor binding preferences [66], where the loss or acquisition of NLGs has been demonstrated affecting human receptor binding of H1N1 viruses [67].

Particularly, it is crucial to emphasize the NLGs 209 NPTT position, because being a warning predicted glycosylation, it appeared and disappeared among diverse isolates, though theoretically NLG with proline is strictly excluded, since the presence of proline blocks glycosylation completely, [68] for structural constraints [52,69]. So, what if hypothetically, this NLGs 209 NPTT might be functional. What if changes in the conformational structure of HA, or in the interaction receptor-glycans of different length may regulate the virus attachment and influence directly the virus entry as previously described [70].

On the other hand, in the case of NA, it's worth highlighting the detection of NLGs 50 NQSV predicted site from two mammal recent isolates (accompanied by a slight increase in the glycosylation potential predicted), as well as the substitution from 68 NISN to NISS, and the extra finding of 70 NNTN. In addition, the loss of 88 NSLL in 1 sample (accompanied by a specific mutation), as well as the substitution of 235 NGSC to 221 NNTL. Considering that, whichever of these viruses have been achieved successful in making illness up to a lethal level, in several species not only birds, it give us that impression that this particular protein region have a current biological relevance. This pending research also includes finding out the variability in the glycosylation potential sites between the isolates. Apparently, this seems to be a progressive adaptive change of Influenza virus. Furthermore, findings of repeated predictions 68 NISS in recent isolates of mammals and birds simultaneously must be further analysed. NLGs are an essential component in the adaptation of Influenza viruses to new hosts (Kim et al., 2018b); and in silico predictions help us to detect some of these variations. The latter could possibly be somehow different in a real trial. Nowadays, it is evident that H5N1 viruses have characteristics to explore in NLGs that may be facilitating the transmission interspecies.

Furthermore, it is necessary to point out the evaluation of the glycosylation findings in HA and NA, should be done jointly, due to the opposing roles of these proteins, the work synchronously they do, during the infection cycle: the stability between HA binding and NA cleavage action is base for overcoming host barriers and adaptation to new host species. The counterbalanced can be disrupted with the addition or NLGs removal, reducing directly the viral fitness. Changes observed in glycosylation patterns should be considered as a predicting feature of future pandemic/panzootias of Influenza viruses [66]. Additionally, posttranslational modification occurring in the other proteins such as PB2 should be taken into account [62].

Our understanding how glycosylations affect viral adaptative achievement, will therefore provide light on the dynamics of IAV adaptative evolution; nevertheless, knowledge in this subject is still limited; thus, it is recommended to carry out complementary studies to the glycosylation findings, as well as protein modeling studies and mass spectrophotometry based on comparative proteomics [72] to discern more about all the NLGs differences found, it is important to highlight that the emergence of extra possible glycosylation sites might suggest that new glycosylation forms might appear and occur sudden pandemics/panzootic in a near future [53] .

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5. Conclusions

The unprecedented spreading of the H5N1 strains of Influenza virus globally has led to catastrophic impacts on wildlife, including the devastating impact in seabirds and pinnipeds colonies that occurred in South America. The interconnectedness existing between humans, nature and the environment, makes us unable to ignore the alarming increase in the circulation of these highly pathogenic viruses that are acquiring new mutational adaptations, and favoring their expansion toward new mammal/bird hosts. Given the critical role of mutations in the adaptative evolution of Influenza viruses, a better understanding of biological properties of these viral adjustments, within One Health perspective, will help us to dilucidate the existing bond between glycosylation/mutations in the breakdown of the species barrier. Thus, reinforcing public data bases is recommended, as well as updating the H5N1 viruses mutational profiles and including NLGs relevant sites.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, S1: Listing of data sets/ mutations found. S2: Complete mutational data analysis; S3: Flumut data analysis; S4: Predicted N-linked Glycosylations of Influenza H5N1 viruses

Author Contributions: Conceptualization, methodology, S.L; software, D.S.; validation, S.L; formal analysis, S.L, L.A, D.S.; investigation, S.L.; resources, S.L.; data curation, S.L, L.A, D.S.; writing—original draft preparation, S.L; writing—review and editing, E.M, E.M.; visualization, L.A.; supervision, E.M.; project administration, S.L, E.M. All authors have read and agreed to the published version of the manuscript.

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