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Comparative Analysis of Avian and Swine Influenza Viruses Infections of Well Differentiated Lung Epithelial Cells of Turkey

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ABSTRACT

Influenza viruses initiate infection by binding of the viral hemagglutinin to the cellular sialic acid residues. The precision-cut lung slice, as a valuable cultural tool of differentiated respiratory epithelial cells, is characterized by its ability to be viable for at least six days *in-vitro*, mimic *in-vivo* original cells and simply monitored by an inverted microscope. The aims of the study were to analyse the distribution of different sialic acid types in bronchus and parabronchial tissues of Turkey Precision Lung Slices (TPCLS), investigate the infection susceptibility of TPCLS by avian influenza (H9N2 and H7N7) and swine influenza (H3N2) viruses and evaluate the infection expression of TPCLS by different influenza viruses in correlation to the cellular sialic acids distribution after infection. The lectin stains and monoclonal antibodies prepared against nucleoprotein of influenza virus were used for analysing sialic acids distributions and viral antigen detection of TPCLS by immunoflourescent technique. The viral infective particles released from infected TPCLS by different avian and swine influenza viruses were titrated at different time intervals after infection. Both α2,3-linked and α2,6-linked sialic acids were expressed in the bronchus of TPCLS, while only α2,6-linked sialic acid was expressed in the parabronchial tissues. The indirect immunoflourescent technique showed variation of infection susceptibility of TPCLS parts by avian and swine influenza viruses. Infection was expressed in the bronchial epithelium by H9N2, H7N7 and H3N2, while in the parabronchial tissue by H9N2 and H3N2. Titration of the released infective viruses in the supernatant of infected TPCLS revealed that H9N2 could replicate faster than the other influenza viruses. TPCLS is a promising *in-vitro* model for viral infection study of turkey.

Introduction

Influenza viruses cause annual serious health threads worldwide not only for mammals but also for birds with high mortality and great economic losses. Influenza viruses, belong to *Orthomyxoviridae* family, are three types (A, B and C). The host restriction of influenza A is partially determined by distribution of specific receptors on the surface of the susceptible cells. These receptors, known as sialic acids, are composed of nine carbon monosaccharaides on the terminal position of glycan chain (Varki, 2007). Binding of the hemagglutinin (HA) of influenza viruses to sialic acid residues is the initial step of viral infection. Viruses isolated from different host species differ in their binding according to the type of sialic acids, e.g. N-acetylneuraminic acid (Neu5Ac), or the types of linkage connecting the sialic acid molecule to the neighboring sugar. Influenza viruses isolated from mammalian hosts prefer alpha 2,6-linked sialic acids whereas most avian influenza viruses preferentially recognize Neu5Ac attached via an alpha 2,3-linkage to galactose (Rogers and Paulson, 1983; Rogers *et al.*, 1983).

Avian viruses of the H9 subtype are an excep-

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tion, because some strains of H9 influenza viruses have been shown to favor alpha 2,6-linked sialic acids (Matrosovich *et al*., 2001; Saito *et al*., 2001).

Analysis of the sialic acids distribution in different organs of some poultry species showed that duck and geese expressed mostly alpha 2,3 linked sialic acids in the respiratory tract and marginal expression of alpha 2,6 linked sialic acids of the colon, while pheasant, turkey, quail and guinea pig fowl expressed both avian and human influenza receptors in the respiratory and digestive tracts. The presence of both types of sialic acids receptors in poultry species present in farms and markets makes them as potential mixing bowl species (Kimble *et al*., 2010).

Several antigenic changes are usually required for the avian influenza viruses to be able to infect human mostly in the HA gene, either by specific mutations known as antigenic drift or recombination with other virus known as antigenic shift. For these changes to be occurred, mixing bowl species, facilitate the processing of infections, as the mixing bowl is a suitable accommodator for both of alpha 2,3-linked sialic acids and 2,6-linked sialic acids receptors, as in swine (Ito *et al*., 1998; Scholtissek *et al*., 1998; Kimble *et al*., 2010). Detection of natural infection of turkey by swine influenza virus H3N2 has been reported in United States of America at 2004 (Choi *et al*., 2004), while, H1N1 and H3N2 infections have been detected in Minnesota in 2012 (Corzo *et al*., 2012). The epidemiological studies of the infection of turkey and chicken by H7N7 virus in the Netherlands during the outbreaks of 2003, highlighted the high susceptibility of turkey for infection by H7N7 (Elbers *et al*., 2010).

Using of *in-vitro* well differentiated epithelial cells derived from airway tissues has been used as a laboratory model for *in-vivo* target organs to study the viral replication cycles and analyse the respiratory viral infection. Precision-cut lung slices (PCLS) prepared from different animals, i.e. bovine, chicken and swine have been established for infection studies (Goris *et al*., 2009; Abd El Rahman *et al*., 2010; Punyadarsaniya *et al*., 2011).

The objectives of this study were to report a new primary tissue cultures of turkey differentiated respiratory epithelial cells (TPCLS), study the distribution of both alpha 2,3 and alpha 2,6-linked sialic acids on TPCLS epithelial cells and analyze the infections susceptibility of lung epithelial cells by two avian influenza and a swine influenza A viruses.

Materials and methods

Virus propagation

The used avian influenza viruses, of low pathogenic effect, were H9N2 and H7N7 subtypes, while the used swine influenza virus was H3N2 subtype. H9N2 (A/chicken/Saudi Arabia/CP7/98) was kindly provided by Hans-Christian Philipp, Lohmann Tierzucht, Cuxhaven, Germany), H7N7 (A/duck/Potsdam/15/80) was given by Friedrich-Loeffler-Institut, Insel Riems, German and H3N2 (A/sw/Bissendorf/IDT1864/2003) was provided by Ralf Dürrwald, IDT Biologika GmbH, Dessau-Rosslau, Germany.

All used viruses were propagated by allantoic cavity inoculation of 100 µl viral stock suspension using specific pathogen free chicken eggs of 10 days old embryos (VALO Biomedia, Cuxhaven, Germany). The collected allantoic fluids were centrifuged at 2000 rpm/min for 10 min., and the supernatants were re-centrifuged at 2000 rpm/min for 10 min., and kept at -80ºC.

Plague assay for viral titration

Eagle's minimal essential medium (EMEM) containing acetyl trypsin 1mg/ml (Sigma-Aldrich, Munich, Germany), 5% fetal calf serum (Biochrom AG, Berlin, Germany), penicillin and streptomycin was used for cultivation of MDCK cells at the bottom of wells plates. For cell infection, the collected supernatants were serially diluted, inoculated in MDCK cells for one hour on a shaker and incubated for 2-3 days. EMEM supplemented with avicel microcrystalline cellulose RC 581 (2.5%; FMC Biopolymer, Brussels), glutamine (GIBCO BRL Life technologies) and bovine serum albumin fraction V (0.2%; AppliChem) was added for each well (3 ml/well). Formaldehyde containing 1% crystal violate was used for fixation and staining of the infected MDCK cells. The calculation of the vial titers was expressed as plaque-forming units per ml (PFU/ml) according to Punyadarsaniya *et al*. (2011).

Preparation of Turkey Precision-cut lung slices (TPCLS)

The embryos of 24 days-old turkey eggs were decapitated and carefully injected by low melted agarose, using a needle fixed in the trachea, to the lungs of the embryos. The injected lungs were kept on ice to stabilize the lung tissue. Using a Krumdieck tissue slicer machine, the lungs were cut into approximately 200-µm-thick slices. The slices were washed thrice by PBS to remove the agarose and incubated at 37°C in eDulb medium (Gibco/Invitrogen). As described by Goris *et al*. (2009), TPCLS viability was analyzed by monitoring the ciliary activity using a light microscope (Zeiss Axiovert 35) with an ORCA C4742-80 digital camera (Hamamatsu), SIMPLE-PCI and analysis software (Compix Imaging systems). Some slices of clear bronchial lumen were selected for analyzing the physiological bronchoconstriction caused by methacholine (acetyl-ß-methylcholine chloride, Sigma Aldrich). The integrity of the cells of TPCLS was also investigated using a Live/Dead viability/cytotoxicity kit (Fluo Probes, FPBE4710).

Viral infection

To analyze the affinity of TPCLS cells for infection by avian and swine viruses, four TPCLS, per each virus, were kept separately in wells of a 24-well plastic plate and infected by different avian influenza viruses (H9N2 and H7N7) and swine influenza virus (H3N2) in a volume of 500 µl/slice at $1x10^5$ PFU/ml in eDulb medium. After incubation for 24 hours at 37°C, the slices were mounted on a small filter paper with tissue-freezing medium (Jung, Heidelberg, Germany), frozen in liquid nitrogen and kept at -80°C. Cryosections of TPCLS were cut at 10 um thickness by a cryostat machine (Reichert-Jung, Nußloch, Germany). The sections were dried overnight at room temperature and kept frozen at -20°C until staining (Punyadarsaniya *et al*., 2011).

Analysis of cryosections by Immunofluorescence assay

The fixed cryosections, by 3% Paraformaldehyde solution for 20 min., were permeabilized by 0.2% Triton X-100 for 5 min. and washed three times with PBS. For detection of infection, a monoclonal antibody against NP of influenza virus prepared in mice (AbDSeroTec, Düsseldorf, Germany) was diluted as 1:750 in 1% bovine serum albumin (BSA) and incubated on the slides for 1 hour at room temperature in a humid chamber followed by washing three times by PBS. Diluted FITC-conjugated anti-mouce IgG antibody (AbDSeroTec, Düsseldorf, Germany) in BSA was added for 1 hour in a humid dark chamber at room temperature. FITC labeled Sambucus nigra agglutinin (SNA) (Vector laboratories, Burlungame, USA) was used for one hour in dark humid chamber for detection of α2,6-linked sialic acids, while the biotinylated Maackia amurensis lectin II (MAAII) was used to determine a2,3-linked sialic acids after pre-incubation with the Avidin/Biotin blocking kit (both from Vector Laboratories, USA). The binding of biotinylated antibodies were detected by slides incubation with streptavidin-Cy3-FITC (Sigma-Aldrich). Antibodies, prepared against cellular ß-tubulin and mucin, were used for staining of ciliated and mucus-producing cells of TPCLS, respectively (Castrucci *et al*., 1994; De Jong *et al*., 1997; Marozin *et al*., 2002; Schrader and Suss, 2003). The slides were incubated with a Cy3-labeled monoclonal antibody (Sigma- Aldrich) recognizing β-tubulin. Goblet cells were stained by mucin-5AC antibody (Santa Cruz Biotechnology), followed by incubation with an anti-rabbit IgG secondary antibody (Sigma-Aldrich). All slides were washed three times by PBS, embedded in Mowiol and examined under the confocal microscope.

Analysis of Viral shedding

To assess viral shedding, 100 µl of the supernatant were collected and pooled from viral infected and uninfected TPCLS (5 slices for each virus) at different interval times (0, 8, 24, 48, 72 and 96) hours post-infection and stored at -80°C. All supernatants were titrated on MDCK cells in 96-well plates by plaque assay as described by Goris *et al*. (2009).

Results

Establishing of well differentiated respiratory epithelial cells from the lung of Turkey's embryos (TPCLS)

Well differentiated epithelial cells of the respi-

ratory system parts as trachea and lung of some animal species and chicken have been modulated. For scoping on the role of turkey in infection and transmission of influenza viruses, we have used the technique of precision-cut lung slices (PCLS) in turkey. The *in-vitro* system of TPCLS represents the natural cell types and closely mimics the *in-vivo* arrangement of the cells. We have prepared the TPCLS from embryos immediately before hatching and analyzed the viability of the cells via daily observation of ciliary activity, which expressed complete ciliary movement for seven days post preparation with a day interval medium changing. By live/dead viability/cytotoxicity assay, the cellular viability of TPCLS expressed mostly living cells in both of bronchus and the parabronchial tissues and rare expression for the dead cells at different time intervals after preparations (data not shown). Furthermore, detecting the physiological response of the sections, as a criterion for the vitality of TPCLS, was analyzed using methacholine. TPCLS responded to the action of methacholine as bronchoconstriction that can be induced in a reversible manner (Fig. 1A & B). Addition of methacholine on TPCLS resulted in bronchoconstriction of the TPCLS bronchus within 30 seconds after addition (Fig. 1B). When the effect of the methacholine was finished from the sections, the bronchodilataion appeared again in few seconds (Fig. 1A). The cellular epithelium of the bronchus of TPCLS has been characterized by immunoflourescent technique for β- tubulin which showed ciliated cells stained by red color (Fig. 1C), and the anti-mucin for detecting mucous producing cell which appeared green in color (Fig. 1D), these cells showed clear mucous production.

Lectin histochemistry of TPCLS showed different distributions of sialic acids types

Expression of different types of sialic acids either α2,3-linked or α2,6-linked sialic acids was observed in different parts of TPCLS at the level of bronchus and parabronchial tissues (Fig. 2A-2F). The bronchus of TPCLS showed abundant expression of both types of sialic acids using MAA II for α2,3-linked sialic acids appeared as red in color and SNA for α2,6-linked sialic acids, which appeared as green in color (Fig. 2A). The apical surface of the ciliated cells expressed mostly α2,3-linked sialic acids (Fig. 2B), while the lower cells of the bronchial epithelium expressed mostly α2,6-linked sialic acids guided by the ciliated cells which appeared as red in color (Fig. 2D). The parabronchial epithelium of TPCLS showed no expression for α2,3-linked sialic acids (Fig. 2C) while showed abundant expression of α2,6-linked sialic acids (Fig. 2E). Analysis of sialic acids expression by immunoflourescent techniques at the bronchus and the parabronchial levels of TPCLS showed abundant expression of both types of α2,3-linked and α2,6-linked sialic acids in the bronchus of TPCLS than in the parabronchial tissues as well, only α2,6-linked sialic acids expressed abundantly in the parabronchial tissues and no expression of α2,3-linked sialic acids.

Susceptibility of TPCLS for infection by avian and swine influenza viruses

TPCLS were infected by different avian and swine influenza viruses, comparatively evaluated for their susceptibility for infection by these viruses

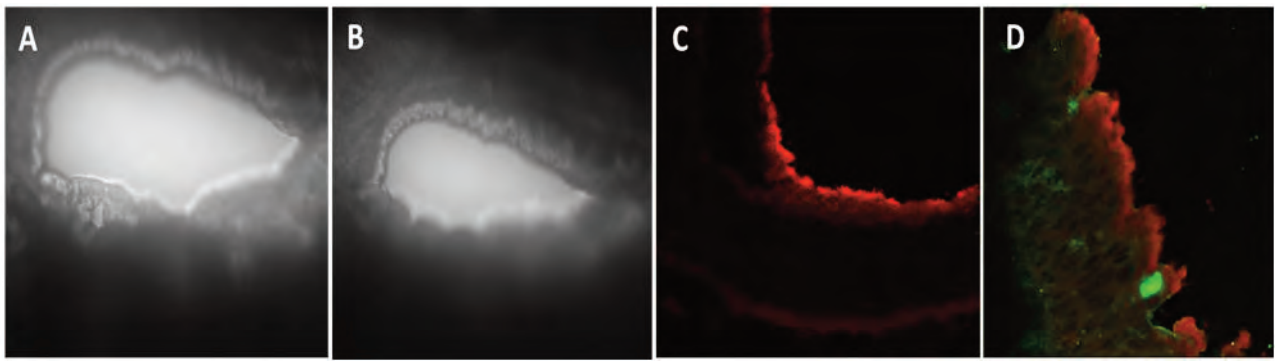

Fig. 1. Characterization of Turkey Precision Cut Lung Slices (TPCLS): A) Bronchodilatation of TPCLS. B) Bronchoconstriction after Methacoline addition. C) The ciliated cells distribution on the bronchus of TPCLS (red). D) The mucous producing cell on the bronchus of TPCLS (green) and the ciliated cells appear (red) in color.

at the level of bronchus and parabronchial tissues and recognized the sialic acids expression post infection. Interestingly, TPCLS could be infected by both avian and swine influenza viruses with differences in the affinity of the parts of TPCLS for infection and the abundance of infections. Infection of TPCLS at the level of bronchial epithelial cells was guided by ciliated cells appeared as red in color and infection appeared as green in color (Fig. 3), H9N2 could infect TPCLS bronchial epithelium extensively comparable to H7N7 and H3N2, and closer to the apical cells of the bronchus, while H7N7 and H3N2 infections were relatively at lower bronchial epithelium (Fig. 3, upper panel), non-infected control slices showed no viral antigen expression (Fig. 3, upper panel). The infection of TPCLS by influenza viruses was detected not only in the bronchus but also in the parabronchial tissues, H3N2 showed extremely more infection than H9N2, while H7N7 showed no infection in the

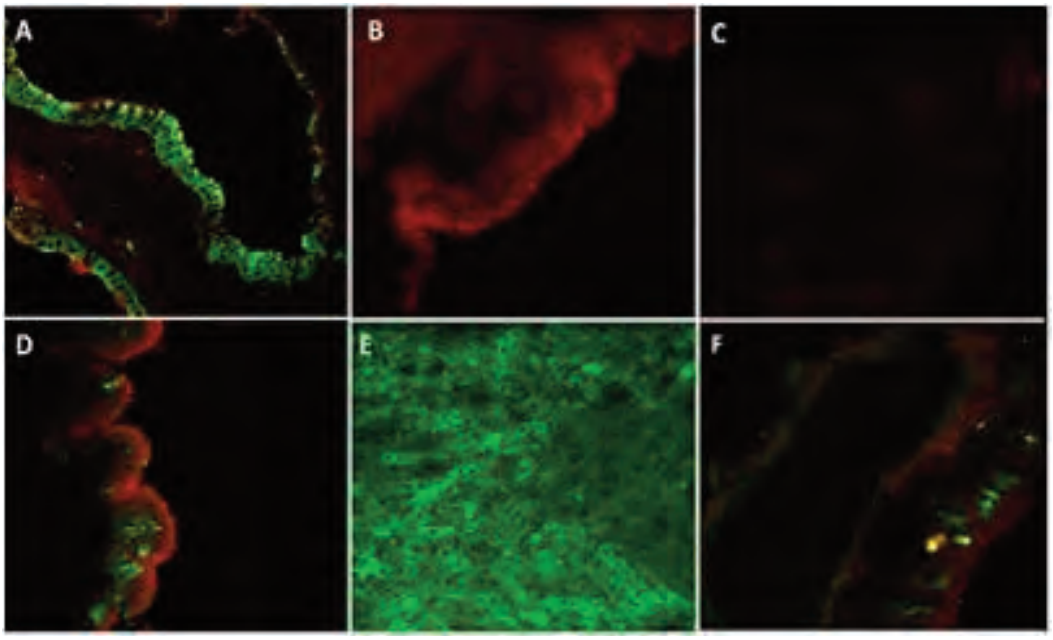

Fig. 2. Distribution of sialic acids types in the bronchus and parabronchial tissues of TPCLS: A) α2,3-linked (red) and α2,6-linked (green) sialic acids expression in bronchus of TPCLS. B) Expression of α 2,3-linked sialic acids (red) in the epithelial cells of the bronchus C) The parabronchial epithelium of TPCLS showed no expression for α2,3-linked sialic acids D) The lower cells of the bronchial epithelium expressed mostly α2,6-linked sialic (green) and the ciliated cells (red). E) Abundant expression of α2,6-linked sialic acids (green) in the parabronchial tissues. F) The mucous producing cell of the bronchus (red) expressed α2,6-linked sialic of bronchioles (green).

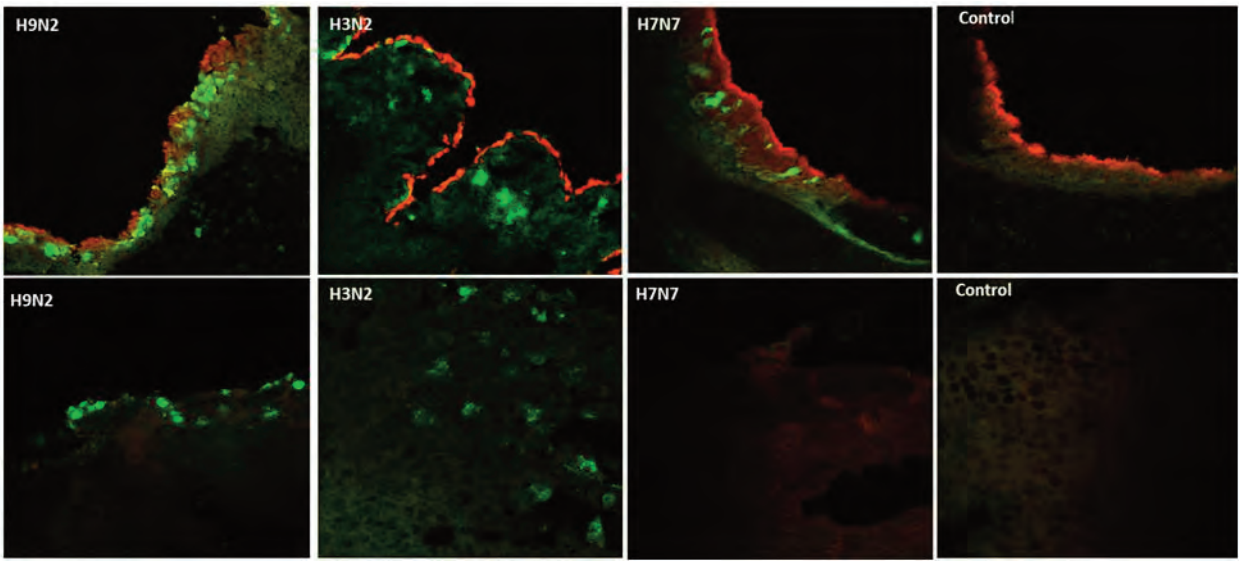

Fig. 3. Infection of TPCLS by influenza viruses at the bronchus and parabronchial tissues: Upper panel; Infection of TPCLS bronchial epithelial cells (green) referred by ciliated cells (red). Lower panel; Infection of TPCLS (green) at the parabronchial epithelial cells guided by MAAII (red).

parabronchial tissues (Fig. 3, lower panel). Comparing the infection of different influenza viruses in the bronchus and parabronchial tissues of TPCLS, revealed abundant infection of bronchus than parabronchial tissue by avian influenza viruses, while more infection was observed in the parabronchial tissues by swine influenza virus (Table 1).

Expression of sialic acids after infection of TPCLS by influenza viruses

The lectin histochemistry expression of α2,3-linked and α2,6-linked sialic acids in TPCLS was correlated with viral infection of TPCLS by H9N2, H7N7 and H3N2 (Figs. 4 & 5). Based on fluorescent analysis of the infected bronchus of TPCLS (green) and MAA II (red), infected TPCLS by H9N2 expressed lower signals of α2,3-linked sialic acids comparable to infections by H7N7 and H3N2. The bronchial epithelium of infected TPCLS by H3N2 showed no clear reduction in the expression of α2,3-linked sialic acids (Fig. 4). The reduced expression of α2,3-linked sialic acids in TPCLS bronchus post infection by different influenza viruses was scored (Table 1). The expression of α 2,6-linked sialic acids (green) was observed in the infected bronchial epithelial cells of TPCLS (red) (Fig. 5, upper panel) and infected parabronchial tissue (red) (Fig. 5, lower panel). We could detect low expression of SNA at the epithelial cells of bronchus and parabronchial tissues infected by H9N2 and H3N2. TPCLS infected by H7N7 expressed no signals for infection in the parabronchial tissue although clear infection was detected in the bronchial epithelial cells with no reduction for α2,6-linked sialic acids at the apical surface of the bronchus.

TPCLS were highly infected by H9N2 than other influenza viruses

To study the efficiency of TPCLS for viral infection and compare the virulence of the different avian and swine influenza viruses, the infectious particles released in the supernatant of infected

Table 1. Scoring the infection susceptibility of TPCLS by influenza viruses at bronchial and parabronchial tissues and the sialic acid expression s post infections.

Influenza subtype	Bronchial infection	Parabronchial infection	α2,3-linked sialic acid	α2,6-linked sialic acid	
			Bronchus	Bronchus	Para-bronchus
H9N2	+++	+	+	+	++
H7N7	++	–	+	++	+++
H3N2	+	++	++	+	+
Control	–	–	–	++	+++

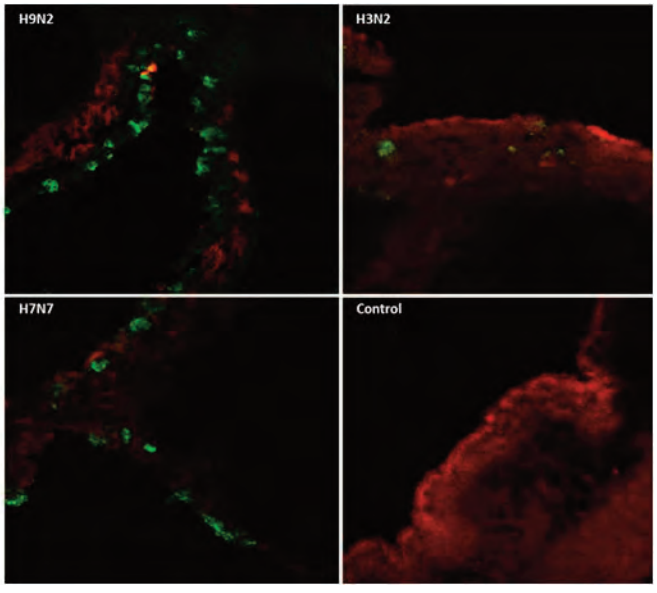

Fig. 4. Infection of the bronchial epithelium of TPCLS by influenza viruses (green) with expression of α2,3-linked (red).

TPCLS were titrated at different time intervals 0, 8, 24, 48, 72 and 96 hours post infection (Fig. 6). The avian influenza (H9N2) serotype showed higher production of infective viral outcome in infected TPCLS comparable to the other influenza viruses. At 24 hours post infection, both of H9N2 and H7N7 showed significant increase in the released virus particles comparable to H3N2, ranging from $1x10^5$ to $1x10^7$ $TCID_{50}$/ml while H3N2 showed about $0.5x10^4$ $TCID_{50}$ /ml. The maximum titer for both avian influenza viruses ($1x10^6$ to $1x10^8$ $TCID_{50}$/ml) was produced at 72 hours post infection, showing that H7N7 expressed two 10-log fold serial dilutions lower than H9N2, while the maximum titer of the swine influenza virus was produced at 48 hours post infection as $1x10^5$ $TCID_{50}$/ml then started to decrease. Comparing the viral outcome of infected TPCLS by H9N2 virus to the other avian influenza H7N7 and swine influenza H3N2 viruses, showed that H9N2 is the fastest and highest virulent serotypes to TPCLS than the other viruses.

Discussion

Investigating the role of turkey as possible mixing bowls in transmission of influenza viruses to human and emerging of highly pathogenic influenza viruses still needs more explanation and evaluation for the distribution of the sialic acids receptors of influenza viruses in turkey. In this study, we have established a new model of well differen-

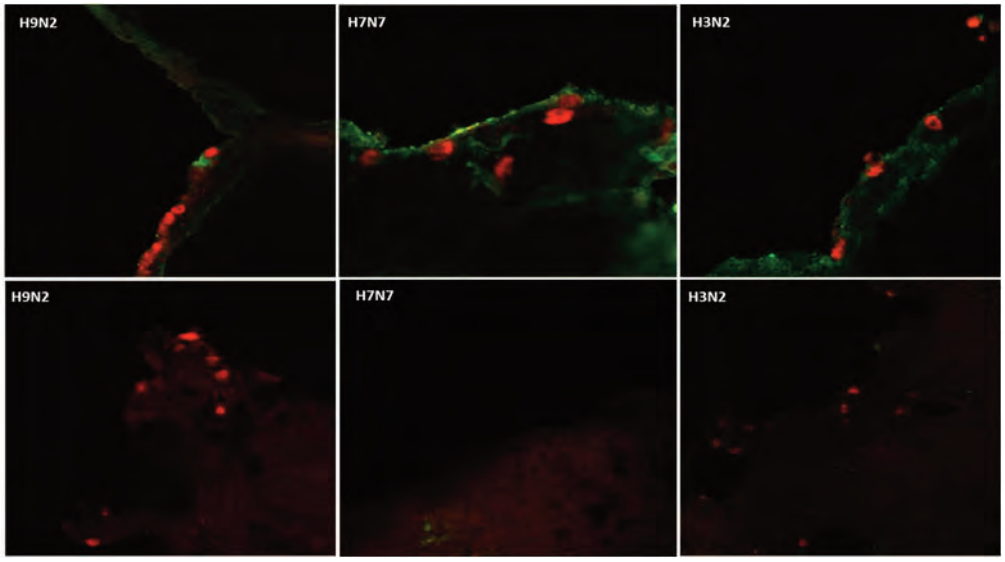

Fig. 5. TPCLS infected by influenza viruses in the bronchus and parabronchial tissues with expression of α2,6-linked sialic acids: Upper panel; Infection of TPCLS bronchial epithelial cells (red) and α2,6-linked sialic acids (green). Lower panel; Infection of TPCLS at the parabronchial epithelial cells (red) and α2,6-linked sialic acids (green).

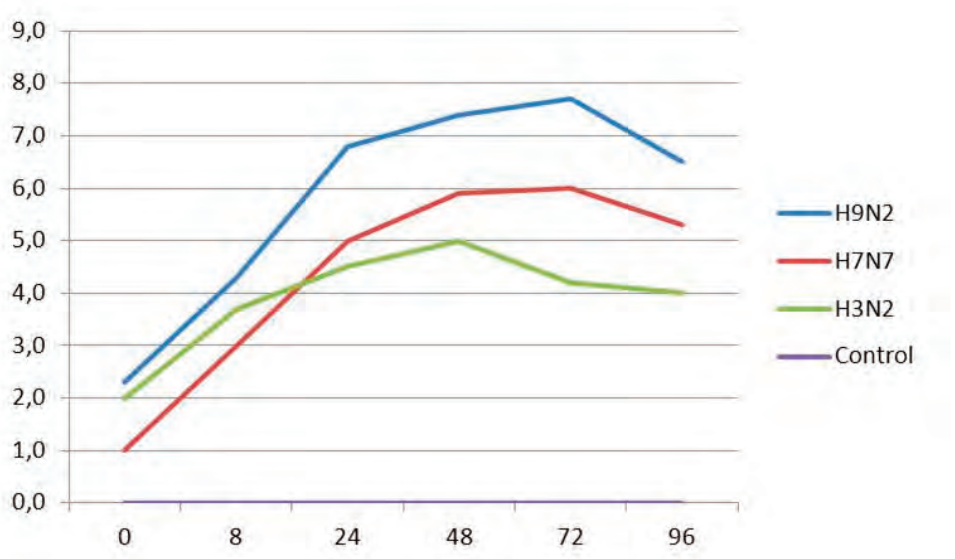

Fig. 6. The titration curve of infectious viruses released in the supernatants of TPCLS infected by Influenza viruses

tiated epithelial cells from the lung of the turkey embryos (TPCLS) as an *in-vitro* system to be used for host-virus relationship studies. Our new model showed all the anatomical, physiological and biological properties of the *in-vivo* lung of turkey (Goris *et al*., 2009; Abd El Rahman *et al*., 2010; Punyadarsaniya *et al*., 2011). The viruses used for the infection of the TPCLS are avian influenza viruses (H9N2 and H7N7) and swine influenza virus (H3N2), all of current circulating viruses that cause economic losses in farms (Homme and Easterday, 1970; Haesebrouck *et al*., 1985). The lectin staining of the bronchus and parabronchial tissues used for characterizing the distribution of both α2,3-linked and α2,6-linked sialic acids in the tissues of TPCLS showing that turkey had the two types of sialic acids in different ratios as obtained from *in-vivo* characterization of distribution pattern of influenza virus receptors by Kimble *et al*. (2010) and Pillai and Lee (2010). More expression of α2,3-linked sialic acids appeared in the bronchus but no signals appeared in the parabronchial tissues (Costa *et al*., 2012), while α2,6-linked sialic acids appeared more in the parabronchial tissue than in bronchial tissues of TPCLS. This might explain the ability of turkey to be infected by both avian and swine influenza viruses, and alert from the role of turkey to act as a possible vector for different Influenza viruses. TPCLS model showed expression of infection for all used influenza viruses with differences in the susceptibility to the parts of lung tissues reported in an *in-vivo* experimental infection of turkey by avian-origin H5N4 and human-origin H1N1 influenza viruses (Costa *et al*., 2012). Some field isolates of H9N2 could acquire human virus-like receptor specificity, preferentially binding α2,6-linked sialic acids (leucine (L) at position 226 in the Haemagglutinin (HA), in contrast to the classic avian virus-like receptor specificity that preferentially binds α2,3-linked sialic acids [glutamine (Q) at position 226 in the HA] (Matrosovich *et al*., 2001; Saito *et al*., 2001; Choi *et al*., 2004; Wan and Perez, 2007). H7N7 influenza virus could infect mainly the bronchial epithelium of TPCLS which expressed abundant α2,3-linked sialic acids specially at the apical border of the bronchus expressing no infection signals at the parabronchial tissues, while H3N2 swine influenza virus expressed more infection in the parabronchial tissues which is mostly rich by α2,6-linked sialic acids. The possibility of TPCLS to be infected by both of avian and swine Influenza viruses might highlight the potential role of turkey in emergency of new high pathogenic viruses due to re-assortment of influenza viruses from different origins which in turn resulting in emergency of new strains of altered receptor specificity including human-like receptor binding (Wan *et al*., 2008). A clear reduction of both types of sialic acids after infection by different viruses at the level of bronchial and parabronchial tissues of TPCLS, this might indicate successful binding of the avian influenza viruses and consuming the sialic acids distributed on the cell membrane of TPCLS during the replication steps of influenza viruses. Studying the outcome of this replication was investigated by titrating the infectious virus particles released in the supernatant of TPCLS infected by different influenza viruses which confirmed higher susceptibility of H9N2 to TPCLS system more than H7N7 and H3N2 at different time intervals post infections. The "mixing vessels" host for influenza viruses was mainly reported in pig but our results showed also the possibility of turkey to act the same role of pigs.

Conclusion

TPCLS as a well-differentiated epithelial cell culture of turkey's lung is a useful tool representing the *in-vivo* original tissues, could be used for infection studies and is recommended to be used for other pharmacological and toxicological studies. The susceptibility of TPCLS to infection by avian and swine Influenza viruses may highlight the role of turkey to be as a potential vector of new variant influenza viruses production.

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