Differential Diagnosis and Molecular Characterization of Avian Influenza Viruses

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CERTIFICATE

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DEDICATED TO MY LOVING AMMI
AND PAPA

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List of Abbreviations

Af Strlng	African Starling
Ag	Antigen
AI	Avian Influenza
AIV	Avian Influenza Virus
Asn	Asparagine
B	Basic residue
Ban	Bantam
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
BSL	Bio Safety Level
Cat#	Catalogue number
cDNA	Complimentary Deoxyribonucleic Acid
CHO sites	Glycosylation sites
Ck	Chicken
Con	Conure
cRNA	Cellular Ribo Nucleic Acid
CSIRO	Commonwealth Scientific and Industrial Research Organization
D	Aspartic Acid
Dbai	Dubai
DNA	Deoxyribo Nucleic Acid
E	Glutamic Acid
Ed	Editor
EDTA	Ethylene Diamine Tetra Acetic acid
F	Phenylalanine
Fy bb	Fairy bluebird
g	Gravity

G	Glycine
Gal	Galactose
Gln	Glutamine
Glu	Glutamic Acid
Gn fwl	Guinea Fowl
GP	Glycoprotein
H5	Hemagglutinin 5
H7	Hemagglutinin 7
H9	Hemagglutinin 9
HA	Hemagglutinin
HAU	Hemagglutination Unit
Heb	Hebei
Hnan/Hen	Henan
Hnrdrf	Heinersdorf
Hok	Hokkaido
HPAI	Highly Pathogenic Avian Influenza
HRSV	Human Respiratory Syncytial Virus
Hub	Hubei
I	Isoleucine
IB	Infectious bronchitis
IBV	Infectious Bronchitis Virus
ILT	Infectious laryngotracheitis
ILTV	Infectious Laryngo Tracheitis Virus
INF α/β	Interferon α/β
Irlnd	Ireland
Isrl	Israel
Itly	Italy
IVPI	Intra Venous Pathogenicity Index
Ja	Jena
K	Lysine
kb	Kilobase

KPK	Khyber Pakhtoon Khuwa
Kor	Korea
L	Leucine
lb	Pound
LBM	Live Bird Market
Leu	Leucine
LPAI	Low Pathogenicity Avian Influenza
Lpzg	Leipzig
M	Molar
mA	milli ampere
Mcw	Macaw
MDCK	Madin-Darby Canine Kidney
MEGA	Molecular Evolutionary Genetics Analysis
MG	Mycoplasma gallisepticum
M-Gene	Matrix Gene
Mglia	Mongolia
min	Minute
Mnglia	Mongolia
mPCR	mutiplex polymerase chain reaction
MS	Mycoplasma synoviae
N	Asparagine
N1	Neuraminidase 1
N2	Neuraminidase 2
N3	Neuraminidase 3
NA	Neuraminidase
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
Nchng	Nanchang
ND	Newcastle disease
NDV	Newcastle Disease Virus
Neu Ac	Neuraminidase acyl

NeuAc	N-Acetylsialic acid
nm	nano mole
Non-psitcne	Non-psittacine
Nor	Norway
NP	Nucleoprotein
NP	Nucleocapsid Protein
NS1	Non-structural 1
P	Proline
PA	Polymerase Protein
Pak	Pakistan
PB	Polymerase Protein
PEG	Poly Ethylene Glycol
Pk Dk	Pekin Duck
PKR	RNA-activated protein kinase
Pn	Pigeon
Poly A	Poly Adenine
Prg falcn	Peregrine Falcon
Prkt	Parakeet
Prmrie	Primorie
Prt	Parrot
Pstdm	Postdam
Pt	Pintail
Q	Glutamine
Qa	Quail
Qlnd	Queensland
R	Arginine
RNA	Ribo Nucleic Acid
RNP	Ribo Nucleo Protein
rpm	revolutions per minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
sec	Second

Sftbill	Softbill
Sgr	Singapore
Shmne	Shimane
Shndng	Shandong
Shntu	Shantou
SIV	Swine Influenza Virus
SJCH	Saint Jude's Children Hospital
SPF	Specific Pathogen Free
sRT-PCR	Single Reverse Transcriptase-Polymerase Chain Reaction
Sw	Swan
T	Threonine
Taq	Thermus aquaticus
TE	Tris EDTA
Tky	Turkey
Tr	Tern
Twn	Taiwan
UK	United Kingdom
V	Volts
VI	Virus Isolation
VNT	Virus Neutralization Test
vRNA	Viral Ribo Nucleic Acid
WHO	World Health Organization
Wk	Week
X	Non-basic residue
YC	Yan Chen
YN/Yn	Yunnan
Zmb	Zimbabwe
α	Alpha
µg/ml	microgram/milliliter
µl	Microliters

List of Publications

Rashid S, Naeem K, Ahmed Z, Siddique N, Abbas MA and Malik SA. Multiplex Polymerase Chain Reaction for the Detection and Differentiation of Avian Influenza Viruses and Other Poultry Respiratory Pathogens. (Published, Poultry Science, December, 2009. 88:2526-2531. doi:10.3382/ps.2009-00262, 2009 Poultry Science Association) (See Annexure).

ABSTRACT

During the Avian Influenza (AI) outbreaks in different areas of Pakistan (2003 - 06), a number of Avian Influenza Virus (AIV) isolates were recovered from the clinical samples. The samples were subjected to comparative diagnostic evaluation using *in-ovo* propagation, Virus Neutralization Test (VNT), rapid detection kits and Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR). The data revealed that RT-PCR technique was most sensitive and specific for the detection of Avian Influenza Virus subtypes and for differentially diagnosing it from other avian respiratory pathogenic viruses. These isolates were further utilized for the development of multiplex RT-PCR. A multiplex reverse transcriptase polymerase chain reaction (**mRT-PCR**) was developed and standardized for the detection of type A influenza viruses, Avian Influenza Virus (**AIV**) subtype H7, H9 and H5 haemagglutinin gene with simultaneous detection of 3 other poultry respiratory pathogens Newcastle disease virus (**NDV**), infectious bronchitis virus (**IBV**) and infectious laryngotracheitis virus (**ILTV**). Seven sets of specific oligonucleotide primers were used in this study for the M-Gene of AIV and haemagglutinin gene of subtypes of H7, H9 and H5 of AIV. Three sets of other specific oligonucleotide primers were used for the detection of avian respiratory pathogens other than AIV. The mRT-PCR DNA products were visualized by Agarose Gel Electrophoresis and consisted of DNA fragments of 1023bp for M-Gene of AIV, 149bp for IBV, 320bp for NDV and 647bp for ILTV. The second set of primers used for m-RT-PCR of H7N3, H9N2 and H5N1 provided DNA products of 300bp for H7, 456bp for H5 and 808bp for H9. The mRT-PCR products for the third format consisted of DNA fragments of 149bp for IBV, 320bp for NDV, 647bp for ILTV, 300bp for H7, 456bp for H5, 808bp for H9. The sensitivity and specificity of mRT-PCR was determined and the test was found to be sensitive and specific for the detection of AIV and other poultry respiratory pathogens. In the present study, multiplex PCR technique has been developed to simultaneously detect and differentiate three most important subtypes of AIV's alongwith 3 most common avian respiratory pathogens prevalent in poultry in Pakistan.

The non-structural 1 (NS1) protein of avian influenza viruses has been earlier described as a remarkably conserved protein amongst type A influenza viruses, however with

subsequent findings of is truncation during extensive circulation in poultry has led to further investigate its mutation in association with point mutations simultaneously occurring in more variable genes such as HA and NA. Apart from affecting any of the biological functions of these viruses, these mutations may affect the immunogenic component(s) of these viruses, affecting the efficacy of prevalent vaccines. To establish if Pakistani H7N3 Avian influenza viruses undergo any truncation in non-structural genes, the non-structural gene 1 (NS1) of 22 H7N3 Avian influenza A viruses isolated from commercial and domestic poultry was sequenced and compared phylogenetically. The isolates included in the present study were both of low pathogenecity (LPAI) and highly pathogenic strains (HPAI) of H7N3 avian influenza viruses as observed in the field with regards to their mortality rates. These isolates circulated in N.W.F.P, Punjab, and Sindh areas of Pakistan from 1995 to 2005. Size variation in the predicted amino acid sequence of each NS1 was revealed with two different levels of carboxy-terminal truncation in those isolates. Of the 22 isolates analyzed, 02 isolates A/Chicken/Pakistan/NARC-100/04 and A/Chicken/Pakistan/NARC-1282/04 encoded a full length NS1 protein of 230 amino acids, whereas 20 encoded a truncated protein of 217 amino acids. The isolates exhibiting the truncated carboxy terminal NS1 protein, clustered together and appeared to be closest to A/Duck/Jiang Xi/6146/03 (H5N3), A/Duck/Hong Kong/610/79 (H9N2) and A/Aquatic Bird/Korea/CN-1/04 (H3N6) at the nucleotide level and amino acid level. In contrast, the nucleotide sequence of one of the isolates with the full length NS1 protein (A/Chicken/Pakistan/NARC-1282/04) showed 99.9% nucleotide homology and 99.6% homology to a set of Italian H7N3 isolates of Turkey from 2002 at the NS1 gene e.g A/turkey/Italy/8912/2002(H7N3) and A/turkey/Italy/214845/02(H7N3). The other isolate (A/Chicken/Pakistan/NARC-100/04) with the full length NS1 protein showed the highest homology (96%) with the NS1 gene of an H5N7 subtype virus A/mallard/Denmark/64650/03.

Out of these 22 H7N3 isolates sequenced for the NS1 gene, 6 isolates from the Northern Parts of Pakistan were further sequenced for the HA and NA genes. One of the isolates had an untruncated NS1 whereas 5 were truncated. The 5 H7N3 isolates with truncated NS1 sequenced were HPAI, for the HA gene and showed the presence of typical highly pathogenic pattern of deduced amino acid sequence at the HA cleavage site. The

phylogenetic analysis of these H7N3 isolates indicated a close resemblance to other Pakistani isolate sequences in the GenBank, with the next closest resemblance to the H7N3 isolate from a Peregrine Falcon in U.A.E in the GenBank besides the other Pakistani isolates. The untruncated isolate for the NS1 gene, A/Chicken/Pakistan/NARC-1282/04, showed a typical low pathogenicity cleavage site sequence at the HA cleavage site. Phylogenetic Analysis of this isolate indicated a close resemblance to Italian H7N3 isolates especially A/Chicken/Italy/682/2003 (H7N3) and A/turkey/Italy/8535/2002 (H7N3). The NA gene was analyzed for the presence or absence of a stalk region in the isolates sequenced. The 5 truncated H7N3 isolates for the NS1 Gene and HP for HA gene had a stalked NA protein as in H7N3 isolates reported in wild birds showing a close resemblance to other previously sequenced H7N3 Pakistani isolate sequences in the GenBank, whereas the untruncated NS1 H7N3 isolate also showing a LPAI cleavage site sequence A/Chicken/Pakistan/NARC-1282/04 had a deleted NA stalk region, deduced amino acid sequence showing a deletion of 24 amino acids in concordance with other Italian H7N3 isolates reflecting a probable introduction of a highly circulating virus in domestic poultry. It was concluded from the present study that the H7N3 isolates from Pakistan show slow antigenic drift and continue to evolve in a slow manner during a ten year period in the poultry population. With information obtained from the data on NS1, HA1 and NA, continuous monitoring of circulating viruses is possible and subsequent production of homologous vaccines from field strains is key to the control of HPAI in poultry.

INTRODUCTION

INTRODUCTION

Influenza is an infection and/or disease syndrome caused by any type A influenza virus, a member of the Orthomyxoviridae family. Influenza A viruses are accountable for major disease problems in birds, as well as in humans and lower mammals (Easterday *et al.*, 1997).

Historical Aspects

The "fowl plague", now known to be caused by highly pathogenic avian influenza viruses, was first described in 1878 (Perroncito, 1878) as a disease affecting chickens in Italy. The causative agent was eventually isolated from a chicken in 1902 A/Chicken/Bresica/1902 (H7N7), marking the first recognized isolation of influenza virus. Similar outbreaks were observed in Europe and then all over the globe, with ensuing isolation of several fowl plague viruses (H7 subtypes). On the other hand, the first human influenza was not isolated until 1933 (Smith *et al.*, 1933). The propagation of the influenza virus in embryonated chicken eggs greatly improved the effectiveness of virus isolation, allowing the preparation of high titer virus stocks (Burnet, 1936).

In 1941, the hemagglutination activity of influenza virus was discovered (Hirst, 1941) and in 1955 it was verified that fowl plague virus was a member of the influenza A virus group (Schafer, 1955). The emergence of human pandemic strains in 1957 and in 1968 encouraged extensive study of the ecology of influenza viruses in animals as a mean of gaining insight into the origin of pandemic strains (Kaplan and Beveridge, 1972). During this surveillance, numerous nonpathogenic avian influenza A viruses were isolated from avian species, including wild birds, captive and caged birds, and domestic ducks, chickens and turkeys (Slemons, *et al.*, 1974; Hinshaw *et al.*, 1980). These isolations led to the comprehension that such viruses are widely distributed among birds and probably omnipresent in waterfowl. Highly pathogenic strains belonging to H5 subtypes were later found in chickens in Scotland [A/Chicken/Scotland/59 (H5N1)] and Terns [A/Tern/South Africa/61 (H5N3)] (Becker, 1966). These findings clarified the presence of two types of avian influenza A viruses based on their virulence: a highly pathogenic virulent type that causes fowl plagues and an avirulent type that causes only mild disease or asymptomatic

infection. In rare instances, however, viruses with low pathogenicity in the laboratory cause outbreaks of severe disease in the field. Such viruses are economically important and (Poss and Halvorson, 1987) includes H7 and H9 subtypes. However, the morbidity and mortality associated with these viruses tend to be much lower than those caused by deadly viruses (Horimoto and Kawaoka, 2001).

Structural Aspects of Avian Influenza Viruses

Antigenic differences based on nucleoprotein (NP) and matrix (M1) protein form the basis for the classification of influenza viruses into types A, B and C. Each type of avian influenza viruses have been categorized as type A. Two surface glycoproteins, Hemagglutinin (HA) and Neuraminidase (NA) are antigenic determinants which further subtype the avian influenza viruses (Murphy and Webster, 1996). These transmembrane glycoproteins are capable of eliciting subtype-specific and immune responses which are significantly protective within, but only partly protective across, different subtypes. On the basis of antigenicity of these glycoproteins, influenza A viruses at present group into sixteen HA (H1 – H16) and nine NA (N1 – N9) subtypes. This grouping comes into the spotlight when phylogenetically analyzing the nucleotide and deduced amino acid sequences of the HA and NA genes (Fouchier, *et al.*, 2005)

A similarity of structures is seen among influenza A viruses (Lamb and Krug, 1996). Electron microscopy has revealed that the viruses are pleomorphic, with virions that are roughly spherical in shape (approximately 120nm in diameter) and filamentous being also included in this category. Two different types of spikes (approximately 16nm in length), representing the HA and NA molecules, are a feature of the surface of the virions. The HA spike sticks out from the envelope as a trimer with a rod shape (Wilson *et al.*, 1981); the NA spike appears as a mushroom shaped tetramer (Colman *et al.*, 1987). Short sequences of hydrophobic amino acids (transmembrane region) anchor the two glycoproteins to the lipid envelope derived from the plasma membrane of host cells. HA is a type 1 glycoprotein (having an N-terminal ectodomain and a C-terminal anchor), whereas NA is a type II glycoprotein (having an N-proximal anchor and a C-terminal ectodomain). HA is responsible for the attachment of the virion to cell surface sialyl-

oligosaccharides (Paulson, 1985) and is the reason behind its hemagglutinating activity (Hirst, 1981). HA invokes the production of virus-neutralizing antibodies that are important in defense against infection. NA is an enzyme, sialidase (Gottschalk, 1957) that does not favour virion aggregation, infact prevents it, rendering the release of virions from the cell, by removing cell and virion surface sialic acid-the primary moiety in sialyloligosaccharides recognized by HA (Paulson, 1985). Antibodies to NA play an important role in protecting hosts (Webster *et al.*, 1988).

A limited number of M2 proteins are also an integral part of the virion envelope besides HA and NA (Zebedee and Lamb, 1988). These M2 proteins form tetramers, have H^+ ion channel activity, and, when activated by the low pH in endosomes, acidify the inside of the virion, resulting in its uncoating (Pinto *et al.*, 1992). Assembly and budding of virions is controlled by the M1 protein that lies within the envelope. The viral envelope encloses eight segments of single-stranded RNA molecules which are negative sense, or complementary to mRNA. These RNA segments are in association with Nucleocapsid Protein (NP) and three subunits of viral polymerase (PB1, PB2 and PA), which together constitute a ribonucleoprotein (RNP) complex that has a pivotal role in RNA replication and transcription.The NS2 (Non-structural 2) protein has been found out to be present internally in virions (Richardson and Akkina, 1991; Yasuda *et al.*, 1993), and is contemplated to play a role in the export of RNP from the nucleus (O'Neill *et al.*, 1998) via an interaction with M1 protein (Ward *et al.*, 1995). NS1 protein, the only non-structural protein of influenza A viruses, has manifold functions, which include regulation and control of splicing and nuclear export of cellular mRNA's as well as providing a stimulus for translation (Lamb and Krug, 1996). One of the prime functions of NS1 protein seems to be to offset the interferon activity of the host, as an NS1 knockout virus was functional although it grew with less vigour than the parent virus in interferon-nondefective cells (Garcia-Sastre *et al.*, 1998).

Replication of AIV

Receptor-mediated endocytosis is responsible for the entery of the virus into the cell after binding to sialic acid-containing receptors on the membrane surface. A conformational change in HA is elicited by low pH in the endosome (Bullough *et al.*, 1994), followed by an event involving membrane fusion between the viral envelope and the endosomal membrane. Within the endosome, the viral core is locally exposed to low pH by the M2 proton channel, resulting in the separation of M1 from RNP and leading to a release of RNP to the cytoplasm. Nuclear localization signals located within the RNP complex [PB1 (Nath and Nayak, 1990), PB2 (Mukaigawa and Nayak, 1991) and NP (Neumann *et al.*, 1997; Wang *et al.*, 1997)] result in the transportation of RNP to the nucleus. The mechanism of viral RNA transcription is exceptional. A viral endonuclease cleaves the 5' cap from cellular mRNAs and used as a primer for transcription by the viral transcriptase (Krug *et al.*, 1979; Plotch *et al.*, 1981). Transcription of six of eight RNA segments into mRNAs is carried out in a monocistronic mode resulting in subsequent translation into HA, NA, NP, PB1, PB2, and PA. On the other hand, splicing produces two mRNAs from 2 RNA segments by transcription of each of these segments (Lamb and Krug, 1996). In the case of both the M and NS genes, these mRNAs are translated in differential reading frames, resulting in the production of M1 and M2 proteins and NS1 and NS2 proteins, respectively. Free NP is believed to be the cause for a shift from mRNA synthesis to cRNA and vRNA synthesis (Shapiro and Krug, 1988). Those vRNAs which are freshly synthesized are encapsidated alongwith NP in the nucleus, functioning as templates for secondary transcription of viral mRNAs (Horimoto and Kawaoka, 2001). As infection proceeds to a late stage, the major translation products are M1, HA, and NA. Glycosyl moieties are attached to HA and NA in the rough endoplasmic reticulum, with further processing occurring in the Golgi complex. Glycosylated and processed HAs are then transported to the cell surface, where they amalgamate into the cell membrane and become integral parts of this outer most boundry. Nuclear localization inherent in M1 and NS2 proteins is vital for the migration of RNP out of the nucleus for assembly into descendant viral particles cytoplasmically (Martin and Helenius, 1991; O'Neill *et al.*, 1998). The RNP-M1 complex most likely interacts with M1 proteins that are linked with the plasma membrane and then buds outward through the cell membrane, encapsulating

itself within a bubble of membrane (envelope) studded with spikes HA and NA glycoproteins (Horimoto and Kawaoka, 2001).

Changes within Antigenic Constituents of AIVs

The antigenic capacity of influenza viruses changes slowly but surely by point mutation referred to as antigenic drift or radically by genetic reassortment referred to as antigenic shift (Murphy and Webster, 1996). Antigenic drift is the resultant of selective immunological pressure on HA and NA. Hence, vaccine strains need to be replaced every several years for human influenza viruses as their antigenicity changes. Antigenic drift has also been detected amid avian influenza viruses, but to a lesser degree than in human viruses (Austin and Webster, 1986; Kida *et al.*, 1987). This could be the result of limited immunological pressure in birds having a short life span. Antigenic shift is the resultant of either conduction of nonhuman influenza viruses to the human population or the mixing by reassortment of genes from two dissimilar influenza viruses that are occupying a single cell via a combined infection (Webster *et al.*, 1982). A theoretical calculation indicates that 256 diverse combinations of RNA can be fashioned from the shuffling of the eight diverse genomic segments of the virus. Genetic reassortment is a fact which is well recognized both in vitro and in vivo under laboratory conditions (Webster and Laver, 1975). More significantly obvious are mixed infections which occur relatively frequently in nature and can be a factor in leading towards genetic reassortment (Young and Palese, 1979; Bean *et al.*, 1980; Hinshaw *et al.*, 1980). Another mechanism by which Antigenic shift can occur is by the reemergence of highly circulating viruses. An example supporting this fact is of the H1N1 Russian influenza virus, which was a highly circulating virus during 1950's (Nakajima *et al.*, 1978, Scholtissek *et al.*, 1978), later reemerged in 1977 in the immunologically naïve human populations. These human populations had never been exposed to a virus of this particular subtype, especially the younger population, who had never been infected with H1N1 virus in the past (Kung *et al.*, 1978).

Reservoirs of Influenza A Viruses and their Evolutionary Pathways

All subtypes of influenza A viruses are found in aquatic bird populations, for the most part in various species of ducks, shore-birds, and gulls. Influenza A viruses have been isolated from a range of animals, including humans, pigs, horses, marine mammals, and various birds (Webster *et al.*, 1992). In the diverse varieties of aquatic fowl, influenza viruses have a preference for replication in the gut especially in the intestinal part, ensuing in excretion of high-titer viruses in the excrement. Thus, the water-oral route which is fecally contaminated plays a major role in viral spread among the waterfowl (Horimoto and Kawaoka, 2001). Seven host-specific phylogenetic lineages have come up for NP genes from various hosts occupying diverse niches in different geographical regions: two equine lineages known as old equine 1 and recent equine 2, one in gulls, one in North American birds, one in Eurasian birds (including avian-like swine virus), one in swine and one in humans (Gorman *et al.*, 1991). The human and classical swine viruses form a genetically connected "sister group" association probably reflecting the sharing of a common ancestor derived from an avian virus (Horimoto and Kawaoka, 2001). Eurasian and American sub-lineages of AIVs have been identified which reflect two widely separated geographical areas. This conclusion has been drawn by genetic studies of all the gene segments of AIVs (Donis *et al.*, 1989). The fact demonstrates that migratory birds with routes between Northern and Southern Hemispheres play a pivotal role in the transmission of avian influenza viruses. Influenza viruses in waterfowl appear to be approaching or to have reached a finest state of adaptation, in which amino acid changes provide no selective advantage (Gorman *et al.*, 1991). This interpretation together with the truth that waterfowl infected with influenza viruses rarely show disease signs, point to an undeniable fact that influenza viruses have achieved a state of evolutionary equilibrium in these birds, making them the eventual primary natural reservoir (Horimoto and Kawaoka, 2001).

Pathogenesis of Avian Influenza Viruses

Avian influenza viruses generate an assortment of syndromes in avian species, ranging from asymptomatic to mild upper respiratory tract disease to complete or partial loss of

egg production to speedily lethal systemic disease (Easterday *et al.*, 1997). A multitude of factors control severity of the infection including the virulence or disease causing ability of the virus, the immunological level and diet of the host, supplementary bacterial infections, and stress level of the host. Pathogenicity in avian species being the main determinant, the AIVs are classified as virulent (causing avian influenza) or avirulent (causing mild/asymptomatic infection). Isolations of most AIVs in the field show an avirulent nature, with practically no isolations of virulent viruses from apparently vigorous aquatic fowl species, exception being isolates collected from ducks or geese from nearby avian influenza outbreak areas (Kawaoka *et al.*, 1987; Rohm *et al.*, 1996). Excrement of infected birds harbours Influenza viruses where they are secreted from the intestinal tract (Webster *et al.*, 1978; Kida *et al.*, 1980). Transmissibility can be direct or indirect including aerosol contact and by handling of virus-infected materials. Fecal material of infected birds is the biggest source of contamination (e.g., feed, water, equipment and cages) as it is excreted in large quantities and contributes to spread of the virus. Waterborne spread of disease could present a mechanism for the year-to-year continuation of spread of avian influenza viruses in aquatic ecosystems harbouring waterfowl habitats. AIVs were readily isolated from Alaskan lakes that serve as breeding areas for migratory aquatic fowl (Ito *et al.*, 1995).

The characteristic signs and symptoms shown by poultry with a systemic infection with highly pathogenic avian influenza (HPAI) viruses include decreased egg production, respiratory distress, rales, extreme lacrimation, sinusitis, cyanosis of unfeathered skin (particularly the combs and wattles), edema of the head and face, ruffled feathers, diarrhea, and nervous system disorders. The number of presenting signs depends on the species and age of the bird, the strain of infective virus, and supplementary bacterial infections (Webster and Kawaoka, 1988; Easterday *et al.*, 1997).

Molecular Basis of Pathogenicity

The HA glycoprotein has a critical role in the polygenic virulence of AIV's (Garten and Klenk, 1999; Steinhauer, 1999). HA has a lead role in primarily initiating infection by attaching the virus to cell receptors and by promoting release of the viral RNP by the

process of membrane fusion (White *et al.*, 1982). Host cell proteases mediate the the posttranslational proteolytic activation of the precursor HA molecule (HA0) into HA1 and HA2 subunits by producing a fusogenic domain at the amino terminal end of HA2, which functions in the fusion between the viral envelope and the endosomal membrane (Klenk *et al.*, 1975; Lazarowitz and Chopin, 1975) and is necessary for infectivity potential because virus-cell fusion is mainly due the presence of free amino terminus of HA2 (Webster et al., 1992). Hence viral dissemination is attained throughout the host's body (Garten and Klenk, 1999). A very inadequate number of cell types are responsible for the necessary cleavage of HA in the case of avirulent AIV's with the subsequent result that such viruses cause only mild or asymptomatic infections. These infections are localized in the respiratory or intestinal tract, or both. On the other hand, the HAs of virulent avian viruses are cleaved in a wide category of variable host cells and hence are able to cause lethal systemic disease in poultry (Bosch *et al.*, 1979; Horimoto and Kawaoka, 2001).

Sequence Determinants for Hemagglutinin Cleavability and Virulence Potential

Two structural aspects of the HA protein establish HA cleavability: the particular sequence of amino acids at the HA cleavage site and the carbohydrate in the environs of the cleavage site. Comparison of amino acid sequences among naturally occurring avirulent and virulent avian influenza viruses have revealed that HAs with limited cleavability (avirulent type) typically have a single arginine (R) whereas those with high cleavability (virulent type) have multiple basic amino acid residues found immediately upstream of the cleavage site (Bosch *et al.*, 1981). The bulk of influenza A viruses have arginine (R) at the carboxyl terminus of HA1 and glycine (G) at the amino terminus of HA2, while some have lysine (K) at the former position (Kawaoka *et al.*, 1990; Gunther *et al.*, 1993). In H5 viruses, proline (P) and glutamine (Q), are always situated proximally upstream of the HA1 carboxyl terminus, hence showing conservation. Between, the Q and G residues lies an area designated the connecting peptide (-P-Q-X-...-X-R//G-, where // points to the cleavage site flanked by HA1 and HA2 and X is any nonbasic amino acid). The sequence in this region has varialble nature and number of amino acids contained, depending on the virus strain (Kawaoka *et al.*, 1984). Vulnerablity to cleavage

depends upon the interaction between cleavage site sequences and a carbohydrate side chain, in close proximity of the HA, in reference to omnipresent proteases. Contact of HA to proteases leading to cleavage is interfered by the presence of carbohydrate side chain (Desphande *et al.,* 1987; Kawaoka and Webster, 1988; Kawaoka and Webster, 1989).

The HA1 carboxyl terminus among H7 viruses has conserved residues immediately upstream of this terminus: -P-E-X-P-X-...-X-R//G- or P-E-P-S-X-...-X-R//G- motifs are usually found. The connecting peptide is formed of residues between the P or S and G. The majority of naturally occurring avirulent H7 viruses have a K-X-R motif. Amino acid sequence alignments of the connecting peptides from virulent viruses and avirulent mutants point to the fact that R-X-B-R is the minimal sequence requirement for H7 virulent-type HAs. Whether K at the fourth position upstream of the cleavage site is good enough for high HA cleavability depends on the HA. Avirulent mutants have K-K-K-E-K-R at the cleavage site (Vey *et al.,* 1992), while virulent avian influenza viruses have HA cleavage site sequences K-K-K-K-R or K-K-K-K-K-K-R (Rohm *et al.,* 1996). Acidic amino acids (E) at position -3 may negatively manipulate protease recognition and proteolytic catalysis (Horimoto and Kawaoka, 2001). The reverse genetics approach helped in proving the direct link between HA cleavability and virulence of AIVs, which resulted in the production of viruses with mutations at the HA cleavage site but otherwise matching genetic backgrounds. That is, a modification of just the HA cleavage site sequence from a virulent type (R-R-R-K-K-R) to a typical avirulent type (R-E-T-R) resulted in chief reduction of the disease causing ability (Horimoto and Kawaoka, 1994). Certain structural features limit the presence of multiple basic residues at the HA cleavage site and such viruses other than H5 and H7 are not very commonly observed (Horimoto and Kawaoka, 2001).

Specific Host Cell Proteases Responsible for Hemagglutinin Cleavage

Two categories of proteases emerge to be primarily responsible for HA cleavage (Klenk and Garten, 1994; Rott *et al.,* 1995). One includes enzymes able to cleave avirulent type HAs with just a single arginine at the HA cleavage site, as well as virulent-type HAs with

multiple basic residues at this site. Such proteases are frequently called trypsin-like enzymes. Exogenous proteases like trypsin have been reported to cause multiple replications of avirulent viruses and mammalian viruses (with the exception of H7N7 equine viruses) in cells growing in tissue culture (Klenk *et al.*, 1975; Tobita *et al.*, 1975). Embryonated chicken eggs are the perfect natural medium for the growth of all avian influenza viruses, where a prothrombin family component protease comparable to the blood-clotting factor Xa, carries out HA cleavage in allantoic fluid, allowing virus replication *in ovo* (Gotoh *et al.*, 1990).

A second group of omnipresent proteases cleave only virulent-type HAs with multiple basic residues at the cleavage site because virulent viruses can replicate in the majority of tissues and cells. Medial or trans-Golgi apparatus sequester this group of enzymes, being calcium dependent and with an acidic pH optimum (Walker *et al.*, 1992). Endoproteolysis is the major method by which a multitude of bioactive peptides and proteins are produced from large precursor molecules, a process that typically occurs at paired or multiple basic amino acids, carried out by mammalian subtilisn related endoproteases (Barr, 1991; Seidah and Chretien, 1997) like furin, the creation of the *fur* gene. Furin has been primarily identified as a cleavage enzyme for the HAs of virulent avian viruses, where furin identifies the B-X-B-R motif (Steineke-Grober *et al.*, 1992; Walker *et al.*, 1994). Its cleavage site is directly downstream of arginine at the B-X-X-R or B-X-X-X-B-R motif, depending on additional structural features of the substrates (Molloy *et al.*, 1992; Brennan and Nakayama, 1994). Another mammalian subtilisin-related endoprotease, PC6, which is found to be functionally active in many tissues and has a sequence obligation similar to that of furin, can also turn on virulent avian viruses, pointing towards the existence of multiple HA cleavage enzymes in mammals (Horimoto *et al.*, 1994; Horimoto and Kawaoka, 2001).

Host array limitation – A Polygenic Trait

Certain host range limitations are present, although interspecies conduction of influenza viruses has been demonstrated. This was firstly reported by Murphy *et al.*, in 1982 that avian influenza viruses do not replicate efficiently in human nor in non-human primates.

Hinshaw *et al.*, in 1983 reported that human influenza viruses do not have the tendency to successfully grow and thrive in ducks. Beare and Webster pointed out in 1991 that, avian influenza viruses do not replicate efficiently in humans.

Host cell recognition being a pivotal role of the HA glycoprotein hence HA is obviously a primary determinant of host range restriction. The property of receptor specificity in influenza viruses depends upon the host from which viral isolation has taken place. Human influenza viruses have a predilection for the recognition of sialyloligosaccharides ending in N-acetylsialic acid linked to galactose by the α2,6 linkage (NeuAcα2,6Gal), on the other hand avian and equine viruses recognize N-acetylsialic acid linked to galactose by the α2,3 linkage (NeuAcα2,3Gal). Such predilection for recognition has been observed as an actuality for humans (Rogers *et al.*, 1983; Rogers and D'Souza, 1989; Connor *et al.*, 1994; Suzuki, 1994; Gambaryan *et al.*, 1997; Matrosovich *et al.*, 1997). Viral replication sites with major sialic acid-galactose linkages of sialyloligosaccharides in epithelial cells are determined by host animal species. For example, the human tracheal epithelial cells majorly possess NeuAcα2,6Gal (Couceiro *et al.*, 1993). On the other hand those in the horse trachea and duck intestine (primary avian viruses replication areas) contain mainly NeuAcα2,3Gal linkages. Pig trachea has epithelial cells which contain both NeuAcα2,6Gal and NeuAcα2,3Gal linkages (Ito *et al.*, 1998), illuminating the high susceptibility of swine to both human and avian influenza viruses. Consequently, the host specificities of influenza viruses could be affected by the profusion of these two types of sialic acid-galactose linkages on cell surface sialyloligosaccharides (Horimoto and Kawaoka, 2001). Structure of the HA receptor-binding site determines such differences in specificity; for example, Leu-226 in human H3 viruses, instead of Gln-226 as in avian and horse viruses, bestows NeuAcα2,6Gal specificity (Rogers *et al.*, 1983; Naeve *et al.*, 1984). A variety of additional amino acids have also been considered as determinants of HA receptor specificity (Horimoto and Kawaoka, 2001).

Genetic studies of the HA gene of the pandemic 1968 Hong Kong virus indicated that it was derived from an avian virus (Scholtissek *et al.*, 1978). Analysis of the predicted amino acid sequence of the hypothetical precursor strain that immediately preceded the pandemic strain indicated that less than six amino acids in the HA changed during the

avian-human shift. Each of these amino acid changes modified the head portion of the HA molecule, as well as the area in the environs of the receptor-binding pocket (Bean *et al.*, 1992). Horimoto and Kawaoka suggested in 2001 that these mutations were a sign of adaptation to a novel host. On the other hand, they also suggested that the onset of antigenic drift was reflected since all of the changes occurred inside an antigenic site, preceding detection of the virus.

Hinshaw *et al.*, in 1983 highlighted the role of NA in host range discrimination, for example, a reassortant virus that possesses all duck virus genes apart from a human virus NA does not replicate in ducks. Efficient virus multiplication appears to be controlled by the substrate specificity of NA as is the case with HA. The significance of both glycoproteins was emphasized by Baum and Paulson in 1991 when they pointed out that NA of an N2 avian virus, while being highly specific for hydrolyzation of the NeuAcα2,3Gal linkage, attained NeuAcα2,6Gal specificity throughout its evolution in humans. This attainment matches up to the specificity of the human virus HA.

Internal proteins encoded by genes of influenza A viruses influence host specificity. Reassortant viruses having internal proteins encoded by avian virus genes, with all other genes reassorting from a human virus, demonstrate attenuated replication in squirrel monkeys in contrast with that of the parental human viruses (Snyder *et al.*, 1987; Treanor *et al.*, 1989). It had been previously reported by Scholtissek *et al.*, in 1978 that reassortant viruses having fowl plague virus HA and M genes and other genes from a human virus (together with NP) failed to multiply in chickens and in chicken embryo fibroblasts. They however replicated soundly in MDCK cells. Such informational data point towards the importance of NP and other internal proteins in host range restriction. Reassorted assemblage of the three polymerase genes (PA, PB1, PB2), occasionally also including NP, have been linked with attenuation of virulence or replicative potential in specific hosts (Rott *et al.*, 1979) for example, replacement of avian for human PB1 in a human virus slows down its multiplication in MDCK cells and squirrel monkeys but not in chicken kidney cells (Snyder *et al.*, 1987), while the PB2 gene of fowl plague virus seems to determine its capability to replicate in mammalian cell cultures (Almond, 1977; Israel, 1980). Likewise replacement of some avian NS genes (B allele) for human NS in a

human virus results in attenuation in squirrel monkeys (Treanor *et al.*, 1989). Hence all this information points to the fact that host range discrimination is a polygenic trait (Webster *et al.*, 1992).

Mutations – A Mechanism for the Emergence of Virulent Avian Influenza Viruses

The attainment of high HA cleavability is a vital occurrence in the conversion of avirulent avian influenza viruses to virulent strains. As only a few mutations are needed to change an avirulent phenotype to a more virulent strain, the most rational mechanism for this change is the introduction of mutations by error-prone RNA polymerase followed by the selection of viruses with highly cleavable HAs. This prospect was fruitfully tested in trypsin-free chicken embryo fibroblast cultures by Ohuchi *et al.*, in 1989 when they used an avirulent virus isolated during 1983 to 1984 Pennsylvania outbreak. All of the mutants studied had augmented numbers of basic amino acids at the HA cleavage site but not possess changes in the glycosylation site at Asn-11, distinguishing it from findings in virulent viruses produced naturally in the Pennsylvania outbreaks. Therefore, selective pressures working in cell culture environment vary from those in natural settings (Horimoto and Kawaoka, 2001). A clue to the mechanism of emergence of virulent avian viruses in nature was provided by the finding that virulent mutants can be readily selected in 14-day-old but not 10-day-old embryonated chicken eggs inoculated with the 1983 Pennsylvania avirulent isolate (Brugh and Beck, 1992; Horimoto and Kawaoka, 1995). Virulent variants are found out to be in the majority after only three passages of the avirulent virus in 14-day-old eggs, pointing towards a replicative benefit of virulent viruses in older eggs (Horimoto and Kawaoka, 1995). Sequencing of the virulent-type HA indicated towards a loss of the glycosylation site at Asn-11, being the identical mutation found in virulent Pennsylvania isolates. It was stated by Horimoto and Kawaoka in 2001 that this role of older eggs in selection of virulent mutants in nature was uncertain

Mechanism for acquirement of multiple basic amino acid residues at the HA cleavage site was postulated by Perdue et al., in 1997 who stated the mechanism as direct duplication of a fraction of the purine-rich region at the cleavage site. Furthermore RNA recombination ensuing in a large insertion of 28S host rRNA (Khatchikian *et al.*, 1989) or

a viral NP gene (Orlich *et al.*, 1994) sequence immediately upstream of the HA cleavage site leads to the production of a highly cleavable HA, inspite of the fact that equivalents of these mutants have yet to be recognized in nature (Horimoto and Kawaoka, 2001).

Phenomenon of Evolutionary Divergence among Influenza A Viruses

Webster *et al.*, in 1992 explained that evolutionary divergence of viruses was attributed to interspecies transmission, combined with isolation of host species, because of the separation of host-specific virus gene pools. Separation of progeny and parent virus gene pools was responsible for the independent evolution of host-specific strains, which is preserved by barriers to regular interspecies transmissions. These barriers might be in the shape of infrequent likelihood of transmission because of dissimilar ecologies of host species, a lack of infectivity potential of the virus in novel hosts, or interference from established viruses interceded by the host immunological status. Geographic separation of waterfowl populations by having different flyways and breeding and overwintering grounds results in the partitioning of avian influenza virus gene pools. The subdivision of host populations offers a great deal of heterogeneity to virus populations and boosts the conservation of a large number of virus subtypes.

Role of Immunological Selection Pressures in Evolutionary Constraints on Influenza Viruses

Gammelin *et al.*, in 1990 and Gorman *et al.*, in 1991 reported the differential evolution of each virus gene because of variable selective pressures and evolutionary restrictions. Genes that code for surface proteins (HA and NA) might be subject to strong selection pressure by neutralizing antibodies of host immune systems. Genes coding for internal proteins (e.g., NP) may not be subjected to strong host immune selection pressure but are thought to experience significant host-specific adaptive evolution. Internal protein genes such as those coding for polymerases (e.g., PB2) may have virus-specific functional constraints on evolution (Gorman *et al.*, 1990). Because of host immune selection pressure, surface proteins are expected to evolve more rapidly and to be replaced by reassortment more frequently. Reassortant viruses with new genes for surface proteins have a selective advantage over the parent virus to which the host has had considerable

antigenic contact the new viruses are able to escape (at least temporarily) the host immune response. If these new viruses are adequately infectious, they can produce pandemics and swap earlier strains, resulting in antigenic shifts (Webster and Laver, 1975). Therefore a long evolutionary history may not precede surface protein genes within hosts that make the virus undergo considerable immune selection pressure e.g., humans. Frequent reassortment is not expected of internal protein genes (e.g., the NP gene) that demonstrate a high degree of host-specific evolution and are the reason for virus attenuation when experimentally reassorted with a multitude of host-specific viruses (Tian *et al.*, 1985; Snyder *et al.*, 1987). However, internal protein genes (e.g., PB1 genes in human viruses) that evolve slowly and do not show a high degree of host-specific evolution might be replaced more repeatedly since new reassorted viruses are not liable to show any attenuation (Kawaoka *et al.*, 1989). Webster *et al.*, in 1992 commented on the conserved nature of these proteins, pointing out the case of PB1 genes, virus-specific constraints on the viral polymerases may avert any significant host-specific divergent evolution. Thus, the conserved nature of these proteins does not present a barrier to reassortment i.e., relative robustness of reassorted viruses is not lost in nature.

Major Outbreaks due to Low Pathogenicity Avian Influenza (LPAI) and Highly Pathogenic Avian Influenza (HPAI) Viruses

Avian influenza viruses of the H5 and H7 subtypes are the only known subtypes so far with the risk of becoming highly pathogenic in chickens and turkeys (Starick and Werner, 2003). Major outbreaks of H5 and H7 reported so far have been extensively reviewed in a number of publications. Following is an account of some major outbreaks of H7 subtype of AIV's.

From the end of March till the beginning of December 1999, an epidemic of low pathogenicity avian influenza (LPAI) affected the industrial poultry population of northern Italy. The virus accountable for the epidemic was subtyped as H7N1 with an intravenous pathogenicity index (IVPI) of 0.0 and a deduced amino acid sequence of the region coding for the cleavage site of HA molecule characteristic of low pathogenicity viruses. The circulation of the virus in a vulnerable population for several months caused

the emergence of a highly pathogenic virus with an IVPI of 3.0 and the presence of multiple basic amino acids in the deduced amino acid sequence at the HA cleavage site distressing over 13 million chickens and turkeys. Immense economic losses were caused by the epidemic (Capua *et al.*, 2000 a).

Capua *et al.*, in 2000 b reported an outbreak of HPAI in intensively farmed ostriches (*Struthio camelus*). Virus isolation yielded an influenza A virus of the H7N1 subtype with a deduced cleavage site motif containing multiple basic amino acids, typical of highly pathogenic viruses. The findings reported indicate that ostriches are susceptible to highly pathogenic avian influenza.

During the winter of 1979 to 1980, an H7N7 influenza virus had been related with a severe outbreak of pneumonia in the New England seal population (Geraci *et al.*, 1982). The H7N7 seal virus was shown to have taken up all of its genome RNA segments from avian influenza strains. Poor replication of the H7N7 seal virus was observed, if at all, in avian species and was not enterotropic in birds (Webster *et al.*, 1981 a). Nevertheless, this virus replicated quite well in a variety of mammals, including squirrel monkeys (Murphy *et al.*, 1983), and was responsible for conjunctivitis in humans (Webster *et al.*, 1981 b), signifying that the host range of H7N7 seal virus was not restricted to marine mammals (Hinshaw *et al.*, 1984).

In 2003, the Netherlands experienced an epizootic of H7N7 highly pathogenic avian influenza (HPAI) A, amid the poultry population in which commercial and pet poultry holdings were infected and millions of animals culled (Den Boer *et al.*, 2004). RT-PCR and virus culture in the laboratory detected H7 infection in humans (Fouchier *et al.*, 2004; Koopmans *et al.*, 2004). The majority of virus isolates obtained from humans had not collected noteworthy mutations except from a fatal case displaying 14 amino acid substitutions. Because H7N7 viruses have caused disease in mammals, including horses, seals, and humans, on numerous instances in the past, they may be bizarre in their zoonotic potential and, thus, pose a pandemic risk to humans (Fouchier *et al.*, 2004).

The H7N2 subtype of avian influenza virus (AIV) has been identified to be continually present within the live bird markets of the northeastern United States since 1994 and on

domestic poultry holdings of the region since 1995 (Panigraphy *et al.*, 2002). The H7N2 AIV has been responsible for several disease outbreaks in this region in recent years. Between 1996 and 1998, an H7N2 outbreak took place that affected layers, turkey flocks, quails flock and a mixed species flock in Pennsylvania (Henzler *et al.*, 2003). From December 2001 to summer of 2002, H7N2 outbreaks in Pennsylvania and Virginia affected broiler breeder flocks initially that afterwards infected commercial broiler flocks (Akey, 2003; Dunn *et al.*, 2003). All field isolates acquired during these H7N2 outbreaks were classified as LPAI viruses on the basis of pathogenicity tests and sequencing analysis of HA gene (Program Committee, 5th international symposium, 2003)

Outbreaks of H7N3 in Pakistan

Outbreak of 1995 and 2000

A highly pathogenic avian influenza virus subtype H7N3 was first diagnosed in Pakistan in 1995. The outbreak to begin with started in the cold northern section of the country where the migratory birds come in every year from China and Russia at the inception of the winter season. The disease affected largely a population of 3.2 million birds, chiefly breeders and broilers within a radius of 100 km. While typically breeder stocks are reared in this part of the country and they were the ones affected badly, some broiler and layer farms also got affected at some additional places. The harshness of the disease started going downhill within 5 months as it descended towards the southern parts of the country. A mass vaccination as well as biosecurity program was initiated in and around the affected area, which ultimately helped to overcome the disease syndrome. Molecular characterization of the field isolates for HA cleavage site motif indicated some changes in the sequence over a period of 4 months. All of the isolations from the field were typed as H7N3 subtype. Sequencing analysis of the field isolates for the HA cleavage site indicated a highly pathogenic pattern for H7 viruses. Epidemiological inquiry pointed towards the information that the virus may have been introduced earlier in the flock through wild birds, such as waterfowl, which habitually migrate to the areas in the months of October-November from the cold parts of Russia. The subsequent outbreak in other flocks might have spread afterwards by flock movement and negligible biosecurity

measures. It was further stated that due to known potential of H5 and H7 virus for point mutations to enhance pathogenicity, it was possible that a virus of H7 subtype with low virulence subsisted previously in the affected area which finally reverted and caused the outbreak (Naeem, 1997).

The outbreak of H7N3 in 2000 was by a LPAI virus subtype H7N3 in the northern areas of the country, primarily affecting broiler-breeders. The disease stayed endemic and was controlled by the introduction of rigorous bio-security measures along with the use of ring vaccination around the affected areas. This helped in the management and control of avian influenza in the affected areas (Naeem and Siddique, 2006).

Epidemic of 2003-2004

Throughout the year 2003, Pakistan was highly affected by a devastating epidemic of HPAI, caused by H7N3 that was a product of mutation of an LPAI virus of the same subtype. The LPAI virus was initially isolated in the southern parts of Pakistan in April 2003, in the coastal town of Karachi, which is the prime area for rearing of some 70% of the country's commercial layers. The initially affected flocks by LPAI of subtype H7N3 showed a 70% decline in production and 20% mortality. The LPAI virus was continually isolated from ten other flocks in the environs but no controlling measures could be implemented as the virus did not meet the criteria under the international standards of controllable avian influenza (Naeem and Siddique, 2006)

In November 2003, the HPAI subtype H7N3 materialized in Karachi area, with an abrupt boost in mortality at some farms. The virus was established as HPAI with an IVPI index of 2.8. At some places, LPAI virus subtype H9N2 was also isolated from the same flocks. The H7N3 subtype was found to affect commercial layers particularly since no outbreak was recorded from broiler flocks raised in the close vicinity. During the next four weeks, the disease had spread all the way through to the commercial layer estates inside a radius of 80 km. This ended in very heavy mortality among the affected flocks ranging from 70-80%. A disease control strategy was developed which included depopulation, vaccination and setting up of a national AIV surveillance program (Naeem and Siddique, 2006).

AIV Genome

The viral genome is composed of eight segments of single-stranded, negative sense (complementary to mRNA) RNA molecules (Easterday *et al.*, 1997; Horimoto and Kawaoka, 2001). Those eight segments (HA, NA, NP, M1, M2, PB1, PB2 and PA) code for 10 viral proteins, eight of which are constituents of the virions (Easterday *et al.*, 1997), whereas a ninth one NS2 is also now known to exist in virions in association with M1 (Richardson and Akkina, 1991; Yasuda *et al.*, 1993; Horimoto and Kawaoka, 2001). NS1 is the only non-structural protein of influenza A viruses.

RNA genome segments and coding assignments are as follows (CSIRO, Diagnostic Manual):

(1) Segment 1, **PB2** = Polymerase Basic protein 2 (2341bp)

(2) Segment 2, **PB1** = Polymerase Basic protein 1 (2341bp)

(3) Segment 3, **PA** = Polymerase Acid protein (2233bp)

(4) Segment 4, **HA** = Hemagglutinin (1778bp)

(5) Segment 5, **NP** = Nucleoprotein (1565bp)

(6) Segment 6, **NA** = Neuraminidase (1413bp)

(7) Segment 7, **MA** = Matrixprotein M1+M2 (1027bp)

(8) Segment 8, **NS** = Nonstructural protein NS1+NS2 (890bp)

PB1, **PB2** and **PA** are the three subunits of viral polymerase which in conjunction with NP participate in RNA replication and transcription (Easterday *et al.*, 1997; Horimoto and Kawaoka, 2001)

HA enables the virion to attach to host cell surface sialyloligosaccharides (Paulson, 1985) and is accountable for its hemagglutinating activity (Hirst, 1941). HA is responsible for

eliciting the production of virus neutralizing antibodies that are important in protection against infection (Easterday *et al.*, 1997)

NP is contained within the viral envelope in association with the eight RNA segments of the virus and is a part of the ribonucleoprotein (RNP) complex that participates in RNA replication and transcription (Horimoto and Kawaoka, 2001)

NA is a sialidase (Gottschalk, 1957) and it prevents virion aggregation by removing cell and virion surface sialic acid (Paulson, 1985). Antibodies to NA are also important in protecting hosts (Webster *et al.*, 1988)

M1 protein in a major structural protein underlying the viral envelope, which surrounds the RNA molecules in association with the NP and three large proteins (PB1, PB2, and PA) of polymerase (Easterday *et al.*, 1997). M1 protein is thought to have a major role in assembly and budding (Horimoto and Kawaoka, 2001)

M2 proteins are incorporated into virions where they form tetramers with H^+ ion channel activity and make possible the uncoating of virions in host cell endosomes (Zebedee and Lamb, 1988; Pinto *et al.*, 1992)

NS1 is the only non-structural protein of influenza A viruses. **NS2** is also now known to exist in virions in association with M1 (Richardson and Akkina, 1991; Yasuda *et al.*, 1993; Horimoto and Kawaoka, 2001).

NS1 Gene of Avian Influenza Viruses in Detail

Replication of viruses can stimulate fundamental changes in the metabolism of the infected host cell. Present day molecular biological techniques have facilitated the examination of the replication cycle of viruses with the recognition and study of viral gene products which alter and affect cellular functions (Knipe, 1996). A well studied example is the NS1 protein of influenza A viruses. The NS1 protein is the only nonstructural protein of the virus and is plentifully expressed in infected cells (Lazarowitz *et al.*, 1971) but it has not been identified and detected in virions (Krug and Etkind, 1973). It is a multifunctional protein, normally possessing 230 amino acid

residues (Krug *et al.*, 2003). More than a few regulatory roles of the NS1 protein have been recognized. The NS1 protein manipulates a multitude of steps regarding the phenomenon of gene expression, including pre-mRNA splicing (Fortes *et al.*, 1994; Lu *et al.*, 1994), nucleocytoplasmic transport of poly A RNA (Fortes *et al.*, 1994; Qiu and Krug, 1994), and translation (Enami *et al.*, 1994; De La Luna *et al.*, 1995). Besides these facts, it was recently revealed that NS1 can obstruct the activation of the double-stranded RNA-activated protein kinase (PKR), most probably due to it double-stranded RNA binding activity. The activation of PKR results in a downregulation of translation and is a component of the cellular antiviral defense system. The NS1 protein may offset this cellular response in order to synthesize high levels of viral proteins in the infected cell (Lu *et al.*, 1995).

The phylogenetic relationships of the NS genes have exposed two different gene lineages, referred to as groups or alleles A and B. AIVs as well as mammalian influenza viruses fall into the A group, while the B group contains practically all AIV strains (Treanor *et al.*, 1989; Ludwig *et al.*, 1991; Suarez and Perdue, 1999). The NS1 protein is synthesized at the primary stage in an infection and has been implicated in inhibition and resultant downfall of the host antiviral defense mediated by alpha and beta interferons (Garcia-Sastre *et al.*, 1998). The interferon antagonist properties of NS1 protein depend on its capability to bind double-stranded RNA, a known strong inducer of interferon. Selected amino acid residues within the NS1 protein take part in a vital role in the ability of this protein to bind to double-stranded RNA, and mutations of only two amino acids amongst these selected ones can result in an attenuated virus (Donelan *et al.*, 2003). This might partly explain the high degree of NS1 conservation among influenza A virus strains (Buonagurio *et al.*, 1986; Ludwig *et al.*, 1991; Suarez and Perdue, 1999) and provides an antigenic marker for influenza virus infections (Birch-Machin *et al.*, 1997; Ozaki *et al.*, 2001). The NS1 protein, double stranded RNA binding property and ensuing prevention of synthesis of INF-α/β has been mapped to the amino-terminal domain of the NS1 protein (Wang *et al.*, 2000). The RNA-binding actions of NS1 are based on the interaction of two functional domains: an RNA-binding domain at the amino end of the protein (amino acids 19-38) that binds to poly A sequences in mRNAs (Qiu and Krug, 1994; Suarez and Perdue, 1998) and an effector domain (amino acids 134 to 161) that

interacts with cellular proteins to stop the nuclear export of mRNA (Qian *et al.*, 1994). These domains are extremely conserved within the NS1 gene (Wang and Krug, 1996; Kawaoka *et al.*, 1998), suggestive of a high level of evolutionary conservation of NS1. Besides that, the carboxy-terminal domain of the NS1 also contributes to its IFN-antagonistic properties, probably by enhancing NS1 stability and dimerization (Nemeroff *et al.*, 1995; Wang *et al.*, 2002) and by attenuating the gene expression in host cells (Noah *et al.*, 2003). The interaction of the influenza virus NS1 protein with the antiviral immune defense of the cell is expected to play a primary role in the virulence potential of the virus and also most likely to regulate viral mutiplication in the host.

NS1 Gene of AIV H7 Subtype

Dundon *et al.*, in 2006 reported the sequencing and phylogenetic analysis of the non-structural gene 1 (NS1) of 40 influenza A viruses isolated from a range of avian populations. The isolates were both LPAI and HPAI viruses of the H7 subtype and were the strains which represented avian influenza viruses that had circulated in northern Italy from 1999 to 2003 spanning a period of four years. The predicted amino acid sequences showed a size variation in the protein. Each NS1 was discovered with two different levels of carboxy-terminal truncation. Of the 40 isolates analysed 16 had a full-length NS1 protein of 230 amino acids, 6 had a truncated protein of 220 amino acids and 18 had an intermediate level of truncation resulting in a protein of 224 amino acids. The entire isolated H7N1 HPAI population possessed the intermediate carboxy-terminal truncation and a novel isoleucine residue at position 136. Besides this fact, the entire H7N1 LPAI viruses circulating at the start of the epidemic had a full length NS1 whereas those circulating near the end of the epidemic period had a truncated protein (Dundon *et al.*, 2006). It was hence reported that in the case of widespread viral circulation in poultry, the NS1 protein is not as conserved as was initially believed. Variation in NS1 sequence length has been formerly reported in influenza A viruses isolated from birds, pigs, horses and humans (Suarez and Perdue, 1999). In the afore mentioned study the NS1 protein of 3 out of 65 avian influenza isolates was truncated. Two of these isolates had a predicted NS1 protein of 217 amino acids while one had a truncated protein of 124 amino acids. A more recent report by Guan *et al.*, 1999 identified a comparable 13 amino acid truncated

NS1 protein in five out of fourteen avian influenza H9N2 isolates analyzed. In addition, 19 influenza isolates of the H9N2 subtype from Southern China have the 13 amino acid carboxy-terminal truncation. These data verify the fact that truncation in the NS1 protein is found in nature and is a renowned occurrence. The Italian epidemic reported beforehand showed the truncations to be only identified in the H7N1 isolates and not in H7N3 isolates (Dundon *et al.*, 2006).

HA Gene of Avian Influenza Viruses

Virulent viruses found in nature contain multiple basic amino acid motifs at the HA cleavage site, while most avirulent viruses do not (Bosch *et al.*, 1979; Kawaoka *et al.*, 1987; Vey *et al.*, 1992; Wood *et al.*, 1993). The presence of basic residues (B) at the HA cleavage site e.g. B-X-B-R, B(X)-X(B)-B-X-B-R, or B-B-B-B-X-R (where B is a basic and X is a non-basic residue) for virulent H5 strains; presence of basic residues (B) as in R-X-B-R at the cleavage site for virulent H7 strains; R-E-T-R cleavage site sequence for avirulent H5 strains; and P-K-G-R for avirulent H7 strains have been identified by sequencing. Therefore meticulous attention must be paid to avirulent viruses with multiple basic residues at the HA cleavage site (Kawaoka *et al.*, 1984).

NA Gene of Avian Influenza Viruses

Adaptation of wild avian strains to land-based domestic or backyard poultry has been credited to the role played by the NA protein (Matrosovich *et al.*, 1999; Banks *et al.*, 2001; Campitelli *et al.*, 2004). Work by Campitelli *et al.*, 2004 accounted for an NA stalk deletion in the NAs of avian viruses isolated from terrestrial and raised aquatic birds which included chickens, turkeys, quail, pheasants, *teal* and *chukar*, and the important point stressed was that this deletion was never observed in wild avian strains. The point emphasized in the study was that the observation of a lack of a long stretch of amino acids in the stalk of NA protein was a trait associated with early adaptation of wild avian viruses to farm based poultry such as turkeys and chickens. Earlier studies have established the fact that viruses with NA stalk deletions are inclined to compensate for their low NA activity potential (that negatively influence the budding of progeny virions from infected cells) by the mechanisms of restoration of NA stalk by RNA-RNA

recombination or by lessening the HA binding affinity to sialic acid by increasing the glycosylation of the HA globular head (Mitnaul *et al.*, 2000; Baigent and McCauley, 2001; Campitelli *et al.*, 2004). This prototype of increased glycosylation seems to be working in nature also as several chicken and turkey viruses with NA stalk deletions have additional probable CHO sites on their HA1 subunits. Hence work by Campitelli *et al.*, 2004 indicates that NA stalk deletion is likely to be vital for efficient virus multiplication in the poultry host, even though its functional relevance remains obscure and mutations in the affinity of HA for virus receptors may arise afterwards to compensate the reduced NA activity.

Diagnosis of Avian Influenza Viruses

Avian influenza is diagnosed by either virus isolation and identification or serological methods.Virus isolation (VI) in embryonating chicken eggs and ensuing HA and neuraminidase subtyping by serological methods constitute the gold standard for AIV detection and subtype identification (Spackman *et al.*, 2002). After isolation of any AIV it is mandatory to characterise the pathogenicity potential of the isolate. Two different methods are prescribed by the European Economic Community; the determination of the intravenous pathogenicity index (IVPI) in 6-wk-old chickens and nucleotide sequencing of the region encoding the cleavage site of the hemagglutinin (HA) gene (CEC, Council Directive, 1992).

Over the last decade, PCR has allowed the speedy and precise detection of numerous pathogens, even at low pathogen counts. The swiftness of this molecular diagnostic tool has been shown to be clinically helpful for both making a positive diagnosis and ruling out infection (Jeffery *et al.*, 1997). The reverse transcriptase PCR (RT-PCR) technique has been successfully employed to identify influenza viruses in throat or nasal specimens collected from humans and pigs (Yamada *et al.*, 1991; Claas *et al.*, 1993; Schorr *et al.*, 1994). This molecular diagnostic tool is not only fast but also more sensitive and specific than conventional laboratory procedures. Standard RT-PCR has been previously applied for the detection of avian influenza virus (Suarez, 1997; Starick *et al.*, 2000; Lee *et al.*,

2001; Munch *et al.*, 2001) and each of the hemagglutinin subtypes (Lee *et al.*, 2001; Munch *et al.*, 2001).

Molecular Diagnostics of Avian Influenza Viruses: Differential Diagnosis - Multiplex RT-PCR (mRT-PCR)

Apart from specific diagnosis of AIV, it is essential to differentiate it from other closely related diseases to avoid delayed virus isolation and typing procedures. In developed countries it is essential to look for some other diseases suspected in the field, while attempting to find AIV infection. In this regard multiplex RT-PCR/PCR has been developed by various groups.

Diagnosis of influenza A virus infection is routinely done by the isolation and identification of the virus. Serotyping is required to differentiate the subtypes of the AI viruses and is laborious and time-consuming. Although single band PCR has been used to detect and differentiate different subtypes, it only recognizes one specific subtype at a time (Lee *et al.*, 2001). Standard RT-PCR has been previously applied to the detection of avian influenza virus (Starick *et al.*, 2000; Munch *et al.*, 2001) and each of the HA subtypes (Lee *et al.*, 2001). In addition, real-time RT-PCR assays for influenza virus have been developed for the detection of influenza virus types A and B (Van Elden *et al.*, 2001) and differentiation of two subtypes H5 and H7 (Spackman *et al.*, 2002). Equipment costs and specific technical training requirements limit usefulness of these assays as routine laboratory tests. Hence Xie *et al.*, in 2006 developed a specific and sensitive multiplex RT-PCR that could simultaneously detect and differentiate the three most important subtypes of avian influenza viruses.

Choi *et al.*, in 2002, developed a multiplex reverse-transcription-polymerase chain reaction assay to detect and identify two subtypes of HA, which were H1 and H3 swine influenza virus (SIV). Two oligonucleotide primer sets were synthesized using published primer sequence data for H1N1 and H3N2. The unique PCR products of each subtype were sequenced and the sequences were confirmed to be subtype specific for the HA gene 1 or 3. The multiplex RT-PCR was applied to 30 SIV isolates subtyped by hemagglutination inhibition (HI) test. Forty-three positive and 20 negative swine field

samples for SIV by virus isolation were also tested. Of these 73 SIV-positive samples tested, H1 and H3 were identified in 38 and 28 samples by the multiplex RT-PCR, respectively. The remaining 7 samples were positive for both H1 and H3 genes. No positive reaction was found for the 20 SIV-negative field samples. Afterward, 235 random field samples from pigs with respiratory distress symptoms were tested by the multiplex RT-PCR, and 26 and 13 samples were found to be positive for H1 and H3, respectively. Results of this multiplex RT-PCR were analogous with those of the HI test. These results advocate the fact that multiplex RT-PCR can be a useful diagnostic test at the molecular level for speedy detection and subtyping of SIV in clinical samples.

Boivin *et al.*, in 2004 used the technique of multiplex real-time PCR for the detection of influenza viruses A and B and the human respiratory syncytial virus (HRSV). Detection of each viral product and of an internal control was based on determination of specific melting temperatures by LightCycler software. The lower limit of detection in the multiplex PCR assay was found to be 50 copies for each viral target. In an evaluation of nasopharyngeal samples collected from hospitalized children with acute respiratory tract infections during the winter of 2001 and 2002, a viral pathogen was detected by the multiplex PCR test in 139 (66.8%) of 208 cases, including 45 (21.6%) influenza A virus infections, no (0%) influenza B virus infections, 106 (51%) HRSV infections, and 12 (5.8%) coinfections. The mutiplex PCR test was compared to rapid antigen detection assays for influenza viruses A and B and HRSV. The sensitivity and specificity of multiplex PCR assays for influenza A virus were respectively100 and 97.7% compared to 43.6 and 98.5% for the antigenic test. Similar was the case with HRSV multiplex assay and its antigenic test. So the multiplex assay combined both rapidity and sensitivity for the detection of the most important respiratory viral pathogens in children.

PCR assays have been developed for many respiratory viruses, allowing detection of small amounts of viral nucleic acid in clinical samples. In the so-called "multiplex" format, PCR assays have been designed to amplify more than one respiratory viral target in the same PCR test (Ellis *et al.*, 1997; Fan *et al.*, 1998; Osiowy, 1998; Grondahl *et al.*, 1999; Liolios *et al.*, 2001). However, most multiplex PCR assays reported require separate steps for the amplification and detection of viral genes, which greatly increases

the assay's turnaround time and the risk of amplicon contamination. Real-time RT-PCR's (which use TaqMan, LightCycler and SmartCycler chemistries) for the detection of influenza viruses in clinical samples have been described (Schweiger *et al.*, 2000; van Elden *et al.*, 2001; Smith *et al.*, 2002; Habib-Bein *et al.*, 2003). These assays use a fluorescent probe for simultaneous amplification and detection of the PCR products (Hindiyeh *et al.*, 2005).

Hindiyeh *et al.*, in 2005 modified a multiplex real-time reverse transcriptase PCR assay (using TaqMan chemistry) and carried out its evaluation and its ability to detect and at the same time differentiate influenza A and B in influenza patients during the 2001-2002 influenza season in Israel. A comparative detection of the TaqMan assay was carried out with those of a multiplex one-step RT-PCR involving gel detection, a shell vial immunofluorescence assay and virus isolation using tissue culture. Sensitivity and specificity of the TaqMan assay was found out to be high for the detection of influenza viruses compared to that of tissue culture. This assay had a faster turnaround time (4.5h) in relation to it's performance and a low cost making it a routine method in place of tissue culture.

Optimization of a mRT-PCR for the detection of influenza A virus and the H5 and H9 subtypes was carried out by Saberfar *et al.*, in 2007. Conserved regions of the matrix gene were targeted using influenza type A specific primers. Hemagglutinin (HA) gene regions which were highly conserved among H5 and H9 subtypes were also amplified specifically. Hence a multiplex format detected these three gene regions simultaneously using the selected primers sets. Cloacal and tracheal swabs of poultry with influenza symptoms were used indicating the usefulness of the mRT PCR assay as a useful diagnostic tool for the subtyping of AIV in clinical cases.

Xie *et al.*, 2006 optimized a mRT-PCR for the detection of type A influenza virus alongwith avian H5, H7 and H9 hemagglutinin subtypes. They used specific primers for these genes. The mRT-PCR products were visualized using Agarose gel electrophoresis and the product sizes were 860 bp for H5, 634 bp for H7, 488 bp for H9 hemagglutinin subtypes. The product size for influenza A was 244 bp. The assay was found out to be

sensitive and specific for the detection of influenza A and the three hemagglutinin subtypes. The detection limit for the PCR amplified DNA products was 100 pg for the subtypes H5, H7 and H9 and 10 pg for type A influenza virus.

A new avenue to rapid detection of important poutry respiratory pathogens in one reaction was opened by Chen *et al.*, in 2008 when they described a mRT-PCR assay for the detection of AIV subtype H5 and H9 alongwith the respiratory pathogen NDV. Three sets of specific primers were applied in the assay based on the sequences of the hemagglutinin gene of H5-AIV, H9-AIV and fusion protein gene of NDV. Clinical samples were throat washes, oral swabs and cloacal scrapings which were detected by mRT-PCR and single RT-PCR (sRT-PCR). The results indicated that the sensitivity and specifity of mRT-PCR were in accordance with sRT-PCR.

Molecular Characterization of H7 subtype of AIVs

DNA sequencing reflects the most important and the well used technique by which the precise order of nucleotides in a piece of DNA can be determined. DNA sequencing methods have continuously been developed for a number of years resulting in the simultaneous development of two different techniques – the chain termination method by F.Sanger and A. R. Coulson in the UK, and the chemical degradation method by A. Maxam and W. Gilbert in the USA. The two techniques are quite different but carry equal weightage in scientific research. Both allow DNA sequences of several kilobases in length to be determined in the minimum of time (Brown, 2001).

The sequences of H7 avian influenza viruses involved in three different outbreaks in commercial poultry in the United States were analysed by Spackman *et al.*, in 2003. Molecular characterization of hemagglutinin (HA) and neuraminidase (NA) genes of H7 avian influenza virus (AIV) isolated between 1994 and 2002 from live-bird markets (LBMs) in the northeastern United States and from three outbreaks in commercial poultry was carried out. A close phylogenetic relationship was observed between the HA and NA genes of the isolates from commercial poultry with those circulating in the LBMs. AIV lineage, since 1994, indicated striking features in the genes studied in the form of a deletion of 17 amino acids and a deletion in HA1 protein in a part of the receptor binding

region. In poultry, the NA stalk deletion in AIV's had previously been observed with different lengths and positions, but the HA1 stalk deletion was a unique occurance in the HA gene of this particular lineage (Zhou *et al.*, 1999; Banks *et al.*, 2001). It has been shown that the enzymatic potential of the NA protein is adversely affected by the deletions in the NA stalk and it is presumed that the spread of the virus to naïve uninfected cells is affected in an adverse manner (Luo *et al.*, 1993). The receptor binding affinity of the HA gene has been described to be decreased as a compensatory change due to a change in the glycosylation pattern to a higher level near the receptor binding site (Matrosovich *et al.*, 1999; Wagner *et al.*, 2000). Only the current lineage of AIVs in the LBMs possessed the eight-amino acid deletion representing the putative receptor binding site (Suarez *et al.*, 1999) with a basis in the structure of the H3 HA gene (Weiss *et al.*, 1988) and the HA gene may have this mutation as a compensation for the deletion in the NA stalk (Spackman *et al.*, 2003). A progression towards a more highly pathogenic cleavage site sequence was observed at the HA cleavage site, with the cleavage site being an indicator of pathogenicity in chickens and turkeys (Suarez *et al.*, 1999).

Pasick *et al.*, in 2005 reported a highly pathogenic avian influenza outbreak in British Columbia where subsequent investigations pointed towards a HPAI H7N3 virus as the causative agent of the outbreak, emerging from a LPAI predecessor It was important to analyse the HA genes of the LPAI and HPAI viruses from the affected farm. It was revealed after the sequencing of the HA genes, that the only difference between the two types of viruses was a 21 nucleotide insert at the HA cleavage site of the HPAI virus. The deductions were that the insert most probably arose as a result of non-homologous recombination between the HA and matrix (M) genes of the same virus. From the total outbreak, 37 viruses were reported to have inserts and 3 were without them. It was presumed that the reported events had great similarity with the Chilean outbreak of 2002 where there was a virulence shift of another H7N3 virus attributed to the intersegmental recombination between HA and NP genes.

The two most important mechanisms of genetic and phenotypic variation of influenza A viruses are high mutation rates and genetic reassortment among different viruses, the causes of antigenic drift and antigenic shift respectively. Non-homologous recombination

is hardly ever reported (Fields and Winter, 1982; Khatchikian *et al.*, 1989; Orlich *et al.*, 1990; Bergman *et al.*, 1992; Orlich *et al.*, 1994; Suarez *et al.*, 2004; Pasick *et al.*, 2005).

Fouchier *et al.*, in 2005 identified a novel hemagglutinin subtype in black headed gulls. Influenza A viruses carrying 15 antigenic subtypes of hemagglutinin (HA) and 9 antigenic subtypes of neuraminidase (NA) have been described for wild aquatic birds and in the poultry population of the world. In Sweden a new and unidentified HA antigenic subtype (H16) was detected among black-headed gull population. No agreement was found for the criteria to detect antigenic subtypes by the hemagglutinin inhibiton and immunodiffusion assays and these tests failed to detect the reaction between H16 and the well known subtypes H1 to H15. It was further found that the H16 HA was genetically distantly related to H13 HA, which was an AIV subtype exclusively of shorebirds. The receptor binding region of H13 and H16 HA's had an amino acid sequence which was quite distinct from all the HA subtypes in circulation among ducks and geese. A similarity between the NA genes of these H16 viruses was detected with Eurasian shorebirds but a genetic distinction was found from the N3 genes of other birds of variable geographical zones. Other genes, like PB2, NP and NS were distinct from other influenza A viruses of these gull viruses of Europe. More information on the whole spectrum of avian influenza A viruses and the creation of diagnostic agents were required for the control of an outbreak and general surveillance of the disease for animal and public health. Fouchier *et al.*, 2005 stressed the need for the sequencing of HA and NA genes of influenza A viruses as a rapid identification measure for new and already existing subtypes.

Phylogenetic Analysis/Characterization

Campitelli *et al.*, in 2004, highlighted the great ecological niche of the domestic birds in the ecology of AIV subtype H7N3, by studying the homology in the sequences of seven out of 8 genes among domestic and wild bird population. The study found out that domestic species, like chickens and turkeys harboring and maintaining several influenza serotypes, where the source of these AIVs is the wild bird reservoir. Hence the study was conducted to identify the direct precursor of AIVs in the domestic bird species and for

that purpose the molecular characterization of the surface receptor proteins of H7N3 viruses from wild ducks in Italy in 2001 was carried out and it was compared with that of circulating AIV H7N3 in domestic turkeys in 2002 – 2003. A strict relation was found at the phenotypic and genetic levels in the AIV strains of both wild and domestic bird species. Seven of the genes studied had homology percentages of 99.8% for PB2 and 99.1% for the M gene. A 23 amino acid deletion was observed at the deduced amino acid level for the NA gene at the NA stalk level. Rest of the molecule showed a 99% similarity in both the virus groups studied. Such findings indicated that AIVs circulating in the wild waterfowl were precursors of the H7N3 strains circulating in the domestic turkey population thus providing information towards comprehending the mechanisms of interspecies transmission and the emergence of pathogenic strains having a pandemic potential.

Evolutionary relationships amongst taxa are represented hypothetically by Phylogenetic trees. Taxa connected by the shortest mutational path will provide information about a hypothetical evolutionary pathway and phylogenetic analysis provides that information. In a phylogenetic analysis no other assumptions are made based on historical evidence, no indication of dates of isolation or measures relating to similarities at the antigenic and molecular levels (Webster *et al.*, 1992).

Evolutionarily speaking, mammalian influenza viruses currently circulating, point towards influenza RNA genome having an avian origin in some of the RNA segments. Genetic and phylogenetic analyses have finally proven that the mammalian gene pool in past centuries has genes of avian origin indicating the effectiveness of such analyses (Webster *et al.*, 1992). In order to get the total history of virus evolution, full viral gene phylogenies provide all the information, whereas, partial information is obtained when a single gene phylogeny is traced. Information from the branching pattern of phylogenetic trees among influenza viruses indicates the reassortment of avian with non avian viruses as an event occurring repeatedly over the evolutionary history of influenza viruses. Gene lineages having different positions relative to each other in the case of nonavian viruses indicate the different relative ages of the genes of host specific viruses. Hence, probably the phenomenon of extinction of some strains or their genes has resulted in the

introduction of new reassorted viruses. Ultimately, phylogenetic analyses give information about how avian viruses as a whole can be transmitted to naïve hosts, how endemic strains are displaced and independent evolution from avian ancestors (Gorman *et al.*, 1990; 1991).

Information about the origin of specific strains of avian influenza viruses, and the epidemiology and spread of the disease is provided by Phylogenetic analysis. In a brief report Banks *et al.*, in 2000 reported the phylogenetic analysis and comparisons of H7 viruses belonging to specific continents at specific nucleotide regions with the Genbank. A 945bp nucleotide region, spanning bases 76 to 1020 was sequenced for the HA1 part of the HA gene for 31 influenza viruses of the H7 subtype isolated primarily from Europe, Asia and Australia over a period of 20 years. The sequences of the HA1 obtained were analyzed phylogenetically and compared with sequences of the same region from 23 H7 subtype viruses available in the GenBank. The gross results indicated the presence of two geographically distinct lineages belonging to North America and Eurasia while major sublineages were also defined which included Australian, historical European and equine viruses. Among these major groups, geographical and temporal parameters were reflected in genetically related sublineages and clades rather than the host avian species. Phylogenetic branches were shared by viruses of low and high pathogenicity giving support to the theory that viruses of high virulence are not maintained separately in the waterfowl population. The HA cleavage site motif was consistent in LPAI viruses and in this case the viruses of low and high virulence shared the phylogenetic tree branches hence supporting the theory that once LPAI viruses reach poultry and circulate, only then HPAI viruses arise by mutation and are not maintained as a separate entity in waterfowl or other birds.

Work by Spackman *et al.*, 2006 also stressed the importance of sequencing and phylogenetic analysis in identifying reassortant genes in H7N3 AIV genome and in the identification of viral lineages. An H7N3 AIV was isolated from Cinnamon Teal (*Anas cyanoptera*) during a survey of wild waterfowl in Bolivia in 2001. The NA and M genes had the maximum identity with North American wild bird isolates, the NS subtype A was most closely related to an equine virus and the remaining genes were most highly similar

to isolates from an outbreak of H7N3 in commercial poultry in Chile in 2002. The HA protein cleavage site and the outcome of pathogenesis studies in chickens were consistent with a LPAI virus and the infective dose was 10^5 times higher for chickens than turkeys. The viral isolate was determined to be the H7 HA, N3 NA, and NS type A subtypes by gene sequencing. Based on phylogenetic analysis, the HA, NP, PA, PB1 and PB2 genes were most closely related to the AIV isolates collected from chickens and turkeys during an outbreak in commercial poultry in Chile in 2002. The HA protein cleavage site was consistent with a LPAI virus and identical to the cleavage site of the LPAI virus from commercial poultry in Chile in 2002. The NA and M genes were most closely related to genes from North American wild bird lineages. The NS gene was most closely related to the NS gene of an equine virus. Analysis of deduced protein sequences reveals trends similar to what is seen in the nucleotide sequence analysis. In addition, the general protein sequence features of this virus are consistent with a wild-bird origin virus; residue 627 in PB2 is glutamic acid, there are no substitutions in the M gene consistent with Amantidine resistance, and both the NA stalk region and NS1 proteins are full length.

Work relating to phylogenetic analysis has also been done on H9N2 subtype of AIVs and has been published by Aamir *et al.*, in 2006. The aim of this study was to establish the phylogenetic relation of H9N2 avian viruses in the Middle East to other Asian H9N2 lineages by characterization of 7 viruses isolated from United Arab Emirates (2000-2003). All these viruses had an additional basic amino acid at the hemagglutinin-connecting peptide; 6 contained a mutation associated with increased affinity toward human-like sialic acid substrates. The surface glycoproteins of the viruses and most internal genes were >90% similar to those of A/Quail/Hong Kong/G1/97 (H9N2) lineage. The hemadsorbing site of neuraminidase had up to 4 amino acid substitutions, as do human pandemic viruses. M2 sequence analysis gave information about amino acid changes at 2 positions, with growing resistance to amantidine in cell culture. They multiplied capably in inoculated chickens and were fruitfully transmitted to contacts. They go on to maintain H5N1-like genes and may add to the spread of H5N1 viruses through regional co-circulation and inapparent infection and were suggested to be pandemic potentials.

AIMS AND OBJECTIVES OF MY STUDIES

- To set up RT-PCR for differential diagnosis of AIV H5, H7, H9 and other poultry respiratory pathogens like NDV, IBV, ILTV (DNA PCR). This was required for future undertaking of AIV surveillance in Pakistan.

- To develop multiplex RT-PCR for the simultaneous detection of Influenza A (M-Gene), subtypes H7, H9, H5 of AIV in combination with NDV, IBV and ILTV in three multiplex formats in various combinations. This new approach was required for setting up diagnostics in the light of our national needs, being most sensitive and specific test along with saving test time.

- Regenerating and confirmation of H7N3 isolates from various outbreaks of AI from 1995-2005, for carrying out molecular and biological studies. This included sequencing of NS1 region of the twenty two selected H7N3 isolates, study of their deduced amino acid sequences and conduction of their phylogenetic analysis.

- After screening H7N3 isolates by NS1 gene sequencing, six isolates were to be selected for carrying out HA and NA genes sequencing and study of their deduced amino acid sequences and conducting their phylogenetic analysis. This would lead to devising better disease control strategy in persisting subtypes of AIV.

- The sequencing of NA gene of the selected H7N3 AIV isolates would lead us to determine the source of new viruses or those evolved due to recombinations between AIVs circulating in a particular ecosystem.

MATERIALS AND METHODS

MATERIALS AND METHODS

Sample Collection

Tissue and Swab samples were collected from 150 chicken flocks (Broiler, Breeder, layers) from different areas of Pakistan during the period of 2004-2006.

Tissue samples included lungs, spleen, trachea, caecal tonsils and pancreas of diseased poultry flocks showing symptoms of AI and other respiratory distress symptoms.

Cloacal and Tracheal Swabs were taken from the above referred 150 flocks, by using sterile swabs and carried to the laboratory in transport media like BHI Broth (details available in annexure 1). The above samples were transported in icebox to the laboratory and stored at -20°C until used.

Sample Processing for Virus Isolation (VI)

A) The collected organs were homogenized by blending 20gm of tissues in 80ml of BHI broth (Oxoid) using a homogenizer (STOMACHER, Biomaster, UK) and centrifuged at 1500xg for 10 minutes at 10°C.

B) The swabs dipped in 1ml of BHI broth were spun at 1500xg for 10 minutes.

C) Both the supernatants were filtered through 0.2µm syringe filters (Sartorius, Minisart, Germany) and saved at 4°C till used.

In-ovo Inoculation

The above filtrates were inoculated into the Allantoic cavity of 9-day old embryonated SPF (specific pathogen free) chicken eggs for virus isolation. For this purpose, the SPF chicken eggs were candled for viability of the embryo, blood vessels and air sacs were marked. Broad end of the eggs were disinfected with 70% ethanol. Under the Biosafety Cabinet (BSL 3), a hole was drilled in the middle of the egg shell at the top and with the help of a 3ml disposable syringe, 0.2ml of inoculum was injected through the hole, just penetrating the chorioallantoic membrane right below the air space. The hole was sealed

with glue (UHU glue) and the eggs were incubated at 37°C for 48 hours whereafter these were chilled for 4 hours at 4-8°C (Swayne *et al.*, 1998b). Allantoic fluid was tested for hemagglutination activity and the positive samples were further tested using reference antisera of AIV H9N2, H5N1, and H7N3 in a standard Virus Neutralization Test (VNT) as described below in the relevant section.

Rapid Detection Kit for H5 Avian Influenza Virus

A Kit named H5-HA (Ag) Hemagglutinin Rapid Detection Kit, manufactured by World of Health Biotech, China was used in this test following the protocol recommended by the manufacturer and briefly given below:-

- Tissue homogenate of each sample was mixed in a 1:1 ratio with virus lysis buffer (provided by the manufacturer).
- Mixture was shaken thoroughly at room temperature for 30 min and centrifuged at 800xg for 5 min. The supernatant was used for the test.
- Rapid Detection Kit cassette was used to place 80µl of virus lysate buffer pre-treated sample into the sample window. Cassette was placed on a flat surface and results were read within 30 min.
- A violet line below the test control line in unknown was considered positive.

Preparation of RBCs Suspension

1. Two ml of blood was collected in sterile disposable syringe containing 3ml of Elsevier's solution (for details see annexure 7) from the subclavian vein of a chicken and transferred to a graduated conical test tube of 10ml capacity.
2. The blood was mixed gently, to avoid clotting, and then centrifuged at 800xg, at 10°C for 10 minutes.

3. The supernatant was then removed with a syringe and to this double quantity of 0.1M PBS (MP Biomedicals, France) pH 7.2 (for details see annexure 8) was added. The RBCs were gently resuspended in PBS and re-centrifuged as in step 2.

4. The supernatant was again discarded, repeating the washing cycle with PBS twice.

5. Finally the clear supernatant was discarded and the volume of the remainder washed RBCs was measured. 25% RBC suspension was then prepared by adding three times more Elsevier's solution. It was stored in a refrigerator and used up to 2 days.

Hemagglutination (HA) Test

1. With the help of a multichannel pipetter (Finpipette), 50µl of PBS was added to each of the 1-12 wells of A-H rows of the round bottom micro-titration plate.

2. Then 50µl of harvested allantoic fluid was added to well number 1A.

3. It was mixed well with the micropipetter and 50µl of the mixture was transferred from well number 1A to 2A and mixed. 50µl of mixture was transferred onwards till well 11A. In this way, the virus was diluted two fold from well 1A to 2A i.e. 1:2, 1:4, 1:8, 1:16.....1:2048.

4. AIV was not diluted in the last well of the row as it served as negative control.

5. Now 0.5% RBC suspension was prepared by diluting 100µl of 25% RBC stock into 5ml of 0.1M PBS. 50µl of this working suspension was added to each well in all the rows being tested. The contents were mixed using rotary plate shaker (Vortex Genie 2 Digital, Scientific Industries) and the plate was incubated at 10°C for 25 minutes. The results were recorded accordingly (Beard, 1980).

Haziness was indication of hemagglutination, whereas, in negative cases, a button formation due to settling of RBCs in the center of the well indicated no hemagglutination. The dilution of the well with haziness just before compact RBCs bead was considered as one HA unit of hemagglutinating virus and hence as titer of the hemagglutinating virus.

Hemagglutination Unit (HAU)

A hemagglutinating unit (HAU) is defined as the reciprocal of the highest dilution of the virus causing complete agglutination when reacted with an equal volume of appropriately diluted RBCs.

Virus Neutralization Test (VNT)

Isolates showing positive HA activity in allantoic fluid were typed by virus neutralization test using reference antisera. Alpha (α) neutralization method was used in this study as described below:

1. For the neutralization part, two-fold serial dilutions of the virus were prepared in duplicate, in 96 well micro titration plate.

2. 50µl of negative control serum and 50µl of positive reference sera (H7N3, H9N2, H5N1, NDV) were dispensed in one plate and diluted accordingly.

3. Now, 50µl of each virus dilution was added to rows with negative and positive reference sera in corresponding wells, starting from the lowest virus dilution.

4. The plates were agitated to thoroughly mix virus and serum, and then allowed to stand at 4°C (on ice or refrigerator) for one hour. 50µl of 0.5% RBCs was added to each mixture and plates were incubated at 37°C for 30 min. After incubation the plates were observed for button formation, in rows containing virus and positive known serum. The absence of HA activity indicated the neutralization of virus. The known reference serum that neutralized the HA activity of an isolate established the identification of the isolates (Beard, 1980).

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR)

All the samples were processed for RNA extraction following the protocol given below. Each sample was subjected to the detection of AIV H7, H9, and H5 and their respective Neuraminidase (NA) subtypes along with the detection of other poultry respiratory pathogens by RT-PCR.

RNA Extraction Protocol

The earlier collected filtrate of tissues and swabs was used for this purpose. Allantoic fluid of negative and positive samples was also used for RNA extraction.

Viral RNA from the viral samples was extracted using QIAamp Viral RNA mini kit according to manufacturer's instructions (QIAamp Viral RNA mini kit, QIAGEN, USA, CAT# 52906).

1. 560µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.
2. 140µl of Viral sample was added to the Buffer AVL-carrier RNA in the microcentrifuge tube. It was mixed by pulse-vortexing for 15 seconds.
3. It was incubated at room temperature (15-25°C) for 10 minutes.
4. Microcentrifuge was briefly centrifuged to remove any drops from the inside of the lid.
5. 560µl of ethanol (96-100%) was added to the sample and mixed by pulse-vortexing for 15 seconds. After mixing, the microcentrifuge was spun briefly to remove drops from the inside of the lid.
6. 630µl of the solution from step 5 was carefully applied to the QIAamp Mini spin column (in a 2ml collection tube) without wetting the rim. The cap was closed and it was centrifuged at 6000xg for 1minute. The QIAamp spin column was

placed into a clean 2ml collection tube and the tube containing the filtrate was discarded.

7. The QIAamp Mini spin column was carefully opened and step 6 was repeated.

8. The QIAamp Mini spin column was carefully opened and 500µl of Buffer AW1 was added. The cap was closed and it was centrifuged at 6000xg for 1minute. The QIAamp Mini spin column was placed in a clean 2ml collection tube and the tube containing the filtrate was discarded.

9. The QIAamp Mini spin column was carefully opened and 500µl of Buffer AW2 was added. It was centrifuged at full speed at 20,000xg for 3 minutes.

10. The QIAamp Mini spin column was placed in a new 2ml collection tube and the old collection tube with the filtrate was discarded. It was centrifuged at full speed for 1minute.

11. The QIAamp Mini spin column was then placed in a clean 1.5ml microcentrifuge tube. The old collection tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 60µl of Buffer AVE equilibrated to room temperature was added. The cap was closed and it was incubated at room temperature for 1min. It was then centrifuged at 6000xg for 1 minute.

12. Viral RNA was finally stored at -20°C in the case of short-term storage and in the case of long term storage the extracted material was stored at -70°C.

DNA from ILTV was extracted using Easy DNA Kit (INVITROGEN, USA) following manufacturer's protocol #7.

DNA Extraction Protocol

1. 20% PEG in 1M NaCl was prepared and chilled at +4°C (for details see annexure 6).

2. 100% (used neat) and 70% Ethanol (for details see annexure 9) were prepared and chilled in the -20°C freezer.

3. 750µl of viral sample was transferred to a microcentrifuge tube. The microcentrifuge was centrifuged at 6000xg for 3 minutes at room temperature to pellet the viral particles. The supernatant was transferred to a fresh microcentrifuge tube.

4. 750µl of cold (+4°C) 20% PEG (Polyethylene glycol) in 1M NaCl (for details see annexure 10) was added to the tube. It was mixed three times by inversion, and incubated on ice for 30 minutes.

5. After incubation on ice the microcentrifuge was spun at maximum speed at +4°C for 10 minutes to pellet the viral particles. The pellet was kept and the supernatant was discarded.

6. The microcentrifuge was re-centrifuged at maximum speed for 2 minutes at +4°C. Residual supernatant was removed with a pipette.

7. The viral particles were resuspended in 100µl TE buffer (provided in the kit).

8. 143µl of Solution A (provided in the kit) was added to the resuspended viral particles and vortexed for 1 second to mix.

9. The microcentrifuge was incubated at 65°C for 6 minutes.

10. 58µl of Solution B (provided in the kit) was added to the microcentrifuge tube and vortexed for 5 seconds until the mixture was uniform and no white plug was seen at the bottom of the tube.

11. 258µl of Chloroform (Merck, Germany) was added to the microcentrifuge and vortexed until evenly mixed.

12. The microcentrifuge was centrifuged at maximum speed for 10min at +4°C to separate phases and to create the interface. The upper, aqueous phase was pipetted into a new microcentrifuge.

13. 500µl of 100% ethanol (-20°C) was added to the DNA solution in the new microcentrifuge. The tube was inverted eight times to precipitate the DNA.

14. The microcentrifuge was spun at maximum speed for 5 minutes at +4°C. The pellet was kept and ethanol was decanted.

15. 500µl of 70% ethanol (-20°C) was added to the microcentrifuge tube and spun at maximum speed for 5 minutes at +4°C. The 70% ethanol was removed with a drawn-out pipette.

16. The microcentrifuge was spun at maximum speed for 2 – 3min at +4°C. Residual ethanol was removed with a pipette and the tube was air dried for 5 minutes.

17. The DNA pellet was resuspended in 20µl of sterile water and stored at -20°C until ready for use.

The extracted material was later on quantified by the Bio-Photometer (Eppendorf, Germany) using protocol described by the manufacturer, and described below.

Spectrophotometric Quantification of Nucleic Acids using Biophotometer (Eppendorf, Germany)

- The required dilution of RNA/DNA was prepared in a standard cuvette (Eppendorf, UVette, 220-1600 nm, Germany) in autoclaved nuclease free water (Gibco, Invitrogen, USA).
- Biophotometer was turned on.
- A cuvette with 100µl of autoclaved nuclease free water (GIBCO, Invitrogen, USA) was put in the cuvette slot and 'BLANK' was pressed. The reading should always be 0.00A°.
- The BLANK cuvette was taken out and the sample cuvette was put in the cuvette slot.
- Program to be run was pressed e.g for double stranded DNA, single stranded DNA or RNA.
- 'DILUTION' button was pressed to enter the exact dilution in 'µl' of the sample and the diluent and the dilution used was entered.
- 'SAMPLE' button was pressed and the reading was noted in µg/ml.

RT-PCR Protocol

1µl aliquot of extracted RNA was used for RT-PCR using SuperScript One-Step RT-PCR KIT (Cat# 10928-042, Invitrogen, USA) following maufacturer's protocol as follows:-

a. A reaction mixture was prepared by firstly adding 25µl of 2X Reaction Mixture (containing dNTP mixture, provided in the kit) to the reaction mixture microcentrifuges (0.5 ml/0.2 ml capacity microcentrifuges).

b. 1µl of template RNA (extracted using the procedure mentioned above) was added to the reaction mixture.

c. 1µl of sense (forward) primer and 1µl of anti-sense (reverse) primer were added to the reaction mixture tubes.

d. 1µl of Reverse Transcriptase/Taq polymerase mixture (provided in the kit) was added to each reaction mixture tube to be tested.

e. All were mixed in 21µl of Nuclease free water (GIBCO, Invitrogen, USA).

f. Hence a total of 50µl of reaction mixture was made ready for RT-PCR.

The primers used for the detection of HA, NA and M-Genes are listed in Table 1 and were provided by Dr. Webster, SJCH, Tennessee, USA and from published data (Ng *et al.*, 2006). Initially, M-Gene primers were used for screening the AIV positive isolates and those were further typed for determining the HA and NA subtype in each case. Sequences of primers used for the detection of NDV, IBV and ILTV were obtained from published data (Pang *et al.*, 2002; Callison *et al.*, 2006) and are listed in Table 2.

One Step RT-PCR was done in a thermocycler (Eppendorf, Germany) using the profile for RT-PCR (Table 3) provided by the manufacturer (Invitrogen, USA). The optimized program was 45°C for 25 minutes (Reverse Transcription/cDNA synthesis), 94°C for 2 minutes (initial denaturation). This was followed by 40 amplification cycles (with each cycle consisting of denaturation at 94°C for 15 seconds, Annealing at 58°C for 30 seconds, and extension at 70°C for 1 minute. Amplification was completed with a prolonged synthesis at 72°C for 10 minutes. This was eventually stored at 4°C until used.

Detection of amplified DNA products

DNA amplicons were visualized by Ethidium Bromide (Sigma) Agarose Gel (MP, Biomedicals) Electrophoresis on a 2% gel (for details see annexture 5). Results were observed after 10µl of PCR product was mixed with 2µl of gel loading buffer (Bromothymol blue) to make a total loading solution of 12µl (for details see annexure 11). 1µl of marker (1kb plus DNA step ladder, Invitrogen, USA)

was mixed with 9µl of nuclease free water along with 2µl of loading dye to make the standard marker loading solution (for details see annexure 11). Electrophoresis was done, at a constant voltage of 200 V, constant current of 200 mA, and constant power of 200 watts for 20 minutes and later photographed using Gel Documentation System (Vilber-Lourmat, France) and analyzed with BioCapt Software (Vilber-Lourmat, France). The size of the amplified product was compared with the standard marker and results noted.

Sensitivity of RT-PCR for the detection of AIVs

Sensitivity of RT-PCR for the detection of all subtypes of avian influenza viruses was determined by making 1/10 serial dilutions of RNA recovered from each positive sample of H7N3, H9N2 and H5N1, by making a mixture in a ratio of 1:50 (1µl virus RNA in 50µl of nuclease free distilled water) and measuring it spectrophotometrically in µg/ml (Biophotometer, Eppendorf, Germany) and then making 1/10 dilutions of this stock.

RT-PCR of these dilutions was done using the standard protocol in a thermocycler (Eppendorf, Germany) using the optimized program.

Specificity of RT-PCR for the detection of AIVs

The specificity of RT-PCR for the detection of AIVs was done by keeping PRIMERS constant for each subtype of AIV (H7N3, H9N2 and H5N1) in three separate RT-PCR reactions, and making them react with or amplify RNAs of each subtype including the subtype whose specific primers were being used. Here RNA from IBV (infectious bronchitis virus) and NDV (Newcastle Disease Virus) was also used as negative control.

MULTIPLEX RT-PCR (mRT-PCR)

Source of Viruses and Specimens

The following listed isolates of H7N3 were used in the standardization of mRT-PCR.

1. A/Chicken/Faisalabad/Pakistan/ NARC-N30/05(H7N3)
2. A/Chicken/Karachi/Pakistan/ NARC-100/04(H7N3)
3. A/Chicken/Mansehra/Pakistan/ NARC-74/ 04(H7N3)

Following isolates of H9N2 were used in the standardization of mRT-PCR

1. A/Chicken/Karachi/Pakistan/NARC-4935/06(H9N2)
2. A/Chicken/Abbotabad/Pakistan/NARC-6649/06(H9N2)
3. A/Chicken/Islamabad/Pakistan/NARC-N240/06(H9N2)

Following isolates of H5N1 were used in the standardization of mRT-PCR

1. A/Chicken/Peshawar/Pakistan/NARC-2517/06(H5N1)
2. A/Chicken/Jehlum/Pakistan/NARC-N353/07(H5N1)
3. A/Chicken/Islamabad/Pakistan/NARC-N240/06(H5N1)

The above mentioned viruses were obtained from the repository of National Reference Laboratory for Poultry Diseases (NRLPD) – National Agricultural Research Center (NARC), Islamabad, Pakistan. The viruses procured from the repository were in a lyophilized form. The viruses were reconstituted with nuclease free water and inoculated in 9-day old embryonated chicken eggs upto 3 passages and tested using Hemagglutination (HA) Test (Beard et al., 1980; Swayne et al., 1998). After the standardization of the test the technique was used for the detection of routine field cases of AI.

NDV, IBV and ILTV were extracted from live freeze dried vaccines (Nobilis, Intervet International B. V. Boxmeer, Holland) for the standardization procedure. The few examples of field isolates of NDV, IBV and ILTV identified by mRT-PCR are listed below:-

1. Chicken/Rawalpindi/Pakistan/NARC-303/05 (IBV M-41)

2. Chicken/Karachi/Pakistan/NARC-2344/06 (IBV M-41)
3. Chicken/Rawalpindi/Pakistan/NARC-378/05 (NDV)
4. Chicken/Karachi/Pakistan/NARC-1045/07 (NDV)
5. Chicken/Faisalabad/Pakistan/NARC-N334/06 (ILTV)
6. Chicken/Islamabad/NARC-N767/07 (ILTV)

The tissues selected for mRT-PCR included Trachea, lungs, cecal tonsils and spleen from the diseased chickens showing respiratory tract infection. The samples were pooled and processed for RNA/DNA extraction.

RNA Extraction Protocol

Viral RNA from the viral samples was extracted using QIAamp Viral RNA mini kit according to manufacturer's instructions (QIAamp Viral RNA mini kit, QIAGEN, USA, CAT# 52906) as mentioned above.

DNA Extraction Protocol

DNA from ILTV was extracted using Easy DNA Kit (INVITROGEN, USA) following manufacturer's protocol #7 as mentioned above.

Standardized Protocol for Preparation of Dilutions of Extracted RNA/DNA

1. A series of 2 serial dilutions of the extracted RNA/DNA were made in the ratio of 1:2 for the RNA to the diluent which was nuclease free water (Gibco, Invitrogen, USA).
2. 1µl of each serially diluted RNA was to be subsequently taken for the preparation of 2 reaction mixtures for mRT-PCR per extracted RNA to be tested.

Standardized Protocol for Preparation of Reaction Mixtures for Mutliplex RT-PCR (mRT-PCR)

a. 1µl of each serial dilution of extracted RNA was used for RT-PCR using SuperScript One-Step RT-PCR KIT (Invitrogen, USA).

b. A reaction mixture was prepared by firstly adding 25µl of 2X Reaction Mixture (containing dNTP mixture, provided in the kit) to the reaction mixture microfuges (0.5ml/0.2ml).

c. 1µl of each template RNA serial dilution was added to its respective reaction mixture.

d. 1µl of sense (forward) primer (100pm/µl) and 1µl of anti-sense (reverse) primer (100pm/µl) were added to the reaction mixture tubes.

e. 2.5µl of Reverse Transcriptase/Taq polymerase mixture was added to each reaction mixture tube to be tested.

f. All were mixed in 19.5µl of Nuclease free water (GIBCO, Invitrogen, USA).

g. Hence a total of 50µl of reaction mixture was made ready for mRT-PCR.

Optimization of multiplex RT-PCR (m-RT-PCR)

The mRT-PCR employed here consisted of a one-step procedure, which included reverse transcription (RT) and PCR amplification in a single step. For this purpose an RT-PCR kit (Invitrogen, USA) was used. The mRT-PCR was performed in 50µl volumes, in which the reaction mixture contained 25µl of 2X Reaction Mix consisting of a proprietary buffer system for reverse transcription and PCR amplification, Mg++, deoxyribonucleotide triphosphates (dNTP's), and stabilizers. Template RNA's in various dilutions/concentration which were optimized after a number of mRT-PCR trials were added in 1µl amounts per reaction mixture. 1µl of forward primers of influenza A, H7, H9, H5, IBV, ILTV and NDV were added in optimized concentration of 100

picomoles/μl, 1μl of reverse primers of the above mentioned viruses were added per reaction mixture in optimized concentration of 100 picomoles/μl. 2.5μl of RT/Platinum Taq Mix was added. This amount was optimized after a number of trials, which contains a mixture of SuperScript II Reverse Transcriptase and Platinum Taq DNA Polymerase for optimal cDNA synthesis and PCR amplification. Nuclease free water was added to bring the final volume to 50μl.

After extensive preliminary trials with different denaturation, annealing and extension temperatures and times and with various concentrations of DNA and RNA, the thermal cycler was programmed for optimum conditions. The mRT-PCR was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The cycling protocol consisted of cDNA synthesis at 45°C for 45 minutes, an initial denaturation at 96°C for 5 minutes, then 40 cycles that each consisted of denaturing at 95°C for 1 minute, annealing at 53°C for 5 minutes and extension at 70°C for 1minute, followed by a final extension at 72°C for 10 minutes. Final storage was at 4°C (Table 6). A negative control did not contain template RNA/DNA and consisted of PCR master mix, all six sets of primers and nuclease free water.

Three sets of m-RT-PCR's were carried out, the first set used primers of H7, H9, and H5 with template RNA's of the specific hemagglutinin subtypes utilizing the optimal conditions mentioned above for multiplex reverse transcriptase-PCR.

A second set of m-RT-PCR was developed using primers for influenza A, NDV, IBV and ILTV with template RNA/DNA of these specific viruses utilizing the optimal conditions of m-RT-PCR.

The third set of m-RT-PCR developed used primers of H7, H9, H5, NDV, IBV, and ILTV with the specific template RNA/DNA with hexavalent amplifications being carried out using the optimized conditions.

The primers used for the detection of AIV, H7, H9, H5 in the multiplex format are given in Table 4. The primers used for the detection of NDV, IBV, ILTV in the multiplex format are listed in Table 5.

Standardized Thermocycling Program for Mutliplex RT-PCR

One Step mRT-PCR was done in a thermocycler (Eppendorf, Germany) using optimized profile in the thermocycler (TABLE 6). The optimized program for mRT-PCR was 45°C for 45 minutes, 96°C for 5 minutes (Reverse Transcription/cDNA synthesis and initial denaturation). This was followed by 40 amplification cycles (with each cycle consisting of denaturation at 95°C for 1 minute, Annealing at 53°C for 05 minutes, and extension at 70°C for 1 minute. Amplification was completed with a prolonged final extension at 72°C for 10 minutes. This was eventually stored at 4°C until used. (TABLE 6)

Detection of Amplified Nucleic Acid Products of Multiplex RT- PCR

DNA amplicons were visualized by running a 2% Agarose Gel with Ethidium Bromide using Agarose gel electrophoresis, along with standard 1kb DNA markers (Invitrogen, USA), at, 120V for 20min and later photographed using gel documentation system (Poddar, 2002) as described above

Primer Designs and Selection

Four sets of primers that specifically amplify type A influenza virus and HA Gene of H7, H9 and H5 subtypes of AIV's (Table 4) alongwith three sets of primers for IBV, ILTV and NDV (Table 5) were used. The sequence of the primer for AIV, H7, H9 was provided by Dr. R. G. Webster, SJCRH, Memphis, TN., and the sequence of H5 was obtained from published data in literature (Ng *et al.*, 2006) and synthesized by Operon Biotechnologies, Huntsville, AL., USA. Primer sequences for IBV was obtained from published data (Callison *et al.*, 2006), and for ILTV and NDV were obtained from published data (Pang *et al.*, 2002). The primers were aliquoted to a final concentration of 100pmol/µl and stored at -20°C.

Multiplex RT-PCR Sensitivity and Specificity

Sensitivity of mRT-PCR was determined by examining the extent of the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV and ILTV. The mRT-PCR was tested using other

avian pathogens that produce similar clinical signs or that can be present in mixed infections with AI subtypes. These AIV subtypes and respiratory pathogens are listed in Table 7 and 8 respectively.

To determine the ability of the multiplex PCR assay to detect and differentiate three subtypes of AIV's H7, H9, H5 and NDV, IBV, ILTV in the same reaction, we used a mixture of DNA/RNA concentrations ranging from 500 ng/ml to 10fg/ml DNA/RNA in various combinations of three subtypes of avian influenza viruses and three other respiratory pathogens. Sensitivity of the mRT-PCR for the detection of these six respiratory pathogens was determined by making ten-fold serial dilutions of 100ng/ml of each respiratory pathogen as template RNA/DNA. Multiplex-RT-PCR (mRT-PCR) of these dilutions was done in the thermocycler using the optimized program.

Specificity of mRT-PCR was determined by examining the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV and ILTV. Primers specific for AIV, H7, H9 and H5 were added to the mutiplex format for the amplification of AIV, H7, H9 and H5 to check their reactivity if any to NDV, IBV and ILTV. Similarly, in the multiplex format NDV, IBV and ILTV specific primers were added in the multiplex format to check if they reacted/amplified AIV, H7, H9 and H5 RNA.

SEQUENCING OF AIV H7N3 AT NS, HA AND NA GENES

Source of Samples

1. Samples were obtained from the repository of viruses at the National Reference Laboratory for Poultry Diseases, NARC, Islamabad, Pakistan, belonging to Avian Influenza viruses subtypes H7N3 in a lyophilized form.

2. The viruses were reconstituted with nuclease free water and inoculated in 9-day old embryonated chicken eggs and were passaged until high viral titres were recovered to make working stocks of the virus.

List of AIVs H7N3 used in the study is given in Table 10

The isolates and their accession numbers for the sequences submitted in the GenBank are given in Table 11 (Results Section).

Extraction of RNA

Viral RNA from the viral samples was extracted using QIAamp Viral RNA mini kit according to manufacturer's instructions (QIAamp Viral RNA mini kit, QIAGEN, USA, CAT# 52906) (as mentioned above in the RNA extraction protocol). Viral RNA was finally stored at -20°C in the case of short-term storage and in the case of long term storage the extracted material was stored at -70°C.

Primer Designs and Selection for Initial Amplification by RT-PCR for NS, HA and NA Genes of AIV H7N3

The primers used for initial amplification by RT-PCR at the NS1, HA and NA gene regions are given in Table 9.

RT-PCR Amplification

One Step RT-PCR was done in a thermocycler (Eppendorf, Germany) using optimized profile in the thermocycler (Table 3). Amplicons were eventually stored at 4°C until used.

Detection of Amplified Nucleic Acid Products of RT- PCR

DNA amplicons were visualized by Ethidium Bromide (Sigma) Agarose Gel (MP, Biomedicals) Electrophoresis on a 2% gel (for details see annexure 5). Results were observed after 10µl of PCR product was mixed with 2µl of gel loading buffer (Bromothymol blue) (for details see annexure 11) to make a total loading solution of 12µl. 1µl of marker was mixed with 9µl of nuclease free water along with 2µl of loading dye to make the standard marker loading solution. Standard 1kb plus DNA Step ladder marker (Invitrogen, USA) was used. Electrophoresis was done, at a constant voltage of 200V, constant current of 200mA, and constant power of 200 watts for 20 minutes and later photographed using Gel Documentation System (Vilber-Lourmat, France) and analyzed with BioCapt Software (Vilber-Lourmat, France). The size of the amplified product was compared with the standard marker and results noted.

In case of multiple bands of DNA in the gel the following protocol was used (QIAquick Spin Handbook, 11/2006, QIAGEN, USA)

QIAquick Gel Extraction Protocol (for multiple bands from RT-PCR)

1. The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose.

2. The gel slice was weighed in a microcentrifuge. 3 volumes of Buffer QG (provided by the manufacturer) were added to the volume of gel (100mg ~↔ 100µl). For example, 300µl of Buffer QG was added to each 100 mg of gel. For >2% agarose gels, 6 volumes of Buffer QG was added. The maximum amount of gel slice per QIAquick column was 400 mg.

3. It was incubated at 50°C for 10 minutes (or until the gel slice was completely dissolved). To help dissolve gel, the tube was mixed by vortexing every 2-3 minutes during the incubation. It was important to solubilize agarose completely. For >2% gels, incubation time was increased.

4. After the gel slice had dissolved completely, the color of the mixture was checked if it was yellow (similar to Buffer QG without dissolved agarose). If the color of the mixture was orange or violet, 3 M sodium acetate, pH 5.0, was added and mixed. The color of the mixture turned yellow. The adsorption of DNA to the QIAquick membrane was efficient only at pH <7.5. Buffer QG contained a pH indicator which was yellow at pH <7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. 1 gel volume of isopropanol was added to the sample and mixed. For example, if the agarose gel slice was 100mg, 100µl isopropanol was added. This step increased the yield of DNA. Sample was not to be centrifuged at this stage.

6. A QIAquick spin column was placed in the provided 2ml collection tube.

7. To bind DNA, the sample was applied to the QIAquick column, and centrifuged for 1 minute. The maximum volume of the column reservoir was 800µl.

8. Flow-through was discarded and QIAquick column was placed back in the same collection tube.

9. 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 minute. This step removed all traces of agarose. It was only required when the DNA was subsequently to be used for direct sequencing.

10. To wash, 0.75 ml of Buffer PE (provided by the manufacturer) was added to QIAquick column and centrifuged for 1 minute. The column was allowed to stand for 2-5 minutes after addition of Buffer PE, before centrifuging.

11. Flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute at 17,900xg. Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. The QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, 50µl of Buffer EB (provided by the manufacturer) or water was added to the center of the QIAquick membrane and the column was centrifuged for 1 minute. Alternatively, for increased DNA concentration, 30µl of elution buffer was added to the center of the QIAquick membrane, the column was allowed stand for 1 minute, then centrifuged for 1 minute.

14. For analysis of the purified DNA on a gel, 1 volume of Loading Dye was added to 5 volumes of purified DNA. The solution was mixed by pipetting up and down before loading the gel.

In case of single bands of DNA in the gel the following protocol was used. (QIAquick Spin Handbook, 11/2006, QIAGEN, USA)

QIAquick PCR Purification Kit Protocol (for single bands from RT-PCR)

1. 5 volumes of Buffer PBI were added to 1 volume of the PCR sample and mixed.
2. The color of the mixture was checked if it was yellow. If the color of the mixture was orange or violet, 10µl of 3M sodium acetate was added, pH 5.0 and mixed. The color of the mixture turned to yellow
3. A QIAquick spin column was placed in a 2 ml collection tube.
4. To bind DNA, the sample was applied to the QIAquick column and centrifuged for 30-60 seconds.
5. Flow-through was discarded. The QIAquick column was placed back into the same tube.
6. To wash, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 30-60 seconds.
7. Flow-through was discarded and the QIAquick column was placed back in the same tube. The column was centrifuged for an additional 1 minute.
8. QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, 50µl Buffer EB (10mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1min. Alternatively, for increased DNA concentration, 30µl elution buffer was added to the center of the QIAquick membrane, the column allowed to stand for 1min, and then centrifuged.
10. For the analysis of purified DNA on a gel, 1 volume of Loading Dye was added to 5 volumes of purified DNA. The solution was mixed by pipetting up and down before loading the gel.

Cycle Sequencing of Double-Stranded DNA

Preparation of 96 Well Reaction Plates or Microcentrifuge Tubes

Reactions for 96-Well Reaction Plates or Microcentrifuge Tubes were prepared using BigDye Terminator v3.1 Sequencing Buffer and BigDye Terminator v3.1 Cycle Sequencing Kit (BigDye Terminator v3.1 Cycle Sequencing Kit Protocol, 2002)

REAGENT	CONCENTRATION	VOLUME
Ready Reaction Premix	2.5X	4µl
BigDye Sequencing Buffer	5X	2µl
Primer	3.2pmol/µl	1µl
Template DNA	-	1-2µl
Water	-	To 20µl
Final Volume	**1X**	**20µl**

PCR Profile for Cycle Sequencing

The PCR Program for the synthesis of chain-terminated strands of DNA was as follows (BigDye Terminator v3.1 Cycle Sequencing Kit Protocol, Cycle Sequencing, 2002)

1. 96-well Plates or tubes were placed in the thermocycler.
2. Initial Denaturation at 96°C for 1 minute.
3. The following was repeated for 25 cycles:
    a. 96°C for 10 seconds
    b. 50°C for 5 seconds
    c. 60°C for 4 minutes
4. 4°C –HOLD- until ready to purify
5. The contents of the plate were spun for further purification of the extension products

Purification of Extension Products-Using BigDye XTerminator Purification Kit

(BigDye XTerminator Purification Kit Protocol, Applied Biosystems, USA, 2007)

1. After Cycle Sequencing was completed, the reaction plate was centrifuged at 6000xg for 1 minute.

2. To each well of the reaction plate, the volume of SAM Solution (SAM Solution improves BigDye XTerminator reagent performance and stabilizes the sample after purification) specified below was added using a conventional pipette tip.

Plate Type and Reaction volume/well	**Volume of SAM Solution/Well (µl)**
96-Well , 20µl	90

3. The XTerminator Solution (XTerminator Solution captures unincorporated dye terminators and free salts from the post cycle-sequencing reaction) was added:

    a. XTerminator Solution bulk was vortexed at maximum speed for at least 10 seconds until homogenous.

    b. Using a wide-bore pipette tip the XTerminator Solution was aspirated.

    c. The volume of XTerminator Solution specified below was added to each well.

Plate Type and Reaction Volume/Well	**Volume of XTerminator Solution/Well (µl)**
96-well, 20µl	20

    d. Pipette tip was discarded.

4. The plate was sealed using MicroAmp Clear Adhesive Film (Applied Biosystems) and verified that each well was properly sealed.

5. The plate was vortexed in a vortex (Digital Votex-Genie 2) at 1000xg for 30 minutes.

6. Vortexing was paused after 1 minute and wells examined for the mixing of contents.

7. In a swinging-bucket Centrifuge (5810 R, Eppendorf, Germany) the plate was spun at 1000xg for 2 minutes.

8. The adhesive film was removed from the 96-well reaction plate and it was covered with rubber septa mat and then placed in the 3130 Genetic Analyzer (Applied Biosystems) for DNA Sequencing.

9. Ultra Sequencing Run Module was selected in the Data Collection Sofware Plate Editor.

10. Plate was run.

The following protocol was also used for the post cycle sequencing clean-up (User Bulletin, Precipitation Method to remove unincorporated Dye Terminators from ABI BigDye Terminator v3.0 Cycle Sequencing reactions, Ethanol/Sodium Acetate precipitation in Microcentrifuge tubes, April 11, 2002, pp 16/17 1-20)

Purification of Extension Products–Ethanol/Sodium Acetate Precipitation in Microcentrifuge Tubes (1.5ml)

A cycle sequenced 20µl-reaction mixture in 96-well plate was precipitated as follows:

Step	Action
1	• The 96-well reaction plate was removed from the thermal cycler. • The adhesive cover was removed from the reaction plate
2	The entire contents of each 20µl cycle sequenced reaction mixture of the 96-well plate was pipetted into 1.5ml microcentrifuge tubes.
3	The ethanol/sodium acetate solution was prepared by combining the following for each sample: • 3.0µl of 3M sodium acetate, pH 4.6 (for details see annexure 12) • 62.5µl of nondenatured 95% ethanol • 14.5µl of deionized water The final volume was 80µl for each sample
4	The following was added to each 20µl reaction mixture in the 1.5ml

	microcentrifuge tubes: • 80µl of ethanol/sodium acetate solution (created in step 3 above)
5	The tubes were closed and vortexed briefly.
6	The tubes were left at room temperature for at least 20-30min to precipitate the extension products. **Note**: Precipitation times <15min will result in the loss of very short extension products. Precipitation times >24hrs will increase the precipitation of unincorporated dye terminators.
7	The tubes were placed in a microcentrifuge at room temperature and their orientations were marked. The tubes were spun for 30 minutes at 13200xg. **IMPORTANT:** The next step was proceeded immediately. If this was not possible, then the tubes were spun for 2 minutes immediately before performing the next step.
8	The supernatants were carefully aspirated with a separate pipette tip for each sample and discarded. Pellets may or may not be visible. **IMPORTANT:** The supernatants were to be removed completely and carefully, as unincorporated dye terminators were dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators were to remain in the samples.
9	250µl of 70% ethanol was added to the tubes and vortexed briefly.
10	The tubes were placed in the microcentrifuge in the same orientation as in step 7 above and spun for 5 minutes at the same speed.
11	The supernatants were carefully aspirated, as in step 8.
12	The samples were dried for 1 hour under the hood or kept overnight at room temperature. Not to be overdried.
13	**The pellets were resuspended in 20µl of Hi-Di Formamide (ABI) and transferred to a sequencing plate for sequencing**

The cleaned post-cycle sequenced products were directly sequenced using automated Genetic Analyzer 3130 (Applied Biosystems, USA).

Nucleotide and Deduced Amino Acid Sequence Analysis

The sequence files were created in the ABI file format and were automatically transferred to the Sequence Analysis Software v5.3 (Applied Biosystems, USA) by the Genetic Analyzer 3130 (Applied Biosystems). The sequences were analysed for quality using the

software and the clean sequences were then further processed for the creation of consensus sequences using the Sequencher 4.8 Program.

Phylogenetic Analysis

Consensus sequences were created using Sequencher 4.8 Program (Gene Codes Corporation, MI, USA). The consensus sequences were uploaded in the GenBank and Accession numbers were noted. The consensus sequences were then BLASTed using National Center for Biotechnology Information – NCBI, Basic Local Alignment Search Tool - BLAST and Nucleotide BLAST was chosen in the options. BLAST analysis was used to initially identify the most closely related H7N3 AIVs from the database of GenBank. The most closely related sequences were chosen, analyzed and aligned by CLUSTAL W in BioEdit sequence alignment editor software, version 7.0.9.0. The ends were trimmed to equal lengths. Further analyses were carried out using Molecular Evolutionary Genetics Analysis 4 (MEGA4) program.

Phylogenetic analysis was performed with phylogenetically representative isolates from all the available sequences for each gene. Phylogenetic trees were generated using Neighbourhood Joining Method by a heuristic search with 1000 bootstrap replicates (MEGA4). All the trees were unrooted.

TABLE 1

RT-PCR PRIMERS USED FOR THE DETECTION OF M-GENE, H7, H9 and H5 SUBTYPES OF AIV

Primer Name	Primer's Oligonucleotide Sequence	Product (bp)
AIV		
M-WSN F	5'-GAA GGT AGA TAT TGA AAG ATG- 3'	
M-1023 R	5'-GAA ACA AGG TAG TTT TTT ACT C-3'	1023
Subtype H7		
H7-397 F	5'-ACA TAC AGT GGG ATA AGA ACC -3'	
H7-391 R	5'-TCT CCT TGT GCA TTT TGA TGC C -3'	300
Subtype H9		
H9-1 F	5'-AGC AAA AGC AGG GGA AYW WC-3'	
H9-808 R	5'-CCA TAC CAT GGG GCA ATT AG-3'	808
Subtype H5		
H5-1 F	5'ACTATGAAGAATTGAAACACCT-3'	
H5-2 R	5'-GCAATGAAATTTCCATTACTCTC-3'	456

*Codes for mixed bases position: Y, C/T; R, A/G; W, A/T; B, G/C/T; K, G/T
Reference : Dr. R. G. Webster (SJCRH, Memphis, TN) (H7 and H9)
Ng *et al.*, 2006 (H5)

TABLE 2

OTHER PRIMERS USED IN RT-PCR/ PCR

Primer Name	Primer's Oligonucleotide Sequence	Product (bp)
IBV (Forward)	5'-GCT TTT GAG CCT AGC GTT-3'	
IBV(Reverse)	5'-GCC ATG TTG TCA CTG TCT ATT-3'	149
NDV(Forward)	5'-GGAGGATGTTGGCAGCATT-3'	
NDV (Reverse)	5'-GTCAACATATACACCTCATC-3'	320
ILTV(Forward)	5'-ACGATGACTCCGACTTTC-3'	
ILTV(Reverse)	5'-CGTTGGAGGTAGGTGGTA-3'	647

Reference: Callison *et al.*, 2006 (IBV)
Reference: Pang *et al.*, 2002 (NDV, ILTV)

TABLE 3

RT-PCR PROFILE

S#	**STEPS OF RT-PCR/PCR**	**TEMPERATURES**	**CYCLES**
1.	REVERSE TRANSCRIPTION	45°C for 25 minutes	1
	INITIAL DENATURATION	94°C for 2 minutes	1
2.	**PCR CYCLES**		
	DENATURATION	94°C for 15sec	x40
	ANNEALING	58°C for 30sec	
	EXTENSION	70°C for 1min	
3.	**FINAL EXTENSION**	72°C for 10 min	1
4.	**STORAGE**	4°C until used	1

Ref: SuperScript RT-PCR kit, Invitrogen, USA

TABLE 4

MULTIPLEX RT-PCR PRIMERS

Primer Name	Primer's Oligonucleotide Sequence	Product (bp)
AIV		
M-WSN F	5'-GAA GGT AGA TAT TGA AAG ATG- 3'	
M-1023 R	5'-GAA ACA AGG TAG TTT TTT ACT C-3'	1023
Subtype H7		
H7-397 F	5'-ACA TAC AGT GGG ATA AGA ACC -3'	
H7-391 R	5'-TCT CCT TGT GCA TTT TGA TGC C -3'	300
Subtype H9		
H9-1 F	5'-AGC AAA AGC AGG GGA AYW WC-3'	
H9-808 R	5'-CCA TAC CAT GGG GCA ATT AG-3'	808
Subtype H5		
H5-1	5'ACTATGAAGAATTGAAACACCT-3'	
H5-2	5'-GCAATGAAATTTCCATTACTCTC-3'	456

Codes for mixed bases position: Y, C/T; R, A/G; W, A/T; B, G/C/T; K, G/T
Reference : Dr. R. G. Webster, St. Judes Children Research Hospital, Memphis, TN. (AIV, H7, H9).
Ng *et al.*, 2006 (H5).

TABLE 5

OTHER PRIMERS USED IN MULTIPLEX RT-PCR

Primer Name	Primer's Oligonucleotide Sequence	Product (bp)
IBV (UP)	5'-GCT TTT GAG CCT AGC GTT-3'	
IBV(DOWN)	5'-GCC ATG TTG TCA CTG TCT ATT-3'	149
NDV(UP)	5'-GGAGGATGTTGGCAGCATT-3'	
NDV(DOWN)	5'-GTCAACATATACACCTCATC-3'	320
ILTV(UP)	5'-ACGATGACTCCGACTTTC-3'	
ILTV(DOWN)	5'-CGTTGGAGGTAGGTGGTA-3'	647

Reference: Callison *et al.*, 2006 (IBV)
Reference: Pang *et al.*, 2002 (NDV, ILTV)

TABLE 6

STANDARDIZED MUTIPLEX RT-PCR PROFILE

S#	**STEPS OF MUTIPLEX RT-PCR**	**TEMPERATURES**	**CYCLES**
1.	REVERSE TRANSCRIPTION	45°C for 45 minutes	1
	INITIAL DENATURATION	96°C for 05 minutes	1
2.	**PCR CYCLES**		
	DENATURATION	95°C for 01 minute	x40
	ANNEALING	53°C for 05 minute	
	EXTENSION	70°C for 01 minute	
3.	**FINAL EXTENSION**	72°C for 10 minutes	1
4.	**STORAGE**	4°C until used	1

Ref: SuperScript RT-PCR kit, Invitrogen, USA

TABLE 7
AVIAN INFLUENZA ISOLATES CONFIRMED BY MULTIPLEX RT-PCR

Avian Pathogen	Subtype	Source	Results of mRT-PCR		
			H5	H7	H9
A/Chicken/Chakwal/Pakistan/NARC-BM/00(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK.	-	+	-
A/Chicken/Murree/Pakistan/ NARC-70/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Mansehra/Pakistan/ NARC-74/ 04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Karachi/Pakistan/ NARC-100/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Chakwal/Pakistan/ NARC-148/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Karachi/Pakistan/ NARC-169/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Rawalpindi/Pakistan/NARC-160/ 04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Mansehra/Pakistan/ NARC-178/ 04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Mansehra /Pakistan/ NARC-214/ 04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Murree/Pakistan/ NARC-216/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Tora Bora/ Afghanistan/NARC-483/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Karachi/Pakistan/ NARC-1118/04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Mansehra/Pakistan/ NARC-1282/ 04(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Sumundri/Pakistan/ NARC-N19/05(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Faisalabad/Pakistan/ NARC-N30/05(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Abbotabad/Pakistan/ NARC-2402/ 05(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Abbotabad/Pakistan/ NARC-2419/ 05(H7N3)	H7N3	NRLPD, NARC, ISLAMABAD, PAK	-	+	-
A/Chicken/Peshawar/Pakistan/NARC-2517/06(H5N1)	H5N1	NRLPD, NARC, ISLAMABAD, PAK	+	-	-
A/Chicken/Islamabad/Pakistan/NARC-N240/06(H5N1)	H5N1	NRLPD, NARC, ISLAMABAD, PAK	+	-	-
A/Chicken/Jehlum/Pakistan/NARC-N353/07(H5N1)	H5N1	NRLPD, NARC, ISLAMABAD, PAK	+	-	-
A/Chicken/Karachi/Pakistan/NARC-7512/07(H5N1)	H5N1	NRLPD, NARC, ISLAMABAD, PAK	+	-	-
A/Chicken/ChakShahzad/Pakistan/NARC-9080/07(H5N1)	H5N1	NRLPD, NARC, ISLAMABAD, PAK	+	-	-
A/Chicken/Islamabad/Pakistan/NARC-N372/07(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+

Avian Pathogen	Subtype	Source	Results of mRT-PCR		
			H5	H7	H9
A/Chicken/Rawalpindi/Pakistan/NARC-N374/07(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+
A/Chicken/Abbotabad/Pakistan/NARC-6649/06(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+
A/Chicken/Karachi/Pakistan/NARC-4935/06(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+
A/Chicken/Islamabad/Pakistan/NARC-N240/06(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+
A/Chicken/Muzaffarabad/Pakistan/NARC-4777/06(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+
A/Chicken/Tarlai/Pakistan/NARC-N441/07(H9N2)	H9N2	NRLPD, NARC, ISLAMABAD, PAK	-	-	+

+ Isolates of avian influenza confirmed by multiplex RT-PCR

TABLE 8

OTHER POULTRY RESPIRATORY PATHOGENS CONFIRMED BY MULTIPLEX RT-PCR

Avian Pathogen	Source of sample	Source	Results of mRT-PCR
NDV	NDV-NOBILIS-VACCINE	NRLPD, NARC, ISLAMABAD, PAK	+
IBV	BRONCHITIS VACCINE-MASS TYPE-M-41	NRLPD, NARC, ISLAMABAD, PAK	+
ILTV	ILTV-NOBILIS VACCINE	NRLPD, NARC, ISLAMABAD, PAK	+
Chicken/Rawalpindi/Pakistan/NARC-303/05 (IBV M-41)	FIELD	NRLPD, NARC, ISLAMABAD, PAK	+
Chicken/Karachi/Pakistan/NARC-2344/06 (IBV M-41)	FIELD	NRLPD, NARC, ISLAMABAD, PAK	+
Chicken/Rawalpindi/Pakistan/NARC-378/05 (NDV)	FIELD	NRLPD, NARC, ISLAMABAD, PAK	+
Chicken/Karachi/Pakistan/NARC-1045/07 (NDV)	FIELD	NRLPD, NARC, ISLAMABAD, PAK	+
Chicken/Faisalabad/Pakistan/NARC-N334/06 (ILTV)	FIELD	NRLPD, NARC, ISLAMABAD, PAK	+
Chicken/Islamabad/NARC-N767/07 (ILTV)	FIELD	NRLPD, NARC, ISLAMABAD, PAK	+

TABLE 9

PRIMER SEQUENCES USED FOR RT-PCR AND SEQUENCING OF AIV H7N3 AT NS1, HA AND NA GENES

PRIMER NAME	PRIMER OLIGONUCLEOTIDE SEQUENCE	PRODUCT (bp)	NOTE
H7 NS1 F	5' GTGACAAAAACATAATGGATTCCAAC 3'	850	
H7 NS1 R	5' TCATTAAATAAGCTGAAACGAGAAAG 3'		
H7 NS1 R INTERNAL PRIMER	5' CACAATTGCACCATCTTCT 3'	693	
H7 HA 7S1 F	5' AGCAAAAGCAGGGGATACAAAATG 3'	492	SEG A HA
H7 HA 7S1 R	5' GCATAGAATGAAGA(A/C/T)CCTGATCT 3'		
H7 HA 7S2 F	5' ACATA(C/T)AG(C/T)GG(A/G)ATAAG(A/G)AC(C/T)AAT GG 3'	544	SEG B HA
H7 HA 7S2 R	5' GGACATTTGCCCACTGCCCTGC 3'		
H7 HA 7S3 F	5' ACATACAGTGGGATAAGAACC 3'	380	SEG C HA
H7 HA 7S3 R	5' TCTCCTTGTGCATTTTGATGCC 3'		
H7 NA 19S1 F	5' GCGCGCGGCCGCATGAATCCAAATCAGAAGATAATA 3'	331	SEG A NA
H7 NA 19S1 R	5' GTATTGCATTGTCCTTGTGA 3'		
H7 NA 19S2 F	5' CACTGCCACTGTGCCCCTT 3'	384	SEG B NA
H7 NA 19S2 R	5' TGGAGTCTGTCATTCTCCCTGC 3'		
H7 NA 19S3 F	5' GCTTGGTCGAGTAGCAGTTGC 3'	457	SEG C NA
H7 NA 19S3 R	5' CAGCTGGCCTGGGAGTGTC 3'		
H7 NA 19S4 F	5' AGGGACAATTGGAARGGTT 3'	467	SEG D NA
H7 NA 19S4 R	5' CCACAGAAAGTAACTATACTATTGC 3'		

Table 10

LIST OF AIV ISOLATES USED IN THE STUDY

Isolate Name	Origin	Type	Collection Date
A/Chicken/Pakistan/NARC-01/95	Murree	Breeder	1995
A/Chicken/ Pakistan/NARC-02/95	Murree	Breeder	1995
A/Chicken/Pakistan/NARC-BM/00	Chakwal	Broiler	15/04/00
A/Chicken/ Pakistan/NARC-35/01	Chakwal	Layer	2001
A/Chicken/ Pakistan/NARC-43/ 03	Sheikhupura	Broiler	28/12/03
A/Chicken/ Pakistan/NARC-23/03	Karachi	Layer	2003
A/Chicken/Pakistan/NARC-70/04	Murree	Breeder	14/04/04
A/Chicken/Pakistan/NARC-74/ 04	Mansehra	Breeder	14/04/04
A/Chicken/Pakistan/NARC-100/04	Karachi	Layer	27/04/04
A/Chicken/ Pakistan/NARC-108/ 04	Mansehra	Breeder	05/05/04
A/Chicken/ Pakistan/NARC-143/ 04	Mansehra	Breeder	11/05/04
A/Chicken/Pakistan/NARC-169/ 04	Karachi	Layer	13/05/04
A/Chicken/ Pakistan/NARC-160/ 04	Rawalpindi	Broiler	14/05/04
A/Chicken/Pakistan/NARC-214/ 04	Mansehra	Breeder	25/05/04
A/Chicken/Afghanistan/NARC-483/ 04	Tora Bora	Broiler	29/07/04
A/Chicken/Pakistan/NARC-178/ 04	Mansehra	Breeder	31/08/04
A/Chicken/Pakistan/NARC-1118/04	Karachi	Layer	04/11/04
A/Chicken/Pakistan/NARC-1282/ 04	Mansehra	Breeder	07/12/04
A/Chicken/Pakistan/NARC-N30/05	Faisalabad	Layer	27/04/05
A/Chicken/Pakistan/NARC-2419/05	Abbotabad	Breeder	25/05/05
A/Chicken/Pakistan/NARC-2402/05	Abbotabad	Breeder	20/05/05
A/Chicken/Pakistan/NARC-N19/05	Sumundri	Broiler	02/02/05

RESULTS

RESULTS

RESULTS OF RT-PCR/PCR

RT-PCR was conducted for the detection of Avian Influenza Viruses subtypes H7N3, H9N2 and H5N1, from field samples of poultry, from various outbreaks of Pakistan during the period from 2004-2006.

RT-PCR and conventional PCR were also carried out to detect the presence of other respiratory poultry pathogens like NDV, IBV and ILTV from field samples.

All field isolates of AIV from various regions of Pakistan, confirmed by RT-PCR, were further used as follows:

- Conventional methods of virus isolation were compared with detection of viruses with RT-PCR, proving the efficacy of the latter as a better molecular diagnostic tool.
- Sensitivity and specificity of RT-PCR for the detection of AIV was also determined.
- Multiplex RT-PCR was developed as a differential diagnostic tool for the simultaneous detection of H7, H9, H5, NDV, IBV and ILTV.
- Sequencing of the NS1 Gene of 22 H7N3 isolates was carried out.
- Subsequently, 6 H7N3 isolates were sequenced at the HA and NA gene level which were isolated from the Northern Areas of Pakistan.

Comparison of conventional methods of virus isolation and detection with RT-PCR

Comparison of conventional methods of virus isolation and detection with RT-PCR showed the following results:-

- A total of 150 field samples, from various outbreaks from various areas of Pakistan during the period of 2004-2006 were processed and studied by various laboratory techniques and RT-PCR.

- Out of these 150 samples, 5 (3.33%) of the field samples (tissue homogenates of trachea, lungs, pancreas and cecal tonsils) were found to be positive for H5N1 by Rapid Detection Kit Test (Figure 1a, b).

- A total of 25 samples (16.6%) were found positive by HA Test using tissue homogenates and swabs (Figure 1a, b).

- After in-ovo inoculation as first passage the figure increased to 42 (28%) by HA Test, and this figure included the 5 field samples of H5N1 which came up as positive by Rapid Kit Test. The percentage of positive samples by HA after first passage was hence greater (Figure 1a, b).

- VNT was performed on the 42 positive samples, 14 were found to be H7N3, 12 as H9N2, 06 as H5N1 and 10 were identified as NDV (Figure 1a, b).

- RT-PCR of these 42 HA+ve isolates was done for M-Gene. Out of the 42 positive samples by HA Test, 32 (76.1%) were found out to be positive for M-Gene, and 10 (23.8%) were negative for M-Gene (Figure 2a, b).

- Out of these 32 M-Gene positive samples confirming the presence of AIV, further RT-PCR for HA and NA genes confirmed the presence of 14 H7N3, 12 H9N2 and 06 H5N1 subtypes of AIVs (Figure 2a, b).

- 10 samples which were M-Gene –ve were found to be NDV positive by RT-PCR using NDV specific primers (Figure 2a, b).

- RT-PCR of 108 HA –ve samples was done for M-Gene and out of these, 27 (25%) came up as +ve whereas 81 (75%) were negative for M-Gene (Figure 3a, b).

- RT-PCR of these positive samples was done for H7N3, H9N2, H5N1, NDV and IBV using specific primers and 11 came up as H7N3, 14 as H9N2, 02 as H5N1 (Figure 3a, b).

- RT-PCR using NDV and IBV specific primers showed that 13 samples were positive for NDV and 05 for IBV which were negative for M-Gene (Fig 4a, b).
- PCR of 63 RT-PCR –ve samples (tissue homogenates) was done for ILTV using ILTV specific primers, 03 (4.76%) were found to be positive for ILTV (Figure 5a, b).
- 63 samples were totally negative for AIV, NDV and IBV.

Hence, out of a total of 150 samples, 25 were H7N3, 26 H9N2, 08 H5N1, 23 NDV, 05 IBV and 03 as ILTV by RT-PCR/PCR proving that RT-PCR/PCR is a better molecular diagnostic tool as compared to other routinely used laboratory procedures for detecting the presence of AIVs and other respiratory poultry pathogens (See figures of routine amplifications of AIV for M-gene, H7N3, H5N1, H9N2, NDV, IBV and ILTV Figures 12 – 18).

Data Calculation for Comparative Laboratory Techniques and RT-PCR

1. TOTAL SAMPLES PROCESSED= **150**
2. RAPID KIT TEST= **05** +VE REMAINING= **145** **%RKT= 5/150=3.33%**
3. IN-OVO INOCULATION= **25**+VE REMAINING= **125** HA+VE= **25/150=16.6%**
4. IN-OVO INOCULATION (P1)= **42**+VE HA –VE= **108** **42/150=28%**
5. VNT= **42** (H7N3 **14**, H9N2 **12**, H5N1 **06**, NDV **10** +VE SAMPLES) **42/42=100%**
6. RT-PCR OF **42** HA +VE ISOLATES FOR M-GENE
7. **(M-GENE +VE)= 32**+VE **32/42=76.1%**
8. M-GENE –VE=**10** **10/42=23.8%**

9. RT-PCR FOR **32** AIV(H7, H9, H5) AND **10** NDV=**(H7 14, H5 6, H9 12, NDV 10)=42/42=100%**

10. RT-PCR OF HA –VE SAMPLES FOR M-GENE=**108**

11. M-GENE +VE= **27= 27/108=25%** M-GENE –VE =**81=81/108=75%**

12. RT-PCR 27 M-GENE +VE SAMPLES FOR AIV (H7, H9, H5), NDV AND IBV **(H7N3 11, H9N2 14, H5N1 02, NDV 13, IBV 5)= 45+VE=45/108=41.66% 63-VE**

13. PCR OF **63** RT-PCR –VE SAMPLES (TISSUE HOMOGENATES) FOR ILTV **03+VE FOR ILTV= 03/63=4.76%**

Hence the total number of viruses found positive by RT-PCR were as follows:-

H7N3= **25**+VE

H9N2= **26**+VE

H5N1= **08**+VE

NDV= **23**+VE

IBV= **05**+VE

14. TOTAL NUMBER OF SAMPLES +VE BY PCR

ILTV= **03**+VE

Sensitivity of RT-PCR/PCR

As regards its sensitivity limit it was found out to be 10ng/ml for AIV H9 (Figure 7), 5.24ng/ml for AIV H5 (Figure 6) and H7 (Figure 8).

Specificity of RT-PCR/PCR

None of the H5, H7 and H9 primers reacted with the RNA from IBV and NDV, likewise none of the primers for IBV and NDV reacted with RNA of H5, H7 and H9 (Figure 9, 10 and 11).

RESULTS MUTIPLEX RT-PCR

Multiplex RT-PCR was optimized to detect and simultaneously differentiate three hemagglutinin subtypes of H7, H9, H5. This was named as trivalent mRT-PCR (Figure 19).

It was also optimized to detect and differentiate influenza A and three other common respiratory pathogens NDV, IBV and ILTV. This was named as tetravalent mRT-PCR (Figure 20).

Multiplex RT-PCR was also optimized to detect and differentiate three haemagglutin subtypes of H7, H9 and H5 with the three common respiratory pathogens NDV, IBV and ILTV. This was called hexavalent mRT-PCR (Figure 21).

Throughout the development of mRT-PCR, various modifications were made to the annealing temperature, extension time, cycle quantity, primer concentration and template dilutions. The multiplex-PCR products consisted of 300bp for H7, 808bp for H9, 456bp for H5, 149bp for IBV, 320bp for NDV and 647bp for ILTV and were visualized by gel electrophoresis (Fig. 19, 20 and 21). The standardized test was employed to test various field cases (listed in Table 7 and 8). Some isolates were used for the standardization procedure and these were the viruses that were obtained from the repository of NRLPD-NARC, Pakistan or from live virus vaccines from the market. The detection of the virus by mRT-PCR is indicated by a + sign when used with the specific primers in the three formats of multiplex RT-PCR standardized.

Sensitivity of mRT-PCR

The sensitivity of mRT-PCR, depicting the limit of the method by visualization of PCR-amplified DNA products was Ing/ml for the hemagglutinin subtypes of AIV's (H7, H9 H5), and type A AIV's. The detection limit was 100pg/ml for IBV, NDV, and ILTV. No spurious PCR amplification reactions were observed in the detection of AIV subtypes H7, H9 and H5 and other respiratory pathogens with various concentrations of DNA and RNA mixtures. All the negative controls used were negative. Negative controls had no template RNA /DNA with just the primers and buffering system provided by the manufacturer in the kit, added (SuperScript One-Step RT-PCR with Platinum Taq, Cat# 10928-042, Invitrogen, USA).

Specificity of mRT-PCR

Specificity of mRT-PCR was determined by examining the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV and ILTV. The specificity of the primers used to detect amplified DNA's was hence determined as no specific amplification bands of the same sizes (300, 808, 456) could be amplified for RNA/DNA of other poultry respiratory pathogens (NDV, IBV, ILTV), and likewise no amplification bands of the sizes (320, 149, 647bp) could be amplified for RNA for AIV's.

RESULTS OF SEQUENCING OF AIV H7N3 ISOLATES AT THE NS1, HA1 AND NA GENE LEVELS

The complete coding sequences for the NS1 gene segment of 22 H7N3 AIV isolates from various areas of Pakistan during a ten year period were sequenced (Figure 58 – 79). The HA1 coding sequence (Figure 80 – 85) and the complete coding sequence for the NA gene segments (Figure 86 – 91) of six of these 22 H7N3 isolates typically belonging to the Northern Areas of Pakistan were sequenced subsequently. List of AIV H7N3 used in the study with reference to information regarding their dates/year of isolation for NS1, HA, NA genes and GenBank Accession numbers are given in Table 10 and 11.

Genomic Sequence and Phylogenetic Analyses for NS1 gene

Nucleotide and predicted amino acid sequences for the NS1 gene from various influenza A isolates from the GenBank were examined including the 22 newly sequenced isolates over 693 nucleotides in length. Size variation in the predicted amino acid sequence of each NS1 was revealed with two different levels of carboxy-terminal truncation in those isolates (Table 12). Of the 22 isolates analyzed, 02 isolates A/Chicken/Pakistan/NARC-100/04 (EF688541.1) and A/Chicken/Pakistan/NARC-1282/04 (EF688535.1) encoded a full length NS1 protein of 230 amino acids, whereas 20 encoded a truncated protein of 217 amino acids (Table 12). The isolates exhibiting the truncated carboxy terminal NS1 protein clustered together and appeared to be closest to A/Duck/Jiang Xi/6146/03(H5N3) (EF597369.1), A/Duck/Hong Kong/610/79(H9N2) (AF523517.1) and A/Aquatic Bird/Korea/CN-1/04(H3N6) (EU301337.1) at the nucleotide level and amino acid level (Table 14 and Figure 22). In contrast, the nucleotide sequence of one of the isolates with the full length NS1 protein (A/Chicken/Pakistan/NARC-1282/04) showed 99.9% nucleotide homology and 99.6% deduced amino acid level homology to a set of Italian H7N3 isolates of Turkey from 2002 at the NS1 gene e.g A/turkey/Italy/8912/2002(H7N3) (CY020609.1) and to A/turkey/Italy/214845/02(H7N3) (AY586444.1) (Table 14, Figure 23). This isolate formed a separate clade with Italian H7N3 viruses at the NS1 gene and didn't resemble any Pakistani H7N3 sequenced. The other isolate (A/Chicken/Pakistan/NARC-100/04) with the full length NS1 protein showed the highest nucleotide homology (95.9%) with the NS1 gene of an H5N7 subtype virus A/mallard/Denmark/64650/03(H5N7) (DQ251446.1) and showing 95% amino acid homology with it (Table 14, Figure 24). It showed no resemblance to any Pakistani H7N3 isolates at the NS1 gene. Furthermore, homologies at the nucleotide and amino acid levels among the Pakistani H7N3 isolates were calculated (Table 14).

The isolate A/Chicken/Pakistan/NARC-BM/00(H7N3) with a truncated NS1 protein, isolated in the spring of 2000 from Chakwal, Punjab province, showed a 100% homology at the nucleotide and deduced amino acid level with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), and A/Chicken/Pakistan/NARC-1/95(H7N3) for the NS1 gene, whereas it showed a 96.4% nucleotide homology and

95% deduced amino acid homology with A/Duck/Jiang Xi/6146/03(H5N3). Hence, for the NS1 gene, the H7N3 viruses from later years resembled the isolate from year 2000 and were probably of the same lineage and did not undergo any change while circulating in the Punjab and Northern Areas of Pakistan (Table 14, Figure 25). The NS1 gene of H7N3 AIV resembled NS1 of other AIV subtypes from Eurasia, China and South East Asia from later years, probably sharing an ancestral virus strain.

The isolate A/Chicken/Pakistan/NARC-214/04(H7N3) with a truncated NS1 protein, isolated at the start of the summer season from Mansehra, KPK (Khyber Pakhtoon Khuwa), in 2004, showed a 99.9% nucleotide homology and 99.6% deduced amino acid homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), A/Chicken/Pakistan/NARC-BM/00(H7N3), whereas it showed a 97.8% nucleotide level and 95.5% deduced amino acid level homology with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03(H5N3). The H7N3 isolates circulating in Punjab and Northern Areas of the years 1995, 2000 and 2001 showed slight changes at the NS1 gene from the isolate of Mansehra in 2004 hence were ancestral viruses for A/Chicken/Pakistan/NARC-214/04(H7N3). The 2005 isolate from Abbotabad carried over these differences later on. Hence these were viruses belonging to the same lineage and had been circulating in the area for nearly a decade (Table 14, Figure 26).

The isolate A/Chicken/Pakistan/NARC-108/04(H7N3), isolated at the start of the summer season from Mansehra, KPK (Khyber Pakhtoon Khuwa), in 2004, showed a 100% nucleotide and deduced amino acid level homology with A/Chicken/Pakistan/NARC-74/04(H7N3) and A/Chicken/Pakistan/NARC-143/04(H7N3) hence were probably the same set of viruses circulating in the Northern Areas of Pakistan. Whereas it showed a 98.3% nucleotide and 95.5% deduced amino acid level homology with A/Duck/Jiang Xi/6146/03(H5N3) and 98.2% nucleotide and 95.5% deduced amino acid level homology with A/Duck/Hong Kong/610/79 (H9N2). The NS1 gene showed some resemblance to AIV subtypes from Eurasia, China and South East Asian region. They probably shared the same ancestral stock (Table 14, Figure 27).

The isolate A/Chicken/Pakistan/NARC-2402/05(H7N3) with a truncated NS1 protein, isolated at the start of the summer season from Abbotabad, KPK (Khyber Pakhtoon Khuwa), in 2005, showed a 100% nucleotide and 100% deduced amino acid level homology with A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), A/Chicken/Pakistan/NARC-BM/00(H7N3). A 97.7% nucleotide and 95% deduced amino acid level homology with A/Aquatic Bird/Korea/CN-1/04 (H3N6) and A/Duck/Jiang Xi/6146/03 (H5N3) was seen. Hence, the H7N3 viruses from previous years resembled the isolate from year 2005 and were probably of the same lineage and did not undergo any change at the NS1 gene while circulating in Punjab and Northern Areas. The NS1 gene resembled other AIV subtypes circulating in Eurasia, China and South East Asia (Table 14, Figure 28).

The isolate A/Chicken/Pakistan/NARC-74/04(H7N3) with a truncated NS1 protein, isolated in the spring season from Mansehra, KPK (Khyber Pakhtoon Khuwa), in 2004, showed a 100% nucleotide and deduced amino acid level homology with A/Chicken/Pakistan/NARC-108/04(H7N3) and A/Chicken/Pakistan/NARC-143/04(H7N3). These were of the same lineage, circulating in the same area and undergoing no change at NS1 gene level. A 96.4% nucleotide and 95.5% deduced amino acid level homology was seen with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03(H5N3), whereas a 96.3% nucleotide and 95.5% deduced amino acid level homology was seen with A/Duck/Hong Kong/610/79(H9N2). A 96.1% nucleotide and 95.5% deduced amino acid level resemblance was observed with A/Duck/Jiang Xi/1742/03 (H7N7) showing resemblance at the NS1 gene with these AIV subtypes from Eurasia, China and South East Asia (Table 14, Figure 29).

The isolate A/Chicken/Pakistan/NARC-1/95(H7N3) with a truncated NS1 protein, isolated in 1995 from Murree, Punjab province but adjacent to KPK (Khyber Pakhtoon Khuwa), showed a 100% nucleotide and deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) showing that this was the ancestral strain to these viruses isolated in later years from the Punjab and Northern Areas of Pakistan and no change occurred at the NS1 gene level. A 98.1% nucleotide and 95% deduced

amino acid level homology was seen with A/Aquatic Bird/Korea/CN-1/04 (H3N6) and some sharing of nucleotide sequence was observed (Table 14, Figure 30).

The isolate A/Chicken/Afghanistan/ NARC-483/ 04(H7N3) with a truncated NS1 protein, isolated in the midsummer season from Tora Bora, Afghanistan, an area adjacent to the border of Pakistan with Afghanistan, in 2004, showed a 99.9% nucleotide and 100% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) showing the circulation of the same viral stock in the Punjab, Northern areas of Pakistan and adjacent border areas with Afghanistan. This isolate showed a 97.6% nucleotide and 95% deduced amino acid level homology with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03 (H5N3) with some resemblance at the NS1 gene level with other AIV subtypes from Eurasia, China and South East Asia (Table 14, Figure 31).

The isolate A/Chicken/Pakistan/NARC-N19/05(H7N3) with a truncated NS1 protein, isolated in the winter season of 2005 from Sumundri, Punjab province, showed a 99.7% nucleotide and 99.6% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/ 05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), A/Chicken/Pakistan/NARC-BM/00(H7N3) showing the circulation of the same viral stock in the Punjab and Northern Areas of Pakistan with slight changes in the NS1 gene. A 96.3% nucleotide and 95.5% deduced amino acid level homology was seen with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03(H5N3) indicating the resemblance at NS1 gene of various subtypes of AIVs (Table 14, Figure 32).

The isolate A/Chicken/Pakistan/NARC-35/01(H7N3) with a truncated NS1 protein, isolated from Chakwal, Punjab in 2001, showed a 100% nucleotide and deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), and A/Chicken/Pakistan/NARC-BM/00(H7N3) indicating the circulation of the same set of H7N3 AIVs in Punjab and Northern Areas of Pakistan. A 97.8% nucleotide and 95% deduced amino acid level homology was seen

with A/Aquatic Bird/Korea/CN-1/04(H3N6) showing some resemblance with Eurasian, Chinese and other South East Asian AIV subtypes at the NS1 gene (Table 14, Figure 33).

The isolate A/Chicken/Pakistan/NARC-178/04(H7N3) with a truncated NS1 protein, isolated in the autumn of 2004 from Mansehra, KPK (Khyber Pakhtoon Khuwa), showed a 99.8% nucleotide and 99.1% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/ 05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), and A/Chicken/Pakistan/NARC-BM/00(H7N3) showing the circulation of the same set of H7N3 AIVs with slight changes at the NS1 gene from the Punjab and North of Pakistan. It showed a 97.5% nucleotide and 95.1% deduced amino acid level homology with A/Duck/Jiang Xi/6146/03 (H5N3) indicating the circulation of various AIV subtypes showing some resemblance at the NS1 gene belonging to Pakistan, Eurasia, China and South East Asia (Table 14, Figure 34).

The isolate A/Chicken/Pakistan/NARC-N30/05(H7N3) with a truncated NS1 protein, isolated in the spring season of 2005 from Faisalabad, Punjab province, showed a 99.3% nucleotide and 100% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) indicating the circulation of AIV H7N3 with slight changes at the NS1 gene in the Punjab and North of Pakistan. A 95% nucleotide and deduced amino acid level homology was seen with A/Duck/Hong Kong/610/79 (H9N2) showing resemblance with other AIV subtypes from Eurasia, China and South East Asia with some conservation of nucleotide sequence for the NS1 gene (Table 14, Figure 35).

The isolate A/Chicken/Pakistan/NARC-160/04(H7N3) with a truncated NS1 protein, isolated from Rawalpindi in the province of Punjab during the summer of 2004, showed a 99.9% nucleotide and 99.6% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) showing slight variation in NS1 gene for these H7N3 AIVs from Punjab and the North of Pakistan. A 96.4% nucleotide and 95% deduced amino acid level

homology was seen with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03(H5N3) indicating the sharing of some sequence of the NS1 gene with other AIV subtypes from Eurasia, China and South East Asia (Table 14, Figure 36).

The isolate A/Chicken/ Pakistan/NARC-23/03(H7N3) with a truncated NS1 protein, isolated in the winter season from Karachi, Sindh province in 2003, showed a 99.9% nucleotide and 99.6% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) indicating the circulation of nearly the same set of viruses all over the country. A 98.1% nucleotide and 95% deduced amino acid level homology was seen with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03(H5N3) indicating the resemblance of isolates from the Sindh province with other AIV subtypes from Eurasia, China and South East Asia (Table 14, Figure 37).

The isolate A/Chicken/Pakistan/NARC-2419/05(H7N3) with a truncated NS1 protein, isolated in the summer season of 2005 from Abbotabad, KPK (Khyber Pakhtoon Khuwa), showed a 99.9% nucleotide and 99.6% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/ 05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) showing the circulation of the same set of H7N3 AIV stock for nearly a decade with slight changes at the NS1 gene. A 97.6% nucleotide and 95.5% deduced amino acid level homology was seen with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/6146/03(H5N3) indicating some resemblance of Pakistani H7N3 with other AIV subtypes from Eurasia, China and South East Asian region (Table 14, Figure 38).

The isolate A/Chicken/Pakistan/NARC-143/04(H7N3) with a truncated NS1 protein, isolated from Mansehra, KPK (Khyber Pakhtoon Khuwa), in the summer season in 2004, showed a 100% nucleotide and deduced amino acid level homology with A/Chicken/Pakistan/NARC-74/04(H7N3) and A/Chicken/Pakistan/NARC-108/04(H7N3) indicating the circulation of the same set of H7N3 viruses in the Northern areas of

Pakistan showing remarkable conservation at the NS1 gene. It showed a 97.8% nucleotide and 95.5% deduced amino acid level homology with A/Duck/Jiang Xi/6146/03(H5N3) showing resemblance with other AIV subtypes from Eurasia, China and South East Asia (Table 14, Figure 39).

A/Chicken/Pakistan/NARC-70/04(H7N3) with a truncated NS1 protein, isolated in the spring of 2004 from Murree, Punjab province, showed a 99.7% nucleotide and 99.6% deduced amino acid level homology with A/Chicken/Pakistan/NARC-2402/ 05(H7N3), A/Chicken/Pakistan/NARC-35/01(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3) and A/Chicken/Pakistan/NARC-BM/00(H7N3) showing the circulation of relatively conserved NS1 among the H7N3 from the Punjab and Northern areas of Pakistan. It also showed a 96.3% nucleotide and 95.5% deduced amino acid level homology with A/Aquatic Bird/Korea/CN-1/04(H3N6) and A/Duck/Jiang Xi/1742/03(H7N7) showing the sharing of some sequence with NS1 of other AIV subtypes from Eurasia, China and South East Asia (Table 14, Figure 40).

A Phylogenetic tree of all Pakistani H7N3 AIV isolates in the GenBank for the NS1 gene was constructed to show the relative resemblance level with each other (Figure 41). The cladogram showed that A/Chicken/Pakistan/NARC-100/04(H7N3) occupied a separate branch on the unrooted tree indicating no resemblance with other Pakistani H7N3 isolates for the NS1 gene. Same was the case with A/Chicken/Pakistan/NARC-1282/04(H7N3), which showed no resemblance with other Pakistani H7N3 isolates for the NS1 gene and occupied a separate branch on the cladogram. Rest of the H7N3 AIVs from the year 1995-2005 isolated from various poultry farms all across the country resembled each other indicating the circulation of the same set of viruses during the 10 year period indicating a certain level of conservation in the NS1 gene. These viruses had a truncated NS1 protein and supplanted the growth of untruncated strains hence were more common in circulation.

Genome Analysis of NS1 gene

The gene sequence of the NS1 gene for the 22 H7N3 AIV isolates was analyzed. For the 217 amino acids truncated protein, a C (Cytosine) to T (Thymine) transversion was

observed at the nucleotide position 652 resulting in a TAG stop codon leading to a truncated NS1 protein of 217 amino acids. The untruncated strains had a C (Cytosine) at position 652 hence had a full length NS1 protein of 230 amino acids. This indicated a point mutation at position 652 at the nucleotide level in the truncated H7N3 strains sequenced and hence - Genetic Drift (Table 13).

Study of specific NS1 protein domains in comparison to Consensus A sequence

Specific regions of the NS1 protein for the 22 H7N3 Pakistani isolates were compared to Consensus A sequence published previously, including two nuclear localization signals, an effector domain, and the RNA binding site (Table 15, 16, 17 and 18). The RNA binding domain stretching from amino acid 19 - 38 of all the isolates studied were similar to the consensus sequence showing conservation in this region (Table 15). The nuclear localization signal 1 domain spanning amino acid 34 – 38 showed complete identity with the consensus A sequence for both the truncated and the untruncated strains hence showing conservation in this region (Table 16). The effector domain defining amino acids 138 – 147 of the NS1 protein showed an N (Asparagine) at position 143 instead of T (Threonine) in the truncated H7N3 isolates sequenced showing that they were variants, whereas the untruncated strains sequenced showed T (Threonine) matching with the consensus A. The untruncated isolate A/Chicken/Pakistan/NARC-100/04 H7N3 had D (Aspartic Acid) instead of E (Glutamic Acid) at position 142 showing a variation from the consensus A sequence (Table 17). The nuclear localization signal 2 domain spanning amino acids 216 – 221 of the NS1 protein showed a truncation at position 218 for the truncated H7N3 isolates showing a variation from consensus A sequence which was showing a complete protein of 230 amino acids. The untruncated strains sequenced showed a match for this nuclear localization signal 2 site with consensus A having Q (Glutamine) at position 218 (Table 18). In total more variation was seen in the truncated H7N3 strains as compared to the untruncated ones for the specific NS1 protein domains.

Genomic Sequence and Phylogenetic Analyses for HA gene

Out of these 22 H7N3 isolates sequenced for the NS1 gene, 6 isolates from the Northern parts of Pakistan were further sequenced for the HA genes and were compared with other

Pakistani H7N3 sequences in the GenBank for sequence identity. Strains with higher sequence identities are generally thought to share a relatively recent common ancestor. One of the isolates had an untruncated NS1 whereas 5 were truncated.

The 5 H7N3 isolates A/Chicken/Pakistan/NARC-1/95(H7N3), A/Chicken/Pakistan/NARC-70/04(H7N3), A/Chicken/Pakistan/NARC-74/04(H7N3), A/Chicken/Pakistan/NARC-2419/05(H7N3) and A/Chicken/Pakistan/NARC-214/04(H7N3) with truncated NS1 were HPAI, for the HA gene (Table 11) and showed the presence of typical highly pathogenic pattern **PETPKRKRKR*GLF** of deduced amino acid sequence at the HA cleavage site with the multiple presence of basic amino acids K(Lysine) and R(Arginine). Each of these H7N3 isolates were from disease outbreaks on large chicken farms, in breeder flocks, the viruses caused facial swelling, dark combs and variable mortality between 30-50% (personal communication).

One H7N3 isolate, A/Chicken/Pakistan/NARC-1282/04(H7N3) with untruncated NS1, showed a typical LPAI HA cleavage site sequence **PEIPKGR*GLF** of deduced amino acids (Table 11). This virus was isolated from breeder flock in Mansehra (KPK (Khyber Pakhtoon Khuwa)) where there was a 20 - 35% drop in egg production, no apparent respiratory disease signs and no mortality (personal communication).

The phylogenetic analyses of these H7N3 isolates for the HA gene indicated a close resemblance to other Pakistani isolate sequences in the GenBank, with the next closest resemblance to the H7N3 isolate from a Peregrine Falcon in U.A.E in the GenBank, A/Peregrine Falcon/U.A.E/188/2384/98H7N3. Nucleotide and deduced amino acid homologies were calculated with other GenBank sequences.

For the HA gene, A/Chicken/Pakistan/NARC-1/95(H7N3) isolated from Murree in 1995 showed 99.8% nucleotide level homology and 100% deduced amino acid level homology with A/Chicken/Pakistan/34669/95 (H7N3) (CY015033.1) and was probably the same lineage of viruses circulating in 1995. A 99.9% nucleotide and 99.7% deduced amino acid homology was observed with A/Chicken/Rawalpindi/NARC68/02(H7N3) (CY035823.1) and A/Chicken/Rawalpindi/NARC72/02(H7N3) (CY035839.1) as it shared a similar HPAI pattern at the HA cleavage site with all these isolates. This isolate

also showed a 97.7% nucleotide level and 98.8% deduced amino acid level homology (Table 20, Figure 42) with A/Peregrine Falcon/U.A.E/188/2384/98(H7N3) but it is unlikely that birds of prey were involved either in the introduction or spread of the H7N3 viruses. In the cladogram this isolate clustered with other Pakistani isolates from 1995, indicating the circulation of viruses of the same lineage in 1995 upto 2002 in the Northern areas and adjacent Rawalpindi. The motif **PETPKRRNR*GLF** reported for another 1995 isolate A/Chicken/Pakistan/16/95 (Banks *et al.*, 2000), was not observed in the recent isolates.

Another AIV A/Chicken/Pakistan/NARC-74/04(H7N3) isolated from Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan in 2004 showed 99% nucleotide and 98.9% amino acid homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) and A/Chicken/Rawalpindi/NARC72/02 (H7N3). All 3 isolates shared a HPAI deduced amino acid sequence at the HA cleavage site, and indicated the circulation of HPAI H7N3 in the Rawalpindi and Northern areas of Pakistan for 2 years, probably being viruses of the same lineage showing some point mutations in the HA gene. The cladogram indicates a clustering of Pakistani isolates from the years 1995 and 2002. The isolate A/Chicken/Pakistan/NARC-74/04(H7N3) clustered with these isolates but occupied a separate sub-branch indicating some differences in the HA gene from the other Pakistani isolates. A 97% nucleotide and 97.7% amino acid homology was found with A/Peregrine Falcon/U.A.E/188/2384/98 H7N3 (Table 20, Figure 44). Although phylogenetic analysis of the HA gene indicates that these strains are most closely related to A/Peregrine Falcon/UAE/188/2384/98 (H7N3) (Manvell *et al.*, 2000), it is unlikely that birds of prey were involved either in the introduction or spread of the H7N3 viruses.

Another isolate sequenced was A/Chicken/Pakistan/NARC-70/04(H7N3) which was isolated from Murree in the spring of 2004, showed 99.7% nucleotide and 99.1% amino acid level homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) and A/Chicken/Rawalpindi/NARC72/02(H7N3). It showed a 97.3% nucleotide and amino acid level homology with A/Peregrine Falcon/U.A.E/188/2384/98 (H7N3) (Table 20, Figure 46). A/Chicken/Pakistan/NARC-70/04(H7N3) clustered with other Pakistani isolates from 1995 and 2002 in the cladogram and had a cleavage site sequence belonging

to HPAI with the other clustering H7N3 viruses with some differences in the exact pattern.

Another AIV sequenced was A/Chicken/Pakistan/NARC-214/04(H7N3) which was isolated from Murree at the start of the summer season in 2004, showed a 98.9% nucleotide and 98.6% amino acid level homology with A/Chicken/Pakistan/CR2/95(H7N3) (AF202230), 98.9% nucleotide and 98.6% amino acid homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) and A/Chicken/Rawalpindi/NARC 72/02(H7N3). Here, a clustering was seen in the cladogram with other Pakistani isolates from 1995 and 2002 but the isolate in question occupied a separate sub-branch indicating a certain level of differences at the HA gene level. A 96.9% nucleotide and 97.4% deduced amino acid level homology was seen with A/Peregrine Falcon/U.A.E/188/2384/98 (H7N3), again it seems unlikely that birds of prey were involved (Table 20, Figure 47).

An isolate A/Chicken/Pakistan/NARC-2419/05(H7N3), from the Abbotabad region, isolated at the start of the summer season in 2005, showed a 98.8% nucleotide and 97.7% deduced amino acid level homology with A/Chicken/Pakistan/447/95(H7N3) (AF202226), a 98.8% nucleotide and 97.4% amino acid level homology with A/Chicken/Pakistan/CR2/95(H7N3) and A/Chicken/Pakistan/16/99/95(H7N3) (AF202233.1), a 98.8% nucleotide homology and 97.1% deduced amino acid level homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) and A/Chicken/Rawalpindi/NARC72/02(H7N3). Hence in the cladogram a clustering of the isolate in question was seen with the other Pakistani isolates from the years 1995 and 2002 but it occupied a separate sub-branch indicating differences in the HA gene due to circulation. A 96.9% nucleotide and 97.1% deduced amino acid level homology with A/Peregrine Falcon/U.A.E/188/2384/98(H7N3) was observed (Table 20, Figure 45).

Another isolate sequenced for the HA gene was A/Chicken/Pakistan/NARC-1282/04(H7N3) which was isolated from Mansehra in the winter season of 2004, showed exclusive nucleotide and deduced amino acid level homologies with a clade of Italian H7N3 viruses isolated from chicken and turkeys from various outbreaks in 2002 and

2003. The closest resemblance of 99.4% at the nucleotide level and 98.8% deduced amino acid level homology with A/Chicken/Italy/682/03(H7N3) was seen (Table 20, Figure 43). No resemblance to any of the Pakistani H7N3 isolates was observed.

A Phylogenetic tree was constructed for the HA gene, of all the H7N3 AIVs sequenced with other GenBank sequences (Fig 56). This cladogram showed a tight clustering of A/Chicken/Pakistan/NARC-2419/05(H7N3), A/Chicken/Pakistan/NARC-1/95(H7N3), A/Chicken/Pakistan/NARC-74/04(H7N3) and A/Chicken/Pakistan/NARC-70/04(H7N3) showing a close resemblance of these isolates with each other at the HA gene. The isolate A/Chicken/Pakistan/NARC-214/04(H7N3) occupied a branch some distance away from these isolates and showed a relative clustering with other Pakistani H7N3 isolates from the years 1995 and 2002.

Amino Acid Changes at Specific Sites in HA Protein

When compared to 1995 H7N3 AIV's there were novel substitutions observed in the amino acid residues at various positions. Some novel substitutions observed for the sequenced viruses as compared to the 1995 strains previously sequenced were as follows:- A/Chicken/Pakistan/NARC-1282/04 (G183D, P29S, I56V, I131M, T143A, S146A, P315S) (H7 numbering); A/Chicken/Pakistan/NARC-214/04: (I14N, N46D, I56F, R58S, A168D); A/Chicken/Pakistan/NARC-2419/05 (N46D, R58S, R139M, G214E, G279E); A/Chicken/Pakistan/NARC-74/04 (N46D, R58S); A/Chicken/Pakistan/NARC-70/04 (N46D, R58S) and A/Chicken/Pakistan/NARC-1/95 resembled A/Chicken/Pakistan/34669/95 previously sequenced (Table 22). The sequenced viruses probably showed an accumulation of a random set of mutations which did not contribute conclusively to a genetic divergence from the other previously sequenced Pakistani H7N3 viruses.

Glycosylation pattern of H7N3 AIV isolates for the HA gene

The HAs of the 6 H7N3 isolates had no differences in glycosylation patterns, with 3 potential sites at positions 12, 28 and 231 of the HA1 (H7 numbering). No additional glycosylation site was observed in these viruses. The glycosylation site at position 231 is

located on the globular head of the HA1. The inclusion of a glycosylation site at the globular head has been suggested to contribute, but does not conclusively designate viruses as highly pathogenic (Banks *et al.*, 2000).

Genomic Sequence and Phylogenetic Analyses for NA gene

The neuraminidase sequences of the Pakistan H7N3 isolates were compared to all of the completed available N3 sequences with special attention to changes in the NA protein indicative of adaptation to land based poultry (deletions in the NA stalk and the reduction in glycosylation) (Matrosovich *et al.*, 1999; Banks *et al.*, 2000). The 5 truncated H7N3 isolates for the NS1 Gene had a stalked NA protein as in H7N3 isolates reported in wild birds showing a close resemblance to other previously sequenced H7N3 Pakistani isolate sequences in the GenBank, whereas the untruncated NS1 H7N3 isolate A/Chicken/Pakistan/NARC-1282/04 had a deleted NA stalk region, deduced amino acid sequence showing a deletion of 24 amino acids in concordance with other Italian H7N3 isolates reflecting a probable introduction of a highly circulating virus in domestic poultry.

For the NA gene, A/Chicken/Pakistan/NARC-1/95(H7N3) isolated from Murree in 1995 showed a 99.7% nucleotide and a 99.3% deduced amino acid homology with A/Chicken/Pakistan/34668/95(H7N3) (CY035833.1) showing the circulation of the viruses of the same lineage in 1995. It showed a 94.6% nucleotide and a 94.2% deduced amino acid homology with A/Duck/Postdam/1689/85 (H2N3) (AY207509.1) from Germany (Table 21, Fig 48). The isolate clustered with other H7N3 isolates from Pakistan indicating a resemblance showing the circulation of viruses from the same lineage in the Northern Areas and adjacent Rawalpindi region.

Another AIV A/Chicken/Pakistan/NARC-74/04(H7N3) isolated from Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan in the spring of 2004, showed 98.1% nucleotide and 95.7% amino acid level homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) (CY035825.1) and A/Chicken/Rawalpindi/NARC72/02(H7N3) (CY035841.1). The isolate clustered with other Pakistani isolates from 1995 and 2002 (Table 21, Figure 50).

A 94.6% nucleotide and 87.6% deduced amino acid level homology was seen with A/Mallard/Italy/208/00(H5N3) (AY586414).

Another isolate sequenced was A/Chicken/Pakistan/NARC-70/04(H7N3) which was isolated from Murree in the spring of 2004, and it's level of homology was 99.6% at the nucleotide level and 99.5% at the amino acid level with A/Chicken/Pakistan/34668/95(H7N3), A/Chicken/Pakistan/34669/95(H7N3) (AY207504), A/Chicken/Rawalpindi/NARC72/02(H7N3) and A/Chicken/Rawalpindi/NARC68/02(H7N3). The isolate clustered with other Pakistani isolates from 1995 and 2002 showing strong resemblance to them and little change at the NA gene level from 1995 till 2004. It also showed a 94.4% nucleotide and 94.8% amino acid level homology with A/Duck/Postdam/1689/85 H2N3 from Germany (Table 21, Figure 52).

Another AIV sequenced was A/Chicken/Pakistan/NARC-214/04(H7N3) which was isolated from Murree at the start of the summer season in 2004, and for the NA gene it showed a 96.8% nucleotide and 92.5% amino acid level homology with A/Chicken/Pakistan/34669/95(H7N3), 96.8% nucleotide and 92.8% amino acid level homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) and A/Chicken/Rawalpindi/NARC72/02(H7N3). In the cladogram the isolate in question clustered with other Pakistani H7N3 isolates from the years 1995 and 2002 but it occupied a separate branch showing some differences at the NA gene level (Table 21, Figure 53). It also showed a 96% nucleotide level and 90.8% amino acid level homology with A/Mallard/Italy/43/01(H7N3), and a 95.8% nucleotide and 90.8% amino acid level homology with A/Mallard/Italy/33/01(H7N3).

An isolate A/Chicken/Pakistan/NARC-2419/05(H7N3), from the Abbotabad region at the start of the summer season in 2005, showed 99.6% nucleotide and 99.4% amino acid level homology with A/Chicken/Pakistan/34668/95(H7N3) and A/Chicken/Pakistan/34669/95(H7N3), whereas a 99.6% nucleotide level and 99.2% amino acid level homology with A/Chicken/Rawalpindi/NARC68/02(H7N3) and A/Chicken/Rawalpindi/NARC72/02(H7N3). In the cladogram, the isolate in question

tightly clustered with other Pakistani H7N3 isolates from 1995 till 2002 showing a close resemblance at the NA gene level. This further indicated the circulation of viruses of the same lineage in a 10 year period in the Northern Areas and adjacent Rawalpindi region. A 94.4% nucleotide and 93.4% amino acid level homology was seen with A/Duck/Postdam/1689/85 H2N3 from Germany (Table 21, Figure 51).

The isolate A/Chicken/Pakistan/NARC-1282/04(H7N3) which was isolated from Mansehra in the winter season of 2004, showed a nucleotide and amino acid level resemblance with a set of AIV's from Italy which were isolated from turkeys in 2002, 2003 (Table 21, Figure 49) e.g it showed a 99.8% nucleotide and 99.4% amino acid level homology with A/Turkey/Italy/214845/02(H7N3), A/Turkey/Italy/1010/03(H7N3), A/Turkey/Italy/8912/02(H7N3), A/Turkey/Italy/220158/02(H7N3) and A/Turkey/Italy/8535/02(H7N3). In the cladogram it showed a tight clustering with these isolates indicating a strong lineage with these Italian H7N3 viruses.

Another Phylogenetic tree was constructed for the NA gene, of all the H7N3 AIVs sequenced with other GenBank sequences (Figure 57). The cladogram showed a close resemblance between A/Chicken/Pakistan/NARC-214/04(H7N3) and A/Chicken/Pakistan/NARC-74(H7N3) showing a clustering with other Pakistani H7N3 in the GenBank. A/Chicken/Pakistan/NARC-70/04(H7N3) clustered with A/Chicken/Pakistan/NARC-01/95(H7N3), whereas A/Chicken/Pakistan/NARC-2419/05(H7N3) occupied a separate branch showing some differences at the NA gene level.

Amino Acid changes at Specific Sites in NA Protein

The H7N3 Pakistani isolates acquired a few amino acid changes in NA as compared to 1995 H7N3 Pakistani sequences in the GenBank: A/Chicken/Pakistan/NARC-2419/05 had T466I; A/Chicken/Pakistan/NARC-74 had A135P, N145K, K205E, E223K, G250S, S265D, K270E, K277T, R280G; A/Chicken/Pakistan/NARC-70/04 had T466I; A/Chicken/Pakistan/NARC-214/04 had A135P, N145K, K205E, E223K, G250S, S265D, K270E, K277T, R280G, R331S, C344R, D377N, A427T, S432G; A/Chicken/Pakistan/NARC-01/95 had in comparison to A/Chicken/Pakistan/34668/95

D98V and S469L; A/Chicken/Pakistan/NARC-1282/04 had N145K, K205E, E223K, G250S, S265D, K270E, K277T, R280G, R331S, C344R, D377N, A427T, S432G with a 24 amino acid deletion in the NA stalk region spanning amino acids 56 to 80.

Analysis of the NA glycosylation sites showed that all the isolates except A/Chicken/Pakistan/NARC-1282/04(H7N3) had 8 potential glycosylation sites at positions 14, 57, 66, 72, 143, 146, 308, and 435. Thus, these isolates showed no loss of potential glycosylation sites as seen in the land based isolates from the Italian lineage like A/Chicken/Pakistan/NARC-1282/04 which missed three potential sites at positions 57, 66 and 72 due to the aforementioned sequence deletion. Analysis of conserved regions coding for the hemadsorbing (HB) site of NA and enzymatic site were found to be conserved (Colman *et al.,* 1983; Kobasa *et al.,* 1997).

Phylogenetic analysis of the NA genes of the Pakistani isolates show that they all belong to the Eurasian branches of influenza viruses. The NA genes are part of an isolated clade in the overall polyphyletic Eurasian clade separated by some distance from H7N3 isolates from Italy and the H7N3 viruses from China and South East Asia, with the isolate A/Chicken/Pakistan/NARC-1282/04 being a part of the Italian cluster. Based on the homology and phylogenetic data, it is concluded that all these chicken isolates share a common ancestor, with regard to NA gene, possibly derived from other Eurasian groups that have diversified and evolved within the region. A/Chicken/Pakistan/NARC-1282/04(H7N3) was probably a chance introduction in the already circulating lot.

Genomic Sequence and Phylogenetic Analyses among the Pakistani H7N3 sequenced for HA and NA genes

Another comparison was done among the 6 H7N3 Pakistani isolates from the Northern Areas of Pakistan, sequenced for their HA and NA's at the nucleotide and deduced amino acid levels (Table 19).

The isolate A/Chicken/Pakistan/NARC-1/95(H7N3) had the closest resemblance to A/Chicken/Pakistan/NARC-70/04(H7N3), with a 99.6% nucleotide level and 98.9% deduced amino acid level homology for the HA gene (Table 19, Figure 54). For the NA

gene, this isolate showed a 99.1% nucleotide and 99% amino acid level homology with A/Chicken/Pakistan/NARC-70/04(H7N3) (Table 19, Figure 55).

The isolate A/Chicken/Pakistan/NARC-70/04 (H7N3) showed highest homology for the HA gene with A/Chicken/Pakistan/NARC-1/95(H7N3), with a 99.6% nucleotide level and 98.9% deduced amino acid level homology (Table 19, Figure 54). For the NA gene this isolate showed highest homology with A/Chicken/Pakistan/NARC-2419/05(H7N3) with 99.6% nucleotide and 99.5% deduced amino acid levels (Table 19, Figure 55).

For the HA gene the isolate A/Chicken/Pakistan/NARC-1282/04(H7N3), showed the highest resemblance to A/Chicken/Pakistan/NARC-1/95(H7N3), with a 79.6% nucleotide level and 80.6% deduced amino acid level homology (Table 19, Figure 54). For the NA gene, this isolate showed the highest homology with A/Chicken/Pakistan/NARC-214/04(H7N3), with a 90.5% nucleotide level and a 90.4% deduced amino acid level homology (Table 19, Figure 55).

The isolate A/Chicken/Pakistan/NARC-214/04(H7N3) for the HA gene showed a 99.9% nucleotide level and 99.7% deduced amino acid level homology with A/Chicken/Pakistan/NARC-74/04(H7N3) (Table 19, Figure 54). For the NA gene, the highest homology was seen with the same isolate with a 97.9% nucleotide level and 96.8% deduced amino acid level homology (Table 19, Figure 55). Identical was the level of similarity for the isolate A/Chicken/Pakistan/NARC-74/04(H7N3) for the HA and NA genes with A/Chicken/Pakistan/NARC-214/04(H7N3) (Table 19, Figures 54, 55)

A/Chicken/Pakistan/NARC-2419/05(H7N3) showed the highest homology for the HA gene with A/Chicken/Pakistan/NARC-1/95(H7N3) and A/Chicken/Pakistan/NARC-70/04(H7N3) with a 98.9% nucleotide level and 97.6% deduced amino acid level homology (Table 19, Figure 54). For the NA gene this isolate showed the highest resemblance to the same above mentioned isolates with a 94.7% nucleotide level and 94.6% deduced amino acid level homology (Table 19, Figure 55).

The overall study indicates a continued circulation of an antigenically and molecularly homogenous group of highly pathogenic H7N3 influenza viruses for a period of 10 years

from chicken farms in Pakistan's Northern areas indicating that the virus is not under pressure to change.

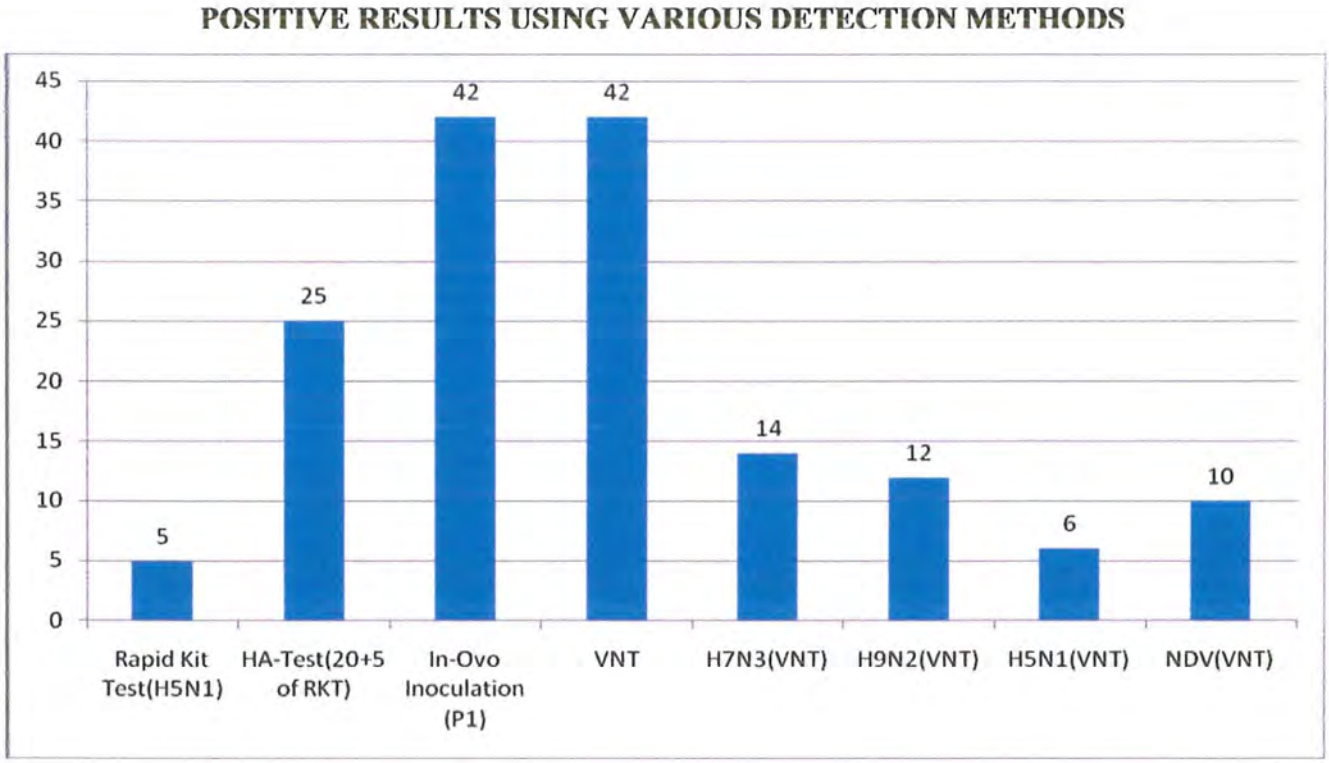

Figure-1 (a): Bar Graph showing results of RKT, HA Test, in-ovo inoculation and VNT of AIV samples analyzed

Total No of Samples Processed = 150

X-axis = Lab Diagnostic Procedures

Y-axis = No of Samples

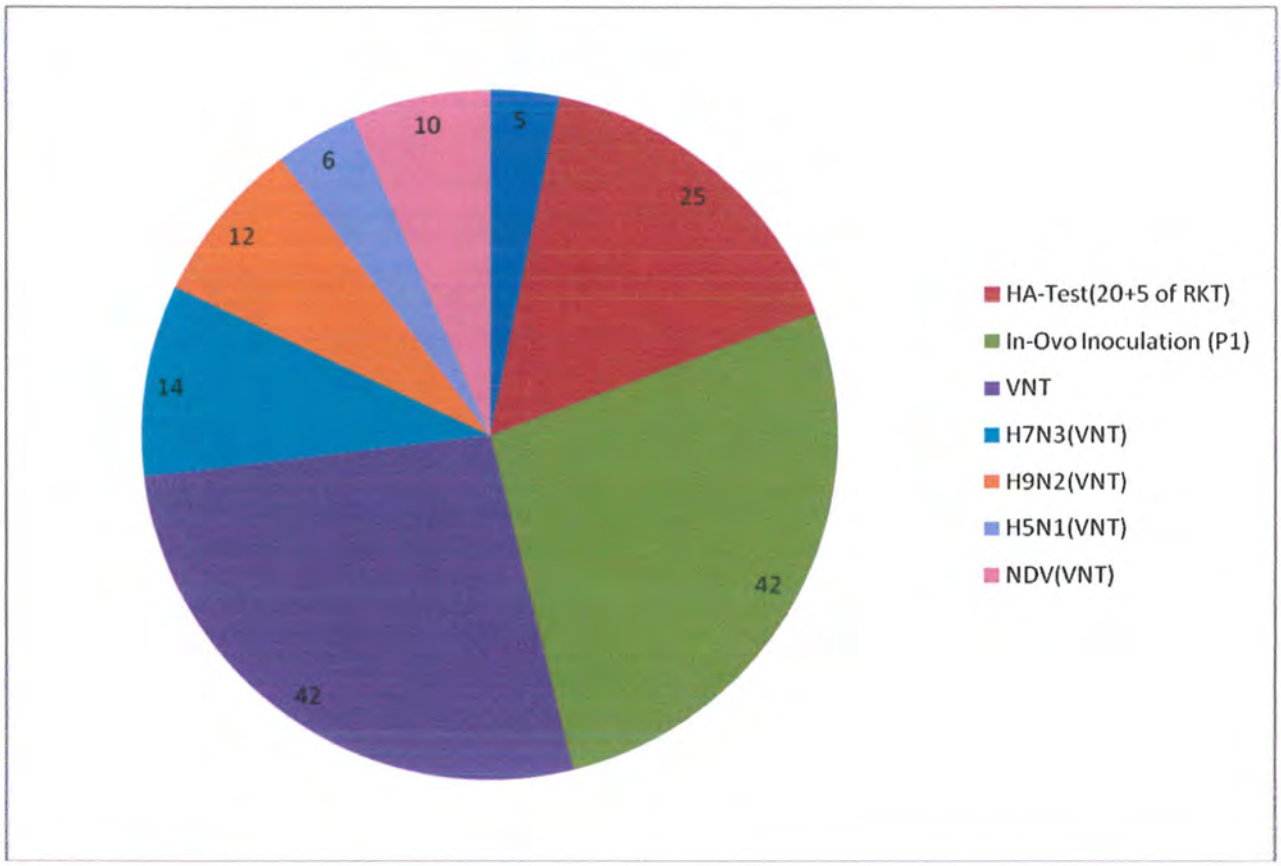

Figure-1 (b) Pie Chart showing results of RKT, HA Test, in-ovo inoculation and VNT of AIV samples analyzed (Proportionate share of Lab Diagnostic Procedures with total sample size of 150)

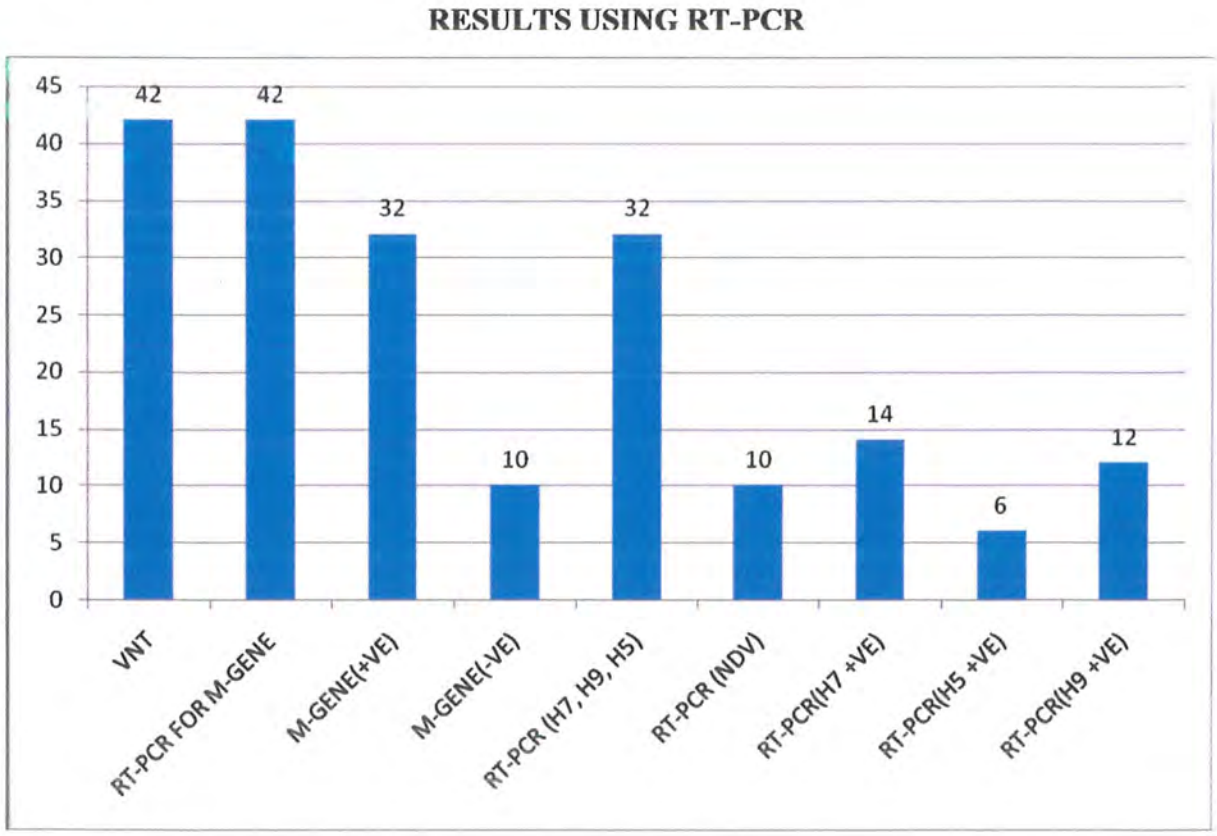

Figure-2 (a) Bar Graph showing results of RT-PCR for M-Gene, H7, H9, H5 subtypes of AIV and NDV

X-axis = Lab Diagnostic Procedures

Y-axis = No of Samples

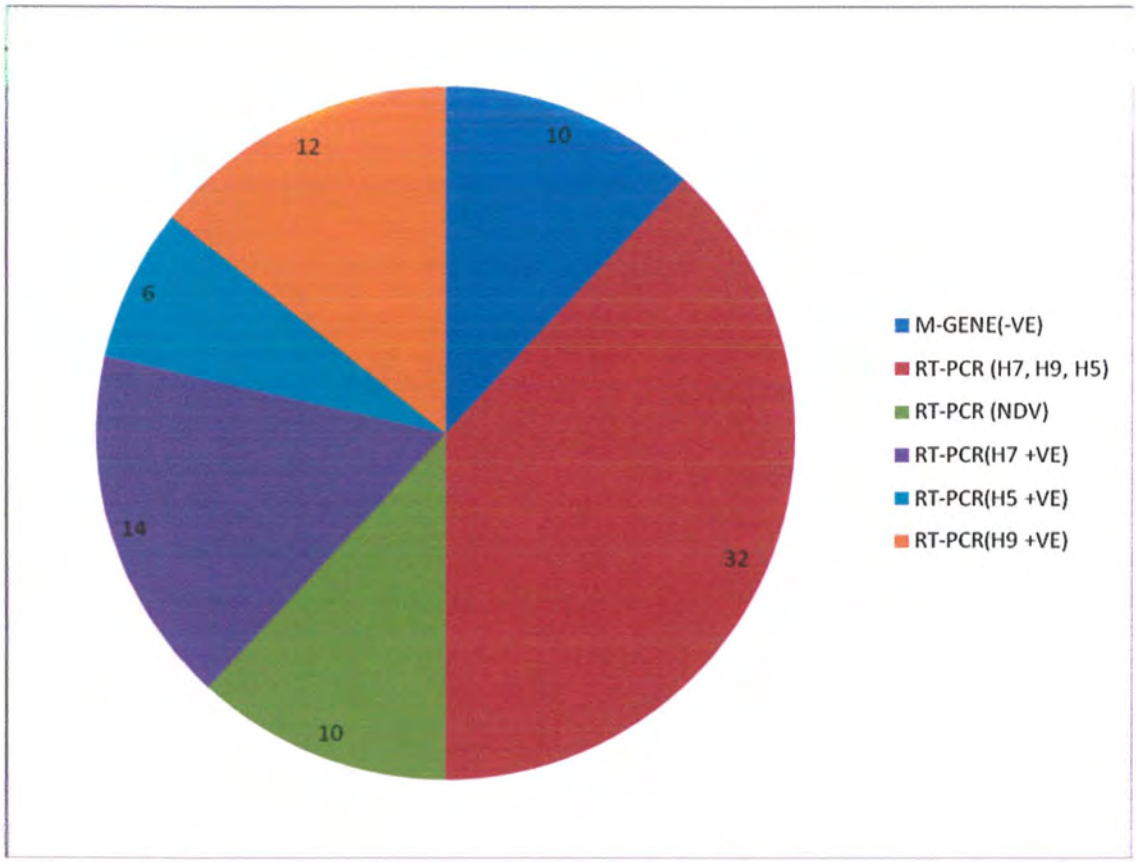

Figure-2 (b) Pie Chart showing results of RT-PCR for M-Gene, H7, H9, H5 subtypes of AIV and NDV

RT-PCR RESULTS SHOWING RESULTS FOR M-GENE AND HA GENE

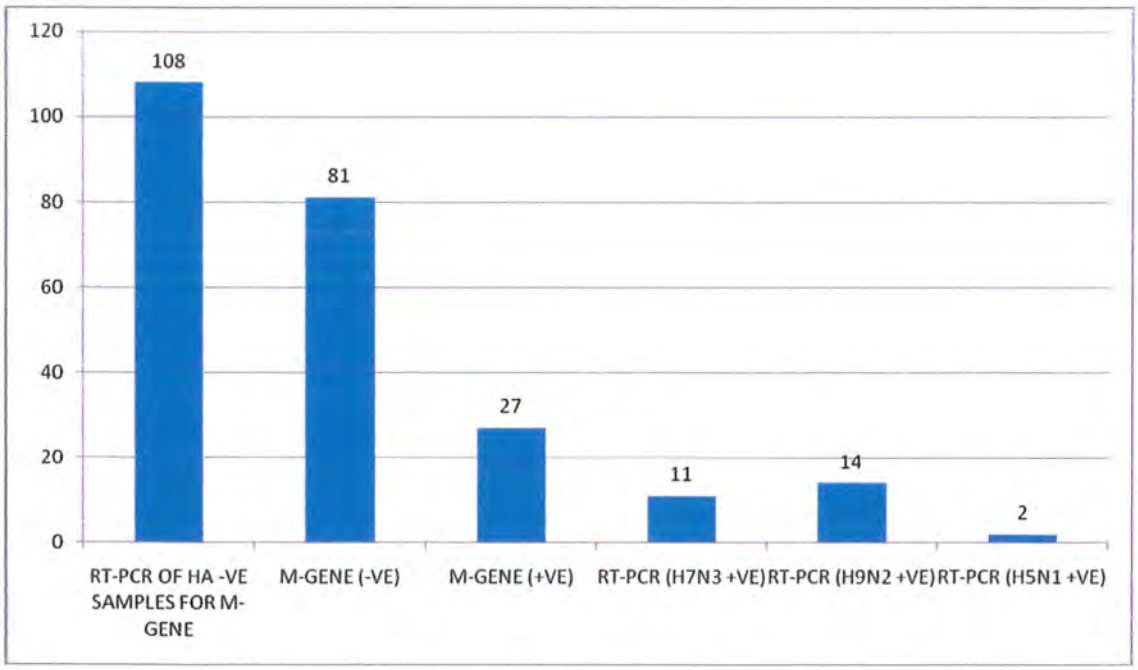

Figure-3 (a) Bar Graph showing results of RT-PCR of –ve samples for M-Gene. RT-PCR results of subsequent M-Gene +ve samples for H7, H9, H5 subtypes of AIV.

X-axis = Lab Diagnostic Procedures

Y-axis = No of Samples

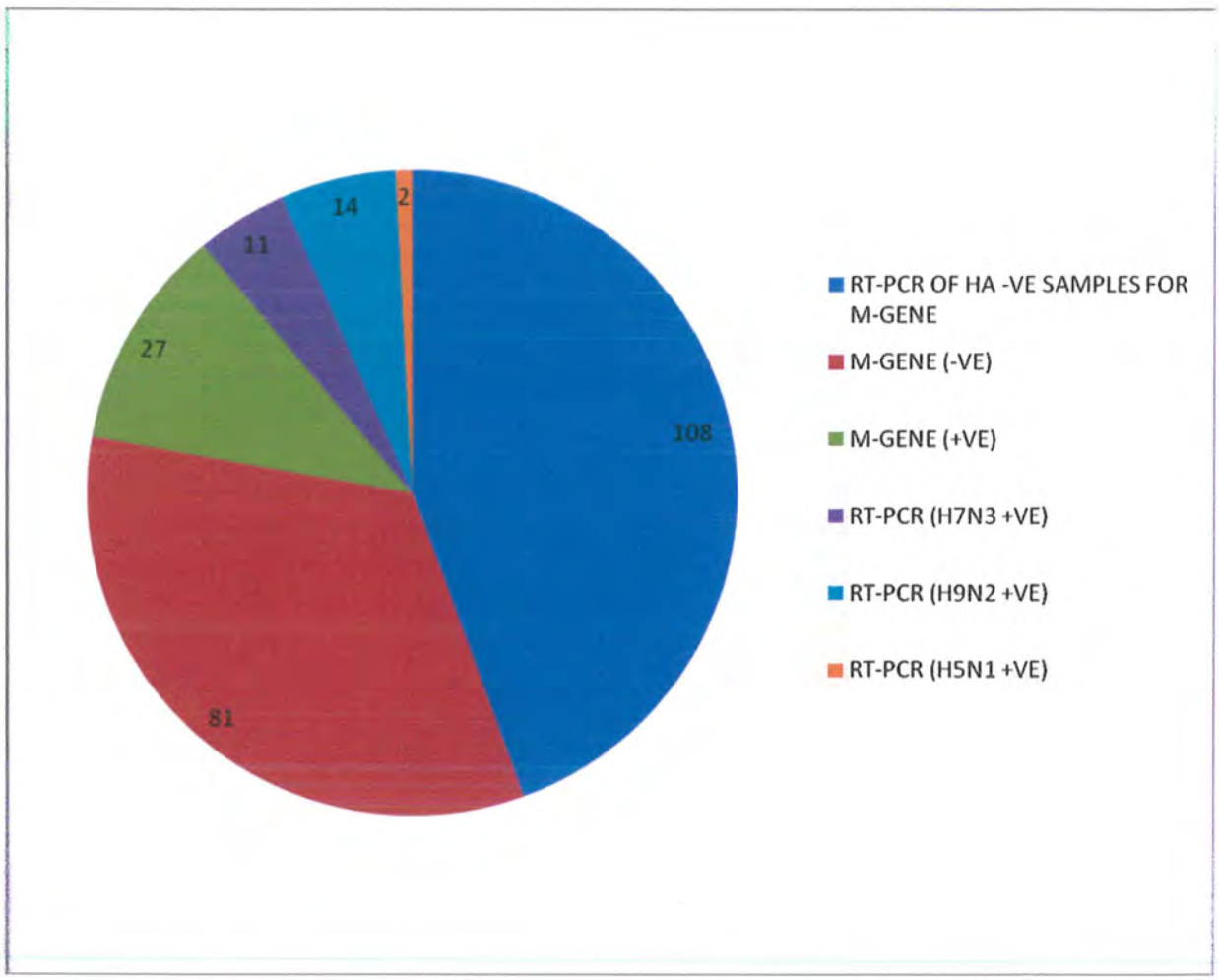

Figure-3 (b) Pie Chart showing results of RT-PCR of –ve samples for M-Gene. RT-PCR results of subsequent M-Gene +ve samples for H7, H9, H5 subtypes of AIV.

RT-PCR RESULTS FOR POSITIVE AND NEGATIVE SAMPLES

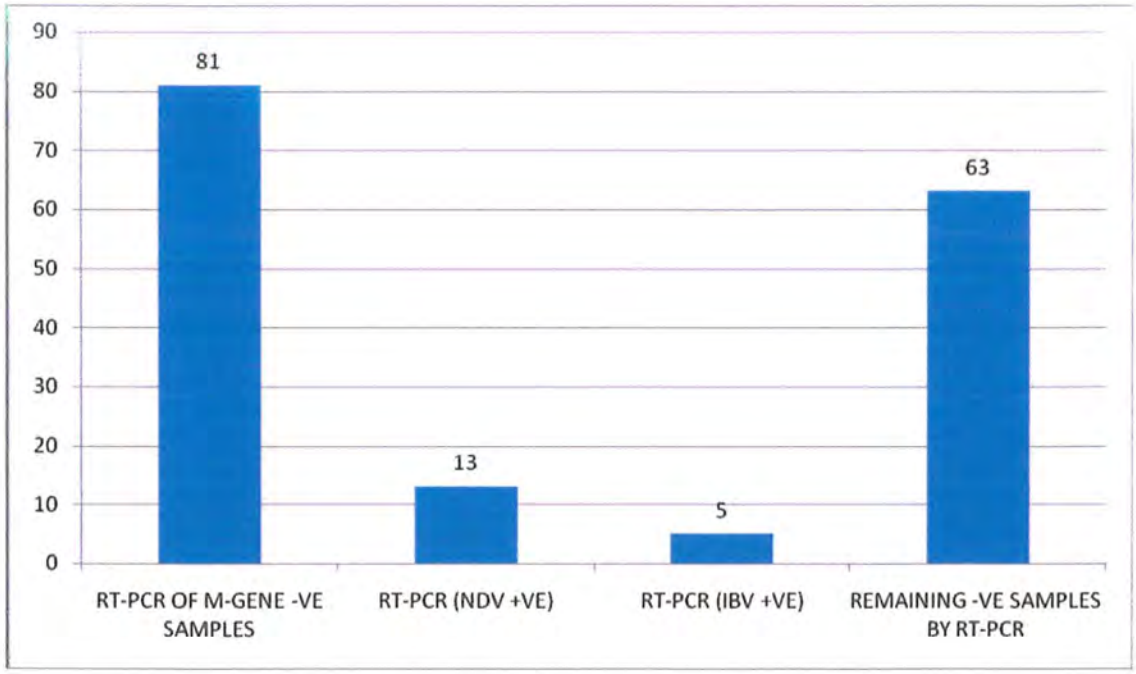

Figure-4 (a) Bar Graph showing RT-PCR of M-Gene –ve samples, subsequent +ve results of RT-PCR for NDV, IBV and remaining –ve samples by RT-PCR.

X-axis = Lab Diagnostic Procedures

Y-axis = No of Samples

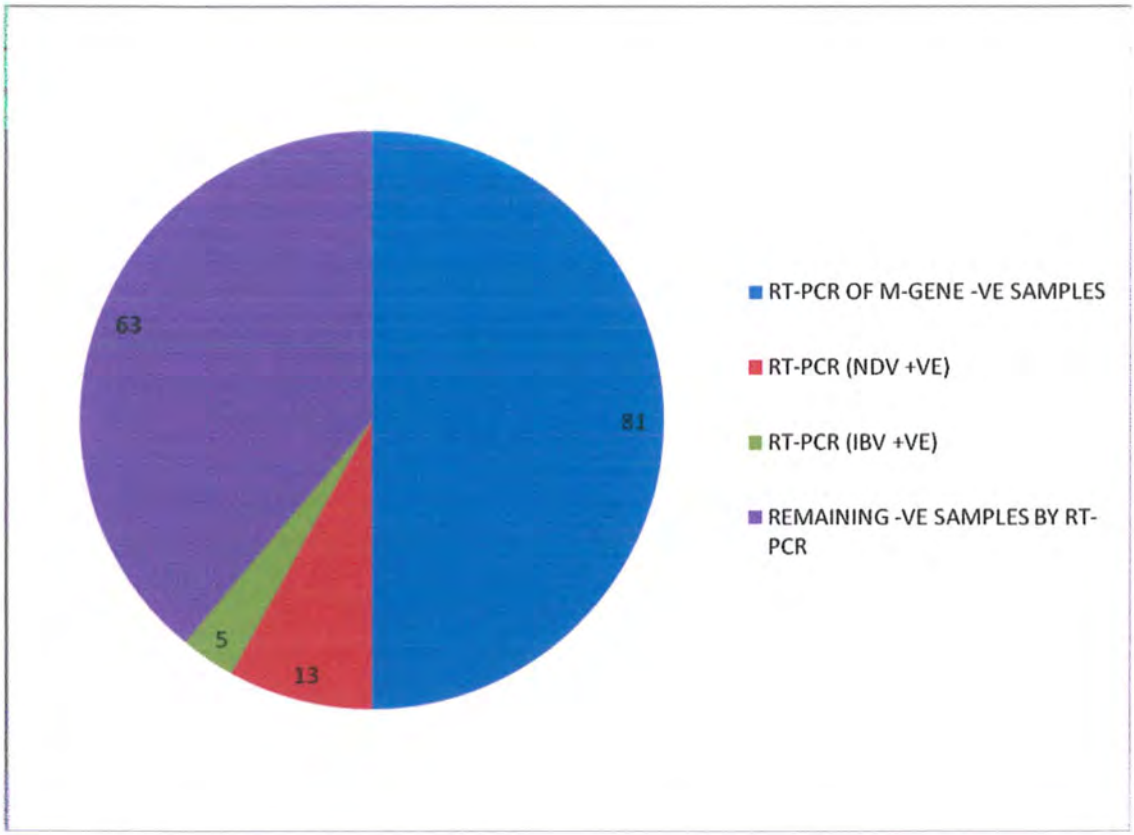

Figure-4 (b) Pie Chart showing RT-PCR of M-Gene –ve samples, subsequent +ve results of RT-PCR for NDV, IBV and remaining –ve samples by RT-PCR.

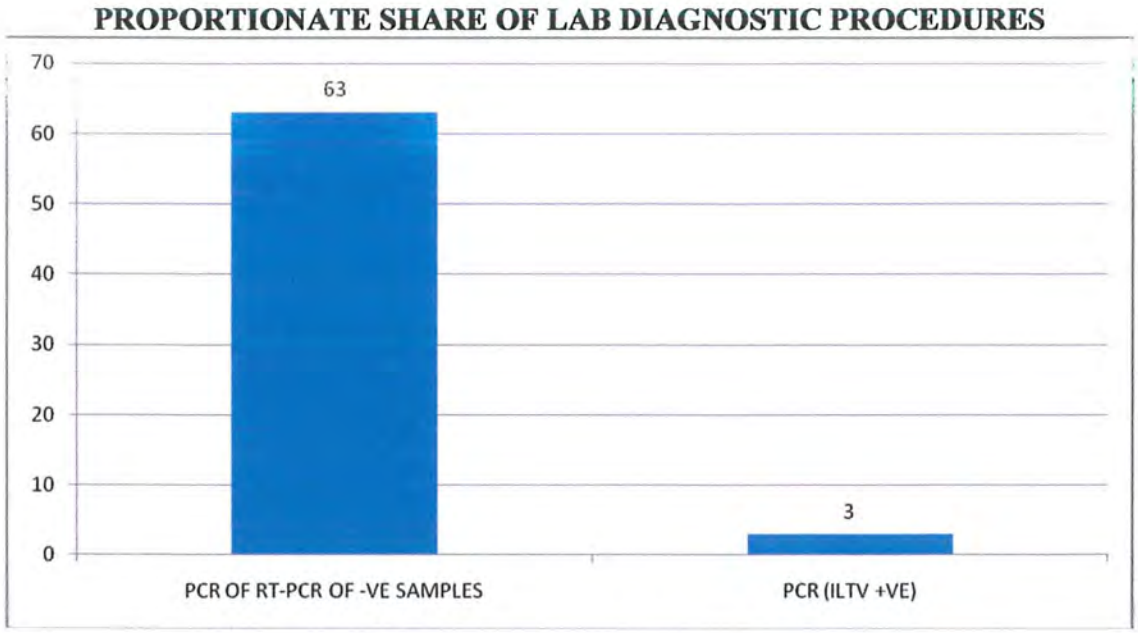

Figure-5 (a) Bar Graph showing results of PCR of RT-PCR –ve samples for ILTV

X-axis = Lab Diagnostic Procedures

Y-axis = No of Samples

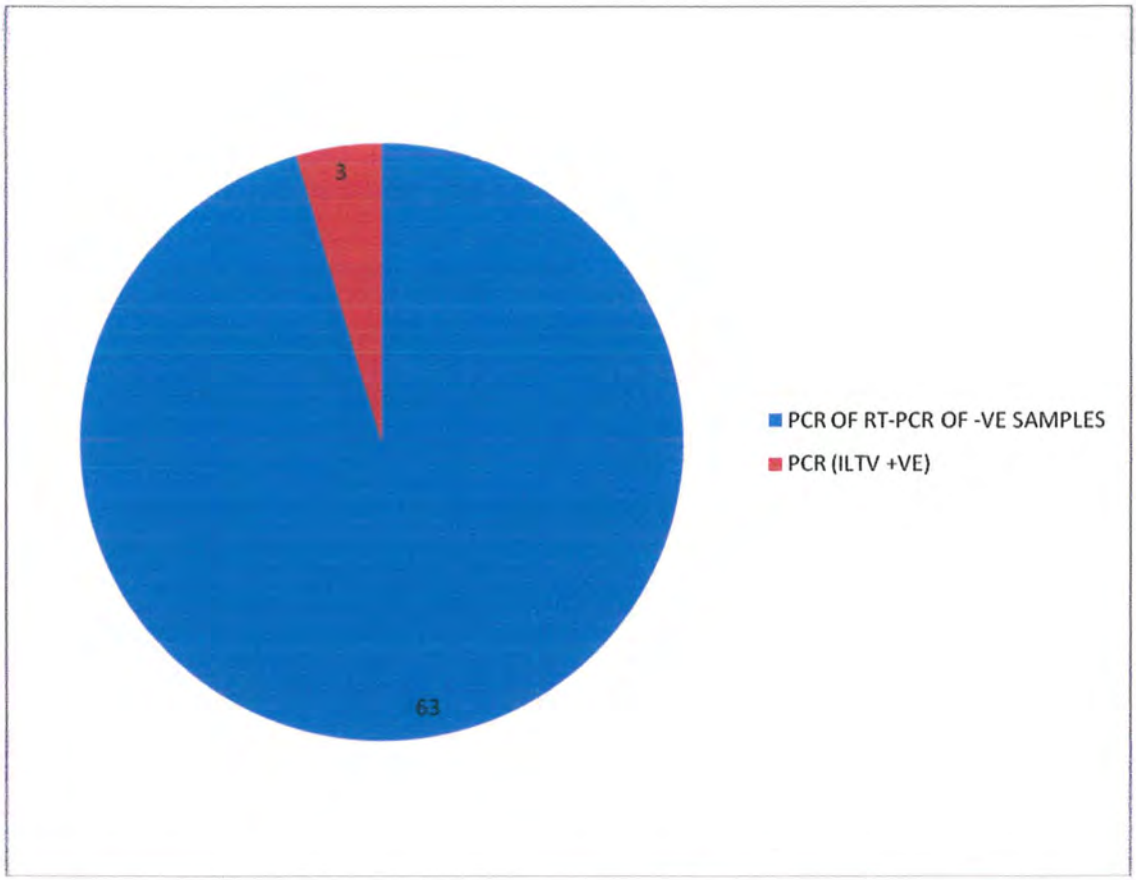

Figure-5 (b) Pie Chart showing results of PCR of RT-PCR –ve samples for ILTV

X-axis = Lab Diagnostic Procedures
Y-axis = No of Samples

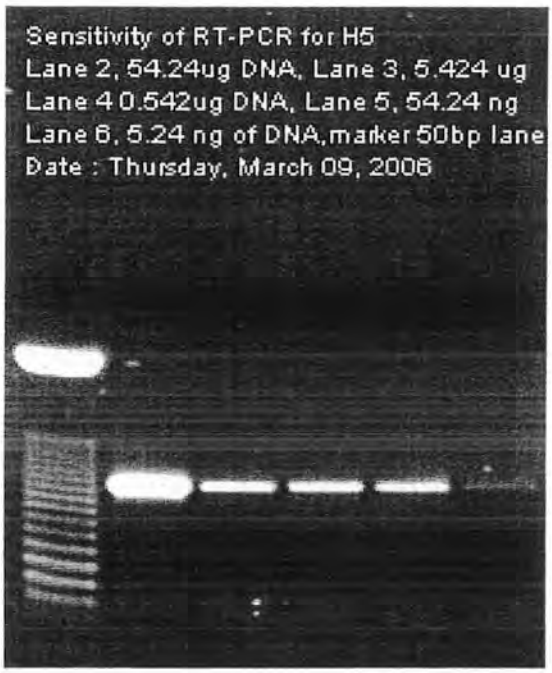

Figure 6 Sensitivity of RT-PCR for the Detection of H5

Lane 1: Marker (DNA step ladder 50bp); Lane 2: 54.24μg/ml DNA

Lane 3: 5.424μg/ml DNA; Lane 4: 0.542μg/ml DNA;

Lane 5: 54.24ng/ml DNA; Lane 6: 5.24ng/ml DNA.

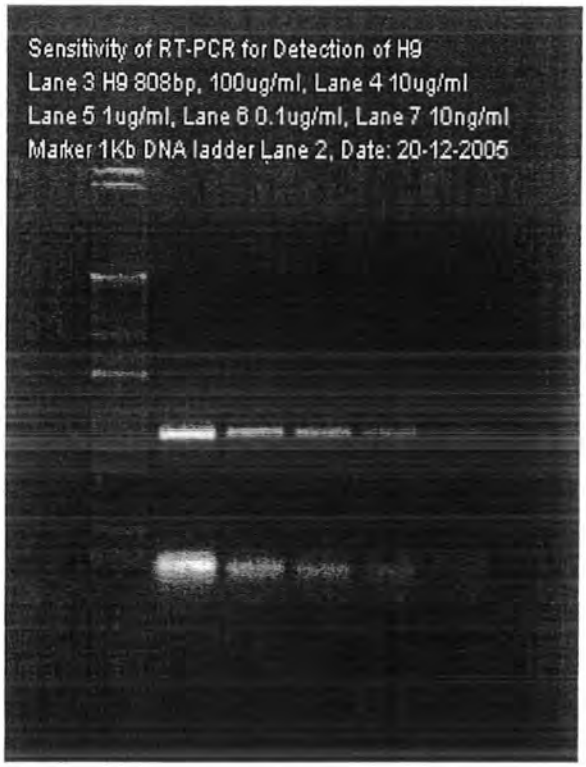

Figure 7 Sensitivity of RT-PCR for the Detection of H9

Lane 2: Marker (1kb plus DNA step ladder); Lane 3: 100µg/ml DNA

Lane 4: 10µg/ml DNA; Lane 5: 1µg/ml DNA;

Lane 6: 0.1µg/ml DNA; Lane7: 10ng/ml DNA.

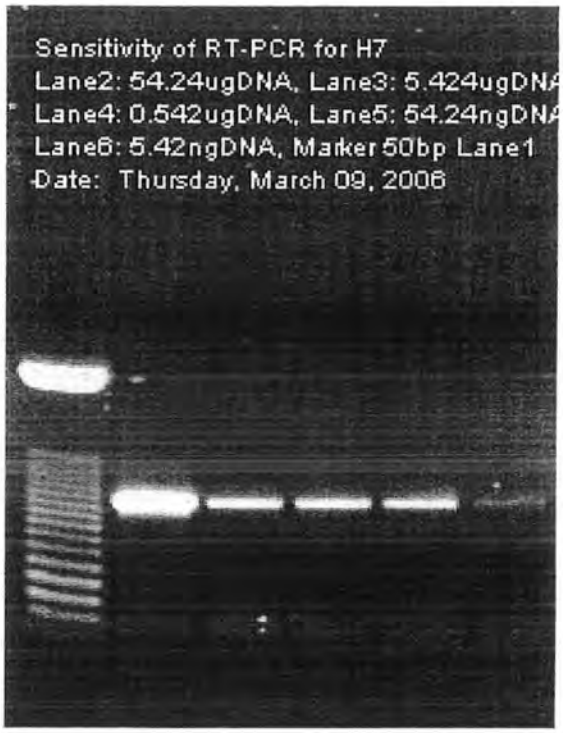

Figure 8 Sensitivity of RT-PCR for the Detection of H7

Lane 1: Marker (DNA step ladder 50bp); Lane 2: 54.24μg/ml DNA

Lane 3: 5.424μg/ml DNA; Lane 4: 0.542μg/ml DNA;

Lane 5: 54.24ng/ml DNA; Lane 6: 5.24ng/ml DNA.

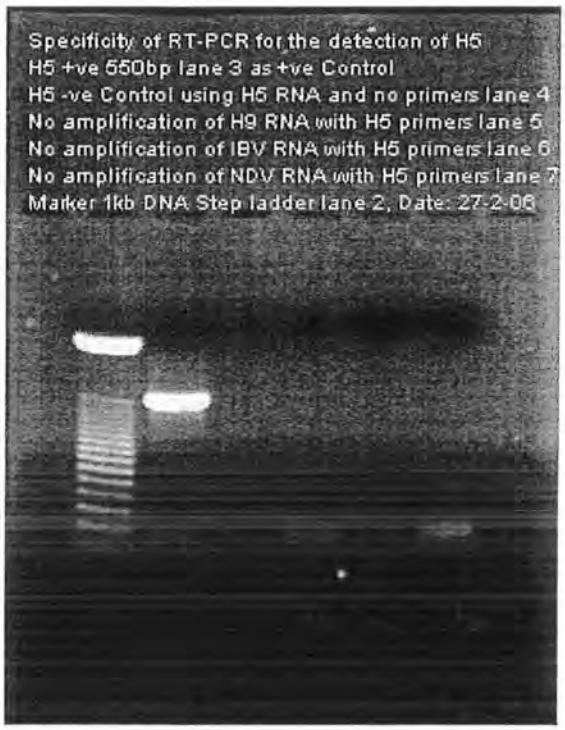

Figure 9 Specificity of RT-PCR for the Detection of H5

Lane 2: Marker (DNA step ladder 50bp); Lane 3: H5 +ve 550bp (+ve Control)

Lane 4: H5 –ve (-ve Control) using H5 RNA and no primers of H5

Lane 5: No amplification of H9 RNA with H5 primers

Lane 6: No amplification of IBV RNA with H5 primers

Lane 7: No amplification of NDV RNA with H5 primers

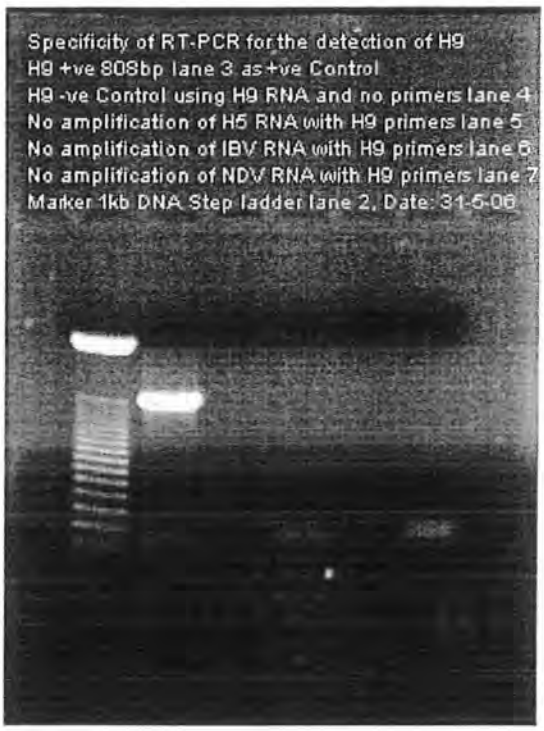

Figure 10 Specificity of RT-PCR for the Detection of H9

Lane 2: Marker (DNA step ladder 50bp); Lane 3: H9 +ve 808bp (+ve Control)

Lane 4: H9 –ve (-ve Control) using H9 RNA and no primers of H9

Lane 5: No amplification of H5 RNA with H9 primers

Lane 6: No amplification of IBV RNA with H9 primers

Lane 7: No amplification of NDV RNA with H9 primers

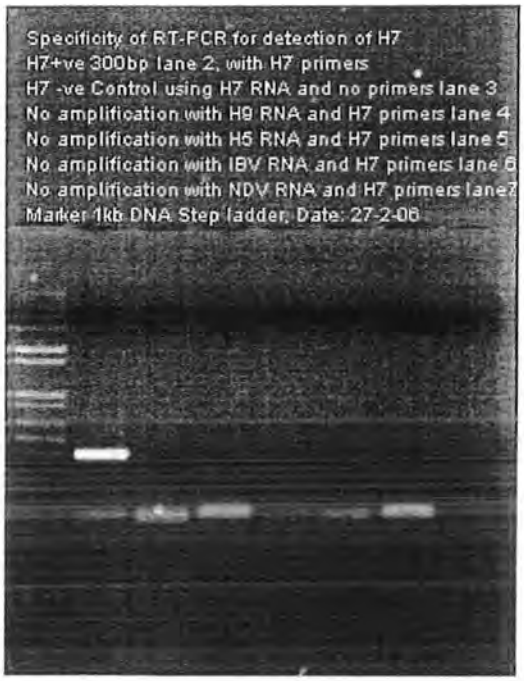

Figure 11 Specificity of RT-PCR for the Detection of H7

Lane1: Marker (1 kb DNA step ladder); Lane 2: H7 +ve 300bp (+ve Control)

Lane 3: H7 –ve (-ve Control) using H7 RNA and no primers of 7

Lane 4: No amplification of H9 RNA with H7 primers

Lane 5: No amplification of H5 RNA with H7 primers

Lane 6: No amplification of IBV RNA with H7 primers

Lane 7: No amplification of NDV RNA with H7 primers

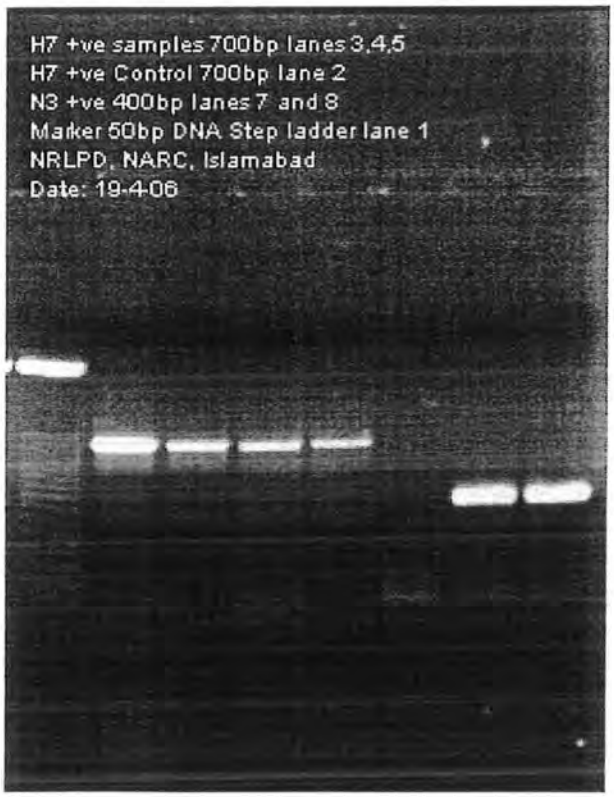

Figure 12 Standard pattern of amplification of H7N3 for HA and NA genes

Lane 1: Marker (DNA step ladder 50bp); Lane 2: H7 HA +ve Control 700bp

Lane 3, 4, and 5: H7 +ve samples 700bp; Lane 6: H7 HA –ve Control

Lane 7: NA N3 +ve Control 400bp; Lane 8: NA N3 sample 400bp

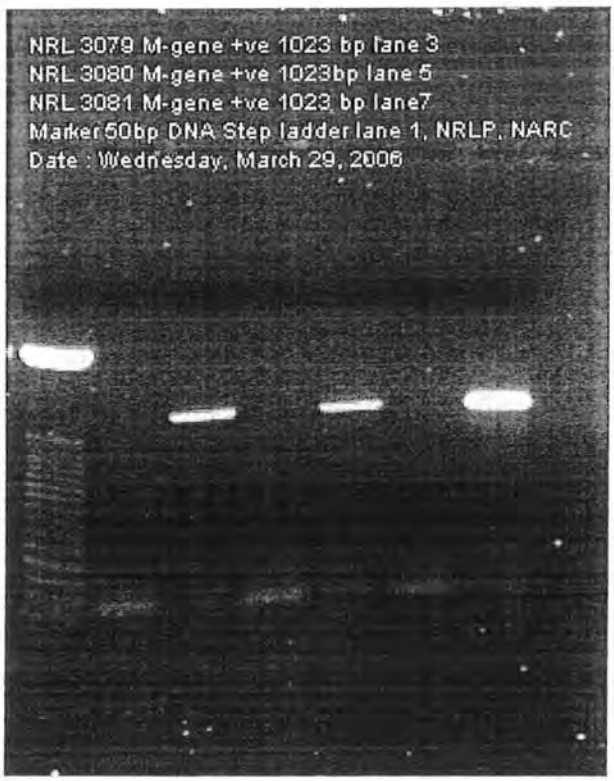

Figure 13 Standard pattern of amplification of AIV (M-Gene)

Lane 1: Marker (DNA step ladder 50bp); Lane 2: M-Gene (–ve Control)

Lane 3: M-Gene +ve Control 1023bp; Lanes 5 and 7: M-Gene +ve

samples 1023bp

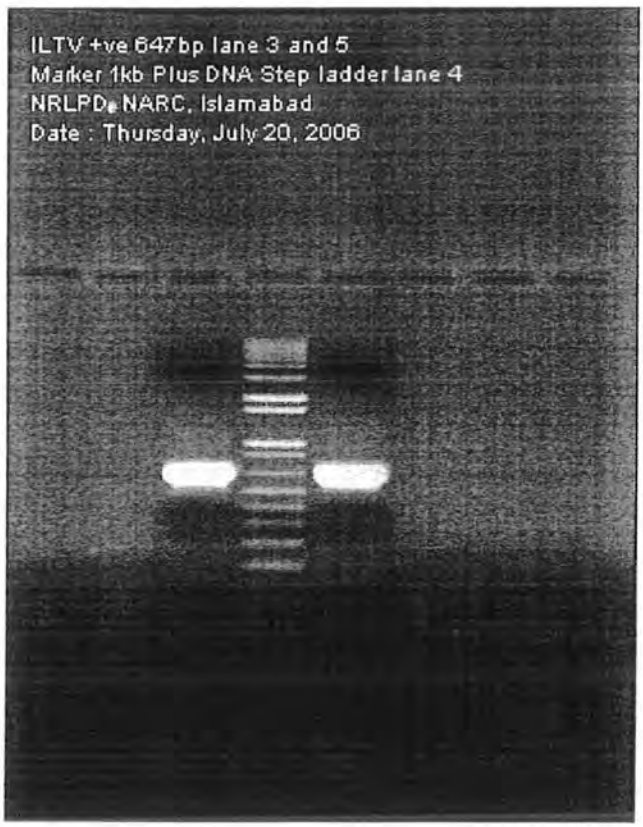

Figure 14 Standard pattern of amplification of ILTV

Lane 4: Marker (1kb plus DNA step ladder); Lane 3: ILTV +ve Control 647bp

Lane 5: ILTV +ve sample 647bp

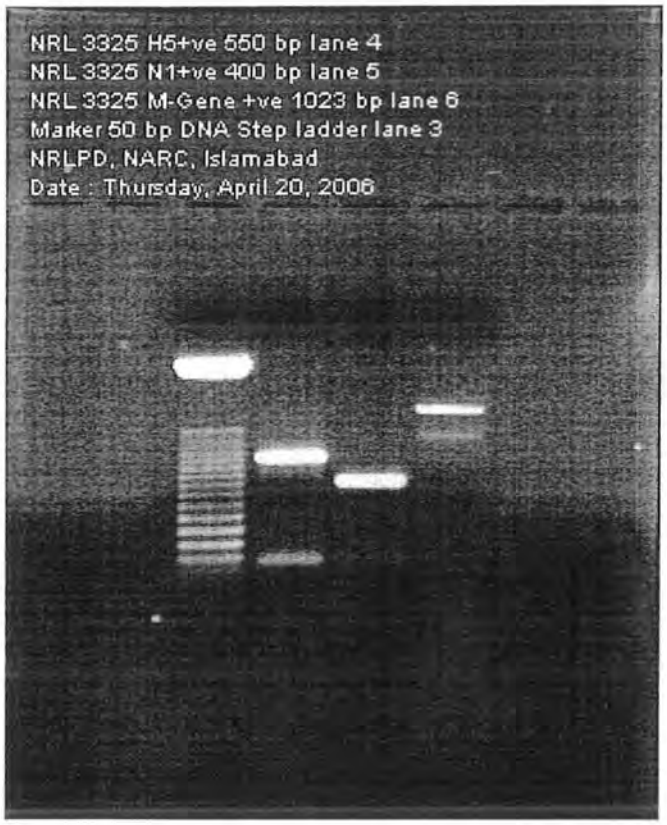

Figure 15 Standard pattern in the amplification of H5N1 for HA, NA and M-Genes

Lane 3: Marker (DNA step ladder 50bp); Lane 4: H5 (HA) +ve 550bp

Lane 5: H5 (NA) N1 +ve 400bp; Lane 6: M-Gene +ve 1023bp

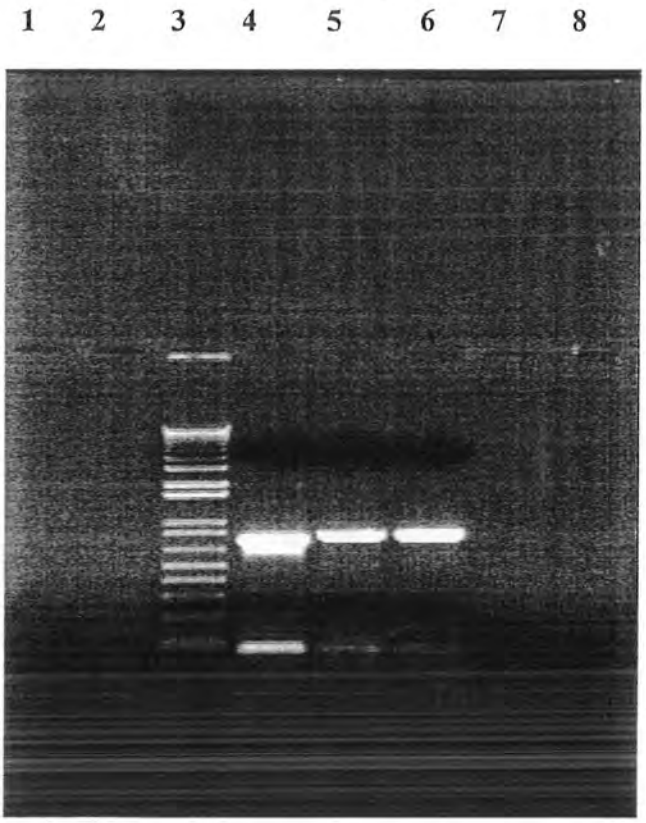

Figure 16 Standard pattern of amplification of H9N2 for HA gene

Lane 3: Marker (1kb plus DNA step ladder); Lane 4: H9N2 HA +ve Control 808bp; Lane 5 and 6: H9N2 HA +ve samples 808bp

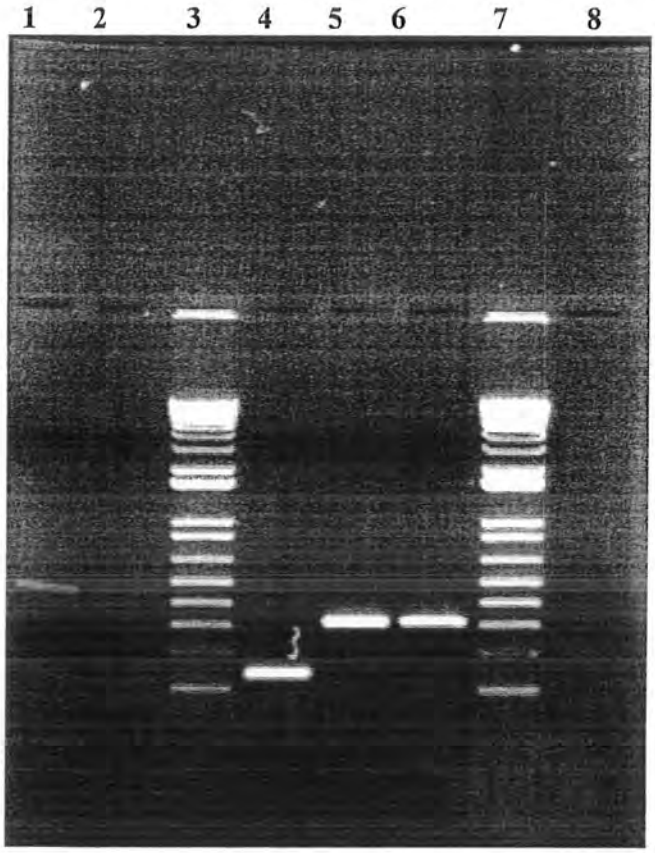

Figure 17 Standard pattern of amplification of IBV and NDV

Lane 3 and 7: Marker (1kb plus DNA step ladder); Lane 4: IBV +ve 149bp

Lane 5: NDV +ve Control 320bp; Lane 6: NDV +ve sample 320bp

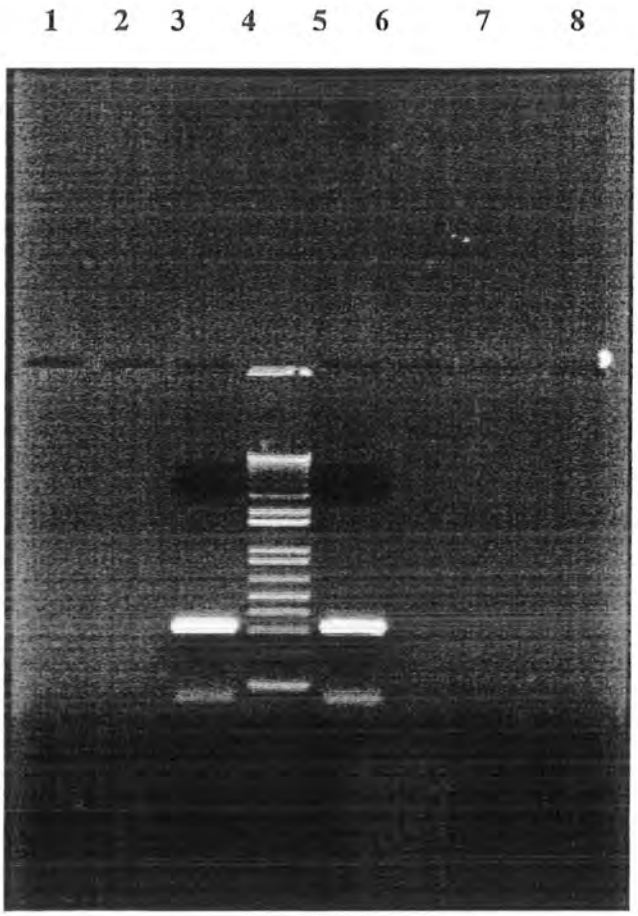

Figure 18 Standard pattern of amplification of NDV

Lane 4: Marker (1kb plus DNA step ladder); Lane 3: NDV +ve Control 320bp; Lane 5: NDV +ve sample 320bp

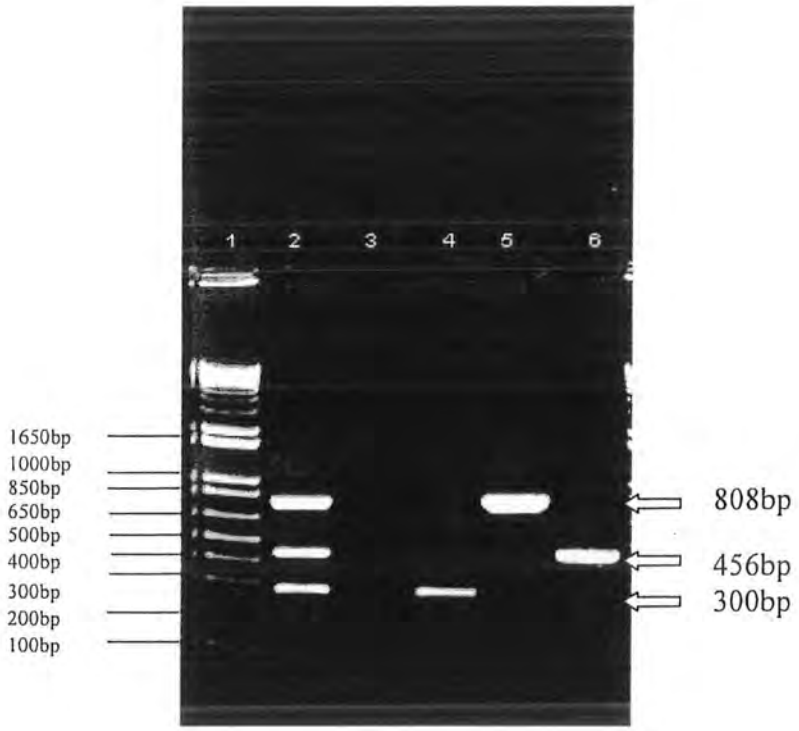

Figure 19 Agarose gel electrophoresis of Trivalent multiplex RT-PCR amplified products from purified RNA of known avian influenza subtypes. Lane 1: molecular size marker; Lane 2: H7N3, H5N1, H9N2 subtypes of AIV; Lane 3: PCR reagent buffer as a negative control; Lane 4: H7N3 300bp; Lane 5: H9N2 808bp; Lane 6: H5N1 456bp.

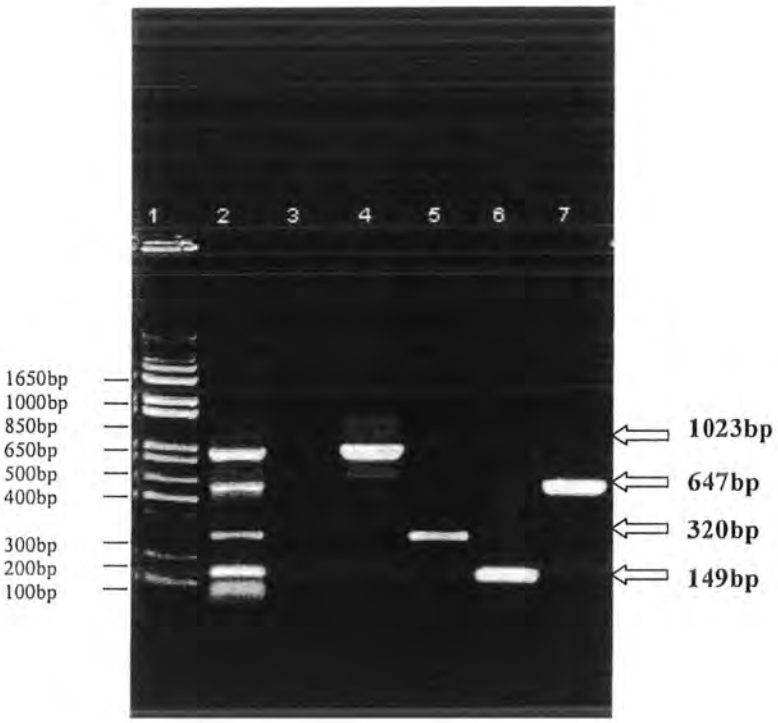

Figure 20 Agarose gel electrophoresis of Tetravalent multiplex RT-PCR amplified products from purified RNA and DNA of known avian influenza subtype and other avian respiratory pathogens. Lane 1: molecular size marker; Lane 2: AIV, NDV, IBV, ILTV; Lane 3: PCR reagent buffer as a negative control; Lane 4: AIV 1023bp; Lane 5: NDV 320bp; Lane 6: IBV 149bp; Lane 7: ILTV 647bp.

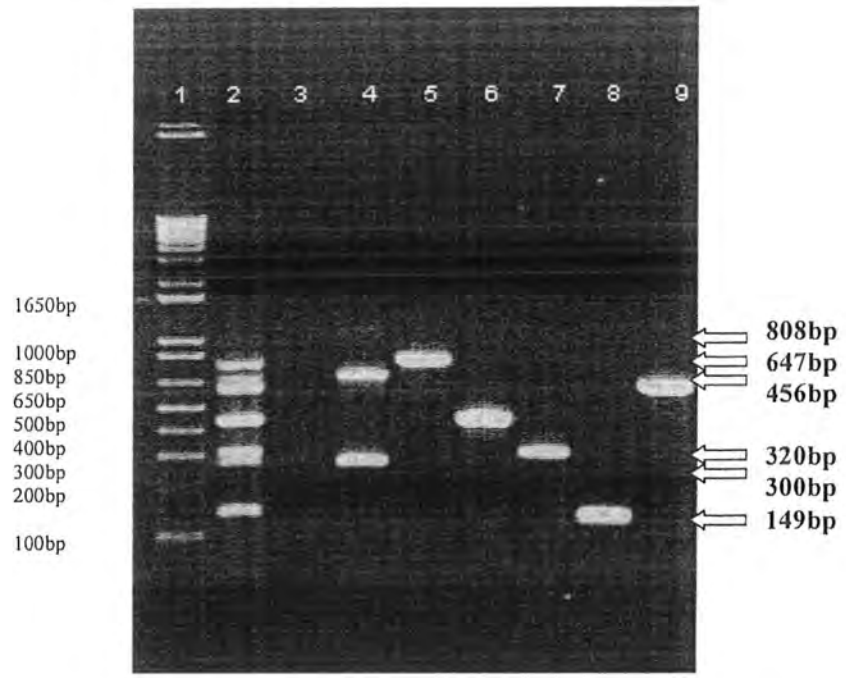

Figure 21 Agarose gel electrophoresis of Hexavalent multiplex RT-PCR amplified products from purified RNA and DNA of known avian influenza subtypes and other avian respiratory pathogens. Lane 1: molecular size marker; Lane 2: H7N3, H5N1, H9N2, NDV, IBV, ILTV; Lane 3: PCR reagent buffer as a negative control; Lane 4: H7N3 300bp; Lane 5: H9N2 808bp; Lane 6: H5N1 149bp; Lane 7: NDV 320bp; Lane 8: IBV 149bp; Lane 9: ILTV 647bp.

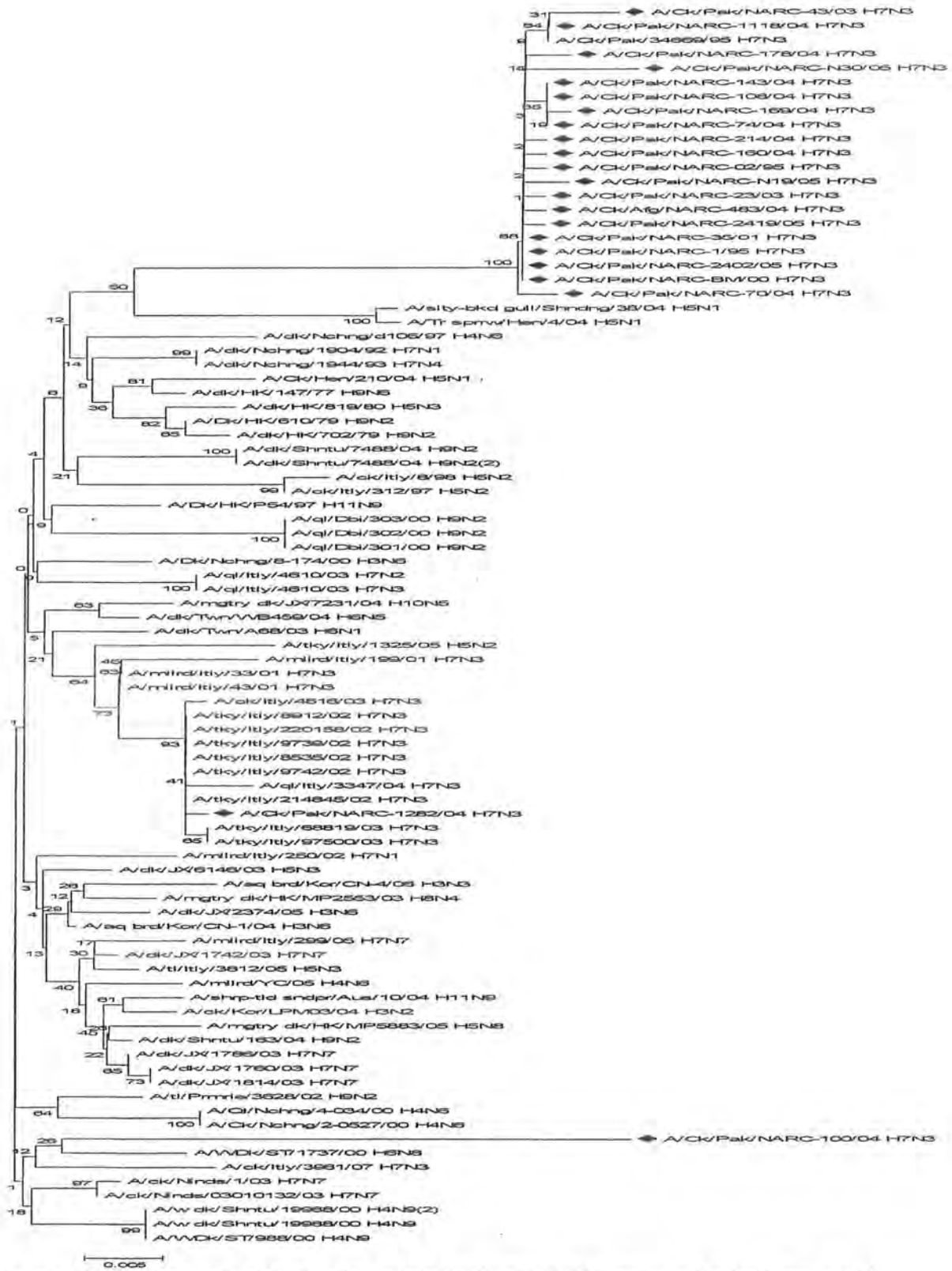

Figure 22: Phylogenetic Relationship of all Pakistani H7N3 isolates for NS1 gene with other GenBank sequences

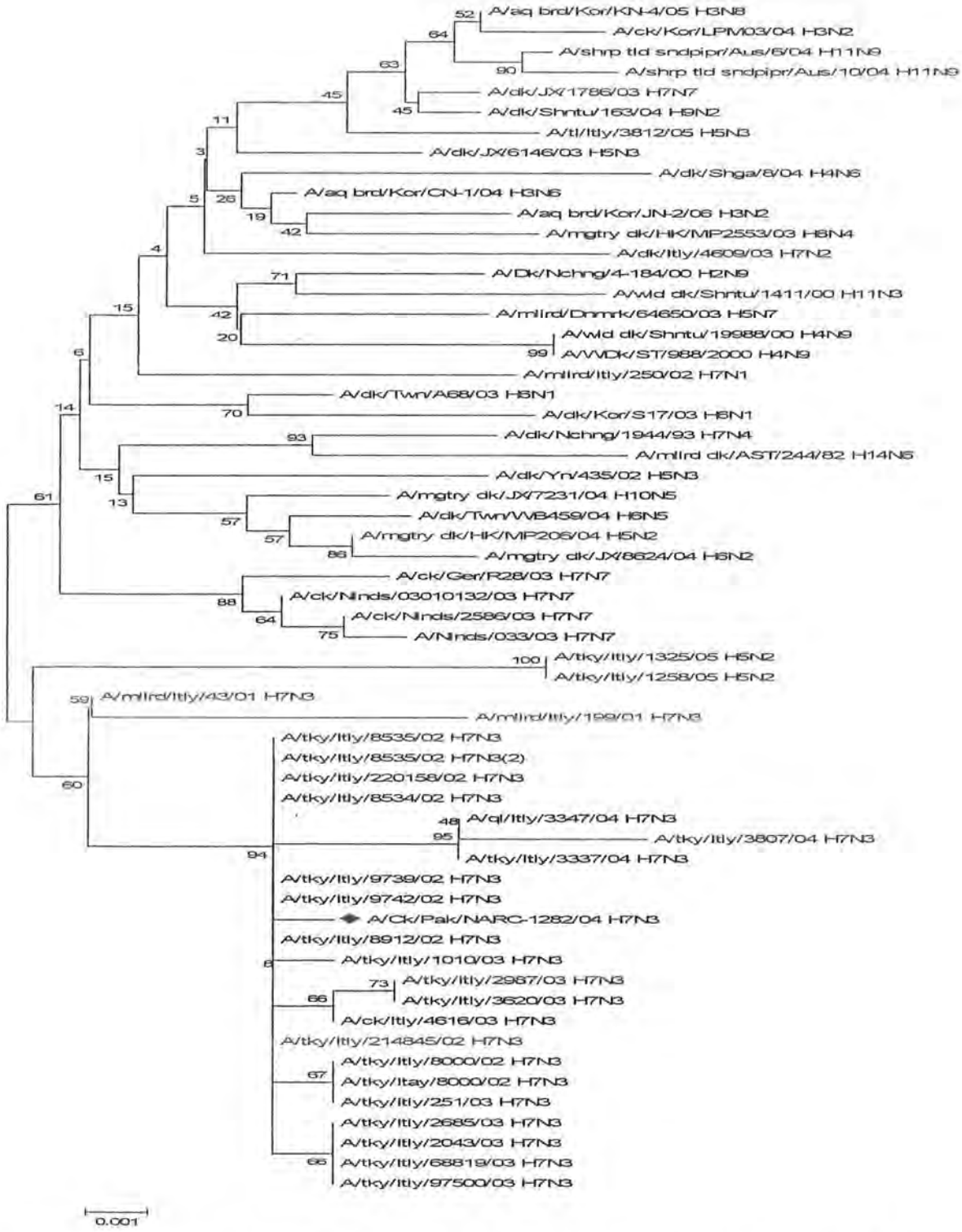

Figure 23: Phylogenetic tree of A/Chicken/Pakistan/NARC-1282/ 04(H7N3) with other GenBank sequences for the NS1 gene

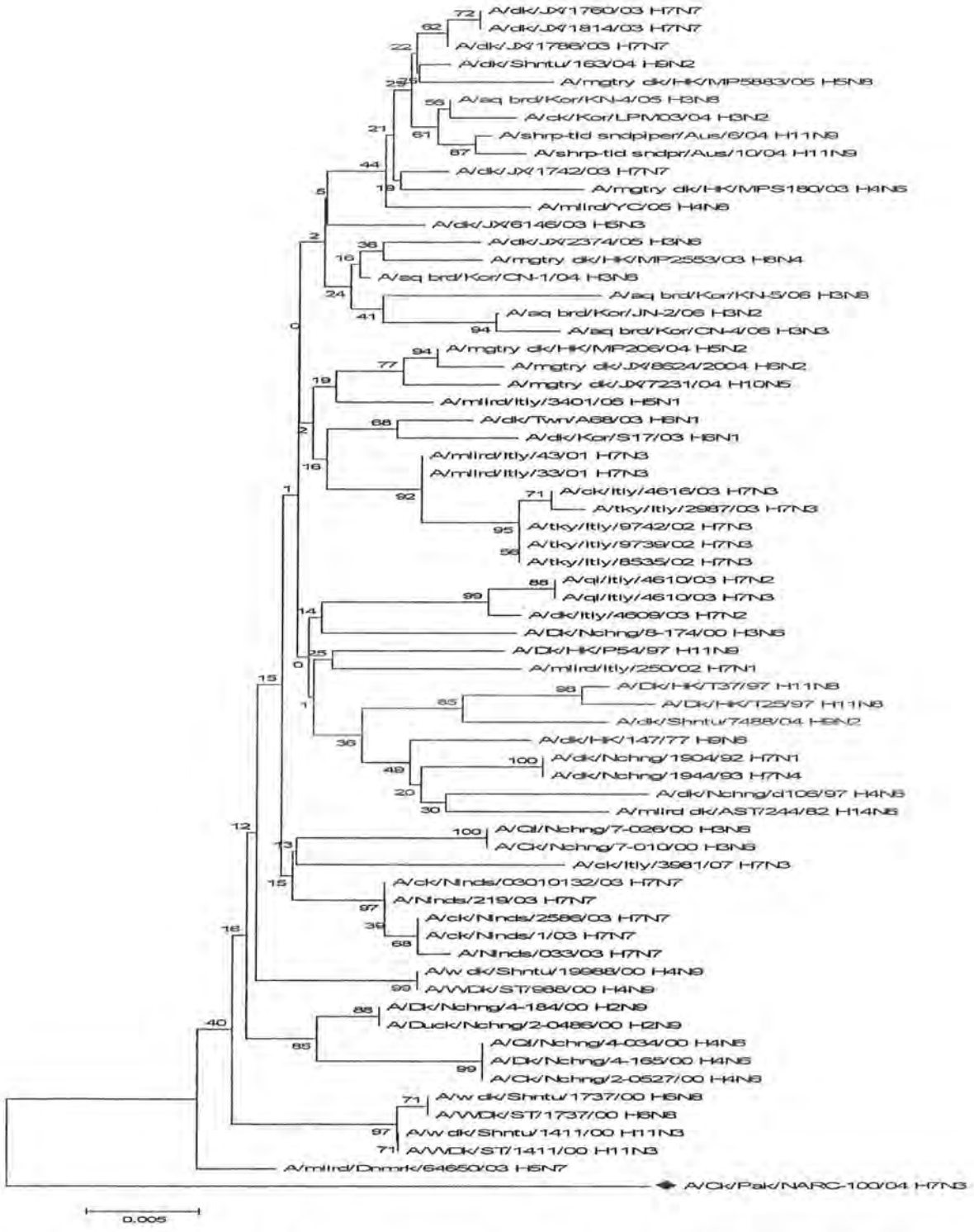

Figure 24: Phylogenetic Tree of A/Chicken/Pakistan/NARC-100/04(H7N3) with other GenBank Sequences for the NS1 gene.

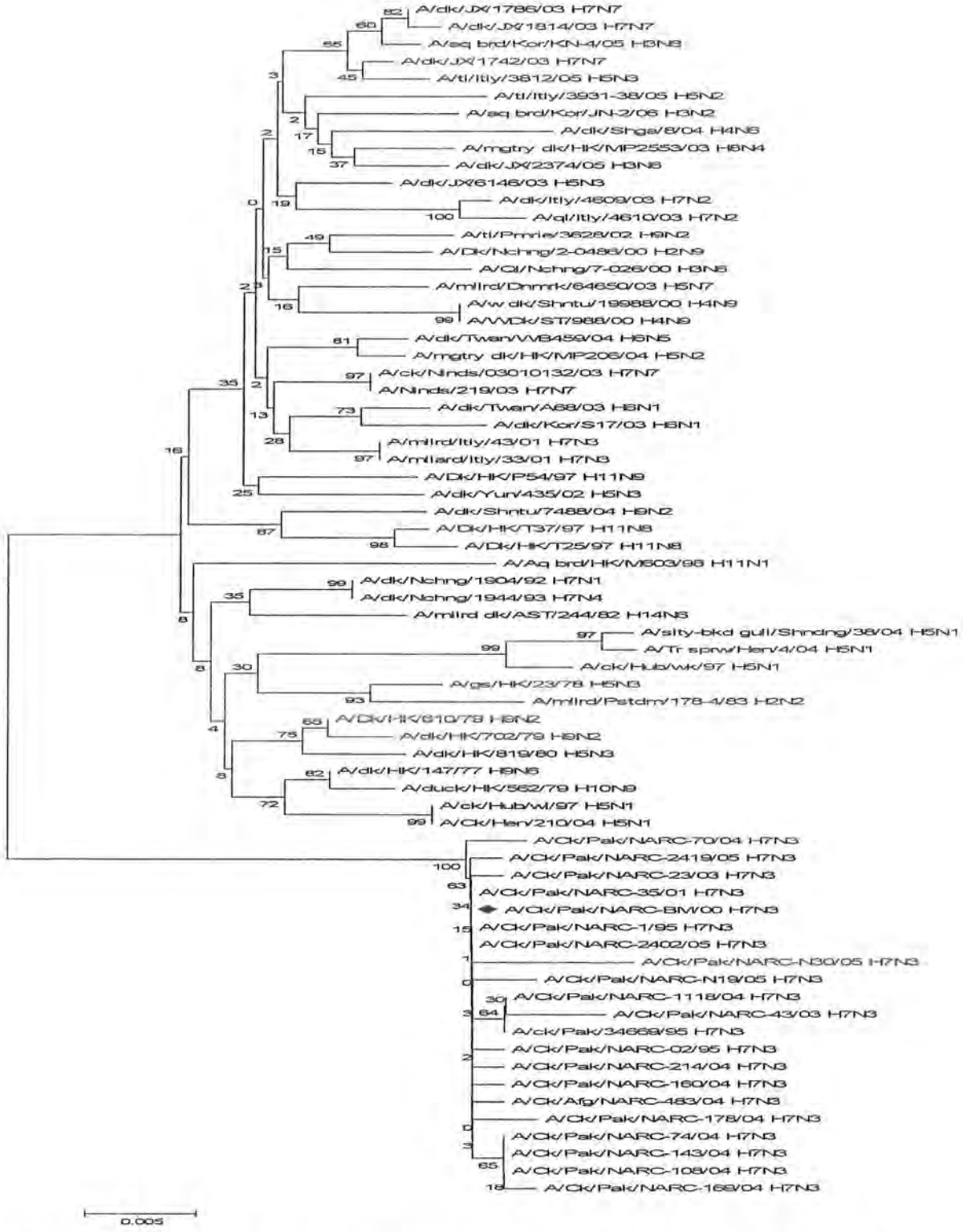

Figure 25: Phylogenetic tree of A/Chicken/Pakistan/NARC-BM/00(H7N3) with other GenBank sequences for the NS1 gene

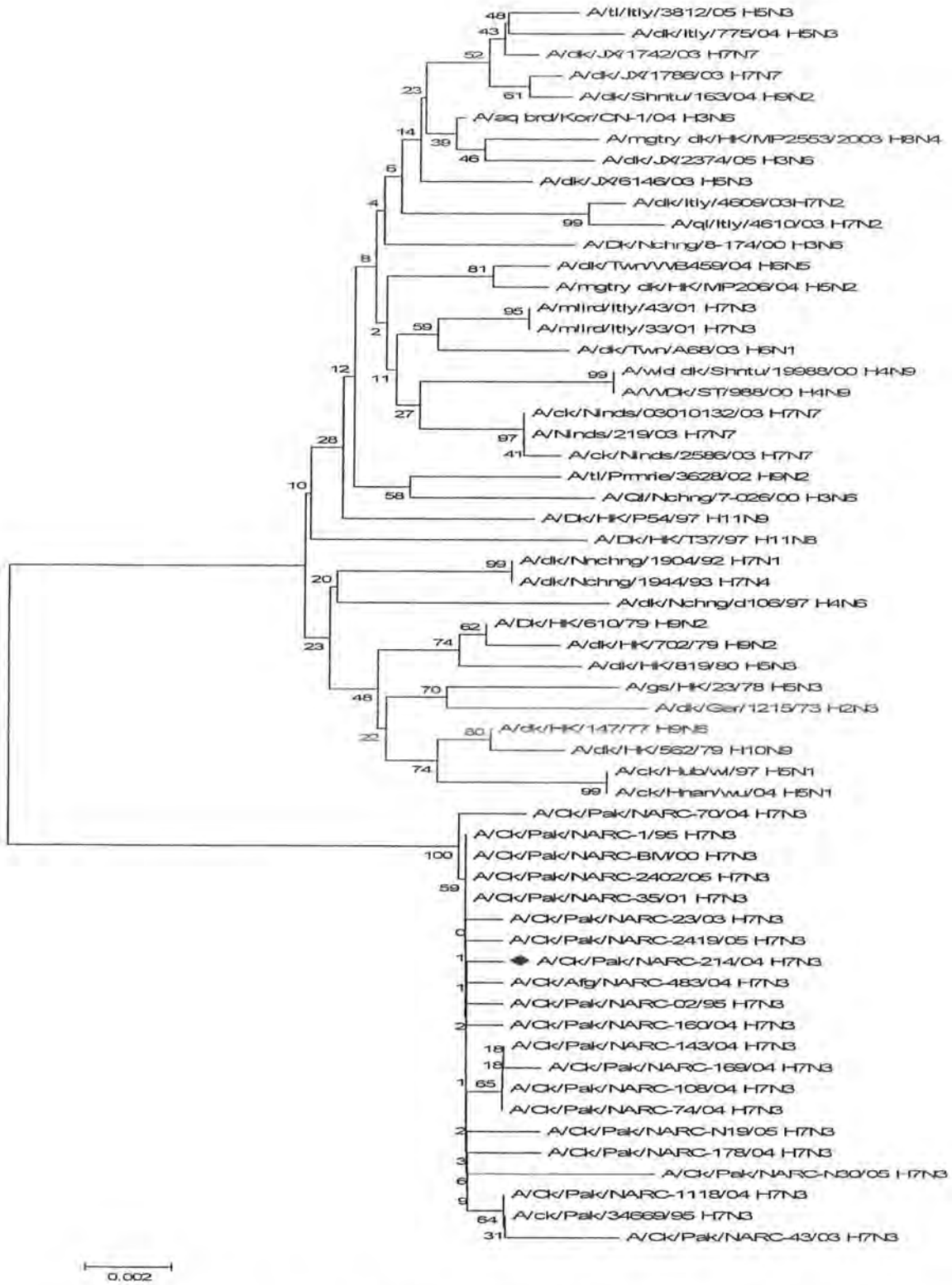

Figure 26: Phylogenetic Tree of A/Chicken/Pakistan/NARC-214/ 04(H7N3) with other GenBank sequences for the NS1 gene

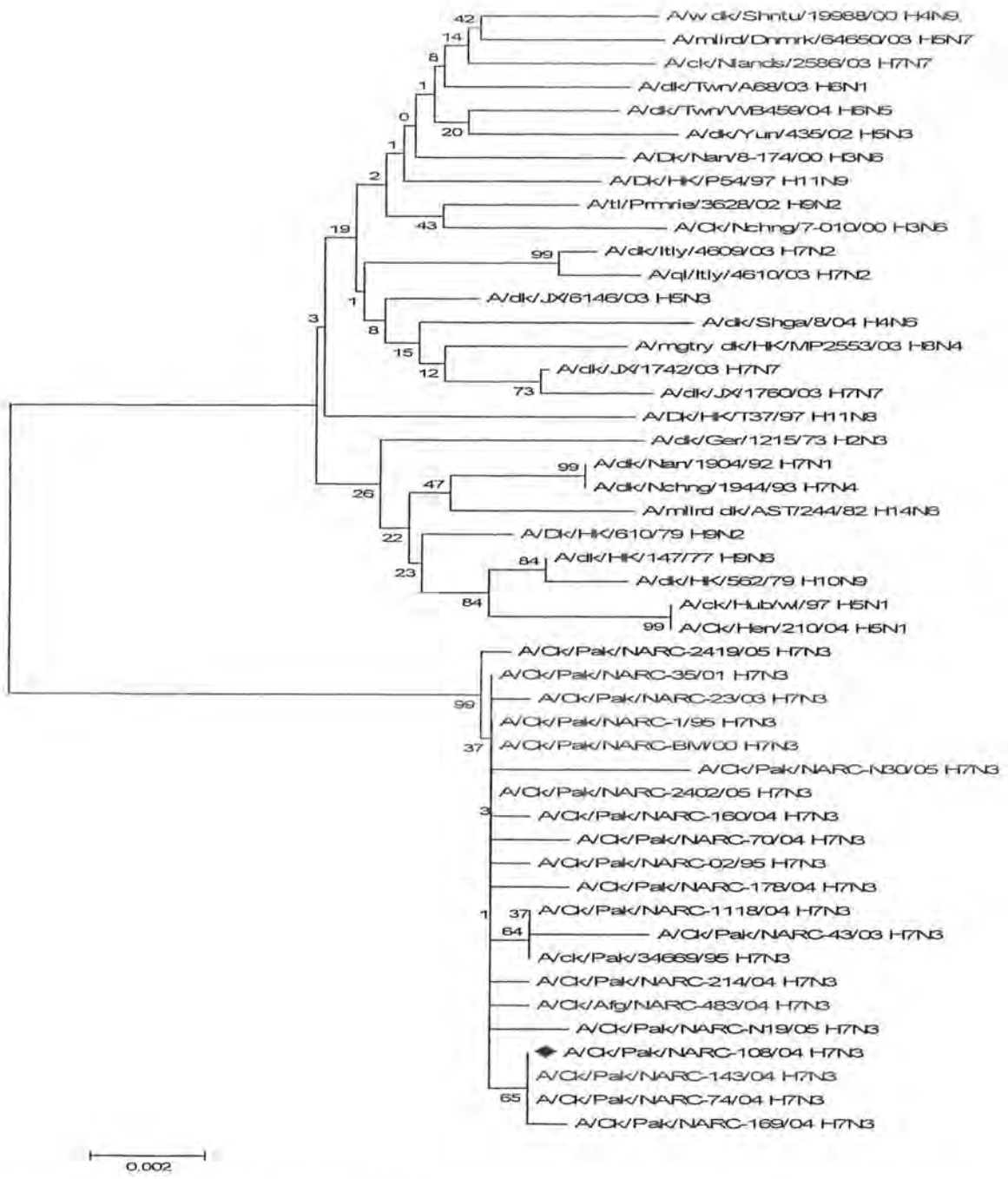

Figure 27: Phylogenetic tree of A/Chicken/Pakistan/NARC-108/ 04(H7N3) with other GenBank sequences for the NS1 gene

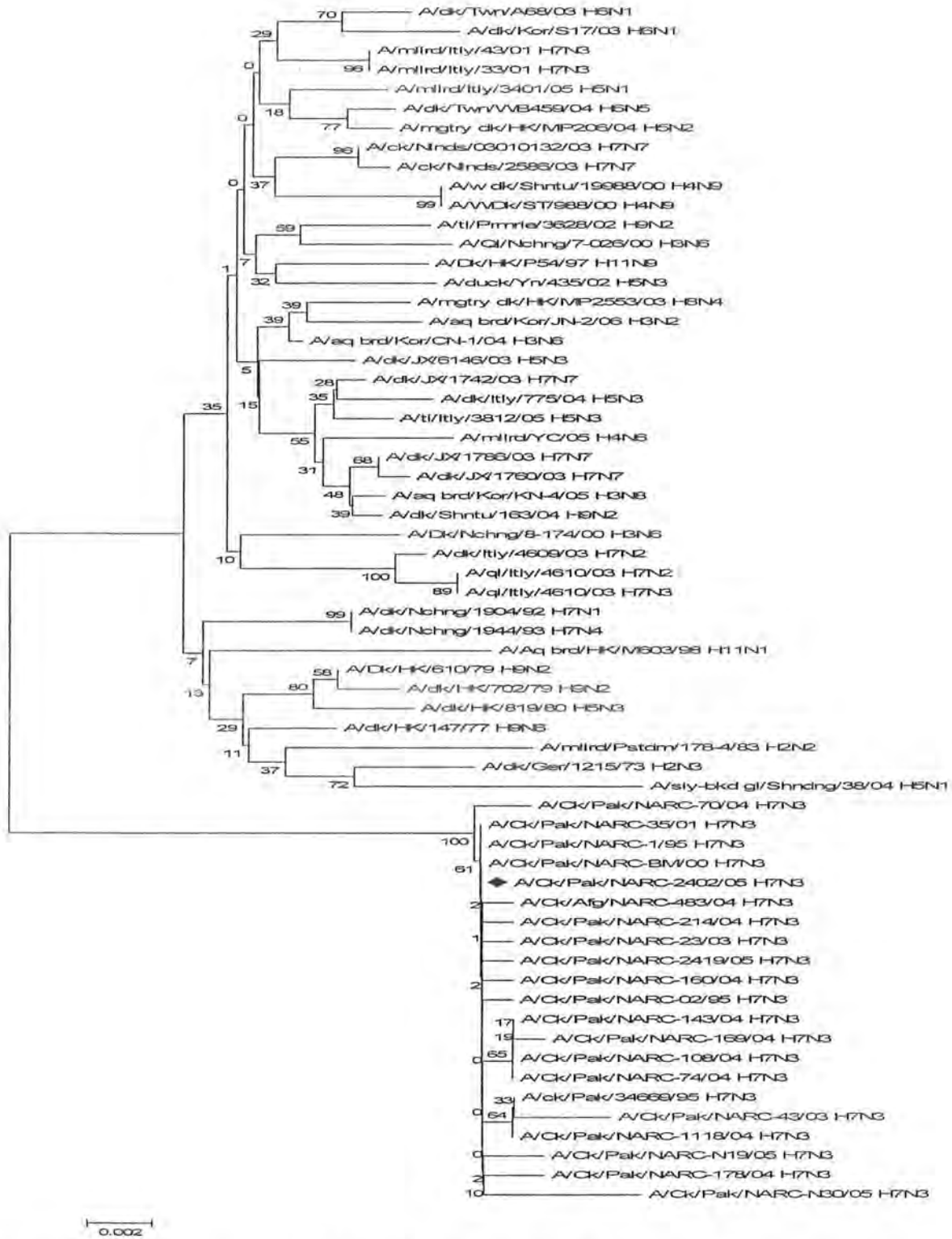

Figure 28: Phylogenetic tree of A/Chicken/Pakistan/NARC-2402/ 05(H7N3) with other GenBank sequences for the NS1 gene

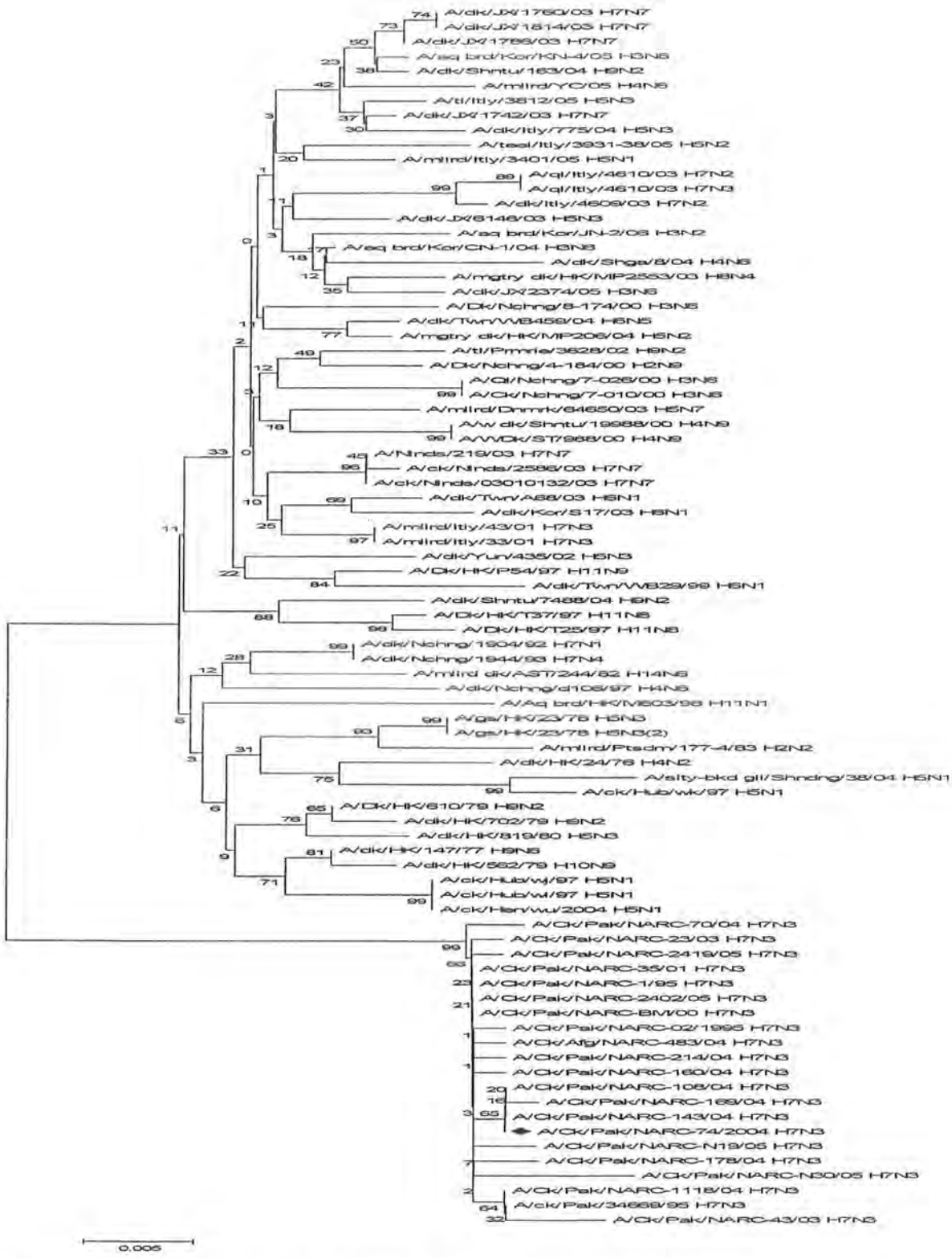

Figure 29: Phylogenetic tree of A/Chicken/Pakistan/NARC-74/ 04(H7N3) with other GenBank sequences for the NS1 gene

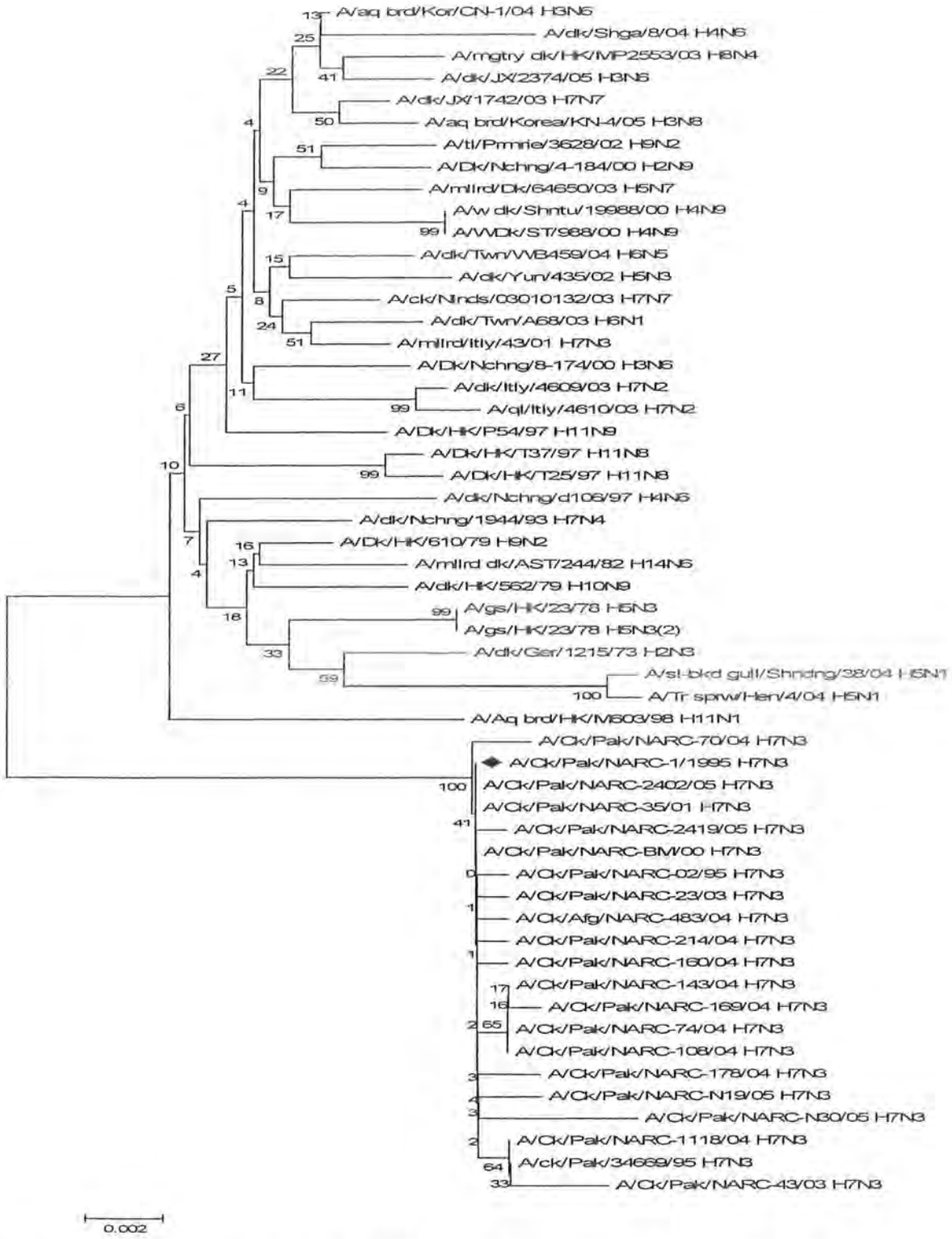

Figure 30: Phylogenetic tree of A/Chicken/Pakistan/NARC-1/95(H7N3) with other GenBank sequences for the NS1 gene

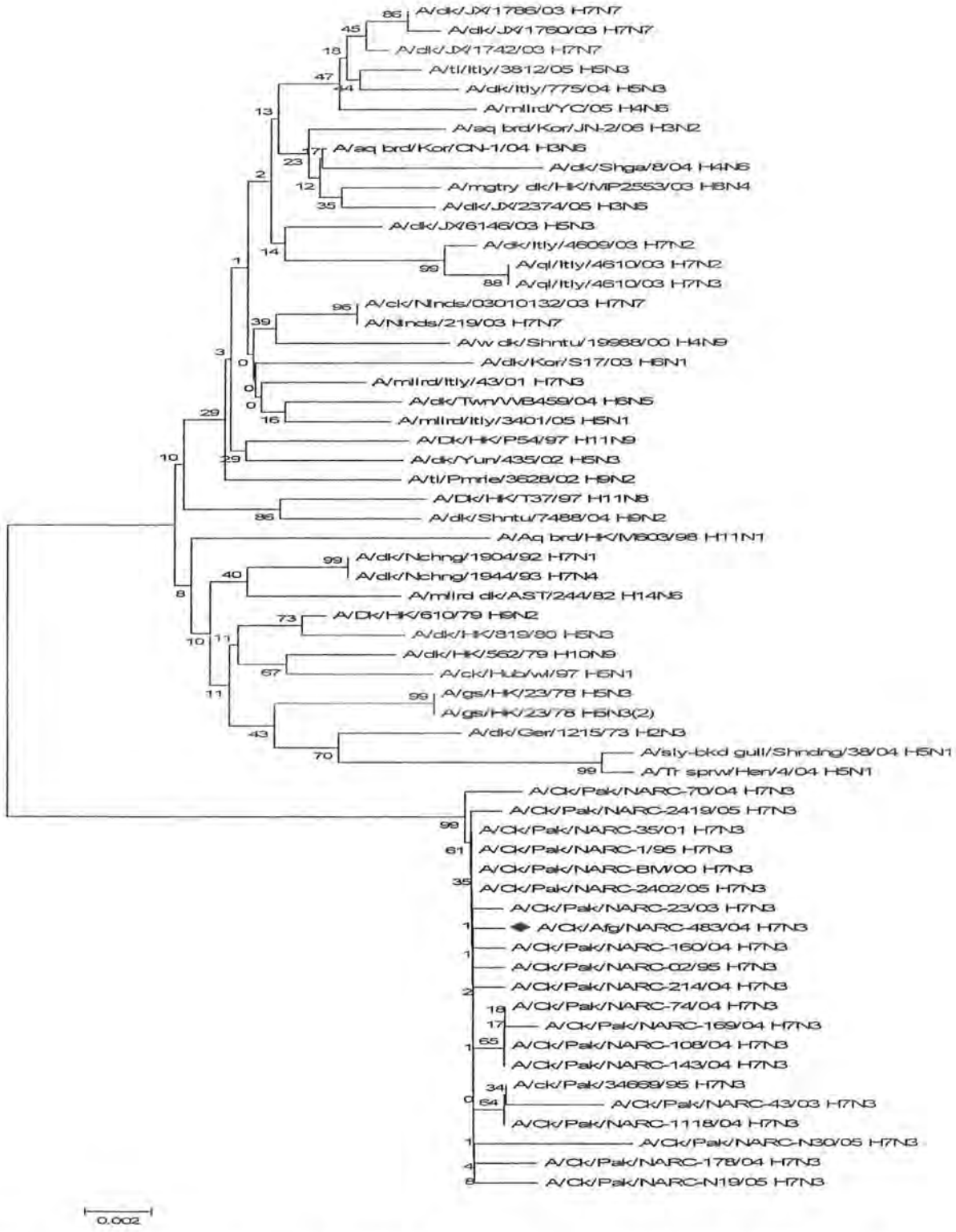

Figure 31: Phylogenetic tree of A/Chicken/Afghanistan/ NARC-483/ 04(H7N3) with othe GenBank sequences for the NS1 gene

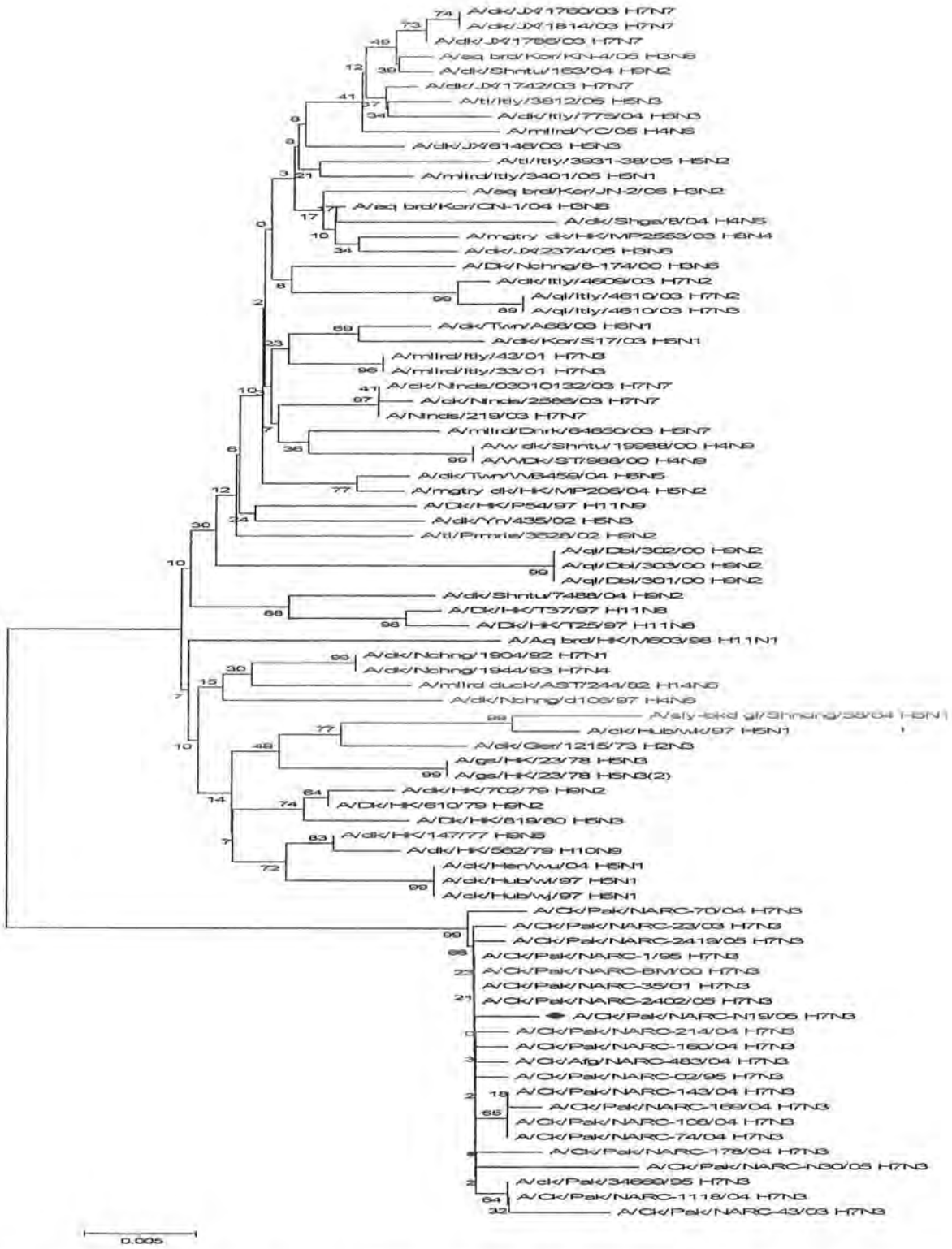

Figure 32: Phylogenetic tree of A/Chicken/Pakistan/NARC-N19/05(H7N3) with other GenBank sequences for the NS1 gene

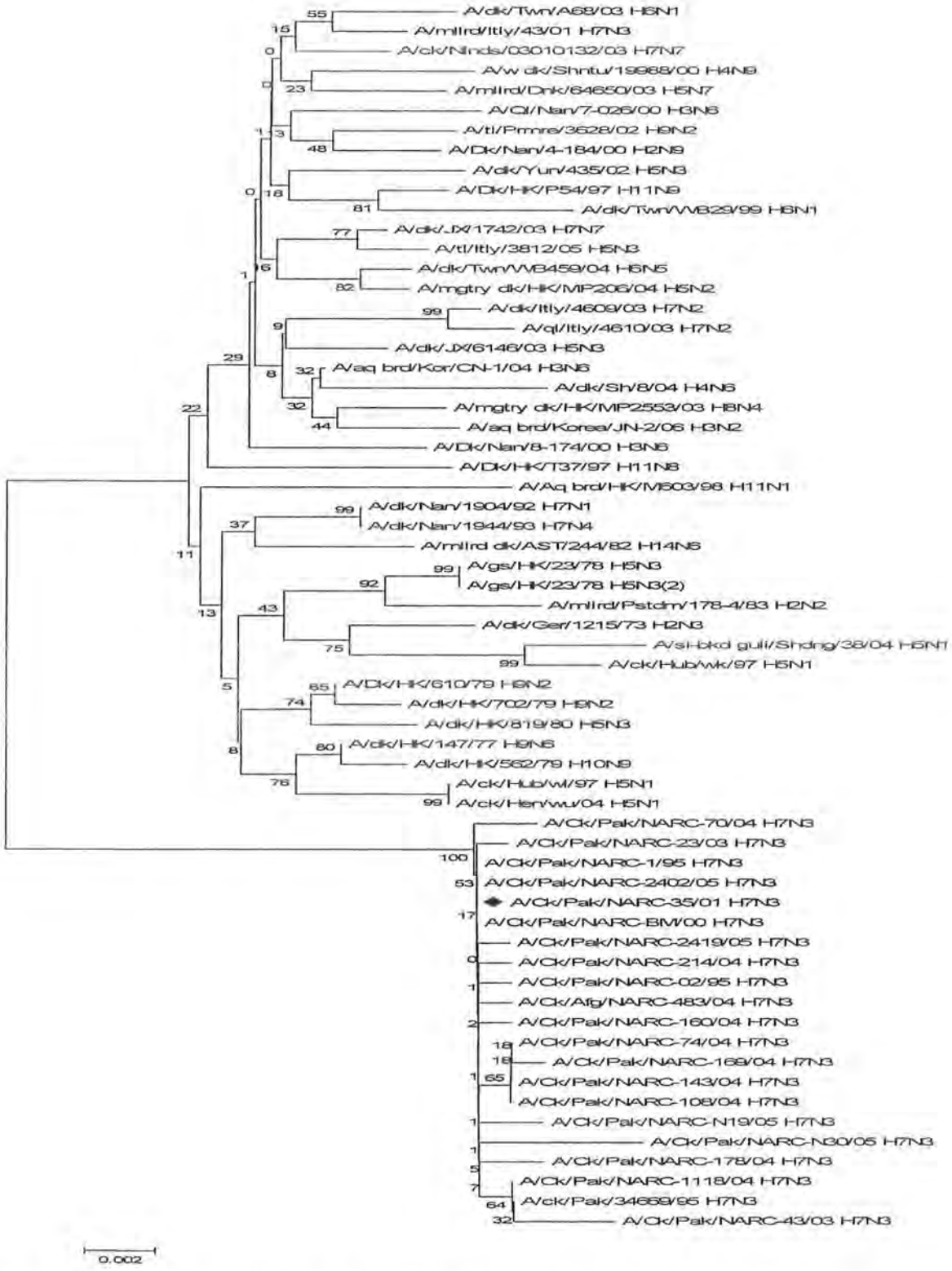

Figure 33: Phylogenetic tree of A/Chicken/Pakistan/NARC-35/01(H7N3) with other GenBank sequences for the NS1 gene

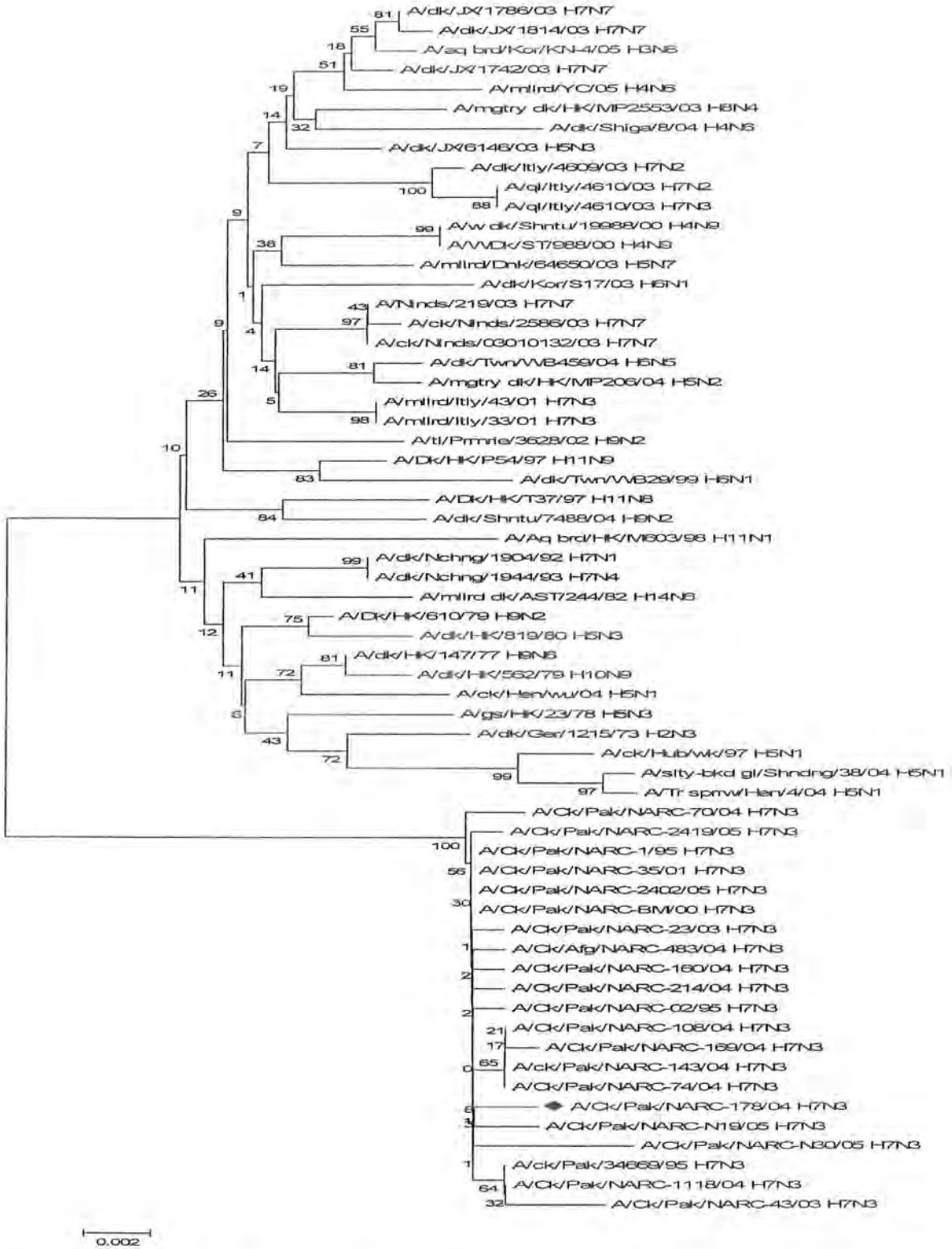

Figure 34: Phylogenetic tree of A/Chicken/Pakistan/NARC-178/ 04(H7N3) with other GenBank sequences for the NS1 gene

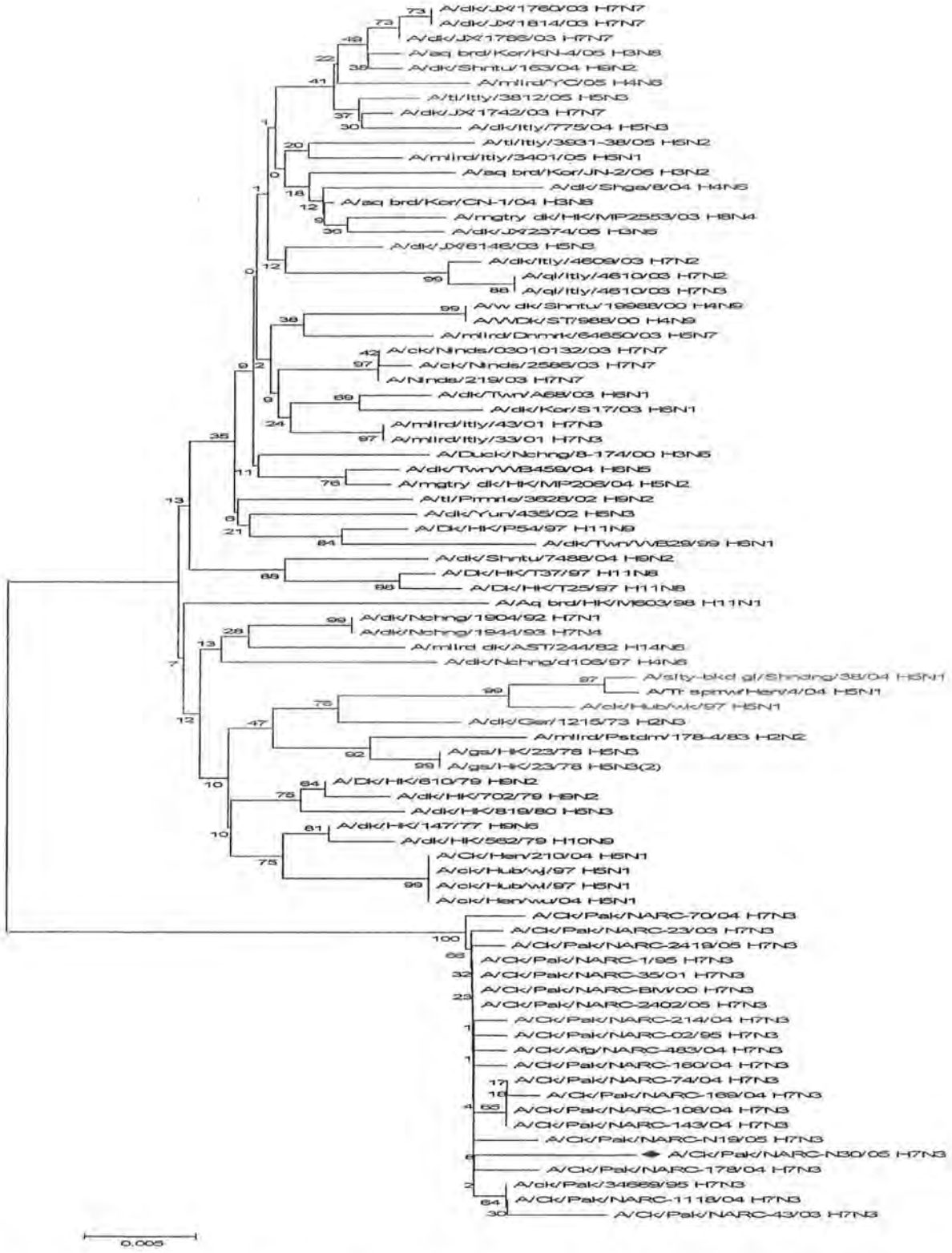

Figure 35: Phylogenetic Tree of A/Chicken/Pakistan/NARC-N30/05(H7N3) with other GenBank sequences for the NS1 gene

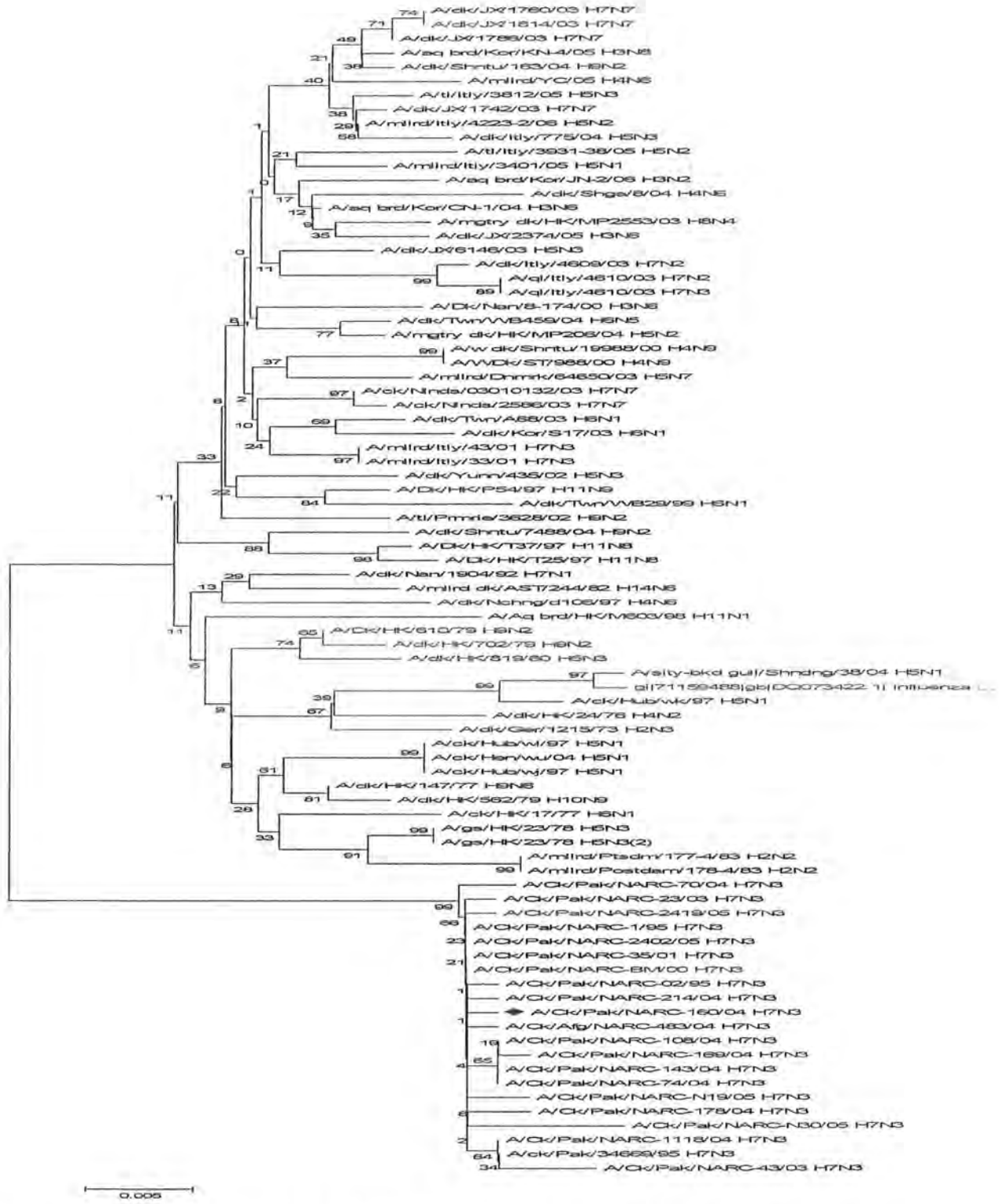

Figure 36: Phylogenetic tree of A/Chicken/Pakistan/NARC-160/ 04(H7N3) with other GenBank sequences for the NS1 gene

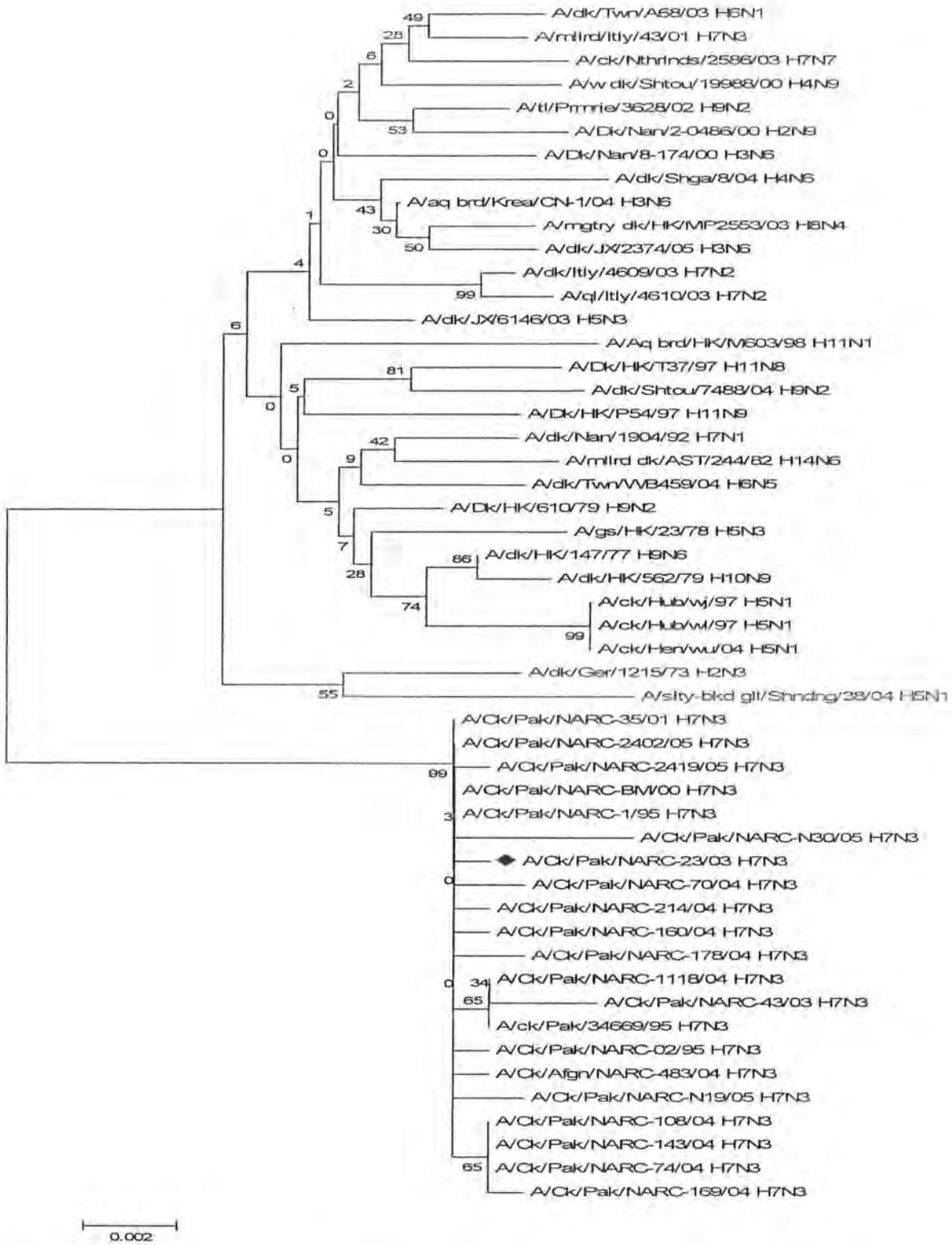

Figure 37: Phylogenetic tree of A/Chicken/Karachi/ Pakistan/NARC-23/03(H7N3) with other GenBank sequences for the NS1 gene

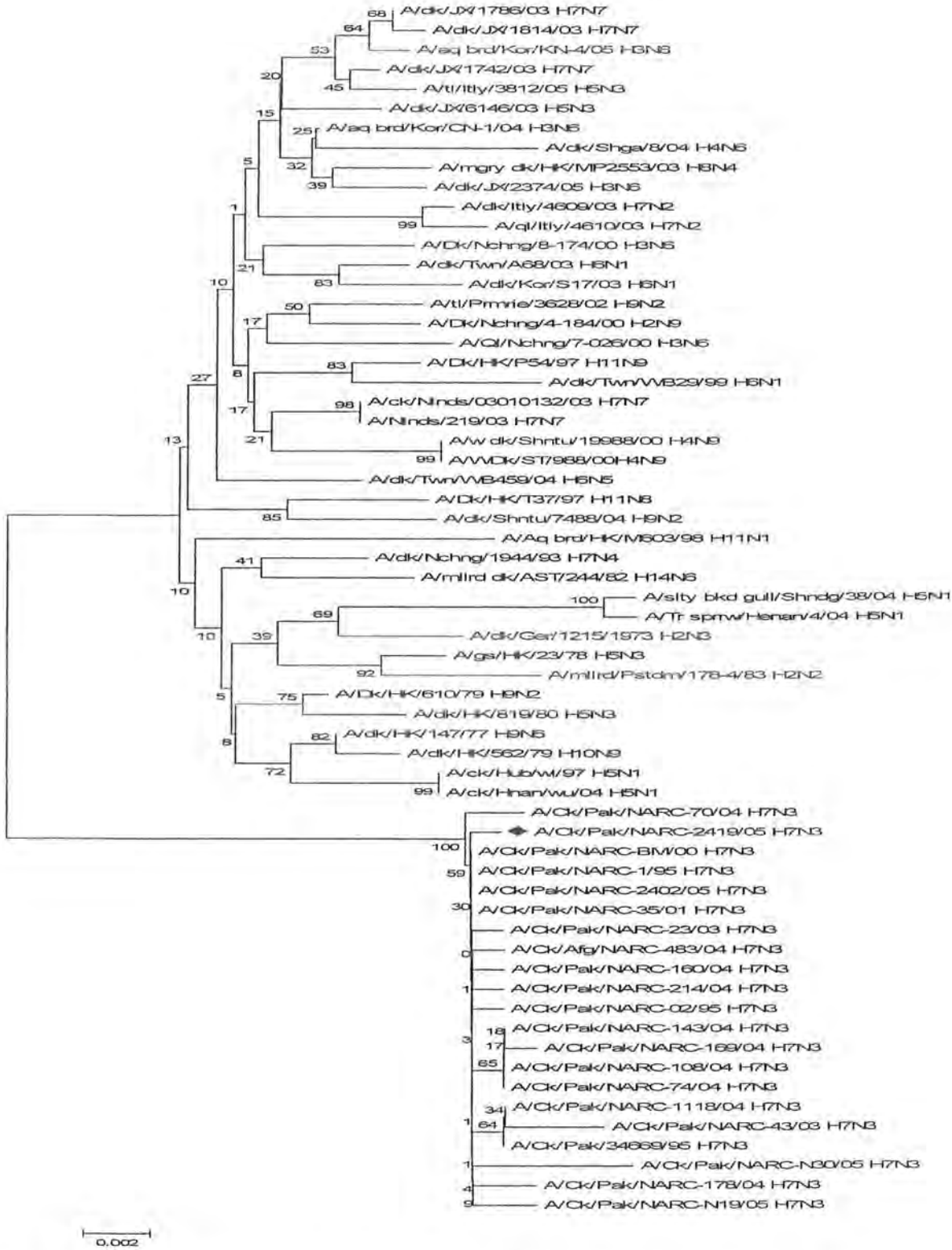

Figure 38: Phylogenetic tree of A/Chicken/Pakistan/NARC-2419/ 05(H7N3) with other GenBank sequences for the NS1gene

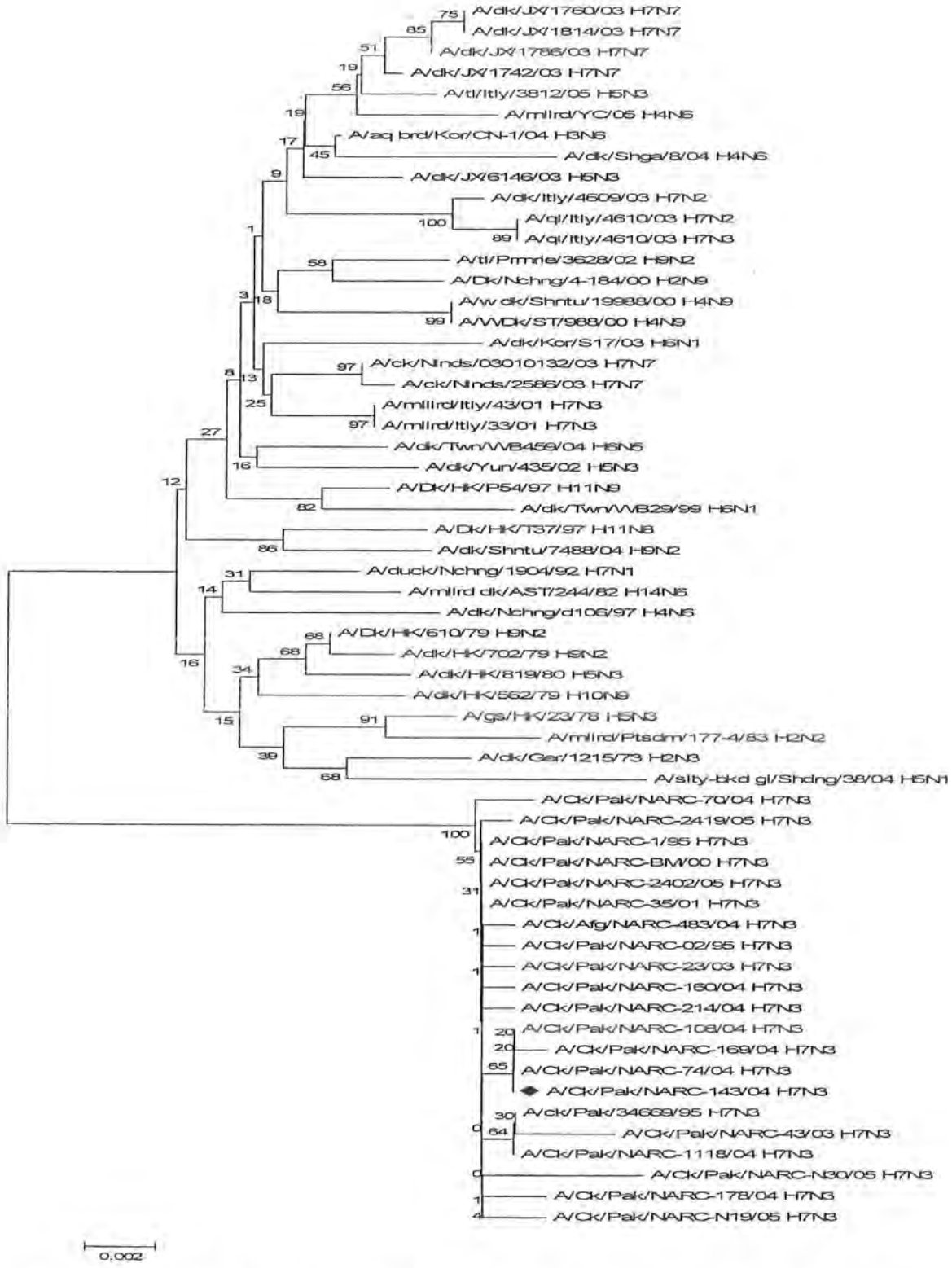

Figure 39: Phylogenetic tree of A/Chicken/Pakistan/NARC-143/ 04(H7N3) with other GenBank sequences for the NS1 gene

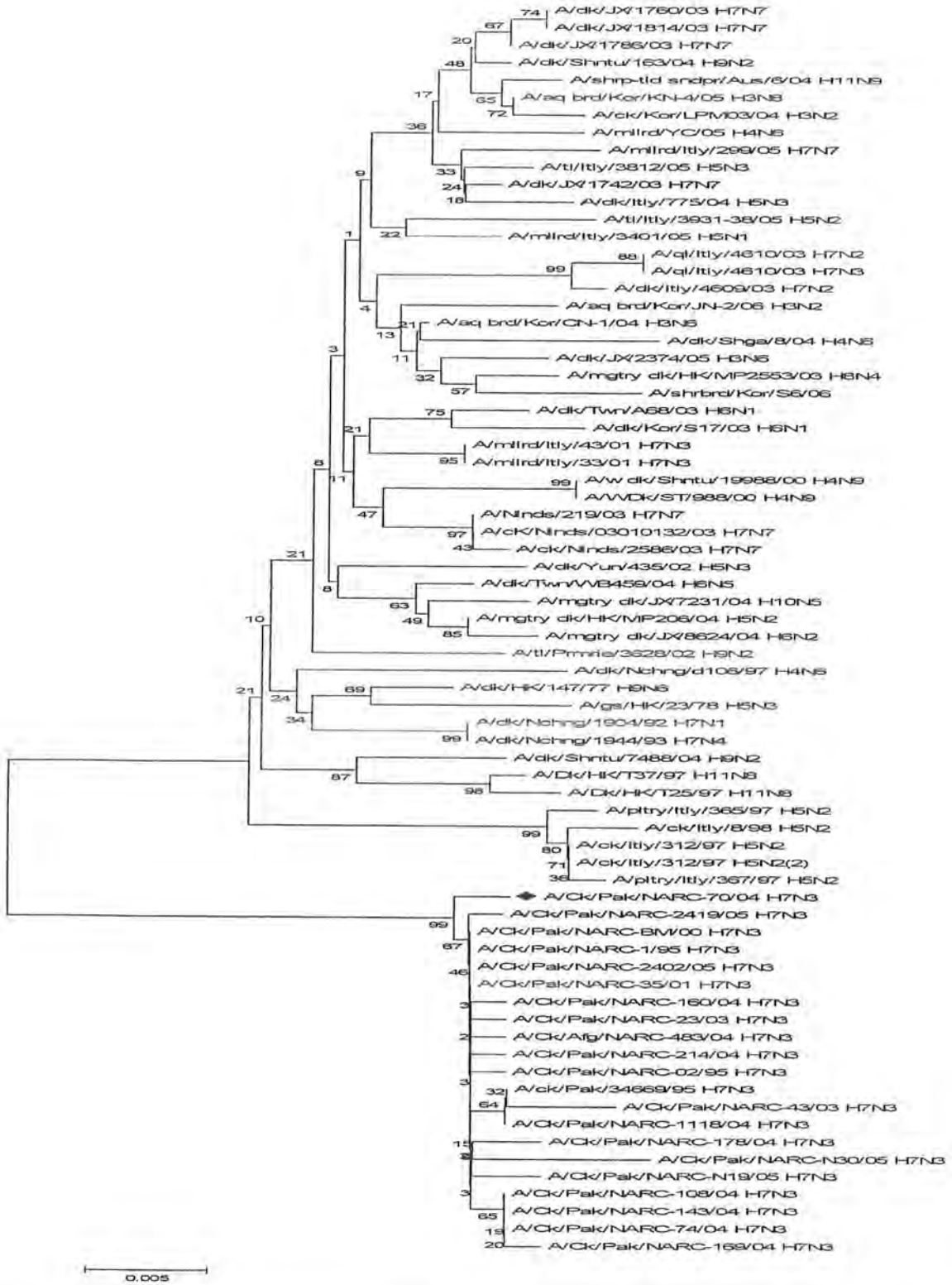

Figure 40: Phylogenetic tree of A/Chicken/Pakistan/NARC-70/04(H7N3) with other GenBank sequences for the NS1 gene

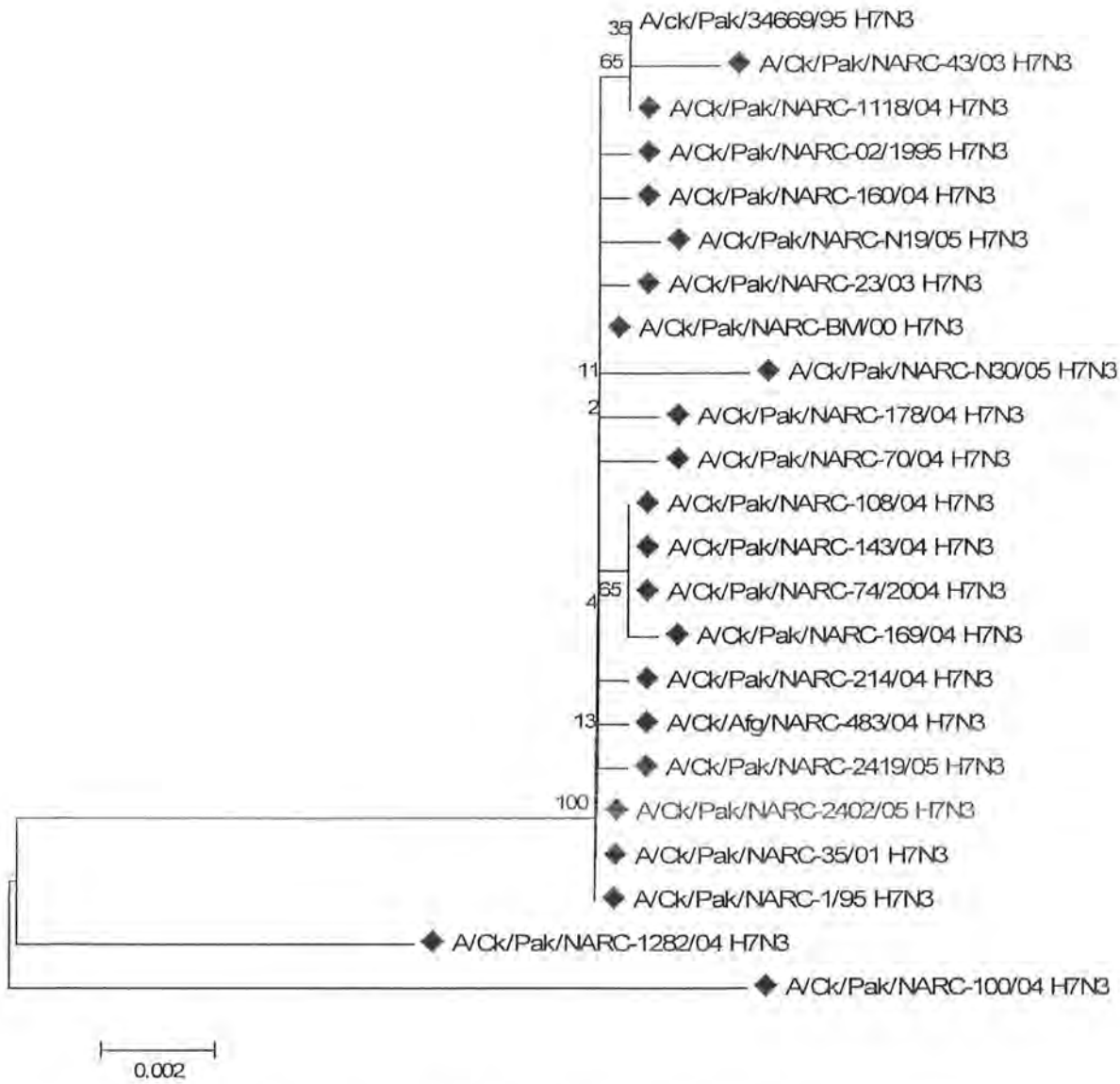

Figure 41: Phylogenetic tree of all Pakistani H7N3 AIV isolates for the NS1 gene

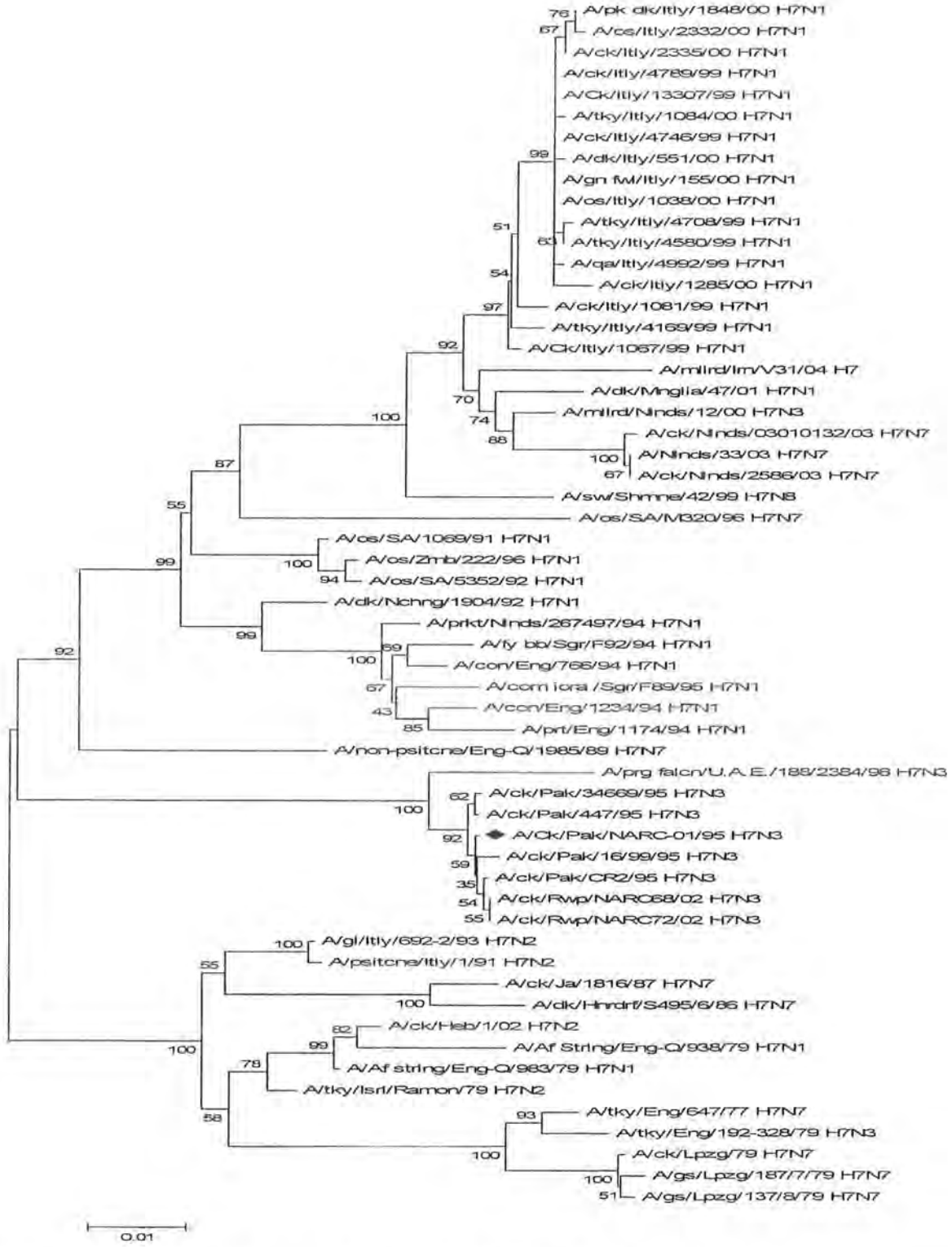

Figure 42: Phylogenetic tree of A/Chicken/Pakistan/NARC-1/95(H7N3) with other GenBank sequences for the HA gene

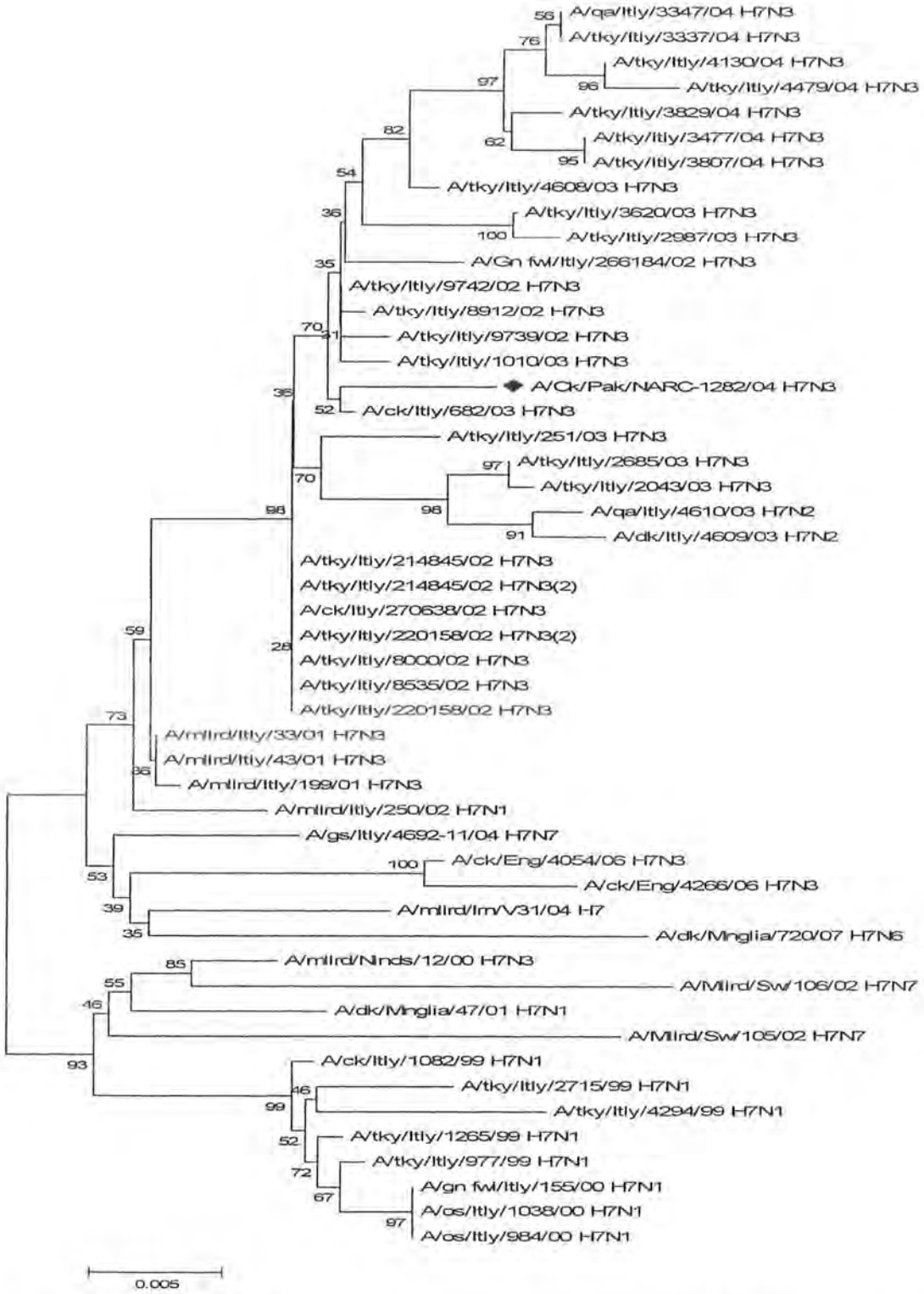

Figure 43: Phylogenetic Tree of A/Chicken/Pakistan/NARC-1282/ 04(H7N3) for the HA gene

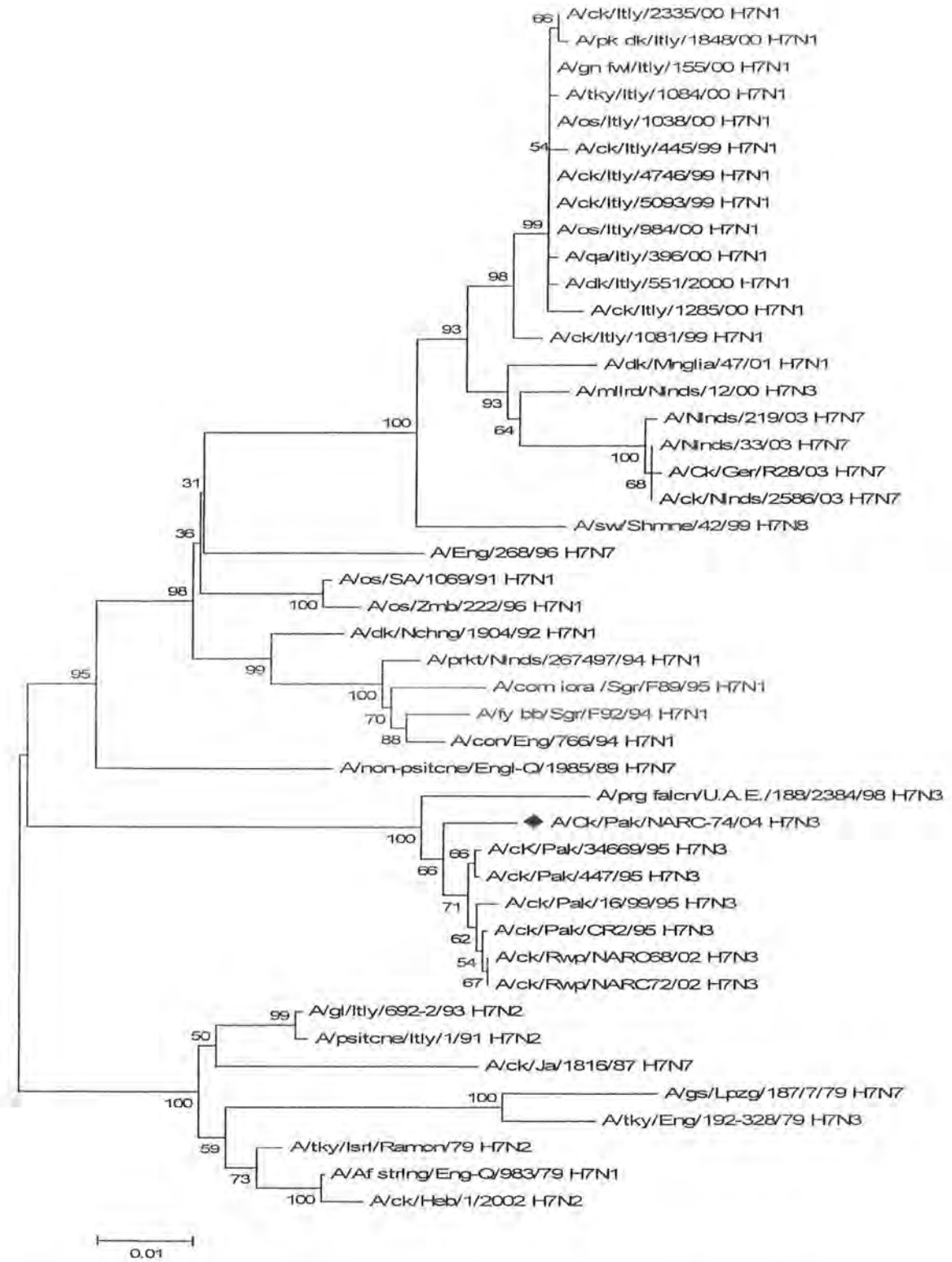

Figure 44: Phylogenetic Tree of A/Chicken/Pakistan/NARC-74/04 H7N3 with other GenBank sequences for the HA gene

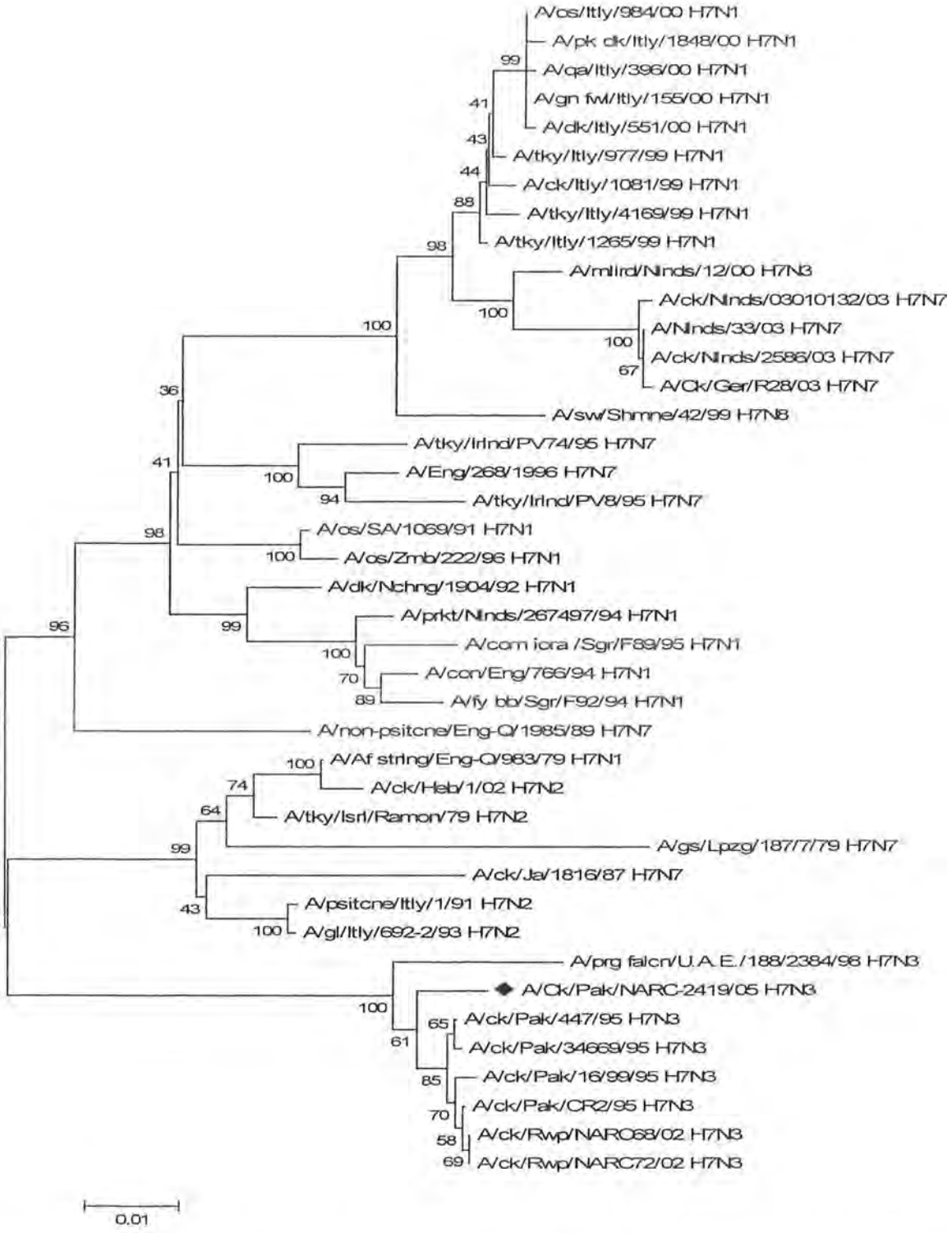

Figure 45: Phylogenetic Tree of A/Chicken/Pakistan/NARC-2419/ 05(H7N3) with other GenBank sequences for the HA gene

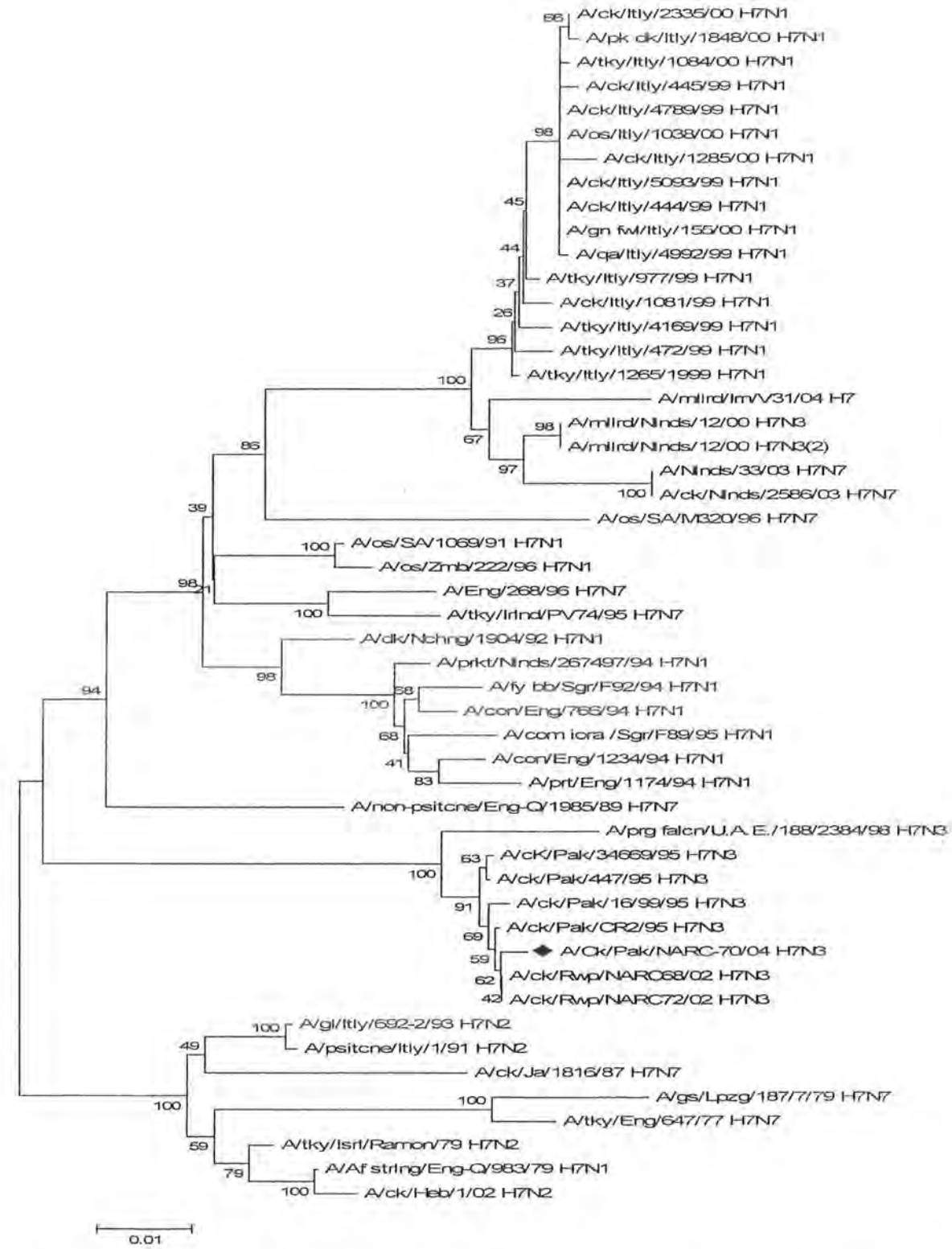

Figure 46: Phylogenetic tree of A/Chicken/Pakistan/NARC-70/04(H7N3) with other GenBank sequences for the HA gene

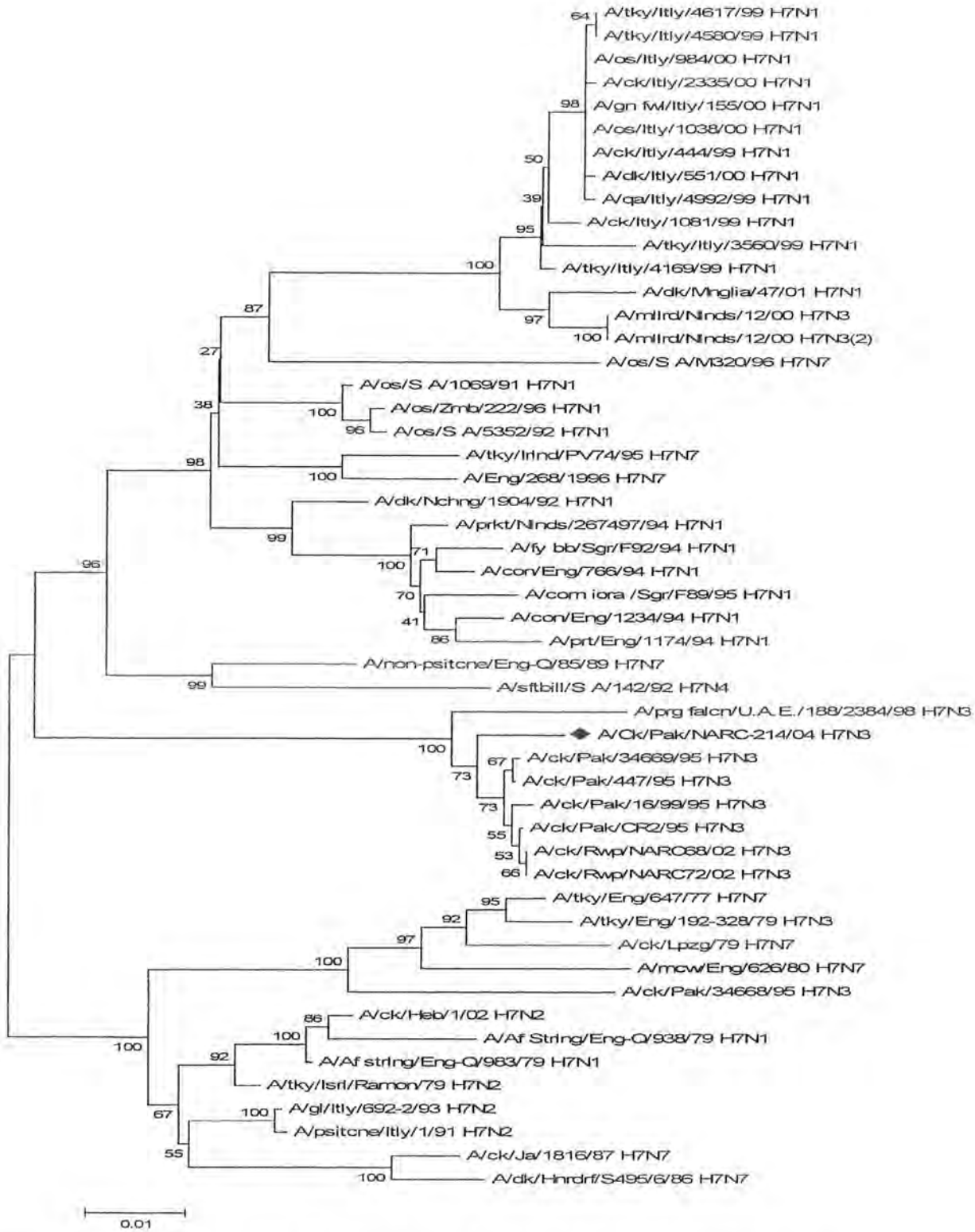

Figure 47: Phylogenetic tree of A/Chicken/Pakistan/NARC-214/ 04(H7N3) with other GenBank sequences for the HA gene

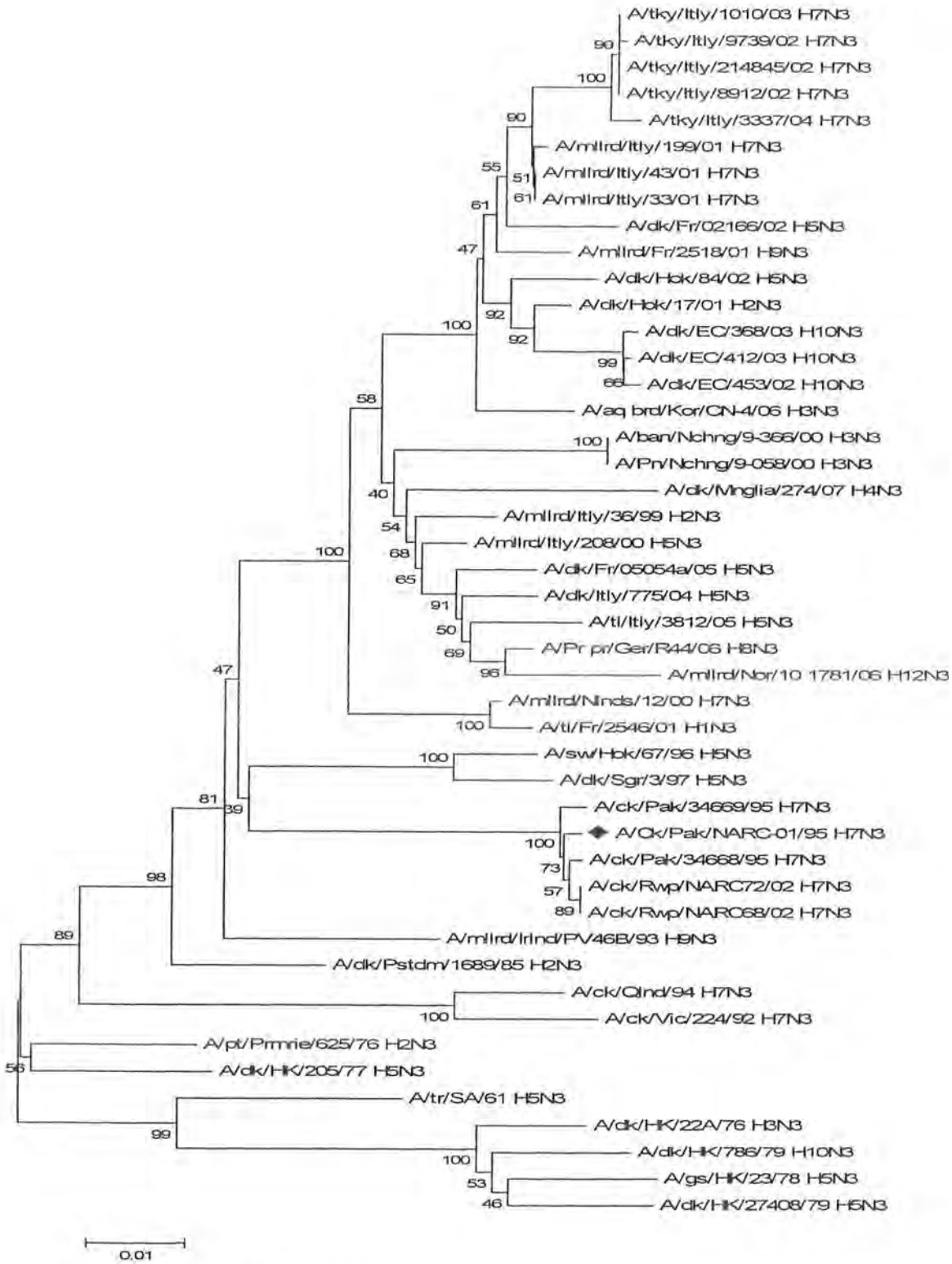

Figure 48: Phylogenetic Tree of A/Chicken/Pakistan/NARC-1/95(H7N3)01 with other GenBank sequences for the NA gene

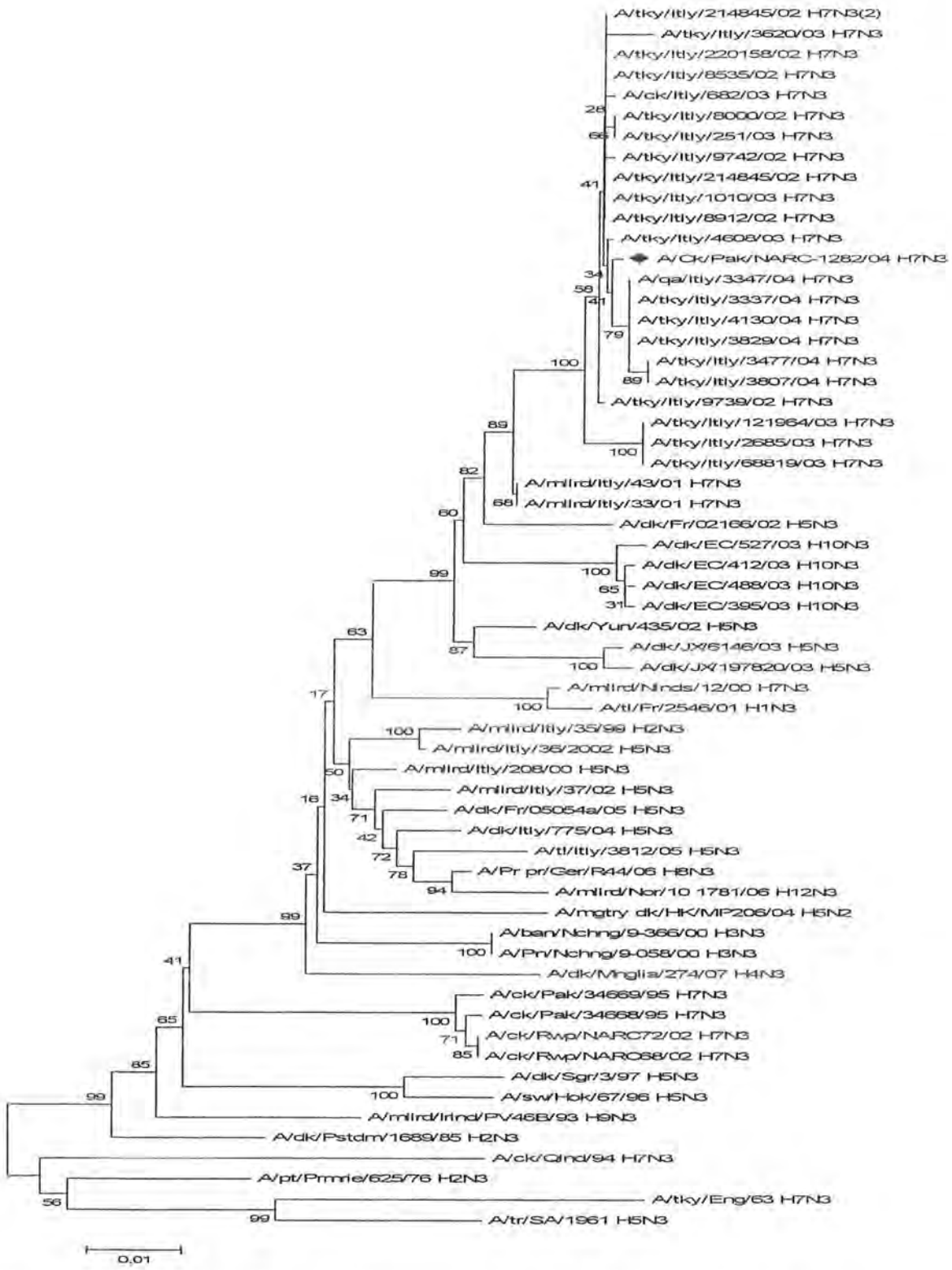

Figure 49: Phylogenetic Tree of A/Chicken/Pakistan/NARC-1282/ 04(H7N3) with other GenBank sequences for the NA gene

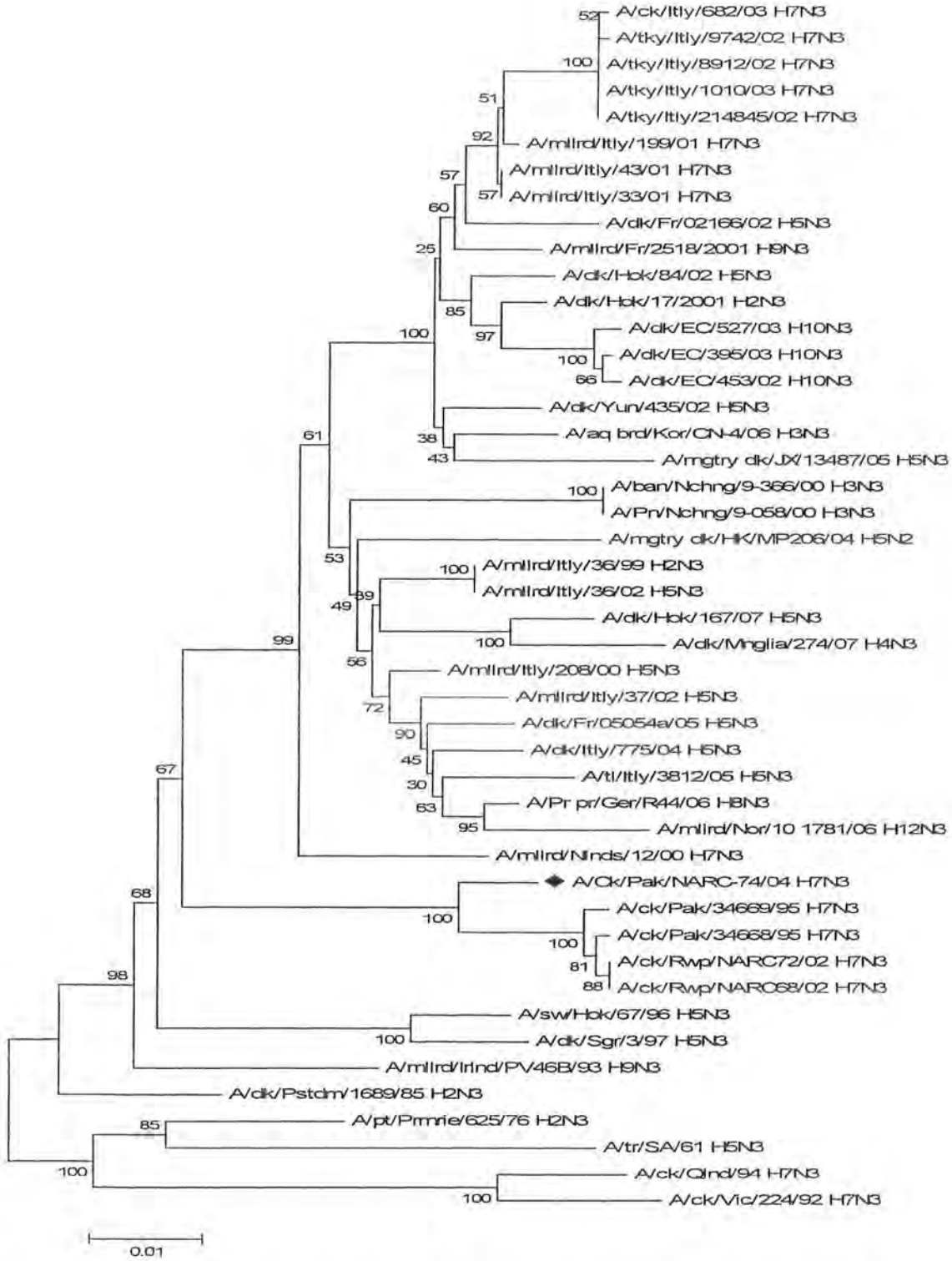

Figure 50: Phylogenetic Tree of A/Chicken/Pakistan/NARC-74/04 H7N3 with other GenBank sequences for the NA gene

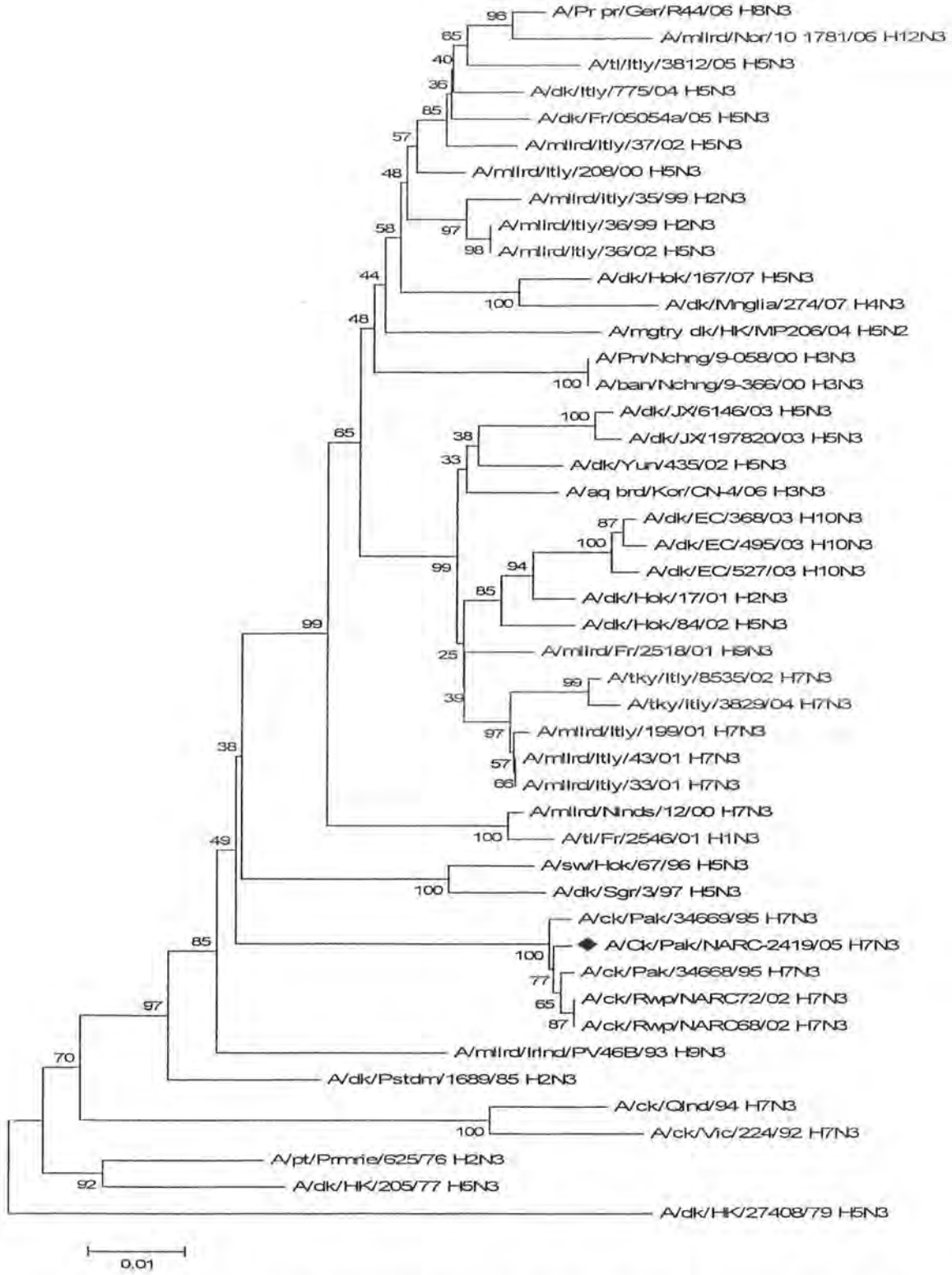

Figure 51: Phylogenetic Tree of A/Chicken/Pakistan/NARC-2419/ 05(H7N3) with other GenBank sequences for the NA gene

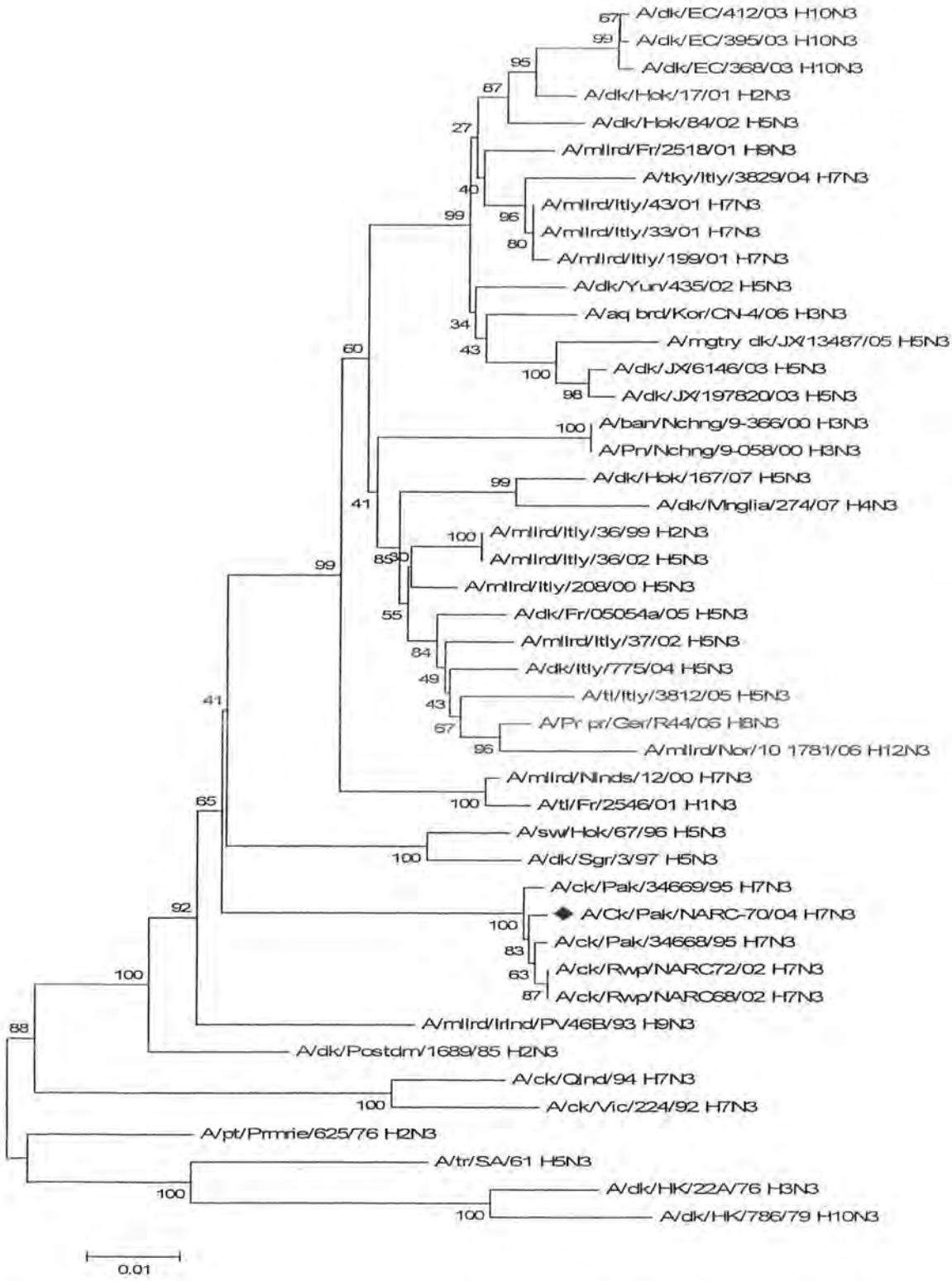

Figure 52: Phylogenetic tree of A/Chicken/Pakistan/NARC-70/04(H7N3) with other GenBank sequences for the NA gene

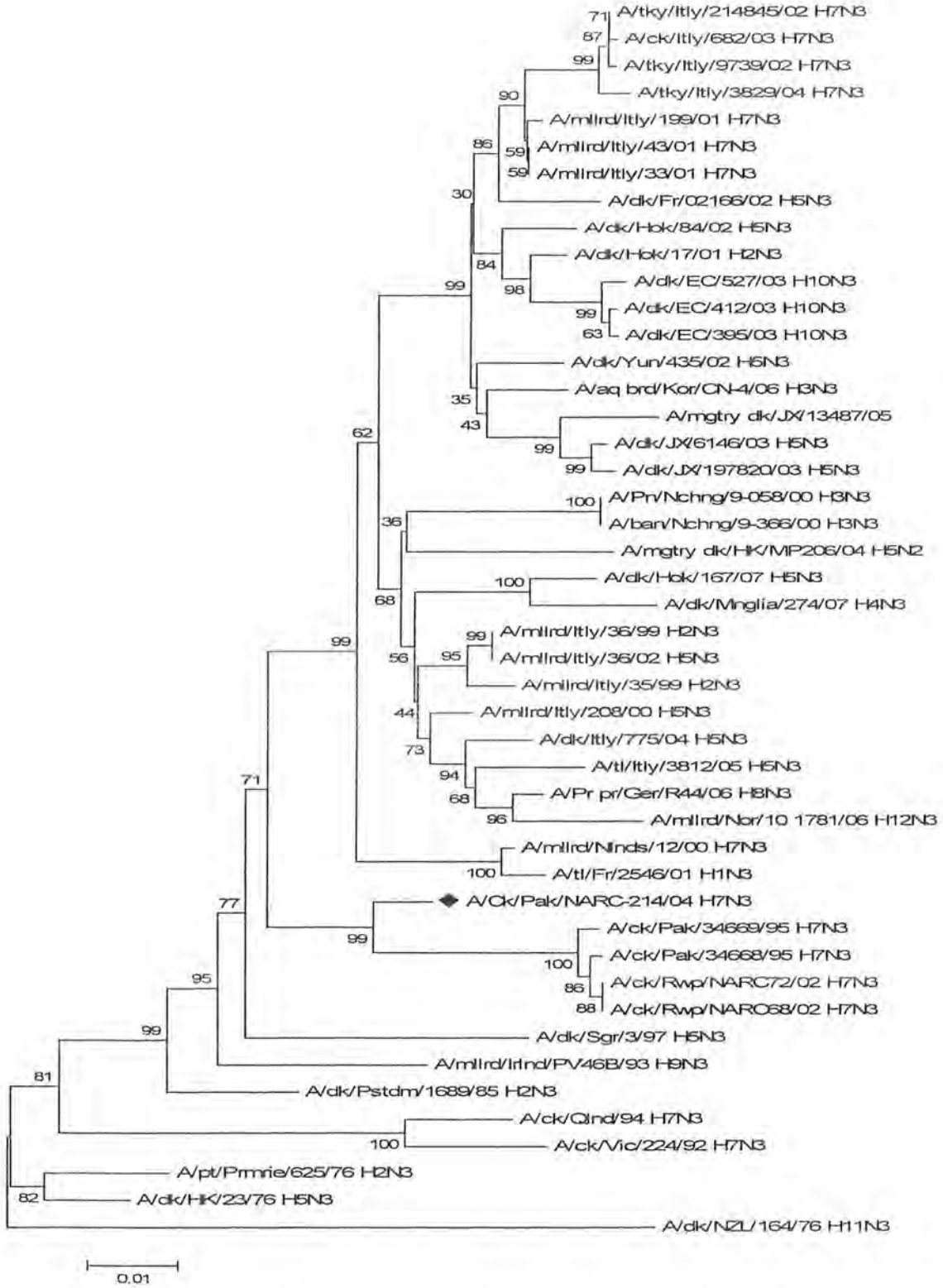

Figure 53: Phylogenetic tree of A/Chicken/Pakistan/NARC-214/ 04(H7N3) with other GenBank sequences for the NA gene

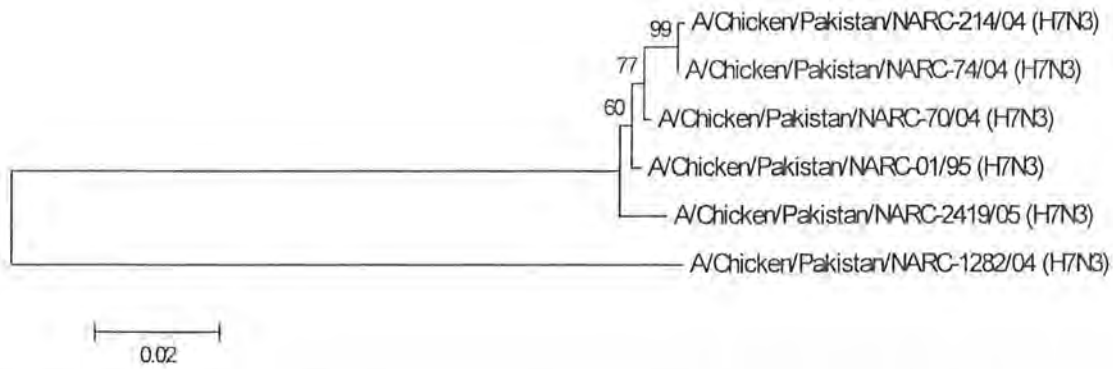

Figure 54: Phylogenetic Tree of Pakistani H7N3 isolates sequenced for the HA gene

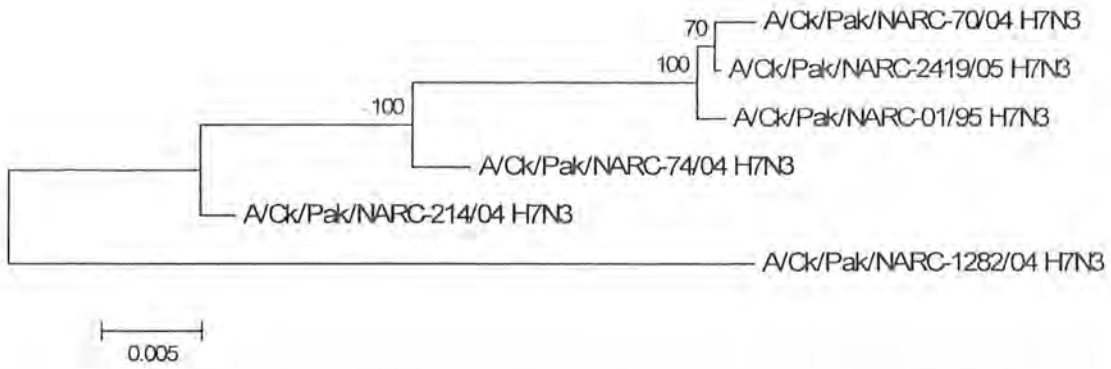

Figure 55: Phylogenetic tree of Pakistani H7N3 isolates sequenced for the NA gene

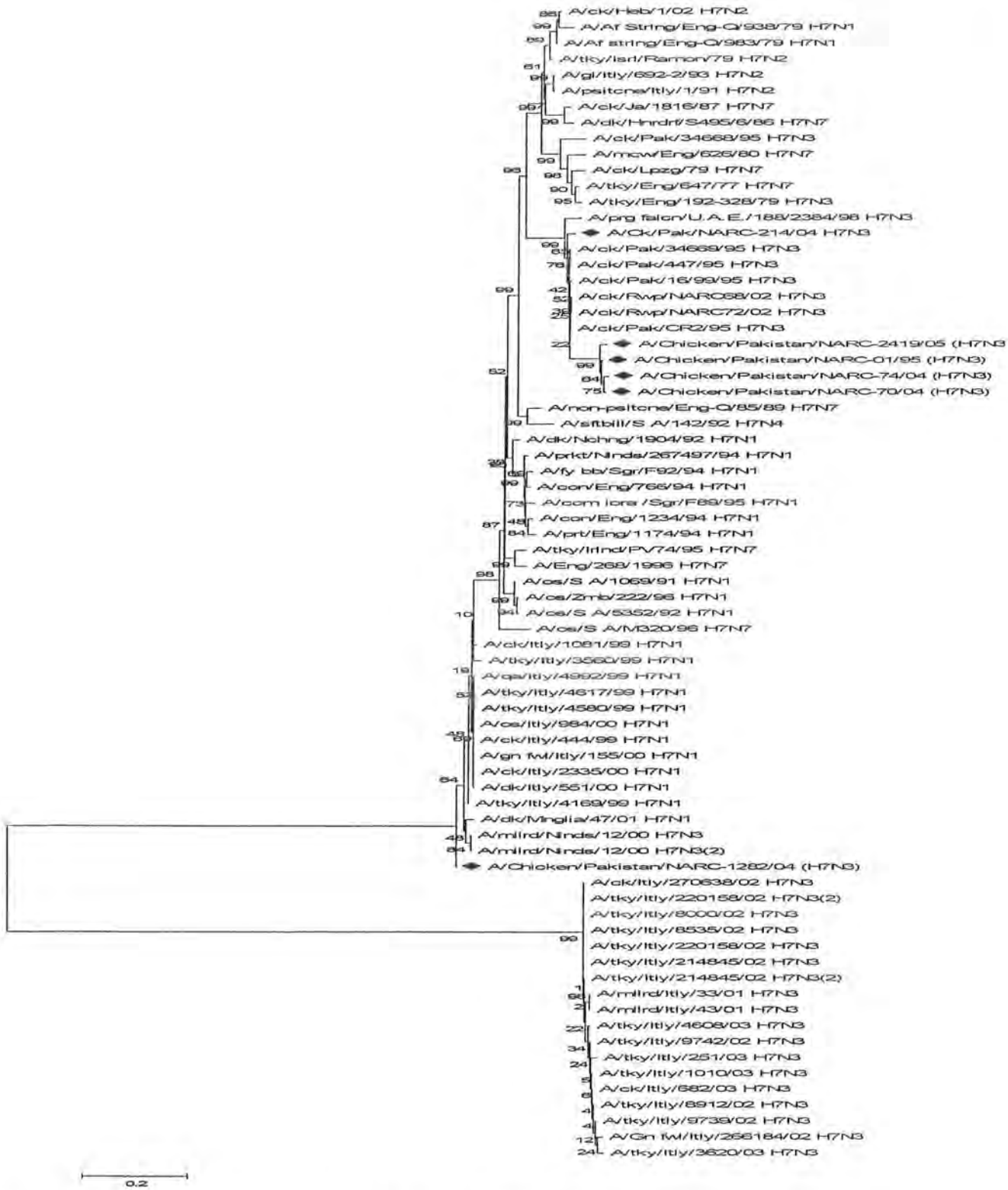

Figure 56: Phylogenetic tree of all Pakistan H7N3 isolates sequenced with other GenBank sequences for the HA gene

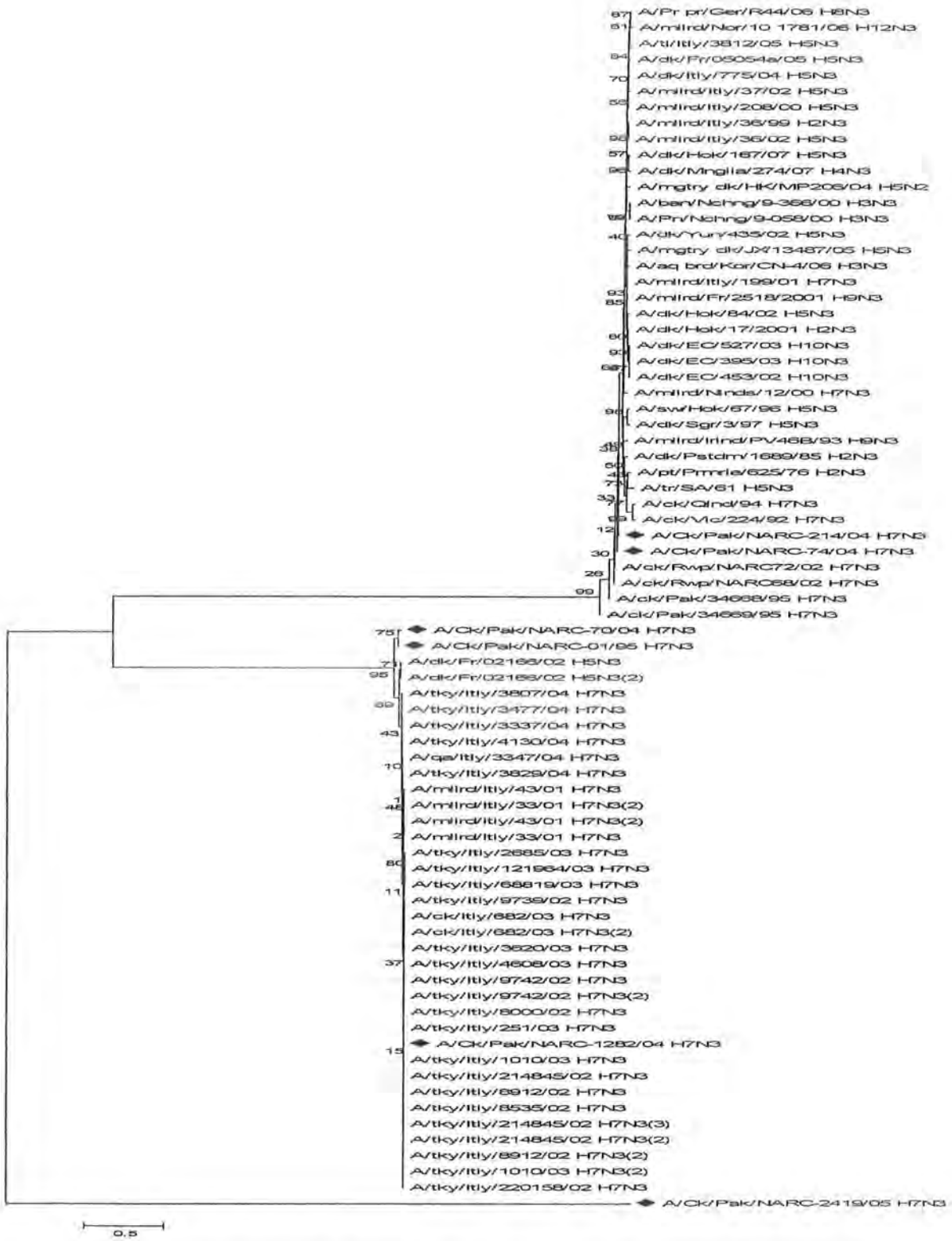

Figure 57: Phylogenetic tree of all Pakistani H7N3 sequenced with GenBank sequences for the NA gene

NUCLEOTIDE SEQUENCES OF NS1 GENE FOR PAKISTANI H7N3 ISOLATES

A/Chicken/Pakistan/NARC-160/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAGCCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 58: Nucleotide sequence of A/Chicken/Pakistan/NARC-160/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688552.1**)

A/Chicken/Pakistan/NARC-1118/04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACCCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 59: Nucleotide sequence of A/Chicken/Pakistan/NARC-1118/04(H7N3) for NS1 gene (**GenBank Acc no: EF688553.1**)

A/Chicken/Pakistan/NARC-108/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTctttggCATGTCCG
CAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGGCTC
CGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGGACA
TCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGAGG
AAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCACGT
TACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCTCAT
GCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCAATA
ATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACCGGCT
GGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGGGA
GAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTCAAAA
ATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAGTTCG
AGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAATGGG
AGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGGGAACAATTGGGT
CAGAAGTTTGA

Figure 60: Nucleotide sequence of A/Chicken/Pakistan/NARC-108/ 04(H7N3) for NS1 gene **(GenBank Acc no: EF688555.1)**

A/Chicken/Pakistan/NARC-N19/05(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTCCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCCTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 61: Nucleotide sequence of A/Chicken/Pakistan/NARC-N19/05(H7N3) for NS1 gene **(GenBank Acc no: EF688542.1)**

A/Chicken/Pakistan/NARC-1/95(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 62: Nucleotide sequence of A/Chicken/Pakistan/NARC-1/95(H7N3) for NS1 gene (**GenBank Acc no: EF688543.1**)

A/Chicken/Pakistan/NARC-BM/00(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 63: Nucleotide sequence of A/Chicken/Pakistan/NARC-BM/00(H7N3) for NS1 gene (**GenBank Acc no: EF688540.1**)

A/Chicken/Pakistan/NARC-2419/ 05(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAaCGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGGC
TCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGGA
CATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGAG
GAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCAC
GTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCTC
ATGCCAAAGCAgAAAGTGGCGGGTCCCCTTTGCATCAAAATGGACCAGGCAA
TAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGtGATTTTTGACCGG
CTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGTGG
GAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTCAA
AAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAGTT
CGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAATG
GGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATTG
GGTCAGAAGTTTGA

Figure 64: Nucleotide sequence of A/Chicken/Pakistan/NARC-2419/ 05(H7N3) for NS1 gene (**GenBank Acc no: EF688534.1**)

A/Chicken/Pakistan/NARC-35/01(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 65: Nucleotide sequence of A/Chicken/Pakistan/NARC-35/01(H7N3) for NS1 gene (**GenBank Acc no: EF688544.1**)

A/Chicken/Pakistan/NARC-214/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGtc
cgCaAacgaTTtGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGGCTCC
GCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGGACAT
CGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGAGGA
AGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCACGTT
ACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCTCAT
GCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCAATA
ATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACCGGCT
GGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAGTTGTGGGA
GAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTCAAAA
ATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAGTTCG
AGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAATGGG
AGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATTGGGT
CAGAAGTTTGA

Figure 66: Nucleotide sequence of A/Chicken/Pakistan/NARC-214/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688538.1**)

A/Chicken/Afghanistan/NARC-483/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTAG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCaCCGTTATCTTCTcTTCCAGgACATacTGAAGAGGAtGTCAAA
AATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAGTTC
GAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAATGG
GAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATTGG
GTCAGAAGTTTGA

Figure 67: Nucleotide sequence of A/Chicken/Afghanistan/NARC-483/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688539.1**)

A/Chicken/Karachi/ Pakistan/NARC-23/03(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTAAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 68: Nucleotide sequence of A/Chicken/Karachi/ Pakistan/NARC-23/03(H7N3) for NS1 gene (**GenBank Acc no: EF688549.1**)

A/Chicken/Pakistan/NARC-02/95(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTGTCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 69: Nucleotide sequence of A/Chicken/Pakistan/NARC-02/95(H7N3) for NS1 gene (**GenBank Acc no: EF688550.1**)

A/Chicken/Pakistan/NARC-74/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGGGAACAATT
GGGTCAGAAGTTTGA

Figure 70: Nucleotide sequence of A/Chicken/Pakistan/NARC-74/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688551.1**)

A/Chicken/Pakistan/NARC-70/04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
AGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATGTTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 71: Nucleotide sequence of A/Chicken/Pakistan/NARC-70/04(H7N3) for NS1 gene (**GenBank Acc no: EF688545.1**)

A/Chicken/Pakistan/NARC-178/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAGTCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGGGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 72: Nucleotide sequence of A/Chicken/Pakistan/NARC-178/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688546.1**)

A/Chicken/Pakistan/NARC-N30/05(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTCCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGGAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTCGAAGAAATGTCAAGGGACTGGTTCATGC
TCATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATTAGAATGGACCAGGC
AATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGAC
CGGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTG
TGGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAG
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 73: Nucleotide sequence of A/Chicken/Pakistan/NARC-N30/05(H7N3) for NS1 gene (**GenBank Acc no: EF688547.1**)

A/Chicken/Pakistan/NARC-143/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGGGAACAATT
GGGTCAGAAGTTTGA

Figure 74: Nucleotide sequence of A/Chicken/Pakistan/NARC-143/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688548.1**)

A/Chicken/Pakistan/NARC-1282/ 04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCACGT
CCGCAAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGACCGG
CTTCGCCGAGATCGGAAGTCCCTAAGAGGAAGGGGCAGCACTCTTGGTCTGG
ACATCGAGACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGGATTCTGGA
GGAAGAATCTGATGAGGCACTTAAAATGACTATTACTTCAGTGCCGGCTTCA
CGCTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGC
TCATGCCCAAGCAGAAAGTGGCAGGTTCCCTTTGCATCAGAATGGACCAGGC
AATAATGGATAAGAACATCAAATTGAAAGCAAACTTCAGTGTGATTTTTGAC
CGGCTGGAAACCCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTG
TGGGAGAAATCTCACCATTACCTTCTCTTCCAGGACATACTGATGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAATGATAACACAG
TTCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGGA
TGGGAGACCTCCACTCCCTCCAAAACAGAAACGGAAAATGGCGAGAACAAT
TGAGTCAGAAGTTTGA

Figure 75: Nucleotide sequence of A/Chicken/Pakistan/NARC-1282/ 04(H7N3) for NS1 gene (**GenBank Acc no: EF688535.1**)

A/Chicken/Pakistan/NARC-2402/ 05(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 76: Nucleotide sequence of A/Chicken/Pakistan/NARC-2402/ 05(H7N3) for NS1 gene (**GenBank Acc no: EF688554.1**)

A/Chicken/Pakistan/NARC-43/ 03(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACCCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGGCTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTTCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATATTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCAAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGAGAACAATT
GGGTCAGAAGTTTGA

Figure 77: Nucleotide sequence of A/Chicken/Pakistan/NARC-43/ 03(H7N3) for NS1 gene (**GenBank Acc no: EF688536.1**)

A/Chicken/Pakistan/NARC-169/04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTTCAGGTAGACTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAATTGGGTGATGCCCCATTCCTTGACCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ACATCGAAACAGCTACTCGTGCGGGAAAGCAGATAGTGGAGCGAATTCTGGA
GGAAGAATCCGACGAGGCACTTAAAATGACCATTGCTTCTGTGCCCGCTTCA
CGTTACCTAACTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGCT
CATGCCAAAGCAGAAAGTGGCGGGTCCCCTTTGCATCAGAATGGACCAGGCA
ATAATGGATAAAACCATCATATTGAAAGCAAACTTCAGTGTGATTTTTGACC
GGCTGGAAAATCTAATACTACTTAGAGCTTCCACAGAAGAAGGAGCAATTGT
GGGAGAAATCTCACCGTTATCTTCTCTTCCAGGACATACTGAAGAGGATGTC
AAAAATGCAATTGGGGTCCTCATCGGAGGATTTAAATGGAATGATAACACAG
TTCGAGTCTCTGAAAATCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGAA
TGGGAGACCTCCACTCCCTCCAAAGTAGAAACGGAAAATGGCGGGAACAATT
GGGTCAGAAGTTTGA

Figure 78: Nucleotide sequence of A/Chicken/Pakistan/NARC-169/04(H7N3) for NS1 gene (**GenBank Acc no: EF688537.1**)

A/Chicken/Pakistan/NARC-100/04(H7N3)

ATGGATTCCAACACTGTGTCAAGCTTCCAGGTAGATTGCTTTCTTTGGCATGT
CCGCAAACGATTTGCAGACCAAGAACTGGGTGATGCCCCATTCCTTGATCGG
CTCCGCCGAGATCAGAAGTCCCTAAGAGGAAGAGGCAGCACTCTTGGTCTGG
ATCTCGAGACAGCTACTCGTGCGGGAAGGCAAATAGTGGAGAAGATTCTGGA
GGAAGAATCTGATGAGGCACTTAAAATGACTATCGCTTCTGTGCCAACTTCA
CGCTACCTGAGTGACATGACTCTTGAAGAAATGTCAAGGGACTGGTTCATGC
TCATGCCAAAGCAGAAAGTGGCTGGTTCCCTGTGCATCAGAATGGATCAGGC
AATAATGGATAAAACCATCATATTGAAAGCAAATTTCAGTGTGATTTTTGACC
GGCTGGACACCTTAATACTACTTAGAGCTTTCACAGAAGAGGGAGCAATTGT
GGGAGAAATCTCACCGTTACCTTCTCTTCCAGGACATACTGATGAGGATGTCA
AAAATGCAATTGGGGTCCTCATCGGAGGACTTGAATGGAATGATAACACAGT
TCGAGTCTCTGAAACTCTACAGAGATTCGCTTGGAGAAGCAGTAATGAGGAT
GGGGGGACCTCCATTCCCTCCAAAGCAGAAACGGAAAATGGCGAGAACAATT
AAGTCAGAAGTTTGA

Figure 79: Nucleotide sequence of A/Chicken/Pakistan/NARC-100/04(H7N3) for NS1 gene (**GenBank Acc no: EF688541.1**)

A/Chicken/Pakistan/NARC-214/04 (H7N3)

ATGAACAATCAAATCCTAATACTCGCTCTTGTGGCGATAAACCCGACAAATG
CAGACAAGATTTGCCTTGGGCATCATGCCGTGCCAAACGGAACTAAGGTAAA
CACACTGACTGAAAGAGGAGTGGAAGTTGTTGATGCAACTGAAACAGTGGA
ACGAACAAGCTTTCCCAGTATCTGCTCAAAAGGAAAAAGGACAATTGACCTT
GGCCAATGTGGACTGCTGGGAACAGTCACTGGGCCACCCCAATGTGACCAAT
TTCTAGAGTTTTCGGCAGATTTAATCATTGAAAGGCGGGAAGGAAATGATGT
CTGTTATCCTGGGAAATTTGCGAATGAGGAAGCTCTGAGGCAAGTTCTCAGG
GAATCAGGCGGAATTGAGAAGGAGACAATAGGATTCACATACAGCGGAATA
AGGACTAATGGAACAACCAGTTCATGTAGGAGATCAGGATCTTCATTCTATG
CAGAGATGAAATGGCTTCTGTCAAATAATGACAATGATGCTTTCCCGCAGAT
GACTAAGTCATACAGAAACACAAGGAAGGGTCCAGCTCTAATAATTTGGGGA
ATCCATCATTCCGGATCAACTACTGAACAGACCAGATTATACGGGAGTGGGA
ACAAACTAATAACAGTTGGGAGTTCCAATTATCAACAGTCCTTTGTACCGAGT
CCAGGAGCGAGACCACAAGTGAATGGCCAATCTGGGAGGATCGACTTCCATT
GGCTGATGCTAAACCCCAATGACACAGTAACTTTCAGCTTCAATGGGGCTTTC
ATAGCTCCAGATCGTGTGAGTTTTCTGAGAGGGAAATCTATGGGAATCCAGA
GTGGAGTACAGGTTGATGCCAGTTGTGAGGGAGATTGCTATCATAGTGGAGG
AACAATAATAAGTAATTTGCCTTTTCAAAACATCAATAGCAGAGCAGTAGGG
AAATGTCCGAGGTATGTTAAACAAGAGAGCCTGCTACTGGCAACTGGGATGA
AGAACGTTCCTGAAACTCCAAAAAGAAAAAGAAAAAGAGGCCTATTTGGTG
CCATAGCGGGATTCATTGAAAATGGATGGGAAGGTTTGATTGATGGGTGGTA
TGGCTTCAGGCATCAAAATGCACAAGGAGAGGGAACTGCTGCAGATTACA

Figure 80: Nucleotide sequence of A/Chicken/Pakistan/NARC-214/04 (H7N3) for HA1 subunit of HA gene

A/Chicken/Pakistan/NARC-2419/05 (H7N3)

ATGAACAATCAAATCCTAATACTCGCTCTTGTGGCGATAATCCCGACAAATG
CAGACAAGATTTGCCTTGGGCATCATGCCGTGCCAAACGGAACTAAGGTAAA
CACACTGACTGAAAGAGGACTGGAAGTTGTTGATGCAACTGAAACAGTGGAA
CGAACAAGCATTCCCAGTATCTGCTCAAAAGGAAAAAGGACAATTGACCTTG
GCCAATGTGGACTGCTGGGAACAGTCACTGGGCCACCCCAATGTGACCAATT
TCTAGAGTTTTCGGCAGATTTAATCATTGAAAGGCGGGAAGGAAATGATGTC
TGTTATCCTGGGAAATTTGCGAATGAGGAAGCTCTGAGGCAAGTTCTCAGAG
AATCAGGCGGAATTGAGAAGGAGACAATAGGATTCACATACAGCGGAATAA
TGACTAATGGAGCAACCAGTTCATGTAGGAGATCAGGATCTTCATTCTATGC
AGAGATGAAATGGCTTCTGTCAAATAATGACAATGCTGCTTTCCCGCAGATG
ACTAAGTCATACAGAAACACAAGGAAGGGTCCAGCTCTAATAATTTGGGGAA
TCCATCATTCCGGATCAACTACTGAACAGACCAGATTATACGGGAGTGGGAA
CAAACTAATAACAGTTGAGAGTTCCAATTATCAACAGTCCTTTGTACCGAGTC
CAGGAGCGAGACCACAAGTGAATGGCCAATCTGGGAGGATCGACTTCCATTG
GCTGATGCTAAACCCCAATGACACAGTAACTTTCAGTTTCAATGGGGCTTTCA
TAGCTCCAGATCGTGTAAGTTTTCTGAGAGGGAAATCTATGGGAATCCAGAG
TGAAGTACAAGTTGATGCCAATTGTGAAGGAGATTGCTATCATAGTGGAGGA
ACAATAATAAGTAATTTGCCTTTTCAAAACATCAATAGCAGAGCAGTAGGGA
AATGTCCGAGGTATGTTAAGCAAGAAAGTCTGCTACTGGCAACTGGGATGAA
GAACGTTCCTGAAACTCCAAAAAGAAAAAGAAAACGAGGCCTATTTGGTGCC
ATAGCAGGATTCATTGAAAATGGATGGGAAGGTTTGATTGATGGGTGGTATG
GCTTCAGGCATCAAAATGCACAAGGAGAGGGAACTGCTGCAGATTACAAGA
GCACCCAGTCAGCAATT

Figure 81: Nucleotide sequence of A/Chicken/Pakistan/NARC-2419/05 (H7N3) for HA1 subunit of HA gene

A/Chicken/Pakistan/NARC-74/04 (H7N3)

ATGAACAATCAAATCCTAATACTCGCTCTTGTGGCGATAATCCCGACAAATG
CAGACAAGATTTGCCTTGGGCATCATGCCGTGCCAAACGGAACTAAGGTAAA
CACACTGACTGAAAGAGGAGTGGAAGTTGTTGATGCAACTGAAACAGTGGA
ACGAACAAGCTTTCCCAGTATCTGCTCAAAAGGAAAAAGGACAATTGACCTT
GGCCAATGTGGACTGCTGGGAACAGTCACTGGGCCACCCCAATGTGACCAAT
TTCTAGAGTTTTCGGCAGATTTAATCATTGAAAGGCGGGAAGGAAATGATGT
CTGTTATCCTGGGAAATTTGCGAATGAGGAAGCTCTGAGGCAAGTTCTCAGG
GAATCAGGCGGAATTGAGAAGGAGACAATAGGATTCACATACAGCGGAATA
AGGACTAATGGAACAACCAGTTCATGTAGGAGATCAGGATCTTCATTCTATG
CAGAGATGAAATGGCTTCTGTCAAATAATGACAATGATGCTTTCCCGCAGAT
GACTAAGTCATACAGAAACACAAGGAAGGGTCCAGCTCTAATAATTTGGGGA
ATCCATCATTCCGGATCAACTACTGAACAGACCAGATTATACGGGAGTGGGA
ACAAACTAATAACAGTTGGGAGTTCCAATTATCAACAGTCCTTTGTACCGAGT
CCAGGAGCGAGACCACAAGTGAATGGCCAATCTGGGAGGATCGACTTCCATT
GGCTGATGCTAAACCCCAATGACACAGTAACTTTCAGCTTCAATGGGGCTTTC
ATAGCTCCAGATCGTGTGAGTTTTCTGAGAGGGAAATCTATGGGAATCCAGA
GTGGAGTACAGGTTGATGCCAGTTGTGAGGGAGATTGCTATCATAGTGGAGG
AACAATAATAAGTAATTTGCCTTTTCAAAACATCAATAGCAGAGCAGTAGGG
AAATGTCCGAGGTATGTTAAACAAGAGAGCCTGCTACTGGCAACTGGGATGA
AGAACGTTCCTGAAACTCCAAAAAGAAAAAGAAAAAGAGGCCTATTTGGTG
CCATAGCGGGATTCATTGAAAATGGATGGGAAGGTTTGATTGATGGGTGGTA
TGGCTTCAGGCATCAAAATGCACAAGGAGAGGGAACTGCTGCAGATTACAAG
AGCACCCAGTCAGCAATTAATTAATCAAATAACAGGGAAATTAAACCGGCTC
ATAGAG

Figure 82: Nucleotide sequence of A/Chicken/Pakistan/NARC-74/04 (H7N3) for HA1 subunit of HA gene

A/Chicken/Pakistan/NARC-01/95 (H7N3)

ATGAACAATCAAATCCTAATACTCGCTCTTGTGGCGATAATCCCGACAAATG
CAGACAAGATTTGCCTTGGGCATCATGCCGTGCCAAACGGAACTAAGGTAAA
CACACTGACTGAAAGAGGAGTGGAAGTTGTTAATGCAACTGAAACAGTGGA
ACGAACAAGCATTCCCAGGATCTGCTCAAAAGGAAAAAGGACAATTGACCTT
GGCCAATGTGGACTGCTGGGAACAGTCACTGGGCCACCCCAATGTGACCAAT
TTCTAGAGTTTTCGGCAGATTTAATCATTGAAAGGCGGGAAGGAAATGATGT
CTGTTATCCTGGGAAATTTGCGAATGAGGAAGCTCTGAGGCAAGTTCTCAGG
GAATCAGGCGGAATTGAGAAGGAGACAATAGGATTCACATACAGCGGAATA
AGGACTAATGGAACAACCAGTTCATGTAGGAGATCAGGATCTTCATTCTATG
CAGAGATGAAATGGCTTCTGTCAAATAATGACAATGCTGCTTTCCCGCAGAT
GACTAAGTCATACAGAAACACAAGGAAGGGTCCAGCTCTAATAATTTGGGGA
ATCCATCATTCCGGATCAACTACTGAACAGACCAGATTATACGGGAGTGGGA
ACAAACTAATAACAGTTGGGAGTTCCAATTATCAACAGTCCTTTGTACCGAGT
CCAGGAGCGAGACCACAAGTGAATGGCCAATCTGGGAGGATCGACTTCCATT
GGCTGATGCTAAACCCCAATGACACAGTAACTTTCAGCTTCAATGGGGCTTTC
ATAGCTCCAGATCGTGTAAGTTTTCTGAGAGGGAAATCTATGGGAATCCAGA
GTGGAGTACAGGTTGATGCCAGTTGTGAAGGAGATTGCTATCATAGTGGAGG
AACAATAATAAGTAATTTGCCTTTTCAAAACATCAATAGCAGAGCAATAGGG
AAATGTCCGAGGTATGTTAAGCAAGAAAGTCTGCTACTGGCAACTGGGATGA
AGAACGTTCCTGAAACTCCAAAAAGAAAAAGAAAAAGAGGCCTATTTGGTG
CCATAGCAGGATTCATTGAAAATGGATGGGAAGGTTTGATTGATGGGTGGTA
TGGCTTCAGGCATCAAAATGCACAAGGAGAGGGAACTGCTGCAGATTACAAG
AG

Figure 83: Nucleotide sequence of A/Chicken/Pakistan/NARC-01/95 (H7N3) for HA1 subunit of HA gene

A/Chicken/Pakistan/NARC-70/04 (H7N3)

ATGAACAATCAAATCCTAATACTCGCTCTTGTGGCGATAATCCCGACAAATG
CAGACAAGATTTGCCTTGGGCATCATGCCGTGCCAAACGGAACTAAGGTAAA
CACACTGACTGAAAGAGGAGTGGAAGTTGTTGATGCAACTGAAACAGTGGA
ACGAACAAGCTTTCCCAGTATCTGCTCAAAAGGAAAAAGGACAATTGACCTT
GGCCAATGTGGACTGCTGGGAACAGTCACTGGGCCACCCCAATGTGACCAAT
TTCTAGAGTTTTCGGCAGATTTAATCATTGAAAGGCGGGAAGGAAATGATGT
CTGTTATCCTGGGAAATTTGCGAATGAGGAAGCTCTGAGGCAAGTTCTCAGG
GAATCAGGCGGAATTGAGAAGGAGACAATAGGATTCACATACAGCGGAATA
AGGACTAATGGAACAACCAGTTCATGTAGGAGATCAGGATCTTCATTCTATG
CAGAGATGAAATGGCTTCTGTCAAATAATGACAATGATGCTTTCCCGCAGAT
GACTAAGTCATACAGAAACACAAGGAAGGGTCCAGCTCTAATAATTTGGGGA
ATCCATCATTCCGGATCAACTACTGAACAGACCAGATTATACGGGAGTGGGA
ACAAACTAATAACAGTTGGGAGTTCCAATTATCAACAGTCCTTTGTACCGAGT
CCAGGAGCGAGACCACAAGTGAATGGCCAATCTGGGAGGATCGACTTCCATT
GGCTGATGCTAAACCCCAATGACACAGTAACTTTCAGCTTCAATGGGGCTTTC
ATAGCTCCAGATCGTGTAAGTTTTCTGAGAGGGAAATCTATGGGAATCCAGA
GTGGAGTACAGGTTGATGCCAGTTGTGAAGGAGATTGCTATCATAGTGGAGG
AACAATAATAAGTAATTTGCCTTTTCAAAACATCAATAGCAGAGCAATAGGG
AAATGTCCGAGGTATGTTAAGCAAGAAAGTCTGCTACTGGCAACTGGGATGA
AGAACGTTCCTGAAACTCCAAAAAGAAAAAGAAAAAGAGGCCTATTTGGTG
CCATAGCAGGATTCATTGAAAATGGATGGGAAGGTTTGATTGATGGGTGGTA
TGGCTTCAGGCATCAAAATGCACAAGGAGAGGGAACTGCTGCAGATTACAAG
AGCACCCAGT

Figure 84: Nucleotide sequence of A/Chicken/Pakistan/NARC-70/04 (H7N3) for HA1 subunit of HA gene

A/Chicken/Pakistan/NARC-1282/04 (H7N3)

ATGAACACTCAAATCCTGGTATTCGCTCTGGTGGCGATCATTCCGACAAATGC
AGACAAGATCTGCCTTGGGCACCATGCCGTGTCAAACGGGACTAAAGTAAAC
ACATTAACTGAAAGAGGAGTAGAAGTCATTAACGCAACTGAAACGGTGGAA
CGAACAAACGTCCCCAGGATCTGCTCAAAAGGGAAAAGGACAGTTGACCTC
GGTCAATGTGGACTTCTGGGAACAATCACTGGGCCACCCCAATGTGACCAAT
TCCTAGAATTTTCAGCCGATTTAATTATTGAGAGGCGAGAAGGAAGTGATGT
CTGTTATCCTGGGAAATTCGTGAATGAAGAAGCTCTGAGGCAAATTCTCAGG
GAGTCAGGCGGAATTGACAAGGAGACAATGGGGTTCACATACAGCGGGATA
AGGACTAATGGAGCAACCAGTGCATGTAGGAGATCAGGATCTTCATTCTATG
CAGAGATGAAATGGCTCCTGTCAAACACAGACAATGCTGCTTTCCCGCAGAT
GACTAAGTCATACAAAAACACAAGGAAAGACCCAGCTCTGATAATATGGGG
AATCCACCATTCCGGATCAACCACAGAACAGACCAAGCTATACGGGAGTGGA
AACAAATTAATAACAGTTGGGAGTTCTAATTACCAACAGTCCTTTGTACCGA
GTCCAGGAGCGAGGCCACAAGTGAATGGCCAATCTGGAAGAATTGACTTTCA
TTGGCTAATGCTAAACCCCAATGACACAGTTACTTTGAGTTTCAATGGGGCCT
TCATAGCTCCAGACCGTGCAAGCTTTCTGAGAGGAAAGTCTATGGGAATTCA
GAGTAGTGTACAGGTTGATGCCAATTGTGAAGGAGATTGCTATCATAGTGGA
GGGACAATAATAAGTAATTTGCCCTTTCAGAACATAAATAGCAGGGCAGTGG
GAAAATGCTCAAGATATGTTAAGCAAGAGAGTCTGATGCTGGCAACAGGAAT
GAAGAATGTTCCCGAAATCCCAAAGGGAAGAGGCCTATTTGGTGCTATAGCG
GGTTTCATTGAAAATGGATGGGAAGGTCTGATTGACGGGTGGTATGGCTTCA
GGCATCAAAATGCACAAGGGGAGGGAACTGCTGCAGATTACAAAAGCACCC
AATCAGCAATTGATC

Figure 85: Nucleotide sequence of A/Chicken/Pakistan/NARC-1282/04 (H7N3) for HA1 subunit of HA gene

A/Chicken/Pakistan/NARC-74/04 (H7N3)

ATGAATCCAAATCAGAAGATAATAACAATTGGTGTAGTGAATACTACTCTAT
CAACAATAGCCCTTCTTATTGGAATTGGGAATCTGGTTTTCAACACCGTTATA
CATGAGAAAGTAGGAGAACAAAAAACTGTGGCATATCCAACAGTAACATCC
CCAGTGGTACCAAACTGCAGTGACACCATAATTACCTACAACAGCACTGTAG
TAAACAACATAACAACAACAATAGTAACTGAAGCGGAAAGGCATTTCAAGTC
CTCACTGCCACTGTGCCCCTTCCGAGGTTTCTTCCCTTTTCACAAGGACAATG
GCAATACGATTGGGTGAGAATAAAGACGTAATAGTCACAAGGGAGCCTTATG
TCAGTTGTGACTATAATGATTGCTGGTCTTTTCCTCTCGCCCAAGGGGCTCTA
CTGGGGACTAAACACAGCAATGGAACCATCAAAGACAGGACACCATATAGA
TCGCTAATTCGGTTCCCAATAGGAGTAGCTCCAGTGCTGGGTAATTACAAGG
AGATATGTGTTGCTTGGTCAAGTAGCAGTTGCTTCGATGGAAAGGAATGGAT
GCATGTTTGCATGACTGGGAACGACAATGATGCGAGTGCCCAAATAATATAT
GCAGGGAAAATGACAGACTCCATTAAATCATGGAGAAAGGACATACTAAGA
ACTCAAGAGTCTGAATGTCAATGCATTGACGGGACTTGTGTTGTCGCTGTTAC
AGATGGTCCTGCAGCTAATAGTGCAGACCACCGAATTTACTGGATACGAAAA
GGGAAGATAATAAAGTATGAGAACATTCCCAAAACAAAGATACAACATTTG
GAGGAGTGTTCTTGTTATGTGGACATTGATGTGTACTGCATATGTAGGGACAA
TTGGAAAGGTTCCAACAGGCCTTGGATGAGGATCAACAATGAGACCATACTA
GCACCGGGGTATGTATGCAGCAAATTCCATTCAGACACTCCCAGGCCAGATG
ATCCTTCAACAGTATCATGCGATTCCCCAAGTAATGTCAATGGAGGACCCGG
AGTCAAAGGATTTGGCTTCAAAACGGGTAATGATGTATGGTTGGGAAGGACT
GTATCCAACAGTGGAAGATCAGGCTTTGAGATCATCAAAGTCACAGAGGGGT
GGATCAACTCCCCCAATCATGCCAAATCAGTTACACAAACATTGGTGTCAAA
CAATGATTGGTCAGGTTACTCAGGGAGTTTCATTGTTGAGAGCAATGGATGTT
TCCAGCCCTGCTTCTATATTGAGCTTATAAGGGGGAAGCCCAATAAGAATGA
TAACGTTTCCTGGACAAGCAATAGTATAGTTACTTTCTGTGGACTAGACAATG
AACCTGGATCGGGAAATTGGCCTGATGGTTCCAACATTGGGTTTATGCCCAA
GTAAC

Figure 86: Nucleotide sequence of A/Chicken/Pakistan/NARC-74/04 (H7N3) for NA (N3) gene

A/Chicken/Pakistan/NARC-70/04 (H7N3)

ATGAATCCAAATCAGAAGATAATAACAATTGGTGTAGTGAATACTACTCTAT
CAACAATAGCCCTTCTTATTGGAATTGGGAATCTGGTTTTCAACACCGTTATA
CATGAGAAAGTAGGAGAACAAAAAACTGTGGCATATCCAACAGTAACATCC
CCAGTGGTACCAAACTGCAGTGACACCATAATTACCTACAACAGCACTGTAG
TAAACAACATAACAACAACAATAGTAACTGAAGCGGAAAGGCATTTCAAGTC
CTCACTGCCACTGTGCCCCTTCCGAGGTTTCTTCCCTTTTCACAAGGACAATG
CAATACGATTGGGTGAGAACAAAGACGTAATAGTCACAAGGGAGCCTTATGT
TAGTTGTGACTATAATGATTGCTGGTCTTTTGCCCTCGCCCAAGGGGCTCTAC
TGGGGACTAACCACAGCAATGGAACCATCAAAGACAGAACACCGTATAGAT
CGCTGATTCGGTTCCCAATAGGAGTAGCTCCAGTGCTGGGTAATTACAAGGA
GATATGTGTTGCTTGGTCAAGTAGCAGTTGCTTCGATGGAAAGGAATGGATG
CATGTTTGCATGACTGGGAACGACAATGATGCAAGTGCCCAAATAATATATG
CAGGGAAAATGACAGACTCCATTAAATCATGGAGGAAGGACATACTAAGAA
CTCAAGAGTCTGAATGTCAATGCATTGACGGGACTTGTGTTGTCGCTGTTACA
GATGGTCCTGCGGCTAATAATGCAGACCACCGAATTTACTGGATACGAAAAG
GGAGAGTAATAAAGTATGAAAACATTCCCAAAACAAAGATAAAACATTTAG
AGGAGTGTTCTTGTTATGTGGACATTGATGTATACTGCATATGCAGGGACAAT
TGGAAAGGTTCCAACAGGCCTTGGATGAGGATCAACAATGAGACCATACTAG
AAACGGGGTATGTATGCAGCAAATTCCATTCAGACACTCCCAGGCCAGATGA
TCCTTCAACAGTATCATGCGATTCCCCAAGTAATGTCAATGGAGGACCCGGA
GTCAAAGGATTTGGCTTCAAAACGGGTAATGATGTATGGTTGGGAAGGACTG
TATCCAACAGTGGAAGATCAGGCTTTGAGATCATCAAAGTCACAGAGGGGTG
GATCAACTCCCCCAATCATGCCAAATCAGTTACACAAACATTGGTGTCAAAC
AATGATTGGTCAGGTTACTCAGGGAGTTTCATTGTTGAGAGCAATGGATGTTT
CCAGCCCTGCTTCTATATTGAGCTTATAAGGGGGAAGCCCAATAAGAATGAT
AACGTTTCCTGGACAAGCAATAGTATAGTTACTTTGTGTGGACTAGACAATG
AACCTGGATCGGGAAATTGGCCTGATGGTTCCAATATTGGGTTCATGCCCAA
GTAACAG

Figure 87: Nucleotide sequence of A/Chicken/Pakistan/NARC-70/04 (H7N3) for NA (N3) gene

A/Chicken/Pakistan/NARC-214/04 (H7N3)

ATGAATCCAAATCAGAAGATAATAACAATTGGTGTAGTGAATACTACTCTAT
CAACAATAGCCCTTCTTATTGGAATTGGGAATCTGGTTTTCAACACCGTTATA
CATGAGAAAGTAGGAGAACAAAAAACTGTGGCATATCCAACAGTAACATCC
CCAGTGGTACCAAACTGCAGTGACACCATAATTACCTACAACAGCACTGTAG
TAAACAACATAACAACAACAATAGTAACTGAAGCGGAAAGGCATTTCAAGTC
CTCACTGCCACTGTGCCCCTTCCGAGGTTTCTTCCCTTTTCACAAGGACAATG
GCAATACGATTGGGTGAGAATAAAGACGTAATAGTCACAAGGGAGCCTTATG
TTAGTTGTGACTATAATGATTGCTGGTCTTTTCCTCTCGCCCAAGGGGCTCTA
CTGGGGACTAAACACAGCAATGGAACCATCAAAGACAGGACACCATATAGA
TCGCTAATTCGGTTCCCAATAGGAGTAGCTCCAGTGCTGGGTAATTACAAGG
AGATATGTGTTGCTTGGTCAAGTAGCAGTTGCTTCGATGGAAAGGAATGGAT
GCATGTTTGCATGACTGGGAACGACAATGATGCGAGTGCCCAAATAATATAT
GCAGGGAAAATGACAGACTCCATTAAATCATGGAGAAAGGACATACTAAGA
ACTCAAGAGTCTGAATGTCAATGCATTGACGGGACTTGTGTTGTCGCTGTTAC
AGATGGTCCTGCAGCTAATAGTGCAGACCACCGAATTTACTGGATACGAAAA
GGGAAGATAATAAAGTATGAGAACATTCCCAAAACAAAGATACAACATTTG
GAGGAGTGTTCTTGTTATGTGGACATTGATGTGTACTGCATATGTAGGGACAA
TTGGAAAGGTTCCAACAGGCCTTGGATGAGGATCAACAATGAGACCATACTA
GAAACGGGGTATGTATGCAGCAAATTCCATTCAGACACTCCCAGGCCAGCCG
ATCCTTCAACAGTATCGTGTGATTCCCCAAGTAACGTCAATGGAGGACCCGG
AGTCAAAGGTTTTGGCTTCAAAACGGGTAATGATGTATGGTTGGGAAGGACT
GTATCAACTAGTGGAAGATCAGGCTTTGAAATCATCAAAGTCACAGAGGGGT
GGATCAACTCCCCCAATCATGCCAAATCAGTTACACAAACATTAGTGTCAAA
CAATGATTGGTCAGGTTACTCAGGGAGCTTCATTGTTGAGAACAATGGATGTT
TCCAGCCCTGCTTCTATATTGAACTTATAAGGGGGAGGCCCAATAAGAATGA
TGACGTTTCCTGGACAAGCAATAGTATAGTTACTTTCTGTGGACTAGACAATG
AACCTGGATCGGGAAATTGGCCTGATGGTTCCAACATTGGGTTTATGCCCAA
GT

Figure 88: Nucleotide sequence of A/Chicken/Pakistan/NARC-214/04 (H7N3) for NA (N3) gene

A/Chicken/Pakistan/NARC-2419/05 (H7N3)

ATGAATCCAAATCAGAAGATAATAACAATTGGTGTAGTGAATACTACTCTAT
CAACAATAGCCCTTCTTATTGGAATTGGGAATCTGGTTTTCAACACCGTTATA
CATGAGAAAGTAGGAGAACAAAAAACTGTGGCATATCCAACAGTAACATCC
CCAGTGGTACCAAACTGCAGTGACACCATAATTACCTACAACAAGCACTGTA
GTAAACAACATAACAACAACAATAGTAACTGAAGCGGAAAGGCATTTTTGCA
AGTCCTCACTGCCAACTGTGCCCCTTCCGAGGTTTCTTCCCTTTTCACAAGGA
CAATGCAATACGATTGGGTGAGAACAAAGACGTAATAGTCACAAGGGAGCC
TTATGTTAGTTGTGACTATAATGATTGCTGGTCTTTTGCCCTCGCCCAAGGGG
CTCTACTGGGGACTAACCACAGCAATGGAACCATCAAAGACAGAACACCGTA
TAGATCGCTGATTCGGTTCCCAATAGGAGTAGCTCCAGTGCTGGGTAATTACA
AGGAGATATGTGTTGCTTGGTCAAGTAGCAGTTGCTTCGATGGAAAAGGAAT
GGATGCATGTTTGCATGACTGGGAACGACAATGATGCAAGTGCCCAAATAAT
ATATGCAGGGAGAATGACAGACTCCATTAAATCATGGAGGAAGGACATACTA
AGAACTCAAGAGTCTGAATGTCAATGCATTGACGGGACTTGTGTTGTCGCTGT
TACAGATGGTCCTGCGGCTAATAATGCAGACCACCGAATTTACTGGATACGA
AAAGGGAGAGTAATAAAGTATGAAAACATTCCCAAAACAAAGATAAAACAT
TTAGAGGAGTGTTCTTGTTATGTGGACATTGATGTATACTGCATATGCAGGGA
CAATTGGAAAGGTTCCAACAGGCCTTGGATGAGGATCAACAATGAGACCATA
CTAGAAACGGGGTATGTATGCAGCAAATTCCATTCAGACACTCCCAGGCCAG
ATGATCCTTCAACAGTATCATGCGATTCCCCAAGTAATGTCAATGGAGGACC
CGGAGTCAAAGGATTTGGCTTCAAAACGGGTAATGATGTATGGTTGGGAAGG
ACTGTATCCAACAGTGGAAGATCAGGCTTTGAGATCATCAAAGTCACAGAGG
GGTGGATCAACTCCCCCAATCATGCCAAATCAGTTACACAAACATTGGTGTC
AAACAATGATTGGTCAGGTTACTCAGGGAGTTTCATTGTTGAGAGCAATGGA
TGTTTCCAGCCCTGCTTCTATATTGAGCTTATAAGGGGGAAGCCCAATAAGAA
TGATAACGTTTCCTGGACAAGCAATAGTATAGTTACTTTCTGTGGACTAGACA
ATGAACCTGGATCGGGAAATTGGCCTGATGGTTCCAATATTGGGTTTATGCCC
AAGTAAA

Figure 89: Nucleotide sequence of A/Chicken/Pakistan/NARC-2419/05 (H7N3) for NA (N3) gene

A/Chicken/Pakistan/NARC-01/95 (H7N3)

ATGAATCCAAATCAGAAGATAATAACAATTGGTGTAGTGAATACTACTCTAT
CAACAATAGCCCTTCTTATTGGAATTGGGAATCTGGTTTTCAACACCGTTATA
CATGAGAAAGTAGGAGAACAAAAAACTGTGGCATATCCAACAGTAACATCC
CCAGTGGTACCAAACTGCAGTGACACCATAATTACCTACAACAGCACTGTAG
TAAACAACATAACAACAACAATAGTAACTGAAGCGGAAAGGCATTTCAAGTC
CTCACTGCCACTGTGCCCCTTCCGAGGTTTCTTCCCTTTTCACAAGGACAATG
CAATACGATTGGGTGAGAACAAAGACGTAATAGTCACAAGGGAGCCTTATGT
TAGTTGTGACTATAATGATTGCTGGTCTTTTGCCCTCGCCCAAGGGGCTCTAC
TGGGGACTAACCACAGCAATGGAACCATCAAAGACAGAACACCGTATAGAT
CGCTGATTCGGTTCCCAATAGGAGTAGCTCCAGTGCTGGGTAATTACAAGGA
GATATGTGTTGCTTGGTCAAGTAGCAGTTGCTTCGATGGAAAGGAATGGATG
CATGTTTGCATGACTGGGAACGACAATGATGCAAGTGCCCAAATAATATATG
CAGGGAGAATGACAGACTCCCTTAAATCATGGAGGAAGGACATACTAAGAA
CTCAAGAGTCTGAATGTCAATGCATTGACGGGACTTGTGTTGTCGCTGTTACA
GATGGTCCTGCGGCTAATAATGCAGACCACCGAATTTACTGGATACGAAAAG
GGAGAGTAATAAAGTATGAAAACATTCCCAAAACAAAGATAAAACATTTAG
AGGAGTGTTCTTGTTATGTGGACATTGATGTATACTGCATATGCAGGGACAAT
TGGAAAGGTTCCAACAGGCCTTGGATGAGGATCAACAATGAGACCATACTAG
AAACGGGGTATGTATGCAGCAAATTCCATTCAGACACTCCCAGGCCAGATGA
TCCTTCAACAGTATCATGTGATTCCCCAAGTAATGTCAATGGAGGACCCGGA
GTCAAAGGATTTGGCTTCAAAACGGGTAATGATGTATGGTTGGGAAGGACTG
TATCCAACAGTGGAAGATCAGGCTTTGAGATCATCAAAGTCACAGAGGGGTG
GATCAACTCCCCCAATCATGCCAAATCAGTTACACAAACATTGGTGTCAAAC
AATGATTGGTCAGGTTACTCAGGGAGTTTCATTGTTGAGAGCAATGGATGTTT
CCAGCCCTGCTTCTATATTGAGCTTATAAAGGGGGAAGCCCAATAAGAATGA
TAACGTTTCCTGGACAAGCAATAGTATAGTTACTTTCTGTGGACTAGACAATG
AACCTGGATCGGGAAATTGGCCTGATGGTTCCAACATTGGGTTTATGCCCAA
GTAAC

Figure 90: Nucleotide sequence of A/Chicken/Pakistan/NARC-01/95 (H7N3) for NA (N3) gene

A/Chicken/Pakistan/NARC-1282/04 H7N3

ATGAATCCAAATCAGAAGATAATAACAATTGGTGTAGTGAATACTACTCTAT
CAACAATAGCCCTTCTTATTGGAGTTGGGAATCTGATTTTCAACACTGTTATA
CATGGGAAAATAGGGGACCACCAAACTGTGGTATATCCAACAGTAACAGCCC
CGGTGGTA----------
ACTAAAGCGGAACCGCACTTCAAGTCCTCACTGCCACTGTGCCCCTTCCGAG
GTTTCTTCCCCTTTCACAAGGACAATGCAATACGATTGGGTGAAAACAAAGA
CGTAATAGTCACAAGGGAGCCTTATGTCAGTTGTGACAATGATGATTGCTGG
TCCTTTGCTCTTGCCCAAGGGGCTCTAGTGGGGACTAAACACAGCAATGGAA
CCATCAAAGACAGGACGCCATATAGATCGCTAATCCGGTTCCCAATAGGGAC
TGCTCCAGTACTGGGTAATTACAAGGAGATATGTGTTGCTTGGTCAAGTAGC
AGCTGCTTCGATGGAAAGGAATGGATGCATGTTTGCATGACTGGGAACGACA
ATGATGCGAGTGGCCAAATAATATATGCAGGGAGAATGACAGACTCCATTAA
ATCATGGAGAAAGGATATACTAAGAACTCAAGAGTCTGAATGTCAATGCATT
GATGGGACCTGTGTAGTCGCTGTTACAGATGGTCCTGCAGCTAATAGTGCAG
ACCACCGAATTTACTGGATACGAGAAGGGAAGATAATAAAGCATGAGAACA
TTCCCAAGACAAAGATACAACATTTGGAGGAGTGCTCTTGTTATGTGGACAT
CGATGTGTACTGCATATGTAGGGACAATTGGAAAGGTTCCAACAGGCCTTGG
ATGAGGATCAACAATGAGACCATATTAGAAACAGGGTATGTATGTAGTAAAT
TCCATTCAGATACCCCCAGGCCAGCCGATCCTTCAACAGTATCGTGTGATTCT
CCAAGTAACGTCAATGGAGGACCTGGAGTCAAAGGTTTTGGCTTCAAAAAGG
GTAATGATGTATGGTTGGGAAGGACTGTATCAACTAGTGGAAGATCGGGCTT
TGAAATCATCAAAGTCACAGAGGGGTGGATTAACTCCCCCAATCATGCCAAA
TCAGTTACACAAACATTAGTGTCAAACAATGATTGGTCAGGTTACTCAGGGA
GTTTCATTGTTGAGAACAATGGCTGTTTTCAGCCCTGCTTCTATATTGAACTTA
TACGGGGAAGGCCCAATAAGAATGATGACGTTTCTTGGACAAGCAATAGTAT
AGTTACTTTCTGTGGACTAGACAATGAACCTGGATCGGGAAATTGGCCTGAT
GGTTCCAACATTGGGTTTATGCCCAAGTAACAGAAA

Figure 91: Nucleotide sequence of A/Chicken/Pakistan/NARC-1282/04 (H7N3) for NA (N3) gene

TABLE 11

SUMMARIZED H7N3 PROFILE

S. no	AIV Isolate	Subtype	Date /yr isolated	Place of Isolation	Presence or absence of Truncation(NS Protein)	Cleavage Site Sequence (HA protein)	Presence or absence of Stalk (NA protein)	GenBank Accession Nos (NS, HA, NA)
1	A/Chicken/Pakistan/NARC-1/95(H7N3)	H7N3 NS	1995	Murree, Punjab, Pakistan	Truncated	PETPKRKRKRG HPAI	Stalked NA	EF688543.1
2	A/Chicken/Pakistan/NARC-02/95(H7N3)	H7N3 NS	1995	Murree, Punjab, Pakistan	Truncated	-	-	EF688550.1
3	A/Chicken/Pakistan/NARC-35/01(H7N3)	H7N3 NS	2001	Chakwal, Punjab, Pakistan	Truncated	-	-	EF688544.1
4	A/Chicken/Pakistan/NARC-43/ 03(H7N3)	H7N3 NS	28/12/03 2003	Sheikhupura, Punjab, Pakisatan	Truncated	-	-	EF688536.1
5	A/Chicken/Pakistan/NARC-70/04(H7N3)	H7N3 NS	14/04/04 (2004)	Murree, Punjab, Pakistan	Truncated	PETPKRKRKRG HPAI	Stalked NA	EF688545.1

S. no	AIV Isolate	Subtype	Date /yr isolated	Place of Isolation	Presence or absence of Truncation(NS Protein)	Cleavage Site Sequence (HA protein)	Presence or absence of Stalk (NA protein)	GenBank Accession Nos (NS, HA, NA)
6	A/Chicken/Pakistan/NARC-74/ 04(H7N3)	H7N3 NS	14/04/04 (2004) 2004	Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan	Truncated	PETPKRKRKRG HPAI	Stalked NA	EF68855 1.1
7	A/Chicken/Pakistan/NARC-100/04(H7N3)	H7N3 NS	27/04/04 (2004)	Karachi, Sindh, Pakistan	Untruncated	-	-	EF68854 1.1
8	A/Chicken/Pakistan/NARC-160/ 04(H7N3)	H7N3 NS	14/05/04 (2004)	Rawalpindi, Punjab, Pakistan	Truncated	-	-	EF68855 2.1
9	A/Chicken/Pakistan/NARC-1282/ 04(H7N3)	H7N3 NS	07/12/04 (2004)	Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan	Untruncated	PEIPKGRG LPAI	Deleted NA Stalk	EF68853 5.1
10	A/Chicken/Pakistan/NARC-N19/05(H7N3)	H7N3 NS	02/02/05 (2005)	Sumundri, Punjab, Pakistan	Truncated	-	-	EF68854 2.1

S. no	AIV Isolate	Subtype	Date /yr isolated	Place of Isolation	Presence or absence of Truncation(NS Protein)	Cleavage Site Sequence (HA protein)	Presence or absence of Stalk (NA protein)	GenBank Accession Nos (NS, HA, NA)
11	A/Chicken/Pakistan/NARC-2419/05(H7N3)	H7N3 NS	25/05/05 (2005)	Abbotabad, KPK (Khyber Pakhtoon Khuwa), Pakistan	Truncated	PETPKRKRKRG HPAI	Stalked NA	EF688534.1
12	A/Chicken/Pakistan/NARC-108/04(H7N3)	H7N3 NS	05/05/04 (2004)	Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan	Truncated	-	-	EF688555.1
13	A/Chicken/Pakistan/NARC-143/04(H7N3)	H7N3 NS	11/05/04 (2004)	Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan	Truncated	-	-	EF688548.1
14	A/Chicken/Pakistan/NARC-2402/05(H7N3)	H7N3 NS	20/05/05 (2005)	Abbotabad, KPK (Khyber Pakhtoon Khuwa), Pakistan	Truncated	-	-	EF688554.1
15	A/Chicken/Pakistan/NARC-1118/04(H7N3)	H7N3 NS	04/11/04 (2004)	Karachi, Sindh, PK	Truncated	-	-	EF688553.1

S. no	AIV Isolate	Subtype	Date /yr isolated	Place of Isolation	Presence or absence of Truncation(NS Protein)	Cleavage Site Sequence (HA protein)	Presence or absence of Stalk (NA protein)	GenBank Accession Nos (NS, HA, NA)
16	A/Chicken/Pakistan/NARC-169/04(H7N3)	H7N3 NS	13/05/04 (2004)	Karachi, Sindh, Pakistan	Truncated	-	-	EF688537.1
17	A/Chicken/Pakistan/NARC-214/04(H7N3)	H7N3 NS	25/05/04 (2004)	Mansehra, KPK (Khyber Pakhtoon Khuwa), Pakistan	Truncated	PETPKRKRKRG HPAI	Stalked NA	EF688538.1
18	A/Chicken/Pakistan/NARC-BM/00(H7N3)	H7N3 NS	15/04/00 (2000)	Chakwal, Punjab, Pakistan	Truncated	-	-	EF688540.1
19	A/Chicken/Afghanistan/ NARC-483/04(H7N3)	H7N3 NS	29/07/04 (2004)	Tora Bora, Afghanistan	Truncated	-	-	EF688539.1
20	A/Chicken/Pakistan/NARC-N30/05(H7N3)	H7N3 NS	27/04/05 (2005)	Faisalabad, Punjab, Pakistan	Truncated	-	-	EF688547.1
21	A/Chicken/Pakistan/NARC-178/04(H7N3)	H7N3 NS	31/08/04 (2004)	Mansehra, KPK (Khyber	Truncated	-	-	EF688546.1

				Pakhtoon Khuwa), Pakistan				
22	A/Chicken/Karachi/ Pakistan/NARC-23/03(H7N3)	H7N3 NS	03-1-03 (2003)	Karachi, Sindh, Pakistan	Truncated	-	-	EF688549.1

TABLE 12: AIV H7N3 ISOLATES SHOWING CARBOXY-TERMINAL ENDS OF NS1 PROTEIN

S. No	Name of isolate	Deduced amino acid sequences	Length of Protein
1	A/Ck/Pak/NARC- 74/2004 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMAGTIGSEV*	217aa
2	A/Ck/Pak/NARC -108/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMAGTIGSEV*	217aa
3	A/Ck/Pak/NARC- 143/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMAGTIGSEV*	217aa
4	A/Ck/Pak/NARC- 2402/05 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
5	A/Ck/Pak/NARC-35/01 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
6	A/Ck/Pak/NARC-1/95 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
7	A/Ck/Pak/NARC-BM/00 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
8	A/Ck/Pak/NARC-169/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMAGTIGSEV*	217aa
9	A/Ck/Pak/NARC-1118/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
10	A/Ck/Pak/NARC-160/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
11	A/Ck/Pak/NARC-02/95 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
12	A/Ck/Pak/NARC-23/03 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
13	A/Ck/Afg/NARC-483/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
14	A/Ck/Pak/NARC-214/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
15	A/Ck/Pak/NARC-2419/05 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
16	A/Ck/Pak/NARC-178/04 H7N3	KWNDNTVRVSENLQRFAWGSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
17	A/Ck/Pak/NARC-70/04 H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
18	A/Ck/Pak/NARCN19/05H7N3	KWNDNTVRVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
19	A/Ck/Pak/NARC-43/03 H7N3	KWNDNTVQVSENLQRFAWRSSNENGRPPLPPK*KRKMARTIGSEV*	217aa
20	A/Ck/Pak/NARC-N30/05 H7N3	KWNDNTVRVSENLQRFAWRSSNESGRPPLPPK*KRKMARTIGSEV*	217aa
21	A/Ck/Pak/NARC-100/04 H7N3	EWNDNTVRVSETLQRFAWRSSNEDGGPPFPPKQKRKMARTIKSEV*	230aa
22	A/Ck/Pak/NARC-1282/04 H7N3	EWNDNTVRVSETLQRFAWRSSNEDGRPPLPPKQKRKMARTIESEV*	230aa

TABLE 13

NUCLEOTIDE BASES OF NS1 GENE SHOWING C TO T TRANSVERSION AT POSITION 652

	NUCLEOTIDE BASES OF NS1 GENE SHOWING C TO T TRANSVERSION AT POSITION 652									
AIV H7N3 ISOLATES	**648**	**649**	**650**	**651**	**652**	**653**	**654**	**655**	**656**	**657**
A/Ck/Pak/NARC- 74/2004 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC -108/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC- 143/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC- 2402/05 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-35/01 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-1/95 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-BM/00 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-169/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-1118/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-160/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-02/95 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-23/03 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Afg/NARC-483/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-214/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-2419/05 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-178/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-70/04 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARCN19/05 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-43/03 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-N30/05 H7N3	A	A	A	G	T	A	G	A	A	A
A/Ck/Pak/NARC-100/04 H7N3	A	A	A	G	C	A	G	A	A	A
A/Ck/Pak/NARC-1282/04 H7N3	A	A	A	G	C	A	G	A	A	A

TABLE 14

PAIRWISE DISTANCE CALCULATION BY MAXIMUM COMPOSITE LIKELIHOOD MODEL FOR NS1 GENE

S. No	Name of isolate	Closest Resemblance to AIV isolate by Phylogenetic Analysis	NS Nucleotide level homology (%)	NS Amino Acid level homology (%)
1	A/Ck/Pak/NARC-74/04 H7N3	A/Chicken/Pakistan/NARC -108/04 H7N3	100%	100%
		A/Chicken/Pakistan/NARC- 143/04 H7N3	100%	100%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	96.4%	95.5%
		A/Duck/Jiang Xi/6146/03 H5N3	96.4%	95.5%
		A/Duck/Hong Kong/610/79 H9N2	96.3%	95.5%
		A/Duck/Jiang Xi/1742/03 H7N7	96.1%	95.5%
2	A/Ck/Pak/NARC -108/04 H7N3	A/Chicken/Pakistan/NARC- 74/04 H7N3	100%	100%
		A/Chicken/Pakistan/NARC- 143/04 H7N3	100%	100%
		A/Duck/Jiang Xi/6146/03 H5N3	98.3%	95.5%
		A/Duck/Hong Kong/610/79 H9N2	98.2%	95.5%
3	A/Ck/Pak/NARC-143/04 H7N3	A/Chicken/Pakistan/NARC -108/04 H7N3	100%	100%
		A/Chicken/Pakistan/NARC- 74/04 H7N3	100%	100%
		A/Duck/Jiang Xi/6146/03 H5N3	97.8%	95.5%
4	A/Ck/Pak/NARC-2402/05 H7N3	A/Chicken/Pakistan/NARC-35/01 H7N3	100%	100%
		A/Ck/Pak/NARC-1/95 H7N3	100%	100%
		A/Ck/Pak/NARC-BM/00 H7N3	100%	100%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	97.7%	95%
		A/Duck/Jiang Xi/6146/03 H5N3	97.7%	95%
5	A/Ck/Pak/NARC-35/01 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	100%	100%
		A/Ck/Pak/NARC-1/95 H7N3	100%	100%
		A/Ck/Pak/NARC-BM/00 H7N3	100%	100%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	97.8%	95%
6	A/Ck/Pak/NARC-1/95 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	100%	100%
		A/Chicken/Pakistan/NARC-35/01 H7N3	100%	100%
		A/Ck/Pak/NARC-BM/00 H7N3	100%	100%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	98.1%	95%
7	A/Ck/Pak/NARC-BM/00 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	100%	100%
		A/Chicken/Pakistan/NARC-35/01 H7N3	100%	100%
		A/Chicken/Pakistan/NARC-1/95 H7N3	100%	100%

		A/Duck/Jiang Xi/6146/03 H5N3	96.4%	95%
8	A/Ck/Pak/NARC-160/04 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-BM/00	99.9%	99.6%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	96.4%	95%
		A/Duck/Jiang Xi/6146/03 H5N3	96.4%	95%
9	A/Ck/Pak/NARC-23/03 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.9%	99.6%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	98.1%	95%
		A/Duck/Jiang Xi/6146/03 H5N3	98.1%	95%
10	A/Ck/Afg/NARC-483/04 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.9%	100%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.9%	100%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.9%	100%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.9%	100%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	97.6%	95%
		A/Duck/Jiang Xi/6146/03 H5N3	97.6%	95%
11	A/Ck/Pak/NARC-214/04 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.9%	99.6%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	97.8%	95.5%
		A/Duck/Jiang Xi/6146/03 H5N3	97.8%	95.5%
		A/Duck/Hong Kong/610/79 H9N2	97.8%	95.5%
12	A/Ck/Pak/NARC-2419/05 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.9%	99.6%
		A/Chicken/Pakistan/NARC-BM/00	99.9%	99.6%

		H7N3		
		A/Aquatic Bird/Korea/CN-1/04 H3N6	97.6%	95.5%
		A/Duck/Jiang Xi/6146/03 H5N3	97.6%	95.5%
13	A/Ck/Pak/NARC-178/04 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.8%	99.1%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.8%	99.1%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.8%	99.1%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.8%	99.1%
		A/Duck/Jiang Xi/6146/03 H5N3	97.5%	95.1%
14	A/Ck/Pak/NARC-70/04 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.7%	99.6%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.7%	99.6%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.7%	99.6%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.7%	99.6%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	96.3%	95.5%
		A/Duck/Jiang Xi/1742/03 H7N7	96.3%	95.5%
15	A/Ck/Pak/NARCN19/05H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.7%	99.6%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.7%	99.6%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.7%	99.6%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.7%	99.6%
		A/Aquatic Bird/Korea/CN-1/04 H3N6	96.3%	95.5%
		A/Duck/Jiang Xi/6146/03 H5N3	96.3%	95.5%
16	A/Ck/Pak/NARC-N30/05 H7N3	A/Chicken/Pakistan/NARC-2402/05 H7N3	99.3%	100%
		A/Chicken/Pakistan/NARC-35/01 H7N3	99.3%	100%
		A/Chicken/Pakistan/NARC-01/95 H7N3	99.3%	100%
		A/Chicken/Pakistan/NARC-BM/00 H7N3	99.3%	100%
		A/Duck/Hong Kong/610/79 H9N2	95%	95%
17	A/Ck/Pak/NARC-100/04 H7N3	A/Mallard/Denmark/64650/03 H5N7	95.9%	95%
18	A/Ck/Pak/NARC-1282/04 H7N3	A/Turkey/Italy/9739/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/8535/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/9742/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/8912/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/8535/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/8534/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/220158/02 H7N3	99.9%	99.6%
		A/Turkey/Italy/214845/02 H7N3	99.9%	99.6%

TABLE 15

RNA BINDING DOMAIN IN NS1 PROTEIN OF PAKISTANI H7N3 ISOLATES COMPARED TO CONSENSUS A REGION

	AMINO ACIDS																			
RNA BINDING DOMAIN	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
CONSENSUS A	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC- 74/2004 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC -108/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC- 143/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC- 2402/05 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-35/01 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-1/95 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-BM/00 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-169/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-1118/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-160/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-02/95 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-23/03 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Afg/NARC-483/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-214/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-2419/05 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-178/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-70/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARCN19/05H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-43/03 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-N30/05 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-100/04 H7N3	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R
A/Ck/Pak/NARC-1282/04	R	K	R	F	A	D	Q	E	L	G	D	A	P	F	L	D	R	L	R	R

TABLE 16

NUCLEAR LOCALISATION SIGNAL 1 IN NS1 PROTEIN OF PAKISTANI H7N3 ISOLATES COMPARED TO CONSENSUS A REGION

	AMINO ACIDS				
NUCLEAR LOCALISATION SIGNAL 1	**34**	**35**	**36**	**37**	**38**
CONSENSUS A	D	R	L	R	R
A/Ck/Pak/NARC- 74/2004 H7N3	D	R	L	R	R
A/Ck/Pak/NARC -108/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC- 143/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC- 2402/05 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-35/01 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-1/95 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-BM/00 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-169/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-1118/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-160/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-02/95 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-23/03 H7N3	D	R	L	R	R
A/Ck/Afg/NARC-483/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-214/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-2419/05 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-178/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-70/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARCN19/05H7N3	D	R	L	R	R
A/Ck/Pak/NARC-43/03 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-N30/05 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-100/04 H7N3	D	R	L	R	R
A/Ck/Pak/NARC-1282/04	D	R	L	R	R

TABLE 17

EFFECTOR DOMAIN IN NS1 PROTEIN OF PAKISTANI H7N3 ISOLATES COMPARED TO CONSENSUS A REGION

	AMINO ACIDS									
EFFECTOR DOMAIN	**138**	**139**	**140**	**141**	**142**	**143**	**144**	**145**	**146**	**147**
CONSENSUS A	F	D	R	L	E	T	L	I	L	L
A/Ck/Pak/NARC- 74/2004 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC -108/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC- 143/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC- 2402/05 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-35/01 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-1/95 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-BM/00 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-169/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-1118/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-160/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-02/95 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-23/03 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Afg/NARC-483/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-214/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-2419/05 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-178/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-70/04 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARCN19/05H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-43/03 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-N30/05 H7N3	F	D	R	L	E	N	L	I	L	L
A/Ck/Pak/NARC-100/04 H7N3	F	D	R	L	D	T	L	I	L	L
A/Ck/Pak/NARC-1282/04	F	D	R	L	E	T	L	I	L	L

TABLE 18

NUCLEAR LOCALISATION SIGNAL 2 IN NS1 PROTEIN OF PAKISTANI H7N3 ISOLATES COMPARED TO CONSENSUS A REGION

	AMINO ACIDS					
NUCLEAR LOCALISATION SIGNAL 2	**216**	**217**	**218**	**219**	**220**	**221**
CONSENSUS A	P	K	Q	K	R	K
A/Ck/Pak/NARC- 74/2004 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC -108/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC- 143/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC- 2402/05 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-35/01 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-1/95 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-BM/00 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-169/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-1118/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-160/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-02/95 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-23/03 H7N3	P	K	*	K	R	K
A/Ck/Afg/NARC-483/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-214/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-2419/05 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-178/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-70/04 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARCN19/05H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-43/03 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-N30/05 H7N3	P	K	*	K	R	K
A/Ck/Pak/NARC-100/04 H7N3	P	K	Q	K	R	K
A/Ck/Pak/NARC-1282/04	P	K	Q	K	R	K

TABLE 19
PAIRWISE DISTANCE CALCULATION BY MAXIMUM COMPOSITE LIKELIHOOD MODEL FOR HA AND NA GENES AMONG THE PAKISTANI H7N3 ISOLATES SEQUENCED

S. No	AIV Isolates	Closest Resemblance to AIV Isolate	HA Homology		NA Homology	
			Amino Acid level	Nucleotide level	Amino Acid level	Nucleotide level
1	A/Chicken/Pakistan/NARC-1/95 H7N3	A/Chicken/Pakistan/NARC-214/04 H7N3	98.9%	98.4%	94.7 %	94.6 %
		A/Chicken/Pakistan/NARC-70/04 H7N3	99.6%	98.9%	99.1 %	99%
		A/Chicken/Pakistan/NARC-2419/05 H7N3	98.9%	97.6%	99.6 %	99.5%
		A/Chicken/Pakistan/NARC-1282/04 H7N3	79.6%	80.6%	78.9%	85.6%
		A/Chicken/Pakistan/NARC- 74/04 H7N3	98.0%	98.7%	96.9%	96.8%
2	A/Chicken/Pakistan/NARC-70/04 H7N3	A/Chicken/Pakistan/NARC-1/95 H7N3	99.6%	98.9%	99.1%	99%
		A/Chicken/Pakistan/NARC-214/04 H7N3	99.3%	99.5%	94.2%	94.1%
		A/Chicken/Pakistan/NARC-2419/05 H7N3	98.9%	97.6%	99.6%	99.5%
		A/Chicken/Pakistan/NARC-1282/04 H7N3	79.2%	79.6%	89.4%	85%
		A/Chicken/Pakistan/NARC- 74/04 H7N3	99.4%	99.7%	96.4%	96.3%
3	A/Chicken/Pakistan/NARC-1282/04 H7N3	A/Chicken/Pakistan/NARC-1/95 H7N3	79.6%	80.6%	78.9%	85.6%
		A/Chicken/Pakistan/NARC-214/04 H7N3	78.8%	79.6%	90.5%	90.4%
		A/Chicken/Pakistan/NARC-70/04 H7N3	79.2%	79.6%	89.4%	85%
		A/Chicken/Pakistan/NARC-2419/05 H7N3	79.1%	80%	85.7%	85.6%
		A/Chicken/Pakistan/NARC- 74/04 H7N3	78.9%	80%	87.7%	87.5%
4	A/Chicken/Pakistan/NARC-214/04 H7N3	A/Chicken/Pakistan/NARC-1/95 H7N3	98.9%	98.4%	94.7%	94.6%
		A/Chicken/Pakistan/NARC-70/04 H7N3	99.3%	99.5%	94.2%	94.1%
		A/Chicken/Pakistan/NARC-2419/05 H7N3	98.3%	97.6%	94.7%	94.6%
		A/Chicken/Pakistan/NARC-1282/04 H7N3	78.8%	79.6%	90.5%	90.4%
		A/Chicken/Pakistan/NARC- 74/04 H7N3	99.9%	99.7%	97.9%	96.8%
5	A/Chicken/Pakistan/NARC-2419/05 H7N3	A/Chicken/Pakistan/NARC-1/95 H7N3	98.9%	97.6%	99.6%	99.5%
		A/Chicken/Pakistan/NARC-214/04 H7N3	98.3%	97.6%	94.7%	94.6%
		A/Chicken/Pakistan/NARC-70/04 H7N3	98.9%	97.6%	99.6%	99.5%
		A/Chicken/Pakistan/NARC-1282/04 H7N3	79.1%	80%	85.7%	85.6%
		A/Chicken/Pakistan/NARC- 74/04 H7N3	98.4%	97.9%	96.9%	96.8%
6	A/Chicken/Pakistan/NARC-74/04 H7N3	A/Chicken/Pakistan/NARC-1/95 H7N3	99%	98.7%	96.9%	96.8%
		A/Chicken/Pakistan/NARC-214/04 H7N3	99.9%	99.7%	97.9%	96.8%
		A/Chicken/Pakistan/NARC-70/04 H7N3	99.4%	99.7%	96.4%	96.3%
		A/Chicken/Pakistan/NARC-1282/04 H7N3	78.9%	80%	87.7%	87.5%
		A/Chicken/Pakistan/NARC-2419/05 H7N3	98.4%	97.9%	96.9%	96.8%

TABLE 20
PAIRWISE DISTANCE CALCULATION BY MAXIMUM COMPOSITE LIKELIHOOD MODEL FOR HA GENE OF PAKISTANI H7N3 WITH GENBANK SEQUENCES

S. No	Name of isolate	Closest Resemblance to AIV isolate by Phylogenetic Analysis	HA Nucleotide level homology (%)	HA Amino Acid level homology (%)
1	A/Ck/Pak/NARC- 74/04 H7N3	A/Chicken/Rawalpindi/NARC 68/02 H7N3	99%	98.9%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	99%	98.9%
		A/Peregrine Falcon/U.A.E/188/2384/98 H7N3	97 %	97.7 %
2	A/Ck/Pak/NARC-1/95 H7N3	A/Chicken/Pakistan/34669/95 H7N3	99.8%	100%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	99.9%	99.7%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	99.9%	99.7%
		A/Peregrine Falcon/U.A.E/188/2384/98 H7N3	97.7%	98.8%
3	A/Ck/Pak/NARC-214/04 H7N3	A/Chicken/Pakistan/CR2/95 H7N3	98.9%	98.3%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	98.9%	98.6%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	98.9%	98.6%
		A/Peregrine Falcon/U.A.E/188/2384/98 H7N3	96.9%	97.4%
4	A/Ck/Pak/NARC-2419/05 H7N3	A/Chicken/Pakistan/447/95 H7N3	98.8%	97.7 %
		A/Chicken/Pakistan/CR2/95 H7N3	98.8%	97.4%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	98.8%	97.1%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	98.8%	97.1%
		A/Chicken/Pakistan/16/99/95 H7N3	98.8%	97.4%
		A/Peregrine Falcon/U.A.E/188/2384/98 H7N3	96.9%	97.1%
5	A/Ck/Pak/NARC-70/04 H7N3	A/Chicken/Rawalpindi/NARC 68/02 H7N3	99.7%	99.1%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	99.7%	99.1%
		A/Peregrine Falcon/U.A.E/188/2384/98 H7N3	97.3%	93.7%
6	A/Ck/Pak/NARC-1282/04 H7N3	A/Chicken/Italy/682/03 H7N3	99.4%	98.8%

TABLE 21
PAIRWISE DISTANCE CALCULATION BY MAXIMUM COMPOSITE LIKELIHOOD MODEL FOR NA GENE OF PAKISTANI H7N3 WITH GENBANK SEQUENCES

S. No	Name of isolate	Closest Resemblance to AIV isolate by Phylogenetic Analysis	NANucleotide level homology (%)	NA Amino Acid level homology (%)
1	A/Ck/Pak/NARC- 74/04 H7N3	A/Chicken/Rawalpindi/NARC 72/02 H7N3	98.1%	95.7%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	98.1%	95.7 %
		A/Mallard/Italy/208/00 H5N3	94.6%	87.6%
2	A/Ck/Pak/NARC-1/95 H7N3	A/Chicken/Pakistan/34668/95 H7N3	99.7 %	99.3%
		A/Duck/Postdam/1689/85 H2N3	94.6%	94.2%
3	A/Ck/Pak/NARC-214/04 H7N3	A/Chicken/Pakistan/34669/95 H7N3	96.8%	92.5%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	96.8%	92.8%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	96.8%	92.8 %
		A/Mallard/Italy/43/01 H7N3	96%	90.8%
		A/Mallard/Italy/33/01 H7N3	95.8%	90.8%
4	A/Ck/Pak/NARC-2419/05 H7N3	A/Chicken/Pakistan/34668/95 H7N3	99.6%	99.4%
		A/Chicken/Pakistan/34669/95 H7N3	99.6%	99.4%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	99.6%	99.2%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	99.6%	99.2%
		A/Duck/Postdam/1689/85 H2N3	94.4%	93.4%
5	A/Ck/Pak/NARC-70/04 H7N3	A/Chicken/Pakistan/34668/95 H7N3	99.6%	99.5%
		A/Chicken/Rawalpindi/NARC 72/02 H7N3	99.6%	99.5%
		A/Chicken/Rawalpindi/NARC 68/02 H7N3	99.6%	99.5%
		A/Chicken/Pakistan/34669/95 H7N3	99.6%	99.5%
		A/Duck/Postdam/1689/85 H2N3	94.4%	94.8%
6	A/Ck/Pak/NARC-1282/04 H7N3	A/Turkey/Italy/214845/02 H7N3	99.8%	99.4%
		A/Turkey/Italy/1010/03 H7N3	99.8%	99.4%
		A/Turkey/Italy/8912/02 H7N3	99.8%	99.4%
		A/Turkey/Italy/220158/02 H7N3	99.8%	99.4%
		A/Turkey/Italy/8535/02 H7N3	99.8%	99.4%

TABLE 22
AMINO ACID CHANGES IN PAKISTANI H7N3 ISOLATES FOR HA GENE

	AMINO ACID POSITIONS OF HA GENE														
AIV H7N3 ISOLATE	**14**	**29**	**46**	**56**	**58**	**131**	**139**	**143**	**146**	**168**	**183**	**214**	**259**	**279**	**315**
A/Chicken/Pakistan/NARC-01/95	I	P	N	I	R	I	R	T	S	A	G	G	A	G	P
A/Chicken/Pakistan/NARC-214/04	N	P	D	F	S	I	R	T	S	D	G	G	A	G	P
A/Chicken/Pakistan/NARC-2419/05	I	P	D	I	S	I	M	A	S	A	G	E	A	E	P
A/Chicken/Pakistan/NARC-74/04	I	P	D	F	S	I	R	T	S	D	G	G	A	G	P
A/Chicken/Pakistan/NARC-70/04	I	P	D	F	S	I	R	T	S	D	G	G	A	G	P
A/Chicken/Pakistan/NARC-1282/04	I	S	N	V	R	M	R	A	A	A	D	G	A	S	S
A/Chicken/Pakistan/CR2/95	I	P	N	I	R	I	R	A	S	D	G	G	A	G	P
A/Chicken/Pakistan/34669/95	I	P	N	I	R	I	R	T	S	A	G	G	A	G	P
A/Chicken/Pakistan/447/95	I	P	N	I	R	I	R	A	S	A	G	G	A	G	P
A/Chicken/Rawalpindi/NARC68/02	I	P	N	I	R	I	R	T	S	D	G	G	A	G	P
A/Chicken/Rawalpindi/NARC72/02	I	P	N	I	R	I	R	T	S	D	G	G	A	G	P
A/Chicken/Pakistan/16/99/95	I	P	N	I	R	I	R	A	S	A	G	G	S	G	P

TABLE 23 AMINO ACID CHANGES IN PAKISTANI H7N3 ISOLATES AT NA GENE

	AMINO ACID CHANGES AT NA GENE LEVEL																
AIV H7N3 ISOLATE	98	135	145	205	223	250	265	270	277	280	331	344	377	427	432	466	469
A/Chicken/Pakistan/NARC-74/04	V	P	K	E	K	S	D	E	T	G	R	C	D	A	S	T	L
A/Chicken/Pakistan/NARC-70/04	V	A	N	K	E	G	S	K	K	R	R	C	D	A	S	I	S
A/Chicken/Pakistan/NARC-214/04	V	P	K	E	K	S	D	E	T	G	S	R	N	T	G	T	L
A/Chicken/Pakistan/NARC-01/95	V	A	N	K	E	G	S	K	K	R	R	C	D	A	S	T	L
A/Chicken/Pakistan/NARC-2419/05	V	A	N	K	E	G	S	K	K	R	R	C	D	A	S	I	L
A/Chicken/Pakistan/NARC-1282/04	V	A	K	E	K	S	D	E	T	G	S	R	N	T	G	T	L
A/Chicken/Rawalpindi/NARC72/02	D	A	N	K	E	G	N	K	K	R	R	C	D	A	S	T	S
A/Chicken/Rawalpindi/NARC68/02	D	A	N	K	E	G	N	K	K	R	R	C	D	A	S	T	S
A/Chicken/Pakistan/34668/95	D	A	N	K	E	G	S	K	K	R	R	C	D	A	S	T	S
A/Chicken/Pakistan/34669/95	V	A	N	K	E	G	S	K	K	R	R	C	D	A	S	T	L

DISCUSSION

DISCUSSION

The present part of the study relating to RT-PCR was conducted to utilize this important molecular diagnostic tool for the identification of field samples of Avian Influenza Viruses. This study was undertaken to evaluate RT-PCR for the detection of Avian Influenza Viruses subtypes H7N3, H9N2,H5N1 and other poultry respiratory pathogens like NDV, IBV and ILTV from field samples of poultry, from various outbreaks in various areas of Pakistan during the period from 2004-2006.

While spread of influenza viruses of swine or avian origin to humans is uncommon, such transmissions can result in elevated mortality and have the impending potential for the generation of pandemic viruses. There is an utmost importance of early rapid detection and characterization of newly emerging AIV variants (Zambon, 1998).

All of the sixteen influenza A virus subtypes (Fouchier, 2005) have been found in aquatic and domestic birds, but only a small number of subtypes have been isolated or identified from mammals and humans (Osterhaus, 2000).

In the present study, investigations were carried out to detect the presence of AIV subtypes and other poultry respiratory pathogens in clinical specimens from poultry.

Rapid Kit Test was employed for AIV H5 subtype detection and the results indicated that out of 150 samples studied 05 were found positive for AIV H5, hence the detection level of Rapid kits is calculated to be only 3.33% (Figure 1a and 1b). Obviously Rapid detection kit for H5 is not quite sensitive. It was hence advisable to re-test the negative samples by other standard laboratory diagnostic techniques such as *in-ovo* inoculation, VNT, and finally RT-PCR/PCR.

In-ovo inoculation of 150 samples showed 25 as positive. Thus sensitivity of the test was calculated to be 16.6% including the ones which came up as H5 positive by the Rapid Kit Test (Figure 1a and 1b). This shows that the egg inoculation test is considerably more sensitive for the detection of AIV's than Rapid Kit Test.

A number of RT-PCR methods for the detection of influenza viruses A & B have been described in literature (Fouchier *et al.*, 2000). These methods depend on the analysis of the PCR product size by Gel Electrophoresis. The One-Step RT-PCR employed here is considered one of the most sensitive molecular diagnostic tools for the detection of AIV subtypes (Figure 2a and 2b). Furthermore, as the detection of viral presence by in-ovo inoculation is based on livability of virus in the specimen and other conditions for its propagation, the in-ovo inoculation does not recover any damaged or inactivated virus particle. This makes the RT-PCR as a more sensitive technique. This is evident from another 45 (41.66%) positive samples out of 108 samples declared negative by other techniques (Figure 3a and 3b). So in summary, it is evident that sensitivity of RT-PCR is manifold higher than Rapid Kit Test and in-ovo inoculation used in this study (Figures 4a, 4b, 5a and 5b).

As far as specificity level of RT-PCR is concerned, it is evident from this data that samples found positive for H7 were never found positive by using HA-primers of H9 or H5, samples found positive for H9 were never found positive by using HA-primers of H7 or H5 and samples found positive for H5 were accordingly never found positive with HA-primers of H7 and H9. On the other hand, the approach of screening the samples by using M-gene primers for the detection of AIV was found to be successful. This could be followed by detecting H7, H9 or H5 subtypes among the positive samples. This approach makes it more economical to carry out regular RT-PCR technique for the virological surveillance of AIV's.

The present study also indicated that 63 samples which were negative by all the available laboratory diagnostic techniques including RT-PCR were tested by PCR using primers specific for ILTV and 03 out of those came up as positive for ILTV showing a percentage of 4.76% (Figures 5a and 5b). Hence PCR was proven to be a better molecular diagnostic tool for DNA containing virus detection which are pathogenic to poultry, in addition to other respiratory pathogens.

The AIV's and other poultry respiratory pathogens isolated from the above mentioned work were further utilized as standards for the extraction of RNA's and DNA's. These were further used in subsequent work relating to the development of multiplex RT-PCR.

Avian influenza (AI) is a viral disease stretching worldwide in its distribution and the causative virus belongs to family Orthomyxoviridae. Influenza viruses are classified into 16 subtypes on the basis of the surface glycoproteins (GP): haemagglutinin (HA), and nine subtypes on the basis of GP neuraminidase (NA). These two proteins are extremely capricious, as a result of which a great number of AIV subtypes occur (Fouchier *et al.*, 2005). Although the majority of AIV's in chickens cause mild and localized infections of the respiratory and intestinal tracts, highly pathogenic subtypes of AI become dispersed all through the body causing viraemia and produce an acute, systemic, and frequently fatal syndrome (Wood *et al.*, 1996; Senne *et al.*, 1996). Traditionally, highly pathogenic avian influenza (HPAI) viruses of fowl are notoriously belonging to the H5 and H7 haemagglutinin subtypes. Therefore, since there is a greater danger for these subtypes to turn out to be highly pathogenic for humans, it is vital to recognize them particularly in surveillance programs (Webster and Kawaoka, 1987; Starick *et al.*, 2000). The H9 subtype has been seen to cause infections in poultry and more recently in humans (Cameron *et al.*, 2000; Butt *et al.*, 2005).

An important previous work relating to mRT-PCR was pioneered by Pang et al., 2002 where a multiplex PCR (mPCR) was developed and standardized to simultaneously detect 6 avian respiratory pathogens, with specific primers for IBV, AIV, ILTV, NDV, MG and MS. Sensitivity and specificity of the test was also determined. Chickens were experimentally infected with respiratory pathogens and in experimental infections the multiplex PCR was able to detect all the infected chickens in each group at 1 to 2 weeks postinfection as compared to serologic tests at 2 weeks postinfection that confirmed the presence of specific antibodies. The multiplex PCR was also able to detect and differentiate coinfections with two or more pathogens.

A mRT-PCR was developed by Xie *et al.*, 2006 and optimized for the detection of type A influenza virus; the assay at the same time differentiated avian H5, H7 and H9

hemagglutinin subtypes. Four sets of precise oligonucleotide primers were employed in this test for type A influenza virus, H5, H7 and H9 hemagglutinin subtypes. The mRT-PCR assay developed in this study was established to be sensitive and specific.

In recent times, infections from AI subtypes H5, H7 and H9 have been repeatedly reported in poultry in Pakistan, especially, since 2004 (Naeem *et al.*, 2007). Unfortunately due to very close resemblance of lesions produced by a number of other pathogens in poultry, it is difficult to differentially diagnose clinical manifestations of any of the above AI infections from diseases such as infectious laryngotracheitis (ILT), Newcastle disease (ND) and infectious bronchitis (IB). The ILT, ND and IB infections are prevalent at a rate of 10-15% in commercial poultry in Pakistan, despite the use of live and inactivated vaccines against such diseases.

In this scenario, it becomes very much essential to develop some sensitive diagnostic technique to differentially diagnose the above mentioned six diseases. One of such techniques is PCR. In this present study, multiplex PCR technique has been developed to simultaneously detect and differentiate the three most important subtypes of AIV's along with 3 most common avian respiratory pathogens prevalent in poultry in Pakistan.

The multiplex RT-PCR was developed in three formats to detect and differentially diagnose in a single reaction, influenza A, avian influenza hemagglutinin subtypes H7, H9, H5 and three respiratory pathogens NDV, IBV and ILTV.

Influenza infection is diagnosed in combination with virus isolation and correct identification by serological tests. Nevertheless, virus isolation and identification is tedious and time consuming and serological tests are less useful if mass vaccination is the usual practice (Reina *et al.*, 1996). This situation does exist in Pakistan where apart from the prevalence of H7, H9 & H5 in the field, the prevalence of NDV, IBV and ILTV is well documented. This makes it very difficult to diagnose any respiratory infection of poultry under field conditions. More recently, PCR assays have been developed for many respiratory viruses, allowing detection of small amounts of viral nucleic acid in clinical samples. In the so-called "multiplex" format, PCR assays have been designed to amplify

multiple respiratory viral targets in the same PCR test (Ellis *et al.*, 1997; Fan *et al.*, 1998; Osiowy, 1998; Grondahl *et al.*, 1999; Liolios *et al.*, 2001; Xie *et al.*, 2006).

Therefore, a mRT-PCR which can rapidly differentiate between AIV subtypes H7, H9, H5 and NDV, IBV and ILTV will be very important for the control of disease transmission in poultry and in humans along with the identification of three of the most common respiratory pathogens often seen as mixed infections in poultry. Economic losses will be reduced in poultry associated with AIV outbreaks with the use of this assay. Although we did not compare the assay developed and reported here with the conventional technique of RT-PCR, it can be safely assumed that this assay is superior to conventional techniques because viral RNA can be directly detected in clinical and field samples in a few hours, all together, as in the case of multiple infections, therefore, it is less time consuming as compared to other diagnostic methods which would take a longer time to detect each and every pathogen in mixed infections. This mRT-PCR is sensitive, specific and it may be used in diagnosis, screening and surveillance of poultry, including the live bird market population which makes up a substantive part of nearly all the major cities of our country Pakistan. This assay has the added benefit of being less time consuming and using single step procedure for PCR with the use of fewer reagents and the simultaneous detection of AIV with other respiratory poultry pathogens. This assay has been developed specially for the developing countries where it is very difficult to buy very expensive laboratory instruments like quantitative Real Time-PCR machines, which are frequently being used in developed countries for the detection of viral nucleic acids.
Future work is needed to expand the panel of viral pathogens detected by such rapid molecular methods in order to eventually circumvent the need for viral cultures. Also, future evaluation of the multiplex PCR assay is warranted.
Influenza A viruses are responsible for natural infections in a multitude of avian and mammalian hosts. Influenza A viruses of avian origin have been implicated to cause outbreaks in mammals, humans as well as in domestic poultry (Webster *et al.*, 1992; Ito *et al.*, 1995; Gauthier-Clerc *et al.*, 2007; Pereda *et al.*, 2008). To date, 16 HA (H1-H16) and 9 NA (N1-N9) antigenic subtypes have been detected in avian species and can be found in multiple combinations (Fouchier *et al.*, 2005; Capua and Alexander, 2006; Olsen *et al.*, 2006)

The natural host and reservoir for influenza virus is allegedly wild waterfowl, gulls, and shorebirds (Slemons *et al.*, 1974; Kawaoka *et al.*, 1988; Stallknecht, 1998) with poultry not being considered a usual host for the virus (Davidson *et al.*, 1988; Hopkins *et al.*, 1990; Easterday *et al.*, 1997; Garcia *et al.*, 1997). Customary transfer of avian influenza viruses (AIV's) from wild bird reservoir to poultry including turkeys, game-birds, domestic ducks, ratites, and other commercially raised birds has been reported. Data supports the fact that manifold subtypes of influenza viruses can create stable phylogenetic lineages in domestic poultry population (Suarez *et al.*, 1999; Webby *et al.*, 2002; Liu *et al.*, 2003), with subsequent materialization of AIVs in chickens, turkeys and other farm-raised birds (Alexander, 2000) being thought to be the consequence of primary virus introduction from the wild bird genetic reservoir. In chickens and turkeys, viruses appear to experience swift evolution, a warning pointing towards the fact that they are adapting to the new host (Garcia *et al.*, 1996; Zhou *et al.*, 1999). Symptoms of the AIV syndrome in poultry may range from asymptomatic or mild respiratory disease to ruthless systemic infections with elevated mortality (Easterday *et al.*, 1997; Suarez *et al.*, 1999; Alexander, 2000). In particular, AIV isolates of the H5 and H7 subtypes, which possess a high rate of mutational capacity to change the HPAI phenotype in domestic poultry, cause somber public health concern for the reasons that firstly these AIV isolates can cause heavy economic losses in poultry industry and secondly might not only be transmissible to humans but also provoke severe and often fatal outcomes in humans as reported (Guan *et al.*, 2003; Osterhaus, 2003). This has led to the hypothesis that domestic poultry, like swine, might act as an intermediary host for the spread of viruses from aquatic birds to humans as recent studies in chickens have established the actuality that birds possess dual receptors in the form of α2,3 and α2,6 sialic acid (SA) receptors (the ones recognized by human viruses) on their epithelial cells and chicken viruses have an intermediate binding level to NeuAc-terminated ganglioside receptors as compared to human and duck viruses (Gambaryan *et al.*, 2002). Consequently chicken viruses as compared to duck viruses are less controlled in humans and have a higher potential to cause infections in humans (Campitelli *et al.*, 2004).

AIV's classified as HPAI can cause systemic disease in poultry with mortality levels reaching nearly 100%. Only H5 and H7 subtypes have been implicated with this property

with every HP lineage preceded by an immediate non-pathogenic precursor virus strain (Alexander, 2007). HPAI H7 viruses are not as a rule present in the wild-bird populations but crop up from LP influenza viruses introduced into poultry flocks from wild bird interactions (Alexander, 2007; Rohm et al., 1995). AIV's are responsible for human infections and can potentially produce pandemic strains by adaptive mutation or genetic reassortment with human influenza viruses (Fouchier et al., 2004). Earlier human pandemics have been thought to have evolved through this reassortment or by direct transfer (Kawaoka et al., 1989; Taubenberger, 2006). The AIV genome has eight gene segments encoding a total of ten proteins, from which HA and NA genes are known to be mutating at a relatively superior rate while circulating in the host population. This may also result in effecting some of the biological functions of AIVs. Amid the other typically conserved gene sequences, it is now known that regular truncation phenomenon has been recorded in the NS1 gene segment after extended circulation of AIV H7 in a poultry population during outbreaks (Ludwig *et al.*, 1991; Dundon *et al.*, 2006).

In the present study a number of H7N3 AIV's isolated from poultry flocks in Pakistan from 1995 to 2005 were studied and characterized at NS1, HA1 and NA gene levels.

Ten proteins are encoded by a total of eight different RNA gene segments in Influenza A viruses. The smallest gene segment, segment 8, encodes two proteins, non-structural protein 1 (NS1) and non-structural protein 2 (NS2). These two NS proteins have ten amino terminal amino acids in common amongst them and the NS2 gene is spliced together with a downstream sequence (Lamb and Krug, 1996). Immediately subsequent to the translation process, the NS1 protein localizes to the nucleus where it is thought to carry out quite a few regulatory activities. Primarily, it attaches to poly A tails of some mRNA, including NS2 mRNA, and stops them from leaving the nucleus (Alonso-Caplen *et al.*, 1992; Fortes *et al.*, 1994; Qiu and Krug, 1994). Secondly, NS1 protein acts to translationally control other influenza proteins including the nucleoprotein (NP) and matrix proteins (Enami *et al.*, 1994; Luna *et al.*, 1995). Thirdly, the NS1 protein stalls pre-mRNA splicing (Lu *et al.*, 1994). Fourthly, the NS1 protein can also bind to double stranded RNA and small nuclear RNA, hindering their potent interferon inducing activities (Hatada and Fukada, 1992; Lu *et al.*, 1995; Qiu *et al.*, 1995).

The NS1 protein had formerly been described as an extraordinarily conserved protein amongst type A influenza viruses (Ludwig *et al.*, 1991; Suarez and Perdue, 1998; Tumpey *et al.*, 2005). However, the study by Dundon *et al.*, in 2006 showed that in case of pervasive viral circulation in poultry, the NS1 protein was not as conserved as was initially believed and it was observed during the H7 epidemic of Northern Italy during the years 1999 to 2003, and that the phenomenon of truncation was a progressive incident. It was observed that all LPAI viruses circulating at the start of the H7N1 epidemic had a full length NS1, whereas the protein got progressively more truncated in the LPAI viruses that were circulating later in the epidemic and that HPAI had an intermediate level truncation.

The NS gene segment phylogenetically divides into two groups, referred to as groups or Alleles A and B (Treanor *et al.*, 1989; Ludwig *et al.*, 1991). The human, swine, most equine and several avian influenza isolates belong to the A subtype referring to the nomenclature used for neuraminidase and hemagglutinin genes, while one equine and various avian influenza isolates are found belonging to the B subtype (Ludwig *et al.*, 1991; Guo *et al.*, 1992). Formerly, the entire highly pathogenic avian influenza isolate population had been characterized as subtype A viruses, but some have been identified in the B subtype grouping as well, providing proof that NS subtype B influenza viruses possess the potential for pathogenicity (Suarez and Perdue, 1998).

The NS1 gene of 40 influenza A viruses isolated from a spectrum of avian species was sequenced and compared phylogenetically including both LPAI and HPAI belonging to the H7 subtype representing AIVs that circulated in Northern Italy from 1999 to 2003. Size difference in the deduced amino acid sequence exposed two different levels of carboxy-terminal truncations, a full length NS1 protein of 230 amino acids and truncated protein of 220 and 224 amino acids. The entire H7N1 HPAI isolates possessed the 224 amino acid truncation level with a novel isoleucine residue at position 136. All the H7N1 LPAI circulating at the commencement of the outbreak had a full length NS1 while those isolated at the end of the epdemic had a truncated NS1 protein. The observed carboxy-level truncation of the NS1 protein was the consequence of antibody selection pressure. All of the H7N3 were located in the subtype A clade of the phylogenetic trees while

H7N1 subtypes were found in subtype B clade. Assessment of the nucleotide sequence revealed that the observed truncations in the H7N1 isolates were due to a single nucleotide change at the respective positions. For the 220 amino acid truncated protein a C to A tranversion was observed at the nucleotide position 663bp while for the 224 amino acid protein a C to T transversion was observed at nucleotide position 673bp ensuing in a TAA and a TGA stop codon respectively. It was finally reported that no truncation was observed in the NS1 gene of the H7N3 Italian AIVs (Dundon *et al.*, 2006). Size variation of different NS genes has been reported previously too of NS1 and NS2 proteins in human, swine and other AIVs in poultry (Norton *et al.*, 1987). Widespread circulation of avian influenza viruses in the poultry population has been known to cause mutation in any of its 8-segmented genome. Apart from affecting any of the biological roles of these viruses, these mutations might affect the immunogenic component(s) of these viruses, affecting the effectiveness of prevalent vaccines. Dundon *et al.*, 2006, revealed that H7N3 showed no truncation in Italian H7N3 isolates.

The aim of our study was to determine the presence and absence of truncation at the amino acid level in the NS1 gene of Pakistani H7N3 AIVs and to investigate their phylogenetic relationships with other avian viruses. Sequencing of the AI H7N3 Pakistani isolates showed the presence of a 13 amino acid truncation in the NS1 protein in the viruses from various areas of Pakistan, hence demonstrating the occurance of the truncation phenomenon in the H7N3 for the first time in Pakistan. It was reported by Dundon *et al.*, 2006, that truncation could be correlated with increased virulence of the H7N1 AIV strains in the Northern Italy epidemics of 1999-2003 but it was not a conclusive observation. The possibility of the removal of the carboxy terminal end of the NS1 protein could help the virus in escaping the immune system of the host, which could be a result of the extensive circulation of LPAI prior to mutation to HPAI. These 22 isolates sequenced for the NS1 gene had a 13 amino acid truncation in the NS1 protein in 20 of the H7N3 isolates resulting in a protein of 217 amino acids. Two isolates showed a full length protein of 230 amino acids. The truncated viruses eluded the immune system and supplanted the growth of untruncated H7N3 AIV strains in the H7N3 outbreaks in various areas of Pakistan.

In Pakistan, after the brief outbreak of HPAI H7N3 during 1995, a major outbreak of H7N3 was recorded in 2004 which lasted more than a year. Two major episodes were recorded, first in southern part of the country, primarily affecting commercial layers, followed by Northern part among broiler-breeders (Naeem *et al.*, 2007). This prompted the present study and the aim to initiate a detailed analysis of the isolates recovered from these outbreaks to assess the effect of prolonged circulation of AIV H7N3 in chickens on their NS1, HA and NA genes. For this purpose, 22 isolates of AIV H7N3, grouped as Southern region isolates and northern region isolates, were evaluated by sequencing their NS1 genes. This was followed by further evaluation of HA and NA genes of Northern isolates in comparison to the previous isolates of Northern region outbreak in 1995. The effect of prolonged circulation on the gene sequences of these field isolates has been evaluated.

The isolates included in the present study of the NS1 gene were both of low pathogenicity avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) strains of H7N3 AIVs. These isolates circulated in N.W.F.P, Punjab, and Sindh areas of Pakistan from 1995 to 2005. Size variation in the predicted amino acid sequence of each NS1 was revealed with two different levels of carboxy-terminal truncation in those isolates. Of the 22 isolates analyzed, 02 isolates A/Chicken/Pakistan/NARC-100/04 and A/Chicken/Pakistan/NARC-1282/04 encoded a full length NS1 protein of 230 amino acids, whereas 20 encoded a truncated protein of 217 amino acids. The isolates exhibiting the truncated carboxy terminal NS1 protein clustered together and appeared to be closest to A/Duck/Jiang Xi/6146/03 (H5N3), A/Duck/Hong Kong/610/79 (H9N2) and A/Aquatic Bird/Korea/CN-1/04 (H3N6) at the nucleotide level and amino acid level. In contrast, the nucleotide sequence of one of the isolates with the full length NS1 protein (A/Chicken/Pakistan/NARC-1282/04) showed 99.9% nucleotide homology and 99.6% homology to a set of Italian H7N3 isolates of Turkey from 2002 at the NS1 gene e.g. A/turkey/Italy/8912/2002(H7N3) and to A/turkey/Italy/214845/02(H7N3). The other isolate (A/Chicken/Pakistan/NARC-100/04) with full length NS1 protein showed the highest homology (96%) with the NS1 gene of an H5N7 subtype virus A/mallard/Denmark/64650/03 and were probably independent new introductions in the already circulating lot of H7N3 AIV's in Pakistan. The sequence data from the 22

Pakistani isolates is in keeping with previous work suggesting that the truncation of NS1 protein may be associated with the extensive circulation of AI in domestic poultry populations. Results of NS-1 sequencing indicate that un-truncated strains of 230 amino acids e.g A/Chicken/Pakistan/NARC-100/04(H7N3) isolated from Karachi, from the province of Sindh, probably remained endemic in the area of Karachi. The epidemic of HPAI H7N3, which primarily affected commercial layers and broiler breeders, occurred in 2003-2004 in Pakistan, caused by H7N3 that originated from the mutation of a LPAI of the same subtype. The LPAI virus was first isolated in the south in April 2003 in the coastal town of Karachi where 70% of the country's commercial layers are raised showing 70% decline in production and 20% mortality. In November 2003, the HPAI subtype H7N3 appeared in the Karachi area, with an abrupt increase in mortality on some farms. Throughout the next few weeks, the disease spread on a large scale in commercial layer farms resulting in 70% to 80% mortality in the layer farms (Naeem *et al.*, 2007). In 2003, no vaccination was done for a few months preceding the H7N3 epidemic in Karachi. Such isolates were most likely introduced from the wild bird population in the wetlands of Sindh Area. Phylogenetic Analysis of this isolate for the NS1 gene indicated a close resemblance (96%) with the NS1 gene of an H5N7 subtype virus A/mallard/Denmark/64650/03.

Afterwards, the infection broadened itself to encompass the northern part of the country where 80% of the broiler-breeder stocks of poultry were reared. A vaccination campaign alongwith improved biosecurity measures assisted in controlling its added spread to other parts of the country (Naeem *et al.*, 2007). Those strains of H7N3 which spread to the Northern parts of the country and were introduced in an already vaccinated population were found truncated in the NS-1 region, except for the isolate A/Chicken/Pakistan/NARC-1282/04 (H7N3), these were immunologically selected and supplanted the growth of untruncated strains. A/Chicken/Pakistan/NARC-1282/04 (H7N3) was probably an introduction in the poultry population of the northern areas of Pakistan from the wild migratory birds which come to spend the summer and winter seasons in those areas. Birds migrating from Siberia to Pakistan and India during September to November start arriving in the North of Pakistan. Birds start arriving in Pakistan as the winter season starts in Siberia (Russia) and this activity often gains

momentum if rains are received here (Chief Conservator KPK (Khyber Pakhtoon Khuwa) Wildlife Department). Millions of migratory birds head towards Pakistan from Europe and Central Asia to escape winter, said wildlife experts. Experts said that every year birds from Europe and Central Asian states came to the Pakistani wetlands for winter. They said that migratory birds flew over Karakorum, Suleiman Ranges and Hindukush along the Indus River, adding that the route was famously known as flyway 4 because of the number of birds using it (there are seven flyways worldwide). They said the route was commonly called the Indus flyway. Migratory birds start reaching Pakistan as winter approaches and keep migrating from September to February and fly back in March, said the experts. The migration journey starts from Siberia to Pakistan and ends in India at Bahartpure. This route is known in the international migration routes as 'Indus Fly-way No. 4' or 'Green Route'. The distance covered by birds during migration is around 4500 kilometers. The migratory birds fly from Siberia to Afghanistan, Karakorum range, across river Indus in Pakistan (*Shoaib Ahmed*, Migratory Birds decreasing, Daily Times, Sunday, September 18, 2005). A/Chicken/Pakistan/NARC-1282/04 (H7N3) isolated from Mansehra in the winter season of 2004, was probably a new introduction into the naïve poultry population of the Abbotabad area in the north of Pakistan. This isolate was untruncated at NS1hence was probably a new introduction from the wild bird/migratory bird population where no truncation is reported in nature.

It was hence concluded that A/Chicken/Pakistan/NARC-100/04(H7N3) showed no resemblance with other Pakistani H7N3 isolates for the NS1 gene. Same was the case with A/Chicken/Pakistan/NARC-1282/04(H7N3), which showed no resemblance with other Pakistani H7N3 isolates for the NS1 gene. Rest of the H7N3 AIVs from the year 1995-2005 isolated from various poultry farms all across the country resembled each other indicating the circulation of the same set of viruses during the 10 year period indicating a certain level of conservation in the NS1 gene. These viruses had a truncated NS1 protein and supplanted the growth of untruncated strains hence were more common in circulation.

Although the cause of truncation and its repercussions are yet to be recognized, it appears that in the midst of sequences present in public databases, only a small percentage of

viruses isolated from wild birds (0.8%) have a truncated NS1, while 19.7% of viruses isolated from poultry demonstrate a truncated protein.

Comparisons of definite regions of the NS1 protein have been performed previously in areas that had previously been described as being important for having a specific function, specifically, two nuclear localization signals, an effector domain, and the RNA binding site (Greenspan *et al.,* 1988; Qian *et al.,* 1994; Suarez and Perdue, 1998). The comparisons of Consensus A subtype virus and the NS1 protein of H7N3 isolates sequenced are presented in Tables (9, 10, 11, 12). The nuclear localization signal 1 appeared to be well conserved between Consensus A and the isolates sequenced, whereas the nuclear localization signal 2 showed a variation between Consensus A and the truncated strains sequenced where these strains showed a truncation of NS1 protein at amino acid position 218 while the untruncated strains appeared well conserved. As previously reported, the RNA binding domain was not as well characterized, having only been localized from amino acids 19 – 38 (Suarez and Perdue, 1998). No variation was observed between Consensus A sequence and the 22 isolates sequenced, for amino acids 19 – 38, hence it was well conserved. Although, amino acids 34 – 38 overlap nuclear localization signal 1, the carboxy-terminal half would appear to be the more important segment of this domain (Suarez and Perdue, 1998). The effector domain was previously pinpointed to 10 amino acids (position 138 – 147) by mutagenesis analysis, with leucines at position 144 and 146 thought to be required for function (Qian *et al.,* 1994), however, comparisons of Consensus A and Consensus B as previously reported, demonstrate that all subtype B viruses have a serine rather than a leucine at position 146, suggesting that the leucine at position 146 is either not vital or other amino acids might substitute for its role (Suarez and Perdue, 1998). The isolates sequenced had leucines at these critical positions showing these well conserved positions with Consensus A.

The present study has shown that in case of extensive circulation in poultry (1995-2005) in Pakistan, the NS-1 protein is not conserved. Variability in NS-1 sequence length has been observed and the present investigation has shown that the truncation of H7N3 is a progressive occurrence during an epidemic and is an indicator of Antigenic Drift. Hence, immunological pressure on NS-1 Gene is thought to drive Antigenic Drift. Two isolates

with NO truncation indicate they were probably introduced form wild birds, where no truncation is reported in nature whereas rest of the truncated H7N3 AIV's from the year 1995 – 2005 isolated from various poultry farms all across the country resembled each other indicating the circulation of the same set of viruses during the ten year period showing a certain level of conservation in the NS1 gene.

The outbreak of H7N3 in chickens in Pakistan started in the northern part of the country in 1995, where it caused extensive disease and mortality. To manage the epidemic, an autologous inactivated vaccine was prepared and used with quarantine measures and controlled marketing (Naeem and Hussain, 1995). It extended and spread in the next 9 years to the central and southern parts of the country where no noticeable antigenic-drift variant selection was reported (Aamir et al., 2009). The epidemic of HPAI H7N3, which chiefly affected commercial layers and broiler breeders, occurred in 2003-2004 in Pakistan, where the causative agent was identified as AIV H7N3 that originated from the mutation of a LPAI of the same subtype. The LPAI virus was primarily isolated in the South in April 2003 in the coastal town of Karachi where 70% of the country's commercial layers are raised showing 70% decline in production and 20% mortality. In November 2003, the HPAI subtype H7N3 emerged in the Karachi area, with an unexpected boost in mortality on a few farms. During the next few weeks, the disease spread on a large scale in commercial layer farms resulting in 70% to 80% mortality. In 2003, no vaccination was done for a few months prior to the H7N3 outbreak in Karachi. Afterwards, the infection extended to the northern part of the country where 80% of the broiler-breeder stocks of poultry were reared. A vaccination campaign followed by better biosecurity measures helped in controlling its added spread to other parts of the country (Naeem *et al.*, 2007).

It was reported previously that truncation in NS1 gene could be correlated with increased virulence of the H7N1 AIV strains. Hence, six isolates from the Northern Parts of Pakistan were further sequenced for HA and NA genes. The phylogenetic analysis of the 5 HPAI H7N3 isolates indicated a close resemblance to other Pakistani isolate sequences in the GenBank, with the next closest resemblance to the H7N3 isolate from a Peregrine Falcon in U.A.E A/Peregrine Falcon/UAE/188/2384/98 (H7N3) (Manvell *et al.*, 2000),

although it is unlikely that birds of prey were involved in the introduction of the H7N3 viruses (Aamir *et al.*, 2009). Examination of the connecting peptide of the HA in the Pakistani H7N3 isolates sequenced revealed 2 patterns: Five HPAI isolates from the years 1995 – 2005 had connecting peptides of **PETPKRKRKR*GLF** and one LPAI isolate A/Chicken/Pakistan/NARC-1282/04 showed the pattern **PEIPKGR*GLF** resembling other Italian H7N3 AIV's. Other Pakistani isolates in the GenBank had connecting peptides showing three patterns: The 1995 isolates had connecting peptides of **PEIPKGR*GLF** and **PETPKRKRKR*GLF** and the remaining isolates had connecting peptides of **PETPKRRKR*GLF** showing a conservation of HA genes over a period of 8 years (Aamir *et al.*, 2009). This conservation of the HA genes in a period of 10 years indicates that continuing evolution has probably not occurred at the connecting-peptide region. These additional basic residues K (lysine) and R (Arginine) are considered a marker of high virulence in domestic poultry. The **PEIPKGR*GLF** motif is commonly found in LP H7 strains belonging to the Eurasian Lineage (Campitelli *et al.*, 2004). The results of our study demonstrates that domestic poultry viruses of the H7N3 subtype, which were responsible for the 2003-2005 H7N3 outbreak in Pakistan in the South and subsequently in the North were similar in their pathogenicity levels, being HPAI with a pattern similar to the H7N3 outbreak in 1995. This indicates that the 1995 strains of H7N3 were precursors of the subsequent isolates at the HA gene level, both at the nucleotide and deduced amino acid level excluding the isolate A/Chicken/Pakistan/NARC-1282/04 (H7N3). Comparison with other GenBank sequences showed a high level of homology with other Pakistani isolates circulating in the outbreaks of H7N3 from 1995 – 2005. This indicates that the 1995 H7N3 viruses were the ancestral viruses of these subsequent ones at the HA gene level. Influenza virus strains have also been isolated sporadically from birds in the U.A.E including H7N3, H7N1 and H9N2 (Wernery and Manvell 2003; Kent *et al.*, 2006; Obon *et al.*, 2007). Only the H7N3 strain, isolated from a peregrine falcon (*Falco peregrinus*) was conclusively found out to be highly pathogenic for the poultry population (Manvell *et al.*, 2000; Obon *et al.*, 2007). Earlier reports of appearance of HPAI H7N3 influenza viruses in Chile and two provinces of Canada (British Columbia and Saskatchewan) have implicated non-homologous recombination with inserts of amino acids at the HA cleavage site of the HA

gene resulting in the conversion of LPAI to HPAI (Pasick *et al.*, 2005; Suarez *et al.*, 2004).

The results of our study demonstrates that domestic poultry viruses of the H7N3 subtype, which were responsible for the 2003-2005 H7N3 outbreak in Pakistan in the South and subsequently in the North were similar in their pathogenicity levels, being HPAI with a pattern similar to the H7N3 outbreak in 1995. This indicates that the 1995 strains of H7N3 were precursors of the subsequent outbreak isolates at the HA gene level, both at the nucleotide and deduced amino acid level with a high degree of identity ranging from 98 – 99.6% at the nucleotide level and 97.6 – 98.9% at the amino acid level excluding the isolate A/Chicken/Pakistan/NARC-1282/04 (H7N3). Comparison with other GenBank sequences showed a 97 – 99.9% homology at the nucleotide level with other Pakistani isolates circulating in the outbreaks of H7N3 from 1995 – 2005, whereas a 93.7 – 100% similarity at the amino acid level was seen with these AIV's. This indicates that the 1995 H7N3 viruses were the ancestral viruses of these subsequent ones at the HA gene level.

The NA gene was analyzed for the presence or absence of a stalk region in the isolates sequenced. The 5 truncated H7N3 isolates for the NS1 Gene and HPAI for the HA gene had a stalked NA protein as in H7N3 isolates reported in wild birds showing a close resemblance to other previously sequenced H7N3 Pakistani isolate sequences in the GenBank, whereas the untruncated NS1 H7N3 isolate A/Chicken/Pakistan/NARC-1282/04 had a deleted NA stalk region, deduced amino acid sequence showing a deletion of 24 amino acids in concordance with other Italian H7N3 isolates reflecting a probable introduction of a highly circulating virus in domestic poultry or an introduction of a wild-bird isolate in the already existing land-based poultry.

The wild and domestic avian strains appeared firmly related at both phenotypic and genetic level with the NA genes differing mainly in a 23 amino acid deletion in the NA stalk. Outside this region of the molecule, the NAs of the Italian isolates showed 99% similarity indicating the origin of the poultry H7N3 from the wild waterfowl reservoir. The NA protein has lately been found out to be involved in the adaptation of wild avian strains to land-based poultry as indicated by a number of studies (Matrosovich *et al.*,

1999; Banks *et al.*, 2001). N3 coded for a polypeptide 470 amino acids long, with a 23 amino acid deletion (positions 56-78) in the stalk region of Italian turkeys. Terrestrial or raised aquatic birds including chickens, turkeys, quail, pheasants, teal and chukar showed this NA stalk deletion in N1, N2 and N3 NAs in avian viruses isolated from them but this type of deletion has never been recognized in wild avian strains. This deletion of a long sequence of amino acids in the stalk is a characteristic associated with early adaptation of wild avian viruses to turkeys and chickens (Campitelli *et al.*, 2004). The Pakistani H7N3 isolates sequenced showed a stalked NA protein in the case of 5 of the isolates sequenced with the probable explanation that these were highly circulating viruses in the poultry population. The isolate A/Chicken/Pakistan/NARC-1282/04 (H7N3) which resembled Italian viruses of 2002-2003 had a deleted stalk region in the deduced amino acid sequence of the NA protein, showing a 24 amino acid deletion like the Italian viruses and was probably an example of an early adaptation of a wild avian virus in poultry or of a highly circulating virus entering in the poultry population as a chance introduction.

The extraordinary difference between the highly pathogenic H7N3 influenza viruses that have been carried on in the domestic poultry population in Pakistan for over 10 years and the HP H5N1 that has been perpetuated in Asia for over a decade is that the H7N3 virus from Pakistan has behaved more like classical H5 and H7 viruses and has shown negligible evolution after acquiring the HP trait after circulation and this Pakistani H7N3 viral gene pool is antigenically highly conserved. Distinct from this is the case of the Eurasian HP H5N1 virus which has undergone rapid evolution and has developed into at least 10 antigenically distinct clades with multiple subclades (Peiris *et al.*, 2007). One likely explanation for the complex diversity pattern of the H5N1 as compared with the H7N3 virus is the failure of H7N3 influenza virus from Pakistan to spread to other variable hosts. In vivo experiments in mice and ferrets have revealed some replicative potential of Pakistani H7N3 viruses and their conserved evolutionary prototype is a sign pointing to their carrying less pandemic capacity than other HP H7 viruses (Aamir *et al.*, 2009).

The above data indicates that persistence of original AIV H7N3 and non-introduction of any new variant would have helped to overcome the infection of HPAI H7N3 in the local

poultry population. As a matter of fact, vaccines prepared from the original isolate of 1995 was reintroduced in 2003 and the vaccine prepared from H7N3 (1995) appears to have possessed high immunogenicity and similar antigenicity as the new isolates. The continuous usage of this vaccine along with improved bio-security measures resulted in the elimination of HPAI H7N3 from the poultry population in Pakistan. The above information reflects that continuous monitoring of circulating viruses in terms of their molecular and antigenic variability and subsequent production of homologous vaccines from field strains is key to the control of HPAI in poultry. The overall study indicates a continued circulation of an antigenically and molecularly homogenous group of highly pathogenic H7N3 influenza viruses for a period of 10 years from chicken farms in Pakistan's Northern areas indicating that the virus is not under pressure to change.

Finally this study provides a unique opportunity to follow the H7N3 outbreak in a decade in Pakistan to see whether AIVs evolve in poultry host and to continue to monitor these viruses for changes in pathogenicity. The availability of additional sequence and epidemiological data from wild and domestic bird isolates will be instrumental in understanding this and other aspects of the pathobiology of influenza A viruses.

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ANNEXURES

Annexure 1: BHI BROTH (Brain Heart Infusion Broth)

BHI Broth	37 gm
Water	1000 ml

37 gm of the powder was suspended in 1 liter of purified water. It was mixed thoroughly. Mixture was heated with frequent agitation and boiled for 1 min to completely dissolve the powder. It was autoclaved at 121°C for 15 min and cooled before used (Manufacturer's Protocol, BBL BHI Broth, Becton Dickinson, Cat # 211059).

Annexure 2: ETHIDIUM BROMIDE (10mg/ml)

Ethidium Bromide	1 gm
Water	100 ml

1 gm of ethidium bromide was added to 100ml of water. Mixture was stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The container was wrapped in aluminium foil or transferred to a dark bottle and stored at 4°C (Sigma, St Louis, MO, USA, Cat # E-8751).

Annexure 3: 0.5M EDTA pH 8.00 (Ethylene Diamine Tetra Acetic Acid)

EDTA	186.1 gm
Water	80 ml

186.1 gm of EDTA (MP Biomedicals, Cat# 195173) was added to 80ml of pure water. It was stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.00 with NaOH

(Merck) pellets (20 gm of NaOH pellets). The solution was dispensed in aliquots after being autoclaved at 121°C for 15 minutes.

Annexure 4: TRIS-BORATE-EDTA (TBE) BUFFER (5X)

Stock solution of 5X/liter was prepared by adding the following:-

TRIS Pure (Research Organics, Cat # 30950T)	54 gm
Boric Acid (Fisher Scientific, Cas # 10043-35-3)	27.5 gm
0.5M EDTA (pH 8.00) (MP Biomedicals, Cat# 195173)	20 ml

The above mentioned chemicals were dissolved in 980 ml of pure distilled water.

Working solution was prepared as follows:-

1X TBE was prepared by dissolving 200 ml of 5X Stock solution in 800 ml of distilled water.

Annexure 5: AGAROSE GEL (1%) FOR ELECTROPHORESIS

Agarose	0.4 gm
1X TBE Buffer	40 ml
Ethidium Bromide	2µl

0.4 gm of Agarose was dissolved in 40 ml of 1X TBE Buffer and 2µl of Ethidium Bromide. The mixture was heated until it boiled and was poured in a mould to set with a gel comb.

Annexure 6: POLYETHYLENE GLYCOL (PEG 8000)

Polyethylene Glycol	20 gm
NaCl	5.84 gm

1. For 100ml, 20 gm PEG 8000 and 5.84 gm NaCl was dissolved in 100 ml water.
2. The solution was autoclaved for 20 min at 15lbs/sq. in.
3. While the solution was still warm (approx 55°C), it was swirled carefully to mix thoroughly. (Manufacturer's protocol, Easy DNA Kit, Invitrogen, USA)

Annexure 7: ALSEVER'S SOLUTION

Sodium Citrate	8 gm
Sodium Chloride	4.2 gm
Citric Acid	0.55 gm
Dextrose	20.5 gm
Distilled Water	1000 ml

The chemicals were mixed in water and the solution was autoclaved at 121°C for 15 minutes at 15 lb/square inch pressure.

Annexure 8: PHOSPHATE BUFFER SALINE (PBS) SOLUTION

1 PBS tablet/100 ml of water (manufacturers protocol, MP Biomedicals)

Annexure 9: PREPARATION OF 70% ETHANOL

Ethanol 100%	70 ml
Water	30 ml

Ethanol and water were mixed and bottle was sealed properly.

Annexure 10: 1M SODIUM CHLORIDE (NaCl)

Sodium Chloride	5.85 gm
Water	100 ml

Solution was prepared by dissolving sodium chloride in water.

Annexure 11: PREPARATION OF LOADING SOLUTION FOR AGAROSE GEL ELECTROPHORESIS

PCR product	10µl
Loading Dye (Bromothymol blue)	2µl

The above mentioned contents were mixed to get a final loading solution of 12µl/well of the Agarose Gel.

Water	9µl
1kb DNA Step ladder	1µl
Loading Dye (Bromothymol blue)	2µl

The above mentioned contents were mixed to get the DNA Marker.

Annexure 12: PREPARATION OF 3M SODIUM ACETATE (pH 4.6)

Sodium Acetate	24.609 gm
Water	100 ml

Solution was prepared by dissolving sodium acetate in water. The pH of the solution was adjusted to 4.6.

Multiplex polymerase chain reaction for the detection and differentiation of avian influenza viruses and other poultry respiratory pathogens

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ABSTRACT A multiplex reverse transcription-PCR (mRT-PCR) was developed and standardized for the detection of type A influenza viruses, avian influenza virus (AIV) subtype H7, H9, and H5 hemagglutinin gene with simultaneous detection of 3 other poultry respiratory pathogens, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), and infectious laryngotracheitis virus (ILTV). Seven sets of specific oligonucleotide primers were used in this study for the M gene of AIV and hemagglutinin gene of subtypes H7, H9, and H5 of AIV. Three sets of other specific oligonucleotide primers were used for the detection of avian respiratory pathogens other than AIV. The mRT-PCR DNA products were visualized by agarose gel electrophoresis and consisted of DNA fragments of 1,023 bp for M gene of AIV, 149 bp for IBV, 320 bp for NDV, and 647 bp for ILTV. The second set of primers used for m-RT-PCR of H7N3, H9N2, and H5N1 provided DNA products of 300 bp for H7, 456 bp for H5, and 808 bp for H9. The mRT-PCR products for the third format consisted of DNA fragments of 149 bp for IBV, 320 bp for NDV, 647 bp for ILTV, 300 bp for H7, 456 bp for H5, and 808 bp for H9. The sensitivity and specificity of mRT-PCR was determined and the test was found to be sensitive and specific for the detection of AIV and other poultry respiratory pathogens. In this present study, multiplex PCR technique has been developed to simultaneously detect and differentiate the 3 most important subtypes of AIV along with the 3 most common avian respiratory pathogens prevalent in poultry in Pakistan. Therefore, a mRT-PCR that can rapidly differentiate between these pathogens will be very important for the control of disease transmission in poultry and in humans, along with the identification of 3 of the most common respiratory pathogens often seen as mixed infections in poultry, and hence economic losses will be reduced in poultry.

Key words: avian influenza virus, multiplex reverse transcriptase polymerase chain reaction, poultry respiratory pathogen, Pakistan

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INTRODUCTION

Avian influenza (AI) is a viral disease spread worldwide and is caused by influenza A viruses of the family Orthomyxoviridae. Influenza viruses are classified into 16 subtypes on the basis of the surface glycoprotein hemagglutinin and 9 subtypes on the basis of the glycoprotein neuraminidase. These 2 proteins are highly variable; therefore, a great number of AI virus (AIV) subtypes occur (Fouchier et al., 2005). Although most AIV in chickens cause mild and localized infections of the respiratory and intestinal tracts, highly pathogenic strains become dispersed throughout the body causing viraemia and produce an acute, systemic, and often fatal disease (Senne et al., 1996; Wood et al., 1996). Historically, highly pathogenic AIV of poultry belong to the H5 and H7 hemagglutinin subtypes. Because there is a greater risk for these subtypes to become highly pathogenic for humans, it is important to identify them specifically in surveillance programs (Webster and Kawaoka, 1987; Starick et al., 2000). The H9 subtype has also caused infections in poultry and humans (Cameron et al., 2000; Butt et al., 2005).

Recently, infections from AI subtypes H5, H7, and H9 have been repeatedly reported in poultry in Pakistan, especially since 2004 (Naeem et al., 2007). Unfortunately, due to very close resemblance of lesions produced by several other pathogens in poultry, it is difficult to differentially diagnose clinical manifestations of any of the above AI infections from diseases such as infectious laryngotracheitis (ILT), Newcastle disease, and infec-

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tious bronchitis. The ILT, Newcastle disease, and infectious bronchitis infections are prevalent at a rate of 10 to 15% in commercial poultry in Pakistan, despite the use of live and inactivated vaccines against such diseases. Therefore, it becomes essential to develop sensitive diagnostic techniques to differentiate between these 6 diseases. In this study, a multiplex PCR technique has been developed to simultaneously detect and differentiate the 3 most important subtypes of AIV along with the 3 most common avian respiratory pathogens prevalent in poultry in Pakistan.

MATERIALS AND METHODS

Source of Viruses and Specimens

The following listed isolates of H7N3 were used in the standardization of multiplex reverse transcription-PCR (**mRT-PCR**):

1. A/Chicken/Faisalabad/Pakistan/NARC-N30/05 (H7N3)
2. A/Chicken/Karachi/Pakistan/NARC-100/04 (H7N3)
3. A/Chicken/Mansehra/Pakistan/NARC-74/04 (H7N3).

The following isolates of H9N2 were used in the standardization of mRT-PCR:

1. A/Chicken/Karachi/Pakistan/NARC-4935/06 (H9N2)
2. A/Chicken/Abbotabad/Pakistan/NARC-6649/06 (H9N2)
3. A/Chicken/Islamabad/Pakistan/NARC-N240/06 (H9N2).

The following isolates of H5N1 were used in the standardization of mRT-PCR:

1. A/Chicken/Peshawar/Pakistan/NARC-2517/06(H5N1)
2. A/Chicken/Jehlum/Pakistan/NARC-N353/07(H5N1)
3. A/Chicken/Islamabad/Pakistan/NARC-N240/06(H5N1).

The above-mentioned viruses were obtained from the repository of the National Reference Laboratory for Poultry Diseases, National Agricultural Research Center, Islamabad, Pakistan. The viruses procured from the repository in a lyophilized form were propagated in 9-d-old embryonated chicken eggs up to 3 passages and tested using hemagglutination test (Beard, 1980; Swayne et al., 1998). After the standardization of the test, the technique was used for the detection of routine field cases of AI.

Newcastle disease virus (**NDV**), infectious bronchitis virus (**IBV**), and ILT virus (**ILTV**) were extracted from live freeze-dried vaccines (Nobilis, Intervet International B.V., Boxmeer, the Netherlands) for the standardization procedure. The few examples of field isolates of NDV, IBV, and ILTV identified by mRT-PCR are listed below:

1. Chicken/Rawalpindi/Pakistan/NARC-303/05 (IBV M-41)
2. Chicken/Karachi/Pakistan/NARC-2344/06 (IBV M-41)
3. Chicken/Rawalpindi/Pakistan/NARC-378/05 (NDV)
4. Chicken/Karachi/Pakistan/NARC-1045/07 (NDV)
5. Chicken/Faisalabad/Pakistan/NARC-N334/06 (ILTV)
6. Chicken/Islamabad/NARC-N767/07 (ILTV).

The tissues selected for mRT-PCR included trachea, lungs, cecal tonsils, and spleen from the diseased chickens showing respiratory tract infection. The samples were pooled and processed for RNA-DNA extraction.

Extraction of RNA and DNA

Viral RNA from the viral samples (tissue homogenates, allantoic fluids, and vaccines) was extracted using QIAamp Viral RNA Mini Kit, according to instructions of the manufacturer (52906, Qiagen Inc., Valencia, CA).

Deoxyribonucleic acid from ILTV was extracted using Easy DNA Kit (Invitrogen, Carlsbad, CA) following protocol 7 of the manufacturer. The concentrations of RNA or DNA were determined using a Bio-Photometer (Eppendorf, Hamburg, Germany).

Primer Designs and Selection

Four sets of primers that specifically amplify type A influenza virus (M gene) and the H gene of H7, H9, and H5 subtypes of AIV are listed in Table 1. Three sets of primers for IBV, ILTV, and NDV are listed in Table 2. The sequence of the primers for H7, H9, and H5 was obtained from already published data in literature and synthesized by Operon Biotechnologies (Huntsville, AL). Primer sequences for IBV were obtained from published data (Callison et al., 2006), and primer sequences for ILTV and NDV were obtained from published data (Pang et al., 2002). The primers were aliquoted to a final concentration of 100 pmol/μL and stored at −20°C until further use.

Optimization of mRT-PCR

The mRT-PCR employed in the present work consisted of a 1-step procedure, which included reverse transcription (**RT**) and PCR amplification in a single step. For this purpose, an RT-PCR kit (Invitrogen) was used. Following the protocol of the manufacturer, the

Table 1. Multiplex reverse transcription-PCR primers[1]

Primer name[2]	Oligonucleotide sequence of primer	Product (bp)
AIV		
M-WSN F	5'-GAA GGT AGA TAT TGA AAG ATG-3'	1,023
M-1023 R	5'-GAA ACA AGG TAG TTT TTT ACT C-3'	
Subtype H7		
H7-397 F	5'-ACA TAC AGT GGG ATA AGA ACC-3'	300
H7-391 R	5'-TCT CCT TGT GCA TTT TGA TGC C-3'	
Subtype H9		
H9-1 F	5'-AGC AAA AGC AGG GGA AYW WC-3'	808
H9-808 R	5'-CCA TAC CAT GGG GCA ATT AG-3'	
Subtype H5		
H5-1	5'ACT ATG AAG AAT TGA AAC ACC T-3'	456
H5-2	5'-GCA ATG AAA TTT CCA TTA CTC TC-3'	

[1]Codes for mixed bases position: Y, C/T; R, A/G; W, A/T; B, G/C/T; K, G/T. References: R. G. Webster (St. Jude Children's Research Hospital, Memphis, TN; H7 and H9) and Ng et al. (2006; H5).

[2]AIV = avian influenza virus.

mRT-PCR was performed in 50-μL volumes, in which the reaction mixture according to the manufacturer contained 25 μL of 2× Reaction Mix consisting of a buffer system for RT and PCR amplification, optimal Mg++ concentration, deoxyribonucleotide triphosphates, and stabilizers. Template RNA in various dilutions, which were optimized after several mRT-PCR trials, were added in 1-μL amounts per reaction mixture. One microliter of forward and reverse primers of influenza A, H7, H9, H5, IBV, ILTV, and NDV was added in an optimized concentration of 100 pmol/μL and 2.5 μL of RT/Platinum Taq Mix was added. This amount was optimized after several trials, and it contained a mixture of Superscript II Reverse Transcriptase and Platinum Taq DNA Polymerase for optimal cDNA synthesis and PCR amplification. Nuclease-free water was added to bring the final volume to 50 μL.

The mRT-PCR was performed in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA). The cycling protocol consisted of cDNA synthesis at 45°C for 45 min, an initial denaturation at 96°C for 5min, then 40 cycles that each consisted of denaturing at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 70°C for 1 min, followed by a final extension at 72°C for 10 min and final storage at 4°C. A negative control did not contain template cDNA and consisted of PCR master mix, all sets of primers, and nuclease-free water.

Three sets of mRT-PCR were carried out; the first set, referred to as trivalent mRT-PCR, used primers of H7, H9, and H5 with template RNA of the specific hemagglutinin subtypes utilizing the optimal conditions mentioned above for mRT-PCR. A second set of mRT-PCR, called tetravalent mRT-PCR, was developed using primers for influenza A, NDV, IBV, and ILTV with template RNA-DNA of these specific viruses utilizing the optimal conditions of mRT-PCR. The third set of mRT-PCR, referred to as hexavalent mRT-PCR, was developed using primers of H7, H9, H5, NDV, IBV, and ILTV with the specific template RNA-DNA with hexavalent amplifications being carried out using the optimized conditions.

Detection of Amplified Nucleic Acid Products of mRT-PCR

The DNA amplicons were visualized using 2% agarose gels with ethidium bromide with standard 1-kb DNA markers (Invitrogen) at 120 V for 20 min. Gels were photographed using a gel documentation system (Poddar, 2002).

Sensitivity and Specificity of mRT-PCR

The mRT-PCR was tested using other avian pathogens that produce similar clinical signs or that can be present in mixed infections with AI subtypes (Table 1). To determine the ability of the mRT-PCR assay to detect and differentiate 3 subtypes of AIV, H7, H9, and H5, and NDV, IBV, and ILTV in the same reaction, we used a mixture of DNA-RNA concentrations

Table 2. Other primers used in multiplex PCR[1]

Primer name[2]	Oligonucleotide sequence of primer	Product (bp)
IBV (up)	5'-GCT TTT GAG CCT AGC GTT-3'	149
IBV (down)	5'-GCC ATG TTG TCA CTG TCT ATT-3'	
NDV (up)	5'-GGA GGA TGT TGG CAG CAT T-3'	320
NDV (down)	5'-GTC AAC ATA TAC ACC TCA TC-3'	
ILTV (up)	5'-ACG ATG ACT CCG ACT TTC-3'	647
ILTV (down)	5'-CGT TGG AGG TAG GTG GTA-3'	

[1]References: Callison et al. (2006; IBV) and Pang et al. (2002; NDV, ILTV).

[2]IBV = infectious bronchitis virus; NDV = Newcastle disease virus; ITLV = infectious laryngotracheitis virus.

DISCUSSION

The mRT-PCR was developed in 3 formats to detect and differentially diagnose in a single reaction influenza A; AI hemagglutinin subtypes H7, H9, and H5; and 3 respiratory pathogens, NDV, IBV, and ILTV.

Influenza infection is diagnosed by virus isolation and identification or serological tests. However, virus isolation and identification is tedious and time-consuming and serological tests are less practical if mass vaccination is practiced (Reina et al., 1996). This situation does exist in Pakistan where apart from the prevalence of H7, H9, and H5 in the field, the presence of NDV, IBV, and ILTV is well documented. This makes it very difficult to diagnose any respiratory infection of poultry under field conditions. More recently, PCR assays have been developed for many respiratory viruses, allowing detection of small amounts of viral nucleic acid in clinical samples. In the so-called multiplex format, PCR assays have been designed to amplify more than one respiratory viral target in the same PCR test (Ellis et al., 1997; Fan et al., 1998; Osiowy, 1998; Grondahl et al., 1999; Liolios et al., 2001; Xie et al., 2006).

Therefore, a mRT-PCR which can rapidly differentiate between AIV subtypes H7, H9, and H5 and NDV, IBV, and ILT will be very important for the control of disease transmission in poultry and in humans along with the identification of 3 of the most common respiratory pathogens often seen as mixed infections in poultry. Economic losses will be reduced in poultry associated with and AIV outbreak with the use of this assay. Conventional techniques such as RT-PCR, used to detect viral RNA, are time-consuming and perhaps even less sensitive. Although we did not compare the assay developed and reported here with the conventional technique of RT-PCR, it can be safely assumed that this assay is superior to conventional techniques because viral RNA can be directly detected in clinical and field samples in a few hours; therefore, it is less time-consuming as compared with other diagnostic methods. This mRT-PCR may be used in diagnosis, screening, and surveillance of poultry. This assay has the added benefit of being less time-consuming and using a single-step procedure for PCR with the use of fewer reagents and the simultaneous detection of AIV with other respiratory poultry pathogens. This assay has been developed specially for the developing countries where it is very difficult to buy very expensive laboratory instruments like quantitative real time-PCR machines, which are frequently being used in developed countries for the detection of viral nucleic acids.

Future work is needed to expand the panel of viral pathogens detected by such rapid molecular methods to eventually circumvent the need for viral cultures. Also, future evaluation of the multiplex PCR assay is warranted.

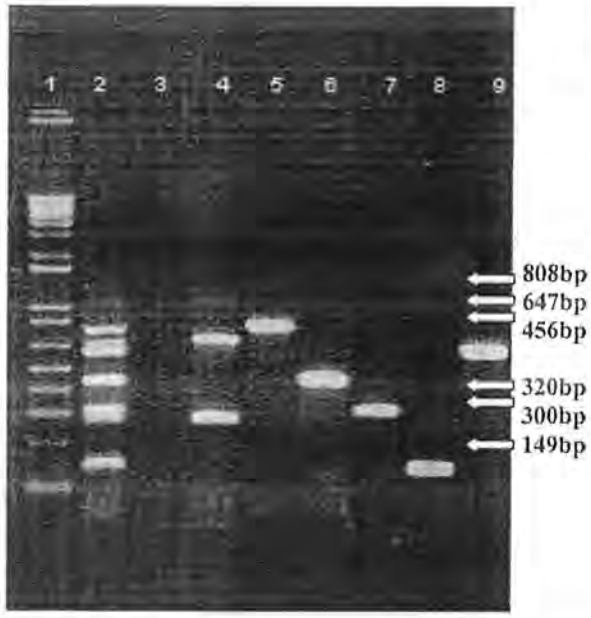

Figure 3. Agarose gel electrophoresis of hexavalent multiplex reverse transcription-PCR-amplified products from purified RNA and RNA of known avian influenza subtypes and other avian respiratory pathogens. Lane 1 = molecular size markers; lane 2 = H7N3, H5N1, H9N2, Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV); lane 3 = PCR reagent buffer as a negative control; lane 4 = H7N3, 300 bp; lane 5 = H9N2, 808 bp; lane 6 = H5N1, 149 bp; lane 7 = NDV, 320 bp; lane 8 = IBV, 149 bp; lane 9 = ILTV, 647 bp.

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ranging from 500 ng to 10 fg of DNA-RNA in various combinations of 3 subtypes of AIV and 3 other respiratory pathogens.

Sensitivity of the mRT-PCR for the detection of these 6 respiratory pathogens was determined by making 10-fold serial dilutions of 100 ng of each respiratory pathogen as template RNA-DNA; mRT-PCR of these dilutions was done in the thermocycler using the optimized program.

Specificity of mRT-PCR was determined by examining the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV, and ILTV. Primers specific for AIV, H7, H9, and H5 were added to the mutiplex format for the amplification of AIV, H7, H9, and H5 to check their reactivity if any to NDV, IBV, and ILTV. Similarly, in the multiplex format, NDV-, IBV-, and ILTV-specific primers were added in the multiplex format to check if they reacted-amplified AIV, H7, H9, and H5 RNA.

RESULTS

Throughout the development of mRT-PCR, various modifications were made to the annealing temperature, extension time, cycle quantity, primer concentration, and template dilutions. The multiplex PCR products consisted of 300 bp for H7, 808 bp for H9, 456 bp for H5, 149 bp for IBV, 320 bp for NDV, and 647 bp for ILTV and were visualized by gel electrophoresis (Figure 1, 2, and 3). The standardized test was employed to examine various field cases. The sensitivity of mRT-PCR, depicting the limit of the method by visualization of PCR-amplified DNA products, was 1 ng for the hemagglutinin subtypes of AIV (H7, H9, and H5) and type A AIV. The detection limit was 100 pg for IBV, NDV, and ILTV. No spurious PCR amplification reactions were observed in the detection of AIV subtypes H7, H9, and H5 and other respiratory pathogens with various concentrations of DNA and RNA mixtures. All negative controls were negative. Negative controls had no template RNA-DNA and just the primers and buffering system provided by the manufacturer in the kit were added (SuperScript One-Step RT-PCR with Platinum Taq, 10928-042, Invitrogen).

Specificity of mRT-PCR was determined by examining the ability of the test to detect type A influenza viruses and differentiate H7, H9, and H5 subtypes of type A influenza viruses along with NDV, IBV, and ILTV. The specificity of the primers used to detect amplified DNA was hence determined because no specific amplification bands of the same sizes (300, 808, and 456 bp) could be amplified for RNA-DNA of other poultry respiratory pathogens (NDV, IBV, and ILTV), and likewise, no amplification bands of the sizes (320, 149, and 647 bp) could be amplified for RNA for AIV.

Figure 1. Agarose gel electrophoresis of trivalent multiplex reverse transcription-PCR-amplified products from purified RNA of known avian influenza subtypes. Lane 1 = molecular size marker; lane 2 = H7N3, H5N1, and H9N2 subtypes of avian influenza virus; lane 3 = PCR reagent buffer as a negative control; lane 4 = H7N3, 300 bp; lane 5 = H9N2, 808 bp; lane 6 = H5N1, 456 bp.

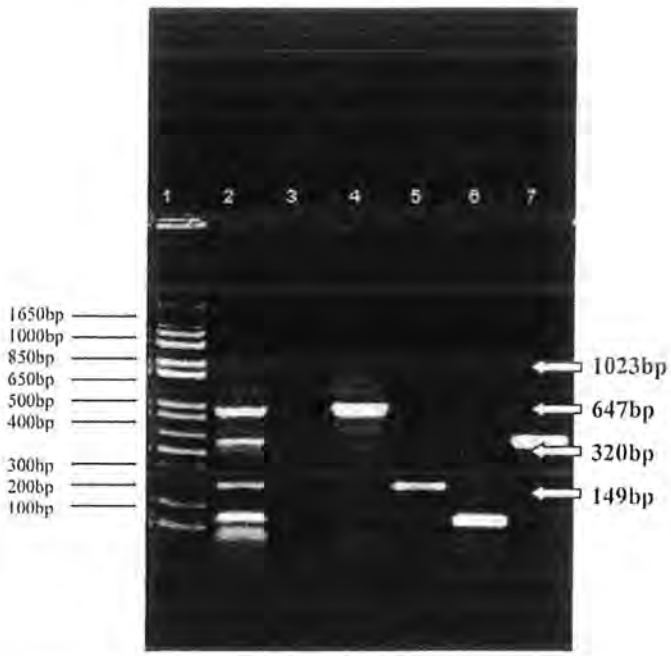

Figure 2. Agarose gel electrophoresis of tetravalent multiplex reverse transcription-PCR-amplified products from purified RNA and DNA of known avian influenza subtypes and other avian respiratory pathogens. Lane 1 = molecular size marker; lane 2 = avian influenza virus (AIV), Newcastle disease virus (NDV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV); lane 3 = PCR reagent buffer as a negative control; lane 4 = AIV, 1,023 bp; lane 5 = NDV, 320 bp; lane 6 = IBV, 149 bp; lane 7 = ILTV, 647 bp.

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