Oseltamivir activity against avian influenza H9N2 strain with different point mutations in their neuraminidase

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Summary
The present study has two aims: to optimize the antiviral activity of oseltamivir in chicken embryos against an avian influenza-H9N2 strain (P0) and to apply the optimized protocol for studying the drug susceptibility of 4 H9N2 mutants (M1, M2, M3, and M4). As for the first aim, oseltamivir antiviral activity was monitored upon its delivery into 9-day-old chicken embryo at a concentration of 0.27 mg/100 µl, against 7 doses of the P0 strain, ranging between 1.2×10^{-5} and 2.0 Hemagglutination (HA) units. Oseltamivir showed its highest efficacy in reduction of viral propagation (95% reduction in HA titer) ($P < 0.05$), when the inoculum level contained a minimum HA units of 1.2×10^{-5}. For the second aim of this study, the application of the 1.2×10^{-5} HA units of the virus in inocula for the evaluation of oseltamivir-antiviral effect against the 4 H9N2 mutants revealed an emergence of a resistant mutant (M1), associated with 2 adjacent point mutations in its neuraminidase (N) amino acid (aa) sequence at positions 46 and 47. The other 3 mutants maintained a variable sensitivity to oseltamivir, resulting in the following reduction in HA titers: M2 (82.9%), M3 (61.5%), and M4 (100.0%). How the point mutations of the neuraminidase sequences affected the susceptibility of H9N2 virus to oseltamivir is still to be determined and deserve further investigations.

Efficacia di oseltamivir sul ceppo H9N2 del virus dell'influenza aviaria con differenti punti di mutazione nella neuraminidasi

Parole chiave
Crescita embrionale,
Emoagglutinazione (HA),
H9N2,
Influenza aviaria,
Mutazione,
Neuraminidasi,
Oseltamivir.

Riassunto
Lo studio ha avuto gli obiettivi di ottimizzare l'attività antivirale di oseltamivir in embrioni di pollo contro ceppi H9N2 del virus dell'influenza aviaria e di applicare il protocollo ottimizzato per lo studio della sensibilità ai farmaci di 4 ceppi H9N2 mutanti (M1, M2, M3 e M4). Per quanto concerne il primo obiettivo, l'attività antivirale di oseltamivir è stata monitorata dal momento dell'inoculazione in uova embrionate, ad una concentrazione di 0,27 mg/100 ul/embrioni di pollo di 9 giorni, e per ognuno dei 7 livelli di densità del ceppo P0, compreso tra $1,2 \times 10^{-5}$ e 2,0 unità di emoagglutinazione (HA). Oseltamivir ha mostrato la sua massima efficacia nella riduzione della propagazione virale (riduzione del 95% del titolo di HA) ($P < 0,05$), quando il livello di inoculazione è risultato contenere un minimo di unità di HA di $1,2 \times 10^{-5}$. Per quanto riguarda il secondo obiettivo dello studio, l'applicazione di $1,2 \times 10^{-5}$ unità di HA del virus in inoculi per la valutazione dell'effetto antivirale dell'oseltamivir nei confronti dei ceppi H9N2 mutanti ha rivelato una mutazione resistente (M1) associata a 2 siti della neuraminidasi alle posizioni 46 e 47 della sequenza aminoacidica. I restanti ceppi mutanti hanno mantenuto

la loro sensibilità variabile all'oseltamivir, generando la seguente riduzione percentuale del titolo HA: M2 (82,9%), M3 (61,5%) e M4 (100,0%). Il numero e la posizione delle mutazioni puntiformi nella neuraminidasi dei 4 ceppi mutanti diversi sembrano determinare la sensibilità del ceppo H9N2 a oseltamivir.

Introduction

The avian influenza (AI) virus, subtype H9N2, is included by the World Health Organization in the list of AI-aetiologies causing zoonoses[1]. The ability of H9N2 virus in adapting to different animal hosts (Parrish and Kawaoka 2005, Peiris *et al.* 2009) and in causing an increasing frequency of human infections, as reported from different parts of the world (Peiris *et al.* 1999, Iqbal *et al.* 1999, Van Kerkhove *et al.* 2011, Hadipour 2011), is alarming.

Most veterinary and human medical laboratories include the antimicrobial susceptibility testing of bacteria and fungi in their routine analytical protocols, while a few laboratories are equipped with facilities for anti-viral susceptibility testing. This fact is hindering the availability of information related to the changing susceptibilities of economic viruses to anti-viral drugs available in the global market (Air and Brouillette 2009).

Different reliable methods for testing the viral susceptibility to available medications have been described in the relevant literature. These include methods like reacting the virus and the medication in cell culture systems (Sgarbanti *et al.* 2011, Muratore *et al.* 2012), in different animal models, including mice (Sauerbrei *et al.* 2003, Sawamura *et al.* 2010), rats (Ottolini *et al.* 2003), hamsters (Renis 1977, Ishii *et al.* 1996, Morrey *et al.* 2004), monkeys (Uckun *et al.* 1997, Annamalai *et al.* 201), and in less costly biological systems, *i.e.* embryos (Haertl *et al.* 2004, Sauerbrei *et al.* 2006). The work of Sauerbrei and colleagues in 2006 was instrumental in justifying the embryo model, due to the reliability of the protocol that utilized embryonated chicken eggs for *in vivo* evaluation of the anti-influenza virus activity of neuraminidase inhibitors (Sauerbrei *et al.* 2006).

Many zoonotic viruses can be propagated in chicken embryos (Sauerbrei *et al.* 2006, Crespo *et al.* 2009, Kwon *et al.* 2009, Boynukara *et al.* 2011), prompting research to develop and adapt this low-cost system to assess the susceptibility of viruses to medications (Rungrotmongkol *et al.* 2009, Stoner *et al.* 2010). Among these, the avian influenza virus can propagate in chicken embryos and could be included in the protocol of antiviral susceptibility assessment in such embryos (Sauerbrei *et al.* 2006, Wang *et al.* 2008). It is always essential to optimize such protocols, due to differences in egg-breed, the nature of the viral strain, and the maternal immunity in the egg embryos (Rungrotmongkol *et al.* 2009, Wang *et al.* 2008). The aim of the optimization is to reach a high sensitivity level able to maximize the reduction of viral replication in the embryonated eggs treated with the antiviral drug compared to the untreated eggs (Rungrotmongkol *et al.* 2009).

Studies conducted in different parts of the world document the ability of H9N2-AI to mutate (Song *et al.* 2011, Tombari *et al.* 2011), especially under the effect of serial passages (Wu *et al.* 2009), vaccination (Lou *et al.* 2009), or other factors (Kimble *et al.* 2001, Peiris *et al.* 2001). Some of the reported H9N2 mutants acquired higher pathogenicity (Shaib *et al.* 2011a, Shaib *et al.* 2011b), while others are emerging as mutants resistant to commonly used anti-influenza drugs (Wang *et al.* 2002, Giria *et al.* 2012).

The emergence of H9N2 strains with mutations in their neuraminidase could result in acquiring resistance to most common drugs used for treating influenza, namely the oseltamivir (Gubareva *et al.* 2002). This situation requires a global search for such strains, to help in building effective strategies to eliminate them from their ecological niches, especially from the poultry host (Swayne and Halvorson 2012). The identification of drug resistant-H9N2 viruses in poultry will help in future development of avian vaccines that include in their seeds the highly pathogenic and drug-resistant strains. The objectives of this research are to optimize the antiviral activity of oseltamivir in chicken embryos against an initial AI-H9N2 virus (P0) (Sauerbrei *et al.* 2006) and to apply the optimized protocol to study the drug susceptibility of four H9N2 mutants, emerged by passaging the P0 strain in adult chicken, thus acquiring different numbers and positions of point mutations in their neuraminidase.

Materials and methods

H9N2 Strains

In 2005 a H9N2 avian influenza virus (P0) was isolated from the trachea of a broiler during an

[1] http://www.who.int/zoonoses/diseases/animal_influenza/en/.

outbreak, which involved farmers and pigs that were in contact with the infected flocks (Barbour *et al.* 2006). Four mutants (M1, M2, M3, and M4) of the initial strain emerged upon different number of passaging of the P0 virus in 21 day-old broilers (Shaib *et al.* 2011b), as demonstrated in Table I. The recovery of the H9N2 mutants was either from lung, trachea, or thoracic air sac of the broilers. The percentage of similarities between the sequence of the mutants' neuraminidase amino acids, located between positions 32 and 104, and those of the P0 was between 83.6-98.6%, with respective different number of neuraminidase-point mutations in mutants M1, M2, M3, and M4 equivalent to 2, 1, 3, and 12 mutations (Table I) (Shaib *et al.* 2011).

Optimization of initial P0 H9N2 susceptibility to oseltamivir

The optimization of the sensitivity of the initial P0-H9N2 isolate to oseltamivir in 9-day-old chicken embryos was accomplished according to the previously reported protocol (Sauerbrei *et al.* 2006), using different titers of H9N2 viral inocula, while keeping constant the amount of oseltamivir injected in each embryonated egg.

Nine day-old chicken embryos were divided into 16 groups, with 4 embryos per group. Seven groups of eggs received oseltamivir (F. Hoffmann. La Roche Ltd, Basel, Switzerland), dissolved in sterile distilled water, delivered through the shell, at the pointed end of the egg, in a constant level equivalent to 0.27 mg/100 µl inoculum/egg, as recommended by the protocol (Sauerbrei *et al.* 2006). The 7 groups received the following respective levels of HA units of the P0-H9N2 through the allantoic route, namely 1.2×10^{-5}, 1.2×10^{-3}, 1.2×10^{-1}, 2.5×10^{-1}, 5.0×10^{-1}, 1.0, and 2.0. The challenge dose was administered 30 minutes after the antiviral treatment. Other 7 groups of the embryonated eggs received the same amount of P0-H9N2, but were deprived of the oseltamivir treatment. Two control groups were included. The embryonated eggs of the first control group received the same constant level of the oseltamivir, while the second control group was deprived of this medicine. Both control groups were deprived of P0-virus inoculation. The 16 groups of embryonated eggs were incubated at 37.7°C and 59% relative humidity for 3 days, then cooled at 4°C for 3 hours. The eggshells were removed from the aircell side and the allantoic fluid was collected from each individual egg, and stored at - 80°C until the determination of the hemagglutination (HA) titer. Each embryo was weighed and the mean weight per each of the 16 treatments calculated.

Table I. *Emerging H9N2 mutants in respiratory organs upon passaging of the initial strain (P0-H9N2) in 21-day-old broilers.*

Initial (P0-H9N2) and its mutants (M)[a]	Number of passages in 21-day-old broilers	Recovery from respiratory organ	% similarity to neuraminidase sequence of initial strain (P0-H9N2)[b]
P0	0	Trachea	-
M1	3	Lung	97.3
M2	3	Trachea	98.6
M3	2	Thoracic air sac	95.9
M4	2	Lung	83.6

[a] P0-H9N2 is a strain isolated from broilers during the first influenza outbreak diagnosed in 2005 (Barbour *et al.* 2006). Mutants M1, M2, M3, and M4 were genetically characterized in our previous work (Shaib *et al.* 2011).
[b] Details of neuraminidase sequences of the H9N2 strains are reported previously (Shaib *et al.* 2011).

HA determination in collected allantoic fluids

Each collected allantoic fluid was thawed and two-fold serially diluted in sterile saline; each dilution of 50 µl was reacted against an equal volume of 1% of washed red blood cells (RBC) of chicken (Swayne *et al.* 1998). The maximum dilution of each allantoic fluid that still resulted in complete agglutination of the RBC suspension was recorded as the HA titer, reflecting the amount of the P0 virus that was able to propagate in presence or absence of oseltamivir.

Mean reduction in HA titer of H9N2-P0

To assess the effect evoked by oseltamivir treatment on H9N2-P0 replication, HA titer was calculated for each level of H9N2-P0 inoculation and applied to the following formula:

$$\text{Mean HA titer reduction (\%)} = \frac{\text{Mean HA (Oselt -)} - \text{Mean HA (Oselt +)}}{\text{Mean HA (Oselt -)}} \times 100$$

where Mean HA (Oselt -) is the mean titer in embryos inoculated with P0 and deprived of oseltamivir treatment. Mean HA (Oselt +) is the mean titer in embryos inoculated with the same amount of P0 and treated with oseltamivir.

Statistical analysis

Comparison of the mean embryo weights and the mean HA titers between oseltamivir treated and untreated groups, inoculated with the same viral concentration were performed using One Way ANOVA followed by Tukey test. The same statistical method was used to compare the two uninfected control groups, the first treated with oseltamivir and the second deprived of it. Significant differences are given at $P<0.05$.

Application of optimized susceptibility protocol on H9N2 mutants

The optimal drug-susceptibility protocol was identified as the maximal difference (reduction) of the viral replication and mean embryo weight between the embryonated eggs treated with the oseltamivir compared to the untreated eggs infected with the same challenge dose of P0. These conditions resulted in the inoculation of 1.2 x 10^{-5} HA units of P0 virus following the administration of 0.27 mg/100 µl of oseltamivir. The protocol was applied to the 4 H9N2 mutants that are detailed in Tables I and II.

This study included 10 groups of 9 day-old chick embryonated eggs, including 8 replicate-eggs/group. Four groups had each embryonated egg treated with 0.27 mg oseltamivir/100µl, while the other 4 groups were left without treatment. Each of the 4 H9N2 strains (M1, M2, M3, and M4) was delivered in 2 groups of eggs, the first group treated with oseltamivir and the second group deprived of it. The inoculum dose of each virus was set at 1.2 x 10^{-5} HA units/100 µl/egg. The last 2 groups were the control, deprived of viral inoculation, with one group treated with oseltamivir and the second control group deprived of the medication. The incubation conditions of the embryonated egg-groups were typical to that used in optimization of the protocol. The same procedures for weighing embryos, testing the HA titer in allantoic fluids, calculation of % reduction in HA titer, and statistical analysis were used in this part of research. This approach aims to the determination of the effect of oseltamivir on H9N2 mutants with different substitutions in their neuraminidase, as characterized in our previous publication (Shaib *et al.* 2011b) and summarized in Tables I and II of this article.

Table II. *Point mutations between 38-70 amino acid positions[a] of neuraminidase enzyme of H9N2 mutants resulting from passaging in 21-day-old broilers.*

H9N2[b] strains	Number of point mutation	Amino acid position in neuraminidase enzyme (38 … 70)
P0	NA	KQNDCTNPRNNQVVPCGPILIERNITEIVHLNN
M1	2	. . . . . P K . . . . .
M2	1	. . . . . P K . . . . .
M3	3	. K . . . . . V . S
M4	12	N A E R M N N . Q I . . . L . . . . . V . S

[a] No mutations did occur between a.a. positions 32-37 and between 71-104 of neuraminidase enzyme. Details of sequences have been reported by Shaib and colleagues (Shaib *et al.* 2011).
[b] P0 is the initial H9N2 strain isolated in the year 2005; M1, M2, M3 and M4 are mutants of the P0 strain, produced upon passaging in 21-day-old broilers.

Results

The result of the optimization of oseltamivir-antiviral effect in chicken embryos, inoculated with variable levels of the initial P0-H9N2 strain, is shown in Table III. The use of different HA units of the P0-H9N2 in the egg-inocula, ranging between 1.2 x 10^{-5} to 2.0 HA units, against constant concentration of oseltamivir, resulted in different reductions of HA titers. The maximum reduction in HA titers induced by antiviral treatment was with a inoculation dose of 1.2 x 10^{-5} HA units, resulting in an significant difference in viral yields (HA titer) between oseltamivir treated (Mean HA titer of 16) and non-treated embryos (Mean HA titer of 320) ($P < 0.05$). In addition, this inoculum resulted in significant difference of mean embryonic weight between the oseltamivir treated and non-treated embryos (4.5 g versus 3.5 g) ($P < 0.05$).

Table III. *Optimization of the protocol for the oseltamivir treatment in chicken embryos, inoculated with different titers of the P0-H9N2 strain. Antiviral efficacy was assessed by quantitative measures of viral propagation (HA titer) and embryonic weights in treated and untreated chick embryos.*

Inoculum-HA titer	Means[a] in chick embryos treated vs. untreated with oseltamivir[b]				% reduction in HA titer due to oseltamivir treatment
	Embryo weight (g)		HA titer[c]		
	oseltamivir treated	oseltamivir untreated	oseltamivir treated	oseltamivir untreated	
1.2 x 10^{-5}	4.50*	3.54*	16*	320*	95.0
1.2 x 10^{-3}	3.97	3.70	64	256	75.0
1.2 x 10^{-1}	3.30	4.00	104	160	35.0
2.5 x 10^{-1}	3.35	3.70	40*	160*	75.0
5.0 x 10^{-1}	3.52	3.63	24*	256*	90.6
1.0	3.73	3.41	136*	384*	64.6
2.0	2.95*	3.62*	32	128	75.0
Controls[d]	4.50	4.30	0	0	Not applicable

[a] Means were measured after 72 hours of P0-H9N2 inoculation. [b] The treatment of the embryos with oseltamivir was fixed at 0.27 mg/100 µl.
[c] The hemagglutination (HA) titer was measured in allantoic fluids of embryos inoculated with H9N2-P0 and in uninoculated controls incubated at 37.7°C for 72 hours.
[d] Controls are embryos that are treated vs. untreated with oseltamivir, but all deprived of viral inoculation.
* The 2 means of embryonic weight or HA titers in a row followed by (*) are significantly different at $P < 0.05$.

Table IV. *Susceptibility to oseltamivir in chicken embryos infected with H9N2 mutants characterized by different point mutations in their neuraminidase enzyme.*

H9N2 strains[a]	Means[b] in chick embryos treated vs. untreated with oseltamivir[c]				% reduction or increase in HA titer	Positions of point mutations in neuraminidase (32-104)
	Embryo weight (g)		HA titer[d]			
	Treated	Untreated	Treated	Untreated		
M1	1.95	2.05	192.0	12.0	1,500 (increase)	46, 47
M2	2.11	2.54	8.2*	48.0*	82.9 (reduction)	46
M3	2.60	2.25	0.5	1.3	61.5 (reduction)	39, 68, 70
M4	2.38	2.27	0.0*	90.7*	100.0 (reduction)	38, 39, 40, 41, 42, 43, 44, 46, 47, 51, 68, 70
Controls[e]	2.91*	2.61*	0.0	0.0	Not applicable	Not applicable

[a] M1 to M4 strains are mutants of the P0 strain, produced upon passaging in 21-day-old broilers. [b] Means were measured after 72 hours of H9N2 inoculation.
[c] The treatment of the embryo with oseltamivir was fixed at 0.27mg/100µl, and the inoculum set at 1.2x10-5 HA units.
[d] HA titer is hemagglutination titer of the allantoic fluids of embryos inoculated with mutants M1 to M4 or uninoculated controls, incubated at 37°C for 72 hours.
[e] Controls are embryos that are treated vs. untreated with oseltamivir, but all deprived of viral inoculation.
* The 2 means of embryonic weight or the two means of HA titers in a row, followed by (*) are significantly different at P<0.05.

The two control groups, deprived of viral inoculation, had no HA activity in their allantoic fluids. Among the viral inoculated groups, the only group that resulted in maximum mean weight of embryos, and maximum reduction in viral propagation, was the oseltamivir treated group that was inoculated with 1.2 x 10^{-5} HA units/embryonic egg. The inoculum of 1.2 x 10^{-5} HA units was applied to the second part of the research, to evaluate the antiviral activity of the oseltamivir on four H9N2 strains, with point mutations in their neuraminidase enzyme.

The oseltamivir reduced the viral propagation in 3 mutants, namely M2, M3, and M4, but not in the M1 mutant (Table IV). Actually, the M1 mutant increased its yield in presence of oseltamivir compared to its yield in the absence of this drug. It is worth stressing that mutants M2 and M4 demonstrated a high sensitivity to the drug, with a reduction in HA titer of 82.9% and 100.0%, respectively. There was no significant effect of oseltamivir administration on the mean embryonic weight in comparison to embryos deprived of the drug and receiving the same H9N2 mutants (P > 0.05). The emergence of the oseltamivir resistant M1 mutant was associated with 2 point mutations at neuraminidase- amino acid (aa) positions 46 and 47, while the other 3 mutants were susceptible, having point mutations at the following aa position(s): M2 (position 46), M3 (positions 39, 68, 70), and M4 (positions 38, 39, 40, 41, 42, 43, 44, 46, 47, 51, 68, and 70) (Table II).

Discussion

The data of the optimization of the initial P0-H9N2 strain susceptibility to oseltamivir in chicken embryos summarized in Table III are in agreement with the protocol documented previously (Sauerbrei *et al.* 2006). The use of P0-H9N2 strain at the lowest level in the inoculum of the egg embryos (1.2 x 10^{-5} HA units) resulted in the highest significant sensitivity of this strain to oseltamivir (95.0% reduction in HA titer), compared to the reduction induced by the other 6 treatments (35.0% -90.6% reduction in HA titers; P < 0.05). This highest reduction in P0-H9N2 strain propagation by oseltamivir was associated with the highest mean embryonic weight compared to embryos inoculated with the same 1.2 x 10^{-5} inoculum but deprived of oseltamivir. The improvement of embryonic weight by oseltamivir treatment correlates well with the significant reduction in propagation of the P0 strain. The interaction between viral inoculation dose and oseltamivir treatment on the embryonic weight resulted in fluctuating figures as shown in Table III, and needs further investigation. It is worth noting that previous works were able to increase the viral yield in egg embryos by lowering the HA units of the influenza virus in the inoculum (Sauerbrei *et al.* 2006). The chance given for adaptation of the fewer viruses in the inoculum to chicken embryonic cells will allow them to improve their propagation ability, enabling the released new particles to effectively infect other cells, resulting in higher yield (higher HA titer) (Sauerbrei *et al.* 2006). However, when a high HA level inoculum is used, it will provide enough non-adapted viral particles to infect most of the available chick embryonic cells, not allowing for adaptability and for higher efficiency of propagation (Leneva *et al.* 2000, Sauerbrei *et al.* 2006). The delivery of low HA units in the inoculum helped in raising the efficacy of the oseltamivir activity against the release of the few propagated H9N2 viruses, thus hindering their propagation into other available non-infected embryonic cells (Sauerbrei *et al.* 2006). This reason is most likely behind the higher sensitivity of P0-H9N2 strain to oseltamivir, when the inoculum was reduced in its HA units to 1.2 x 10^{-5}, leading at the same time to a better significant embryonic growth. It is worth noting that only one other treatment, using 2.0 HA

unit in the inoculum showed a significant reduction in the HA titer ($P < 0.05$), but the reduction was lower (75.0%), compared to the 95.0% reduction obtained by the 1.2×10^{-5} HA units-inoculum.

These data are showed in Table III and allowed us to select the 1.2×10^{-5} HA unit inoculum for its use against a constant oseltamivir administration per embryo (0.27 mg/100 µl) to evaluate the susceptibility of the 4 mutants of H9N2, differing in the number and position of the point mutations of their neuraminidase enzyme (Table IV).

The antiviral effect of oseltamivir was consistent on different H9N2 strains, reducing the propagation in embryos of the susceptible 3 mutants, namely M2, M3, and M4, but not the resistant mutant M1 (Table IV). Actually, the M1 mutant increased its yield in the presence of oseltamivir by 1,500%. This M1 mutant was characterized by 2 point mutations in positions 46 and 47 of neuraminidase enzyme with the arginine (46) and asparagine (47) replaced by proline and lysine, respectively (Table II). The presence of 2 adjacent point mutations in the M1 H9N2 mutant could have induced changes in the configuration of the enzyme hindering the attachment of oseltamivir drug to the molecule, thus inhibiting its antiviral activity and allowing the virus to propagate (Collins *et al.* 2008). It is documented that oseltamivir inhibits the neuraminidase enzyme, due to the drug binding at the enzyme's amino acid positions of histidine 274, asparagine 294, and tyrosine 252 (Bloom *et al.* 2010, Collins *et al.* 2008, Kiso *et al.* 2001). There is a need for future investigation to compare the impact of the changes in the M1 mutant Arg46Pro and Asn47Lys on oseltamivir binding-inhibition to the reported point mutations occurring at histidine, asparagine, and tyrosine, located at respective positions of 274, 294, and 252.

The two H9N2 mutants, M2 and M4, with respective 1 and 12 mutations (Table II) were highly sensitive to the drug (respective reduction in HA units of 82.9% and 100%) (Table IV). The single point mutation in M2 at position 46 of neuraminidase and the 12 point mutations in the M4 strain (Table II) seem to have relaxed the interaction between the oseltamivir and its binding site at the neuraminidase enzyme (Aoki *et al.* 2007), which led to higher susceptibility of these 2 mutants to the drug, compared to the initial P0 strain.

It is worth noting that previous documented literature showed that the influenza viruses could mutate to result in higher susceptibility of the mutants to a drug compared to their parent strain (Yen-Lieberman *et al.* 2010), depending on the nature of change in their amino acid positions (Collins *et al.* 2008) or specific deletions in their neuraminidase structures (Gulati *et al.* 2009).

In conclusion, the H9N2 susceptibility to oseltamivir in chicken embryos is affected by the challenge titre. The use of optimized higher sensitivity protocol uncovered the variation in drug-susceptibilities of H9N2 mutants, differing in number and position of the point mutations in their neuraminidase enzyme. It is recommended in the future to search for oseltamivir resistant mutants of H9N2-zoonotic organism in poultry and other animals, and in ecological systems of our planet, in order to share information with the World Health Organization and the World Animal Health Organization to build a future strategy for vaccine development, targeting the reduction or elimination of these resistant strains, to reach the One Health Global objective for animals, humans, and the environment.

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