UNIVERSITI PUTRA MALAYSIA

COMPARISON OF TWO DIFFERENT DISINFECTANTS IN THE INACTIVATION OF NEWCASTLE DISEASE VIRUS AND INFECTIOUS BURSAL DISEASE VIRUS

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It is hereby certified that we have read this project paper entitled "Comparison of Two Disinfectants in the Inactivation of Newcastle Disease Virus and Infectious Bursal Disease Virus" by Maizatul Amirah binti Man and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Project.

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DEDICATIONS

In the name of Allah, The Most Benevolent, The Most Merciful

My humble effort is dedicated to The Al-Mighty,

All the support and care from my loving family and friends,

Along with all responsible and respected lecturers,

As well as all guidance and assistance from laboratory staff and friends.

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Thank you to Dr. Nor Yasmin Abd Rahman for spending her valuable time listening to my presentation and giving valuable constructive criticism and suggestions. The project would not have been possible without the active cooperation of many people, namely; Mr. Mohd Kamaruddin Awang Isa, Mrs. Siti Khatijah Mohamad, Mr Rusdam and Ms Nur Hidayah who are the staff of Virology Laboratories, Faculty of Veterinary Medicine, Universiti Putra Malaysia. I am very fortunate to receive assistance from them throughout the five weeks.

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LIST OF ABBREVIATIONS

UPM = University Putra Malaysia

FPV = Faculty of Veterinary Medicine

UVH = University Veterinary Hospital

FYP = Final year project

MRC = Manufacturer's Recommended Concentration

NDV = Newcastle Disease Virus

IBDV = Infectious Bursal Disease Virus

AOAC = Association of Official Agricultural Chemists

ATCC = American Type Culture Collection.

% = Percentage

et al. = et al. (abbr. Latin)

ABSTRAK

Abstrak daripada projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir

PERBANDINGAN DI ANTARA DUA DISINFEKTAN DALAM PENTAKAKTIFANVIRUS GUMBORO (INFECTIOUS BURSAL DISEASE VIRUS) DAN VIRUS SAMPAR (NEWCASTLE DISEASE VIRUS)

Oleh

Maizatul Amirah binti Man

2017

Penyelia: Dr. Farina Mustaffa Kamal

Kini, industri ayam terancam oleh pelbagai penyakit sama ada penyakit virus endemik mahupun penyakit baru dan mengakibatkan kerugian ekonomi yang besar kepada industri. Disinfektan yang berasaskan bahan kimia mampu membantu dalam mengganggu penyebaran virus; namun kebanyakan disinfektan ini boleh menimbulkan kesan berbahaya kepada pekerja ladang kerana mempunyai sifat toksik dan menghakis yang tinggi. Oleh itu, disinfektan yang selamat digunakan tanpa menjejaskan keberkesanannya perlu diuji sebagai disinfektan alternatif. Nanopartikel perak telah dikaji terutamanya dalam potensi antimikrobial; walaubagaimanapun, ia juga telah terbukti berkesan terhadap virus. Dalam kajian ini, keberkesanan melumpuhkan virus hasil daripada dua disinfektan

berasaskan ‘nanosilver’; produk A dan produk B telah disiasat terhadap virus Newcastle (NDV) dan virus Gumboro (IBDV). Analisis kuantitatif menggunakan telur telah digunakan untuk menentukan keupayaan disinfektan ini untuk membunuh virus. Kedua-dua produk menunjukkan keupayaan untuk membunuh NDV dalam kepekatan yang berbeza. Namun, keberkesanan kedua-dua disinfektan berbeza apabila diuji terhadap IBDV. Produk A mampu memberikan keberkesanan pentakaktifan virus lebih tinggi terhadap IBDV apabila menggunakan kepekatan disyorkan pengeluar berbanding dengan yang dicairkan 3 kali. Sebaliknya, produk B mempunyai keberkesanan lebih rendah berbanding dengan produk A tetapi keberkesanannya setanding walaupun kepekatan rendah disinfektan digunakan. Kesimpulannya, kedua-dua disinfektan berkesan membunuh virus sampul berbanding viru stidak bersampul. Malah, keberkesanan mereka bergantung kepada kepekatan disinfektan. Produk A dan produk B menunjukkan keberkesanan pentakaktifan virus NDV dan IBDV, jadi penggunaan disinfektan berasaskan ‘nanosilver’ sesuai dan selamat untuk digunakan. Walau bagaimanapun, kepekatan yang berkesan harus dinilai berdasarkan aplikasi khusus dalam industri ternakan.

Kata kunci: Penyakit Virus Gumboro, Penyakit Virus Newcastle, keberkesanan virucidal, analisis kualitatif

ABSTRACT

An abstract from the project paper presented to the Faculty of Veterinary Medicine in the partial fulfilment of course VPD 4999 – Final Year Project

COMPARISON OF TWO DIFFERENT DISINFECTANTS IN THE INACTIVATION OF INFECTIOUS BURSAL DISEASE AND NEWCASTLE DISEASE VIRUSES

By

Maizatul Amirah binti Man

2017

Supervisor: Dr. Farina Mustaffa Kamal

Co-supervisor: Dr. Nor Yasmin Abd. Rahaman

The poultry industry is currently threatened by either endemic or emerging viral diseases associated with major economic losses to the industry. Chemical disinfectants may help in disrupting the transmission of viral pathogen; however many of these disinfectants could pose harmful effect to the farm workers due to its toxicity and corrosive rating. Hence, disinfectant that has less adverse health effect without compromising its efficacy need to be tested as potential alternative disinfectant. Silver nanoparticles have been studied mainly for their antimicrobial

potential; however, it has also been proven to be effective against viruses. In this study, the virucidal efficacy of two nanosilver-based disinfectants, product A and product B were investigated against the Newcastle Disease Virus (NDV) and Infectious Bursal Disease Virus (IBDV). Quantitative analysis using embryonated eggs was used to determine the ability of these disinfectants to inactivate the viruses. Both disinfectants showed the ability to inactivate NDV in different concentrations. However, there was reduced efficacy of both disinfectants when tested against IBDV. Product A has greater virucidal efficacy against IBDV when using manufacturer's recommended concentration compared to when using 3-fold diluted concentration. In contrast, Product B has lower virucidal efficacy compared to product A but the efficacy was comparable between the two concentrations tested. It was concluded that both disinfectants effectively inactivate enveloped virus compared to non-enveloped virus and their efficacies are concentration-dependent. As product A and product B showed virucidal efficacy against NDV and IBDV, the use of nanosilver-based disinfectant provides a less hazardous choice of disinfectant; however, the effective concentration need to be further evaluated based on its specific application in the poultry industry.

Key words: Nano-silver disinfectant, Infectious Bursal Disease Virus, Newcastle Disease Virus, Virucidal efficacy, Qualitative analysis

1.0 INTRODUCTION

1.1 Background of the study

Poultry sector is the biggest component of livestock industry in Malaysia, supplying about 81% of the total meat demand and almost 111% egg demand by the domestic market. The local poultry industry also exports to Singapore, Brunei, Hong Kong, and Japan (Veterinary Research Institute, 2013). The biggest threat to the poultry industry is due to either epidemic or endemic disease outbreak. The common diseases encountered are Newcastle diseases (ND), infectious bursal disease (IBD) infectious bronchitis, infectious coryza, fowl cholera, fowl pox, Mareks disease, lymphoid leucosis, pullorum, chronic respiratory disease and coccidiosis. Other health problems are caused by endoparasites, ectoparasites, haemoprotozoa and to a lesser extent microfilaria infection (Aini, 1990). However, viral infections have led to numerous diseases in poultry industry due to its high morbidity.

In order to control the spread of the pathogens, an immediate control such as vaccination of uninfected flocks and culling of infected flocks should be in place to prevent a disease outbreak. Once the birds are removed, facilities, equipment, vehicles, and their litters need to be treated with the aim of inactivating the infectious agent which can be achieved by a proper disinfection. The objective of disinfection is to prevent disease transmission and thus, an outbreak can be either

avoided or controlled. Secondary spread occurs mainly because of human-related behaviour and it is connected with movement of people and equipment. Therefore, chemical disinfectants are important in disrupting the transmission of viral infectious disease and aiding in containment and eradication efforts. While there is considerable published information and disinfection efficacy data regarding bacteria and fungi, the efficacy of chemical disinfectants against viruses has not been well studied. The need for a standard disinfectant test against viruses has long been recognised (McDuff & Gaustad, 1976; Chen & Koski, 1983). Existing literature in the area of virucidal disinfectant efficacy is of limited value by virtue of the diverse testing methodologies used (Bieker, 2016).

Silver nanoparticles have been widely studied especially the bactericidal properties. However, it has also been proven to be effective against viruses. Mehrbod *et al*. (2009) had conducted a study investigating the antiviral activity of nano-silver by using biochemical and molecular method and suggested that the potential of nano-silver as disinfectant to prevent the viral transmission. Hence, it is possible to study the virucidal activity of silver nanoparticles.

1.2 Overall Objectives of the study

1. To compare the efficacy of two nanosilver-based disinfectants in the inactivation of NDV and IBDV.

2. To determine the optimal concentration sufficient to inactivate NDV and IBDV

1.3 Justifications of the study

Silver nanoparticles have been studied on its general efficacy against viruses. However, there is limited data on the efficacy of nanosilver-based disinfectants against enveloped and non-enveloped viruses.

1.4 Hypotheses of the study

H_0: There are no difference in the efficacy between both disinfectants in the inactivation of NDV and IBDV.

H_A: There are difference in the efficacy between both disinfectants in the inactivation of NDV and IBDV

2.0 LITERATURE REVIEW

2.1 Chemical Disinfectant

Disinfecting agents are registered by the Environmental Protection Agency (EPA) as antimicrobial pesticides and used to control, prevent, or destroy harmful microorganisms (i.e., bacteria, viruses, or fungi) on inanimate objects and surfaces. EPA provides a quick reference guide for testing the basic efficacy claims and described in Table 1. There are several types of disinfectants that are commonly used in poultry farm which are phenolic compounds (e.g.: Pine-sol, One Stroke, Osyl), iodine or iodophors, (e.g.: Betadine and Weladol), chlorine compounds (e.g.: Clorox, generic bleach), quaternary ammonium compound (e.g.: Roccal D Plus) and oxidizing compounds (e.g.: Virkon S, Oxy-Sept 333) (Darre, 2014). According to the study conducted by Patnayak (2008), phenolic compounds were found to be the most effective against avian influenza virus (AIV), Newcastle disease virus (NDV) and avian metapneumovirus (aMPV). Phenolic compounds comprises of pure phenol and substitution products with halogens and alkyl groups which act to denature and coagulate proteins. Subsequently, this compound is general protoplasmic poisons. However, phenolic compounds are more difficult to rinse out from equipment than the other disinfectants and they are also not safe for workers. Equipment and devices treated with phenols, particularly para-tertiary amyl phenol, will caused depigmentation of the skin and injury to mucous membranes (Crawford *et al.,* 2000). Thus, applying phenolic compound disinfectant could only be performed by trained workers.

Table 1: Testing for basic efficacy claims (Adapted from: Product Performance Test Guidelines OCSPP 810.2200: Disinfectants for Use on Hard Surfaces—Efficacy Data Recommendations by US EPA)

Level of efficacy	Test Methods		Test Organisms	No. Of Batches/ Carriers	Evaluation of success
Limited spectrum disinfectant/ hard non-porous surfaces	Water solluble powders/liquid	AOAC Use-Dilution Method or AOAC Hard Surrface Carrier Test (distilled water only)	Stapgylococcus aureus (ATCC 6538) or Salmonella enterica (ATCC 10708)	Three batches, one at least 60 days old. 60 carriers against either organism claimed (180 carriers)	59/60 carriers are negative for each batch tested for all methods except AOAC Hard Surface Carrier Test, which is 58/60 carriers are negative for each batches
	Spray products	AOAC Germicidal Spray Products Test			
	Towelletes	Modified AOAC Germicidal Spray Products Test			
Broad-spectrum disinfectant/hard non porous surface	Water solluble powders/liquid	AOAC Use-Dilution Method or AOAC Hard Surrface Carrier Test (distilled water only)	Stapgylococcus aureus (ATCC 6538) or Salmonella enterica (ATCC 10708)	Three batches, one at least 60 days old. 60 carriers against either organism claimed (360 carriers)	59/60 carriers are negative for each batch tested for all methods except AOAC Hard Surface Carrier Test, which is 58/60 carriers are negative for each batches
	Spray products	AOAC Germicidal Spray Products Test			
	Towelettes	Modified AOAC Germicidal Spray Products Test			
Hospital or healthcare disinfectant/ hard non-porous surfaces.	Water solluble powders/ liquid	AOAC Use-Dilution Method or AOAC Hard Surrface Carrier Test (distilled water only)	Stapgylococcus aureus (ATCC 6538) or Salmonella enterica (ATCC 10708)	Three batches,one at least 60 days old.60 carriers against each organism (360 carriers)	59/60 carriers are negative for each batch tested for all methods except AOAC Hard Surface Carrier Test, which is 58/60 carriers are negative against Staphylococcus aureus for each batch, and 57/60 carriers are negative against Pseudomonas
	Spray products	AOAC Germicidal Spray Products Test			

	Towelettes	Modified AOAC Germicidal Spray Products Test			
Fungicidal disinfectant/ hard non-porous surfaces.	Water solluble powders/liquid	AOAC Use-Dilution Test modified for fungi or AOAC Fungicidal Test	Trichophyton mentagrophytes (ATCC 9533)	Two batches,ten carriers per batch for the modified AOAC Use Dilution Test, the modified AOAC Germicidal Spray products Test, and the EPA Towelette Test. Two batches for the AOAC	All fungal spores on all carriers should be killed. For the AOAC Fungicidal Test, all fungal spores should be killed at 10 and 15 minutes to support a 10 minute label claim.
	Spray products	AOAC Germicidal Spray Products Test modified for fungi			
	Towelettes	Modified Germicidal Spray Test			
Virucidal disinfectant/ hard non-porous surfaces.	Water solluble powders/liquid	AOAC Use-Dilution Test modified for viruses or ASTM E1053-	Virus claimed on label or approved surrogate	Two batches. One surface per batch	Complete inactivation of virus. Where cytotoxicity is present, demonstrated $\log_{10}$ reduction.
	Spray products	AOAC Germicidal Spray Products Test modified for viruses or ASTM 31053-			
	Towelettes	Modified Germicidal Spray Test			
Tuberculoidal disinfectant/ hard non-porous surfaces.	Water solluble powders/liquid	AOAC Tuberculocidal Activity of Disinfectant, Quantitative Tuberculocidal Activity Test	Myconbacterium bovis BCG	Two batches. Ten carriers per batch	10/10 carriers are negative for growth and there is no growth in the additional test media. Survival Curve constructed from 4 separate replicates at the 95% confidence level to show probability of one survivor.
	Spray products	AOAC Germicidal Spray Products Test modified for tuberculocidal activity		Two batches, ten carriers per batch	10/10 carriers are negative for growth and there is no growth in the additional test media.
	Towelettes	Modified Germicidal Spray Test		Two batches, ten carriers per batch	10/10 carriers are negative for growth and there is no growth in the additional test media.
Additional bacteria disinfectant/ hard non-porous surfaces	Water solluble powders/ liquid	AOAC Use-Dilution Method or AOAC Hard Surrface Carrier Test (distilled water only)	Bacteria claimed on the label in addition to the base broad spectrum claim	Two batceches, ten carriers for each batch	10/10 carriers are negative for growth of the test organism
	Spray products	AOAC Germicidal Spray Products Test			

2.2 Issues of using disinfectants in poultry farm

Based on Zander *et al.* (1997), the principles of disease prevention and control within the poultry industry are based on flock management, bio-security, preventive vaccination and sanitation. In general, a sanitation program should include safe and easy procedures outlining the correct application of detergents and disinfectants, proper use of sanitation equipment and an efficient monitoring system (Spielholz, 1998). Based on Compendium of Veterinary Standard Precautions for Zoonotic Disease Prevention in Veterinary Personnel (2008), an EPA-registered disinfectant should be used according to label instructions with proper dilution and contact time. Therefore, time allocation and correct concentration of disinfectant are important in order to ensure efficient disinfection to prepare the flock house for the new batch to arrive. Paniago (2010) recommended to keep the facility empty for at least 2 weeks after disinfection to ensure the success of the sanitation program. In addition, surface cleaning with water and detergent is recommended prior to disinfection of equipment and surfaces. As discussed by Ruano et al. (2001), most disinfectant products were not effective at the manufacturer's recommended dilution when tested with organic matter. This proved that some of the disinfectants lose its efficacy in the presence of organic matter. Aini (2000) also stated that lime is usually the common choice among small farmers due to its low cost. However, lime is effective only at controlling certain poultry diseases which are avian influenza, Newcastle disease and ornithosis (Jacob, 2015). Improper disinfection and sanitation procedure

would then lead to severe disease outbreaks resulting in heavy morbidity and mortality of birds (Mathialagan, 2000)

2.3 Silver Nanotechnology in Disinfectant

Nanotechnology is a technology that deals with structures ranging from 1-100 nm in at least one dimension (British Standards Institute [BSI] 2007; Scientific Committee on Emerging and Newly Identified Health Risks [SCENIHR] 2008). The major focus of nanotechnology is on the development and application of materials ranging of between 1 to 100 nm. Currently, nano-silver is a type of nanomaterial that is widely being used. Nano-silver particle is mostly smaller than 100 nm and consists of about 20 to 15,000 silver atoms (Chen & Schluesener, 2008). Pure metallic silver is inert and does not react with human tissue or kill microorganisms until it is ionised. At low chloride concentration, silver ions inhibits the respiratory path of sensitive strain organisms, destroying cell wall, impairs essential enzymes, metabolic activity and/or causes RNA and DNA alteration (Melaiye &Youngs, 2015). The continuous release of silver cations from the nanostructured surface proves to be a notable determinant responsible for efficient antibacterial activity (Malina *et al.,* 2010). Nanoparticles of size less than 10 nm in diameter can bind to bacterial cell wall causing its perforation which finally leads to cell death of gram-positive and gram-negative bacteria as observed by Feng (2000). Nano-silver accumulation within the cell membrane will also lead to rapidly increased cell permeability and ultimately, cell death (Sondi & Salopek-Sondi, 2004). According to Morones *et al*., (2005), particles under 10 nm in size are able to penetrate into cytoplasm where they disturb cell metabolism and

biochemical processes. These particles have influence on the fungus developmental cycle through blocking one-cell to group form such as micelles and thus, deprived them of the capacity to induce infection (Kim *et al.,* 2008). A direct contact of virus with nanoscale silver *in vitro* caused the decline of isolated viral DNA load. The same studies showed that nanoparticles can also prevent binding of viral coat protein to antibodies as well as counteract virus penetration into cells (Mehbrod *et al.,* 2009). Plus, 53% of the EPA-registered biocidal silver products contain nanosilver (Nowack *et al.,* 2013).

2.4 Factors influencing disinfection

The disinfectant effectiveness depends on the chemical category and formulation, correct dilution (dosage), contact time, presence of organic matter, type and quantity of microorganisms, temperature, pH and water hardness (Ruano *et al.,* 2001). Block (2001) reported that during the production of virus pools, mutation in viruses may alter their susceptibility to germicides. Also, different viruses within a population, especially when that population is dried onto a carrier surface, have slightly different environments and degrees of shielding from the nearest neighbours.

Bieker (2006) mentioned that methodologies for evaluating disinfectants for their virucidal efficacy exist both nationally and internationally, in the form of guidelines and published standards, although these differ quite drastically. Among these methodologies, the two predominant methods for evaluating virucidal efficacy of disinfectants are suspension and carrier tests. However, Green and Birkeland (1942) had described the usage of chick embryo in evaluating

disinfectant was possible to determine the accuracy of the comparative value of disinfectants under conditions.

2.5 Virology of viruses:

2.5.1 Infectious Bursal Disease (IBDV)

IBDV is classified in the family *Birnaviridae* which contains single genus, *Birnavirus* (Dobos *et al.,* 1979). The agent is a 55-65 nm diameter non-enveloped virus with single capsid structure showing icosahedral symmetry (Hirai & Shimakura, 1974). There are two serotypes of IBDV which can be distinguished serologically (McFerran *et al.,* 1980). Very virulent strains of IBD virus (vvIBDV) can result in mortality of up to 40% of chicken's population in a flock. This strain commonly affect poultry industry worldwide, including Malaysia. This disease caused serious economic losses especially in commercial poultry industry. (Thangavelu et al., 1998). IBDV can be grown in nine to eleven day-old embryonated eggs. Chorio-allantoic membrane (CAM) or the yolk sac inoculation route is preferable to the classical allantoic route, as the former gives a greater yield of virus (Berg *et al.,* 2000). Embryonic death may occur three to seven days following inoculation. Observation of enlarged blood vessel and oedematous chorioallantoic membranes (CAMs) in inoculated eggs is indicative of positive IBDV infection (Mutinda *et al.*, 2015). IBDV tolerates a wide range of pH, temperature conditions, other environmental stresses, and can persist in farms for months. Some disinfectants, including aldehyde, chlorine, and iodine compounds, and inert soap with added NaOH, can inactivate IBDV but their virucidal effect is greatly decreased at low temperature (Guan *et al.,* 2014).

2.5.2 Virology of Newcastle Disease Virus (NDV)

NDV is a member of the order *Mononegavirales*, family *Paramyxoviridae* and genus *Avulavirus* which is an enveloped virus with negative-sense; non-segmented single stranded RNA genome consisting of 15,586 nucleotides (Phillips *et al.*, 1993). On the surface of NDV particles, there are two important functional proteins, haemagglutinin/neuraminidase (HN) and fusion (F), which can be visualised as spikes under the electron microscope. The fusion (F) protein is responsible in mediating fusion of the viral envelope with cellular membranes and the haemagglutinin-neuraminidase (HN) protein is involved in cell attachment and release (Jaganathan, 2015). The hemagglutination-inhibition (HI) test is used to detect and measure the level of subtype-specific antibodies in serum, plasma, and yolk following infection with NDV. Meanwhile, haemagglutination test (HA) is used to quantify the amount of NDV in a suspension (FAO, 2002) Newcastle Disease (ND) is a highly contagious disease in chicken where outbreaks can cause flock mortality up to 100% and has been one of the major causes of economic losses in the poultry industry (Aldous & Alexander, 2001). Compared to IBDV, NDV is less resistant to environmental stresses and more susceptible to drying as described by Guan *et al.,* (2014). Many common disinfectants including phenolic, aldehyde, and oxidizing compounds can inactivate NDV at temperature above 0°C.

3.0 Materials and Methods

3.1 Disinfectants

Samples from commercially available nanosilver technology-based products were obtained and its efficacy in the inactivation of NDV and IBDV was evaluated. The chemical composition of both disinfectant products were evaluated in Table 2.

Table 2: Active ingredients of commercial disinfectants tested

Disinfectants	Active ingredients	Concentration
Product A	Hydrogen peroxide Silver Phosphoric acid	4-5% Ca. 0.01% 0.05-0.1%
Product B	Hydrogen peroxide, 30% solution Ethanol Potassium nitrate Silver nitrate	1-13% 1-10% 1-5% <0,4%

Since the information of the product A's exact concentration was not readily available, the concentrations tested were based on the manufacturer's recommended concentration (MRC). Therefore, two concentrations were included in the experiment which were the MRC and 3-fold dilution of MRC (diluted MRC). Both disinfectants were diluted with sterile deionized water according to the Table 3:

A useful formula for calculating dilution volumes is $C_1V_1 = C_2V_2$

C_1 = Concentration of stock solution V_1 = Volume of stock solution

C_2 = Concentration of diluted solution V_2= Volume of diluted solution

Table 3: Calculation of dilution of disinfectant products

Disinfectant product	Product A	Product B

Manufacturer's recommended concentration (MRC)	• Consist of 10% solution and it is readily used	• Consist of 2000pm (stock) and the MRC stated is 200ppm • To dilute 2000ppm product B stock into 10ml of 200ppm solution undergoes **10 fold dilution**: 200ppm (10ml)=2000ppm (x) $x = \frac{200\,ppm(10ml)}{2000\,ppm} = 1ml$ To make 10ml of solution, 1ml of product B and 9ml of deionized water mixed together
3-fold dilution of MRC (Diluted MRC)	Asssumption : the product have 100% of 10% solution. Hence, to perform 3-fold dilution of 10% solution undergoes **13 fold dilution**. 100% / 13 = 7.7% 7.7%(10ml) =10% (x) $x = \frac{7.7\%(10ml)}{10\%} = 7.7ml$ To make 10ml of solution, 7.7ml of product A and 2.5ml of deionized water mixed together	To dilute 2000ppm product B stock into 10ml of 150ppm solution undergoes **13 fold dilution**: 150ppm (10ml)=2000ppm (x) $x = \frac{150\,ppm(10ml)}{2000\,ppm} = 0.75ml$ To make 10ml of solution, 0.75ml of product B and 9.25ml of deionized water mixed together

3.2 Embryonated eggs

Nine-day-old embryonated eggs were obtained from Charoen Pokphand Malaysia hatchery farm, Rembau, Negeri Sembilan. Egg candling was performed to ensure the viability of the eggs prior to egg inoculation experiment.

3.3 Preparation of virus titre using 50 percent Embryo Infectious Dose (EID_{50})

The V4 strain of NDV and UPM0081 vvIBDV strain of IBDV were diluted through 10-fold serial dilutions. Each tube was clearly labeled with the dilution factor of the original suspension and 900µL of deionized water was dispensed into each tubes. 100µL of the virus suspension stock (NDV) was taken and transferred into the first tube labeled 10^{-1} and mixed well using pipettes. This procedure was repeated until reaching the 10^{-10} dilution. A new sterile pipette tip was used at each dilution to ensure consistent results and prevent contamination. Each dilutions were then propagated into five 10-day-old embryonated eggs. The air sac of the eggs was determined through candling of the eggs and labelled. A small hole was placed at the air sac for intra-embryonic inoculation. The eggs was inoculated intraembryonically with each dilution (from 10^{-4} to 10^{-10}dilution) by using 1 mL syringe attached with 27 G x 1/2 inch needle as in figure 1:

Figure 3: Intraembryonic inoculation of virus suspension

The previous hole was sealed by using hot wax and the eggs were candled again to ensure that the hole was sealed. The inoculated eggs were incubated for 5-7 days at 37 °C. The viability of the embryo were examined every day by candling method to observe the movement of embryo. Any dead embryo was

placed in 4°C chiller. After incubation, the allantoic fluid from each egg was harvested and test for haemagglutination while IBDV post-inoculation eggs were observed for lesions. The results are tabulated in Table 4 and Table 5 and Reed and Muench mathematical technique was used to calculate the infectivity of tire of the original suspension (Reed & Muench, 1938)

Table 4: EID50/mL of NDV suspension

Dilutions	Number of eggs infected (HA +)	Number of eggs not infected (HA -)	Accumulated numbers			Percentage infected A/(A+B) x 100
			Infected (A)	Not infected (B)	Total number tested (A+B)	
10-8	5	0	12	0	12	100%
10-9	5	0	7	0	7	100%
10-10	2	3	2	3	5	40%

The Reed and Muench formula to calculate the index is:

$$\text{Index} = \frac{(\%\text{ infected at dilution immediately above 50\%}) - 50\%}{(\%\text{ infected at dilution immediately above 50\%}) - (\%\text{ infected at dilution immediately below 50\%})}$$

$$= \frac{(100\%) - 50\%}{(100\%) - 40\%}$$

$$= 0.833$$

The infectivity titre of NDV has been calculated to be $10^{-9.833}$EID50/mL

Table 5: EID50/mL of IBDV suspension

Dilutions			Accumulated numbers	

	Number of eggs infected (inflammation of blood vessel and thickened membrane)	Number of eggs not infected	Infected (A)	Not infected (B)	Total number tested (A+B)	Percentage infected A/(A+B) x 100
10-4	5	0	27	8	35	77%
10-5	5	0	22	8	30	73%
10-6	5	0	17	8	25	68%
10-7	4	1	12	8	20	60%
10-8	4	1	8	7	15	53%
10-9	2	3	4	6	10	40%
10-10	2	3	2	3	5	40%

The Reed and Muench formula to calculate the index is:

$$\text{Index} = \frac{(\%\text{ infected at dilution immediately above 50\%}) - 50\%}{(\%\text{ infected at dilution immediately above 50\%}) - (\%\text{ infected at dilution immediately below 50\%})}$$

$$\frac{(53\%) - 50\%}{(53\%) - (40\%)}$$

$=0.23$

The infectivity titre of IBDV has been calculated to be $10^{-8.23}$EID50/mL

3.4 Disinfectant test procedure

Concentration-course experiments were carried out for the evaluation of both disinfectants. For each concentration, petri dish were prepared in duplicate. Virus inoculum containing 10 µl of approximately $10^{-9.833}$EID50/mL of NDV or $10^{-8.23}$EID50/mL of IBDV were applied to a marked circle area which was approximately half of the surface of each petri disk (30mm x 7.5mm). It was then air-dried in a biosafety cabinet for 15 minutes. The circled area was wiped with disinfectant using sterile cotton sticks as shown in figure 2:

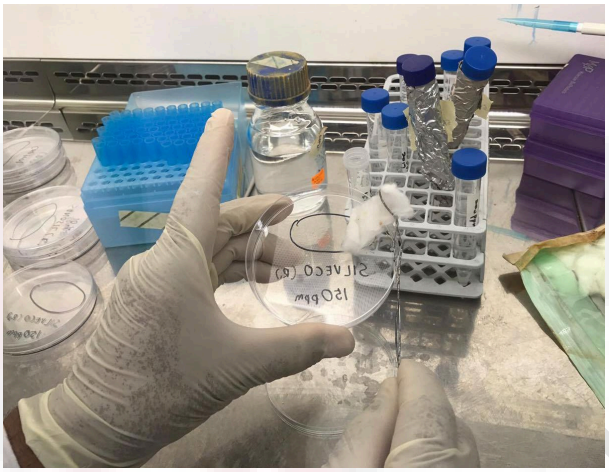

Figure 4: Wiping the sterile gauze soaked with disinfectant at the circled area

As for positive control, deionized water was used instead of disinfectant to wipe the area containing the virus. Empty petri dish without virus and wiped with disinfectant was designated as negative control. The petri dish was left for 3 hours in the biosafety cabinet with normal room temperature to allow the virus inactivation to occur. Next, 1 ml of deionized water was added into each wiped petri dish. The deionized water was aspirated out for the estimation of virucidal activity by inoculating 0.1 ml of the deionized water into each of the 10 eggs. The eggs were incubated at 37 °C for 5 days for NDV and 8 days for IBDV.

3.5 Evaluation of disinfectant against viruses

In order to test the presence of NDV-infected embryo, hemagglutination test was done by obtaining defibrinated 10% blood of one-day old chicken aseptically. The chicken RBC were washed three times in at least 10 parts of phosphate-buffered solution (PBS) (Fisher Scientific, Hanover Park, IL) to 1 part of RBC and centrifuged at 1500 rpm for 10 minutes. The hemagglutination (HA) test was done by placing a drop of the RBC suspension on a white tile and mixed with a drop of allantoic fluid from the egg to be tested. The tile was rocked from

side to side for 1 minute. This produces hemagglutination activity (HA), or clumping of cells that is visible to the naked eye (Swayne & King, 2003) due to the binding of hemagglutinin or neuraminidase viral protein to receptors on the RBC membranes as shown in Figure 3 and Figure 4:

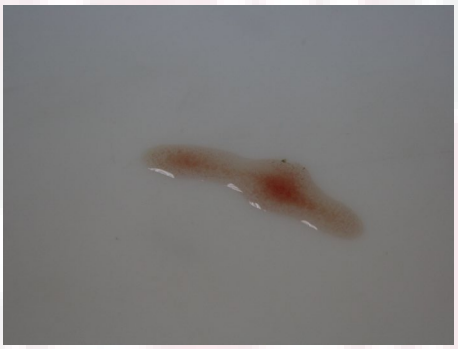

Figure 3: HA positive

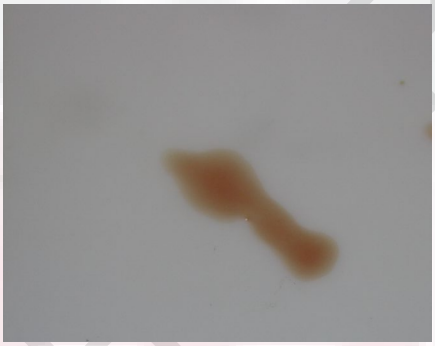

Figure 4: HA negative

In contrast, at 8th day post-inoculation, enlarged blood vessel and oedematous chorioallantoic membranes (CAMs) were observed with congestion or haemorrhages indicating positive IBDV-infected eggs (Mutinda *et al., 2015*) as shown in figure 5:

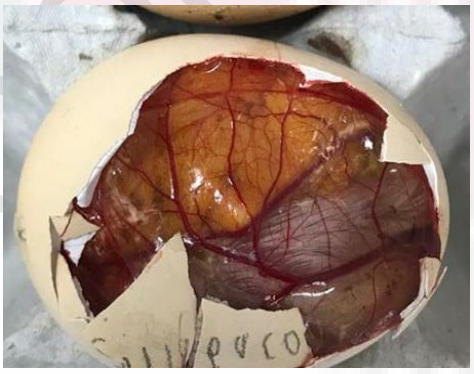

Figure 5: Positive IBDV infected egg

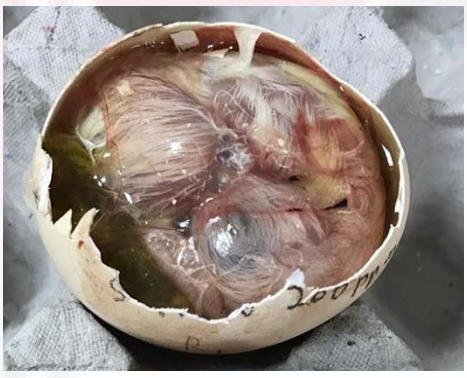

Figure 6: Negative IBDV infected egg

3.6 Data Analysis

The percentage of virus-infected eggs was calculated by using mathematical formula described below:

R_1 % = number of eggs inoculated and viable until the end of experiment

– (number of non-infected eggs) x 100

To compare between the two different disinfectants efficiency in the inactivation of both viruses, the average percentage of total virus infected eggs was calculated by this mathematical formula: $\left[\frac{R1\% + R2\%}{2}\right]$

The disinfectant efficacy is measured by comparing the average percentage of total virus-infected eggs.

4.0 RESULT AND DISCUSSION

The virucidal efficacy of both disinfectant products can be evaluated by comparing the average percentage of the infected eggs after the treatment. Table 6 lists the virucidal activity against NDV for the two products used as disinfectants. The average percentage of infected eggs in both disinfectant in MRC and diluted MRC were 0%. Both products managed to inactivate the virus despite using different concentrations as all embryos were tested negative in the HA test. This finding indicates that both disinfectants have high virucidal efficacy against NDV. Projections from NDV envelope known as spikes or peplomers comprises of functional proteins making the virus less virulent and sensitive to heat, acids and lose its efficacy after drying. NDV is known to be susceptible to many chemical disinfectants (Guan *et al.,* 2014).

The virucidal efficacy against IBDV was different between the two disinfectant products. Based on Table 3, it was shown that product A has the lowest percentage of infected egg (20%) at MRC which indicates the highest virucidal efficacy. This is in agreement with the study conducted by Stevens (1998) which stated that a disinfectant is most effective at the manufacturer's recommended level. In contrast with product B, their virucidal efficacy did not have significant differences compared to product A despite of their different concentrations. MRC of product B gave an average percentage of infected eggs of 55%, while diluted MRC has an average percentage of infected eggs of 63.3%. IBDV is non-enveloped virus with single capsid structure showing icosahedral symmetry and it is more virulent and stable in the environment. Results from both products against IBDV show that concentration affects the disinfectant efficacy. Ruano *et al.,* 2001

reported that among the different factors that could affect the efficacy of disinfectant, the most important factors are chemical category and correct dilution (dosage) which are in line with this study.

In addition, a total of four contaminated eggs was observed during the disinfectant testing against IBDV coming from positive control group, replicate 1 in product A (diluted MRC) group, replicate 2 in product A (diluted MRC) group and replicate 1 in product B (diluted MRC) group. This is probably due to technical error during egg inoculation as direct puncture through air sac may ruptured the blood vessels.

Furthermore, ten-day-old old embryonated eggs were inoculated with 0.1 ml of each product of the two tested concentrations and incubated until hatched. This acts as negative control to determine whether the disinfectant products have direct effects on th embryonated eggs. Chicks hatched from these eggs were observed to be weak regardless of product concentrations compared to the eggs inoculated with deionized water. Two embryonated eggs inoculated with product B of MRC were found dead before hatching. The cause of death of embryonated eggs inoculated with product B may be due to the ethanol present in product B. Presence of ethanol may caused growth suppression in embryonic chicks (Bupp & Shibley, 2002).

Table 6: Disinfectant efficacy against NDV after 3 hours of contact time

Products	(HA+) after treated		(HA-) after treated		Percentage infected eggs (%)		Average percentage of infected eggs (%)
	R_1	R_2	R_1	R_2	R_1	R_2	
A (MRC)	0	0	10	10	0	0	0
A (Diluted MRC)	0	0	10	10	0	0	0
B (MRC)	0	0	10	10	0	0	0
B (Diluted MRC)	0	0	10	10	0	0	0
Deionized water (virus only)	10	10	0	0	100	100	100

Table 7: Disinfectant efficacy against IBDV after 3 hours of contact time

Products	Number of infected eggs		Number of non-infected eggs		Total eggs remaining		Percentage infected eggs (%)		Average percentage of infected eggs (%)
	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	
A (MRC)	2	2	8	8	10	10	20	20	20
A (Diluted MRC)	6	7	3	2	9	9	66.67	77.78	72.22
B (MRC)	6	5	4	5	10	10	60	50	55
B (Diluted MRC)	6	6	3	4	9	10	66.67	60	63.33
Deionized water (virus only)	9	10	0	0	9	10	100	100	100

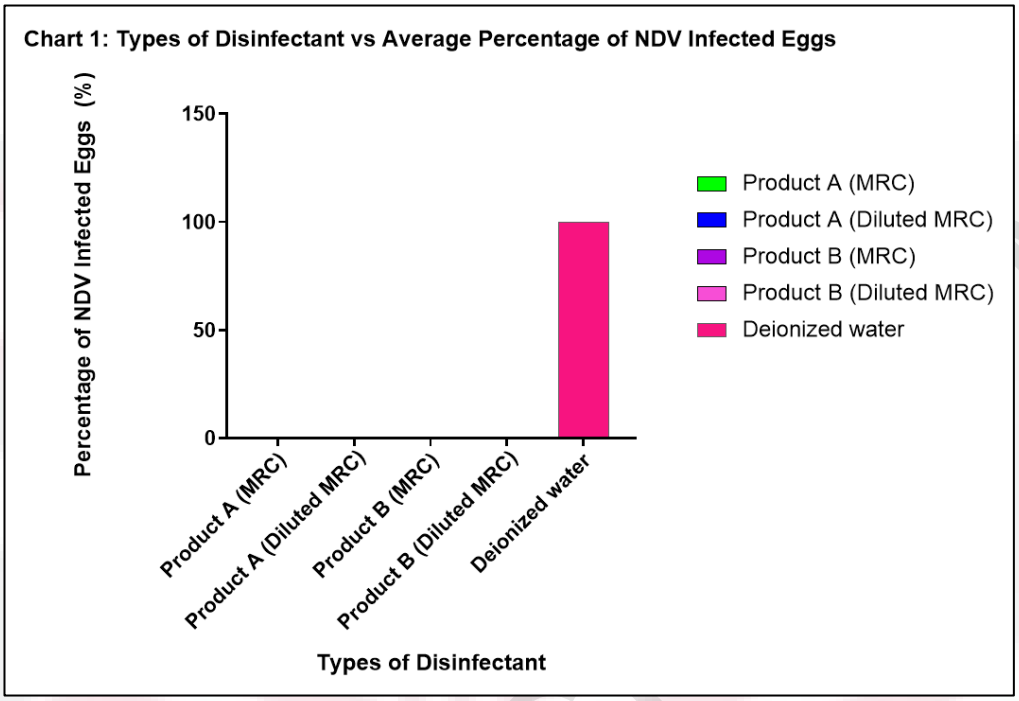
Chart 1: Types of Disinfectant vs Average Percentage of NDV Infected Eggs
Percentage of NDV Infected Eggs (%)
150
100
50
0
Product A (MRC)
Product A (Diluted MRC)
Product B (MRC)
Product B (Diluted MRC)
Deionized water
Types of Disinfectant
Product A (MRC)
Product A (Diluted MRC)
Product B (MRC)
Product B (Diluted MRC)
Deionized water

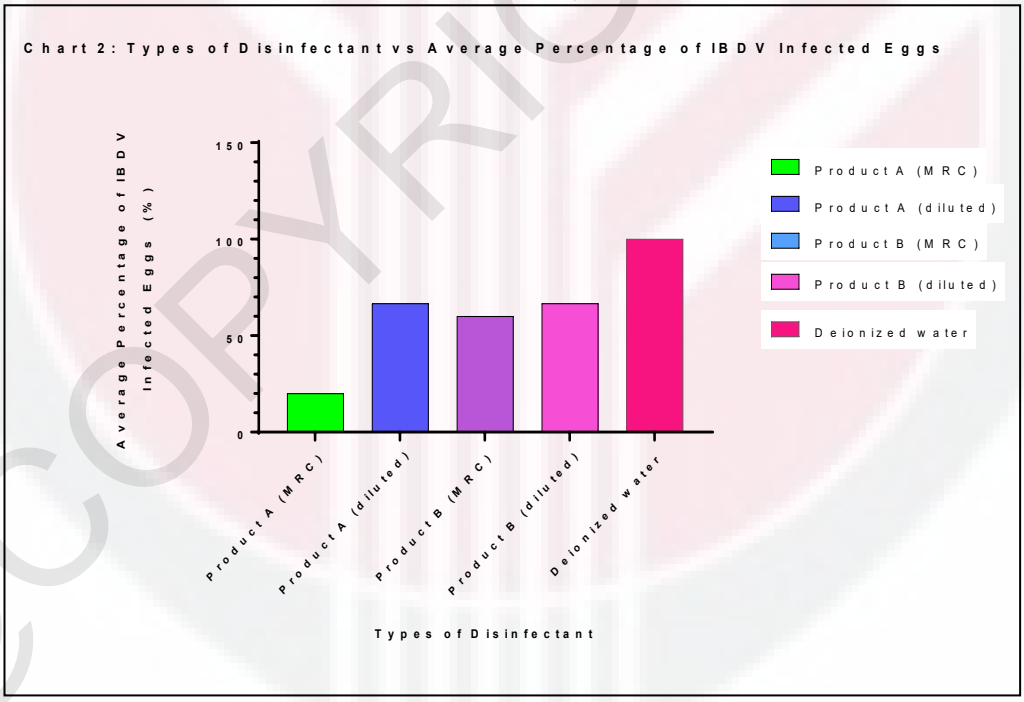
Chart 2: Types of Disinfectant vs Average Percentage of IBDV Infected Eggs
Average Percentage of IBDV Infected Eggs (%)
150
100
50
0
Product A (MRC)
Product A (diluted)
Product B (MRC)
Product B (diluted)
Deionized water
Types of Disinfectant
Product A (MRC)
Product A (diluted)
Product B (MRC)
Product B (diluted)
Deionized water

6.0 CONCLUSION AND RECOMMENDATIONS

Overall, it is observed that both disinfectant products were able to inactivate enveloped virus but there were reduced in efficacy against non-enveloped virus. Based on the result obtained against IBDV, both disinfectants were able to inactivate the virus but their efficiency depends primarily on concentrations. Disinfectant efficacy was the highest when using the disinfectants at the manufacturer's recommended level.

However, the effective concentration of these two disinfectants against non-enveloped virus need to be further evaluated based on its specific application in the poultry industry. Thus, a study testing wide range of concentrations in nano-silver based disinfectants is recommended to determine the optimal concentration against non-enveloped virus. Other factors that commonly affects disinfectant efficacy should also be investigated such as contact time, presence of organic matter, type and quantity of microorganisms, temperature, pH and water hardness.

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