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STANDARDIZATION OF A PROTOCOL FOR INACTIVATION OF PULVERIZED AVIAN INFLUENZA VIRUS (AIV) BY ESSENTIAL OILS

by
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"Hallowed be His name"

The LORD by wisdom founded the earth;
By understanding He established the heavens;
By His knowledge the depths were broken up,
And clouds drop down the dew.
(Proverbs 3:19-20)

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AN ABSTRACT OF THE THESIS OF

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Title: Standardization of a Protocol for Inactivation of Pulverized Avian Influenza Virus (AIV) by Essential Oils

The purpose of this thesis is to construct an apparatus and standardize a protocol that enables to study the effect of an equivalent blend of eucalyptus and peppermint essential oils on viability of air suspended avian influenza of H9N2 subtype. The first six experiments studied the impact of air suspension time of pulverized virus, the volume of pulverization chamber, the density and volume of the pulverized AIV H9N2, the nature of pulverized viral carrier, the vacuum pressure, and the quantity of transport medium in the collection flask, on recovery of the virus, and the assessment of its viability in chicken embryos. The seventh experiment studied the weight loss of essential oils subjected to evaporation at a temperature of 35 ˚C. The result showed that the refined constructed apparatus that allows the 100% recovery of pulverized influenza is constituted of a pulverization vessel of 2100 c.c., a recovery vessel of 250 c.c., a negative pressure of 6 mbar, and an air-suspension time of pulverized influenza of 1.5 minutes. This apparatus was used to generate data related to anti-influenza, with H9N2 suspended in the air at 1.2×10^7 particles / c.c., and vaporized blend of essential oils equivalent to 0.10 µl / c.c., resulting in H9N2-recovery equivalent to 12.5% after a contact time of 1.5 minutes, compared to control apparatus, deprived of the vaporized essential oils blend (50.0% recovery). In conclusion, the use of the refined-constructed apparatus at the above mentioned conditions, allowed for determining the reduction of the viral viability by vaporized blend of essential oils.

Keywords: AIV H9N2 aerosols, essential oils of eucalyptus and peppermint vapors, constructed apparatus, protocol, and inactivation of AI viral aerosols.

CONTENTS

ILLUSTRATIONS

TABLES

ABBREVIATIONS

AIV	Avian Influenza Virus
P0 AIV	Avian Influenza Virus (zero passage in chicken trachea)
ECE	Embryonated Chicken Eggs
HA	Haemaglutinin
HPAIV	Highly Pathogenic Avian Influenza Virus
IBDV	Infectious Bursal Disease Virus
LPAIV	Low Pathogenic Avian Influenza Virus
MG	Mycoplasma Gallisepticum
NA	Neuraminidase
NDV	Newcastle Disease Virus
QACs	Quaternary Ammonium Compounds
RNA	Ribonucleic Acid
$TCID_{50}$	Tissue Culture 50% Infectious Dose
MDCK	Madin- Darby Canine Kidney
SDS	Sodium Dodecyl Sulfate

CHAPTER I

INTRODUCTION

The scientists are continuously working to solve global issues in all fields related to human concern. Influenza viruses are of three types A, B, and C where the A type infects both humans and avian spp. (birds) while the B & C types infect only humans. The influenza A virus is a major pathogen capable to create pandemics causing human sickness and death, especially during favorable seasons for viral particles survival and transmission (Tellier, 2006). It is an economic disease for poultry producers all over the world who suffer losses caused by the avian influenza virus (AIV), such as H9N2 (Nili and Asasi, 2002). The evolving viruses, through change of genetic material by antigenic drift or shift, produce new escape mutants from the immune system, providing quick spread and transmission of these particles (Lee and Saif, 2009). Fear of pandemics threatening human health and poultry sector is a prevailing situation where the highly pathogenic strains of influenza A viruses are found in Asia, Europe, and Africa. The major route of acquiring influenza virus infection is through the airborne large-droplet transmission as indicated by many researchers (Tellier, 2006; Verreault *et al.*, 2008; Yang *et al.*, 2011). The survival and transmission of the virus in the air is affected by several environmental factors mainly temperature, humidity, UV light, ionizing radiation, and air turbulence (Barreca & Shimshack, 2012; Yang & Marr, 2011; Xie *et al*. 2007; Muhamad *et al.*, 2001).

Many researches introduced several ways to control the evolving avian influenza viruses, among which the AIV (H9N2). Several groups of active disinfectants against AI viruses are used including alcohols, soaps and detergents, acids, alkalis,

chlorine and chlorine compounds, oxidizing agents, aldehydes, quaternary ammonium compounds (QACs) and phenols. Their efficacy in the field is highly influenced by many factors like the environmental temperature and organic matter presence (Benedictis *et al.,* 2007). Antiviral drugs belonging to M2 inhibitors and the neuraminidase (NA) inhibitors classes are used in treatment (Wutzler *et al.,* 2004). However, the continuously evolving influenza viruses mutants are found to be building resistance against these drugs (Nicholson *et al.*, 2003), which is making it necessary to find other approaches for their control.

The essential oils have direct inhibitory activity against influenza viruses (Wu *et al.,* 2010). They were found to interfere with the function of the outer viral envelope (Hayashi *et al.,* 1995). The Mentofin®, a 100% natural herbal additive product consisting of a blend of essential oils extracted from Eucalyptus and Peppermint, was found effective against the AIV H9N2 (Barbour *et al.,* 2006; Barbour *et al.,* 2010; Barbour *et al.,* 2011; Rehman *et al*., 2013). The efficacy of Mentofin® against H9N2 virus could be due to the presence of numerous components such as, 10 % eucalyptus oil, 10% menthol, 47% saponins, and 33% liquid builders (Rehman *et al*., 2013). The objectives of the study are to:

- Develop an apparatus and standardize a protocol for the assessment of essential oils efficacy against AIV H9N2 aerosols.

- Test the efficacy of the natural essential oils of Mentofin® vapors in inactivating the low pathogenic AIV H9N2 aerosols in the air.

CHAPTER II

LITERATURE REVIEW

A. Avian influenza virus (AIV)

Avian influenza virus (AIV) causes economic diseases that were reported in the Middle East and worldwide. The mild and virulent strains of the well-known AIV H9N2 have led to high mortality on broiler farms. The problem is incremented especially when associated with other respiratory diseases, in what is known as a co-infection condition (Nili and Asasi 2002). The antigenic drift, where permanent alteration in the genetic material, and the antigenic shift at which genetic mix and recombination among subtypes take place to form different strains of viruses, result in viruses that are constantly changing, and rapidly spreading due to reduced immunity to their escape from the immune system . Influenza pandemics are creating a worldwide issue (Cornell University, 2008).

1. Description

Influenza viruses, which belong to the *Orthomyxoviridae* family, are enveloped viruses of negative single-stranded RNA. It is classified into three different types A, B and C based on the differences of nucleocapsid (NP) and matrix (M) proteins. Type A infects mammals (humans, swine, and horses) and bird species; therefore, all of the AI viruses belong to this type. However, types B and C infect only humans (Lee and Saif, 2009; Hatta and Kawaoka 2002). The envelope of the influenza viruses has on its surface the agents of pathogenicity namely, the haemaglutinin (HA) and neuraminidase (NA) glycoproteins whose antigenic relationships divide influenza A viruses into

subtypes. There are 17 different haemaglutinin HA (H1-H17) and 10 neuraminidases NA (N1-N10) discovered of the avian influenza AI viruses (Abdelwhab and Hafez, 2012).

2. Pathogenicity

Based on pathogenicity level, avian influenza viruses are classified into low pathogenic AI (LPAI) and highly pathogenic AI (HPAI) (Lee and Saif, 2009). The viral invasion relies on the presence of the HA and NA. Pathogenicity in terms of infectivity and replication requires the HA, which helps in binding the virus to the host cell, followed by its cleavage into HA1 and HA2. On the other hand, the virions are released from inside the cell by the work of NA that remove the sialic acid residues from the HA binding (Mitnaul *et al.*, 2000; Els *et al.*, 1989).

a. LPAI

The low pathogenic AI (LPAI) causes mild symptoms in infected birds and a drop in eggs of layers. They are never systemic, due to the fact that their HA are not cleaved by the protease enzymes present in the intestinal and respiratory tracts of birds (Garten and Klenk, 1999; Horimoto and Kawaoka, 2001; Lee and Saif, 2009).

b. HPAI

The highly pathogenic AI (HPAI) causes systemic infection due to the cleavage of H-protein in these viruses by the ubiquitous proteases (i.e. furins and PC 6 Proteases) of the Golgi apparatus found in all body cells (Horimoto *et al.*, 1994).

B. Factors affecting transmission, suspension, and viability of influenza viruses' aerosols in the air

In the United State, influenza is estimated to cause between 1,700 and 59,000 human deaths annually (Barreca & Shimshack, 2012). There are four different modes of transmission of influenza virus that are identified: large droplets by coughing or sneezing, airborne through droplet nuclei < 2.5 μm in radius (aerosols), contact transmission by direct physical touch, and indirectly through secretions on fomites (Weber, 2008; Shaman & Kohn, 2009; Yang *et al.*, 2011).

The droplets nuclei are the shrunken diameter of aerosol droplets formed by evaporation; being hygroscopic, they swell back in humid medium such as the lungs. The influenza virus aerosols are suspended in the air or gas of solid or liquid particles that are small enough to remain as airborne structures for prolonged time, due to their low settling velocity. The spherical shaped aerosols, when having same density, settle at different times owing to diameter and air turbulence. From a 3 m fall, particles of 100 μm settle in 10 s, the 20 μm particles settle in 4 min, the 10 μm particles settle in 17 min, the 5 μm particles settle in 62 min, and < 3 μm particles do not settle at all. These results are summarized in Table 1.

Table 1. Settling velocity of the particles with different diameters from a 3 meters height*

Diameter size in µm	Vertical distance in cm	Time in sec	Calculated settling velocity in cm/sec*
100	300	10	30.00
20	300	240	1.25
10	300	1020	0.29
5	300	3720	0.08

*Settling velocity of particles with different diameters from 3 meters height according to Knight 1980 and supported by similar findings by Hinds, 1999; Nicas et al., 2005; Tellier, 2006; and Verreault et al., 2008.
**The velocity is calculated in cm/second after dividing the vertical distance covered in cm by the time in seconds.

Based on settling velocity, the rapidly settling aerosols > 10 µm that do not settle in the lower respiratory tract, are considered large droplets (Knight, 1980; Nicas et al., 2005; Tellier, 2006).

Viable airborne pathogens in < 5 µm particles are removed from the air by particle settling in droplets, die-off, exhaust ventilation, and air disinfection methods. The viability of the Influenza virus aerosols in particular is mainly affected by humidity, temperature, UV, & ionizing radiations (Lowy *et al.*, 2001; Yang & Marr, 2011).

1. Effect of humidity and temperature

Many studies prove that low relative humidity and low temperature favor influenza virus survival and transmission. It is clear to mention that precipitation and cold temperature increase disease transmission, especially when susceptible individuals move indoors. Despite controlling each of temperature or humidity separately, either absolute humidity or temperature was proven as a critical determinant of observed influenza events (Barreca & Shimshack, 2012).

The humidity determines the droplet size of the viruses and affects their inactivation rates, which affects the efficacy of transmission. Recent findings showed that the rate of transmission is 2.4 times more at 10% RH compared to 90% RH. This rate increases as the time increases. So far, studies agree that the lower relative humidity is better for longer survival of airborne influenza viruses. The increasing RH increases the inactivation rate of the Influenza viruses on one hand and accelerates the settling velocity of aerosol viral particles on the other hand, due to the fact that particles retain humidity to become larger droplets, resulting in faster settlement. Research findings suggest that humidification of indoor air may help decrease the spread of influenza viruses, since the amount of water vapor in the air affects viability, which could be explained by the surface inactivation of lipid-containing influenza viruses (Shaman & Kohn, 2009; Yang & Marr, 2011). Absolute humidity (AH) compared to relative humidity (RH) was found more significant to predict influenza virus transmission and survival. Having 50% of transmission variability and 90% of survival variability were explained better in relation to AH; whereas, a respective 12% of transmission variability and 36% of survival variability were better related to RH. Epidemiologic evidence from the U.S. found that a drop in AH can predict the human influenza outbreaks and the resulting excess mortality (Shaman & Kohn, 2009; Barreca & Shimshack, 2012).

The cold temperature is another factor that favors transmission and the seasonality of epidemic of influenza viruses, especially at high RH. It was found that a greater frequency of transmission has occurred at 5 °C compared to 20 °C and 50%-80% relative humidity; whereas the difference was not significant at 35%-65% RH. On the contrary, no transmission occurred at 30 °C and 35% RH. Already mentioned earlier

that higher humidity negatively affects rate of transmission; however, this study deduced that the lower temperature favors transmission at high RH (Lowen et al., 2007). There was no statistically significant relationship between temperature and human mortality due to influenza virus infection at mean daily temperatures below -9.4 °C and above 15.6 °C. The mean daily temperatures associated with significant increase in fatalities due to influenza virus infection were found between -1.1 °C and 15.6 °C with its peak at -1.1 °C (Barreca & Shimshack, 2012).

2. Effect of ultraviolet (UV) and ionizing radiations

Solar insolation was found to reduce influenza virus survival (Barreca & Shimshack, 2012). Inactivation rates of influenza viruses aerosols by greater than 99.9% were obtained after having been passed through the UV cell at flow rates of 100 ft(3)/min and 200 ft(3)/min (Jensen, 1964). However, a study showed that exposure of high pathogenic avian influenza HPAI (H7N3) to UV light for 45 minutes was not enough for inactivation, inferring that ultraviolet radiation is not effective in timely manner. This method is efficacious when the surfaces are well cleaned with disinfectants before the direct and close application (Muhamad et al., 2001; Samberg and Meroz, 1995).

Ionizing radiation is a better method for inactivation of avian influenza viruses compared to ultraviolet light or chemical agents. According to scientific findings ionizing radiation, neutrons (η) and in particular gamma (γ) photons have a superior penetration into biological and other material. The gamma photon radiation was the most efficacious approach for inactivation of human influenza viruses. These studies helped in adoption of such technique for sterilization of laboratory tools and clinical

equipment (Lowy *el al.*, 2001). The genome size of viruses correlates with the needed dose of radiation for inactivation, since the waves target the viral RNA and not the viral proteins (Thomas *et al*., 1982; House *et al*., 1990).

3. Airflow effect

a. Air flow direction and rate effects

The influenza aerosol particles' transmission follows the airflow direction as noted in guinea pig experiments, otherwise, it doesn't occur (Lowen *et al*. 2006). In 1962, a controlled experiment with ventilation rate between 2-30 L/min was performed using mice as a model for influenza viral transmission. The airborne transmission was positively correlated to the low humidity and low rates of airflow (Schulman and Kilbourne, 1962).

b. Factors affecting distance travelled in an air flow

Although not comprehensively confirmed, the three-feet rule that is found in infection control guidelines for influenza, stated that large droplets do not travel farther than 3 ft. (Tellier, 2009). However, according to Xie *et al*. model, the behavior of respiratory droplets is assumed to be like that of 0.9% NaCl droplets in water. The movement of respiratory droplets, after exhaled in the air, is directed forward in a jet. The very large droplets quickly fall down on the ground, followed by intermediate size droplets that leave the jet, either desiccated partially or completely, according to their size in the ambient air. The small droplets, however, completely desiccate in the jet. An experiment was accomplished using a 2 m height trajectory and 50% RH, it was found that particles of initial diameter of 30–50 μm travelled horizontally at an initial 1 m s^{-1}

of normal breathing velocity, equivalent to a distance that is less than a meter. Whereas at the sneezing velocity 50 m s^{-1}, the travelled particles exceeded 6 m distance. The horizontal distance travelled by respiratory droplets is increased by decreased RH, increased initial velocity, and reduced particle size. On the other hand, the larger particles at increased relative humidity and decreased initial velocity will travel less (Xie *et al*. 2007).

C. Impact of essential oils on Influenza viruses

The antiviral effect of essential oils is perceived in nature where plants use chemical components to protect them from disease causing insects, bacteria, and viruses (Wu *et al.,* 2010).

The water soluble extract of *Sanicula europaea* was found to completely inhibit the growth of AI virus (slight extension) (Turana *et al.,* 1996). An in vitro study evaluated a blend of essential oils that combines a mixture of wild orange, clove, cinnamon, eucalyptus and rosemary that could significantly attenuate influenza virus PR8 infectivity in Madin- Darby canine kidney (MDCK) cells. These oils didn't affect viral binding or cellular internalization in MDCK cells. Researchers suggested that antiviral effect may be due to inhibition of viral protein expression, given that the treated viruses continued to express viral mRNAs while having minimal expression of viral proteins (Wu *et al.,* 2010). Earlier, essential oils of *Houttuynia cordata* (Saururaceae) were found to interfere with the function of virus envelope, possessing a direct inhibitory activity against influenza virus (Hayashi *et al.,* 1995).

1. *Impact of essential oils sprays/vapors on human influenza viruses*

French victims of the influenza 1918-19 epidemic were subjected to varied extensive therapies, among which the daily fumigation of bed space with eucalyptus vapours (Hildreth, 1991).

An invention of a composition of terpenes by Lanny Franklin in U.S, having different formulations, can be applied in spray form into the nasal cavities to reduce the amount of microorganisms responsible of infections caused by bacteria, viruses, and fungi. The prevention and treatment of a respiratory infection is by the inhalation of a solution containing a single bioactive terpene, a bioactive terpene mixture, or a liposome-terpene(s). Terpenes are found mainly in plants as constituents of essential oils, oleoresins and balsams of plants, such as conifers. There are examples of different forms of terpenes; menthol from peppermint oil is an alcohol form of terpene, citronellal is an aldehyde terpene, and also found in ketone form (Franklin, 2003: Patent number US 2003/0180349 A1).

2. Impact of essential oils on avian influenza virus H9N2

Having a worldwide distribution, avian influenza virus is one of the most devastating viral diseases in the poultry industry. High mortality caused by avian influenza virus H9N2 type was observed commonly on broiler farms of the Middle East region. The raising concerns of induced pandemic spread of AIV H9N2, has set high importance to control it (Barbour et al., 2006). There are two classes of commercial antiviral drugs for influenza treatment namely, the M2 inhibitors adamantanes (Amantadine and Rimantadine), and the Neuraminidase (NA) inhibitors (Oseltamivir and Zanamivir) (Wutzler et al., 2004). Due to resistance of the avian influenza viruses

to these known antiviral drugs (Nicholson et al., 2003, Webster et al., 1985, Beard et al., 1984), essential oils are experimented in this perspective.

A disinfectant invention, containing a combination of essential oils and chemicals, by Robert Bowker in US, was tested for virucidal efficacy against Avian Influenza A virus, Turkey/W is/66 strain (H9N2). The germicidal spray composition consists of 60% distilled water, 10% polysorbate 80, 5% orange Valencia oil, 10% orange terpene oil, and 15% of a 35% Hydrogen peroxide. The avian influenza virus was inactivated and no virus was detected after exposure to the germicidal spray composition for 10 minutes at ambient room temperature 22˚C (Bowker, 2009, Patent number US7,578,970 B2).

D. Mentofin®

Mentofin® is a 100% natural herbal additive developed by EWABO, a German company. It is a blend of essential oils containing 10 % eucalyptus oil, 10% menthol, 47% saponins, and 33% liquid builders (Rehman *et al*., 2013). Mentofin® can be administered through spraying, drinking water of animals and birds, or in the feed. It is applied in water for the general prevention of respiratory problems of most livestock, and for boosting and homogeneity of vaccine responses. Extensive research on the product revealed its antimicrobial activity, positive influence on immunity, and improvement of performance in poultry flocks.

1. Mentofin® effects on immunity

Significant economic losses due to high morbidity, mortality and higher feed conversion ratio (FCR) are encountered in the broilers' industry suffering from co-

infections (Nili and Asasi, 2002; Ley, 2003). High susceptibility however to secondary infections, poor feed conversion ratio and weak response to commonly used vaccines maybe found in immuno-suppressed birds (Sharma *et al*., 2000). There are many factors that lead to immunity failure in chickens; namely, the variety of avian pathogens, biological or chemical induced immune suppression, and irregular use of vaccines (Xie and Song, 2005). To improve the immunity of broilers, immunostimulants must be added. Many herbal products, among which Mentofin®, were proven effective as immunopotentiating agents, able to produce a homogeneity in immune titers of the flock (Carli *et al.,* 2008; Barbour *et al.*, 2011; Rehman *et al*., 2013). Eucalyptus and peppermint oils potentiate both innate-cell mediated and humoral immune responses in poultry birds. They also have a potent immunomodulatory effect on immune response of birds to vaccines (Awaad *et al.,* 2010). It was found to improve immunity of broilers by a consistent higher magnitude of acquired humoral responses to the IBDV and NDV (Barbour *et al.*, 2008).

2. Mentofin® Activity against animal respiratory pathogens

a. Newcastle Disease (ND) virus

Mentofin® has antimicrobial activity against Newcastle Disease (ND) virus. It inactivated the lentogenic strain of ND virus within 15 minutes of interaction at 0.5 % concentration and 37 ˚C (Rehman *et al*., 2013). From a different *in vitro* study, the concentration of Mentofin® for the inactivation of a lentogenic strain of ND virus was found inversely proportional to time. The virus was inactivated at 37 ˚C in 5 minutes at 0.75% Mentofin® concentration, 10 minutes at 0.5%, and 15 minutes at 0.25% (Barbour *et al.* 2010). It reduces the host reactions produced by Newcastle disease (ND)

vaccination (Carli *et al.,* 2008). The effect was significant on antibody response of Mentofin® treated broilers to Newcastle Disease (ND) virus vaccine showing higher consistent anti-NDV HI antibody titer as compared to untreated broilers (Barbour *et al.,* 2008; Awaad *et al.,* 2010; Rehman *et al*., 2013). The level of protection against ND virus was found 87% in 35 days old broilers Mentofin® treated and NDV-vaccinated or only NDV-vaccinated, 37% in only Mentofin® treated, and 25% in untreated and unvaccinated birds (Rehman *et al*., 2013). Whereas, in a different study using a velogenic strain of NDV, the level of protection was 35% in Mentofin® treated and NDV-vaccinated broilers, 25% in untreated but vaccinated, and 0% in untreated and unvaccinated broilers (Awaad *et al*., 2010). Apparently, eucalyptus and peppermint oils increase the non-specific resistance of chickens (Rehman *et al*., 2013).

b. Activity against Infectious Bronchitis virus (IBV)

It was found that Mentofin® reduces the morbidity and specific lesions in broilers challenged with infectious bronchitis virus (IBV) at 15 days post challenge (Barbour *et al*., 2008).

c. Activity against *Escherichia coli* (*E. coli*) bacteria

The ability of Mentofin® was evaluated in a study on commercial broiler chickens to reduce Escherichia coli-related respiratory lesions. Other matters were included in the evaluation as the ability to immunomodulate Newcastle disease (ND) vaccine response, alteration of pharyngeal aerobic bacterial counts, and impacts on specific production parameters. Experimental birds were naturally exposed to infectious bursal disease virus (IBDV) and administered live Newcastle disease virus (NDV)

vaccine. The Mentofin® was able to reduce E. coli-related lesions, as well as, the mortality rate that is caused by the administration of live NDV to broilers with clinical IBDV (Çarli *et al*, 2008).

d. Activity against *Mycoplasma gallisepticum* (MG) bacteria

Mycoplasma gallisepticum (MG) causes chronic respiratory disease in chickens, which is remarkable of high economic significance. It reduces feed intake, and results in egg production reduction, high morbidity, high mortality, and significant degrading effect on meat quality.

The MG was included in a study, testing the alleviation of host injuries by the essential oils of Mentofin® against a serious respiratory disease complex caused by the interaction among *Mycoplasma gallisepticum* MG, IBDV, IBV, and NDV vaccine viruses (Barbour *et al.*, 2008).

e. Activity against other respiratory pathogens

The antibacterial activity of eucalyptus oil was reported against non-spore forming bacterial species such as *Haemophilus influenzae, H. parainfluenzae, S. maltophilia* and S. *pneumonia* (Cermelli *et al.*, 2008).

3. *Impact of Mentofin® on avian influenza virus H9N2*

An in vivo study revealed significant impact of eucalyptus and peppermint essential oils (Mentofin®) in the protection of the respiratory system of broilers against controlled challenges by avian influenza virus H9N2. Histopathological observations revealed a significant reduction ($P < 0.05$) in microscopic tissue lesions of H9N2-

challenged birds specifically in tracheal deciliation, tracheal goblet cells degeneration, tracheal mucus accumulation, and heterophil infiltration (Barbour *et al.,* 2006).

A research study, conducted in 10 days-old embryonated eggs, determined that eucalyptus and peppermint essential oils of Mentofin® had a complete virucidal activity against AIV H9N2 in presence and absence of 1% skimmed milk, at 2.78% Mentofin® concentration, room temperature, and 30 minutes contact time. In fact, organic matter has a neutralizing effect on essential oils; therefore, in the absence of skimmed milk, the virucidal activity of Mentofin® on AIV H9N2 started at $2.78x10^{-1}$ % concentration. The essential oils were found safe to chicken embryoes, resulting in 100% survival when administered in 0.1 ml/chick embryo at concentrations between $2.78x10^{-3}$ and 27.8% (Barbour *et al.,* 2010).

Essential oils of Mentofin® were studied to test their effect on a worldwide poultry condition involving H9N2-avian influenza virus (AIV) in addition to multiple viral and bacterial respiratory infections including Infectious Bursal Disease Virus (IBDV), Newcastle Disease Virus (NDV), *Mycoplasma gallisepticum* (MG), and Infectious Bronchitis Virus (IBV). The treatments with eucalyptus and peppermint essential oils against challenged birds with the viral and bacterial pathogens were found effective in the following: Mortality in challenged and unchallenged birds was reduced, the feed conversion ratio (FCR) in challenged birds was improved, specific signs and lesions were reduced, liver function (serum aspartate aminotransferase) in challenged and unchallenged birds was improved, the creatinine level in challenged birds was homogenized, as indicated by the low coefficient of variation, the immune reaction to the H9N2 challenge and to the IBDV intermediate strain was reduced, and the NDV immunity to the NDV vaccine was boosted (Barbour *et al.,* 2011).

E. Disinfectants against avian influenza viruses

According to Noll and Youngner 1959, the viruses in general are classified into three categories A, B, and C based on their resistance to chemical agents. The A category includes all enveloped viruses of intermediate to large size, among which are the avian influenza (AI) viruses. The viruses' susceptibility to the chemical agents is influenced by the virus size and the presence or absence of viral lipid. It is a fact that the lipids in enveloped viruses are the main factor behind the high susceptibility of these viruses to most disinfectants (Benedictis et al., 2007).

1. Classification of active disinfectants against avian influenza viruses

There are several active disinfectants against AI viruses namely; those grouped under alcohols, soaps and detergents, acids, alkalis, chlorine and chlorine compounds, oxidizing agents, aldehydes, quaternary ammonium compounds (QACs) and phenol compounds. The efficacy of disinfection in the field is highly influenced by the environmental temperature, in which temperatures above 20 ˚C provide the optimum efficacy for the majority of disinfectants (Benedictis et al., 2007).

a. Soaps and detergents

For general cleaning, soaps and detergents are widely used for decontamination of surfaces, through the removal of organic materials and dirt. Owing to their surfactant properties, they are efficacious disinfectants against all enveloped viruses due to their impact on the lipid components of virus particles (Ausvetplan, 2005). They are characterized by producing a curd and leaving a film on surfaces due to the free negatively charged ions that combine to calcium and magnesium in hard water. Soaps

and detergents are efficacious against AIVs based on their effect on lipids (Benedictis et al., 2007).

b. Acids

Acid disinfectants are used for several decontamination purposes, due to their high virucidal activity. They are categorized into two groups, organic and inorganic acids. They are effective on viruses that are sensitive to low pH and in general have slow action activity (Jeffrey, 1995).

Organic acids include formic, citric, lactic, mallic, glutaric and propionic acids (Jeffrey, 1995). By reducing pH and through the interaction of lipophilic structures of organic acids with membranes of enveloped viruses, they can inactivate them (Haas et al., 1995). The combination of organic acids and free anionic detergents or sulphonate compounds has been used in sanitizing formulations that lead to improvement of activity (Jeffrey, 1995). In slurry, the interaction of these acids with protein renders them less effective in inactivating viruses (Haas et al., 1995). A research study on the efficacy of a commercial organic disinfectant (Venno Vet 1 super®) against LPAI virus (H7N1) of titers ranging between 108.1 $TCID_{50}$/ml and 106.8 $TCID_{50}$/ml was conducted. The organic acid disinfectant, composed of 55% formic acid and 7% glyoxylic acid, was investigated at different concentrations (0.1%, 0.5%, 1%, 2%), temperatures (4 ˚C, 10 ˚C and 20 ˚C), and contact times (5, 10, 15, 30, 60 and 120 min). The antiviral activity was evaluated in presence or absence of proteins and in suspension conditions or through carriers. The efficacy of the disinfectant was negatively influenced by the protein load and low temperature, but found effective in suspension without protein at 20 ˚C. (Venno Vet 1 super®), in carrier at 1%

concentration, enabling the inactivation of the AIV at 10 and 20 ˚C temperature and 30 min contact time. At the same 1% concentration in carrier and at 4 ˚C, the contact time was extended up to 120 min to result in complete inactivation of the virus. However, at 2% concentration in carrier and at 4 ˚C, contact time for inactivation was reduced to 15 min. On the other hand, the disinfectant in suspension at 1% concentration was effective at all temperatures within 5 min (Yilmaz et al., 2004). For these reasons, disinfectants again are proved ineffective during winter at temperatures below 20 ˚C (Benedictis et al., 2007). A recent study evaluated the eggshell decontamination by a sanitizer formed by a combination of the novel levulinic acid plus sodium dodecyl sulfate (SDS). Influenza A H3N2 virus was inoculated onto chicken eggshells before treatment with the different rates of the sanitizer. Three treatments with liquid solutions were applied for 1 min, using 5% levulinic acid with 2% SDS, 2% levulinic acid with 1% SDS, and 0.5% levulinic acid with 0.5% SDS. Different inactivation rates of the influenza A H3N2 virus were observed by the various levulinic acid plus SDS concentrations tested ($P \leq 0.05$). It was found that the mixture of 5% levulinic acid with 2% SDS sanitizer provided the greatest level of inactivation (2.23 log PFU) (Aydin et al., 2013).

Inorganic acids include nitric, hydrochloric, sulphuric, phosphoric, and sulphamic acids (Jeffrey, 1995). This group of acids, and only through decreasing pH values, has the ability to inactivate viruses (Haas et al., 1995). The inorganic acids are limited in use, with the exception of hydrochloric and citric acids, owing to their corrosive impact on skin and material. However, hydrochloric acid as a strong acid, and citric acids as a weaker acid are easily available and generally used in decontamination procedures, due to their known safety. The hydrochloric acid doesn’t have a high

toxicity index. The citric acid is found in solid form and can be used for decontamination of clothing (Ausvetplan, 2005).

c. Chlorine and derivates

The chlorine is an anion that denatures proteins by oxidizing the peptide links (Maris, 1995). The hypochloric acid produced at acid pH from chlorine in water, gives this disinfectant group its property. For that reason the stability of this group of disinfectants is pH dependent, showing decreased efficacy at high pH values. The chlorine compounds are less priced and are easily available liquids compared to sodium hypochlorite (household bleach) and the solid form of calcium hypochlorite. They act rapidly, but corrosive and inhibited by organic material. The liquid form of sodium hypochlorite is usually available at 10–12% concentrations, to be diluted into 2–3% in water upon use (Ausvetplan, 2005).

An old study, determined through experiments, the effect of hypochlorous acid gas and hypochlorite mists on influenza A viruses (PR8) aerosols. At a concentration of $1{:}2 \times 10^6$ of hypochlorous acid gas volume to air volume, 99% of aerosol viral particles were deactivated (Edward & Lidwell, 1942). Another study showed that a 0.125% dilution of a commercial available sodium hypochlorite product was able to inactivate LPAI H7N2 of 109.8–109.4 ELD_{50} isolated in Pennsylvania during the 1997–1998 outbreak. The inactivation efficacy of AIV by a mixture of anti-freeze compounds namely ethylene glycol, propylene glycol and methyl alcohol with sodium hypochlorite was investigated in the same study. Disinfectant efficacy decreased with the dilution of sodium hypochlorite with propylene glycol and methylene glycol (50% in 50%); on the

other hand, sodium hypochlorite activity against AIV was not affected upon dilution (30% in 70%) with methyl alcohol (Davison et al., 1999).

In addition, the efficacy of sodium hypochlorite in the inactivation of AI virus was demonstrated in a study using 1 : 10 dilution as recommended by the manufacturer, in addition to a dilution of 1 : 100.Two different LPAI strains H5N9 (A/turkey/Wisconsin/68) and H7N3 (A/turkey/Oregon/71) were included as test particles. The real-time reverse transcriptase polymerase chain reaction (RT-PCR) revealed no detection of viral RNA at 1 : 10 dilution. This indicates that the sodium hypochlorite at manufacturer's dilution 1 : 10 was able to damage the viral RNA (Suarez et al., 2003).

In another study, the efficacy of six types of povidone-iodine products 0.25% PVP-I palm, 0.23% PVP-I gargle, 0.5% PVP-I scrub, 2% PVP-I solution, 0.23% throat spray and 2% PVP-I solution for animals were tested against high and low pathogenic avian influenza viruses. One HPAI virus A/crow/Kyoto/T2/04 (H5N1) of 106.5 ELD_{50}/0.1 ml and three LPAI viruses namely, A/whistling swan/Shimane/ 499/83 (H5N3) 104.8 ELD_{50}/0.1 ml, A/whistling swan/Shimane/42/80 (H7N7) 106.5 ELD_{50}/0.1 ml, and A/duck/Hokkaido/26/99 (H7N7) 104.8 ELD_{50}/0.1 ml. At the ratio of 1:2, each disinfectant product was tested against viruses for 10 s contact time and 25 ˚C temperature, followed by inoculation of embryonated chicken eggs. The virus infectious titres were reduced to levels below the detection limits of virus isolation, which indicated a virucidal activity against all AIVs tested (Ito et al., 2006).

d. Oxidizing agents

The oxidizing agents are active disinfectants, oxidizing lipids and nucleic acids by the free hydroxyl radical that is developed. The surfaces disinfected with oxidizing agents must be previously cleaned since the efficacy of many different groups of disinfectants is decreased in the presence of organic material, which is also true for any oxidizing agent. These agents should be used with awareness because they are corrosive for metals and could cause irritations in the mucous membranes of the eyes and to skin (Muhammad et al., 2001).

At different concentrations and incubation times, Virkon-S activity was tested against Pakistan isolate H7 subtype. The avian influenza (AI) virus was fully inactivated at 0.5% of Virkon-S solution after 90 min, while complete inactivation was achieved at 1% and 2% concentration after only 30 min (Muhammad et al., 2001).

In different studies, Virkon-S disinfecting activity was found to be dependent on the freshness of the solution. That was demonstrated at room temperature, using the recommended dilution of 1% w/v for each of fresh and 10-day-old Virkon-S solutions against two LPAI viruses H5N9 (A/ turkey/Wisconsin/68) and H7N3 (A/turkey/Oregon/71), with contact times of 10 min and 60 min. The real time RT-PCR was used to establish the inactivation efficacy of Virkon-S against AIVs. The results showed that a fresh Virkon-S solution disrupted completely the viral genome, but the 10-day-old solution failed to completely disrupt the nucleic acid of the AIV (Suarez et al., 2003).

As an alternative to the use of toxic disinfectants and polluting gases used to clean inert surfaces, such as glass and steel, the efficacy of hydrogen peroxide that is well known against many microorganisms namely bacteria, yeasts and viruses has been

studied. Nevertheless, the corrosive effects of the hydrogen peroxide due to an accelerated surface oxidation are undesirable on metal surfaces. The efficacy of this agent in inactivating AIVs, however, was not the same in different studies (Benedictis et al., 2007). The hydrogen peroxide can reduce virus infectivity after an exposure for 30 min, but it failed when used at 10% micro-aerosolized to inactivate completely a viral solution of 106 ELD_{50}/ml of H9N2 (A/turkey/Wisconsin/66) (Neighbor et al., 1994). In other studies, exposure to hydrogen peroxide vapour in liquid and in dried status for 30 min inactivated AIV (H5N2 A/ chicken/Pennsylvania/83) of a titre 108.5 ELD_{50}/ml (Heckert et al., 1997).

e. Aldehydes

Aldehydes are organic compounds that have a virucidal effect. The inactivation of viruses is by the alkylation of amino and sulphydrilic groups of viral proteins and purinic bases. The organic matter decreases their efficacy. There are three aldehyde agents that are widely used for disinfection namely, the glutaraldehyde, formaldehyde and formalin (Jeffrey, 1995; Ausvetplan, 2005).

The glutaraldehyde is a chemically stable disinfectant that is used in sterilization of medical instruments, but not in general use due to its cost. It works well as a disinfectant at contact times between10 to 30 min and dilutions between 1% and 2% (Jeffrey, 1995; Ausvetplan, 2005).

Formalin is a toxic compound made of 40% aqueous solution of the more stable compound formaldehyde gas. Formalin is an effective disinfectant against many viruses, belonging to different families, at 8% dilution. It was found that at 0.04% and 0.1% concentrations of formalin at 37 °C and contact of 16 h, can cause the inactivation of

HPAI viruses (H5N2 A/chicken/Pennsylvania/ 1370/83 and H5N9 A/turkey/Wisconsin/68) and LPAI viruses (H9N2 A/turkey/Wisconsin/68). The haemagglutinating activity, however, was preserved (King, 1991). Another study used the following dilutions of formalin 0.06%, 0.12% and 0.24% at contact times 6, 12, 18 and 24 h for testing the efficacy against H7N3. Formalin failed to inactivate AIV at 0.06% and 0.12% concentrations after 6 h, but at 0.24% concentration the virus was completely inactivated. The inactivation of the AI viruses at all tested concentrations of formalin was obtained after 12 h contact time (Muhammad et al., 2001). The viricidal efficacy of aldehydes is lost at temperatures below 20 °C (Yilmaz et al., 2004), which demands the combination of anti-freeze products. For this reason, the propylene glycol has been widely used with formalin to improve dispersion of the disinfectant under low environmental temperatures (Meroz and Samberg, 1995). The commercial product Venno FF super® containing 20% formaldehyde and 12% oligomer pentaerythritose condensate was effective in inactivating a LPAI H7N1 viral stock (A/Carduelis/ Germany/72) of 108.1 to 106.8 $TCID_{50}$/ml density. The test results showed that this product inactivated the virus efficaciously at 1% concentration and at 10 & 20 ˚C temperatures after 60 min contact time. Higher concentrations of 2% and longer contact times of 120 min were recommended to completely inactivate AIV at 4˚C in the carrier-testing system. On the other hand, in suspension test conditions, the disinfectant at 1% and all temperatures was efficacious against AIV within 5 min time. When protein was present, it was found to decrease the efficacy of this product, as it is the case with other disinfectants (Yilmaz et al., 2004).

The formaldehyde gas is toxic and must be given ample attention when used due to exhibiting residual toxic activity. There are certain compounds, but not all, that

are dangerous for having specific agents bound to formaldehyde such as potassium permanganate. On the beneficial side, agents as QACs can be combined with formaldehyde (Jeffrey, 1995). The combination of QACs with 2.28% formaldehyde was effective to inactivate avian influenza H7N2 virus of 109.8 ELD_{50}/ml. This mixture was efficacious when combined equally with the anti-freeze product propylene glycol, but ineffective with methyl alcohol or with ethylene glycol (Davison et al., 1999). A commercial product of the Health Company in Ashland, OH, USA containing 20% propanediol, 2.28% formaldehyde and 3.08% alkyl dimethyl benzyl ammonium chloride has been reported to completely inactivate AIV H7N2 at a final dilution of 1% after 10 min exposure. The viral strains were A/chicken/PA/3972-1/97 with a 105.5 ELD_{50}/ ml titre, A/chicken/PA/3972-2/97 with a 104.5 ELD_{50}/ml titre, A/chicken/PA/3779-1/97 with a 104.5 ELD_{50}/ml titre, and A/chicken/PA/3779-2/97 with a 104.2 ELD_{50}/ml titre (Lu et al., 2003). The equipment material must be kept dry, in which air spaces can be decontaminated using gaseous formaldehyde. However, during an outbreak the optimal conditions such as concentration of the disinfecting gas, distribution, time of contact with surfaces, temperature, and humidity make it difficult for gaseous formaldehyde to be considered sufficient to decontaminate a closed air space. This fact does not exclude gaseous formaldehyde from being used since there are no satisfactory alternatives available except hydrogen peroxide, which is a disinfectant restricted mainly to laboratory environments (Ausvetplan, 2005).

f. Phenol and phenolic compounds

Phenol, which is carbolic acid, is capable of maintaining virucidal activity in the presence of organic matter. It was the first disinfectant used in hospital surgical

antisepsis. Phenol compounds are synthetized of phenols that are being safe in general despite, sometimes, irritation in skin that can occur after exposure to cresol containing products. They are available at low to moderate prices in the market. These agents are active against enveloped viruses where at high concentrations are found capable of denaturing and precipitating the viral proteins. Halogenated phenols have been used as fogging sprays for veterinary purposes (Jeffrey, 1995). Phenol crystals are recently used at poultry farms to sterilize instruments and syringes (Benedictis et al., 2007).

There are three categories of phenol disinfectants namely the clear soluble phenol, white fluid phenol, and black fluid phenol (Jeffrey, 1995). The clear soluble phenol is incompatible with acids or alkalis; it is made of liquid soap containing synthetic phenol. This combination is not possible with soaps that inhibit the action of all phenols; for example those based on tallow, tall oil or oleic acid. White fluid phenol and black fluid phenol are toxic and have a strong smell. These solutions are of different tar acid fractions. The white fluid phenol is soluble in water and can be diluted in seawater or brackish water (Jeffrey, 1995).

The phenol crystal was found to inactivate a Pakistanian isolate of AIV H7N3. The experiment was conducted using 0.1%, 0.2% and 0.4% concentrations at 6, 12, 18 and 24 h of contact time. After 24 h, the 0.1% phenol crystal solution could not inactivate AIV, while after 18 h it killed the AIV using the 0.2% concentration, in which only 12 h contact time was required for the 0.4% phenol crystal solution to be efficacious (Muhammad et al., 2001). Another experiment used two strains of avian influenza viruses H5N9 A/ turkey/Wisconsin/68 and H7N3 A/turkey/Oregon/71. These AIVs were inactivated after 1 h contact time, using 0.1% dilution of phenol disinfectants from USA (Tektrol® of Bio-Tek Industries Inc.) and another (One-stroke

Environ®) from Steris Corporation (Suarez et al., 2003). Studies indicate that phenol compounds' combinations with antifreeze chemicals can be used during winter to prevent freezing of the disinfectant solution (Benedictis et al., 2007). The synthetic phenols combined, at a 1:2 dilution, with anti-freeze compounds namely ethylene glycol or propylene glycol, or with windscreen washer fluid (methyl alcohol), are found effective against AI viruses. This was shown in a test of two commercial disinfectants with 1:256 dilution, that were effective against H7N2 of 109.8 ELD_{50}/ml (Davison et al., 1999). The synthetic phenols can still allow the RNA to be detected by real-time RT-PCR, despite inactivation of the avian influenza viruses (Suarez et al., 2003; Spackman and Suarez, 2005).

g. Quaternary ammonium compounds (QACs)

Quaternary ammonium disinfectants are safe for personal use. They are cationic compounds that contain ammonium ions (NH4+) that are effective against all enveloped viruses. The area of disinfection must be cleaned and rinsed before treatment with QACs, to avoid reduction of efficacy after binding to organic material and soaps. They are available and not expensive. QACs are used in combination with chelating agents, such as EDTA to prevent the decreased activity in the presence of hard water (Jeffrey, 1995). They can be mixed with anti-freeze solution, such as ethylene glycol or propylene glycol, or with windscreen washer fluid solution, such as methyl alcohol, without losing efficacy (Davison et al., 1999).

h. Alcohols

Alcohols are used in general at laboratories, hospitals and staff use for disinfection of skin and laboratory equipment. It is also used as a thinner of other chemical disinfectants and several formulations containing alcohol. They are not recommended to be applied in poultry buildings since they are inflammable and can damage plastic objects. Alcohols are efficacious against enveloped viruses, including AIVs at concentrations above 50%; it is worth noting that its use at lower concentrations decreases its efficacy abruptly (Ausvetplan, 2005).

The virucidal activity of MR-1 disinfectant solution was studied on a human type A influenza virus. This solution consisted of 1.26 g of a non-ionic detergent (Nonidet) and 0.4 g of an ionic detergent (lithium dodecyl sulphate) in 100 ml of ethanol. The mixture is characterized by having a virucidal efficacy and a viral inhibitory activity due to the combination of alcohol and non-ionic detergent and lithium respectively. Inactivation of viruses occurred after 5 min contact time, given 25 ˚C temperature, and an initial concentration of 107 PFU/ml. The MR-1 solution was shown to inactivate 0.1 ml of the viral suspension of 108 PFU/ml on plastic or glass surfaces (Skinner et al., 1998). The activity against four AIV strains of H7N2 namely A/chicken/PA/3972-1/97, A/chicken/PA/3972-2/ 97, A/chicken/PA/3779-1/97, and A/chicken/PA/3779-2/97 was carried out in a different study using another commercial product that contained 20% propanediol, 2.28% formaldehyde and 3.08% alkyl dimethyl benzyl ammonium chloride. At a dilution of 1%, initial titres between 104.2 and 105.5 ELD_{50}/ml, and after 10 min contact time, was able to inactivate the AIVs completely (Lu et al., 2003). The efficacy of a different disinfectant including four alcohols 55% ethanol, 10% propan-1-ol, 5.9% propan-1.2-diol and 5.7% butan-1.3-diol

mixed with 0.7% phosphoric acid was studied against a human type A influenza strain (Aichi/2/68 H3N2). Results revealed after three separate assays, in the presence or absence of protein (0.2% of bovine serum albumin or 10% of foetal calf serum), and an initial viral titre of 107.5 $CCID_{50}$/ml and a 96% dilution of the stock, the virus was inactivated in 30 s while at 20% dilution, its inactivation took 1 min (Kramer et al., 2006). More studies aimed at testing the virucidal activity of alcohol. It was found after 15 min of contact time that 70% concentration of ethanol can inactivate four different AIV strains H7N2 of subtype A/chicken/PA/3972-1/97 with a titre of 105.5 ELD_{50}/ml, A/chicken/PA/3972-2/97 and A/ chicken/PA/3779-1/97 with titres of 104.5 ELD_{50}/ml, and A/ chicken/PA/3779-2/97 with a titre of 104.2 ELD_{50}/ml (Lu et al., 2003). An identical study showed that inactivation of AIV H7N2 subtype in the presence 70% concentration ethanol took only 5 min (Castro et al., 1998).

CHAPTER III

MATERIALS AND METHODS

A. Determination of diameter size of the pulverized particles by the nozzle used in Apparatuses 1&2

The objective of this first experiment is to determine the diameter range and average of pulverized particles using a specific nozzle that is used in the apparatuses that are designed for the experiments aiming at the optimization of H9N2-viral air-suspension time before trapping in collection flasks.

An amount of 500 µl of Bromophenol blue solution was sprayed by the pulverization nozzle using the 195 l/min vacuum pump of 0.7 bar, positive pressure. Droplets were collected at a distance of 8-10 cm over a glass slide. The diameter of 10 droplets was measured under microscope magnification of (100 x) and the minimum and maximum range as well as the average were reported (Table 2).

B. Determination of the evaporation rate of Mentofin through time at 35 °C

This experiment aimed at determining the rate of evaporation of the essential oils of Mentofin® at different times from 0 to 60 min at a fixed temperature of 35°C.

An amount of 0.8276 g of Mentofin® was put in an Erlenmeyer flask (250 ml capacity). The flask was incubated at 35 °C, using a water bath and the weight was recorded at different time intervals (Table 3).

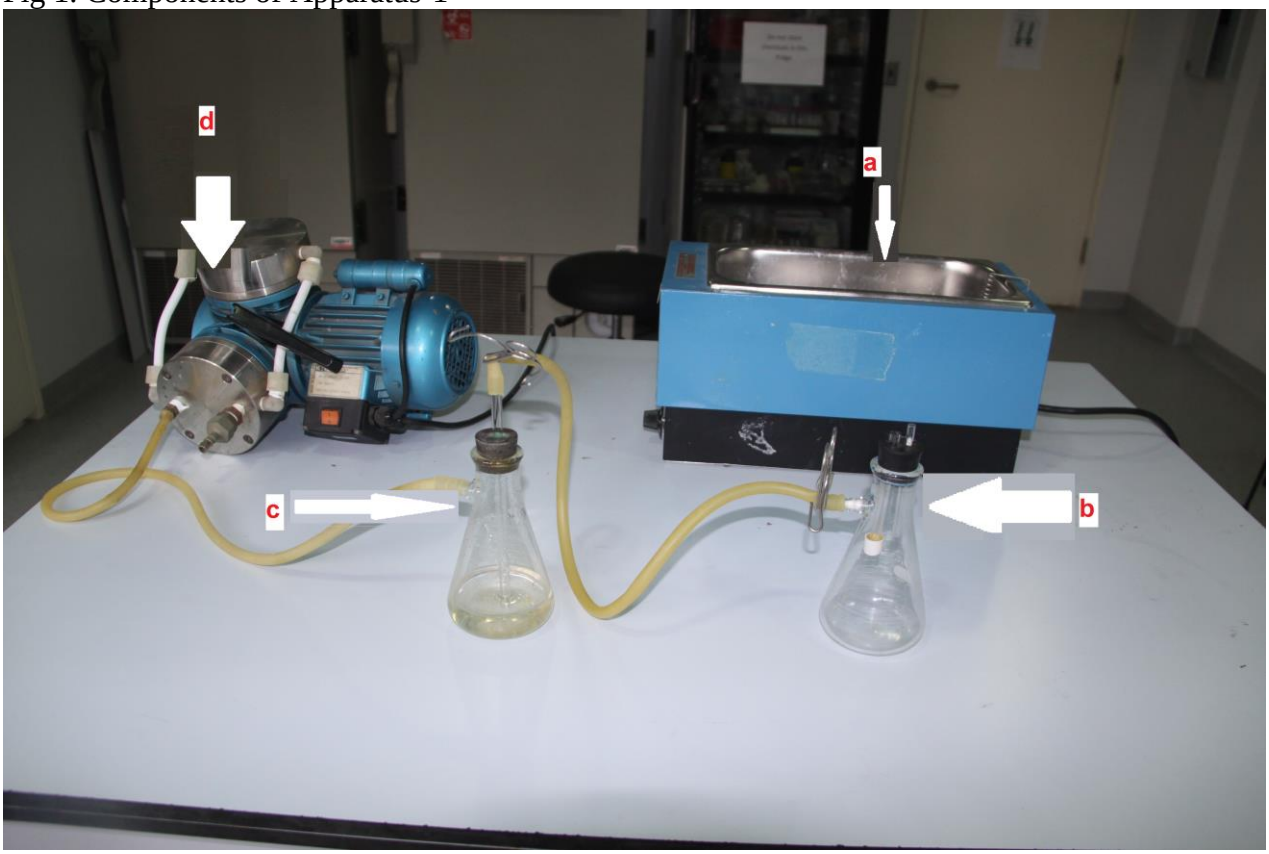
C. APPARATUS-1

1. Description

The Apparatus-1 is designed to be used in experiments aiming at the optimization of H9N2-viral air-suspension time before trapping in collection flasks.

Briefly, it consists of a pulverization chamber (flask) and a collection flask. A negative pressure is generated in the two flasks using a vacuum pump. The description is detailed as per the below, with reference to Fig. 1 that illustrates the different parts of the apparatus.

Fig 1. Components of Apparatus-1

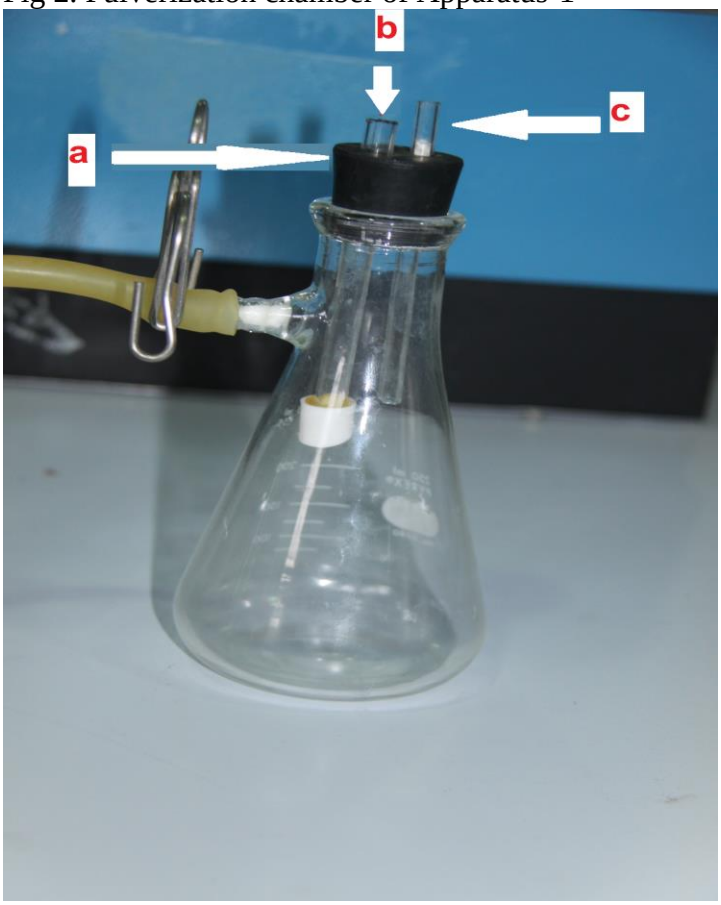


a. Water bath
b. Pulverization chamber (250 ml Erlenmeyer flask)
c. Collection chamber (250 ml Erlenmeyer flask)
d. Vacuum pump (KNF: Model UN035.1.2 STP, suction capacity 56 l/min)

a. Pulverization chamber:

The pulverization chamber consists of a 250 ml capacity Erlenmeyer flask, a rubber stopper with a 9.4 cm length of a Pyrex tube of (6 mm in diameter), partially closed with glass wool for air suction during vacuum pumping, and a pulverizing nozzle, which is described in Fig 2. The pulverizing nozzle, used for loading and pulverizing the viral suspension, consisting of a Pyrex tube of 8.0 mm in diameter, ending at the bottom with a nozzle that pulverizes the viral suspension into particle size of an average of 44.27 µm in diameter (Table 2). In this chamber, the reaction between Mentofin vapor and AI pulverized particles will take place.

Fig 2. Pulverization chamber of Apparatus-1



a. Rubber stopper
b. Pulverizing nozzle
c. Air inlet tube plugged with sterile cotton

b. Collection flask:

The collection flask, which is shown in Fig 1, consists of a 250 ml Erlenmeyer flask having a rubber stopper with a 19.5 cm length and 8 mm diameter of Pyrex tube, in which the upper end lies outside the chamber to connect to the pulverization chamber by means of a rubber tube. The lower end of the Pyrex tube is located inside the collection flask, immersed in 50 ml of transport medium where the viral particles will be presumptively trapped and collected, under negative pressure (maximum vacuum) of 350 mbar. The collection flask connects to the pulverization chamber with a 35 cm length rubber tube and to a vacuum pump with a pressure tube of 30 cm length. The transport medium contains phosphate-buffered saline (PBS) that 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 distilled water. The pH of the PBS is adjusted to 7.4, then the prepared solution is mixed with 500 ml glycerol before the addition of Benzyl penicillin (2×10^6 IU/liter), Streptomycin (200 mg/liter), Polymyxin (2×10^6 IU/liter), Gentamicin (250 mg/liter), and Nystatin (0.5×10^6 IU/liter).

c. Vacuum pump

A vacuum pump (KNF: Model UN035.1.2 STP, VAC 50 Hz 1.8 AMP, suction capacity 56 l/min), which is shown in Fig 1, is connected to the collection flask to ensure a negative pressure (maximum vacuum) of 350 mbar inside the system, for viral suction and collection. The positive pressure (maximum continuous pressure) of 0.5 bar generated by the pump was used first to pulverize the viral suspension through the nozzle in the pulverization chamber.

PS. Clips are used to confine the initial pulverized AI in the pulverization chamber during contact time before the application of vacuum (negative pressure).

2. Standardization of air-suspension timing of P0 AIV in Apparatus-1

a. PBS-Glycerol transport medium preparation:

- The Phosphate-buffered saline (PBS) is prepared as follows: dissolving NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, and KH_2PO_4 0.24 g in one liter of distilled water.
- Autoclaving the PBS and mixing 1:1 with sterile glycerol to make 1 liter
- Addition to the 1 liter PBS/glycerol of the following:
    - Benzyl penicillin (2×10^6 IU/liter)
    - Streptomycin (200 mg/liter)
    - Polymyxin B (2×10^6 IU/liter)
    - Gentamicin (250 mg/liter)
    - Nystatin (0.5×10^6 IU/liter)

b. P0 AIV H9N2

The experimental avian influenza virus H9N2 is labeled P0, denoting zero passage in chicken trachea. It is a mildly pathogenic AIV H9N2 isolated from a broiler farm in Lebanon, propagated in 10-day-old chicken embryos and preserved at – 80 ˚C in transport medium.

c. First experimental protocol

The objective of the experimental protocol was to find out the proper time needed for the sprayed virus to stay in the pulverization chamber, which could be

retrieved efficiently in the transport medium present in collection flask, using Apparatus-1. An amount of 300 µl of P0 AI virus, having an HA titer of 1 : 64 was sprayed and kept in the pulverization chamber for five experimental times namely 2 , 4 , 6 , 8 , and 10 min respectively before it was vacuum-pumped for 2 min using a negative pressure of 350 mbar capacity pump (KNF: Model UN035.1.2 STP) into the collection flask containing 50 ml of the prepared transport medium.

d. Attempt to propagate the retrieved influenza virus from the transport medium

The collected influenza virus in the transport medium is diluted 1/10, 1/100, 1/1000, and 1/10,000 using sterile transport medium. Inoculate 100 µl of each collected and diluted medium in duplicate into allantoic membrane of 9-10 d embryonated eggs and incubate them at 37 ˚C for 3 days, and perform candling to determine the percentage of embryonic mortality. The eggs are put in fridge at 4 ˚C for at least 3 hours, harvesting the allantoic fluid using sterile pipettes, and performing the HA test, to detect the presence of viable H9N2 in the collected diluted fractions.

e. HA test

i. Preparation of 1 % RBCs in saline medium

- Collect chicken blood into heparin containing tubes.
- After 30 min, pool the collected blood & centrifuge at 2000 rpm for 10 min.
- Discard the plasma and buffy coat.
- Wash the RBC twice by suspending in saline and centrifuging at 2000 rpm for 10 min.

- Dilute with saline to 25 % RBCs (stock suspension) then dilute to 1 % (working suspension) to perform HA test.

ii. HA test to quantitate the embryo-propagated AI virus

- Add 50 µl of saline to each of the 96 wells of the microtiter plate using a multichannel pipet.
- Each allantoic fluid sample is serially diluted, in a dilution factor of ½, using one row of the micro-titer plate.
- Add 50 µl of 1 % chicken RBC suspension and incubate at room temp for 30 min.
- Read and record the HA titer, which is the maximum dilution of the propagated virus that still can agglutinate completely the 1 % RBC suspension.

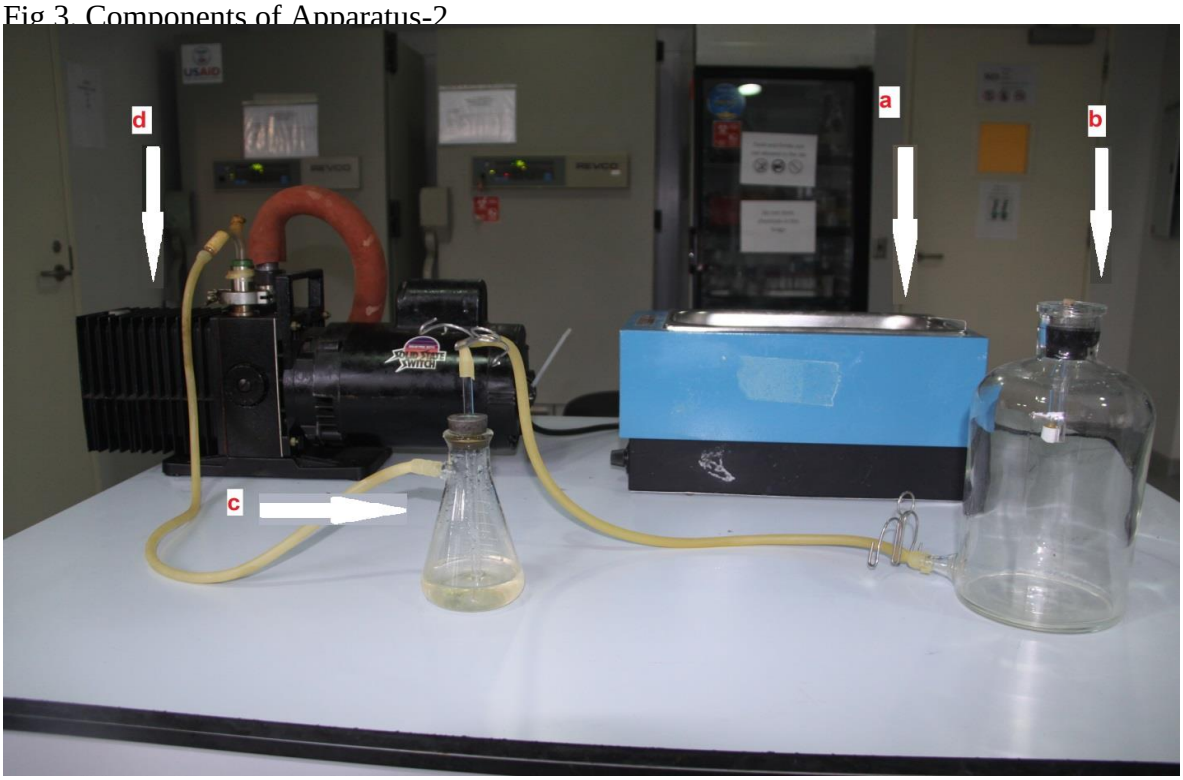
D. Apparatus-2

1. Description

The Apparatus-2 is the 2nd modified apparatus designed to conduct experiments following the failure of the attempt to collect pulverized AIV by Apparatus-1.

Briefly, the 250 ml pulverization chamber in Apparatus-1 was replaced by a 2100 ml capacity flask, and the vacuum pump was replaced by a new pump Model DDC 195, with a suction capacity of 6 mbar negative pressure instead of 350 mbar negative pressure. The description is detailed as per the below, assisted by labeling the apparatus in Fig 3.

Fig 3. Components of Apparatus-2

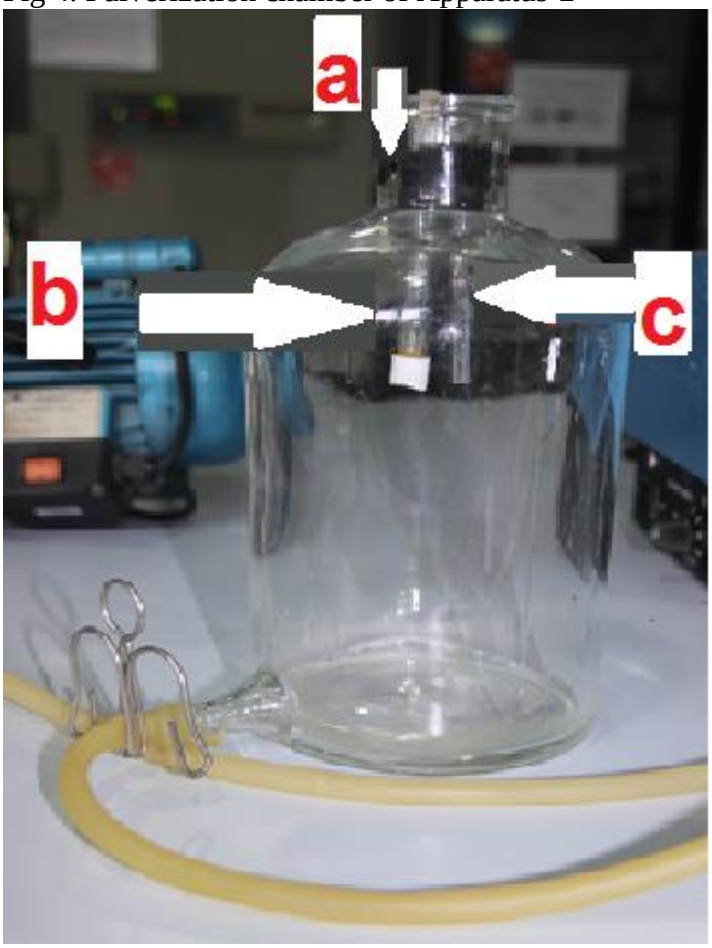


a. Water bath
b. Pulverization chamber (2100 ml volume flask)
c. Collection chamber (250 ml Erlenmeyer flask)
d. Vacuum pump (DDC 195 model)

a. Pulverization chamber:

The pulverization chamber consists of a 2100 ml capacity flask, a rubber stopper with a Pyrex tube of a 9.4 cm length and 6 mm diameter, partially closed with glass wool for air suction during vacuum pumping, and a pulverizing nozzle, as illustrated in Fig 4.

Fig 4. Pulverization chamber of Apparatus-2



a. Rubber stopper
b. Pulverization nozzle
c. Air inlet tube plugged with sterile cotton

The pulverizing nozzle, for loading and pulverizing the viral suspension, consists of a Pyrex tube of eight mm of diameter, ending at the bottom with a nozzle that pulverizes the viral suspension into particle size of average diameter of 44.27 µm (Table 2).

b. Collection flask:

The collection flask of apparatus-2 is similar to that of Apparatus-1, described previously (paragraph C-1-b) in description of apparatus-1; however, it was connected

to a vacuum pump of 6 mbar negative pressure and a pulverization chamber of 2100 cc volume, which is shown in Fig 3.

c. Vacuum pump:

A vacuum pump having a capacity of negative pressure of 6 mbar (Model DDC 195, 195 l/m) is connected to the collection flask to ensure a negative pressure of 6 mbar inside the system, for viral trapping and collection. The positive pressure 0.7 bar generated by the pump was used first to pulverize the viral suspension through the nozzle in the pulverization chamber. The installed pump can be seen in Fig 3.

PS. Clips are used to confine the initial pulverized AI in the pulverization chamber during contact time before the application of vacuum (negative pressure).

2. Standardization of the air-suspension timing of P0 AIV in Apparatus-2

a. P0 AIV H9N2

Same P0 AIV H9N2 used in the first standardization experiment (paragraph A-1-b) is propagated in 10-day-old embryos and preserved in TPB at – 80 ˚C.

b. Second experimental protocol using Apparatus-2

The objective of this experimental protocol was to find out the proper time needed for the virus to stay in the pulverization chamber before it is retrieved efficiently in the transport medium of the collection flask. Inject 300 µl of P0 AIV in TPB (256 HA) into each of the pulverization chambers I, II, III, and IV, then wait 15 sec, 30 sec, 45 sec, and 1 min respectively before vacuuming. Apply vacuum pumping on each of the 4 chambers for a fixed period of 2 minutes, and collect the viral particles into 50 ml

transport medium present in each of four respective collection chambers. The transport medium present in each collection flask, presumptively containing the virus, is kept in separate sterile urine cups and stored at – 80 ˚C for viral propagation in 10 days old embryonated chicken eggs.

c. Attempt to propagate the collected virus and HA test

The attempt to propagate the presumptively collected virus is performed as follows: Inoculate 100 µl of each collected medium in embryonated eggs (two eggs/ treatment). Incubate the eggs at 37 ˚C for 3 days and perform candling to determine the percentage of embryonic mortality. Then put the eggs in fridge at 4 ˚C for at least 3 hours, harvest the allantoic fluid using sterile pipettes, and perform HA test. The HA test used to quantitate the propagated AI viruses is performed as described previously (paragraph A-2-e).

d. Third experimental protocol using Apparatus-2

Same objective and procedures were maintained as in the second experimental protocol (paragraph D-2-b), but with some differences. The air-suspension times in the pulverization chambers I, II, III, IV, and V were 30 sec, 1 min, 2 min, 3 min, and 5 min respectively before vacuuming. The transport medium presumptively containing the collected virus was propagated in triplicates of 9 days old embryonated chicken eggs.

e. Attempt to propagate the collected virus and HA test

The attempt to propagate the presumptively collected virus is performed as follows: Inoculate 100 µl of each collected medium in embryonated eggs (three eggs/

treatment). Incubate the eggs at 37 ˚C for 3 days and perform candling to determine the percentage of embryonic mortality. Then put the eggs in fridge at 4 ˚C for at least 3 hours, harvest the allantoic fluid using sterile pipettes, and perform the HA test. The HA test used to quantitate the propagated AI viruses is performed as described previously (paragraph A-2-e).

f. Fourth experimental protocol using Apparatus-2

Same objective and procedures were maintained as in the second and third experimental protocols (paragraph D-2-b), but with particular differences. The air-suspension time in the pulverization chambers I, II, III, IV, and V were 1 min, 1.5 min, 2 min, 2.5 min, and 3.5 min respectively before vacuuming. The transport medium presumptively containing the collected virus was propagated in 9 replicates of 9 days old embryonated chicken eggs.

g. Attempt to propagate the collected virus and HA test

The attempt to propagate the presumptively collected virus is performed as follows: Inoculate 100 µl of each collected medium in embryonated eggs (nine eggs/ treatment). Incubate the eggs at 37 ˚C for 3 days and perform candling to determine the percentage of embryonic mortality. Then put the eggs in fridge at 4 ˚C for at least 3 hours, harvest the allantoic fluid using sterile pipettes, and perform HA test. The HA test used to quantitate the propagated AI viruses is performed as described previously (paragraph A-2-e).

h. Fifth experimental protocol using Apparatus-2: Reproducibility of air-suspension time for P0 AIV H9N2 at 1.5 min

The objective of this experiment, based on the positive results obtained in the 4th experimental protocol, is to evaluate reproducibility of air-suspension time for P0 AIV H9N2 at 1.5 min of air-suspension in 3 replicates of apparatus-2. The steps were repeated as in the 4th experimental protocol, but using a1.5 min of air suspension time in pulverization chambers I, II, and III before vacuuming.

i. Attempt to propagate the collected virus and HA test

The attempt to propagate the presumptively collected virus is performed as follows: Inoculate 100 µl of each collected medium in embryonated eggs (nine eggs/ treatment). Incubate the eggs at 37 ˚C for 3 days and perform candling to determine the percentage of embryonic mortality. Then put the eggs in fridge at 4 ˚C for at least 3 hours, harvest the allantoic fluid using sterile pipettes, and perform HA test. The HA test used to quantitate the propagated AI viruses is performed as described previously (paragraph A-2-e).

j. Sixth experimental protocol: Reproducibility of the air-suspension time for P0 AIV H9N2 using Apparatus-2 at 1.5 min, following an increase in the volume of pulverized AI and a reduction of the volume of collected transport medium

A total of two runs using Apparatus-2 were used namely I and II. An amount of 500 µl of 256 HA of P0 in TPB were sprayed in pulverization chambers I and II, then incubated at 35 °C for 1.5 min before vacuuming. Vacuum pumping was applied for 2 minutes, presumptively collecting the pulverized viruses into reduced volume of 25 ml transport medium in the two collection chambers I and II connected to their respective pulverization chambers. The transport medium, presumptively containing the virus, was

put in a separate sterile urine cup and kept at -80˚C for viral propagation in 10-days-old embryonated chicken eggs.

k. Attempt to propagate the collected virus and HA test

The attempt to propagate the presumptively collected virus is performed as follows: Inoculate 100 µl of each collected medium in embryonated eggs (twelve eggs/ treatment). Incubate the eggs at 37 ˚C for 3 days and perform candling to determine the percentage of embryonic mortality. Then put the eggs in fridge at 4 ˚C for at least 3 hours, harvest the allantoic fluid using sterile pipettes, and perform HA test. The HA test used to quantitate the propagated AI viruses is performed as described previously (paragraph A-2-e).

l. Seventh experimental protocol: Evaluation of the virucidal efficacy of essential oils in Mentofin® vapors against pulverized AIV particles at 1.5 min air- suspension time

A total of four runs using Apparatus-2 were used namely I, II, III, and IV. An amount of 1 ml of Mentofin® was evaporated in Pulverization chambers I and II for a period of 30 minutes at 35 °C liberating a total of 200 µl of essential oils. An amount of 500 µl of 256 HA of P0 in TPB were then sprayed in pulverization chambers I, II, III, and IV, then incubated at 35 °C for 1.5 min before vacuuming. Vacuum pumping was applied for 2 minutes, presumptively collecting the pulverized viruses into reduced volume of 25 ml transport medium in the four collection chambers I, II, III, and IV connected to their respective pulverization chambers. The transport medium, presumptively containing the virus, was put in a separate sterile urine cup and kept at -80˚C for viral propagation in 10-days-old embryonated chicken eggs.

The calculated amount of evaporated essential oils in pulverization vessel is 200 µl / 2100 c.c. (0.10 µl / c.c.), and the expected viral particles in the same vessel is 256 HA / 2100 c.c. (0.12 HA unit / c.c. = 1.2×10^7 H9N2 particles / c.c.)

m. Attempt to propagate the collected virus and HA test

The attempt to propagate the presumptively collected virus is performed as follows: Inoculate 100 µl of each collected medium in embryonated eggs (twelve eggs/ treatment). Incubate the eggs at 37 ˚C for 3 days and perform candling to determine the percentage of embryonic mortality. Then put the eggs in fridge at 4 ˚C for at least 3 hours, harvest the allantoic fluid using sterile pipettes, and perform HA test. The HA test used to quantitate the propagated AI viruses is performed as described previously (paragraph A-2-e).

3. Statistical analysis

Chi square test was used to compare the frequency of viral recovery between the Mentofin and control treatments ($p = 0.05$).

CHAPTER IV

RESULTS AND DISCUSSION

A. Determination of diameter size of the pulverized particles by the nozzle used in Apparatuses 1&2

The objective of this first experiment is to determine the diameter range and average of pulverized particles using a specific nozzle that is used in the apparatuses that are designed for the experiments aiming at the optimization of H9N2-viral air suspension timing before trapping in collection flasks. An amount of 500 µl of 2% Bromophenol blue solution was sprayed by the pulverization nozzle using the 195 l/min vacuum pump of positive pressure of 0.7 bar Droplets were collected at a distance of 8-10 cm over a glass slide. The diameter of 10 droplets was measured under microscope magnification of (100 x).

The pulverized H9N2 particles' diameter ranged between 33.0 and 59.8 µm with an average of 44.27 µm, it is worth noting that the size of human sneeze particles ranges between 0.1 µm and 1 mm, which includes the range of the pulverized particles (Lindsley *et al.*, 2010).

Table 2. Determination of diameter size of the sprayed particles by the pulverization nozzle into the pulverization chambers of used apparatuses.

Droplets' minimum diameter (µm)	**Droplets' maximum diameter (µm)**	**Droplets' average diameter (µm)$_1$**
33	59.8	44.27

[1]The average of 10 droplets of the bromophenol blue solution, sprayed through the pulverization nozzle over the glass slides, was calculated.

B. Determination of the evaporation rate of Mentofin® through time at 35 °C

This experiment aimed at determining the rate of evaporation of the essential oils of Mentofin® at different times from 0 to 60 min at a fixed temperature of 35°C. An amount of 0.8276 g of Mentofin was put in an Erlenmeyer flask (250 ml capacity). The flask was incubated at 35 °C, using a water bath and the weight was recorded at different time intervals.

The increasing evaporation rate of Mentofin® essential oils was correlated to the exposure time given at temperature of 35 °C as shown in table 3. Increasing rates of evaporation were observed between 0-30 minutes, but seems to reach a plateau after that time. Accordingly, the experiment on inactivation of the H9N2 was set at 35 °C for 30 minutes evaporation of the Mentofin®, which lead to evaporation of 20.8% of the total volume of Mentofin®. It is worth noting that the essential oils of eucalyptus ideally evaporates at temperature of 32 °C, while that of pepper mint essential oil evaporates at 21 °C (Herbert *et al.*, 1987; Imdorf *et al.*, 1995).

Table 3. The evaporation rate of the essential oils of Mentofin® after different incubation times at a temperature of 35 °C.

	Evaporation rate* (Loss of weight x100/ initial weight)
At t=0 min	0.0 %
At t = 5 min	6.4 %
At t = 30 min	20.8 %
At t = 60 min	28.2 %

*Actual temperature of incubation was 35 °C since Menthol starts to evaporate at 21 °C (Herbert *et al.*, 1987), while the Eucalyptus was found to evaporate at 32 °C (Imdorf *et al.*, 1995).

C. Apparatus-1

1. First experimental protocol using Apparatus-1: Standardization of air-suspension timing of P0 AIV in Apparatus-1

The objective of this experiment is to find out the proper time needed for the sprayed virus to stay in the pulverization chamber before it is retrieved efficiently in the transport medium present in collection flask, using Apparatus-1. An amount of 300 µl of P0 AIV in transport medium, having an HA titer of 1 : 64 was sprayed and kept in the pulverization chamber for five experimental times namely 2 , 4 , 6 , 8 , and 10 min respectively before it was vacuum-pumped for 2 min using a negative pressure of 350 mbar capacity pump (KNF: Model UN035.1.2 STP) into the collection flask containing 50 ml of the prepared transport medium.

No HA activity was observed in all allantoic fluids of embryos that were inoculated by different dilutions of the transport medium at the different suspension times.

The failure in Apparatus-1 to recover the viral particles suspended for different periods in the air of the 250 ml Erlenmeyer flask could be due to the short distance of the nozzle to the inside wall of the flask, which could lead to sticking of aerosols to the inner surface. Another reason is the insufficient high vacuum pressure and/ or the long suspension time given to this trial namely, 2, 4, 6, 8 and 10 minutes, which could have led to settling of the viral particles at the wall and bottom of the flask. In addition, the injection of 1 : 64 HA units of the virus present in 300 µl could be low. The different settling of particles depending on the suspension time is explained in many studies that show the relation between size of aerosolized particles and settling velocity. The large droplets settle faster. According to Hinds (1999), the100 µm particles settle at 2.49E-01 m/s, while the 10 µm particles settle at 3.06E-03 m/s. In another study by Knight

(1980), the results are pretty similar where the 100 µm particles settle at 3.0E-01 m/s and the 10 µm particles settle at 2.94E-03 m/s (Knight, 1980; Hinds, 1999; Nicas et al., 2005; Tellier, 2006; Verreault *et al.*, 2008). The effect of viral density used in the injected volume is clear from many previous studies, documenting that the virus concentration is a key factor to increase sampling sensitivity (Meng *et al.*, 2010) and recovery probability (Hermann *et al.*, 2006). It was proven that the efficiency of collection is a function of the aerosol concentration (Jaschhof 1992).

D. Apparatus-2

1. Second experimental protocol using Apparatus-2: Standardization of air suspension timing of P0 AIV in Apparatus-2

The objective of this experimental protocol was to find out the proper time needed for the virus to stay in the pulverization chamber before it is retrieved efficiently in the transport medium of the collection flask. Inject 300 µl of P0 AIV in TPB (256 HA) into each of the pulverization chambers I, II, III, and IV, then wait 15 sec, 30 sec, 45 sec, and 1 min respectively before vacuuming. Apply vacuum pumping on each of the 4 chambers for a fixed period of 2 minutes, and collect the viral particles into 50 ml transport medium present in each of four respective collection chambers. The transport medium present in each collection flask, presumptively containing the virus, is kept in separate sterile urine cups and stored at – 80 ˚C for viral propagation in 10 days old embryonated chicken eggs.

The mortality and HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different suspension times namely 15 s, 30 s, 45 s and 1 minute, using Apparatus-2 are presented in Table 4.

Table 4. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different suspension* times using Apparatus-2

Air suspension times	Average HA titer of a duplicate	Embryonic mortality (no. dead/ 2)
15 sec	0	1
30 sec	0	0
45 sec	0	0
1 min	512	0
Ctrl	0	0

*An amount of 300 µl of P0 AIV (HA titer = 1/256) was pulverized in the pulverization chamber of Apparatus-2. The volume of the transport medium in the collection chamber was 50 ml.

Reducing the vacuum pressure from 350 mbar to 6 mbar, replacing the transport medium carrier of the P0 AIV by TPB, using higher density of the virus (1 : 256) in the same injection volume of 300 µl, and shortening the air-suspension times to 15 sec, 30 sec, 45 sec, and 1 min, gave different titers of recovery of the P0 AIV. The 1 min suspension in 2100 ml Erlenmeyer flask gave a successful recovery of the virus under a higher negative pressure of 6 mbar compared to the previous failing trial of lower vacuum pressure (350 mbar). The effect of the higher vacuum pressure is explained by the studies that involved sampling of airborne viruses by the All-Glass Impingers (AGIs), which are also called Porton impingers, or even AGI-like samplers. These types of samplers are successful in capturing airborne viruses (Verreault *et al.*, 2008); the collection chamber of the apparatuses-1&2, used in this study, had a vacuum pressure less or equal to -0.05 atm, which is equivalent to -50.6625 in mbar (Hermann *et al.*, 2006; Meng *et al.*, 2010). In the study reported by Jaschhof (1992), the increase in flow rate is considered a functional parameter and determinate of efficient collection. This implies that using the higher negative vacuum pressure (6 mbar) of the (DDC 195 model) pump used in the design of Apparatus-2 improved the collection compared to

the lower negative vacuum pressure of 350 mbar in the case of the (KNF: Model UN035.1.2 STP) vacuum pump used in Apparatus-1.

The delay in viral settling is always due to their smaller diameters (Knight 1980), leading to improvement of the recovery of H9N2 viruses. However, although the effect of time of suspension can be a factor affecting recovery of viral particles, it could be also due to other factors documented in literature (Knight, 1980; Hinds, 1999; Nicas et al., 2005; Tellier, 2006; Verreault *et al.*, 2008). In our experiment, the average pulverized droplets size was found to be 44.27 µm, ranging between 33 µm and 59.8 µm in diameter as shown in Table 2. This range in particle size requires between 1 - 16 seconds to freely settle in the bottom of the 20 cm height (2100 ml volume) pulverization chamber of Apparatus-2, and also less than 8 seconds to settle in the bottom of the 10 cm height flask of 250 ml Erlenmeyer flask included in Apparatus-1, as deduced in literature and illustrated in Table 1 (Knight 1980; Hinds, 1999; Nicas et al., 2005; Tellier, 2006; and Verreault et al., 2008).

Table 5. The expected settling time in seconds of the predicted particle diameter AI viral P0-H9N2 particles in the pulverization chambers of Apparatuses-1&2*

Diameter size of particles in µm	**Settling time in seconds in the pulverization chamber of Apparatus-1 having 10 cm height**	**Settling time in seconds in the pulverization chamber of Apparatus-2 having 20 cm height**
100	0.33	0.67
20	8.00	16.00
10	34.48	68.97

*The expected time of settling of the AI viral particles with different diameters extrapolated from Knight, 1980; Hinds, 1999; Nicas et al., 2005; Tellier, 2006; and Verreault et al., 2008.

Based on the extrapolation of the above references, the pulverized P0 AIV H9N2 particles, which are of an average diameter of 44.27 µm, and ranging between 20

- 100 µm diameter, should have settled in the bottom of the pulverization chambers before 16 seconds in the Apparatus-2. This extrapolation contradicts with what was found in this study. The several experiments conducted, successfully recovered the viable viral particles of the P0 AIV H9N2 after suspension times ranging between 30 seconds and 2.5 minutes, where it is expected, according to Knight (1980), to have all of the pulverized viral particles settling before 16 seconds. The experimental results are clear by referring to Tables 4, 6, & 7 of the second, third and fourth experimental protocols using the Apparatus-2, where the recovery was successful at more than 16 seconds period.

It is worth noting that, the high relative humidity (RH) in the chamber may affect negatively the recovery of the viral particles by several ways, mainly by an increase of the size of the particles, due to the condensation of the water vapor on the airborne particles, leading to faster settling of large droplets. Another way is the negative effect of RH that has on damaging the viruses, leading to a loss of infectivity after dissolving the particle nuclei and exposing the viruses to high concentrations of solutes (Verreault *et al.* 2008).

However, the successful recovery of the pulverized P0 AIV H9N2 that took place in the time range between 30 seconds and 2.5 minutes of suspension times can be explained by the several conditions of the experiments, among which are temperature and relative humidity, that could have contributed to the results: The water bath, in which the pulverization chamber was placed, was set at a temperature of 35 °C. The injected 300 µl of P0 AI in TPB (256 HA) into each of the pulverization chambers, should have an expected settling of few seconds and not exceeding the 16 seconds suspension time as illustrated in Table 5. As discussed in the review of Verreault *et al.*

(2008), post settling, the liquid evaporates carrying the viruses and a droplet nucleus is obtained by the concentration of the non-evaporative contents. This implies that in this study, the evaporated medium carrying the AI viral particles could have taken place after 30 seconds at 35 °C followed by aggregation, which possibly lead to re-suspension (re-aerosolization) of the viral particles to remain for several seconds in the air of the chamber before settling again after 2.5 minutes of suspension time. This phenomenon of re-suspension is mentioned as reentrainment in the study of Hermann *et al.* (2006), which supports the obtained data, with highest success of recovery at 1.5 minutes suspension time before settling again.

The effect of the higher vacuum pressure (Hermann *et al.*, 2006; Verreault *et al.*, 2008; Meng *et al.*, 2010), as well as the higher density of the virus suspended in the air (Jaschhof, 1992; Hermann *et al.*, 2006; Meng *et al.*, 2010), and the synchronized air-suspension times between 30 seconds and 2.5 minutes for viruses to be re-suspended in the air of the pulverization chamber (Hermann *et al.*, 2006) improved the recovery of H9N2 viruses in this study.

2. Third experimental protocol using Apparatus-2: Standardization of air suspension timing of P0 AIV in Apparatus-2

The mortality and HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different air-suspension times namely 30 s, 1 min, 2 min, 3 min, and 5 min, using Apparatus-2 are presented in Table 6.

Table 6. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different suspension* times using Apparatus-2

Air suspension times	**Average HA titer**	**Embryonic mortality (no. dead/ 3)**
30 sec	64	2
1 min	128	0
2 min	241.33	0
3 min	0	1
5 min	0	0
Ctrl	0	0

*An amount of 300 µl of P0 AIV (HA titer = 1/256) was pulverized in the pulverization chamber of Apparatus-2. The volume of the transport medium in the collection chamber was 50 ml.

The recovery of the virus under the same conditions and Apparatus-2, as those used in the second experimental protocol was successful given suspension times that are around the 1 min namely, 30 sec, 1 min, and 2 min. The viral particles seem to settle when given a suspension time of 3 min and over, or probably indicating a resettling.

Fixing the parameters of HA units of the virus at 1 : 256, and the volume of the injected virus at 300 µl, and the volume of the Erlenmeyer flask containing the suspended virus at 2100 ml, at higher negative pressure of 6 mbar, allowed for detecting accurately the impact of variation of the air suspension times on recovery. It is worth noting that the mortality of the embryos was not correlated to the density of the propagated virus, which is in agreement with our previous works, proving that the P0 virus is not able to kill the embryos, while passaging the virus will higher its pathogenicity (Shaib *et al.*, 2010).

3. Fourth experimental protocol using Apparatus-2: Standardization of air suspension timing of P0 AIV in Apparatus-2

The HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different suspension times namely 1 min, 1.5 min, 2 min, 2.5 min and 3.5 min, using Apparatus-2 is presented in Table 7, and Figure 5.

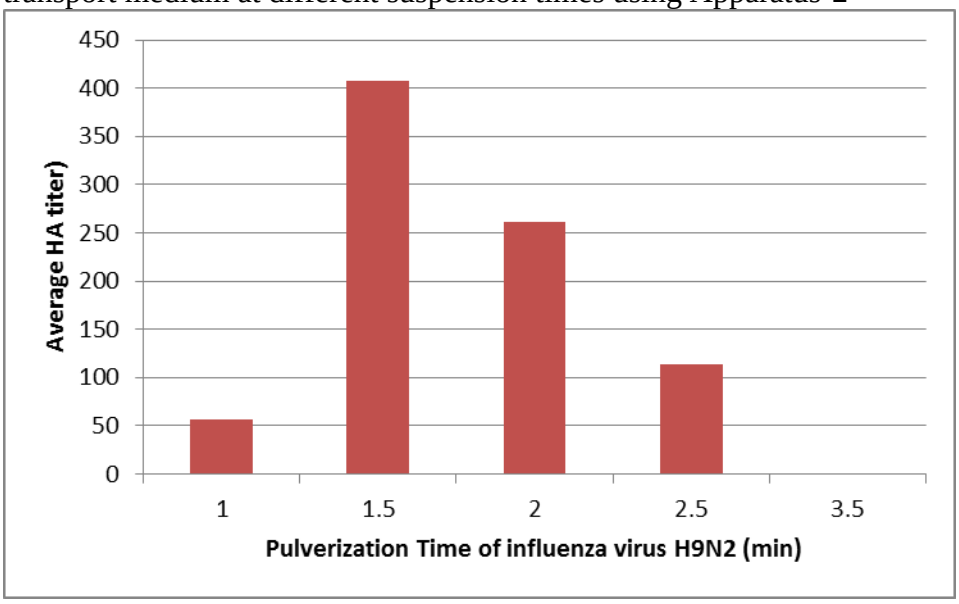
Table 7. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different air-suspension* times using Apparatus-2

Treatment **	**Viral pulverization**	**Suspension times (min)**	**Mean HA titer**
1	+	1	56.89
2	+	1.5	408
3	+	2	260.9
4	+	2.5	113.78
5	+	3.5	0
6	-	0	0

*An amount of 300 µl of P0 AIV (HA titer = 1/256) was pulverized in the pulverization chamber of Apparatus-2. The volume of the transport medium in the collection chamber was 50 ml.

**In each treatment, an amount 300 µl P0 AIV (HA = 1/256) were pulverized in pulverization chambers, except in treatment 6 (negative control) where no viruses were pulverized. A waiting time post-pulverization was set before vacuuming: Treatment 1 = 1 min, treatment 2 = 1.5 min, treatment 3 = 2 min, treatment 4 = 2.5 min, treatment 5 = 3.5 min, and control treatment 6 = 0 min.

Fig. 5. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at different suspension times using Apparatus-2



The use of same experimental conditions as those in the second and third protocols, and varying only the air suspension times using narrower intervals namely, 1 min, 1.5 min, 2 min, 2.5 min, and 3.5 min gave us a better cut point for settlement of the virus at suspension times greater than 2.5 min. This is an indication that the apparatus and the fixed conditions are appropriate for discovering the impact of air suspension time on recovery of the virus.

Previous works standardized the conditions of the apparatus and were able to study the collection efficiencies of different samplers, among which the AGI (all-Glass Impinger). The detection capability of the impinger was equated to 100% success (Jaschhof 1992).

4. Fifth experimental protocol using Apparatus-2: Reproducibility of viral recovery at fixed suspension time of 1.5 m of the P0 AIV H9N2.

The mortality and HA titers of the P0 AIV H9N2 in allantoic fluid of chicken embryos inoculated with the transport medium at 1.5 min in 3 replicates of Apparatus-2 is presented in Table 8.

Table 8. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at constant 1.5 min suspension* time in three replicates of Apparatus-2

Replicates at constant 1.5 min air- suspension time	**Frequency of embryonated eggs that their allantoic fluid showed a positive HA activity**	**Mean HA titer ****	**Embryonic mortality (no. dead/ 9)**
1	2/9	3.56	0
2	0/9	0	0
3	3/9	36	0

*An amount of 300 µl of P0 AIV (HA titer = 1/256) was pulverized in the pulverization chamber of Apparatus-2. The volume of the transport medium in the collection chamber was 50 ml.
**Mean HA titer of 9 embryonated eggs in each replicate.

The recovery of the virus under the same conditions of Apparatus 2 in second, third, and fourth protocols was applied in this fifth experiment, using three replicates at fixed air-suspension time of 1.5 min, in order to assess the reproducibility of this viral recovery. The reproducibility was in 2 out of 3 replicates (~ 67%). The fact that not all inoculated embryos of the same replicate were having successful propagation of the virus is indicative of the low viral count in the small inoculum viral volume of 100 µl/ embryo. It is worth noting that we were using 100 µl out of 50,000 µl as an inoculum. Further work should target a higher reproducibility of viral recovery, by attempting to increase the viral density injected into the suspension flask of 2100 ml. The impact of

viral density in the air-suspension on reproducibility of recovery of the virus was documented previously in the study of Jaschhof, 1992.

5. Sixth experimental protocol using Apparatus-2: Reproducibility of the viral recovery of P0 AIV H9N2 using Apparatus-2 at 1.5 min suspension time, following an increase in the volume of pulverized AIV and a reduction of the volume of collected transport medium

The mortality and HA titers of the P0 AIV H9N2 in allantoic fluid of chicken embryos inoculated with the transport medium at fixed air suspension of 1.5 min in the two replicates of Apparatus-2 are presented in Table 9.

Table 9. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at constant 1.5 min suspension* time in two replicates of Apparatus-2

Replicates at constant 1.5 min air-suspension time	**Frequency of viral recovery****	**Mean HA titer*****	**Embryonic mortality (no. dead/ 12)**
1	5/10	4.2	2
2	4/8	26	4

*An amount of 500 µl of P0 AIV (HA titer = 1/256) was pulverized in the pulverization chamber of Apparatus-2. The volume of the transport medium in the collection chamber was 25 ml.
**The initial number of embryonated eggs/treatment was 12. Embryos that were dead after 24 hours incubation were discarded.
***Mean HA titer of 10 and 8 embryonated eggs in replicates 1 and 2 respectively.

Reproducibility was improved to 100% by increasing the viral injected volume to 500 µl, and reducing the transport medium collecting the virus in a volume of 25 ml instead of 50 ml.

6. Seventh experimental protocol using Apparatus-2: Evaluation of the virucidal efficacy of essential oils in Mentofin® vapors against pulverized AIV particles at 1.5 min

The following experiment involved an increase in the volume of pulverized AIV and a reduction of the volume of collected transport medium. The evaluation of efficacy is represented in Table 10.

Table 10. HA titers of the P0 AIV in allantoic fluid of chicken embryos inoculated with the transport medium at a constant 1.5 min suspension time and evaluation of the virucidal efficacy of essential oils in Mentofin® vapors against pulverized AIV particles using Apparatus-2

Treatment	P0 AIV pulverization[1]	Mentofin® vapors[2]	Frequency of viral recovery[3]	Mean HA titer	Percentage reduction of HA titer by Mentofin®[4]	% frequency of viral recovery in embryonated eggs[5]
Mentofin	+	+	**0/6[a]**	**0**	**73.5%**	**12.5%**
Mentofin	+	+	**¼[ab]**	**8**		
Control	+	-	**5/10[b]**	**4.2**	**0%**	**50.0%**
Control	+	-	**4/8[b]**	**26**		

[1]An amount of 500 microliters of 256 HA of P0 in TPB was sprayed in the pulverization chamber. The volume of transport medium in the collection chamber was 25 ml.
[2]An amount of 1 ml of Mentofin® was vaporized at 35˚C for 30 minutes in the pulverization chamber. Contact time between the virus and Mentofin® vapors was 1.5 minutes.
[3]The initial number of embryonated eggs/treatment was 12. Embryos that were dead after 24 hours incubation were discarded.
[4]The percentage reduction of HA titer by Mentofin® was calculated as follows: The mean HA titer of Mentofin treatment replicates is 4 and the mean HA titer of Control replicates is 15.1; therefore, the Mentofin® treatment reduced the HA titer by 11.1, which comprises 73.5% reduction compared to control groups.
[5]The % reduction in frequency of viral recovery in embryonated eggs is calculated as follows: In Mentofin® treatments (0% + 25%) / 2 = 12.5% and in control treatments (50% + 50%) / 2 = 50.0%

The Mentofin® vapors at 35˚C for 30 minutes were able to inactivate the viral particles suspension in the air, resulting in reduction of the % of HA titer by 73.5% compared to suspension of the same viral loud without that of treatment. In addition, the

recovery of the virus in eggs was reduced by Mentofin® to a 12.5% compared to a recovery of 50.0% in egg embryos.

The Mentofin® essential oils of eucalyptus and peppermint are known for their antiviral activity, specifically against H9N2 subtype of avian influenza viruses (Barbour *et al.,* 2010). The antiviral efficacy of Mentofin® could be due to the presence of numerous components in the essential oils, such as Cineol (eucalyptus) (Kirsch and Buettner, 2013), Menthol, etc. (Rehman *et al*., 2013), prohibiting the virus from developing simultaneous resistance to each compound.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

The first six experimental protocols standardized the air-suspension time of pulverized AIV H9N2 in the constructed apparatuses and adjusted the parameters that affected the recovery of the viable viral particles in the transport medium of the collection chamber. The refined constructed apparatus consisted of pulverization chamber of 2100 c.c., a collection chamber of 250 c.c. containing 25 ml of transport medium, and a DDC 195 vacuum pump creating a negative pressure of 6 mbar (Fig. 3). This apparatus was able to recover 100% of the pulverized H9N2 virus after 1.5 minutes of air-suspension. The optimized conditions of the apparatus were applied to test the impact of essential oils vapors on air-suspended H9N2 viral particles.

The seventh experiment dealt with evaporation of Mentofin® for 30 minutes at 35˚C a concentration of 0.10 µl / c.c. of air, and allowing a constant time of 1.5 minutes with 1.2×10^7 H9N2 particles / c.c., resulting in 12.5% viral recovery, compared to 50% recovery of H9N2 in absence of essential oils.

In addition, the percentage reduction in HA titers of H9N2 by essential oils, compared to that obtained by the control apparatus, was 73.5%.

It is recommended in the future to use a titration scale for the concentration of the evaporated essential oils against a fixed number of viral particles (1.2×10^7 / c.c.) and the same contact time of 1.5 minutes, targeting a complete inactivation of the H9N2. This will help in future application of proper level of the evaporated essential oils to inactivate influenza viruses in closed systems of hospitals, army facilities, pre-schools, and kinder gardens, nurseries, influenza infected farms, and slaughter houses.

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The Lord be with you