**SIMULTANEOUS DETECTION OF ALL AVIAN INFLUENZA VIRUSES AND DIFFERENTIATION OF H5, H7, N1 AND N2 SUBTYPES BY A MICROSPHERE BASED ASSAY**

**by**

**TENEEMA KURIAKOSE**

**(Under the Direction of Mark W. Jackwood)**

## ABSTRACT

Avian influenza viruses are negative sense single stranded RNA viruses. They are deadly pathogens in poultry and rapid identification of these viruses is very important because of the pandemic threat as human adapted viruses can emerge by mutation or reassortment. A multiplex microsphere assay for the simultaneous detection of all avian influenza viruses and differentiation of H5, H7, N1 and N2 subtypes was developed in this study. Multiplex RT-PCR using biotinylated primers specific to the target followed by hybridization with specific oligonucleotide probe coated microspheres in a multiplex format were performed. Streptavidin-R-Phycoerythrin was used as the reporter and the fluorescence intensity was measured by the Bioplex machine. The assay is 97.43% specific and the diagnostic sensitivity is $10^{2.5}$ -$10^{2.8}$ $EID_{50}$ of virus. Validation of the assay was performed with 102 clinical samples. This assay can be used as a rapid, sensitive and specific diagnostic test for avian influenza viruses.

INDEX WORDS: Avian influenza diagnosis, Multiplex Microsphere Assay, Hemagglutinin, Neuraminidase, Subtyping

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## DEDICATION

***This work is dedicated to***
***My parents***
***For their unfailing love, concern and support throughout my life***

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# INTRODUCTION

The family *Orthomyxoviridae* includes single strand negative sense RNA viruses with segmented genomes. They are enveloped viruses with a helical nucleocapsid. The three genera of this family are Influenza viruses A, B and C and they are subdivided based on the antigenic differences in their matrix protein and nucleoprotein (Palese and Shaw 2007; Swayne and Halvorson 2008). These viruses infect humans, birds and a wide range of animal species (Wright *et al*. 2007). Influenza A viruses are the most important pathogen among the three since they cause morbidity and mortality in humans, animals and birds and are responsible for the major pandemics in the last century (Wright *et al*. 2007). Influenza B can cause the same spectrum of illness as Influenza A, but the frequency of severe illness is much lower, causing outbreaks every 2-4 years in humans. Influenza C is associated with sporadic and subclinical infections in humans and swine and is only rarely associated with severe lower respiratory tract infections (Zambon 1999; Sandrock and Kelly 2007; Wright *et al.* 2007).

The Influenza A virus genome has eight segments encoding eleven proteins. The important proteins are matrix proteins (M), hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), nonstructural proteins (NS) and RNA polymerase (PB1, PB2, PA) (Palese and Shaw 2007; Swayne and Halvorson 2008). The virus replicates in the nucleus of the infected cell. Since the cells cannot copy the negative strand RNA, a positive strand mRNA is first synthesized by the viral RNA dependant RNA polymerase. This mRNA is translated into viral proteins and it also acts as template for the synthesis of negative strand genome (Palese and Shaw 2007; Swayne and Halvorson 2008).

Hemagglutinin and neuraminidase are integral membrane glycoproteins of the virus and the classification of Influenza A viruses into different subtypes is based on antigenic and genetic differences of their HA and NA proteins (Wright *et al.* 2007; Swayne and Halvorson 2008). There are 16 known subtypes of HA (1 – 16) and 9 subtypes of NA (1 – 9). HA is the principal antigen on the surface of the virus and it is responsible for virus binding to terminal sialic acid moieties present in host cell surface glycoproteins and glycolipids. After the viral entry into the endosomes, HA facilitates the fusion of viral and endosomal membranes and the release of virion contents into the cytoplasm. HA is also the primary target for neutralizing antibodies. Neuraminidase facilitates the release of the virions from infected cells (Wright *et al.* 2007; Swayne and Halvorson 2008).

Influenza viruses undergo constant antigenic variation by two different mechanisms which help them to escape host immune response – antigenic drift and antigenic shift (Wright *et al.* 2007; Swayne and Halvorson 2008). Mutations may accumulate in the newly replicated viral populations that result in antigenic drift giving rise to new variants that evade immunity as they are immunologically distinct from the previous strains circulating in a population. Antigenic drift is minor, gradual point changes in HA or NA proteins as a result of point mutations. These antigenic drift variants escape neutralizing antibodies and are selected by sequential accumulation of point mutations. Similarly, the segmented genome may allow reassortment of viral segments resulting in the emergence of a new virus with novel proteins (Wright *et al.* 2007). Outbreak of Spanish influenza (H1N1), Asian influenza (H2N2), Hong Kong influenza (H3N2) and Russian influenza (H1N1) were the result of antigenic shift (Wright *et al.* 2007). Pandemic influenza result from this antigenic shift as the population may have no or little immunity against the virus with new HA (Poland *et al.* 2007; Wright *et al.* 2007).

**Avian Influenza**

Avian Influenza or Fowl plague is a contagious disease in poultry caused by Influenza A viruses. Only type A influenza viruses are known to cause natural infections in birds and all different possible combinations of HA and NA subtypes have been isolated from them (Alexander 2000; Wright *et al.* 2007; Alexander 2008). Depending on the ability to cause disease, influenza A viruses in poultry are divided into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) (Alexander 2000; Alexander 2008). Some viruses of the subtypes H5 and H7 cause HPAI and the mortality rate may reach up to 100% (Alexander 2000). All other Influenza A viruses infecting poultry cause LPAI, characterized by a mild respiratory infection and drop in egg production, which can be exacerbated by other conditions (Swayne and Halvorson 2008; Pantin-Jackwood and Swayne 2009). Wild birds of the order *Anseriforms* and *Charadriiforms* are the natural hosts and asymptomatic carriers of avian influenza, and hence are not usually affected by HPAI (Wright *et al.* 2007). However, influenza viruses can be transmitted from wild aquatic to domestic birds, which usually results in LPAI (Alexander 2007). After circulating in domestic poultry, a few H5 and H7 LPAI viruses may mutate into HPAI causing severe systemic disease (Alexander 2007).

The HA gene is the primary determinant of virulence in avian influenza virus (Wright *et al.* 2007; Swayne and Halvorson 2008). The cleavage of HA0 into HA1 and HA2 is essential for the virus to be infectious. In HPAI, the HA cleavage site has multiple basic amino acids that can be recognized and cleaved by ubiquitous proteases like furin and PC6 and hence the virus can enter and replicate in organs throughout the body causing severe clinical disease and death (Stieneke-Grober *et al.* 1992; Wright *et al.* 2007; Swayne and Halvorson 2008). In LPAI, the HA can be cleaved only by trypsin like proteases which are present in restricted sites such as

respiratory and digestive tracts and hence the clinical signs are limited to these sites (Bosch *et al.* 1979; Steinhauer 1999; Wright *et al.* 2007; Swayne and Halvorson 2008).

Wild birds are the natural hosts, but the virus can cross the species barrier and can infect and cause disease in chickens, humans and other mammals, and are hence classified under avian zoonosis (Alexander 2007; Wright *et al.* 2007; Swayne and Halvorson 2008). The species specificity is determined by the HA glycoprotein binding to the sialic acid residues of the host cell. Human and swine H1N1 influenza viruses preferentially recognize receptors with saccharides ending in sialic acid (SA)α 2,6 galactose mainly expressed in tracheal epithelial cells whereas avian and equine viruses prefer those terminating in SAα 2,3 galactose located mainly on tracheal and intestinal epithelial cells (Rogers and Paulson 1983; Wright *et al.* 2007). Initially it was thought that avian influenza cannot cause infection in humans due to the differences in receptor specificities and their location. However that thinking has changed since the Hong Kong outbreak of avian influenza in humans in 1997 with 18 proven cases of which six were fatal. This is likely due to the presence of a minor population of ciliated cells in human tracheal and bronchial tissues (Matrosovich *et al.* 2004) and non-ciliated cuboidal bronchiolar cells (Shinya *et al.* 2006) that contain SAα 2,3Gal oligosaccharides. Transmission of influenza A virus from birds to humans may also be associated with the ability of HA to switch its preference from SAα 2,3Gal to SAα 2,6Gal (Yamada *et al.* 2006). Since the respiratory epithelium of pigs expresses both SAα 2, 3 Gal and SAα 2, 6 Gal, pigs can be infected by both avian and human influenza virus (Kida *et al.* 1994) and is considered as the mixing bowl of infection (Ito *et al.* 1998; Wright *et al.* 2007).

**Economic and public health significance**

The economic impact of avian influenza in the poultry industry is enormous. Since 1955, more than 24 documented epizootics of HPAI have occurred worldwide resulting in the depopulation of millions of birds (Perdue and Swayne 2005). An outbreak of HPAI virus in the Netherlands in 2003 caused by the subtype H7N7, that lasted fewer than 3 months, resulted in culling more than 30 million birds (Van Riel *et al.* 2009). Similarly, more than 13 million birds were affected due to HPAI H7N1 influenza virus in Italy in 1999, which originated as LPAI (Capua *et al.* 2000). In 2004, an HPAI H7N3 virus arose from a LPAI virus in British Columbia resulting in the depopulation of 19 million birds (Tweed *et al.* 2004).

Multiple subtypes of avian influenza have infected and caused disease in humans. In 1997, HPAI infections caused by H5N1 subtypes were transmitted to humans, resulting in 6 deaths (Katz *et al.* 2009). These were the first documented human cases caused by avian influenza viruses. In 2003, the virus again infected humans and spread to multiple Asian countries, Africa and Eurasia affecting approximately 390 persons and causing 245 deaths as of October 2008 (Katz *et al.* 2009). The ability of H5N1, H7N7 and H9N2 to infect humans makes them the most likely avian candidates to cause future pandemics (Lazzari and Stohr 2004).

**Transmission, distribution and spread**

Wild aquatic birds are the natural reservoir for avian influenza (Stallknecht and Shane 1988; Webster *et al.* 1992; Webster 1997; De Marco *et al.* 200; Wright *et al.* 2007; Swayne and Halvorson 2008). They do not cause signs of disease, they replicate in intestinal tract and they are transmitted through feces (Webster 1997). The virus may be transmitted from one bird to another by the fecal-oral route through water (Webster 1997). Viruses in their natural host undergo only limited mutation indicating that they are adapted to the natural reservoirs (Webster

*et al.* 1995). However, when transmitted to another host like domestic poultry, humans or other mammals, they rapidly mutate undergoing antigenic shift and drift, which results in increased virulence and infections (Webster *et al.* 1995). Bird to bird transmission is poorly understood and can be complex depending on the virus strain and species of bird (Alexander 2007). Primary introduction of LPAI viruses into a poultry population depends on contact with wild birds, either through contaminated water sources, direct contact or fomites (Alexander 2007; Swayne and Halvorson 2008). Virulent strains may show poorer transmission than viruses of low pathogenicity in both natural and experimental infections (Alexander 2007). One reason suggested for this observation is that highly virulent viruses rapidly kill the host resulting in little virus excretion (Alexander 2007).

Migratory waterfowl can be an important source of bird-to-bird transmission (Hinshaw *et al.* 1980). A considerable number of viruses excreted in the feces (Hinshaw 1979; Webster *et al.* 1992) may contaminate lakes or ponds where the virus can remain for a long time. Depending on the water temperature, the virus retains infectivity from less than one month to several months (Webster *et al.* 1992; Stallknecht and Shane 1988; Alexander 2007). Avian influenza may be transmitted from infected/contaminated meat to mammals including humans. Domestic cats, pigs, tigers, etc. that had taken raw chicken carcasses can be infected with the virus (Thanawongnuwech *et al.* 2005; Kuiken *et al.* 2004; Kida *et al.* 1994).

Human infection due to avian influenza viruses mainly occurs due to close contact with infected birds especially through the direct contact with the excreta from infected birds and mucous membrane with infected secretions (Tran *et al.* 2004; Koopmans *et al.* 2004; Hayden and Croisier 2005). Personnel involved in processing the birds for consumption have occasionally been infected. The virus may also enter through respiratory tract or conjunctivae (Fouchier *et al.*

2004). Human-to-human transmission may occur with low efficiency which may involve close contact during the early phase of infection (Ungchusak *et al.* 2005; Koopmans *et al.* 2004).

**Pathobiology**

In gallinaceous poultry, highly pathogenic avian influenza produces high morbidity and mortality, and systemic disease with necrosis and inflammation in multiple organs (Swayne and Halvorson 2008; Pantin-Jackwood and Swayne 2009). LPAI mainly affects respiratory system characterized by catarrhal, serofibrinous or muco/fibrinopurulent inflammation (Swayne and Halvorson 2008). Little or no virus is found in other organs except the reproductive organs, which may result in misshapen eggs and lesions in the oviduct. However, HPAI produces lesions in multiple organs characterized by tracheatis, edema, congestion and haemorrhage of lungs, interstitial pneumonia and bronchitis, edema of brain, myocarditis, pancreatitis, lymphoid depletion, phagocytic hyperplasia, necrosis and haemorrhage of bursa and thymus, subcutaneous edema, necrosis of bone marrow, myofibre degeneration of skeletal muscle, hepatocellular necrosis, tubular necrosis and interstitial nephritis (Pantin-Jackwood and Swayne 2009).

In humans, avian influenza mainly causes lesions in the respiratory tract. The lungs show diffuse alveolar damage, interstitial fibrosis, hyperplasia of type II pneumocytes, hemorrhage, pleuritis etc. (Korteweg and Gu 2008: Wright *et al.* 2007). It also causes central lobular necrosis, microvesicular fatty changes, cholestasis and hemophagocytic activity in liver, acute tubular necrosis of kidneys, edema of brain, reactive hemophagocytosis, congestion and depletion of lymphocytes in spleen and hypoplastic or hyperplastic bone marrow (Korteweg and Gu 2008; Wright *et al.* 2007).

**Clinical signs**

Clinical signs are similar to other avian respiratory infections and vary greatly depending on the age and species of poultry infected and the pathogenicity of the virus (Swayne and Halvorson 2008; Pantin-Jackwood and Swayne 2009). LPAI is characterized by low mortality but high morbidity. The clinical signs of LPAI include respiratory distress, coughing, sneezing, tracheal rales, depression, lack of appetite, ruffled feathers, edema of head, comb and wattles and drop in egg production (Swayne and Halvorson 2008; Pantin-Jackwood and Swayne 2009). Sudden onset of high mortality in the flock is the first sign of HPAI. In per acute cases, sudden death without showing clinical signs and the few surviving birds showing lethargy and comatose condition may be noticed. The clinical signs associated with the disease include depression, decreased feed and water intake, drastic drop in egg production and loose feces with mucus. In acute to subacute cases, birds may develop nervous signs including tremors of head and neck, convulsions, paralysis, incoordination, loss of balance etc. (Swayne and Halvorson 2008; Pantin-Jackwood and Swayne 2009).

**Immunity**

Protection against influenza virus can be achieved through the innate immune response or the adaptive immune response (Doherty *et al.* 2009; Swayne and Kapczynski 2008; Wright *et al.* 2007; Suarez and Schultz-Cherry 2000). Toll-like receptors are important in innate immunity as they recognize influenza virus and trigger a cascade of reactions that stimulate interferon and TNF-α (Wright *et al.* 2007). Adaptive immune responses include humoral and cell mediated immunity. When HPAI affects a naive bird, not exposed to the virus before, death occurs in a short period, providing inadequate time to mount an immune response (Swayne and Kapczynski 2008). If sudden death does not occur, a humoral immune response is elicited with the

production of IgM within 5 days post infection and IgY (avian IgG) thereafter (Suarez and Schultz-Cherry 2000; Swayne and Kapczynski 2008). In this case, the humoral immune response in poultry is comparable to other species. Vaccination induces the production of neutralizing IgY antibodies, which are directed against the HA protein and block viral attachment (Chambers *et al.* 1988; Swayne and Kapczynski 2008). Even though antibody titer (measured by an indirect antibody test, the hemagglutinin inhibition test) induced by vaccination is low, antibodies are primary responsible for mediating protection (Chambers *et al.* 1988). Anti-HA antibodies are specific to each of the different HA subtypes (Allan *et al.* 1971; Swayne and Kapczynski 2008). Anti-NA antibodies are only partially protective but may reduce the efficiency of viral release and subsequent spread. They are also specific to each of the different NA subtypes (Allan *et al.* 1971; Swayne and Kapczynski 2008). Thus, antibody raised to one virus can neutralize other viruses of the same HA or NA subtype, but do not cross neutralize viruses of different HA and NA subtypes (Suarez and Schultz-Cherry 2000).

Immune responses against proteins other than HA and NA do not provide significant protection (Swayne and Kapczynski 2008). However, the immune response to the M2 protein can be important. The transmembrane protein M2 forms a proton channel which helps in equilibrating the pH across viral membrane during entry (Pielak and Cho 2010)**.** Vaccination of mice with M2 reduced the amount and duration of virus shedding and provided partial protection from disease (Slepushkin *et al.* 1995). Moreover, passively transferred monoclonal antibody to the M2 protein could inhibit viral replication in mice (Treanor *et al.* 1990). Since the M2 protein is well conserved in all influenza Type A viruses, antibodies to M2 can be protective against all HA and NA subtypes (Slepushkin *et al.* 1995; Treanor *et al.* 1990; Suarez and Schultz-Cherry 2000).

Mucosal immunity may provide some protection from infection and helps in the recovery of infected birds (Suarez and Schultz-Cherry 2000). Intranasal administration of H5N2 inactivated virus along with adjuvants to one day old chicks significantly increased the number of IgA and IgG secreting cells and the number of intraepithelial lymphocytes, CD3(+) T lymphocytes and mast cells, in the respiratory tract indicating that the local immune response in respiratory tract can be beneficial (Xiaowen *et al*. 2009). Similarly, the avian harderian gland may generate mucosal and systemic immunity to AI through the production of IgA and IgG (van Ginkel *et al*. 2009).

Cellular immunity against HPAI in poultry may not be very effective because mortality may occur before cytotoxic T-lymphocyte specific immune responses are mounted (Swayne and Kapczynski 2008). Moreover, inactivated AI vaccine mainly stimulates humoral immunity rather than cell mediated immunity (Swayne and Kapczynski 2008). However, a few studies reported that cellular immunity may also be important in protective immunity (Gao *et al*. 2006; Wu *et al.* 2009). Similarly, adoptive transfer of T lymphocytes or CD8(+) T cells from chickens infected with H9N2 influenza virus to those not exposed to the virus protected them from infection with H5N1 indicating that cellular immunity can be protective against influenza virus (Seo and Webster 2001)

**Diagnosis**

Traditionally, AI viruses are detected by virus isolation (VI) in specific pathogen-free (SPF) eggs or in cell cultures (Swayne *et al*. 2008; Cattoli and Terregino 2008). However, the method is time consuming, not cost-effective, requires expertise and SPF eggs may not always be available. Similarly, handling of the samples should be done with precautions even though there is only one documented case of AI laboratory-acquired infection (Alexander 2006). Thus,

biosafety and biocontainement should be seriously considered due to the emerging zoonotic potential of the virus. In spite of the above disadvantages, VI in SPF eggs still remains the gold standard diagnostic method because of its superior sensitivity (Cattoli and Terregino 2008). Moreover, antigenic variations of the virus and the presence of contaminants or PCR inhibitors may not affect VI but immunoassays and other molecular techniques can be significantly affected (Cattoli and Terregino 2008).

Conventional diagnosis includes virus isolation, virus identification and assessment of pathogenicity (Alexander 2008). Tracheal or cloacal swab suspensions from live birds or tissue samples from dead birds are used for virus isolation (Swayne *et al*. 2008; Alexander 2008). Samples are inoculated into the chorioallantoic sac of 9-11 day old embryonating chicken eggs and incubated at 35–37°C for 4–7 days (Swayne *et al.* 2008; Alexander 2008). Sometimes inoculation either into the yolk sac or onto the chorioallantoic membrane of embryonating chicken eggs may be needed (Woolcock 2001). Chorioallantoic fluid from eggs with dead or dying embryos is harvested and tested for the presence of hemagglutinating antigen (Swayne *et al*. 2008; Alexander 2008).

The HA on AIV interacts with receptors on the surface of red blood cells (RBCs) causing agglutination (Swayne *et al.* 2008). Agglutination of RBCs is the basis of the hemagglutination assay. If chorioallantoic fluid is hemagglutination-positive, it may indicate AI virus but other viruses can also agglutinate RBCs (Newcastle disease virus) so the presence of the AI virus should be confirmed by the immunodiffusion test or other suitable test. The immunodiffusion test uses concentrated virus and antiserum to the nucleocapsid or matrix proteins common to all influenza A viruses (Alexander 2008). The presence of NDV can be evaluated by the hemagglutination-inhibition (HI) test (Swayne *et al*. 2008; Alexander 2008).

Subtype identification of influenza A viruses is usually done with the HI test (Alexander 2008). Inhibition of the agglutination reaction by HA subtype-specific antiserum is the basis of the HI test. Polyclonal chicken antiserum raised against the 16 distinct HA subtypes is used in the HI assay to confirm the HA subtype. Typing is also done using antiserum raised against the different NA subtypes of AI viruses to avoid false-positive reactions (Pedersen 2008; Alexander 2008). However, subtyping is labor intensive and requires large stocks of antiserum. The HI test can also be used to detect and quantitate HA subtype-specific antibodies, which may be detected as early as 7 days, in serum or yolk following infection or vaccination (Swayne *et al.* 2008).

For assessment of pathogenicity, fresh infective allantoic fluid with a HA titer greater than 1/16 is diluted 1/10 in sterile isotonic saline and 0.1 ml is injected intravenously into each of ten 4-8-week old SPF chickens (Alexander 2008; Swayne *et al*. 2008). Birds are examined for 10 days at 24 h interval and scored based on the condition of the birds; normal (0), sick (1), severely sick (2) or dead (3). The IVPI is the mean score per bird per observation (total of all the individual chicken scores divided by the total number of observations) over the 10-day period. IVPI of 3.00 means that all birds died within 24 h, an index of 0.00 means that none of them showed any clinical sign during the 10 days (Alexander 2008). Isolates that are lethal for more than 75% of birds or have an IVPI score of more than 1.2 are considered HPAI (Swayne *et al*. 2008). If the isolate is H5 or H7, amino acid sequence at the cleavage site of HA will be determined. H5 or H7 isolates having an amino acid sequence at the HA cleavage site compatible with HPAI virus are also considered HPAI even if mortality of more than 75% of birds does not occur (Swayne *et al*. 2008).

Even though virus isolation remains the method of choice for the diagnosis of AI, a number of different techniques including molecular methods and antigen detection methods are now routinely used for the detection of AI.

***Immunoassay tests***

Immunoassays are based on antigen capture using monoclonal antibodies. For example Directigen Flu A kit (Becton Dickinson Microbiology Systems), used for the detection of influenza A viruses in poultry uses a monoclonal antibody against the nucleoprotein and hence can be used to detect any influenza A virus (Alexander 2008; Cattoli and Terregino 2008). The main advantage of antigen detection kits are that they provide results in minutes and do not require sophisticated instruments or expertise and hence are desirable when a large number of samples need to be tested in a short time (Lu 2006; Cattoli and Terregino 2008; Alexander 2008). However, the main disadvantage with immunoassay tests is their low sensitivity when compared to VI or molecular methods. They do not indicate the subtype or pathotype involved and may not be able to identify new strains (Lu 2006; Cattoli and Terregino 2008; Alexander 2008). Because of their low sensitivity, large number of samples need to be tested which makes them unsuitable for early detection programs (Cattoli and Terregino 2008).

***RNA detection***

Tracheal, oropharyngeal or cloacal swabs are preferably used as the specimen of choice for detection of viral RNA in gallinacaeceous birds (Swayne and Halvorson 2008; Swayne *et al*. 2008). Reverse transcriptase procedure is used for the detection of AI in clinical samples for which sensitivity is comparable to the virus isolation method (Swayne *et al*. 2008). For this procedure, RNA is isolated from the samples and converted to cDNA using reverse transcriptase which is then amplified using PCR and evaluated for the presence of target DNA sequences.

Both RT-PCR and real time RT-PCR (RRT-PCR) have been used for detection and both have their own advantages and disadvantages (Swayne *et al*. 2008). Whereas RT-PCR is less expensive, it requires more labor and has the problem of cross contamination. RRT-PCR is rapid, less labor intensive and has a lower chance of cross contamination, but it is more expensive. Confirmation of RT-PCR product is done by DNA sequencing, restriction enzyme endonuclease assays or Southern Blot hybridization. In RRT-PCR, the analysis of the amplified product is done with fluorescently labeled probes such as Taqman probes or dyes and special thermocyclers that measure signal levels, which is proportional to the amount of viral RNA in the reaction (Swayne *et al*. 2008).

Horimoto and Kawaoka (1995) first reported the use of direct RT-PCR for the diagnosis of avian influenza using the HA cleavage site sequence, a marker for the virulence potential of avian influenza viruses. On experimentally infected chickens with either virulent or avirulent virus, RT-PCR detected the HA gene, even when specimens were negative by conventional virus isolation in eggs. Moreover, the total time taken by RT-PCR was only 3 days whereas the standard procedure needed for the VI test is 4-7 days (Horimito and Kawaoka 1995). Later, identification and HA-subtyping of avian influenza virus by RT-PCR was done using a set of primers specific to the NP gene of avian influenza virus followed by running simultaneously 15 RT-PCR reactions, each using a set of primers specific to each of the HA subtypes 1 through 15 (Lee *et al*. 2001). Similarly, a one tube NP RT-PCR assay along with the use of primers for amplifying the HA cleavage site for the subtypes H5 and H7 have been described (Munch *et al*. 2001). In addition to HA subtyping, RT-PCR can also be used for NA subtyping (Alvarez *et al*. 2008; Chander *et al*. 2010; Fereidouni *et al*. 2009). However, RRT-PCR is advantageous over RT-PCR in that it is more rapid and could avoid cross contamination of new samples with

previously amplified products (Spackman *et al*. 2002). Real time RT-PCR (RRT- PCR) is also advantageous over VI due to the speed of detection even though the sensitivity is lower than VI. The results of AIV detection and subtyping by RRT-PCR can be obtained in 1 day whereas it may take many days with VI (Spackman *et al*. 2002). Moreover, since the virus is inactivated during RNA extraction, handling of potentially infectious material can be reduced (Spackman *et al*. 2002).

Real time RT-PCR was developed for the detection of influenza A virus using a primer/probe set designed for a region conserved in the matrix gene in all type A influenza viruses and also for the detection of avian H5 and H7 HA subtypes using H5 and H7-specific primer and probe sets based on North American avian influenza virus sequences (Spackman *et al*. 2002; Spackman *et al.* 2003). These assays were comparable to VI and HI in sensitivity since 94% of the samples that were VI positive and 97% that were H7 positive by HI were also positive by RRT-PCR (Spackman *et al*. 2002). However, there is not 100% agreement in the results between RRT-PCR and VI (Elvinger *et al*. 2007). A few VI positive samples can be RRT-PCR negative and vice versa indicating that VI may not be 100% sensitive even though the sensitivity of VI is higher than RRT-PCR (Elvinger *et al*. 2007). The sensitivity of the RRT-PCR relative to VI is 85.1% while the specificity relative to VI was 98.9% (Elvinger *et al*. 2007).

Since the original H5 assay used mainly North-American lineage AIVs (Spackman *et al.* 2002), some modifications have been made to the original H5 assay for the detection of Eurasian H5N1 and Asian lineage H5N1 HPAI which are distinct from American lineages for both H5 and H7 AIVs (Slomka *et al*. 2007). Similarly, a RRT-PCR assay able to detect H5, H7, and H9 subtypes of Eurasian and African lineages have been developed (Monne *et al*. 2008). Recently, three specific and sensitive RRT-PCR assays were developed for detecting type A influenza

virus and for subtyping all avian H5 and H7 hemagglutinin subtypes (Sidoti *et al*. 2010). For detecting H5 and H7 subtypes in a single analytical session, these RRT-PCR assays were designed using the same annealing temperature of 60°C. Similarly, RRT-PCR has also been developed for the detection of N1 gene from AIV (Aguero *et al*. 2007).

***Multiplex PCR for diagnosis***

Multiplex PCR is a PCR test in which simultaneous amplification of many targets of interest can be done in one reaction/one tube using more than one pair of primers (Elnifro *et al*. 2000). This method has been applied in many areas of nucleic acid diagnostics, microsatellite analysis and detection of pathogens (Elnifro *et al*. 2000). However, it is expensive and time consuming and requires expertise. One of the problems with multiplex PCR is the poor sensitivity and specificity due to the presence of more than one primer in the reaction mixture which may give rise to non-specific amplification and the formation of primer dimers (Elnifro *et al*. 2000). Hence designing optimal primer combinations through empirical testing and trial and error methods can be time consuming. In addition, special attention to various primer design parameters needs to be considered when developing multiplex PCR tests. Despite these disadvantages, multiplex PCR has been a valuable diagnostic technique for the identification of many infectious diseases.

The use of multiplex RT-PCR for simultaneous detection of many avian respiratory diseases has been reported previously (Pang *et al*. 2002; Malik *et al*. 2004). A single step multiplex RT-PCR has been used for the detection of influenza A virus subtype H5N1 using primer set specific for M, H5 and N1 (Payungporn *et al*. 2004) or by using primer sets specific for NP, H5 and N1 (Wei *et al*. 2006). Similarly, this method was developed and optimized for simultaneous detection of avian H5 and H7 using two subtype-specific primer sets corresponding

to any AIV type A virus, H5 and H7 subtypes (Thontiravong *et al*. 2007) and for simultaneous detection of H5, H7 and H9 hemagglutinin subtypes using four sets of specific oligonucleotide primers for any type A influenza virus, H5, H7 and H9 hemagglutinin subtypes (Xie *et al*. 2006; Chaharaein *et al*. 2009). Whereas the common primer set for type A influenza virus was able to amplify all subtypes of AIV, the primers for H5, H7 and H9 hemagglutinin subtypes were specific for the subtypes (Xie *et al*. 2006; Chaharaein *et al*. 2009). Another assay specific for eight genomic segments of the currently circulating H5N1 viruses was developed (Auewarakul *et al*. 2007) using primer sets against ,PB1, PB2, PA, NP , HA ,NA, NS and M and the multiplex RT-PCR was carried out in three tubes with combination of primer sets. Even though this assay could theoretically be useful for surveillance of virus resulting from reassortment between human influenza virus and the avian H5N1 virus, the level of sensitivity was not high enough to detect all the genomic segments simultaneously, making it less suitable for direct screening of clinical specimens (Auewarakul *et al*. 2007). Recently, multiplex RT-PCR was developed for typing of influenza A and B virus, and subtyping of influenza A virus into H1, 2, 3, 5, 7, 9, N1 (human), N1 (animal), N2, and N7, which also exhibited excellent analytical sensitivity (He *et al*. 2009)

For the rapid, simultaneous detection of the H5 and H7 avian influenza hemagglutinin (HA) subtypes, multiplex real-time RT-PCR (mRRT-PCR) was developed with hydrolysis type probes labeled with the FAM (H5 probe) and ROX (H7 probe) reporter dyes (Spackman *et al*. 2003). Later, the method was extended using triple fluorescent reporters FAM, VIC and NED to detect M, H5 and N1 genes of HPAI H5N1 simultaneously (Payungporn *et al*. 2006). High specificity for the assay was also reported since there was no cross reaction to human genomic DNA, Newcastle disease virus, respiratory syncytial virus, infectious bursal disease virus or

infectious bronchitis virus. Similarly, there was no cross reaction to other subtypes of influenza A virus (Payungporn *et al*. 2006). The test can also be used for the simultaneous detection and subtyping of H9N2 (Ong *et al*. 2007), influenza Type A and B and subtypes H5 and N1 (Wu *et al*. 2008) and influenza Type A H5 and H9 subtypes (Li *et al*. 2008). Multiplex RRT-PCR for universal detection of influenza A viruses and simultaneous typing of influenza A/H1N1/2009 has also been reported (Gunson *et al*. 2010).

***Microsphere based assays***

Microsphere-based suspension array technologies allow high-throughput detection and quantification of both proteins and nucleic acids, which can be used in a variety of applications (Dunbar 2006; Adams and Thompson 2008; Tait *et al*. 2009). It is rapid, has excellent sensitivity and specificity and has the capability for multiplexed analysis. Its low cost, ease of use, statistical superiority and faster hybridization kinetics makes it more attractive than planar microarrays.

The Luminex xMAP system is a microsphere based suspension array technology, in which up to 100 different reactions can be analyzed in a single reaction, using small polystyrene microspheres containing two spectrally distinct internal fluorochrome dyes (Dunbar 2006; Adams and Thompson 2008). By adjusting the amounts of each of the fluorochromes, an array is created consisting of 100 different sets with specific spectral addresses that can be individually identified by a laser within the analyzer. Since each microsphere set is distinct with their specific spectral addresses, the beads can be coated with different fragments of nucleic acids or proteins and can be combined allowing all the 100 different sets to be measured simultaneously. A third fluorochrome, coupled with reporter molecules like *R*-phycoerythrin, Alexa 532, or Cy3, is reacted with the bead and the biomolecular interaction is quantified using lasers. Two lasers in the Luminex machine identify Microspheres individually. The first laser excites the two

fluorochromes in the microspheres while the second laser excites the reporter fluorochrome bound to the microspheres. Thus the system classifies the microspheres based on their spectral addresses. Luminex xMAP technology has been used in the recent years for both clinical research and diagnostic purposes.

Luminex xMAP technology can be used for single nucleotide polymorphism genotyping, screening of genetic diseases, gene expression profiling, HLA DNA typing and microbial detection (Dunbar 2006). It can be used to detect various microorganisms including bacteria, viruses and fungi. Rapid detection of many fungal pathogens including *Trichosporon* (Diaz and Fell 2004), *Candida* species (Das *et al*. 2006), *Cryptosporidium hominis* and *C. parvum* (Bandyopadhyay *et al*. 2007) and *Asperigillus fumigatus* (Etienne *et al*. 2009 a and b) has been reported using this assay system.

Dunbar *et al*. (2003) reported that Luminex LabMAP system could be used for the simultaneous detection of *Escherichia coli*, *Salmonella*, *Listeria monocytogenes* and *Campylobacter jejuni*. Organism-specific capture probes were coupled to the microspheres. Variable regions of bacterial 2S ribosomal DNA were amplified using universal primers and the varying quantities of targets were hybridized to the microsphere sets. The assay could be completed in 30-40 min post-amplification with a detection limit of $10^6$ to $10^7$ genome equivalents in the hybridization reaction. As few as $10^3$ genome copies in PCR amplification reactions were sufficient for the detection of *E. coli*, *L. monocytogenes* and *C. jejuni*. For Salmonella, $10^5$ genome copies were required. Thus, LabMAP system can be used for rapid, simultaneous, multiplexed detection of DNA from these pathogens. Similarly, *Salmonella* O-group-specific Bio-Plex assay based on *rfb* gene, which can be completed 45 min post-amplification, could be used for the detection of six common serogroups in the United States and

serotype Paratyphi A (Fitzgerald *et al*. 2007). It can also be used in the laboratory diagnosis of bacterial vaginosis, a recurrent bacterial infection characterized by an increase in anaerobic and gram negative organisms including *Gardenella vaginalis* and *Atopobium vaginae* (Dumonceaux *et al*. 2009). The Luminex system has the potential to be used in water quality applications since the Luminex probes can detect DNA from multiple fecal indicating bacteria in environmental samples (Baums *et al*. 2007).

Viral nucleic acids can be detected and quantified using a rapid multiplexed format based on fluorescent detection using FlowMetrix analysis system (Defoort *et al*. 2000; Smith *et al*. 1998; Hindson *et al*. 2008). Oligonucleotide probes specific for human immunodeficiency virus (HIV) and hepatitis C virus (HCV) RNAs and herpes simplex virus (HSV) DNA that were coupled individually to six microsphere sets and mixed to form a multiplexed set could be detected by FlowMetrix system in a rapid and specific manner (Smith *et al.* 1998). Similarly, using multiplex RT-PCR and flow cytometer microsphere-based hybridization assays, quantitation and detection of HIV, HCV and hepatitis B virus (HBV) have been reported (Defoort *et al*. 2000). Multiplexed RT-PCR microsphere assay can also be used for the simultaneous detection of multiple human respiratory pathogens in a rapid, sensitive and specific manner (Letant *et al*. 2007; Lee *et al*. 2007) and to differentiate foot and mouth disease virus from other vesicular diseases like swine vesicular disease virus and vesicular exanthema virus (Hindson *et al*. 2008).

Microsphere immunoassays have also been used to detect serum antibodies to avian influenza virus (Deregt *et al*. 2006). Recombinant influenza A nucleoprotein expressed in baculovirus, conjugated to microspheres and incubated with serum was found to be useful for surveillance and screening of poultry affected with LPAI. Similarly, a triplex bead assay can be

used to detect antibodies against avian influenza by expressing recombinant nucleoprotein, matrix protein and non-structural protein 1 using a baculovirus expression system coupled to fluorescent xMAP microspheres (Watson *et a*l. 2009).

***Serological assays***

Serological assays like agar-gel immunodiffusion (AGID), ELISA, HI and NI are widely used tests for early detection, surveillance and disease control efforts in domestic poultry species and are based on the presence of influenza specific antibodies that first appear around 2 weeks and reach peak levels at 4–7 weeks after initial infection (Swayne *et al*. 2008; Swayne and Halvorson 2008). AGID is the most preferred test and it detects antibodies against nucleoprotein and matrix protein, which are common to all Type A influenza viruses (Swayne *et al*. 2008; Swayne and Halvorson 2008). However, it is only moderately sensitive to gallinaceous poultry and does not produce consistent results with some species like ducks (Brown *et al*. 2009; Spackman *et al.* 2009).

ELISA assays detect antibodies to AI viruses and commercial ELISA kits are available to detect antibodies in serum or chicken egg yolk (Swayne *et al*. 2008; Swayne and Holvorson 2008; Spackman *et al*. 2009). They can be faster and more sensitive than AGID. However, specificity can be poor and can give false positive results. Moreover, commercially available kits are specific to chickens and turkeys and hence may not be useful in other species (Spackman *et al*. 2009). Development of competitive ELISA can result in a single test for all avian species (Shafer *et al*. 1998; Zhou *et al*. 1998; Song *et al*. 2009) and is useful in species where agar-gel immunodiffusion is ineffective due to lack of precipitating antibody.

HI test is useful for identification of antibodies to HA subtype of Al virus in serum or egg yolk specimen by using a panel of viruses belonging to all 16 HA subtypes (Swayne *et al*. 2008:

Spackman *et al*. 2009). HI test may not be very useful in the initial screening of birds. NI test helps in differentiating AI on the basis of the antigenic character of NA.

***Recent trends in the diagnosis of AI***

Isothermal amplification methods can be useful in the diagnosis of influenza. They are similar to conventional PCR in that specifically designed primers are used to amplify target nucleic acid sequence. However, it differs in that the amplification occurs at a single temperature and hence does not require thermocyclers and gel electrophoresis (Charlton *et al*. 2009). Loop-mediated isothermal amplification or LAMP utilizes four primers and a DNA polymerase with high strand displacement activity which ensures high speed and high specificity for target amplification due to target DNA recognition by 6 independent sequences (Pasick 2008). Reverse transcriptase-LAMP assay can be used for detection of influenza viruses with high specificity by including a heat-stable reverse transcriptase in the isothermal reaction. (Imai *et al*. 2007; Jayawardena *et al*. 2007; Belak *et al*. 2009).

Another isothermal amplification method is nucleic acid sequence-based amplification (NASBA) which is a transcription-based method specifically designed for the detection of RNA targets (Deiman *et al*. 2007; Pasick 2008) and can be used for the detection of influenza A virus with high specificity (Collins *et al.* 2002; Lau *et al*. 2004).

Many microarray-based assays can be used for detection and subtyping influenza A virus isolates. The assays use immobilized capture oligonucleotides and have the ability to simultaneously test for thousands of different nucleotide sequences (Charlton *et al*. 2009). RT-PCR amplification of influenza RNA is required before hybridizing with capture oligonucleotides immobilized on the microarray (Pasick 2008). Microarray that have employed low density formats with 15 distinct oligonucleotides designed to target Matrix gene sequences,

which shows slow sequence evolution over time, could accurately subtype H1N1, H3N2, and H5N1 influenza A viruses in less than 12 h (Townsend *et al*. 2006; Dawson *et al.* 2006; Charlton *et al*. 2009). Clinical sensitivity of 97% and a clinical specificity of 100% was obtained when 43 different highly pathogenic A/H5N1 viral isolates where analyzed for detection and subtyping (Dawson *et al*. 2007). The clinical sensitivity and specificity are comparable to that of viral culture and RT-PCR and requires only 7-8 h to complete the analysis (Mehlmann *et al*. 2007). Recently, it was reported that all the subtypes of the influenza A virus could be identified simultaneously by DNA microarrays with high sensitivity (Han *et al*. 2008). Microarray for both HA (Gall *et al*. 2009a) and NA subtyping (Gall *et al*. 2009b) has been developed. Microarrays can also be used for simultaneous detection and differentiation of New Castle disease virus and avian influenza virus, which often display similar signs (Wang *et al*. 2008).

Fluorescent DNA barcode-based immunoassay (Cao *et al*. 2010), pyrosequencing, molecular pathotyping, linear-after-the-exponential polymerase chain reaction (LATE PCR), light upon extension polymerase chain reaction, proximity ligation assay, nanotechnology, liquid-phase microarrays using luminex technology etc. are some of the other technologies that have the potential to be used for the diagnosis of avian influenza in the future (Belak *et al*.2009).

**Vaccination**

Even though inactivated whole AI virus vaccine is effective in preventing clinical signs and mortality, the vaccines are virus-subtype specific and thus, the use of all 16 subtypes as vaccines is not very practical (Swayne and Holvorson 2008). Moreover, it can hide the clinical signs of AI and thus may interfere with the rapid diagnosis of HPAI (van den Berg *et al*. 2008; Capua and Alexander 2008). However, vaccination has been used as a routine management tool and also for preventive and emergency purposes (van den Berg *et al*. 2008; Capua and Alexander

2008). Major types of vaccines used are inactivated whole AI virus, recombinant live virus vectors, subunit AI proteins and naked DNA vaccines (Swayne and Kapczynski 2008; van den Berg *et al*. 2008)

**Treatment**

Antiviral drugs**,** amantadine and rimantadine are currently licensed for prophylactic and therapeutic use against influenza in humans (Wright *et al*. 2007), but are not approved for food animals (Swayne and Halvorson 2008). Neuraminidase inhibitors like oseltamivir have also been approved for use in humans (Wright *et al*. 2007). Currently, no specific treatment against AI virus exists in poultry (Swayne and Halvorson 2008).

## MATERIALS AND METHODS

### Viruses

Avian Influenza H5N1 (A/Black Duck/NC/674-964/06) and Avian Influenza H7N3Mallard duck (A/ Mallard Duck/ MN/A 107 -3431/2007) were propagated in 10 day old embryonating eggs. The 50% embryo infectious dose titer ($EID_{50}$) was calculated by Reed and Muench method. The viruses used in this study are listed in Table 1.

### RNA Extraction, RT-PCR amplification and cloning

Viral RNA was extracted using the High Pure RNA isolation kit (Roche Applied Science, Indianapolis, IN) and the protocol for isolation of total RNA from cultured cells was followed. The RNA was eluted in 50µl of elution buffer. A portion of the influenza virus matrix (M), H5, and H7 genes were amplified using the primers listed in Table 2 (Spackman *et al.* 2002; Spackman *et al.* 2008). The primers were synthesized by Integrated DNA Technologies (Novato, CA). A one step RT-PCR was performed using Titan one tube RT-PCR system (Roche Applied Science, Indianapolis, IN). Amplification was done in 50 µl reactions containing 10 µl of RT-PCR buffer, 10 mM of each dNTPs, 12 pmoles of each primer, 1.5 µl $MgCl_2$, 2.5 µl of DTT, 1 µl of Taq DNA polymerase and 2 µl of viral RNA. The thermocycler conditions were $42^0$C for 60 minutes and $95^0$C for 5 minutes for the RT step. Amplification of the cDNA was completed by 35 cycles of $94^0$C for 30 seconds, $50^0$C for 30 seconds and $68^0$C for 30 seconds. Reactions were performed in DNA Engine, Peltier Thermal Cycler (BIORAD). In the negative control, nuclease free water (IDTE) was used instead of template RNA. The RT-PCR products were electrophoresed on 1% agarose gel and the amplicons were excised and purified using Qiagen

gel extraction kit (Qiagen, Valencia, CA). The amplification products were cloned into the pCR-XL-TOPO vector (Invitrogen Life Technologies, Rockville, MD) following the manufacturer's recommendations. Transformation of TOP10 electrocompetent cells (Invitrogen Life Technologies, Rockville, MD) were performed with the vector and the cells were grown in SOC media for an hour at $37^0$C and plated on LB-kanamycin agar plates. The plates were incubated at $37^0$C overnight. Colonies were grown in LB-Kanamycin media and plasmid DNA was purified from bacterial cultures using the Qiagen Miniprep kit (Qiagen, Valencia, CA) and the accuracy of the inserts was verified by sequencing using M13 Forward primer and the BigDye Terminator v3.1 cycle sequencing kit and ABI 3730 sequencer (Applied Biosystems) as per manufacturer's protocol.

**In vitro transcription**

The plasmids with M, H5 and H7 gene inserts were used to generate runoff RNA transcripts with the Megashortscript kit (Ambion, Austin, TX) using the T7 promoter as per the kit instructions. The plasmids with the gene inserts were linearized with Spe I restriction enzyme (NEB, Beverly, MA). The linearized plasmids were gel purified and used as template for the transcription reactions. The transcripts were purified using the Megaclear purification kit (Ambion) and RNA concentration was determined by spectrophotometer analysis.

**Amplification of Neuraminidase N1 and N2**

RNA was extracted using High Pure RNA isolation kit (Roche Applied Science, Indianapolis, IN) as per the protocol described previously. Amplification of a portion of neuraminidase gene was performed with NA primers listed in Table 2 using Titan One Tube RT-PCR system (Roche Applied Science, Indianapolis, IN). The primers were synthesized by Integrated DNA Technologies (Novato, CA). Amplification was done in 50 µl reactions

containing 10 µl of RT-PCR buffer, 10 mM of each dNTPs, 12 pmoles each of forward and reverse primer, 1.5 µl $MgCl_2$, 2.5 µl of DTT, 1 µl of Taq DNA polymerase and 2 µl of viral RNA. The thermocycler conditions were $50^0$ C for 30 minutes for reverse transcription step followed by denaturation at $95^0$ C for 15 minutes. Amplification was completed by 35 cycles of $94^0$C for 1 minute, $53^0$C for I minute and $72^0$C for 1 minute followed by a final extension step at $72^0$C for 10 minutes (Chander *et al*. 2010). Reactions were carried out in DNA Engine (BIORAD, CA). The amplified product was electrophoresed on 1% agarose gel and purified from the gel using Qiagen gel extraction kit (Qiagen, Valencia, CA). The accuracy of the amplicons was verified by sequencing using the primers used for amplification and the BigDye Terminator v3.1 cycle sequencing kit and ABI 3730 sequencer (Applied Biosystems) as per manufacturer's protocol.

**Strand displacement amplification**

Total RNA extracted from H5N1 and H7N3 virus samples were amplified in a two-step strand-displacement RT-PCR with the TAKARA kit (TAKARA BIO Inc., Otsu, Japan) as per the manufacturer's protocol. The reverse transcription was done using the Genome 5' degenerate primer (5' AGC GGG GGT TGT CGA ATG TTT GAN NNN N-3'). The thermocycler conditions for the RT step were $65^0$C for 10 minutes, $30^0$C for 10 minutes, $42^0$C for 60 minutes, $99^0$C for 5 minutes and $5^0$C for 5 minutes. The cDNA was amplified using the biotinylated Genome 5' primer (5'-/5Biosq/AGC GGG GGT TGT CGA ATG TTT GA-3'). The PCR conditions were $94^0$C for 2 minutes followed by 30 cycles of $94^0$C for 30 seconds, $60^0$C for 30 seconds and $72^0$C for 3 minutes. Reactions were carried out in DNA Engine (BIORAD, CA). The amplified product was concentrated and purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA).The eluted DNA was electrophoresed on 1% agarose gel and purified

from the gel using Qiagen gel extraction kit (Qiagen, Valencia, CA ). The purified cDNA was used in cloning and transformation reactions as described above. The cells in SOC media were used for plating LB-Kanamycin agar Q-trays and were sent to the Plant Genome Mapping Laboratory at the University of Georgia for sequencing. Plasmid DNA from the libraries of cloned cDNA fragments for each virus was isolated using an alkaline lysis method modified for the 96-well format, and incorporating both Hydra and Tomtek robots (http://www.intl-pag.org/11/abstracts/P2c_P116_XI.html). Cycle sequencing reactions were performed using the BigDye™ Terminator® Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA) and MJ Research (Watertown, MA) thermocyclers. Finished reactions were filtered through Sephadex filter plates into Perkin-Elmer MicroAmp Optical 96-well plates. A 1/12-strength sequencing reaction on an ABI 3730 was used to sequence each clone from both the 5' and 3' ends. The sequences were compiled using the SeqMan and EditSeq program (DNASTAR, Inc. Madison, WI). The BlastX program (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) for homologous sequences. 691 sequences were analyzed and the percentage of different influenza genes among the clones was determined.

**Multiplex RT-PCR**

Multiplex RT-PCR was performed using Titan one tube RT-PCR kit, and 3pmoles of M +25 and M –124 primers, 6pmoles of H5 +1456 and H5 – 1685 primers and 12pmoles of H7 PanAm F1515 and H7 PanAmR1628 primers were used in a 50µl reaction. The three reverse primers were biotinylated. The concentration of reagents and thermocycler conditions were kept exactly similar to that of singleplex RT-PCR.

The multiplex RT-PCR of neuraminidase N1 and N2 was performed in a separate reaction. The concentration of reagents and thermocycler conditions were kept exactly similar to the singleplex reaction and equal concentration (12 pmoles) of all the four primers were used for multiplex amplification.

**Design of Neuraminidase probes**

Probes were designed for regions conserved in N1 and N2 neuraminidase genes. The sequences of viruses used in evaluating the N1 and N2 subtype specific primers (Chander *et al*.2010) were aligned using the ClustalW in DNASTAR (Lasergene 8, Madison, WI) and regions specific to each subtype were identified for probe design. 20 to 25 base pair regions were selected and BLAST analysis was performed to verify the specificity. The melting temperature, stability and other factors were evaluated using the PrimerSelect program in DNASTAR.

**Coupling of probes to microspheres**

Oligonucleotide probes based on matrix, H5, H7, N1 and N2 genes were synthesized with an amino-modified 6-carbon spacer at the 5' end of each probe. The sequence of the previously published probes are M +64 5'-/5AmMC6/TCA GGC CCC CTC AAA GCC GA –3'(Spackman *et al.*, 2002), H5 Probe IY 5'-/5AmMC6/TCA ACA GTI GCG AGT TCY CTA GCA –3'(D.Suarez*,* Southeast Poultry Research Laboratory, Athens, Georgia, Personal communication) , and H7 PanAm F1585 5'-/5AmMC6/ CAG ATA GAC CCA GTG AAA TTG AGT-3'(Spackman *et al.*, 2008). We designed the NA N1 Probe 5'-/5AmMC6/AAC YTT CTT TCT AAC TCA RGG GGC-3' and NA N2 Probe 675 5'/5AmMC6/CTC AGA ACT CAG GAG TCA GAG TGC G-3' from alignments of those genes using the MegAlign program in DNASTAR (Lasergene, Madison WI). Carbodimide coupling was used to covalently attach the probes to carboxylated microspheres as per the protocol previously described (Fitzgerald *et al.*

2007). Briefly, 400µl of uncoupled microspheres (BIORAD, Hercules, CA) were pelleted and resuspended in 45 µl of 0.1M MES, pH 4.5 (Teknova, Bert Drive Hollister, CA) by vortexing. One nmol of probe was added to the microspheres and mixed by vortexing. 2.5 µl of freshly prepared 10mg/ml solution of EDC (Pierce, Rockford, IL) was added to the microspheres and vortexed and the microsphere solution was incubated at room temperature in the dark with shaking for 30min. The EDC addition and incubation steps were repeated with another fresh aliquot of EDC. To wash the microspheres, 1ml of 0.02% Tween20 (Sigma, St. Louis, MO) was added to the solution, vortexed and spun for 2min at 13000 rpm and the supernatant was discarded. The microspheres were again washed with 1ml of 0.1% SDS (Sigma, St. Louis, MO). The microspheres were resuspended in 80 µl of TE buffer, enumerated using a hemocytometer and stored at $4^0$C in the dark.

**Evaluation of coupling reaction**

To confirm the coupling of probes to the microspheres, biotinylated oligonucleotides complementary to the probes were synthesized and a coupling confirmation assay was performed. The coupled microspheres were resuspended and a working solution of 150 microsheres/µl in 1.5 X Tetra Methyl Ammonium Chloride (TMAC) (Sigma, St. Louis, MO) hybridization solution was prepared. A 10 femtomol/µl solution of the biotinylated reverse complement of probes were prepared. Then 33 µl of the microsphere solution was mixed with different concentrations of the biotinylated reverse complement of probes and TE buffer to bring the final volume to 50 µl. Negative controls were prepared with the microsphere solution and TE buffer. The mixtures were incubated in a thermocycler at $95^o$C for 5 minutes for denaturation followed by hybridization at $55^o$C for 15 minutes. After incubation, the microspheres were pelleted by centrifugation at 2500rpm for 3 min and the supernatant was removed. The microspheres were

resuspended in 75 µl of detection buffer, which contained 3 µg/ml of streptavidin-R-phycoerythrin, (Invitrogen Molecular Probes, Eugene, OR) in 1X TMAC hybridization solution and incubated the suspension at room temperature for 5 min. Then 50 µl of the reaction was analyzed on the Bioplex machine (Bio-Rad, Hercules, CA) according to the system manual. The analyzer was set to measure the fluorescence for a minimum of 100 microspheres of each set in the reaction. The median fluorescence intensity (MFI) for each microsphere set was automatically calculated and reported by the Bioplex Manager 5.0 software (Bio-Rad, Hercules, CA). A MFI of at least twice that of a negative control with all components of reaction except oligonucleotide was considered as positive and a standard curve was generated to compare the coupling efficiency of different microsphere sets.

**Microsphere assay**

Each of the influenza M, H5, H7, N1 and N2 amplified products (5ul) from a singleplex or multiplex RT-PCR reaction were added to 33 µl of microsphere solution and 12 µl of TE buffer in a single well of a 96 well plate. The reactions were kept at $95^0$C for 10 minutes for denaturation followed by hybridization at $50^0$C for 30 minutes. The assay was performed as described for coupling confirmation and the MFI was calculated. A MFI of at least twice that of background was considered to be positive.

**Multiplex microsphere assay**

We designed and optimized a multiplex microsphere assay by mixing all the five sets of microspheres in the hybridization buffer. The working solution had 150 microspheres of each set/µl. In a single reaction, 33µl of microsphere working solution, 5µl each of post RT-PCR product from the two separate multiplex RT-PCRs and 7µl TE buffer were used and the assay was performed as described above. The MFI was reported for each set of microspheres.

**Sensitivity and limit of detection of the assay**

The sensitivity of the microsphere assay was determined using ten fold serial dilutions of the viruses. RNA was extracted from each dilution using the High Pure RNA isolation kit (Roche), reverse transcribed and amplified by either singleplex RT-PCR in a smartcycler or multiplex RT-PCR using the Titan one step RT-PCR kit as previously described. Then 5µl of the RT-PCR product without any purification was used in the microsphere assay described above. The reaction was analyzed using the Bioplex (Bio-Rad, Hercules, CA) machine and the lowest dilution of the virus giving a positive MFI was identified.

The limits of detection of the probe coupled microspheres for biotinylated amplicons were determined using 2 fold serial dilutions of purified RT-PCR product. The templates were amplified using the specific primer sets (Table 2) with the reverse primer biotinylated at the 5' end. The RT-PCR product was gel purified and the DNA concentration was determined by spectrophotometer. Microsphere assays were conducted and the limit of detection was estimated as the lowest dilution giving positive MFI.

**Specificity of the assay**

To test specificity, singleplex or multiplex RT-PCR and microsphere assays were performed with RNA templates from known positive samples of different influenza subtypes as well as with other avian respiratory tract viruses. RNA was extracted from the samples, reverse transcribed, and amplified in a singleplex or multiplex reaction and the 5µl of RT-PCR product was used in the microsphere assay described above. The specificity of the probe coupled microspheres was tested with both singleplex and multiplex microsphere assays.

**Real Time RT-PCR**

Real time RT-PCR assays were performed as previously described (Spackman *et al.*, 2008;

Spackman *et al.*, 2002). RNA extracted with MagMax 96 total RNA isolation kit (Ambion, Austin, TX) from the samples using KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA)was used as templates along with the specific primers and probes in a 25 µl real time RT-PCR reaction in a Smart Cycler (Cepheid, Sunnyvale, CA).

**Validation of the assay**

We used 102 oropharyngeal or cloacal samples from a previous study to validate the assay. We tested 60 samples from H6N2 infected birds and 42 samples from H9N2 infected birds. RNA was extracted from the samples using MagMax 96 total RNA isolation kit (Ambion, Austin, TX) and KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) as per the manufacturer's recommendations. Two separate multiplex RT-PCRs were performed as described above and 5µl of each RT –PCR product was used in a fiveplex microsphere assay and the MFI was calculated for each set of microspheres. Real time RT –PCR using matrix primers and probe was performed in parallel to determine the false negatives and false positives from the microsphere assay. The false negative samples from the microsphere assay were again tested in a singleplex microsphere assay using M probe after amplification using M primers in the Smartcycler.

Table 1. Viruses used in this study.

| **Virus designation** |
| --- |
| Influenza A Virus H5N1(A/Black Duck/NC/674-964/06) |
| Influenza A Virus H7N3 (A/ Mallard Duck/ MN/A 107-3431/2007) |
| Influenza A Virus H9N9 (A/Ruddy Turnstone/NJ/749/02) |
| Influenza A Virus H4N6 (A/Mallard/MN/198/99) |
| Influenza A virus H5N2 (A/Mallard/MN/1/00) |
| Influenza A Virus H9N2 (A/Mallard/MN/232/98) |
| Influenza A Virus H6N2 (A/CK/NY/14677-13/98) |
| New Castle Disease Virus/Infectious Bronchitis Virus Vaccine |
| (Broilerbron, Schering-Plough Animal health) |
| Infectious Bronchitis Virus Vaccine –Ark Type Live virus (Intervet) |
| Infectious Bronchitis Virus Vaccine – Mass Type Live virus |
| Infectious Bronchitis Virus strain Mass41 |
| Infectious Laryngotracheitis Virus USDA AviServe ILT strain |

Table 2. Primers used in this study.

| Specificity | Primer designation | Primer Sequence (5'–3) |
|---|---|---|
| Influenza A virus[A] | M+ 25 | AGA TGA GTC TTC TAA CCG AGG TCG |
| | M - 124 | TGC AAA AAC ATC TTC AAG TCT CTG |
| Hemagglutinin H5[A] | H5 +1456 | ACG TAT GAC TAT CCA CAA TAC TCA G |
| | H5 - 1685 | AGA CCA GCT ACC ATG ATT GC |
| Hemagglutinin H7[B] | H7 PanAm F1515 | ATG GAG AGC ATA AGG AAC |
| | H7 PanAm R1628 | CCG AAG CTA AAC CAT AAG |
| Neuraminidase N1[C] | NA-1.2 F | CAA AGT GTC ATT ACC TAC GAA AAC |
| | NA- 1.2 R | TTG TCT GGG CCG GAA ATA CC |
| Neuraminidase N2[C] | NA- 2.3 F | CTG GTG GGG ACA TTT GGG TAA C |
| | NA -2.3 R | TAT TCT AGT ATC GGC CTT TCC TG |

A. Spackman, E., *et al.* 2002.

B. Spackman, E., *et al*. 2008.

C. Chander, Y., *et a*l. 2010.

## RESULTS

### Confirmation of coupling

The binding of the probes to each set of microspheres in all coupling reactions was confirmed using dilutions of biotinylated oligonucleotides complementary to the probes. The MFI from the background wells with all reaction components except the target is considered as background fluorescence. For the coupling confirmation assays, the background florescence was negligible when compared to the wells with the lowest dilution of the oligonucleotides and the MFI increased linearly with the quantity of oligonucleotides in the sample (Fig.1). The same pattern was observed with the different microspheres in all coupling reactions.

### Strand Displacement amplification and microsphere assays

The total RNA was extracted and amplified using degenerate primers. Agar gel electropheresis of the PCR product showed a smear of DNA ranging in size from 500 – 1500 bp as expected (Fig.2). Microsphere assays were performed using the gel purified product in a singleplex format with the M probe coupled microspheres. The MFI observed with two different concentrations of the sample was similar to the negative controls (Fig.3). Microsphere assays using H5 and H7 probe coupled microspheres were also performed with strand displacement amplification product from serial dilutions of the H5N1 and H7N3 viruses (Fig.4) without gel purification, and a significantly positive MFI was not observed with any of the samples (Fig.5). Strand displacement amplification with run off transcripts of M, H5 and H7 genes did not show any smear of amplicons on agar gel electrophoresis.

In an attempt to explain the negative results obtained from random amplification and microsphere testing, we examined the number of individual AIV genes amplified in the strand displacement amplification test and the data are presented in Table 3. The sequences of 691 clones were analyzed and 72.21% of the clones contained the PB2 gene. Only 0.29% of the clones had the matrix gene and 9.5% had the hemagglutinin gene.

**Specific amplification of matrix, H5, H7, N1 and N2 genes**

The matrix, H5, H7, N1 and N2 genes were amplified using specific primers and agar gel electrophoresis of the RT–PCR products are shown in Figs.6 and 7. The matrix, H5 and H7 gene amplicons were the predicted size of 100bp, 150bp and 113bp in length respectively. The N1 and N2 gene amplicons were the predicted size of 448bp and 433bp in length. The identity of the RT–PCR product was confirmed by sequencing.

**Limit of Detection**

The limit of detection of the probe coupled microspheres for biotinylated RT–PCR product was estimated by analysis of two fold dilutions of specifically amplified M, H5 and H7 genes. The lowest dilution giving a significantly positive MFI was determined using Student's T test with $p<0.05$. The limits of detection for M (Fig.8), H5 (Fig.9), H7 (Fig.10), N1 (Fig.11) and N2 (Fig.12) probe coupled microspheres were 0.04ng, 0.15ng, 0.17ng, 1.56ng and 1.15ng respectively. The quantity of DNA corresponds to $3.71 \times 10^{8}$, $9.26 \times 10^{8}$, $1.39 \times 10^{9}$, $3.27X10^{9}$, and $2.3\ X10^{9}$ -copies of biotinylated amplicons of M, H5, H7, N1 and N2 respectively. The H7 probe coupled microspheres consistently showed a pattern different from that of M and H5 while determining the limit of detection. With H7, the MFI increased as the concentration of DNA increased and then it leveled off then decreased at higher concentrations of DNA (Fig.10).

The lowest dilution of the virus in allantoic fluid giving a positive MFI in the microsphere assay was estimated from 10 fold serial dilutions of the virus and the data for the H7 virus, which is also representative of the M and H5 assays, are presented in Fig. 13. In singleplex assays, the M, H5 and H7 probe coupled microspheres were able to detect $10^{-1}$, $10^{0}$, and $10^{0}$ $EID_{50}$ viral particles respectively. The sensitivity observed was greater compared to the real time RT–PCR when amplification conditions are the same.

The limit of detection of the microspheres following multiplex RT-PCR was also determined using 10 fold serial dilutions of the virus and the data for the M coupled microspheres, which are also representative of the H5 and H7 coupled microspheres are presented in Fig. 14. All the three probe coupled microspheres were able to detect between $10^{2.5}$ - $10^{2.8}$ $EID_{50}$ of virus particles.

**Specificity of the assay**

The specificity of the M, H5, H7, N1 and N2 coated microspheres was determined using specifically amplified M, H5, H7, N1 and N2 amplicons and the data for M, H5, H7, N1 and N2 are shown in Figs. 15, 16, 17, 18, and 19 respectively. The amplicons generated in singleplex and multiplex RT-PCR, shown in Fig. 20 were tested in a multiplex microsphere assay with M, H5 and H7 coated microspheres and the data are presented in Fig. 21. Also the amplicons generated in singleplex and multiplex RT-PCR, shown in Fig. 22 were tested in a multiplex microsphere assay with N1 and N2 coated microspheres and the data are presented in Fig. 23. No cross reactivity was observed for any of the assays.

Finally, RT-PCR product of RNA extracted from several different avian influenza viruses and other avian respiratory viruses were analyzed with M, H5 and H7probe-coupled microspheres in a multiplexed format and the data is shown in Fig. 24. The specificity of N1 and

N2 coated microspheres were also tested separately and the data is shown in Fig. 25. No nonspecific binding was observed when NDV, IBV, or ILTV samples were tested and no binding was detected for negative controls including negative allantoic fluid. All the influenza viruses were positive with the M probe coupled microspheres. In addition, the H5 viruses were positive with the H5 probe coupled microspheres and not positive against the H7 probe coupled microspheres. Similarly, the H7 viruses were positive with the H7 probe coupled microspheres and not positive with the H5 probe coupled microspheres. Also, the N1 and N2 viruses were positive with N1 and N2 probe coated microspheres only.

**Evaluation of the Assay**

We tested 102 oropharyngeal or cloacal swabs from previous studies using H6N2 and H9N2 viruses to evaluate the assay and the results are shown in Table 4. 94 samples were negative with both real time RT-PCR and multiplex microsphere assay and 5 samples were positive with both the tests. Among the 5 positive samples from the microsphere assay, the M probe coupled microspheres in the fiveplex assay detected all 5 whereas the N2 probe coupled microspheres detected only 4 of the positive samples. Three samples were positive only with real time RT-PCR and these 3 samples gave positive MFI when re-tested with M probe coupled microspheres in a singleplex format. The M, H5, H7 and N2 probes were able to identify the positive controls with H5N1, H5N2 and H7N3 viruses without any cross hybridization and the MFI from the negative controls were negligible. The N1 probe showed some cross reaction with the positive control sample with H5N2 virus in three of the five assays.

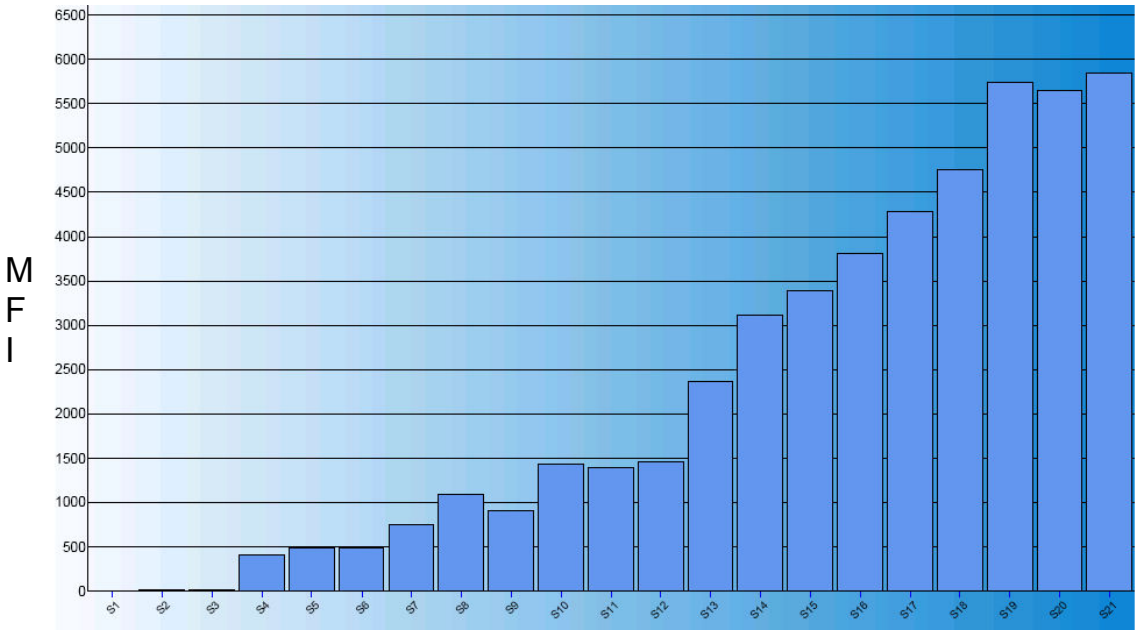



Fig. 1. Confirmation of coupling. The binding of M, H5 and H7 probes to the microspheres were confirmed using the biotinylated oligonucleotides complementary to the probes. The coupling confirmation assays were done using dilutions of the biotinylated oligonucleotides and reactions were set up in triplicate with the concentration ranging from 5 – 200 femtomoles (fm). The median fluorescence intensity (MFI) is shown for negative controls (S1 –S3), 5 fm (S4-S6), 10 fm (S7-S9), 20 fm (S10-S12), 50 fm (S13-S15), 100 fm (S16-S18), 200 fm (S19-S21). A representative graph is shown for the M probe

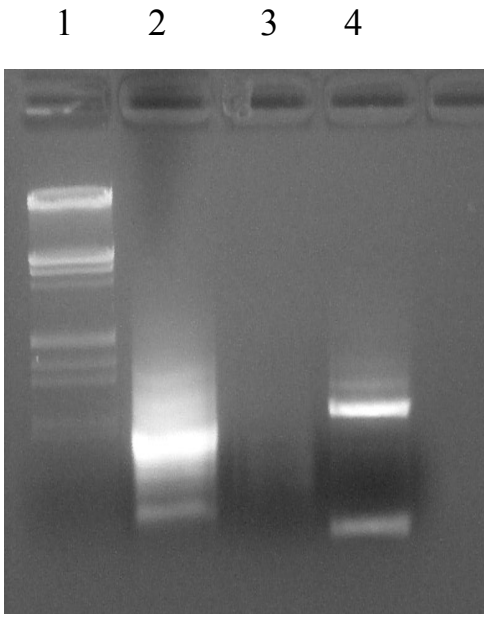



Fig. 2 . Strand displacement amplification of total RNA. Strand displacement amplification of total RNA extracted from allantoic fluid containing H5N1 virus was performed using TAKARA kit and the PCR product was exectrophoresed on a 1% agarose gel. Lane 1: molecular weight marker λ digested with Hind III and EcoRI; lane 2: the strand displacement amplification product; lane 3: empty; lane 4: PCR product from the positive control RNA included in the TAKARA kit.

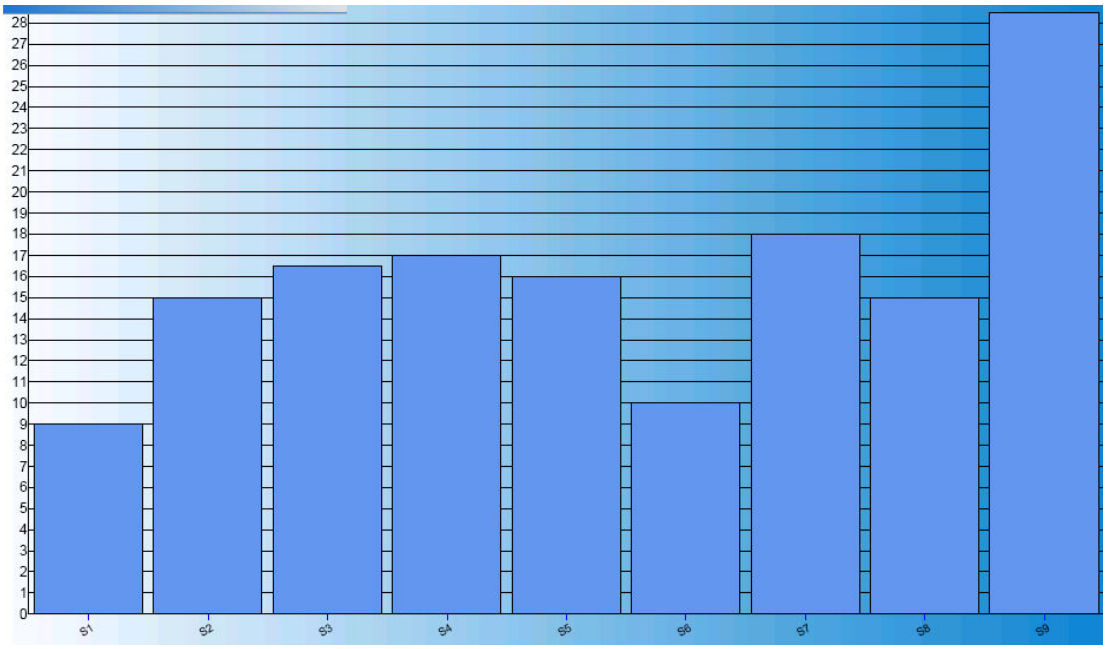



Fig. 3. Singleplex microsphere assay using M probe and strand displacement amplification product. Singleplex microsphere assays were performed using the M probe coupled microspheres and the samples were run in triplicate. Negative controls (S1-S3) were set up with out any PCR product. The microsphere assay was performed with 5µl (S4- S6) and 10µl (S7 –S9) of the gel purified strand displacement amplification product.

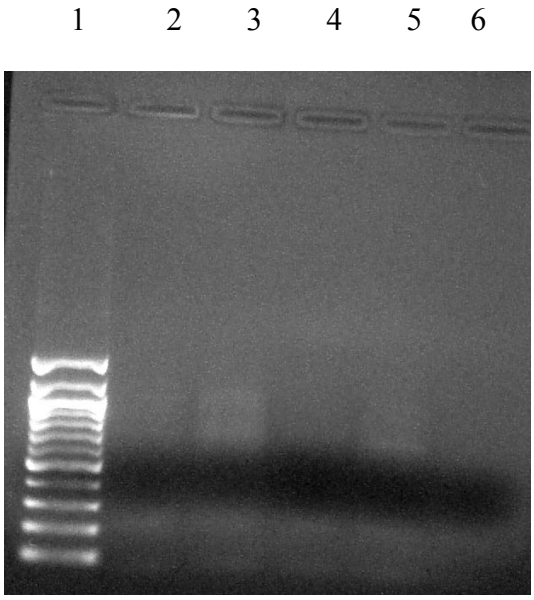



Fig. 4. Strand displacement amplification of RNA extracted from ten fold serial dilutions of H5N1 virus. Strand displacement amplification was performed using TAKARA kit with RNA extracted from ten fold serial dilutions of the H5N1 virus and 20ul of the PCR product was electrophoresed on 1% agarose gel. Lane 1: Molecular weight marker, 1Kb DNA ladder; lane 2: undiluted virus; lane 3: virus diluted $10^{-1}$; lane 4: virus diluted $10^{-2}$; lane 5: virus diluted $10^{-3}$; lane 6: virus diluted $10^{-4}$.

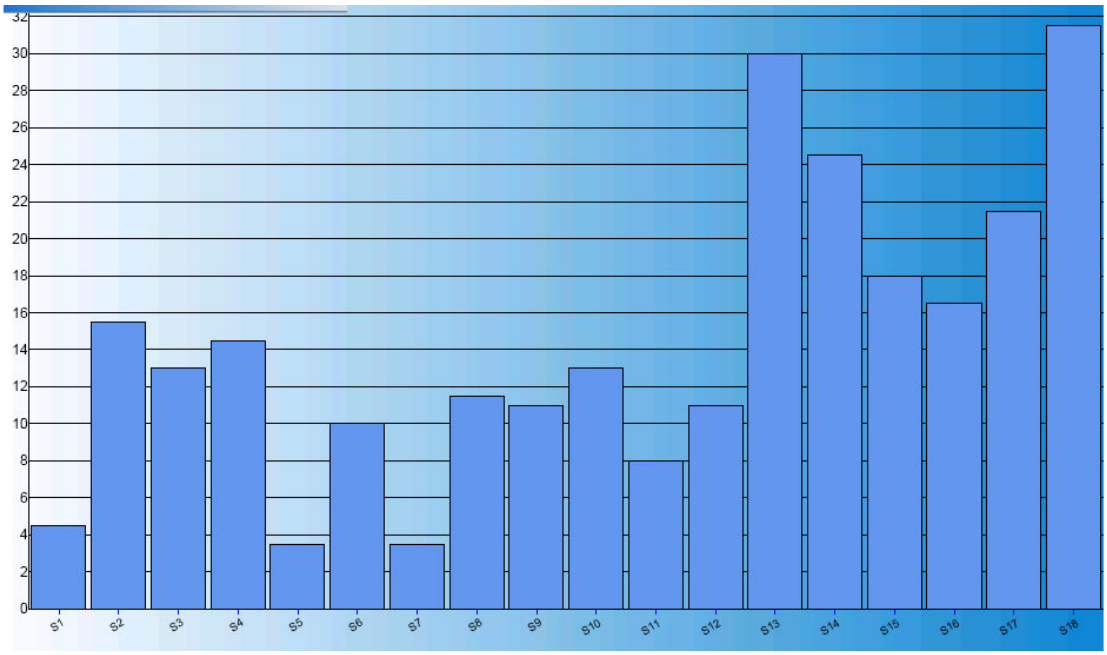



Fig.5. Singleplex microsphere assay using H5 probe and strand displacement amplification product from serial dilutions. Strand displacement amplification was performed with serial dilutions of the H5N1 virus and 10µl of purified PCR product per sample was used in the microsphere assay with H5 probe. Negative controls (S1-S3) were set up with TE buffer instead of cDNA and samples in triplicates (S4 – S6 -$10^{-4}$ dilution; S7 –S9 – $10^{-3}$ dilution; S10 – S12 – $10^{-2}$ dilution; S13 –S15 -$10^{-1}$ dilution; S16 –S18– undiluted virus) were set up for each dilution.

Table 3. Number of clones containing the indicated AIV genes, cloning vector and chicken genes contained in a cDNA library following strand displacement random amplification of the Influenza A Virus H5N1 (A/Black Duck/NC/674-964/06).

| Gene identified | Number of clones /691 | % of total |
|---|---|---|
| PB2 | 499 | 72.21 |
| PB1 | 16 | 2.31 |
| PA | 17 | 2.46 |
| Nucleocapsid | 52 | 7.5 |
| Hemagglutinin | 66 | 9.5 |
| Neuraminidase | 7 | 1.01 |
| Matrix | 2 | 0.29 |
| Cloning Vector | 7 | 1.01 |
| Chicken genes | 25 | 3.62 |

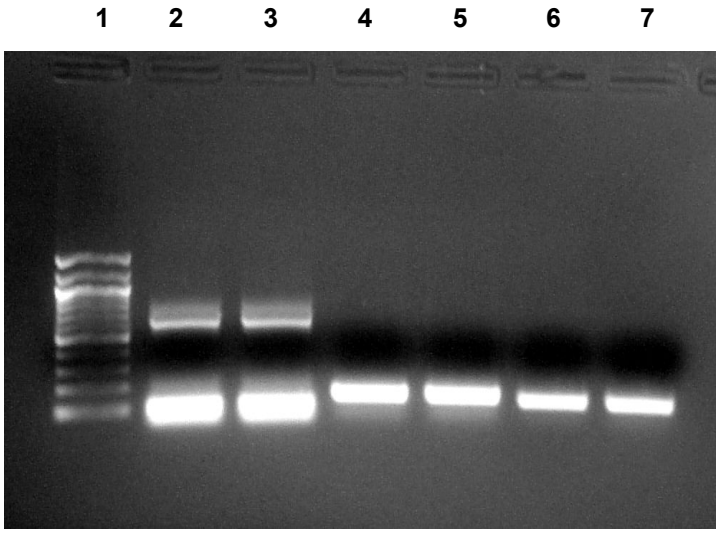



Fig. 6. RT-PCR of RNA extracted from H5N1 and H7N3 viruses with M, H5 and H7 primers. RT- PCR of the RNA extracted from H5N1 and H7N3 viruses were performed and the RT-PCR product was electrophoresed on 1% agarose gel with 100base pair DNA ladder as marker. Lane 1: Molecular weight marker, 100 bp DNA ladder; lane 2 and 3: Matrix gene amplicon; lane 4 and 5: H5 amplicon; lane 6 and 7: H7 amplicon.

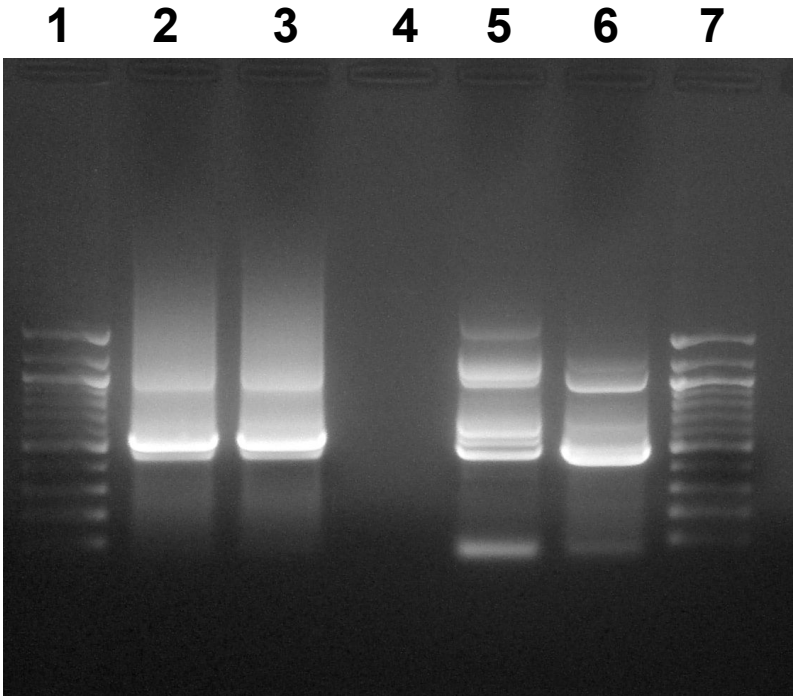



Fig. 7. RT –PCR of RNA extracted from H5N1, H5N2 and H9N2 viruses with N1 and N2 primers. RT- PCR of the RNA extracted from H5N1, H5N2 and H9N2 viruses were performed and the RT-PCR product was electrophoresed on 1% agarose gel with 100base pair DNA ladder as marker. Lane 1: Molecular weight marker, 100 bp DNA ladder; lane 2 and 3: N1 gene amplicon; lane 4: Empty; lane 5: N2 amplicon from H5N2 RNA; lane 6: N2 amplicon from H9N2 RNA; lane 7: Molecular weight marker.

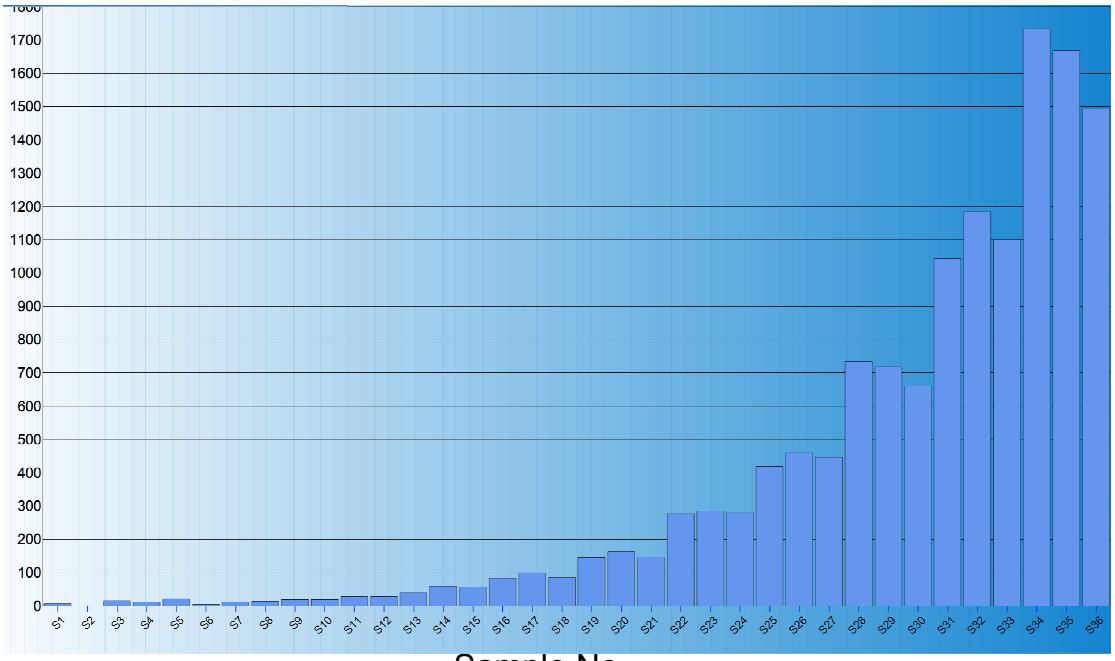



Fig. 8. The limit of detection of M probe coupled microspheres. The limit if detection of M probe coupled microspheres for specifically amplified matrix gene cDNA was estimated by two fold serial dilutions of the gel purified amplicons. Triplicate samples were run for each dilution (S4 – S36). Negative controls (S1 –S3) were run without cDNA.

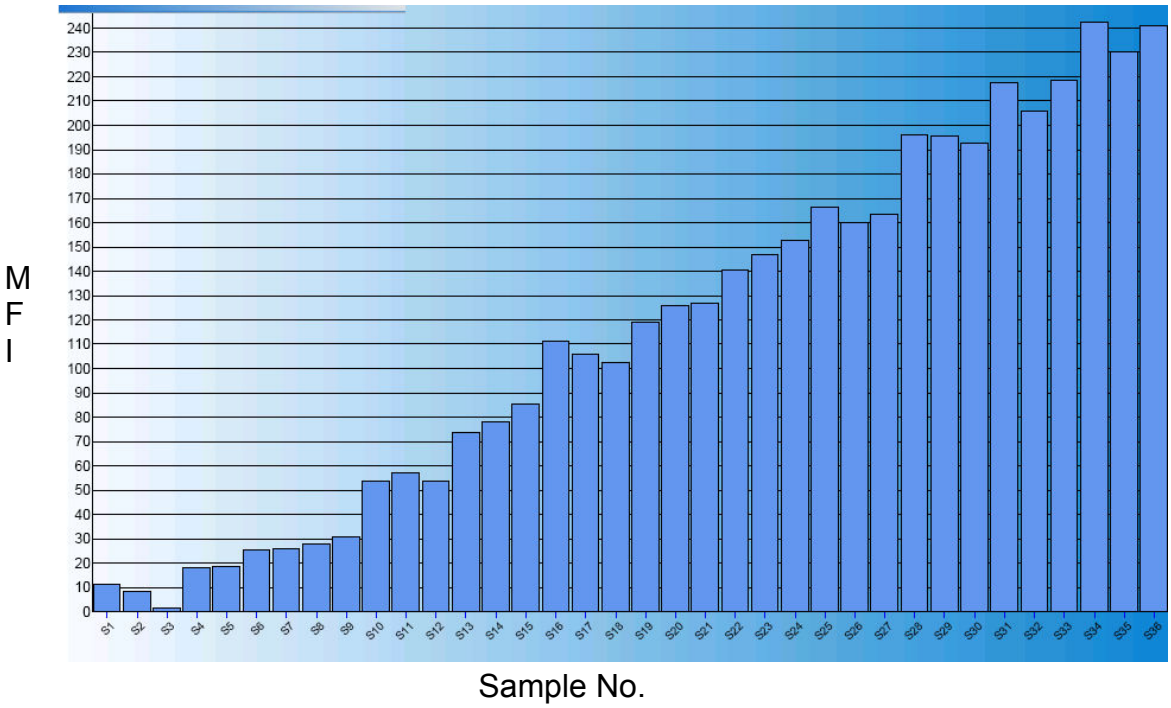



Fig. 9. The limit of detection of H5 probe coupled microspheres. The limit if detection of H5 probe coupled microspheres for specifically amplified H5 gene cDNA was determined by two fold serial dilutions of the gel purified amplicons. Triplicate samples were run for each dilution (S4 –S36). Negative controls (S1- S3) were run without cDNA.

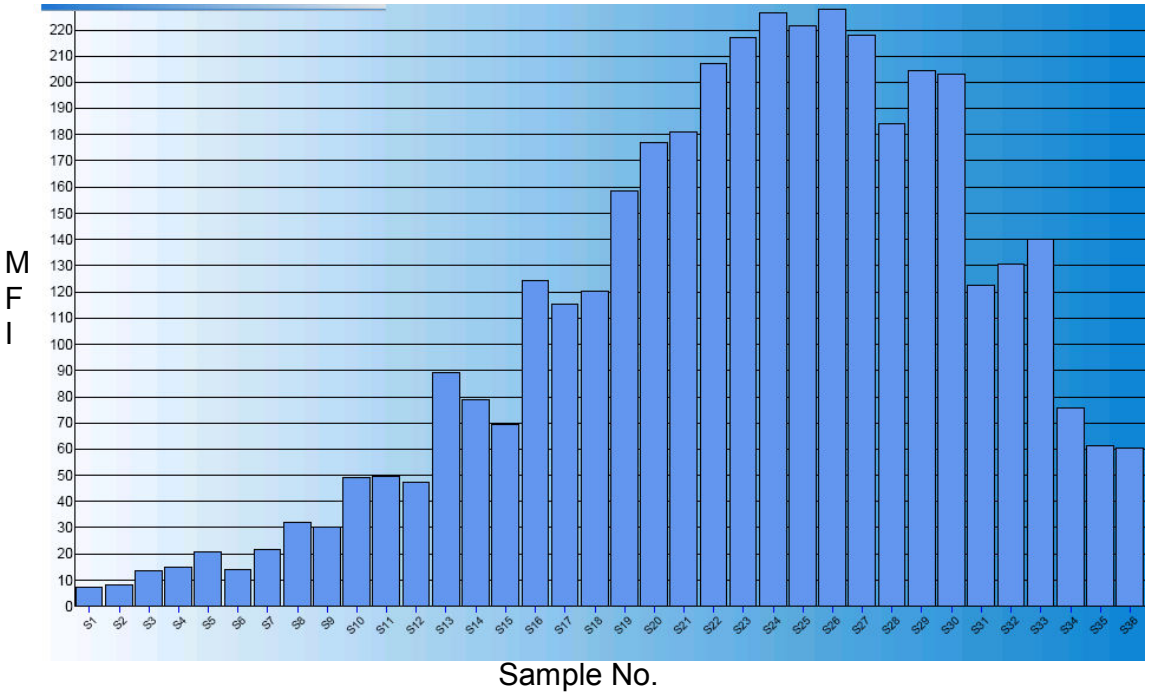



Fig. 10. The limit of detection of H7 probe coupled microspheres. The limit if detection of H7 probe coupled microspheres for specifically amplified H7 gene cDNA was detected by two fold serial dilutions of the gel purified amplicons. Triplicate samples were run for each dilution (S4 –S36). Negative controls (S1 –S3) were set up without cDNA.

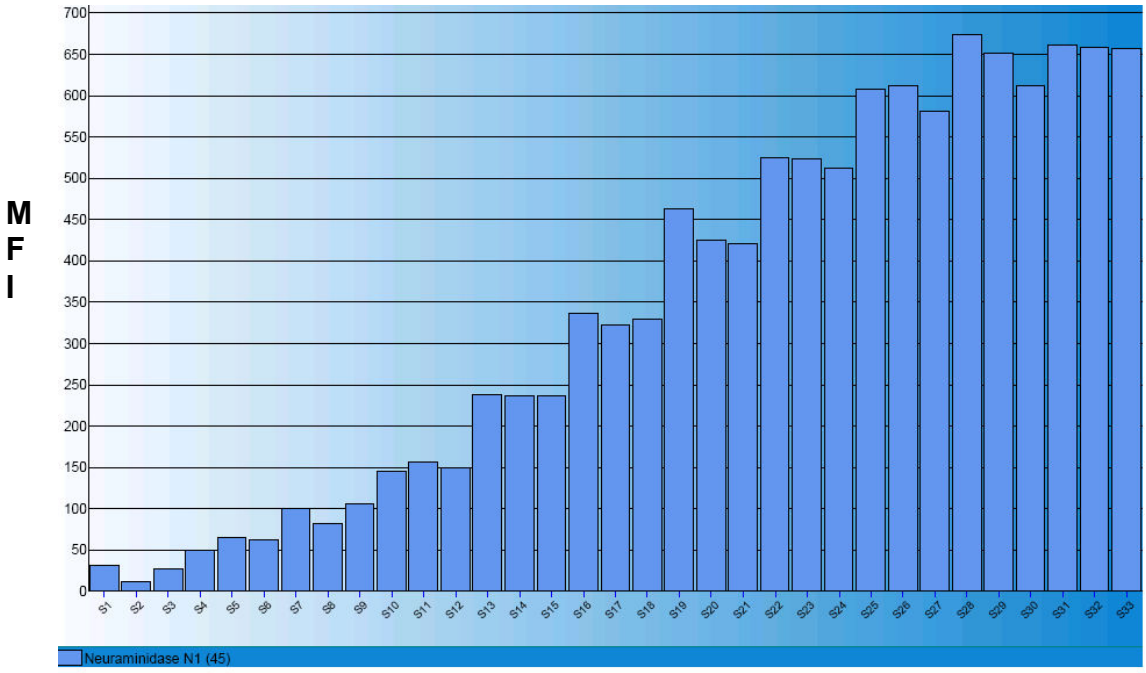



Fig. 11. The limit of detection of N1 probe coupled microspheres. The limit of detection of N1 probe coupled microspheres for specifically amplified N1 gene cDNA was detected by two fold serial dilutions of the gel purified amplicons. Triplicate samples were run for each dilution (S4 –S33). Negative controls (S1 –S3) were set up without cDNA.

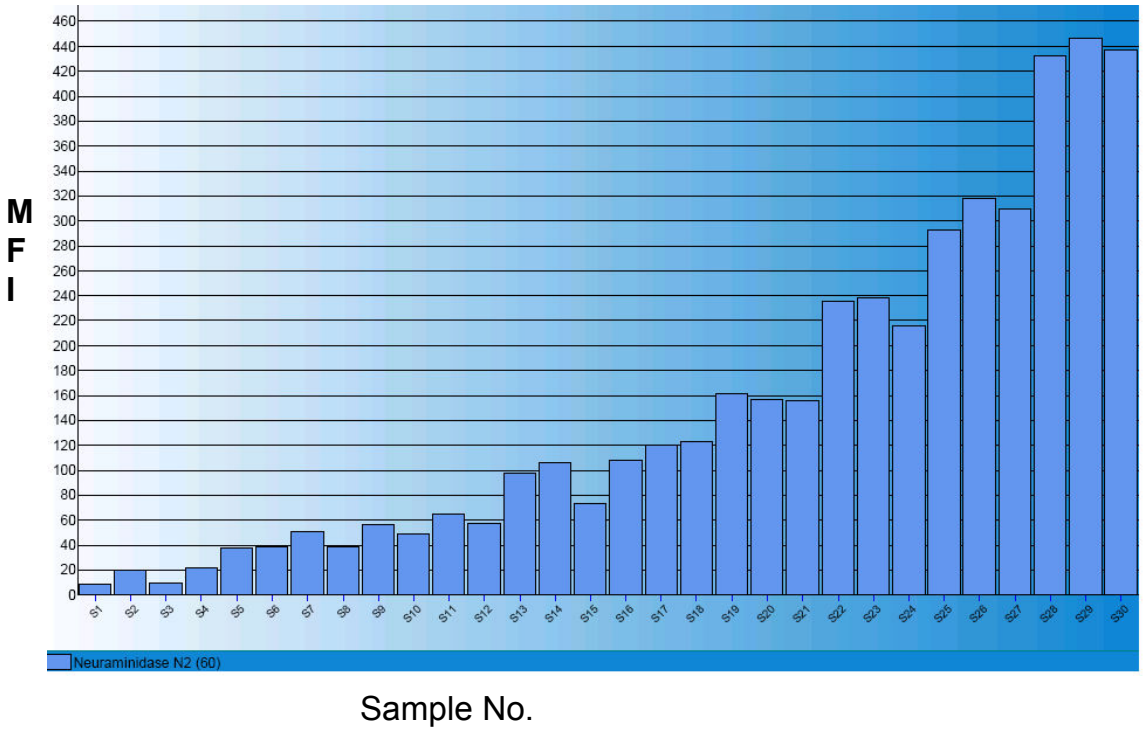
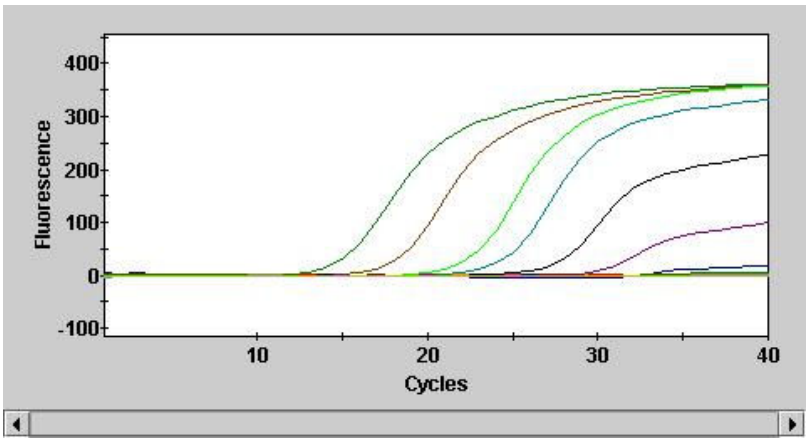



Fig. 12. The limit of detection of N2 probe coupled microspheres. The limit of detection of N2 probe coupled microspheres for specifically amplified N2 gene cDNA was detected by two fold serial dilutions of the gel purified amplicons. Triplicate samples were run for each dilution (S4 – S30). Negative controls (S1 –S3) were set up without cDNA.

**A.**




**B.**

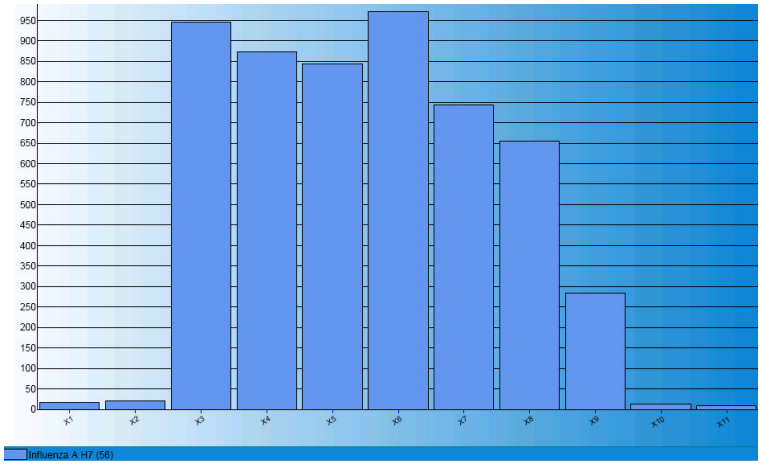



Fig.13. The limit of detection of probe coupled microspheres for virus particles after amplification in Smartcycler. The limit of detection of probe coupled microspheres for virus particles was tested in a singleplex format using ten fold serial dilutions of the viruses. The results of the H7 virus are shown and are representative of the results for M and H5 assays. (A) The RT–PCR was performed in a SmartCycler with specific primers for the H7 gene. The undiluted virus result is indicated, reactions to the right in order represent virus diluted $10^{-1}$ to $10^{-8}$. (B) The singleplex microsphere assay was conducted with the H7 probe coupled microspheres. X1: microsphere control, no amplified product; X2: RT-PCR control, no template RNA; X3: amplified product from undiluted virus; X4 –X11: amplified products from 10 fold serial dilutions of the virus, $10^{-1}$ to $10^{-8}$.

Table 4. Comparison of CT value and MFI from real time RT-PCR and singleplex microsphere assays using ten fold serial dilutions of H5N1 and H7N3 viruses

| Dilutions of viruses | M Assay | | H5 Assay | | H7 Assay | |
|---|---|---|---|---|---|---|
| | CT Value | MFI | CT value | MFI | CT Value | MFI |
| Undiluted | 18.56 | 2242.0 | 18.22 | 2089.0 | 14.99 | 945.5 |
| $10^{-1}$ | 22.67 | 2186.5 | 22.05 | 2244.0 | 18.09 | 874.0 |
| $10^{-2}$ | 27.49 | 2102.0 | 27.07 | 1606.0 | 22.25 | 843.5 |
| $10^{-3}$ | 30.74 | 2170.0 | 30.67 | 816.0 | 24.32 | 971.5 |
| $10^{-4}$ | 35.65 | 1113.5 | 0.00 | 188.0 | 27.98 | 744.0 |
| $10^{-5}$ | 0.00 | 46.5 | 0.00 | 23.5 | 31.76 | 654.0 |
| $10^{-6}$ | 0.00 | 31.5 | 0.00 | 14.0 | 0.00 | 283.0 |
| $10^{-7}$ | 0.00 | 17.0 | ND | ND | 0.00 | 9.5 |
| $10^{-8}$ | ND | ND | ND | ND | 0.00 | 12.0 |

CT Value – Cycle Threshold value
MFI – Median Fluorescence Intensity

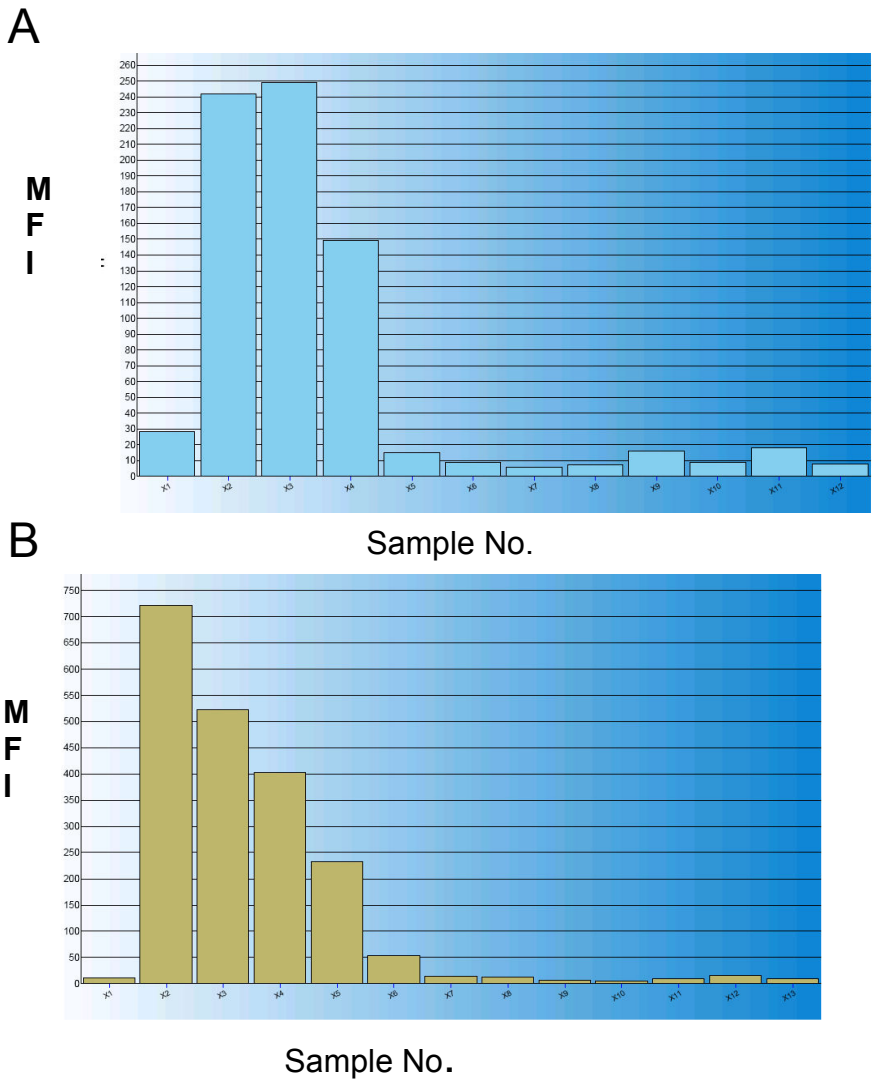



Fig. 14. The limit of detection of probe coupled microspheres for virus particles after multiplex RT-PCR. The limit of detection of multiplex microsphere assay following M, H5 and H7 multiplex RT –PCR. (A) M probe coated microspheres against amplified product from the H5N1 virus ($10^{6.8}$ $EID_{50}$). (B) M probe coated microspheres against amplified product from the H7N3 virus ($10^{8.5}$ $EID_{50}$). X1: microsphere control, no amplified product; X2: amplified product from undiluted virus; X3 –X12: amplified product from10 fold dilutions of the virus, $10^{-1}$ to $10^{-11}$; X13 (B only) $10^{-12}$.

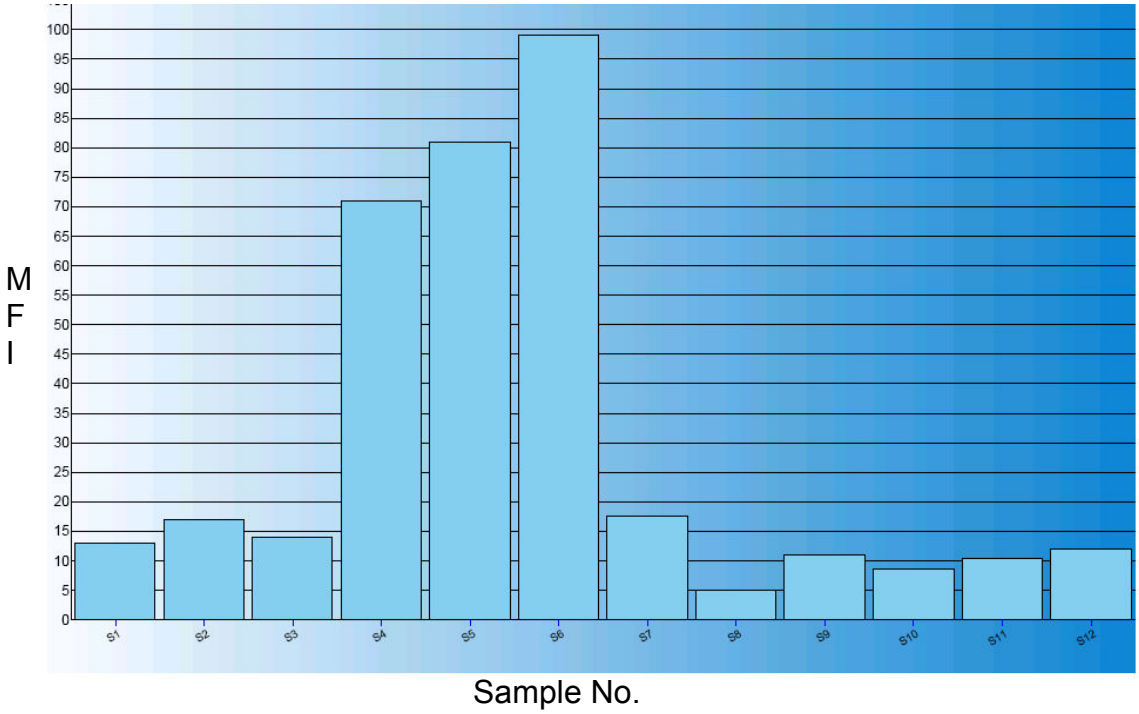



Fig. 15. The specificity of M probe coupled microspheres in singleplex format. The specificity of the M probe coupled microspheres was tested against RT-PCR amplified M, H5, and H7 genes. The assays were run in triplicate with S1-S3: Negative control, no amplified product; S4-S6: M amplified product; S7 –S9: H5 amplified product; S10- S12: H7 amplified product.

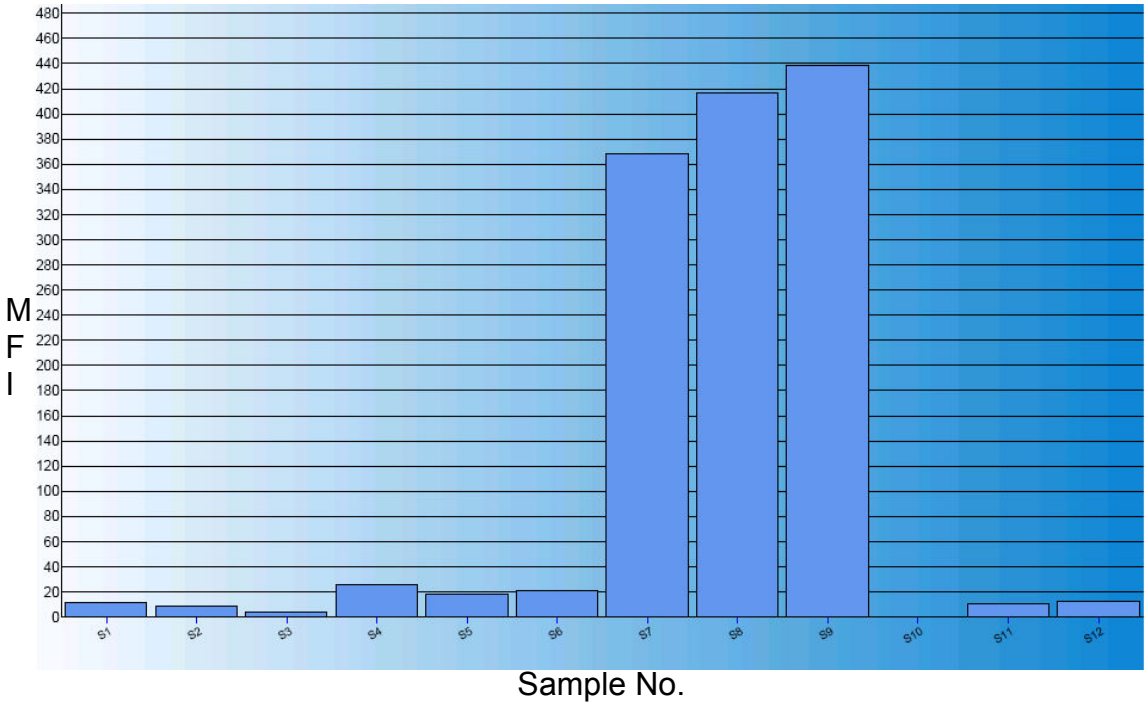



Fig. 16. The specificity of H5 probe coupled microspheres in singleplex format. The specificity of the H5 probe coupled microspheres was tested against RT-PCR amplified M, H5, and H7 genes. The assays were run in triplicate with S1-S3: Negative control, no amplified product; S4-S6: M amplified product; S7 –S9: H5 amplified product; S10- S12: H7 amplified product.

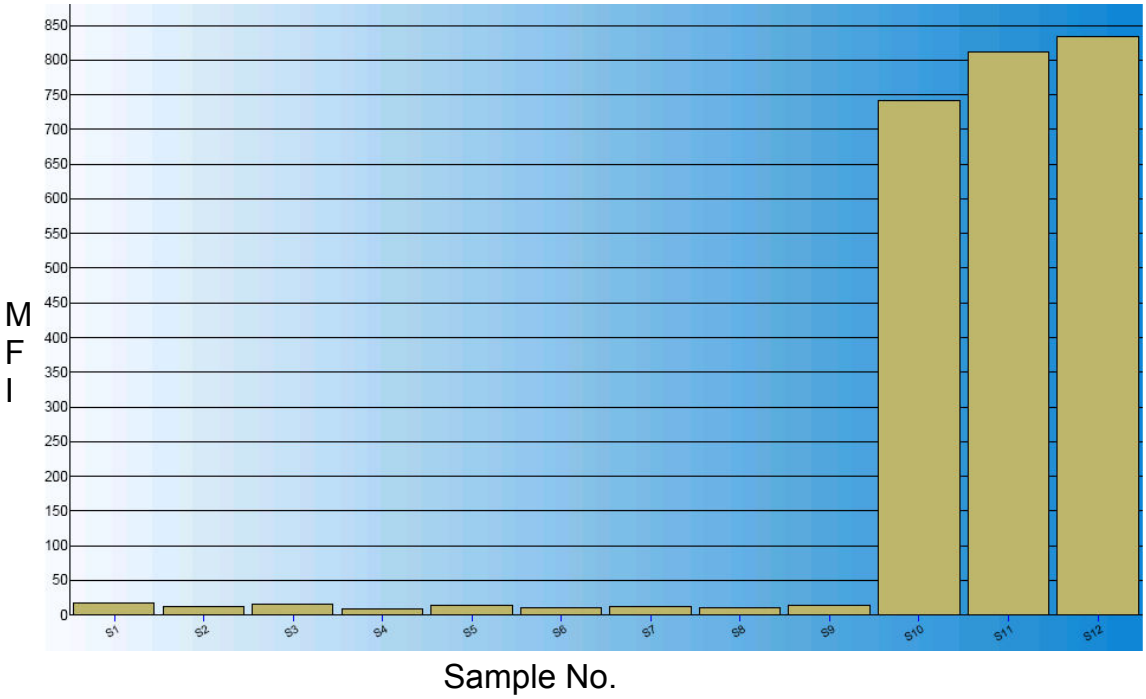



Fig. 17. The specificity of H7 probe coupled microspheres in singleplex format. The specificity of the H7 probe coupled microspheres was tested against RT-PCR amplified M, H5, and H7 genes. The assays were run in triplicate with S1-S3: Negative control, no amplified product; S4-S6: M amplified product; S7 –S9: H5 amplified product; S10- S12: H7 amplified product.

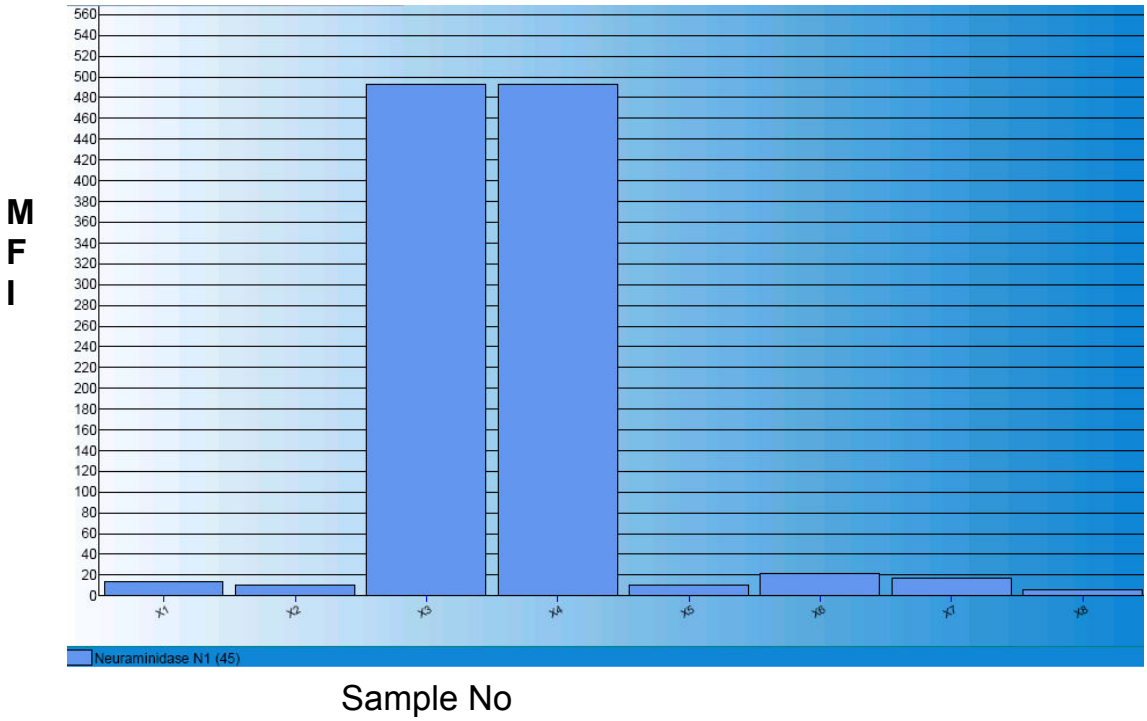



Fig.18. T he specificity of N1probe coupled microspheres in singleplex format. The specificity of the N1 probe coupled microspheres was tested against RT-PCR amplified N1 and N2 genes. The assays were run in duplicate with X1-X2: Negative control, no amplified product; X3-X4: N1 amplified product; X5 –X6: N2 amplified product; X7- X8: N2 amplified product.

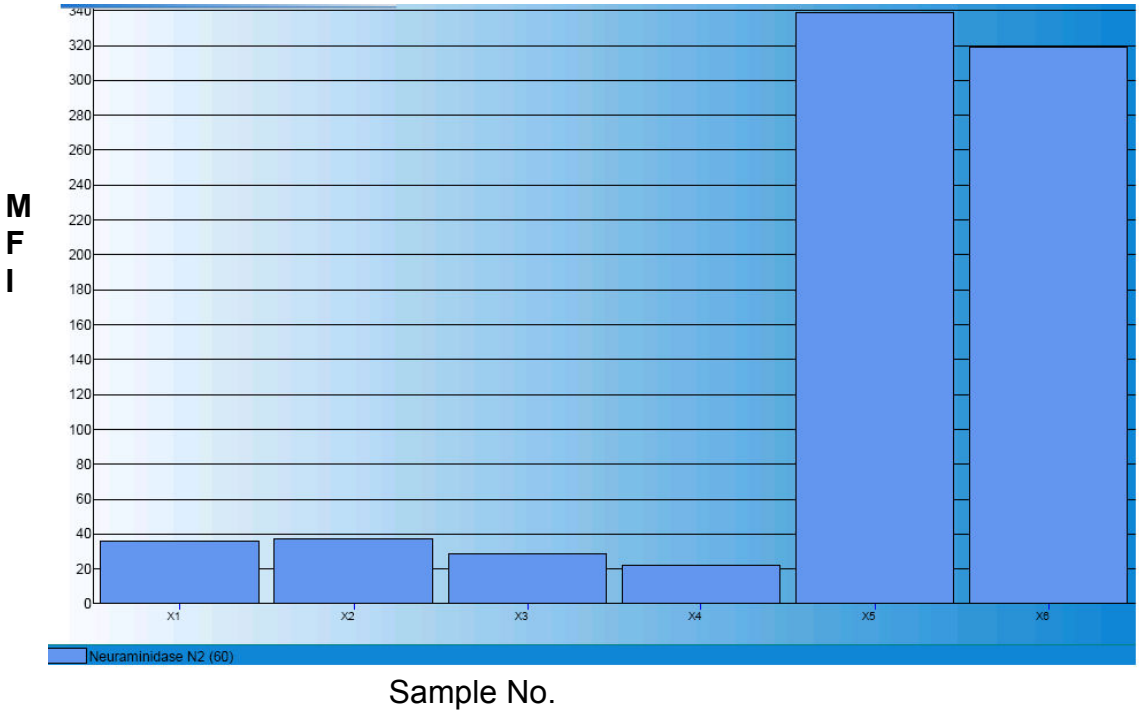



Fig. 19. The specificity of N2 probe coupled microspheres in singleplex format The specificity of the N2 probe coupled microspheres was tested against RT-PCR amplified N1 and N2 genes. The assays were run in duplicate with X1-X2: Negative control, no amplified product; X3-X4: N1 amplified product; X5 –X6: N2 amplified product.

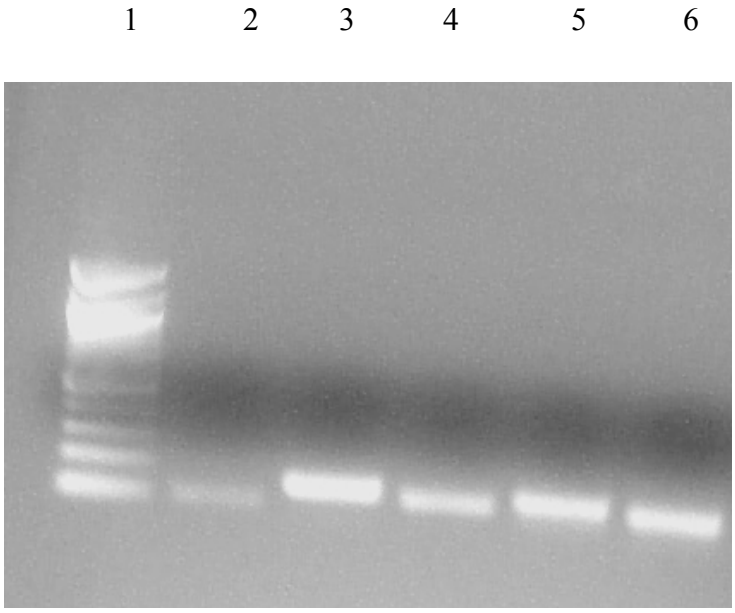
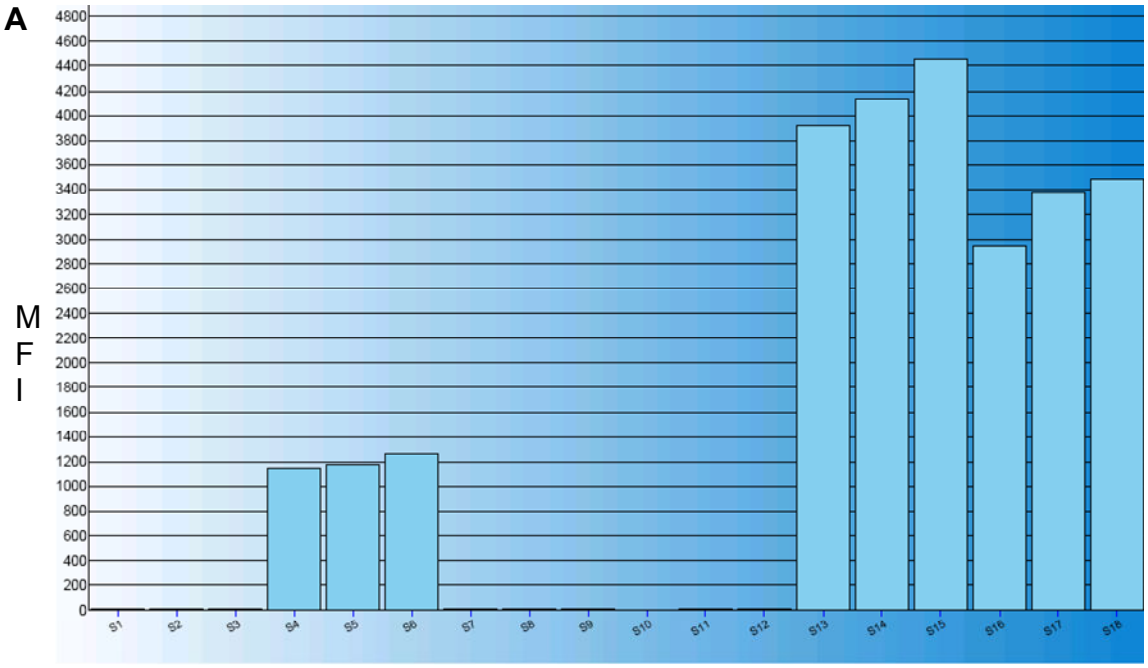


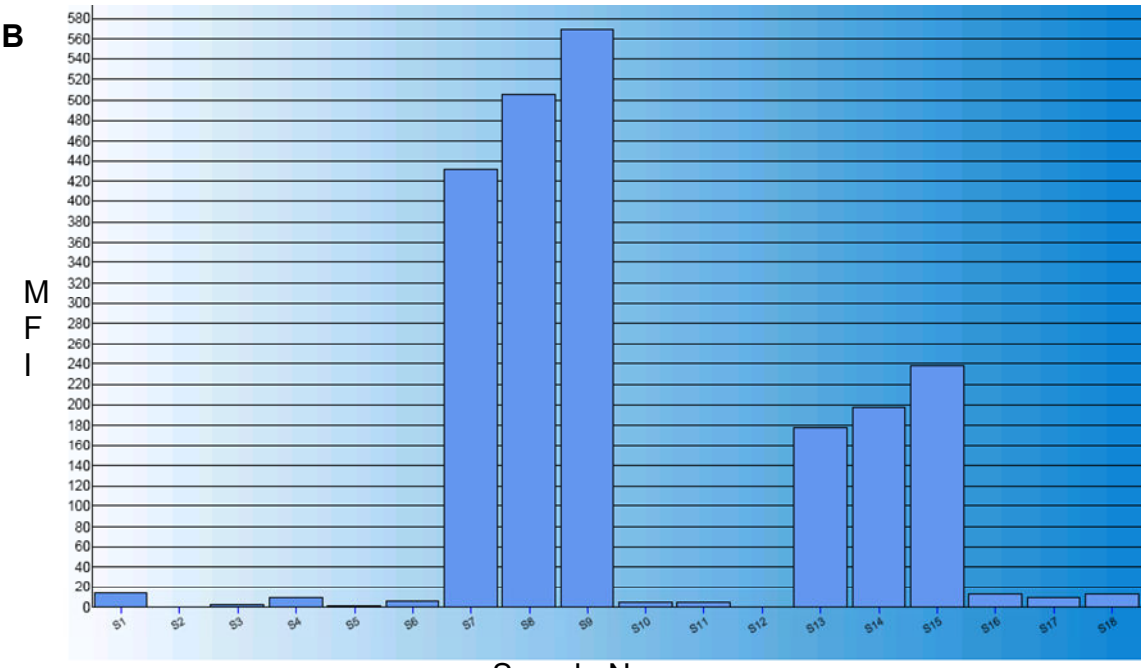

Fig. 20. Singleplex and Multiplex RT –PCR of RNA extracted from H5N1and H7N3 viruses with M, H5 and H7 primers. Both singleplex and multiplex RT–PCR was performed with RNA extracted from H5N1 and H7N3 viruses. The PCR product was electrophoresed on a 1% agarose gel. Lane 1: 100 base pair molecular weight marker; lane 2: M gene amplified product; lane 3: H5 gene amplified product; lane 4: H7 gene amplified product; lane 5: M, H5 and H7 multiplex RT-PCR amplified product from the H5N1 virus; lane 6 M, H5 and H7 multiplex RT-PCR amplified product from the H7N3 virus.

**A**




Sample No.

**B**




Sample No.

C

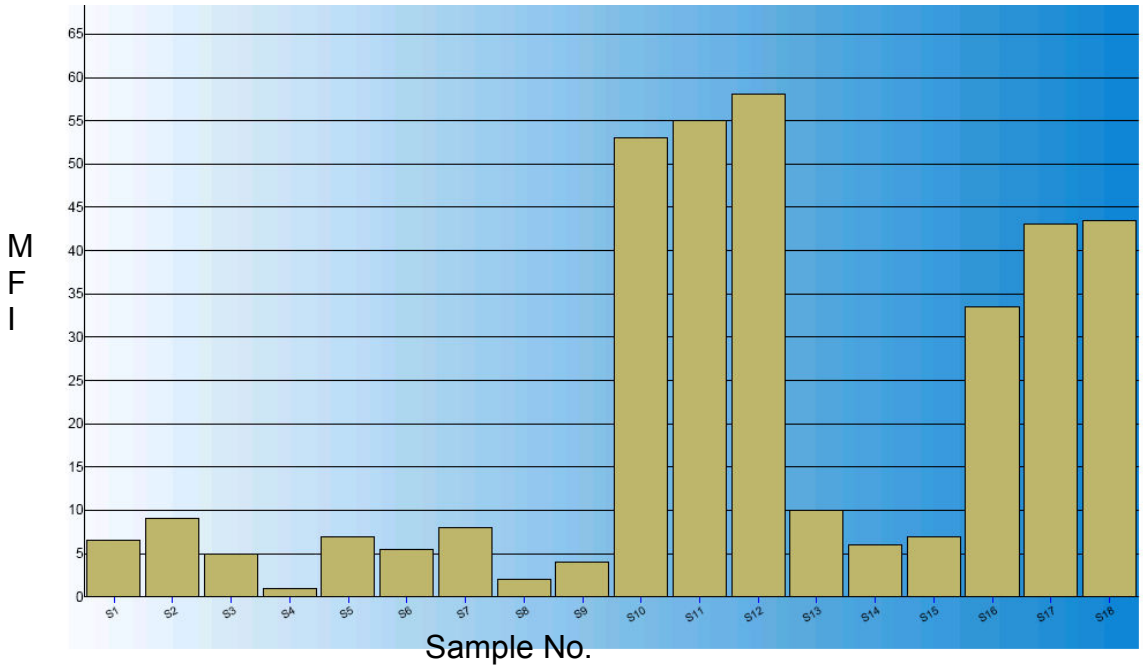



Fig. 21. Multiplex microsphere assay with M, H5 and H7 probe coupled microspheres and singleplex and multiplex RT –PCR product The specificity of the microsphere assay in a multiplex format was tested using amplicons from both singleplex and multiplex RT-PCR. The M, H5 and H7 probe coupled microspheres in a multiplex format was hybridized with amplicons generated in singleplex RT-PCR with M, H5 and H7 primers as well as amplicons generated from multiplex RT-PCR with all the three sets of primers using RNA from H5N1 and H7N3 viruses. (A) Specificity for the M probe. (B) Specificity for the H5 probe. (C) Specificity for the H7 probe. The reactions were run in triplicate; S1-S3: negative controls, no amplified product; S4-S6; amplicons from the singleplex RT-PCR with M primers; S7 –S9: amplicons from singleplex RT-PCR with H5 primers; S10-S12: amplicons from singleplex RT-PCR with H7 primers; S13-S15: amplicons from multiplex PCR using H5N1 RNA; S16-S18: amplicons from multiplex RT-PCR using H7N3 RNA.

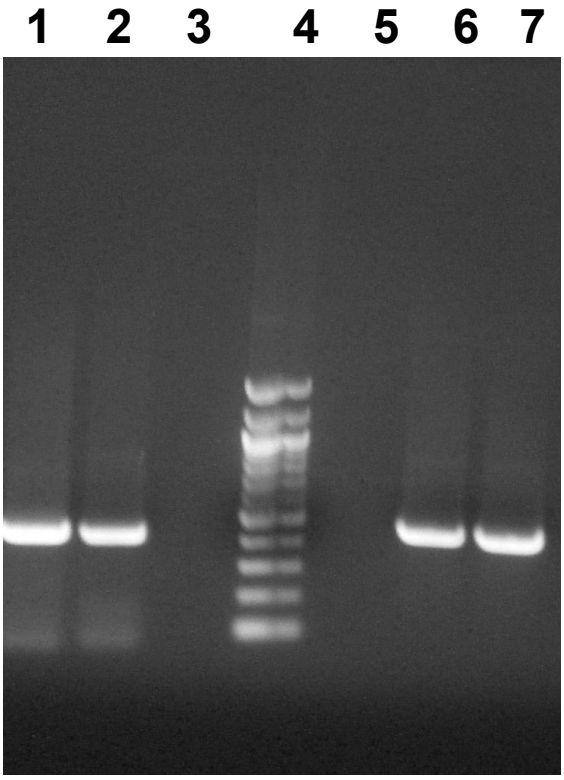
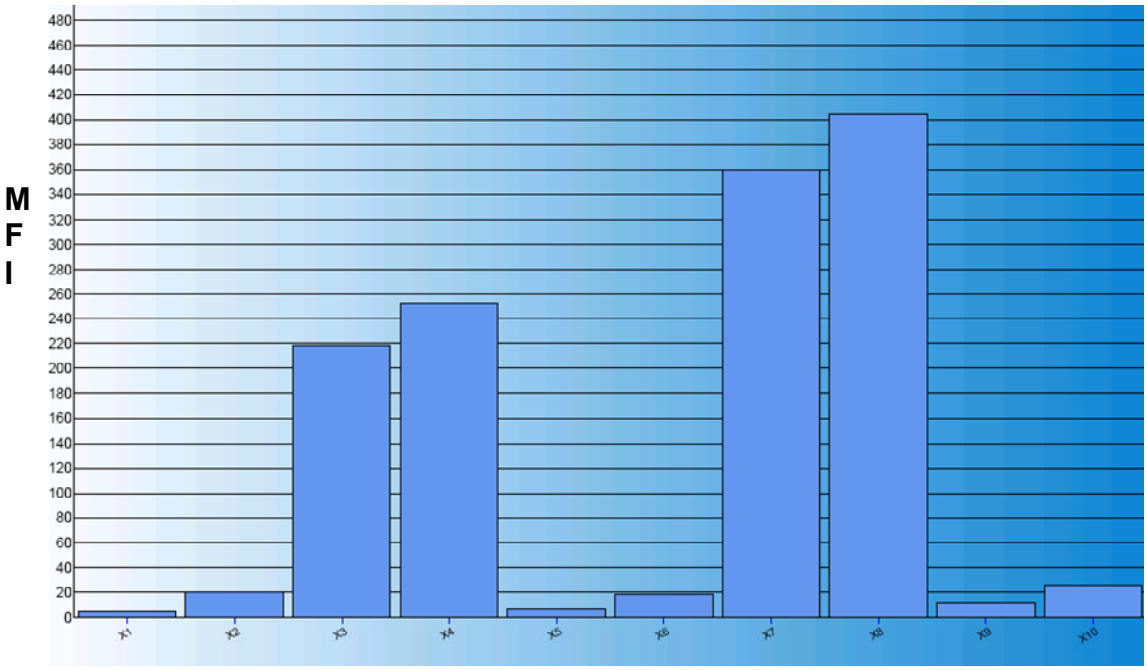



Fig. 22. Singleplex and multiplex RT-PCR of RNA extracted from H5N1and H9N2 viruses with N1 and N2 primers. Both singleplex and multiplex RT–PCR was performed with RNA extracted from H5N1 and H9N2 viruses. The PCR product was electrophoresed on a 1% agarose gel. Lane 1: N1 gene amplified product; lane 2: N2 gene amplified product; lane 3: Empty t; lane 4: 100 base pair molecular weight marker; lane 5: empty; lane 6:N1 and N2 multiplex RT-PCR amplified product from the H5N1 virus; lane 7: N1 and N2 multiplex RT-PCR amplified product from the H9N2 virus.

**A**


MFI
480
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
X1
X2
X3
X4
X5
X6
X7
X8
X9
X10
Sample No.

**B**

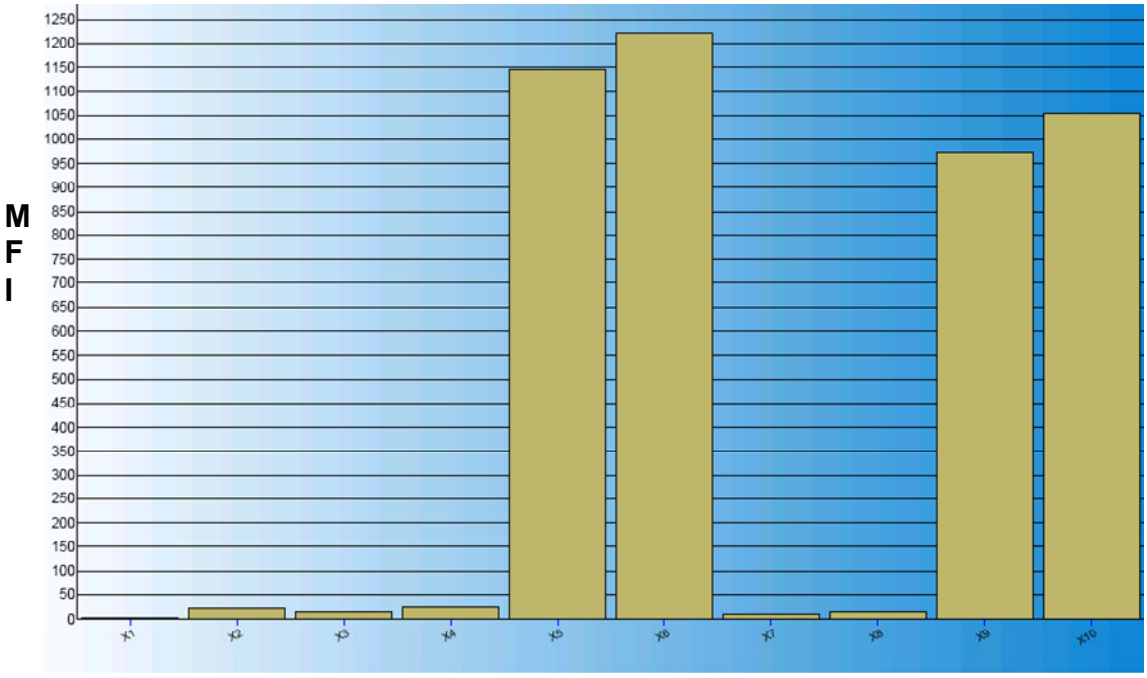
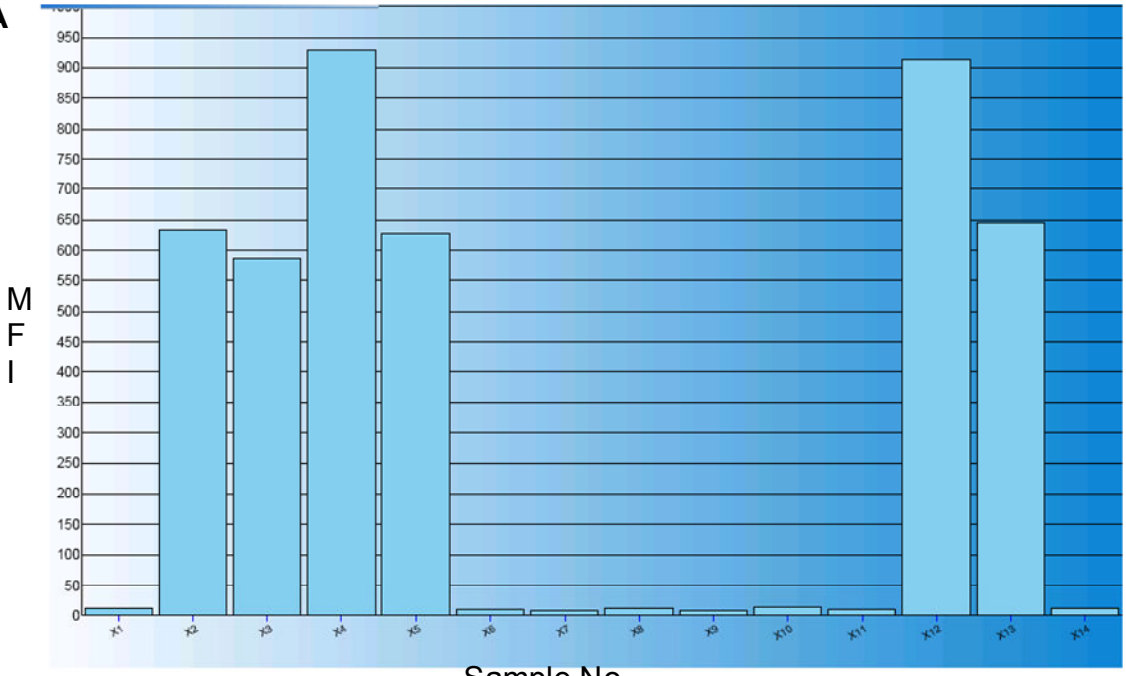

MFI
1250
1200
1150
1100
1050
1000
950
900
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
X1
X2
X3
X4
X5
X6
X7
X8
X9
X10
Sample No.

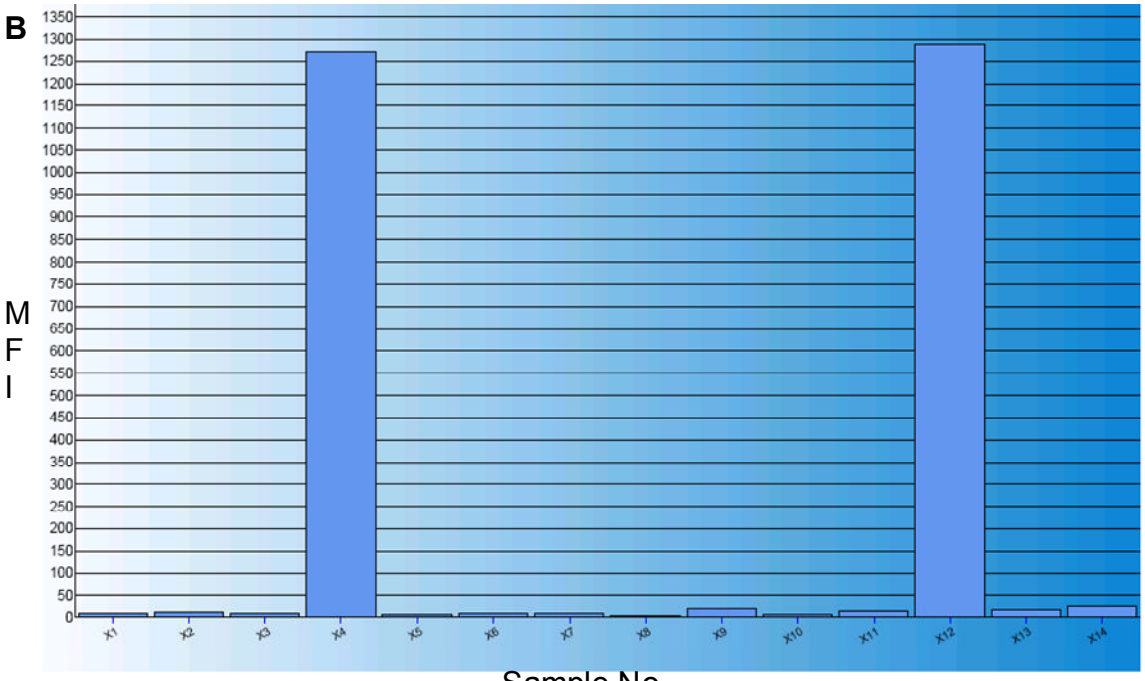
Fig. 23. Multiplex microsphere assays with N1 and N2 probe coupled microspheres and singleplex and multiplex RT –PCR product. The specificity of the microsphere assay in a multiplex format was tested using amplicons from both singleplex and multiplex RT-PCR. The N1 and N2 probe coupled microspheres in a multiplex format was hybridized with amplicons generated in singleplex RT-PCR with N1 and N2 primers as well as amplicons generated from multiplex RT-PCR with all the two sets of primers using RNA from H5N1 and H9N2 viruses. (A) Specificity for the N1 probe. (B) Specificity for the N2 probe. The reactions were run in duplicate; X1-X2: negative controls, no amplified product; X3-X4; amplicons from the singleplex RT-PCR with N1 primers; X5 –X6: amplicons from singleplex RT-PCR with N2 primers; X7-X8: amplicons from multiplex PCR using H5N1 RNA; X9-X10: amplicons from multiplex RT-PCR using H9N2 RNA.

A




Sample No.

B




Sample No.

**C**

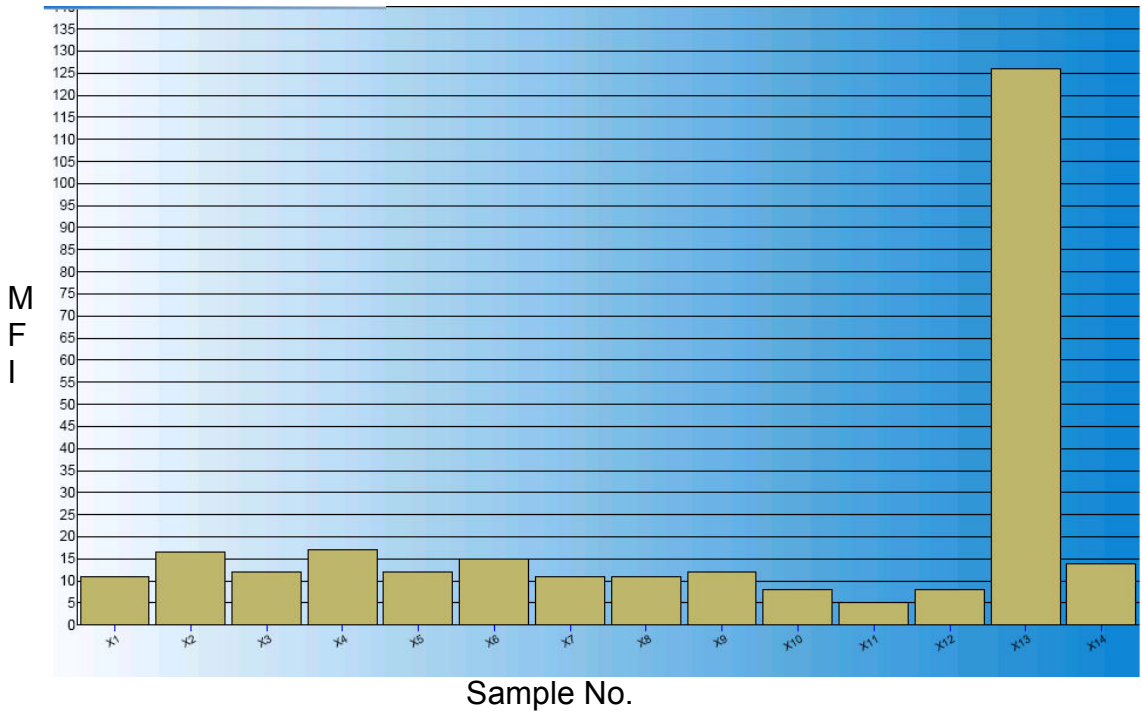



Fig. 24. The specificity of M, H5 and H7 probe coupled microspheres for avian influenza viruses and H5 and H7 subtypes. The specificity of the Microsphere assay for detection of; X1: negative control, microspheres only; X2: H9N9; X3: H4N6; X4: H5N2; X5: H9N2; X6: NDV/IBV vaccine; X7: IBV Ark vaccine; X8: IBV Mass vaccine; X9: IBV Mass 41: X10: ILTV; X11: negative allantoic fluid; X12: H5N1; X13: H7N3; X14: RT-PCR negative control, no RNA template. (A) M probe coupled microspheres. (B) H5 probe coupled microspheres. (C) H7 probe coupled microspheres.

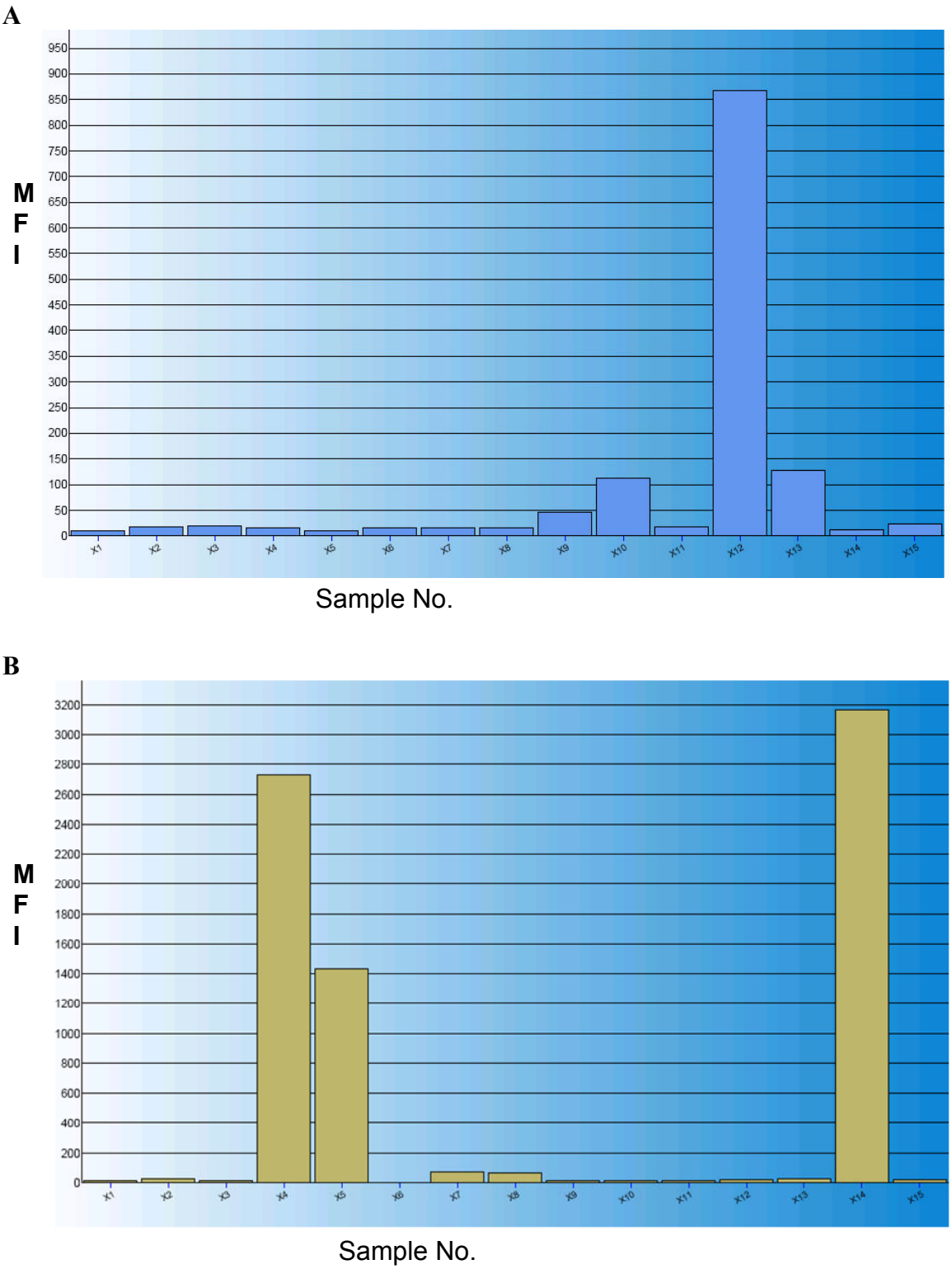



Fig. 25. The specificity of N1 and N2 probe coupled microspheres for avian influenza viruses N1 and N2 subtypes. The specificity of the Microsphere assay for detection of; X1: negative control, microspheres only; X2: H9N9; X3: H4N6; X4: H5N2; X5: H9N2;

X6: NDV/IBV vaccine; X7: IBV Ark vaccine; X8: IBV Mass vaccine; X9: IBV Mass 41: X10: ILTV; X11: negative allantoic fluid; X12: H5N1; X13: H7N3; X14: H6N2; X15: RT-PCR negative control, no RNA template. (A) N1 probe coupled microspheres. (B) N2 probe coupled microspheres.

Table 5. Summary of real time RT-PCR and Microsphere assay results for samples tested

| Virus isolate | Samples negative for avian influenza virus | | Samples positive for Avian influenza virus | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Real time RT-PCR | Microsphere assay, Multiplex | Real time RT-PCR | Microsphere assay | | | | | |
| | | | | Multiplex | | | | | Single plex |
| | | | | M | H5 | H7 | N1 | N2 | M |
| H6N2 | 52/60 | 52/60 | 8/60 | 5/60 | 0/60 | 0/60 | 0/60 | 4/60 | 8/60 |
| H9N2 | 42/42 | 42/42 | 0/42 | 0/42 | 0/42 | 0/42 | 0/42 | 0/42 | 0/42 |

## DISCUSSION

In this study, a multiplex microsphere assay for the simultaneous detection of all avian influenza viruses and differentiation of H5, H7, N1 and N2 subtypes was developed. Rapid identification and subtyping of these viruses are important not only because they are deadly pathogens in poultry but also because of the threat to public health world wide as human adapted viruses can emerge by mutation or reassortment. To our knowledge, this is the first time a microsphere-based assay was developed for the detection and subtyping of avian influenza virus.

The microsphere assay consists of three steps, RNA extraction, RT-PCR amplification, and detection of virus specific amplicons using probe-coupled microspheres. RNA was extracted using High Pure RNA isolation (Roche) and MagMax 96 total RNA isolation (Ambion) kits and both appeared to give equivocal yields. Spackman *et al.* (2002) developed a real time RT –PCR assay which is the current gold standard diagnostic test for AIV. This assay can be used to detect any avian influenza virus using the highly conserved matrix gene as well as H5 and H7 subtypes. Subsequently the Pan-American H7 test was improved using a new set of primers and probe, as the previous test could not detect all H7 AIVs in North and South America (Spackman *et al*. 2008). Similarly, a new H5 probe was developed to improve the sensitivity of the H5 test (D.Suarez, Personal communication). We used these primers for developing the RT-PCR portion of the assay as the sensitivity and specificity were already documented. A multiplex RT-PCR was designed to facilitate the simultaneous amplification of multiple gene targets in a single reaction. To overcome the variation in amplification efficiencies we changed the primer concentrations to optimize the multiplex RT-PCR. The microspheres were coupled with probes

that were designed with the primers for real time RT-PCR. The M probe (Spackman *et al.* 2002) could detect all Influenza A viruses whereas the H5 IY probe (D.Suarez, Personal communication) and H7 PanAm F1585 probe (Spackman *et al.* 2008) could detect H5 and H7 subtypes.

We expanded this microsphere assay to include N1 and N2 subtyping. Amplification of a portion of the N1 and N2 gene segments was accomplished using the primers developed by Chander *et al*. (2010). Since the thermocycler conditions are identical, multiplex RT-PCR using N1 and N2 primers was possible even though these primers were not designed for multiplex RT-PCR. However, we were not able to perform a multiplex RT-PCR using M, H5, H7, N1 and N2 primers in a single reaction since the N1 and N2 primers showed non-specific priming when the optimal thermocycler conditions for the other three primer sets were used. We designed specific probes targeting the conserved sequences in neuraminidase gene of N1 and N2 subtypes using the sequences of the viruses used in evaluating the primers (Chander *et al.* 2010) and the specificity was verified by BLAST analysis. The specificity was also tested with various viruses available in our laboratory and we did not observe any cross hybridization or nonspecific reaction with these probes.

All five probes were amino modified at the 5' end to facilitate the covalent attachment to the carboxylated beads. A 6 carbon spacer was also attached to the 5' end to reduce steric hindrance from microspheres. And all the reverse primers were labeled with biotin at the 5' end since the strand generated by these primers had the complementary sequence to the probes.

Strand displacement amplification with the 5' degenerate primer was used in the early iterations of the assay to avoid multiplex RT-PCR, but the considerable bias in the amplification of various genes with very few copies of the target sequence, resulted in negative results even

with high concentration of virus. The majority of clones from the cDNA library had the polymerase gene and among the 691 sequences analyzed, there were only two copies of the matrix gene and there was no representation of the NS1 gene. Preferred amplification of certain genes was previously reported with strand displacement amplification (Abulencia *et al.* 2006). The size of the template, availability of the random primer and stochastic effects are the possible reasons for this bias. Differential cloning efficiency of the amplicons is another possibility. Interestingly, secondary structures in the genome were not considered as a cause for this bias (Abulencia *et al.* 2006). PCR selection due to the different binding energies of degenerate primers can cause significant bias in amplification of mixed templates. This is caused by the differences in the GC content of template and higher primer affinity for regions with higher GC content (Polz *et al.* 1998). The size of the template also seems to be important in biased amplification as 72% of our clones were PB2, which is the longest gene, and none had NS1, which is the shortest gene of influenza A virus. We are not sure why the length of the gene would bias either amplification or cloning.

A multiplex assay with the M, H5, H7, N1 and N2 probe coupled microspheres is feasible as TMAC in the hybridization buffer will equalize the melting temperatures of probes with different characteristics (Dunbar *et al.* 2006) and hence identical hybridization conditions can be used in the microsphere assay. Signal intensities of the probes in singleplex and multiplex assays were not significantly different. Dunbar (2006) suggested that the length and GC content of the probes and the length of the biotinylated amplicons can affect the outcome of the assay. The probes used in this study were 20 to 25 bases in length and the GC content varied between 41% and 65%, which corresponded to the probes in previous studies. Contradictory reports exist regarding the optimal amplicon length for hybridization. As per Diaz and Fell (2004), efficient

hybridization reaction occurs when the amplicon length is more than 600 bp whereas Etienne *et al.* (2009) reported that a 250 bp amplicon length was optimal for hybridization. Even though the length of the RT-PCR product of the different gene segments in this assay varied between 100 and 450 base pairs, a significant variation in MFI depending on the amplicon length was not noticed. Variation in MFI was observed with all five probes, but the fluorescence was always considerably higher for the positive samples when compared to the negative controls. This variation was not unexpected because it can occur due to differences in RNA extraction, RT-PCR amplification or the microsphere assay. Very high concentration of the amplicons can also result in variation of MFI as competitive hybridization of complementary strands can occur. In the assay described herein, the differences in amplification of different gene segments in the multiplex RT-PCR seems to be the major factor determining the outcome of the assay.

The diagnostic sensitivity of the assay was demonstrated by estimating the limit of detection for viral genome copies and correlated to virus titer in embryonating eggs. We found that the limit of detection of the assay depends mostly on the RT-PCR amplification. The assay developed in this study can detect $10^{2.5} – 10^{2.8}$ $EID_{50}$ of virus, whereas real time RT-PCR using the Matrix probe can detect $10^{-1}EID_{50}$ of virus (Spackman *et al.* 2002). However, when the RT-PCR was performed under similar conditions in a smart cycler, the probe coupled microspheres were found to be more sensitive in a singleplex format than real time RT-PCR. Amplicons in samples that were negative by real time RT-PCR could be detected by the microspheres. This difference between conventional thermocycler and real time RT-PCR is due to the increased amplification resulting from a rapid temperature change in the reaction mixture in the real time RT-PCR reaction tubes due to their high surface area to volume ratio. Moreover, the total volume of the real time RT-PCR reaction mixture is only half of that of conventional

thermocycler and hence the concentration of amplicons will be more in the post PCR sample. Since the amplification conditions for the primers are different, a multiplex real time RT-PCR could not be performed. Thus, we performed two separate reactions in the conventional thermocycler, one with M, H5 and H7 primers and the other with N1 and N2 primers, followed by mixing equal volumes of post RT-PCR product for the multiplex microsphere assay. Even though the amplification was lower with multiplex RT-PCR, the assay developed can detect and subtype avian influenza virus at clinically relevant concentrations.

The microsphere assay was able to detect the target sequence when at least 0.04ng (M) to 1.56g (N1) of cDNA was present. This corresponds to 3.71 $X10^8$, 6.07X $10^8$, 1.39$X10^9$, 3.27$X10^9$, and 2.3 $X10^9$ copies of biotinylated amplicons of M, H5, H7, N1 and N2 respectively. These limits of detection represent the quantity of cDNA in samples where MFI was at least two times higher than that of a negative control. A detection limit ranging from 6.08$x10^7$ to 7.58$x10^8$ copies was previously reported for *Trichosporon* species of yeast (Diaz *et al* 2004). Dunbar and Jacobson (2007) reported a detection limit of 5.9X $10^6$ to 3.2 X $10^7$ copies for *Escherichia coli* and *Vibrio parahaemolyticus* respectively. Our estimated detection limit is lower than these reported values; but when we tested the sensitivity of the microspheres with biotinylated oligonucleotides complementary to the probes, the microspheres where much more sensitive and the MFI with 5 femtomole of target was 45 to 50 times higher than that of the negative controls.

The amplification of the target gene using specific primers along with the detection by sequence specific probes results in almost 100% specificity. The probe coupled microspheres were hybridized with amplicons generated in both singleplex and multiplex RT-PCR using RNA extracted from avian influenza and other respiratory pathogens to test the specificity of the multiplex microsphere assay for the target. Each probe detected and differentiated the

homologous RT-PCR product within the mixture and no probe seems to interfere with the detection of any other probe. The assay appears to be reproducible since repeated assays performed with the same samples generated identical results.

A multiplex microsphere assay was developed as a diagnostic tool to detect avian influenza viruses in clinical samples. Known positive and negative samples from a previous study were used for the evaluation of the assay. From a total of 102 samples tested, 99 gave the same results with both the methods. Overall agreement between tests was calculated as the proportion of samples with concordant results out of the total number tested and the kappa value calculated to estimate the agreement between real time RT-PCR and multiplex microsphere assay was 0.76 showing substantial agreement between both assays (http://en.wikipedia.org/wiki/Cohen%27s_kappa accessed on May 30, 2010). Three samples positive by real time RT-PCR were negative by the microsphere assay. This difference in detection can be due to the higher sensitivity of real time RT-PCR compared to the microsphere assay. The low amplification by multiplex RT-PCR can result in these negative results. But when we conducted the microsphere assay using amplified product from the real time RT-PCR assay, a positive MFI was obtained. It is possible that sufficient quantity of virus was not present in the samples for multiplex amplification. Hence the overall sensitivity of the multiplex microsphere assay seems to be lower than that of the real time RT-PCR. In addition to the detection of avian influenza virus, the multiplex microsphere assay identified the N2 subtype of four of the five positive samples. The potential of the fiveplex assay for simultaneous detection of all avian influenza viruses and H5, H7, N1 and N2 subtypes was demonstrated by the three positive controls with H5N1, H5N2 and H7N3 viruses. The N1 probe showed some cross reaction with the positive control with H5N2 virus. The amplification of N2 gene as well as the MFI from

singleplex microsphere assay using N2 probe was lower for this virus sample when compared to other N2 subtype viruses even though sequence analysis of the amplicons did not show any variation in the sequence. Since this probe did not cross react with the other N2 viruses, we suspect the presence of some N1 virus in the sample. This illustrates the usefulness of the multiplex microsphere assay in diagnosing mixed infections.

The major advantage of the microsphere assay is its potential for multiplexing. Theoretically, 100 different analytes can be incorporated in a single reaction and hence there is considerable room for expansion of the assay. It is faster and easier than the conventional RT-PCR and sequencing to determine the subtype of the virus. It is also less laborious and time consuming than the traditional virus isolation and hemagglutinin inhibition tests. Although the multiplex microsphere assay is more time consuming and less sensitive when compared to the real time RT-PCR for avian influenza detection, it does offer the convenience of typing and subtyping the virus in a single reaction. Since multiple targets can be detected from a single reaction, doing multiple assays with the same sample can be avoided. The microsphere assay is a post RT-PCR detection method and hence the handling of RT-PCR product is necessary and therefore the chances of contamination are increased. Post RT-PCR processing of the sample takes almost 50 minutes and the Bioplex machine analyzes one sample in a fiveplex reaction in 30 seconds. Results from 96 samples can be obtained within 6 -7 hours. Even though the assay is more time consuming than real time RT-PCR, the diagnostic information obtained is significantly greater. Although the initial expenditure for the microsphere suspension array is high, the estimated total cost per sample is only $3.75 to $4.00 (Wallace *et al* 2005; Wilson *et al* 2005).

The ability of the multiplex assay to detect all avian influenza viruses and differentiate the H5, H7, N1 and N2 subtypes demonstrates its potential as a future diagnostic tool. A two step assay to type and subtype the virus will be ideal as it is less laborious and more economical. Samples can be tested for the presence of avian influenza virus using the M primers and probe in a singleplex assay whereas the subtyping of the virus can be performed for positive samples in a separate multiplex assay. Optimization of a multiplex RT-PCR for H5, H7, N1 and N2 will be the major barrier in developing this assay since the primers are not designed for multiplexing and hence further work is required to redesign or optimize the multiplex RT-PCR. Also, asymmetric RT-PCR using a higher concentration of biotinylated primer is recommended, as this will generate larger amounts of biotinylated amplicons and thereby increase the hybridization signal (Das *et al* 2006).

In conclusion, we have developed and evaluated a multiplex method to identify all avian influenza viruses and differentiate H5, H7, N1 and N2 subtypes. This method is rapid, sensitive and specific and at the same time simple to perform. The multiplexing ability of the microsphere system allows the simultaneous detection of multiple organisms or different subtypes of the same organism and the only requirement for expansion of the panel is the addition of specific probe coupled microsphere sets. In the future, this platform could be used to develop a pan respiratory virus assay for routine use in veterinary diagnostic laboratories for detection of all major avian respiratory pathogens.

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