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

Taha Kumosani[1], Youssef Obeid[2], Houssam Shaib[2], Khalid Abualnaja[3], Said Salama Moselhy[4,5], Archana Iyer[6], Rizwana Sultan[7], Asim Aslam[7], Ahsan Anjum[7] and Elie Barbour[8*]

[1]Department of Biochemistry, Faculty of Science, Experimental Biochemistry Unit, King Fahd Medical Research Centre, Production of Bio-products for Industrial Applications Research Group, King Abdulaziz University, Jeddah, Saudi Arabia

[2]Department of Agriculture, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon

[3]Department of Biochemistry, Faculty of Science, Bioactive Natural Products Research Group, King Abdulaziz University, Jeddah, Saudi Arabia

[4]Department of Biochemistry, Faculty of Science, Experimental Biochemistry Unit, King Fahd Medical Research Center, Bioactive Natural Products Research Group, King Abdulaziz University, Jeddah, Saudi Arabia

[5]Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt

[6]Biochemistry Department, Faculty of Science, Production of Bio-products for Industrial Applications Research Group, Vitamin D Pharmacogenomics Research Group and Experimental Biochemistry Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia

[7]Department of Pathology, University of Veterinary and Animal Sciences, Out Fall Road, Lahore, Pakistan

[8]Department of Agriculture, Faculty of Agricultural and Food Sciences, American University of Beirut, Beirut, Lebanon, and Adjunct Professor at Biochemistry Department, Faculty of Science, Experimental Biochemistry Unit, King Fahd Medical Research Centre, Production of Bio-products for Industrial Applications Research Group, King Abdulaziz University, Jeddah, Saudi Arabia

***Corresponding author:** Elie K Barbour, Department of Agriculture, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon, Tel: 4460 +9611350000; Fax: +9611744460; E-mail: eb01@aub.edu.lb

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 at aerosolized viral particles count of 1.2×10^6/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.

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 created an immune pressure in the host against these viruses, that 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 disinfectants 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.
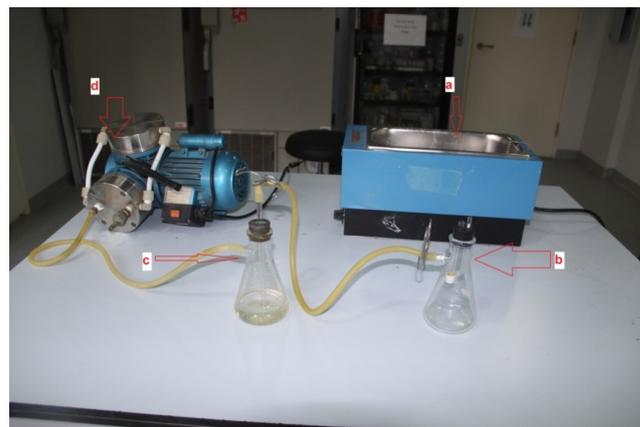

Figure 1 The composition of the impinger: (a) Water bath for heating the pulverization chamber; (b) Pulverization chamber, (c) Collection chamber containing the transport medium; (d) Vacuum pump.

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), Na_2HPO_4 (1.44 g), and KH_2PO_4 (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 sevena 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[b] 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 time 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 Phospate Broth medium; [e]Pulv=Pulverization; [f]HA=Hemagglutination titer of the H9N2 virus

Expt[c] No.	Pulverized H9N2 virus[d]		Pulv[e]. Chamber Vol. (cc)	Transport Medium Vol. (cc)	Vacuum Pump Negative Pressure (mbar)	T (°C)	Air Suspension Time of H9N2 (min or s)
	Vol (μl)	HA[f] (units)					
1	300	1:64	250	50	350	25	2, 4, 6, 8, 10 min
2	300	1:256	2100	50	6	25	15, 30, 45, 60 s
3	300	1:256	2100	50	6	25	30 s, 1, 2, 3, 5 min
4	300	1:256	2100	50	6	25	1, 1.5, 2.0, 2.5, 3.5 min
5	300	1:256	2100	50	6	25	1.5 min
6	500	1:256	2100	25	6	35	1.5 min

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-Terpinene, 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[a], 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[d] 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**

Experiment no.[b]	Air Suspension Time[a] of H9N2 (min or s)	Mean frequency (%) of transport medium-inoculated embryonated eggs showing hemagglutination activity
1	2.0, 4.0, 6.0, 8.0, 10.0 min	0
2	15.0, 30.0,	0
	45.0 s	0
	1.0 min	30
3	30.0 s	0
	1.0 min	30
	2.0 min	60
	3.0 min	0
	5.0 min	0
4	1.0 min	30
	1.5 min	95
	2.0 min	60
	2.5 min	40
	3.5 min	0
5	1.5 min (25°C)	90
6	1.5 min (35°C)	100

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 allontoic 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 allontoic 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 allontoic 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 impinge [6]. The impinger's conditions in the successful 6th experiment were set at aerosolized viral particles count of 1.2×10^6/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 allontoic 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 allontoic fluid; Moreover, there was a reduction in the quantified mean HA titer of allontoic 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

embyonated 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 embryonated 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[a] in reference to the aerosolized virus deprived of EOB

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

Contact time[b] (Min)	Mean % Reduction in frequency of embryonated Eggs[c] showing HA activity	Mean % Reduction in Mean HA titer
0.5	22.3[1]	58.7[1]
1	27.0[1]	63.0[1]
1.5	42.5[2]	84.6[2]

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].

Conflict of Interest

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

References

1. Pascua PNQ, Choi YK (2014) Zoonotic infections with avian influenza A viruses and vaccine preparedness: A game of "mix and match". Clin Exp Vac Res 3: 140-148.

2. Palese P, Shaw ML (2007) Orthomyxoviridae: The viruses and their replication. In: Knipe DM and Howley PM (ed). Fields Virology. Philadelphia, PA: Lippincott Williams & Wilkins, pp: 1647-1689.

3. Alexander DJ (2007) Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002-2006. Avian Dis 51: 161-166.

4. Wan Z, Ye J, Xu L, Shao H, Jin W, et al. (2014) Antigenic mapping of the hemagglutinin of an H9N2 avian influenza virus reveals novel critical amino acid positions in antigenic sites. J Virol 88: 3898-3901.

5. Marois I, Cloutier A, Garneau E, Richter MV (2012) Initial infectious dose dictates the innate, adaptive, and memory responses to influenza in the respiratory tract. J Leukoc Biol 92: 107-121.

6. Hermann JR, Hoff SJ, Yoon KJ, Burkhardt AC, Evans RB, et al. (2006) Optimization of a sampling system for recovery and detection of airborne porcine reproductive and respiratory syndrome virus and swine influenza virus. Appl Env Microbiol 72: 4811- 4818.

7. Liu K, Wen Z, Li N, Yang W, Wang J, et al. (2012) Impact of relative humidity and collection media on mycobacteriophage D29 aerosol. Appl Env Microbiol 78: 1466-1472.

8. Yu KP, Chen YP, Gong JY, Chen YC, Cheng CC (2016) Improving the collection efficiency of the liquid impinger for ultrafine particles and viral aerosols by applying granular bed filtration. J Aerosol Sci 101: 133-143.

9. Alexandersen S, Donaldson AI (2002) Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. Epidemiol Infect 128: 313-323.

10. Miljevic B, Modini RL, Bottle S, Ristovski Z (2009) On the efficiency of impingers with fritted nozzle tip for collection of ultrafine particles. Atmos Environ 43: 1372-1376.

11. Riemenschneider L, Woo MH, Wu CY, Lundgren D, Wander J, et al. (2010) Characterization of reaerosolization from impingers in an effort to improve airborne virus sampling. J Appl Microbiol 108: 315-324.

12. Wei Z, Rosario RC, Montoya LD (2010) Collection efficiency of a midget impinger for nanoparticles in the range of 3-11 nm. Atmos Environ 44: 872-876.

13. Office International Epizootiologie (2015) Avian Influenza (Infection with Avian Influenza Viruses), OIE Terrestrial Manual, 2015, Chapter 2.3.4, pp: 10 - 11.

14. Cage BR, Schreiber K, Barnes C, Portnoy J (1996) Evaluation of four bioaerosol samplers in the outdoor environment. Ann Allergy Asthma Immunol 77: 401-406.

15. Donaldson AI, Ferris NP, Gloster J (1982) Air sampling of pigs infected with foot-and-mouth disease virus: comparison of Litton and cyclone samplers. Res Vet Sci 33: 384-385.

16. Jensen PA, Todd WF, Davis GN, Scarpino PV (1992) Evaluation of eight bioaerosol samplers challenged with aerosols of free bacteria. Am Ind Hyg Assoc J 53: 660-667.

17. Lin WH, Li CS (1998) The effect of sampling time and flow rates on the bioefficiency of the three fungal spore sampling collection. Aerosol Sci Tech 28: 511-522.

18. Lin XJ, Willekew K, Ulevicius V, Grinshpun SA (1997) Effect of sampling time on the collection efficiency of all-glass impingers. Am Ind Hyg Assoc J 58: 480-488.

19. Oh D, Anwar A, Theodore A, Lee JH, Wu CY, et al. (2010) Development and evaluation of a novel bioaerosol amplification unit (BAU) for improved viral aerosol collection. J Aerosol Sci 41: 889-894.

20. Tseng CC, Li CS (2005) Collection efficiencies for aerosol samplers for virus-containing aerosols. J Aerosol Sci 36: 593-607.

21. Minnesota Department of Health, Environmental Health Division, Environmental Surveillance and Assessment Section (2016). Chemicals of High Concern, P.O. Box 64975, St. Paul, MN 55164-0975.

22. Park DU, Choi YY, Ahn JJ, Lim HK, Kim SK, et al. (2015) Relationship between exposure to household humidifier disinfectants and risk of lung injury: A family-based study. PloS One 10: e0124610.

23. Yang HJ, Kim HJ, Yu J, Lee E, Jung YH, et al. (2013) Inhalation toxicity of humidifier disinfectants as a risk factor of children's interstitial lung disease in Korea: A case-control study. PloS One 8: e64430.

24. US Environmental Protection Agency (US EPA) (2013) Green cleaning, sanitizing and disinfecting: A toolkit for early care and education. EPA's Healthy Childcare.

25. US EPA (2011) EPA registration number 84683-3 of Benefect Botanical Daily Cleaner Disinfectant Spray, Office of Prevention, Pesticides and Toxic Substances, Washington, DC 20460.

26. University of California (2013) Green Cleaning, Sanitizing and Disinfecting: A toolkit for Early Care and Education, Berkley Center for Environmental Research and Children's Health, UCSF Institute for Health and Aging.

27. Usachev EV, Pyankov OV, Usacheva OV, Agranovski IE (2013) Antiviral activity of tea tree and eucalyptus oil aerosol and vapor. J Aerosol Sci 59: 22-30.

28. Wei A, Shibamoto T (2010) Antioxidant/lipoxygenase inhibitory activities and chemical compositions of selected essential oils. J Agric Food Chem 58: 7218-7225.

29. Donaldson AI, Gibson CF, Oliver R, Hamblin C, Kiching RP (1987) Infection of cattle by airborne foot-and-mouth disease virus: Minimal doses with O1 and SAT 2 strains. Res Vet Sci 43: 339-346.

30. Gerone PJ, Couch RB, Keefer GV, Douglas RG, Derrenbacher EB, et al. (1966) Assessment of experimental and natural viral aerosols. Bacteriol Rev 30: 576-588.

31. Hogan Jr CJ, Kettleson EM, Lee MH, Ramaswami B, Angenet LT, et al. (2005) Sampling methodologies and dosage assessment techniques for submicrometer and ultrafine virus aerosol particles. J Appl Microbiol 99: 1422-1434.

32. Wichmann HE, Spix C, Tuch T, Wolke G, Peters A, et al. (2000) Daily mortality and fine and ultrafine particles in Erfurt, Germany. I. Role of particle number and particle mass. Res Rep Health Eff Inst 2000: 5-86.

33. Elazhary MA, Derbyshire JB (1979) Effect of temperature, relative humidity and medium on aerosol stability of infectious bovine rhinotracheitis virus. Can J Comp Med 43: 158-167.

34. Ijaz MK, Sattar SA, Alkarmi T, Dar FK, Bhatti AR, et al. (1994) Studies on the survival of aerosolized bovine rotavirus (UK) and a murine rotavirus. Comp Immunol Microbiol Infect Dis 17: 91-98.

35. Karim YG, Ijaz MK, Sattar SA, Johnson-Lussenburg CM (1985) Effect of relative humidity on the airborne survival of rhinovirus-14. Can J Microbiol 31: 1058-1061.

36. Hermann J, Hoff S, Munoz-Zanzi C, Yoon KJ, Roof M, et al. (2007) Effect of temperature and relative humidity on the stability of infectious porcine reproductive and respiratory syndrome virus in aerosols. Vet Res 38: 81-93.

37. Ijaz MK, Karim YG, Sattar SA, Johnson-Lussenburg CM (1987) Development of methods to study the survival of airborne viruses. J Virol Methods 18: 87-106.

38. Schoenbaum MA, Zimmermann JJ, Beran GW, Murphy DP (1990) Survival of pseudorabies virus in aerosol. Am J Vet Res 51: 331-333.

39. Cooper CW (2010) High volume air sampling for viral aerosols: A comparative approach. Thesis, Airforce Institute of Technology, Wright-Patterson Air Force Base, Ohio, USA, 1-177.

40. Agranovski IE, Safatov AS, Pyankov OV, Sergeev AA, Sergeev AN, et al. (2005) Long-term sampling of viable viruses. Aerosol Sci Tech 39: 912-918.

41. Brown JR, Tang JW, Pankhurst L, Klein N, Gant V, et al. (2015) Influenza virus survival in aerosols and estimates of viable virus loss resulting from aerosolization and air-sampling. J Hosp Infect 91: 278-281.

42. Macher JM, Chatigny MA, Burge HA (1995) Sampling airborne microorganisms and aeroallergens. In: Cohen BS and Hering SV (eds). Air Sampling Instruments for Evaluation of Atmospheric Contaminants. Cincinnati, OH: ACGIH, pp: 279-321.

43. Verreault D, Moineau S, Duchaine C (2008) Methods for sampling of airborne viruses. Microbiol. Mol Biol Rev 72: 413-444.

44. Bourgueil E, Hutet E, Cariolet R, Vannier P (1992) Air sampling procedure for evaluation of viral excretion level by vaccinated pigs infected with Aujeszky's disease (pseudorabies) virus. Res Vet Sci 52: 182-186.

45. Hennigson EW, Ahleberg MS (1994) Evaluation of microbiological aerosol samplers-a review. J. Aerosol Sci 25: 1459-1492.

46. Wen-Hai L (1999) Collection efficiency and culturability of impingement into a liquid for bioaerosols of fungal spores and yeast cells. Aerosol Science and Technology 30: 109-118.

47. Ijaz MK, Sattar SA, Johnson-Lussenburg CM, Springthorpe VS, Nair RC (1985) Effect of relative humidity, atmospheric temperature, and suspending medium on the airborne survival of human rotavirus. Can J Microbiol 31: 681-685.

48. Pyankov O, Usachev EV, Agranovski IE (2012) Inactivation of airborne influenza virus by tea tree and eucalyptus oils. Aerosol Sci Tech 46: 1295-1302.

49. Grossgebauer K, Spicher G, Peters J, Kuwert E, Pohle HD, et al. (1975) Experiments on terminal disinfection by formaldehyde vapor in the case of smallpox. J Clin Microbiol 2: 516-519.

50. Sirikanchana K, Shisler JL, Marinas BJ (2008) Effect of exposure to UV-C irradiation and monochloramine on adenovirus serotype 2 early protein expression and DNA replication. Appl Environ Microbiol 74: 3774-3782.

51. Taharaguchi M, Takimoto K, Zamoto-Niikura A, Yamada YK (2014) Effect of weak acid hypochlorous solution on selected viruses and bacteria of laboratory rodents. Exp Anim 63: 141-147.

52. Barbour EK, Yaghi RH, Jaber LS, Shaib HA, Harakeh S (2010) Safety and antiviral activity of essential oil against Avian Influenza and New Castle Disease viruses. Int J Appl Res Vet Med 8: 60-64.

53. US EPA (2016) Efficacy Requirements for Antimicrobial pesticides. pp: 1-10.

54. US EPA (1980) Efficacy data and labelling requirements: Air sanitizers. pp: 1-8.