

Avian influenza virus inactivation by caprylic acid, sodium caprylate, and monocaprylin

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Abstrak

Latar belakang: Flu burung merupakan salah satu penyakit berbahaya yang disebabkan oleh virus RNA dari keluarga *Orthomyxoviridae*. Virus ini sangat menular dan transmisi virus flu burung ke manusia akan mengakibatkan penyakit yang fatal. Caprylic acid, asam lemak alami, dan derivat lainnya seperti sodium caprylate dan monocaprylin diketahui sangat efektif dalam membunuh berbagai bakteri dan virus penyebab penyakit. Penelitian ini bertujuan untuk mengetahui daya hambat caprylic acid, sodium caprylate dan monocaprylin terhadap virus flu burung.

Metode: Uji daya hambat caprylic acid dan monocaprylin menggunakan virus influenza low pathogenic H5N1 dan H5N2. Pengurangan partikel virus pada sel terinfeksi dalam serial pengenceran caprylic acid, sodium caprylate, monocaprylin dan control positif dibandingkan dengan menggunakan metode kuantitatif real-time RTPCR.

Hasil: Virus avian influenza dapat diinaktivasi oleh caprylic acid 0,2% dan 0,4% masing-masing sebesar 2 log dan 3 log. Pada penelitian ini sodium caprylate tidak menunjukkan daya hambat yang bermakna. Sedangkan, monocaprylin memiliki dosis yang lebih efektif untuk mengurangi jumlah partikel virus yang sama (0,08%-2 log dan 0,16%-3 log).

Kesimpulan: Konsentrasi caprylic acid dan monocaprylin yang rendah secara *in-vitro* mampu mengurangi jumlah partikel virus flu burung. Monocaprylin merupakan senyawa yang paling efektif dalam menghambat pertumbuhan virus dibandingkan dengan senyawa uji lain. (*Health Science Indones 2011; 2: 41 - 5*)

Kata kunci. virus flu burung, inaktivasi, caprylic acid, sodium caprylate dan monocaprylin

Abstract

Background: Avian influenza is an important viral disease caused by RNA viruses from the *Orthomyxoviridae* family. The virus is highly contagious, and transmission of the virus to humans resulted in fatal disease. Caprylic acid, a natural fatty acid, and its other chemical forms, namely sodium caprylate and monocaprylin, are highly effective in killing a variety of disease causing bacteria and viruses. This study was conducted to investigate the antiviral effect of caprylic acid, sodium caprylate and monocaprylin against avian influenza virus.

Methods: Low pathogenic avian influenza viruses H5N1 and H5N2 were subjected to caprylic acid, sodium caprylate and monocaprylin. The reduction of viral particles in infected cells maintained in serial dilutions of caprylic acid, sodium caprylate, and monocaprylin and the positive controls were compared by using quantitative real-time RTPCR method.

Results: Avian influenza viruses were inactivated by 0.2% and 0.4% caprylic acid up to 2 logs and 3 logs respectively. Sodium caprylate was not producing significant reduction of viral particles in this study. Whereas, monocaprylin has more effective doses to reduced the similar number of viral particles (0.08% - 2 logs and 0.16% - 3 logs).

Conclusion: Low concentration of caprylic acid and monocaprylin *in-vitro* were able to reduce Avian influenza virus. Monocaprylin is more effective in reducing the viral particles compared to the other compounds. (*Health Science Indones 2011; 2: 42 - 6*)

Key words. avian influenza virus, inactivation, caprylic acid, sodium caprylate, monocaprylin

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In recent years, avian influenza has crossed the species barrier causing human fatal cases in many Asian countries.^{1,2} Based on Indonesia's Agriculture Department record in 2008, the avian influenza H5N1 virus has spread throughout 294 districts in 31 provinces. The number of poultry deaths was more than 46,000. Furthermore, the transmission of the virus to humans highlights the public health significance of the disease in Indonesia.³ Up to February 2010 there were 163 confirmed human cases with 135 fatal cases.⁴

Since the virus is highly virulent, strict hygienic measures employed can greatly help prevent the virus from spreading. The use of effective and safe antimicrobials could greatly reduce the spread of the virus. Caprylic acid, also known as octanoic acid, is a natural eight-carbon fatty acid. Free fatty acids and their monoglycerides have inhibitory effect against an array of pathogenic microorganisms

Caprylic acid and monocaprylin was reported to be effective in killing herpes simplex virus (HSV-1) and respiratory syncytial virus (RSV).⁵ It was also found to have antiviral activity on vesicular stomatitis virus (VSV), but not against poliovirus (a non-enveloped virus).⁶ In addition, sodium caprylate was very effective in inactivating human immunodeficiency virus (HIV-1), bovine viral diarrhoea virus (BVDV) and pseudorabies virus (PRV).⁷ This paper will discuss about avian influenza virus inactivation by caprylic acid, sodium caprylate and monocaprylin.

Determining the effect of these compounds on avian influenza virus may help us to devise new antimicrobial approaches to fight this fatal viral disease affecting the poultry industry and humans. When the compounds are effective in killing the virus in the laboratory model, they could potentially be used to supplement food, thus preventing infection in the poultry and reducing transmission to humans. Furthermore, since the gastrointestinal tract is one of the major sites of avian influenza virus replication in birds, administration of a safe antiviral compound through feed and water could potentially reduced the virus multiplications in the live birds.

This study was conducted to investigate the antiviral effect of caprylic acid, sodium caprylate and monocaprylin against avian influenza virus.

METHODS

Cells

The chicken embryo kidney cells (CEKC), a primary cell line, was maintained in Dulbecco's Modified Eagle's medium (DMEM-Animal Cell Culture Facility, University of Connecticut) supplemented with 10% fetal bovine serum (FBS), 0.35g/ml glucose, 200 mM L-glutamine, 1 mg/ml fungizone, 100U/ml penicillin and 100ug/ml streptomycin (Animal Cell Culture Facility, University of Connecticut). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Viruses preparation

LPAI A/TY/CA/209092/02 (H5N2) and LPAI A/TY/MA/40550/87-Bel/42 (H5N1) strains were obtained from NSVL (Ames, Iowa). Viruses propagation was conducted by using specific pathogen free (SPF) eggs (Charles River SPAFAS Inc., Storrs, Connecticut) through the allantoic cavity route. Titers were calculated using the method of Reed and Muench and expressed as log₁₀ TCID₅₀/ml.

Testing the antiviral capacity of caprylic acid and monocaprylin

Critical concentrations of the compounds that able to reduce plaque forming unit in the plaque assay (16) were used for testing the compounds by using real-time PCR. CEKC were cultured in 12-well plates (BD Falcon, NJ), washed once with 0.9% sodium chloride irrigation solution (Baxter, IL). The cells then infected with 300PFU/well and incubated in 37°C in a humidified 5% CO₂ atmosphere. After 45 min, inocula were withdrawn and washed once with DMEM (Animal Cell Culture Facility, University of Connecticut). The wells were then maintained with DMEM media containing caprylic acid (0.1%, 0.2%, 0.4%), sodium caprylate (0.075%, 0.1%, 0.125%), monocapry-

lin (0.04%, 0.08%, 0.16%) and control with DMEM (Animal Cell Culture Facility, University of Connecticut). Each concentration was triplicate. Upon 24 hours of incubation at 37°C in a humidified 5% CO₂ atmosphere, the supernatants were collected for the RNA extraction.

Development of real-time PCR standard curve

RNA extraction and complementary DNA (cDNA) synthesis

RNA extraction was conducted by using Trizol® reagent (Invitrogen, Carlsbad, CA) method. The cDNA were synthesized with the Uni12 primer (5'-AGCAAAAGCAGG-3') and H5C reverse primers (5'-ACTGCAATCCTTC-AGAATGAGGGG-3') from the extract RNA using Sensiscript Reverse Transcription kit (Qiagen, Valencia, CA). cDNA was amplified using Go Taq® Green Master Mix (Promega, Madison, WI). The cycling condition was as follows: 2 min at 95°C, 35 cycles at 95°C for 30 s 55°C for 45 s 72°C for 1 min, and 10 min at 72°C. Subsequently, the PCR products run in the gel electrophoresis at 107volts and visualized by Gel Doc XR Scanner (Biorad, CA). The correct bands were excised and extracted using Gel Extraction kit (Qiagen, Valencia, CA).

Cloning

The PCR products were cloned directly into pCR®2.1-TOPO plasmid by using TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Plasmid purification was conducted using plasmid purification kit (Qiagen, Valencia, CA). Plasmid concentrations were measured using Nanodrop 1000 Spectrophotometer V3.6 (Thermoscientific, Wilmington, DE). Identification of insert in the plasmid was conducted by amplifying the gene using using Go Taq® Green Master Mix (Promega, Madison, WI). The cycling condition was as follows: 2 min at 95°C, 35 cycles at 95°C for 30 s 55°C for 45 s 72°C for 1 min, and 10 min at 72°C. After that, gel electrophoresis was conducted to verify the size of the PCR products at 107 volts and visualized by Gel Doc XR Scanner (Biorad, CA). Plasmid samples were sent for sequencing

to the DNA Biotechnology Facility University of Connecticut.

Plasmid linearization and *in-vitro* transcription

Plasmids that contained the correct insert were linearized using *Bam*HI restriction enzyme from (Promega, Madison, WI). In-vitro transcription was conducted using RiboMAX™ Large Scale RNA Production System – T7 (Promega, Madison, WI).

Quantitative real-time reverse transcriptase polymerase chain reaction

The testing the caprylic acid and monocaprylin antiviral effect to the viral growth was conducted by using Power SYBR Green® master mix (Applied Biosystem, CA). The standard RNA was serially diluted up to 10⁻⁵ and used as a template for the standard curve. The samples of RNA extract from the treated viruses were directly used as templates without any dilution. The quantification was conducted using standard curve with 7500 real-time PCR system (Applied Biosystem, CA). The viral particles from each sample were calculated by using the standard curve method.

The viral particles of H5N1 and H5N2 affected by specific concentrations of caprylic acid and monocaprylin were examined by ANOVA (SAS 9.1 Software, North Carolina).

RESULTS

The results of Avian influenza viruses (H5N1 and H5N2) which had been incubated with various concentrations of caprylic acid, sodium caprylate and monocaprylin for 24 hours are as follow.

Caprylic acid in the concentration of 0.2% was able to make a viral growth reduction 1 log in H5N1 and 2 logs in H5N2. In the concentration of 0.4%, it was able to make a viral growth reduction 2 logs in H5N1 and 3 logs in H5N2. Although sodium caprylate increased viral growth in 2 treatment groups (0.075%-H5N1 and 0.125%-H5N2), it reduced the viral growth in other treatment groups. Monocaprylin in the

concentration of 0.08% was able to make a viral growth reduction 2 logs in H5N2. In the concentration of 0.16%, it was able to make a viral growth reduction 2 logs in H5N1 and 3 logs in H5N2 (Table 1).

Table 1. Inactivation of avian influenza virus by caprylic acid, sodium caprylate, and monocaprylin

Lipid	Concentration (%)	Reduction of H5N1 titer (in log10)	Reduction of H5N2 titer (in log10)
Caprylic acid	0.1	<1	<1
	0.2	1	2
	0.4	2	3
Sodium caprylate	0.075	*	0
	0.1	<1	0
	0.125	<1	*
Monocaprylin	0.04	*	<1
	0.08	*	2
	0.16	2	3

*The viral particles detected was higher than positive control

DISCUSSION

Caprylic acid and its derivatives are known to have antimicrobial activity against HSV-1, RSV, HIV-1, BVDV, PRV, VSV, fungi, *Salmonella enteritidis*, *Campylobacter jejuni*, *E.coli* O157:H7, *E.coli* O103, *E.coli* DH5 α , *Staphylococcus aureus*, *Cronobacter* spp. (*Enterobacter sakazakii*).^{5,7-16} Most of the susceptible viral pathogens tested were enveloped virus. Avian influenza viruses are also enveloped, therefore caprylic acid, sodium caprylate and monocaprylin are potential to inactivate avian influenza virus.

The results showed that low concentrations of caprylic acid and monocaprylin were able to reduce the number of viral particles significantly. Sodium caprylate was not giving significant effect to the viral growth, although the concentrations tested was based on the critical concentrations from the previous study.¹⁷ This was probably occurred due to shorter incubation period (24 hours) used for real-time RTPCR assay in this study compared

to the incubation period (48-72 hours) used for the plaque assay in the previous study.¹⁷

The concentration needed by monocaprylin to make the same reduction effect to the viral growth was lower than the concentration needed by caprylic acid. This is in accordance with the previous studies results that showed that monocaprylin also more effective in reducing VSV, visna virus, and HSV-1 compared to caprylic acid.^{5,6} Caprylic acid and monocaprylin were also more effective in reducing the viral growth of H5N2 compared to H5N1.

Caprylic acid, also known as octanoid acid, is present in breast milk, bovine milk, and coconut oil.^{18,19} Sodium caprylate is a sodium salt of caprylic acid and monocaprylin is monoglyceride form of caprylic acid. The basic materials for these compounds are easily found in tropical countries like Indonesia (avian influenza endemic). Thus, the application of these compounds for controlling avian influenza virus as poultry food supplement will be simpler compared to vaccination which need complex and costly process.

Overall, caprylic acid and monocaprylin were able to inhibit avian influenza viruses' growth *in vitro*. Based on the real-time RTPCR result monocaprylin is proved as the most effective compound in reducing the viral growth of H5N1 and H5N2. Nevertheless, different effect of the compound occurred in different tests and different avian influenza virus, therefore a further study is needed to investigate the mechanism of the fatty acids as the antiviral compounds. Since the goal of the study is to control AIV infection in poultry, *in-vivo* study is needed to investigate the viral load in the infected chicken feed with various concentrations of these compounds as treatment, based on the results on this study.

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