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Synthesis of von Willebrand Factor Antigen in *Chlamydophila pneumoniae*-Infected Endothelial Cells

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ABSTRACT

Objective: The main objective of our study was to evaluate the response of endothelial cells infected with *Chlamydophila pneumoniae (C. pneumoniae)*, by using von Willebrand factor (vWf) antigen as a marker for endothelial damage and dysfunction. Another objective was to evaluate the effect of cycloheximide on *C. pneumoniae* infectivity and vWf secretion from human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were harvested. After first passage, the HUVECs were inoculated with *C. pneumoniae* in three concentrations of cycloheximide (0, 1, and 2 μg/mL). At 24, 48, and 72 hours post-inoculation, supernatants from each HUVEC culture well were collected and measured for vWf antigen by sandwich ELISA as well as non-infected HUVECs were used as controls. *C. pneumoniae* infectivity was evaluated by indirect immunofluorescence technique and polymerase chain reaction.

Results: The cycloheximide-treated HUVECs resulted in greater infection compared to the non-treated HUVECs. Means of vWf antigens from HUVECs infected with *C. pneumoniae* were not different from those of non-infected HUVECs. However, there was a significant change in vWf secretion when different concentrations of cycloheximide were used in the culture system.

Conclusion: From our study, the results of vWf antigen secreted from HUVECs did not support the direct endothelial damage effect caused by *C. pneumoniae* infection. Therefore, vWf antigen is not a sensitive marker for this event. Furthermore, results from this study supported the infectious ability of *C. pneumoniae* on HUVECs and the importance of cycloheximide in improving the infectivity of this organism. However, the investigators had to use this substance cautiously, especially in protein synthesis study. Nevertheless, a non-variable dose of *C. pneumoniae* and the use of only one surrogate endothelial damage marker may limit the interpretation of this study.

Keywords: Atherosclerosis; *Chlamydophila* (or *Chlamydia) pneumoniae*; cycloheximide; human umbilical vein endothelial cell; von Willebrand factor

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Atherosclerosis is an inflammatory disease of large and medium-sized arteries that causes much morbidity and mortality. The pathogenesis of this condition has been explained by many mechanisms.[1,2] The main mechanisms are the abnormalities in lipid metabolism, endothelial dysfunction, inflammatory and immunologic factors, plaque rupture, and smoking and in recent years, inflammation caused by infection have been proposed. Many organisms are involved in atheroma formation including *Chlamydophila pneumoniae (C. pneumoniae*, previously called *Chlamydia pneumoniae)*, cytomegalovirus, and *Helicobacter pylori*. Some studies add hepatitis A virus and herpes simplex virus type 1 and type 2 as contributors of atherosclerosis.[3-7] Concerning *C. pneumoniae*, there are many evidences on its relationship to atherosclerosis. Firstly, a study by Saikku *et al.* in 1988 demonstrated that patients who had coronary artery diseases (CAD) were more likely to have a significant level of anti- *C. pneumoniae* antibodies than those who did not have a CAD condition.[8] Following this study, several papers have been published to demonstrate the association of *C. pneumoniae* and atherosclerosis and its consequences. Apart from seroepidemiological study, other evidences are used to support the link between *C. pneumoniae* and atherosclerosis such as *in vivo* animal model studies and direct detection of *C. pneumoniae* within atherosclerotic lesions by immunohistochemistry, electron microscopy, *in situ* hybridization or amplification of bacterial DNA by polymerase chain reaction (PCR).[9-13]

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There are many possible mechanisms used to explain the pathogenesis of atherosclerosis caused by *C. pneumoniae*. One of these is the endothelial dysfunction. It is found that infection of this obligate intracellular gram negative bacterium can cause exposure of bacterial endotoxin or lipopolysaccharide (LPS) and heat shock protein 60 to endothelial cell and macrophage which contributes to the stimulation of many inflammatory cytokines that initiate atheroma formation.[14] Following this event, the injured endothelium will produce many adhesion molecules such as E-selectin or endothelial-leukocyte adhesion molecule 1 (ELAM-1), intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) as well as procoagulant-like tissue factor.[15-17]

von Willebrand factor (vWf) is an adhesive glycoprotein stored in specific secretory of endothelial cell organelles known as Weibel-Palade bodies that undergo regulated exocytosis in response to inflammation and vascular injury. It has been estimated that only 5% of newly synthesized vWf is stored in cultured HUVECs but secreted continuously. Previous data demonstrated that plasma vWf level increment is correlated with endothelial dysfunction or damage in atherosclerosis and coronary artery disease.[18-22] Some *in vitro* studies showed that the inflammatory response of endothelial cells from various stimulations can aggravate the production of vWf.[23,24] The effect of *C. pneumoniae* infection and vWf stimulation due to endothelial dysfunction were studied.[25-27] Cook *et al.* investigated the association between *C. pneumoniae* infection and cardiovascular disease as well as various markers including vWf. They demonstrated the correlation of *C. pneumoniae* IgG level and severe hypertension, but not the plasma vWf antigen.[25] Gabriel *et al.* also showed that *C. pneumoniae* infection did not relate to the change of plasma vWf and platelet activity, although other inflammatory markers increased.[26] This result was in consistent with the study by Rothenbacher *et al.* in that various infections were not associated with vWf activity.[27] However, there is no in vitro study about the correlation between vWf secreated from HUVECs and *C. pneumoniae* infection. The sensitive cell lines for cultivation *C. pneumoniae* are cycloheximide-treated HEp-2 cells, NCI-H 292, and HL cell lines.[28,29] Other cell lines such as McCoy, HeLa 229 and BHK-21 are also commonly used. Although HUVECs is not the cell line of choice for *C. pneumoniae* culture, it was shown that this organism can infect HUVECs.[30-34]

In this study, we investigated the association of human umbilical vein endothelial cells (HUVECs) that were infected with *C. pneumoniae* and the production of vWf, one of the markers for endothelial damage *in vitro* in order to show that vWf can be a marker for endothelial damage caused by *C. pneumoniae*. Furthermore, we studied the effect of cycloheximide, a substance that inhibits protein and DNA synthesized of eukaryotic cell in the culture system, on the yield of the infection and the amount of vWf production.

MATERIALS AND METHODS

Human umbilical vein endothelial cell (HUVEC) culture

HUVECs were collected from human umbilical veins according to the Jaffe's method.[10] Human umbilical cord was cut off at the suture marks at both ends and then the umbilical vein was cannulated with the catheters at both sides by aseptic technique. After removing the retained blood clots by perfusing the vein with PBS, the vein was filled with 0.05% trypsin-EDTA and incubated at 37^0C for 20 minutes. The released cells were collected in 50-mL tube and centrifuged at 1,000 rpm for 10 minutes. The cell pellet were suspended in M-199 media (Gibco, USA) supplemented with 20% fetal bovine serum (FBS, Gibco, USA) and cultured in 35 mm tissue culture Petri dishes at 37^0C in a 5% CO_2 humidified atmosphere.

Chlamydophila pneumoniae organisms

C. pneumoniae from stock cultures were inoculated and propagated in a HEp-2 cell monolayer that was cultured in Earle's minimal essential medium (EMEM, Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), streptomycin 50 µg/mL and cycloheximide 2 µg/mL at 37^0C in a 5% CO_2 humidified atmosphere before being inoculated into the endothelial cells. The quantification of organisms was performed by indirect immunofluorescent staining and reported as inclusion forming unit per ml (IFU/mL). In this study, the *C. pneumoniae* quantities of 2.2 x 10^3 IFU/mL were used.

Chlamydophila pneumoniae infection of human umbilical endothelial cells

Upon the confluence of HUVECs, these cells were then trypsinized into a 24-well plate at a concentration of 400,000 to 500,000 cells per well and maintained for 24 to 48 hours in M-199 with 20% FBS. Before being infected with *C. pneumoniae*, the endothelial culture media in each well was removed. Then the 2.2 x 10^3 IFU/mL of *C. pneumoniae* were inoculated into the endothelial cells. Following centrifugation at 800×g and incubation at 37^0C for 1 hour, the inocula were removed. Infected HUVECs were washed twice with phosphate buffered saline (Gibco, USA) and cultured in M-199 media containing 10% inactivated FBS and cycloheximide at the concentration of 0, 1 and 2 µg/mL. After inoculation, the infected HUVECs were incubated at 37^0C in a 5% CO_2 humidified atmosphere for 72 hours. The control, non-infected HUVECs were incubated in the same concentrations and conditions of cycloheximide like those of infected cells. During the incubation, 250 µL of supernatant from each well was collected at the time of inoculation, 24, 48 and 72 hours post-incubation for the detection of von Willebrand factor. After 72 hours, the trypsinized cells from each well were quantitated to determine any change of cell volume by counting on counting chamber.

Infection of *C. pneumoniae* in HUVEC cells at 72 hours post infection (hpi) was confirmed by indirect immunofluorescent staining and nested polymerase chain reaction (PCR). Indirect immunofluorescent staining was performed by trypsinization of HUVECs from each well and stained with monoclonal antibody against *C. pneumoniae* (DAKO, Denmark). The target gene for nested PCR is 16s rRNA gene of *C. pneumoniae*. It was performed by using outer primers: C07 (5′ to 3′: ACG GAA TAA TGA CTT CGG) and C08 (5′ to 3′: TAC CTG GTA CGC TCA ATT). Inner primers were C11 (5′ to 3′: ATA ATG ACT TCG GTT GTT ATT) and D01 (5′ to 3′: CGT CAT CGC CTT GGT GGG CTT). The final amplified products sized 221 base pairs. The PCR programmed was heating at 95^0C for 2 min followed by 35 cycles of 94^0C for 1 min, 55^0C for 30 seconds, and 72^0C for 1 min.

Detection of von Willebrand factor antigen

vWf antigen from each well at different time (24, 48, and 72 hpi) was measured by a sandwich enzyme-linked

immunosorbent assay (ELISA). In short, the 100 µL of supernatant from each well collected at different times was added into the prepared well of the microtiter plate which was coated with rabbit anti-human vWf in duplicate. Then the plate was incubated at 37^0C for 150 minutes and washed for removal of excess antigens. After completing the incubation, 100 µL of anti-human vWf IgG conjugated with peroxidase was added into each well and incubated for 150 minutes at 37^0C. Then the plate was washed and 100 µL of substrate solution was added to each well. The reactions were read by an ELISA reader at λ of 492 nm and reported as OD. The recorded values were calculated into percentage of standard by using a standard curve.

RESULTS

Infection of *C. pneumoniae* on HUVECs

Micrographs of HUVECs before infection and after inoculation with *C. pneumoniae* were recorded under inverted and fluorescent microscopes, respectively. Before inoculation, HUVECs were arranged as monolayers in polygonal morphology with close contact borders in a cobblestone arrangement (Fig 1). After 72 hpi, HUVECs were stained with fluorescent monoclonal antibody to *C. pneumoniae*. Positive cells were presented as single, round and apple-green color in the cytoplasm. Control HUVECs showed no positive infected cell (Fig 2A). Less number and less fluorescent intensity of positive cells were found in the absent of cycloheximide culture (Fig 2B) compared to infected cells in cycloheximide cultures at the concentration of 1 µg/mL (Fig 2C) and 2 µg/mL (Fig 2D). Slightly differences of the number of cells at pre-inoculation and 72 hpi were observed (data not shown).

The infection of HUVECs by *C. pneumoniae* at 72 hpi was then confirmed by using nested PCR. After amplification, HUVECs that were infected with *C. pneumoniae* and gave the DNA products as shown in Fig 3. Non-infected HUVECs showed no product on amplification.

Quantities of von Willebrand factor antigen from HUVECs in various conditions

The data of vWf antigen level was presented in the form of the mean and% coefficient of variation (%CV) from the duplicated experiments. The amounts of vWf antigen production in various conditions at different times after *C. pneumoniae* inoculation are summarized in Fig 4.

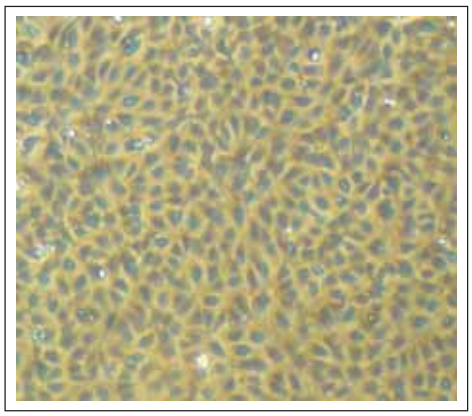


Fig 1. HUVEC monolayer examined under inverted microscope before infected with *C. pneumoniae*. These cells are in large, flat, polygonal-shaped cells with a cobblestone arrangement.

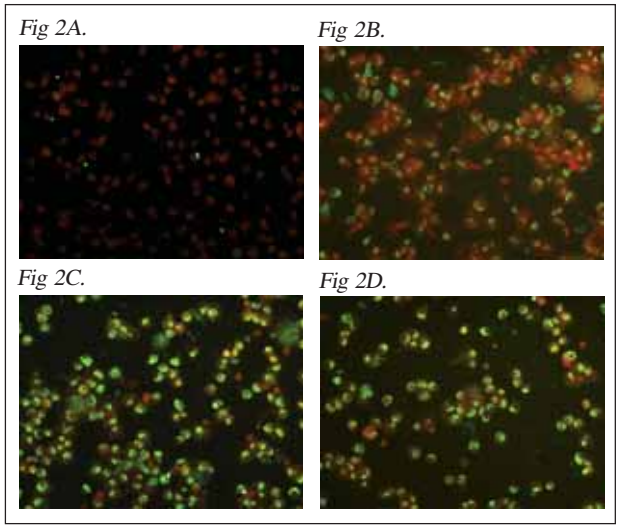


Fig 2. HUVEC culture after 72 hpi examined under fluorescence microscope. No apple green, fluorescent color of HUVECs was observed in control HUVECs (A), *C. pneumoniae*-infected HUVECs in culture without cycloheximide showed weak intensity and less numbers of positive cells (B) compared to infected HUVECs culture in the presence of 1 µg/mL of cycloheximide (C) and 2 µg/mL of cycloheximide (D) which showed strongly intensity and high number of fluorescent positive cells.

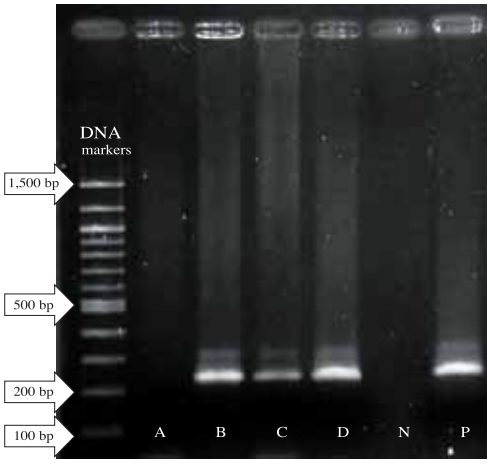


Fig 3. Nested PCR of HUVECs at 72 hpi. Lane N and P represented negative and positive control for 16s rRNA gene of *C. pneumoniae* respectively. Lane A, HUVECs that were not infected with *C. pneumoniae* showed no amplification product. In lane B, C, and D, HUVECs that were infected with *C. pneumoniae* but varied in cycloheximide concentration (0, 1, and 2 µg/mL respectively) showed positive products at the same level.

In this study, the amounts of vWf antigen were continuously increased according to the time after the inoculation. The initial amount of vWf antigen level in each condition was approximately 40 % according to the quantity of vWf antigen found in fetal bovine serum in culture media. At 24 hpi, the levels of vWf in all conditions did not diverge as much as at 72 hpi. The levels of vWf production are higher in the culture system without cycloheximide than in that with cycloheximide. At 72 hpi, the amounts of vWf of HUVECs without cycloheximide were approximately twice of those produced by HUVECs that had cycloheximide in the culture system. The difference in cycloheximide concentration (1 and 2 µg/mL) did not

TABLE 1. Demonstration of means of vWf antigen measured by the sandwich ELISA method in various conditions of HUVECs at 24, 48, and 72 hpi. Results are expressed as mean (and% coefficient of variation).

Concentration of cycloheximide (μg/mL)	Means of vWf antigen (%) and percentage of coefficient of variation (%CV)					
	24 hpi		48 hpi		72 hpi	
	Non-infection	Infection	Non-infection	Infection	Non-infection	Infection
0	62.5 (5.6)	58.5 (8.3)	124.0 (1.1)	139.0 (2.1)	235.0 (9.0)	240.0 (18)
1	39.5 (1.8)	39.7 (4.3)	71.5 (11.3)	70.3 (1.4)	104.5 (14.1)	113.0 (6.6)
2	40.1 (0.4)	47.0 (2.1)	69.0 (2.0)	69.5 (3.8)	84.0 (5.1)	104.0 (3.9)

seem to significantly affect the production of vWf antigen. Moreover, the presence of *C. pneumoniae* infection in HUVECs did not affect the production of vWf antigen.

DISCUSSION

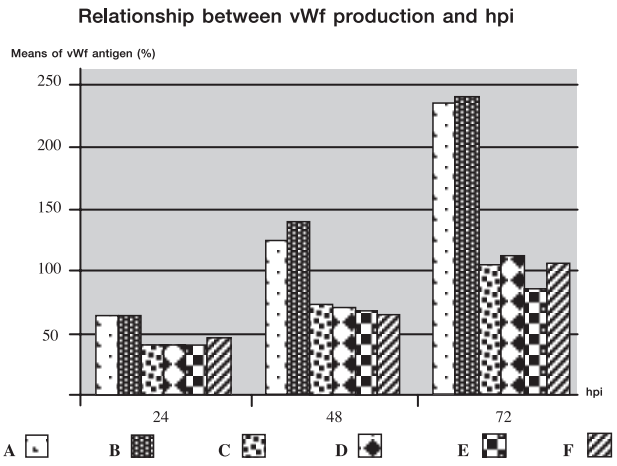


Fig 4. Quantities of vWf antigen in various conditions of HUVECs at 24, 48, and 72 hpi. A and B show the amounts of vWf that produced by non-infected and *C. pneumoniae*-infected HUVECs in the condition which lack of cycloheximide. C and D show the amounts of vWf that produced by non-infected and *C. pneumoniae*-infected HUVECs in the condition which use cycloheximide 1 μg/mL. E and F show the amounts of vWf that produced by non-infected and *C. pneumoniae*-infected HUVECs in the condition which use cycloheximide 2 μg/mL.

Many factors contribute to the pathogenesis of atherosclerosis. One of these is the effect of *C. pneumoniae* infection. In this study, we investigated the correlation between *C. pneumoniae* infection and vWf, i.e., an endothelial marker *in vitro*, and the effect of cycloheximide on the infection and synthesis of vWf in a culture condition. Our study showed the continuously increased level of vWf in both non-infected and infected conditions with *C. pneumoniae* during the time of inoculation, but the differences of vWf level from infected and non-infected HUVECs is not significant. However, vWf levels changed greatly with altered cycloheximide in the culture media. In the conditions without cycloheximide, vWf levels were greater than those with cycloheximide in culture conditions. These data suggest that vWf from HUVECs, which represents endothelial damage or dysfunction, was secreted continuously whether the *C. pneumoniae* infection occurred or not, but the important factor which affected vWf production was the presence of cycloheximide.

From this study, the infection of HUVECs with *C. pneumoniae* was confirmed by using immunofluorescent staining and nested PCR. One of the substances used to improve the ability of *C. pneumoniae* to infect cell culture in many studies is cycloheximide. It is widely used in many researches as a glutarimide antibiotic which inhibits DNA and protein synthesis in eukaryotic cells but does not affect prokaryotic cells. It is suggested that in cultures treated with cycloheximide, the number of chlamydial inclusions is generally higher than those treated by other methods.[29,35,36] In our study, HUVECs which were treated with cycloheximide before inoculation with *C. pneumoniae* gave a stronger intensity of fluorescent staining than the cells that did not use cycloheximide. This confirms that the infectivity of *C. pneumoniae* in condition where cycloheximide is present is better than those without it. We also found that different concentrations of cycloheximide at 1 and 2 μg/mL did not show a more distinctly staining intensity or level of vWf antigen. This demonstrated the inhibitory effect of cycloheximide on vWf synthesis.

A previous study by Paleolog *et al.* also showed that pre-incubation of IL-1 and TNF-α-treated HUVECs with 5 μg/mL of cycloheximide abolished the enhancement of vWf secretion.[37] However, Schorer et al. found that cycloheximide had no effect on vWf secretion of IL-1 and LPS-treated HUVECs.[38] Tsai et al. investigated two forms of vWf from HUVECs and found that cycloheximide inhibits the release of low molecular weight vWf but not the high molecular weight vWf.[39] In our study, so far, we found that cycloheximide was useful for *C. pneumoniae* inoculation to HUVECs, but an appropriate dose of cycloheximide was needed to validate the analysis about protein synthesis study. Moreover, variable ranges of cycloheximide should be further investigated to confirm the lack of cycloheximide dosage effect. This information will be useful for investigators in setting up experiments on protein synthesis in the culture system with cycloheximide.

From these results, we suggested that the infection of HUVECs by *C. pneumoniae* did not affect endothelial damage as demonstrated by vWf secretion compared to non-infected HUVECs. The limitation of this study was the non-variety doses of *C. pneumoniae* and other surrogate markers of endothelial damage and dysfunction which contributed to atherosclerosis were not investigated in this study. Therefore further studies on atherogenic pathogenesis of endothelial damage by *C. pneumoniae* are still needed for the benefit of patient's care and monitoring.

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บทคัดย่อ

การสร้างวอนวิลลีแบรนด์แฟคเตอร์แอนติเจนโดยเซลล์เอนโดธีเลี่ยมที่ติดเชื้อคลามัยโดฟิลา นิวโมนิอิ

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วัตถุประสงค์: เพื่อศึกษาการตอบสนองของเซลล์เอนโดธีเลี่ยมต่อการติดเชื้อคลามัยโดฟิลา นิวโมนิอิ โดยใช้การวัดวอนวิลลีแบรนด์แฟคเตอร์แอนติเจนเป็นตัวแทนของการเกิดการทำลายของเซลล์เอนโดธีเลี่ยม วัตถุประสงค์รองเพื่อศึกษาผลของซัยโคลเฮกซีไมด์ที่มีต่อการติดเชื้อคลามัยโดฟิลา นิวโมนิอิและการสร้างวอนวิลลีแบรนด์แฟคเตอร์แอนติเจน

วิธีการ: ทำการเก็บเซลล์เอนโดธีเลี่ยมและทำการเพาะเลี้ยง จากนั้นนำเซลล์ที่ได้แยกเป็นสองส่วน ส่วนหนึ่งเป็นเซลล์ควบคุมที่ไม่มีการติดเชื้อ อีกส่วนหนึ่งนำมาติดเชื้อคลามัยโดฟิลา นิวโมนิอิ โดยจะแบ่งทั้งสองกลุ่มออกตามความเข้มข้นของซัยโคลเฮกซีไมด์ที่ใช้ ได้แก่ 0, 1, และ 2 ไมโครกรัมต่อมิลลิลิตร ทำการเก็บน้ำเลี้ยงเซลล์หลังจากมีการติดเชื้อแล้ว 24, 48, และ 72 ชั่วโมงเพื่อวัดปริมาณวอนวิลลีแบรนด์แฟคเตอร์แอนติเจน เมื่อครบ 72 ชั่วโมง นำเซลล์เอนโดธีเลี่ยมมาย้อมด้วยวิธีอิมมูโนฟลูออเรสเซนท์และตรวจด้วยวิธีเนสต์พีซีอาร์เพื่อยืนยันการติดเชื้อ

ผลการศึกษา: เซลล์เอนโดธีเลี่ยมในภาวะที่มีซัยโคลเฮกซีไมด์ผสมในน้ำเลี้ยงเซลล์จะแสดงการติดเชื้อคลามัยโดฟิลา นิวโมนิอิได้ดีกว่าเซลล์เอนโดธีเลี่ยมที่ไม่มีซัยโคลเฮกซีไมด์ในน้ำเลี้ยงเซลล์ ค่าเฉลี่ยของวอนวิลลีแบรนด์แฟคเตอร์แอนติเจนจากเซลล์เอนโดธีเลี่ยมที่ติดเชื้อคลามัยโดฟิลา นิวโมนิอิไม่แตกต่างจากเซลล์ที่ไม่ติดเชื้อแต่ค่าดังกล่าวจะต่ำมากในเซลล์เอนโดธีเลี่ยมกลุ่มที่มีซัยโคลเฮกซีไมด์ในน้ำเลี้ยงเซลล์เมื่อเทียบกับกลุ่มที่ไม่มีซัยโคลเฮกซีไมด์ในน้ำเลี้ยงเซลล์

สรุป: จากปริมาณวอนวิลลีแบรนด์แฟคเตอร์แอนติเจนที่วัดได้จากการทดลองนี้ยังไม่แสดงถึงบทบาทการทำลายเซลล์เอนโดธีเลี่ยมโดยตรงโดยเชื้อคลามัยโดฟิลา นิวโมนิอิ แต่การศึกษานี้ได้ยืนยันการติดเชื้อคลามัยโดฟิลา นิวโมนิอิในเซลล์เอนโดธีเลี่ยมภายนอกร่างกาย โดยการมีซัยโคลเฮกซีไมด์ในระบบการติดเชื้อจะช่วยให้การติดเชื้อดีขึ้น อย่างไรก็ตามสารนี้ยังมีผลต่อการทดลองที่เกี่ยวกับการสร้างโปรตีนของเซลล์ด้วย ข้อจำกัดที่สำคัญของการศึกษานี้คือใช้ปริมาณเชื้อคลามัยโดฟิลา นิวโมนิอิที่เป็นปริมาณเดียวและใช้ตัวชี้วัดสำหรับการทำลายเซลล์เอนโดธีเลี่ยมเพียงชนิดเดียว