Standardization of a Protocol for Quantitative Evaluation of Anti-Aerosolized Influenza Virus Activity by Vapors of a Chemically-Characterized Essential Oil Blend

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Abstract

The aim of this research is to standardize the conditions of a constructed impinger, enabling to evaluate quantitatively the anti-aerosolized H9N2 avian influenza virus (AIV) activity by vapors of a chemically-characterized essential oil blend. The standardization resulted in 100% recovery of the aerosolized H9N2 virus when the impinger’s conditions were set as aerosolized viral particles count of 1.2 × 10⁶/p.c.c. of Tryptose Phosphate Broth, temperature of 35°C, average micelle diameter of 44.3 μm, negative pressure of 6 mbar, air-suspension time of H9N2 virus of 1.5 min, collection chamber and its transport medium volumes of 250 cc and 25 cc, respectively. The adoption of the above standardized conditions, with an inclusion of vaporized essential oil (EO) at 1.0 × 10⁻⁴ μL EO/μL volume of the pulverization chamber, and contact times of 0.5-1.5 min with the H9N2 virus, resulted in 84.6% reduction in viral titer at 1.5 min contact time, compared to the control virus, deprived from contact with vaporized EO (P<0.05). This new finding will help in future investigations related to application of safe essential oils in reduction of air-suspended influenza virus in closed systems harboring domestic animals and human populations.

Keywords: Aerosols; Essential oils; Influenza virus; Impinger standardization

Introduction

The avian influenza virus (AIV) of subtype H9N2, listed by the World Health Organization (WHO) under zoonotic subtypes of AIV, had an economic burden on both animals and humans [1]. The airborne nature of H9N2 transmission and infection is documented [2]. The significant economic losses in domestic poultry and other animals, due to H9N2 and other subtypes such as the H5 and H7, resulted in development of related vaccines [3]; The inclusion of such vaccines in control programs led to emergence of escape mutants with higher pathogenicity on both humans and vaccinated animals [4].

This critical public health situation, due to zoonotic influenza viruses, required a supplemental approach to vaccination, attempting to inactivate or reduce significantly the viral load in the air, so that the immune system of the vaccinated host can handle the reduced exposure below the infective dose of these viruses [5]. The research in aerosolized viral inactivation by different reagents are based on standardization of the impinger conditions, to reach to a high recovery efficiency of the
aerosolized virus, that will be subjected to viability evaluation after contact with the antiviral vapors [6-8]. The construction of impingers by many previous investigators required a pulverization chamber to aerosolize the viral particles and to provide contact with vapors of experimental air sanitizers, connected to a collection flask, which in turn is connected to a vacuum pump, creating a converged environmental air stream that delivers the aerosolized particles to a liquid phase in the collection flask [9-12]. The collected virus in the liquid phase will be tested for its viability by propagating it in appropriate cell culture line or in chicken embryos [13]. The major variables that need to be optimized, targeting a maximum recovery of the aerosolized virus, are the viral particle count per unit volume of the pulverization chamber, the magnitude of the negative pressure created by the vacuum pump, air-suspension time of aerosolized virus before it is vacuumed towards the collection flask, and the volumes of the collection chamber and its contained viral transport liquid medium [14-20].

The aerosolized chemical disinfestants used to inactivate viral particles in hospitals, nurseries, schools, army facilities, aeroplanes, airports, cooling towers, abattoirs, and closed farm systems are rejected by many people, due to documented reports about their toxicity [21-23].

The search for natural and safe environmentally friendly-aerosolized substances, aiming at disinfecting the air, has increased in the last decade [24]. In 2011, The US EPA [25] approved an air disinfectant that is formulated from essential oil of thyme, having a commercial name ’Benefect’. In 2013, The UCSF [26] Institute for Health & Aging, University of California, Berkley Center for Environmental Research and Children’s Health developed an educational toolkit entitled ‘Green Cleaning, Sanitizing and Disinfecting: A Toolkit for Early Care and Education’, that was approved later for listing on the Website of the US EPA, 2013. Sporadic reports are documented on the importance of aerosolized essential oils, due to their low boiling points, helping them to vaporize at low temperatures of the indoor spaces. Usachev et al. reported the antiviral activity of tea tree and eucalyptus oil aerosolized vapor against Influenza A and E. coli phage M13 test organisms [27].

The safe vapors of essential oils of eucalyptus and peppermint have been used for long time in folklore medicine, against human cold viruses [28], in the absence of scientific evidence of their anti-aerosolized viral activity.

To our knowledge, this is the first research in optimization of six variables of a constructed impinger aiming at augmenting the efficiency in recovery of pulverized H9N2 virus in the transport medium of the collection chamber; Moreover, the second objective is first of its kind in using the optimized impinger system to determine quantitatively the amount of vaporized chemically defined-blend, of equivalent amount of Eucalyptus and peppermint essential oils, needed to reduce significantly the viability of the aerosolized H9N2 virus.

Materials and Methods

Description of the constructed impinger

The image of the constructed impinger in Figure 1 consisted of a glass pulverization chamber, closed with two holes-rubber stopper; the first hole fitted a Pyrex tube of 9.4 cm length and 6 mm in diameter, plugged with glass wool for air inlet during vacuuming. The second hole fitted a pulverizing Pyrex nozzle of 8.0 mm diameter for aerosolizing the viral suspension at an average particle diameter of 44.3 μm.

Figure 1 The composition of the impinger: (a) Water bath for heating the pulverization chamber; (b) Pulverization chamber, (c) Collection chamber containing the transport medium; (d) Vacuum pump.

The collection glass chamber has also two holes-stoppers. The first hole fitted a Pyrex tube of 19.5 cm length and 8.0 mm diameter connected at one end to the pulverization chamber by a rubber tube while its other end is immersed in the transport medium. This medium will trap the aerosolized virus, after subjecting it to a negative pressure. The negative pressure is achieved by a vacuum pump that is connected to the tube, fitted to the second hole of the collection chamber’s rubber stopper. It is worth noting that the transport medium contained in the collection chamber is prepared by dissolving NaCl (8 g), KCl (0.2 g), Na2HPO4 (1.44 g), and KH2PO4 (0.24 g) in 500 ml of distilled water, then mixed with 500 ml glycerol; the mixed contents are sterilized by autoclaving, cooled, and supplemented with Benzyl penicillin (2 × 10^6 IU/l), Streptomycin (200 mg/l), Polymyxin (2 × 10^6 IU/l), Gentamicin (250 mg/l), and Nystatin (0.5 × 10^6 IU/l).

Optimization of the impinger variables

The optimization of the variables of the impinger, targeting an efficient trapping of the pulverized H9N2 virus in the transport medium of the collection chamber, was determined according to six separate experiments, including in each, different levels of variables, as demonstrated in Table 1. The seven variables used in this optimization included the volume of Tryptose Phosphate Broth (TPB) carrying the H9N2 that will be pulverized, concentration of the H9N2 virus present in TPB given in Hemagglutination Units (HA units), volume of the pulverization...
chamber of the impinger, volume of transport medium in the collection chamber, negative pressure of vacuum pump, temperature inside the pulverization chamber, and different suspension times of the pulverized H9N2 virus before it is vacuumed for collection. The pulverized H9N2 virus in Tryptose Phosphate Broth (TPB) had an average micelle diameter of 44.3 μm, and the vacuuming time following the assigned suspension time of the virus was also constant, set at 2 min.

Each of the six experiments described in Table 1 was repeated twice, except for the 5th experiment that was run in triplicate.

Table 1 A change in seven variables of six experiments targeting the optimization of the impinger to yield an efficient recovery of pulverized H9N2 virus in the collection chamber.

Fixed parameters of the impinger are given in the below footnote of Table 1; Notes: 

a The seven variables were the volume of Tryptose Phosphate Broth carrier of pulverized H9N2 virus, the concentration of the virus measured in Hemagglutination (HA) Units, the volume of the pulverization chamber, the volume of the transport medium in the collection flask, the negative pressure of the vacuum pump, the temperature in the pulverization chamber, and the air suspension time of the pulverized H9N2 before vacuuming it to the collection flask; 
b The fixed parameters of the impinger were: average pulverized particle diameter of 44.3 μm, volume of collection chamber of 250 cc, vacuuming at the end of each air suspension time of the virus of two min; 
c Expt=Experiment; 
d The H9N2 virus is pulverized in Tryptose Phosphate Broth medium; 
e Pulv=Pulverization; 
f HA=Hemagglutination titer of the H9N2 virus.

### Table 1

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>Pulverized H9N2 virus</th>
<th>Pulv. Chamber Vol. (cc)</th>
<th>Transport Medium Vol. (cc)</th>
<th>Vacuum Pump Pressure (mbar)</th>
<th>T (°C)</th>
<th>Air Suspension Time of H9N2 (min or s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300 1:64</td>
<td>250</td>
<td>50</td>
<td>350</td>
<td>25</td>
<td>2, 4, 6, 8, 10 min</td>
</tr>
<tr>
<td>2</td>
<td>300 1:256</td>
<td>2100</td>
<td>50</td>
<td>6</td>
<td>25</td>
<td>15, 30, 45, 60 s</td>
</tr>
<tr>
<td>3</td>
<td>300 1:256</td>
<td>2100</td>
<td>50</td>
<td>6</td>
<td>25</td>
<td>30 s, 1, 2, 3, 5 min</td>
</tr>
<tr>
<td>4</td>
<td>300 1:256</td>
<td>2100</td>
<td>50</td>
<td>6</td>
<td>25</td>
<td>1, 1.5, 2.0, 2.5, 3.5 min</td>
</tr>
<tr>
<td>5</td>
<td>300 1:256</td>
<td>2100</td>
<td>50</td>
<td>6</td>
<td>25</td>
<td>1.5 min</td>
</tr>
<tr>
<td>6</td>
<td>500 1:256</td>
<td>2100</td>
<td>25</td>
<td>6</td>
<td>35</td>
<td>1.5 min</td>
</tr>
</tbody>
</table>

### Anti-pulverized H9N2 virus by vapors of an essential oil blend

The aim of the second objective was to study the effect of timed contact in the pulverization chamber between a known volumes of the Essential Oil blend (EOB), liberated at 35°C, on the inactivation of known concentration of aerosolized H9N2 virus, in relation to same pulverized virus deprived of the contact with the EOB. The EOB was developed and provided thankfully by the Department of R and D of EWABO Co., Chemikalien GmbH, Kolpingstrabe 4, 49835 Wietmarschen, Germany. This blend was made of equivalent volumes of Eucalyptus and Peppermint. The chemical analysis of the blend consisted of two major components namely, 1,8-Cineol (42.2%) and Menthol (48.7%). The other minor active ingredients included gamma-Terpine, Limonene, Menthone, Methofuran, Methyl acetate, Phellandrine, and Pinene.

The optimized conditions of the impinger, obtaining a 100% recovery of pulverized H9N2 in the Transport Medium of the collection chamber (Experiment 6, Tables 1 and 2), was adopted in this second objective. The introduction of the EOB vapor to the pulverization chamber was performed in three different individual experiments, each run in duplicate, aerosolizing the EOB at 0.5 min, 1.0 min, and 1.5 min, before the end of the constant pulverized viral air suspension time of 2.0 min. Vacuuming in all experiments was initiated at the end of the 2.0 min of the viral suspension time. Another control experiment, run in duplicate, included the collection of the EOB vapors without the pulverization of the virus, to assess the safety of the inoculated EOB, carried in its transport medium, on 12 chicken embryos. The total number of transport medium samples resulting from the three experiments, each run in duplicate, was eight.

Table 2 Mean frequency (%) of Transport medium-inoculated embryonated eggs, showing Hemagglutination (HA) activity, under the different air suspension times of pulverized H9N2

Notes: 

a The total air suspension times of H9N2 in the six experiments was twenty-one, resulting in twenty-one trials, each performed in duplicate, except for the 5th experiment that was run in triplicate. The virus was collected in each run in its respective Transport medium. Each of the Transport Medium collection was randomly sampled for twelve times, and each sample was inoculated in embryonate chicken egg to study the presence of the virus and its ability to propagate, resulting in HA titer; 
b The variables and fixed parameters of each of the six experiments are defined in Table 1.
<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Air Suspension Time$^a$ of H9N2 (min or s)</th>
<th>Mean frequency (%) of transport medium-inoculated embryonated eggs showing hemagglutination activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.0, 4.0, 6.0, 8.0, 10.0 min</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>15.0, 30.0, 45.0 s</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0 min</td>
<td>30</td>
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<tr>
<td>3</td>
<td>30.0 s</td>
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<td></td>
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<td></td>
<td>2.0 min</td>
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<td></td>
<td>1.5 min</td>
<td>95</td>
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<td></td>
<td>2.0 min</td>
<td>60</td>
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<tr>
<td></td>
<td>2.5 min</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>3.5 min</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1.5 min (25°C)</td>
<td>90</td>
</tr>
<tr>
<td>6</td>
<td>1.5 min (35°C)</td>
<td>100</td>
</tr>
</tbody>
</table>

Viability of the collected H9N2 virus

The eight transport medium samples were immediately stored in separate sterile cups at -80°C, for testing the viability of the recovered virus, and the safety of the EOB. The viability was tested by attempting to propagate twelve randomly chosen aliquots, each of 100 μl inoculum, in twelve respective embryonated chicken eggs of 10 days old. It is worth noting that two runs were performed per treatment, except for the three runs applied in experiment five, described in Tables 1 and 2. The inoculated embryonated eggs were incubated at 37°C for three days, followed by cooling them in the fridge at 4°C for three hours, harvesting their allantoic fluid using sterile pipettes, and performing the Hemagglutination (HA) test according to a documented protocol by the World Animal Health Organization [13]. Briefly, 50 μl of the allantoic fluid of each egg was diluted serially in sterile saline, using a dilution factor of ½, followed by reacting each viral dilution against equal volume of 1% suspension of chicken red blood cells in Microtiter wells for a period of 45 min, and recording the HA titer. Actually, the HA titer is the maximum dilution of the propagated virus that still has enough viral agglutinin proteins that show complete agglutination of the 1% RBC suspension. The experiment on safety of the EOB was evaluated by checking the survival of the inoculated embryos after 3 days, using candling.

The mean percent reduction in frequency of eggs inoculated with transport medium and showing viral HA activity and reduction in quantified mean HA titers of their allantoic fluid, due to contact between vapor of EOB and aerosolized virus, were determined in comparison to means of Control trials, in which the aerosolized virus was deprived from contact with the EOB.

The safety of collected EOB in transport medium was reported in percentage of surviving embryos after three days of inoculation and incubation.

The statistical analysis for comparison of the means was by one way ANOVA followed by Tukey’s test. Statistical differences are reported at P<0.05.

Results and Discussion

Optimization of the impinger variables

The optimization of seven variables of the impinger, targeting an efficient trapping of the pulverized H9N2 virus in the transport medium of the collection chamber, was determined according to six separate experiments, as demonstrated in Table 1. The standardized variables used in the 6th experiments resulted in 100% recovery of the H9N2 virus from different randomly taken samples of the transport medium present in the collection flask (Table 2, 6th experiment). This obtained data is in agreement with previous documentation, recommending that an air sampling system should be optimized for the target microorganism, and that the obtained results should be interpreted in the context of the determined conditions of the impinger [6]. The impinger’s conditions in the successful 6th experiment were set at aerosolized viral particles count of $1.2 \times 10^6$/c.c. with viral strength in Tryptose Phosphate Broth (TPB) carrier of 1:256 Hemagglutination Units (HA units), contained in
500 µl volume of TPB, pulverized in 2100 c.c. chamber at a temperature of 35°C, under a negative pressure of 6 mbar, an air-suspension time of aerosolized H9N2 virus of 1.5 min, and a collection chamber of 250 c.c., containing 25 ml of a 50% glycerol-transport medium. Previous workers reported the success in recovery of aerosolized viruses based on specific viral density in a certain volume of the aerosolization chamber [29-32], the appropriate nature and volume of the carrier in the aerosolized particles that enable the sustainability of the viral viability [33-35], the role of the temperature in the pulverization chamber [36-38], the appropriate negative pressure that carries the virus from the pulverization chamber towards a proper nature of the transport medium in the collection chamber [13,39].

There was a clear effect of the air suspension time of the virus in the pulverization chamber, before it is vacuumed to the collection flask, on the success of its recovery in the transport medium (Table 2). This effect was observed in Experiments 2, 3 and 4 in which as the air suspension time approached to around 1.5 min, the recovery of the pulverized H9N2 in the transport medium improved significantly. Actually, the essential role of optimization of the air suspension time of the target pathogen, before applying a negative pressure on the aerosolized particles, was documented by Herman et al. [6].

It is worth noting that the fixed parameters of the impinger in all six experiments were an average pulverized particle diameter of 44.3 um, a volume of collection chamber of 250 ml, and a vacuuming time at the end of each air suspension time of the virus equivalent to 2 min.

The preliminary standardization of the impinger conditions, aiming at successful collection of the viable H9N2 virus in the transport medium, was first recognized in the second experiment (Tables 1 and 2), in which 30% of the embryonated chicken eggs, inoculated with the transport medium present in the collection chamber, showed the presence of the hemagglutinating H9N2 virus.

The air suspension time of the H9N2 virus in the pulverization chamber, before it is vacuumed towards the collection chamber, seems to be the most important variable that needed further refining. This is in agreement with previously documented works [40-43]. The success in collection of the virus in the second experiment was obtained at an air suspension time of 1.0 min, while shorter suspension times of 15.0, 30.0, and 45.0 sec failed in recovering the virus from the transport medium.

The recovery of the virus in the embryonated eggs, inoculated by the transport medium of the 2nd experiment, was of a low frequency, equivalent to 30% (Table 2). The 3rd and 4th experiments in Tables 1 and 2 included larger range of air suspension times of the H9N2 virus, targeting higher frequency of recovery of the virus in embryonated eggs. Actually, the 4th experiment raised the viral recovery to 95.0% in the inoculated chicken egg replicates, a figure that fluctuated by 5% from the 90.0% recovery obtained in the 5th experiment that implemented the same values of the seven parameters (Tables 1 and 2).

In an attempt to higher further this recovery, three variables were changed in the final 6th experiment, compared to experiments 4 and 5 namely, the pulverized H9N2 carrier volume that was raised from 300 to 500 µl of Tryptose Phosphate Broth (TPB), the reduction in the volume of the transport medium in the collection chamber from 50 cc to 25 cc, and the raise in the temperature of the pulverization chamber from 25°C to 35°C, while keeping the air suspension time fixed at 1.5 min. This attempt in the 6th experiment raised the success of recovery of the H9N2 virus in embryonated eggs, inoculated by the collecting transport medium, to 100% (Table 2). The raise in recovery of the virus was most likely due to the volume increase of the Tryptose Phosphate Broth carrier that increased the density of the pulverized particles of the H9N2 virus in the pulverization chamber [29,30,32,44,45]; in addition, the higher recovery of the virus in the 6th experiment could have resulted from the 50% decrease in the dilution factor, created by reduction of the volume of the transport medium located in the collection chamber [18,46], while the raise in the temperature of the pulverization chamber could have led to lighter air, and possibly more stability in the viral viability [36,47].

**Anti-pulverized H9N2 virus by vapors of the essential oil blend (EOB)**

The optimized conditions of the impinger, obtaining a 100% recovery of pulverized H9N2 in the transport medium of the collection chamber (Experiment 6, Tables 1 and 2), was adopted in this second objective, aiming at studying the effect of timed contact in the pulverization chamber between a known volume of liberated EOB at 35°C on the inactivation of known concentration of aerosolized H9N2 virus, in reference to Control-pulverized virus, deprived of EOB contact. The pulverized virus, deprived of contact with the EOB, was viable in the transport medium present in the collection flask, as detected by the inoculation of this medium in twelve replicates of chicken embryonated eggs per each of two trials, revealing presence of HA activity in 100% of the embryo replicates. However, the inoculated embryos by the transport medium, collected after the pulverized H9N2 virus was contacted for different times with the EOB, showed an increase in reduction of allantoic fluid-HA activities with an increase in the contact time (Table 3). Actually, there was a significant mean percent reduction in frequency of Transport medium-inoculated embryonated eggs that showed HA activity in their allantoic fluid; Moreover, there was a reduction in the quantified mean HA titer of allantoic fluid of embryonated eggs that were inoculated by collected H9N2 in Transport medium after they were pulverized and contacted with EOB for 1.5 min, compared to the viral HA titer when contacted for shorter times of 0.5 min or 1.0 min (P<0.05).

It is worth noting that the safety experiment related to the EOB showed a 100% survival of the embryos, after three days of inoculation of the transport medium containing the collected EOB vapors in absence of the virus.

The introduction of the vapor of essential oil blend (EOB) to the pulverization chamber at 0.5, 1.0 and 1.5 min, before the end of the air suspension time of 1.5 min, resulted in gradual increase in reduction of the frequency of H9N2 positive
embryoned eggs equivalent to 22.3%, 27.0% and 42.5%, respectively (Table 3). This is in agreement with previous reports documenting the relationship in increments of contact time to the increase in effectiveness of aerosolized sanitizers against the target virus [48]. The obtained relationship in this study between the contact time and the decrease in recovery of the virus from transport medium is indicative of the effectiveness of the EOB against suspended H9N2 in the air. In addition, the increase in reduction of the quantified HA titer in the inoculated embryoned eggs with more elongation of the contact time between the H9N2 virus and the EOB provided evidence on the effectiveness of this blend in reducing the ability of the surviving H9N2 particles to propagate. It is worth noting that other works pointed at sanitizers that injure viruses, thus lowering their ability to multiply in the host cells [49-51]. Moreover, the 100% survival of the embryos inoculated by the transport medium, containing only the collected vapor of the EOB, is indicative of the vapor’s safety, a data that is in agreement with another work that reported the safety of direct inoculation in chicken embryos of high concentrations of the liquid phase of an equivalent ratio of Eucalyptus and peppermint (1/1 V/V) [52].

Table 3 Impact of different contact times with vapors of essential oil blend (EOB) on inactivation of H9N2 virus aerosolized in the optimized impinger in reference to the aerosolized virus deprived of EOB

Notes: aOptimized impinger conditions, concluded from Experiment no. six, and presented in Table 1; bEach contact time trial was run in duplicate, including impinger with EOB vapor contact and Control impinger deprived of EOB vapour; cEach of twelve embryoned chicken eggs was inoculated with 100 μl of the transport medium; 1,2Means in a column followed by different Arabic numeral superscripts are significantly different (P<0.05)

<table>
<thead>
<tr>
<th>Contact time (Min)</th>
<th>Mean % Reduction in frequency of embryonated Eggs showing HA activity Mean % Reduction in Mean HA titer</th>
</tr>
</thead>
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<tr>
<td>0.5</td>
<td>22.31</td>
</tr>
<tr>
<td>1</td>
<td>27.01</td>
</tr>
<tr>
<td>1.5</td>
<td>42.52</td>
</tr>
</tbody>
</table>

Conclusion and Recommendations

To our knowledge, this is the first documentation in literature relating the impact of contact time between the evaporated EOB and the inactivation and reduction in multiplication ability of the aerosolized H9N2 virus.

In conclusion, this study was able to optimize the conditions of the impinger, resulting in 100% recovery of the H9N2 virus from the transport medium of the collection flask. The obtained high recovery was helpful in implementing the optimized conditions of the impinger for studying the impact of the EOB contact time on reduction of the H9N2 recovery from the collection chamber, and on a significant decline in the ability of the surviving virus to propagate in embryonated chicken eggs compared to its propagation in the absence of contact with the EOB.

It is recommended in the future to use the optimized conditions of the constructed impinger for providing data related to efficacy and labeling requirements that are required by authorities for approving the implementation of safe air sanitizers against influenza viruses in domestic animal facilities and human assembling locations [53,54].

Acknowledgement

We acknowledge the cooperation among the different laboratory staff at the Biochemistry Department of King Abdulaziz University, the Agriculture Department at the American University of Beirut, the Department of Pathology, University of Veterinary and Animal Science at Lahore in Pakistan, and the R&D Department at EWABO Co., Wietmarschen, Germany.

Conflict of Interest

The authors declare that they have no actual or potential competing financial interests.

References


