โปรตีนรอพทรีเน็ค-2 ตัวบ่งชี้จำเพาะใหม่ของเชื้อมาลาเรียชนิดไวแวกซ์ระยะไซซอนต์ในตับและฮิปโนซอยต์

Rhoptry-neck Protein-2, a Novel Marker of *Plasmodium vivax* Liver Stage Schizont and Hypnozoite

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บทคัดย่อ

การกำเริบใหม่เป็นปัญหาสำคัญของโรคมาลาเรียชนิดไวแวกซ์ โดยมีสาเหตุจากเชื้อระยะพักตัวที่เรียกว่า ฮิปโนซอยต์ ซึ่งจะฝังตัวอยู่ในเซลล์ตับเป็นเวลาหลายสัปดาห์หรือหลายปี เพื่อรอการกระตุ้นให้เข้าสู่กระบวนการเจริญเติบโตในเซลล์ตับต่อไป ทั้งนี้ชีววิทยาของฮิปโนซอยต์ยังไม่เป็นที่เข้าใจอย่างถ่องแท้ อีกทั้งการศึกษาเชื้อมาลาเรียระยะในตับก็ทำได้ยาก เนื่องจากการขาดโมเดลในการทดลองซึ่งต้องคำนึงถึงความจำเพาะระหว่างเชื้อมาลาเรียและเซลล์ของโฮสต์ ดังนั้นการศึกษาตัวบ่งชี้จำเพาะนี้จึงมีประโยชน์อย่างยิ่งต่อการศึกษาชีววิทยาของฮิปโนซอยต์และกลไกการกำเริบใหม่ของโรค Rhoptry-neck Protein-2 (RON-2) มีการแสดงออกในเชื้อมาลาเรียระยะรุกราน ได้แก่ สปอโรซอยต์ ซึ่งเป็นระยะที่ไชเข้าเซลล์ตับและเมอโรซอยต์ซึ่งเป็นระยะที่ไชเข้าเม็ดเลือดแดง แต่ยังไม่พบว่ามีรายงานการศึกษา RON-2 ในระยะตับของมาลาเรียชนิดไวแวกซ์มาก่อน การทดลองนี้จึงตั้งสมมติฐานว่า RON-2 น่าจะมีการแสดงออกในเชื้อมาลาเรียระยะในตับ ซึ่งจะสามารถใช้เป็นตัวบ่งชี้จำเพาะของเชื้อมาลาเรียระยะไซซอนต์ในตับและฮิปโนซอยต์ได้การทดลองนี้จึงใช้หนูทดลองที่ปลูกถ่ายตับมนุษย์มาทำให้ติดเชื้อมาลาเรียชนิดไวแวกซ์ จากนั้นจึงนำตับที่ติดเชื้อมาใช้ในการศึกษาการแสดงออกของ RON-2 โดยเทคนิคอิมมูโนฟลูออเรสเซนต์ ผลการทดลองแสดงให้เห็นว่าโปรตีน RON-2 มีการแสดงออกอยู่ที่ด้านปลายยอดของเชื้อระยะสปอโรซอยต์ และเมอร์โรซอยต์ที่อยู่ระยะไซซอนต์ในเลือด ในขณะที่พบการแสดงออกของ RON-2 ลักษณะเป็นวงล้อมรอบบริเวณของเชื้อมาลาเรียระยะไซซอนต์ในตับและฮิปโนซอยต์ โดยพบในตำแหน่งเดียวกันกับ Up-regulated in infective sporozoites protein 4 (UIS-4) ที่เป็นตัวบ่งชี้จำเพาะที่เยื่อหุ้มแวคิลโอลของเชื้อมาลาเรียระยะในตับ ทำให้สามารถสรุปได้ว่า RON-2 แสดงออกอยู่ที่เยื่อหุ้มแวคิล-โอลของเชื้อมาลาเรียระยะในตับ และสามารถใช้เป็นตัวบ่งชี้จำเพาะตัวใหม่สำหรับเชื้อมาลาเรียชนิดไวแวกซ์ในระยะ

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ไซซอนต์ในตับ และระยะฮิปโนซอยต์ได้ ทั้งนี้จะเป็นประโยชน์ต่อจำแนกเชื้อมาลาเรียในขั้นตอนการศึกษาวิจัยเพื่อพัฒนาการรักษาและควบคุมการกำเริบใหม่ของมาลาเรียชนิดไวแวกซ์ได้

คำสำคัญ : ฮิปโนซอยต์ มาลาเรียระยะในตับ เยื่อหุ้มแวคิวโอล ไวแวกซ์ RON-2

ABSTRACT

Relapse is a serious problem caused by vivax malaria. The dormancy stage called hypnozoite can be latent in the hepatocyte for several weeks or years, waiting for reactivation before developing into liver stage schizont. The biology of hypnozoite is not well understood. Furthermore, the study of the liver stage of human malaria is also difficult, due to the lack of small laboratory animal models and the restriction of specific binding molecules between human malaria parasites and human host cells. Thus, the study of the hypnozoite marker would be beneficial for further study of hypnozoite biology and the relapse mechanism. Rhoptry Neck Protein-2 (RON-2), the well-known rhoptry marker, was reported for localization in the invasive stage of the malaria parasite including sporozoite and merozoite, the invasive stages for hepatocytes and erythrocyte respectively. However, there are no reports for the expression of RON-2 in the liver stage. Therefore, we hypothesized that RON-2 which was found to be involved in the invasion process would be expressed in liver stage parasites and would be used as a marker for liver stage schizont and hypnozoite. Here, the human hepatocyte chimeric mice were infected with *P. vivax* sporozoites before collecting the infected liver tissue for examination using the immunofluorescent technique. The results showed that RON-2 was expressed at the apical end of *P. vivax* sporozoites and merozoites. However, RON-2 was localized in the surroundings of liver stage schizonts and hypnozoites, and co-localized with Up-regulation in infective sporozoites protein 4 (UIS-4), a well-known form of PVM protein. In conclusion, RON-2 localized at the PVM of the liver stages of schizont and hypnozoite, can be a novel marker for the identification of *P. vivax* liver stage schizont and hypnozoite. This could be beneficial for further investigation on the treatment and control of the relapse caused by vivax malaria.

Keywords: hypnozoite, liver stage malaria, parasitophorous vacuole membrane, *Plasmodium vivax*, RON-2

Introduction

Plasmodium vivax has the high incidence in worldwide, which has been found predominantly in South America and Asia including Thailand (World Health Organization, 2018). To complete its complex life cycle, *P. vivax* requires both of human and female *Anopheles* mosquito. During the bite of infected mosquito, the salivary gland sporozoites are injected and transmitted into the blood circulation of the human. After that, the sporozoites invade into the hepatocyte and develop inside the hepatocyte, called the liver stage. The liver stage parasites develop into schizonts containing with numerous merozoites. Then, the merozoites are released to the blood circulation. They invade and develop inside the red blood cells, called the blood stage. During this stage, the precursor of sexual stage parasites, gametocytes, are also develop and wait for ingestion by female *Anopheles* mosquito for transmission. (Mueller et al., 2019).

However, the considerable stage of *P. vivax* is a hypnozoite which can remain in the hepatocyte as a latent form. The hypnozoite can be reactivated in several weeks, months, or years after the primary infection. The activated hypnozoite can develop into the liver stage schizont and further cause symptom in the patient who had been completely cured by the medical treatment. Many reports suggested that hypnozoite was the most serious problem of vivax malaria treatment and control (Anstey et al., 2009; Mikolajczak et al., 2015; Price et al., 2007).

Plasmodium spp. was categorized as the intracellular parasite which resides in the parasitophorous vacuole (PV) within the host cell. PV formation was initiated during the parasite penetration into the host cell. PV was the cellular structure originated from the host cell membrane with modification by parasite proteins. Therefore, the complete of vacuole membrane formation could be used for identifying the success of parasite infection. The parasite modulated the parasitophorous vacuole membrane (PVM) by parasitic proteins for their growth and replication (Nyboer et al., 2018; Spielmann et al., 2012). These proteins had been contemplated in the blood stage parasites, however, UIS-4 was the only well-known protein for PVM marker of the liver stage parasite and hypnozoite (Mikolajczak et al., 2015). Therefore, the discovery of the novel liver stage schizont and hypnozoite marker would be benefit for the *P. vivax* research.

During the step of invagination into the red blood cell, parasite formed the moving junction (MJ) to anchor between the parasite and host cell which the actin-myosin motor of the parasite was the power for invasion (Lamarque et al., 2011; Shen et al., 2012). Initially, several proteins such as Rhoptry Neck Protein-2 (RON-2) was released from the rhoptry, an apical organelle commonly found in Apicomplexan parasites, during the MJ formation. The RON-2 protein extended to function as a receptor for Apical Membrane Antigen-1 (AMA-1) during further step of invasion (Delgadillo et al., 2016; Salgado-Mejias et al., 2019). Thus, it could be hypothesized that RON-2 would function in the liver stage and might be identified as a novel marker of liver stage schizont and hypnozoite.

In this study, the human hepatocyte chimeric mice had been utilized for *P. vivax* liver stage propagation. The infected liver was then collected for investigating of RON-2 protein localization in the liver stage schizont and hypnozoite.

Methods

Bioinformatics analysis of *Pv*RON-2

The *Pv*RON-2 gene was achieved from *P. vivax* Sal-1 strain genome via PlasmoDB database. The SMART version 7 was used to analyze protein sequence of *Pv*RON2 (Arévalo-Pinzón et al., 2011; Roobsoong et al., 2014).

Animal model and *P. vivax* infection

Human hepatocytes chimeric mice were imported from PhoenixBio Co., Ltd. (Higashi-Hiroshima, Japan). The protocols for study were approved by the Faculty of Tropical Medicine-Animal Care and Use Committee (FTM-ACUC), Mahidol University, Bangkok, Ref No.: FTM-ACUC 003/2016. Briefly, the *P. vivax* sporozoites were collected from the salivary glands of day14 infected *Anopheles* mosquitoes. After that, one million sporozoite were injected to the mouse via intravenously administration.

Specimen preparation

The samples from liver stage parasite, sporozoite and blood stage schizont were prepared in the different protocols. For *P. vivax* sporozoite samples, the infected salivary glands were dissected from mosquitoes. The dissected glands were smashed in the microcentrifuge tube (Axygen, USA) before washing and spotting onto the 8-well slides. Then, the samples were allowed to air dry and fixed by 4% paraformaldehyde (Merck, USA) before performing the immunofluorescent assay. For liver stage parasites and hypnozoites, the liver samples were collected from mice on day8 after *P. vivax* sporozoite inoculation. Then, the liver tissues were fixed with 4% paraformaldehyde and followed by paraffin technique (Cui et al., 2017). The tissue-paraffin blocks were sectioned at 4 μm thickness by microtome (Thermo Scientific, USA), placed onto the glass slides, and dried at 45ºC for overnight. The tissue sections were further proceeded to immunofluorescent assay. For *P. vivax* infected blood samples, the infected blood from the patients which the Ethical Review committee of Faculty of Tropical Medicine, Mahidol University, approved a Human Subjects protocol, was cultured for short-term to harvest the mature blood stage schizont. The cultured blood was spotted onto the glass slides, allowed to air dry and fixed by cold acetone (Roobsoong et al., 2014). The fixed blood samples were air dried and stored at -80 ^{o}C until used for the immunofluorescent assay.

Immunofluorescent assay

The section of liver sample was deparaffinized by xylene (RCL Labscan, Thailand) and rehydrated with ethanol (Merck, USA) and tap water. The sporozoite and infected blood samples were brought out from the storage and prepared at room temperature. After that, the samples were blocked from the non-specific protein binding by 4% bovine serum albumin in PBS at 37ºC for 2 hr. Then, they were incubated at 4ºC for overnight with rabbit anti-*Pv*RON-2 (100 time diluted in PBS; 1:100) and counterstained with the optimized dilution of well-known marker of each parasite stage. The counterstains were mouse anti-*Pv*UIS-4 (1:100) for PVM of liver stage (Mikolajczak et al., 2015), mouse anti-*Pv*CSP (1:20,000) for the sporozoite surface (Swearingen et al., 2016), and mouse anti-Bip (1:250) for endoplasmic reticulum of blood stage schizont (Roobsoong et al., 2014). After that, the samples were washed by PBS containing with 0.05% Tween-20 (PBS-T) before incubating with goat anti-rabbit conjugated with Alexa 488 (Invitrogen, USA), goat anti-mouse conjugated with Alexa 568 (Invitrogen, USA), and 4´, 6-diamidino-2-phenylindole (DAPI) at 37ºC for 1 hr. The sample slides were washed with PBS-T before coversliping with Prolong® gold antifade mountant (Life technologies, USA), and sealed with nail polish. The results of immunostaining were observed and taken for photomicrograph by using the inverted confocal laser scanning microscope Fv1000 (Olympus, Japan).

Results

Bioinformatics analysis of *Pv*RON-2

The PlasmoDB database showed that PVX_117880 was rhoptry neck protein-2 (RON-2) in *P. vivax* Sal-1 strain. This gene was found in chromosome 12 containing 1 exon. The coding sequence was 6,612 base pairs and the protein length was 2,203 amino acids. The signal peptide is localized in amino acid position 1 to18. In addition, the length of transmembrane protein is 22

amino acids which is localized at amino acid position 2,087 to 2,109. This information revealed that *Pv*RON-2 may function at the membrane portion which possibly be organelle membrane, parasite plasma membrane (PPM), parasitophorous vacuole membrane (PVM), or host-cell plasma membrane (HPM).

Expression of *Pv*RON-2 in *P. vivax*

The counterstain with well-known marker of each stage was specifically detected at the cellular compartment of the parasites as observed in the previous reports (Mikolajczak et al., 2015; Roobsoong et al., 2014; Swearingen et al., 2016) *Pv*CSP was found at the peripheral surface of the sporozoite (Figure 1A). *Pv*UIS-4 was presented at the PVM as a circumferential pattern at the peripheral of parasite in liver stage schizont (Figure 1B). Bip was located at the cytoplasm of parasite which limited to the endoplasmic reticulum of the parasite (Figure 1C). The expression of *Pv*RON-2 in sporozoite was clearly detected at the apical tip of sporozoite (Figure 1A). Accordingly, *Pv*RON-2 in blood stage schizont was localized at the apical end of merozoite and presented as the punctate pattern (Figure 1C). Interestingly, *Pv*RON-2 was exhibited as the circumferential pattern at the peripheral of liver stage parasite and co-localized with UIS-4 (Figure 1B).

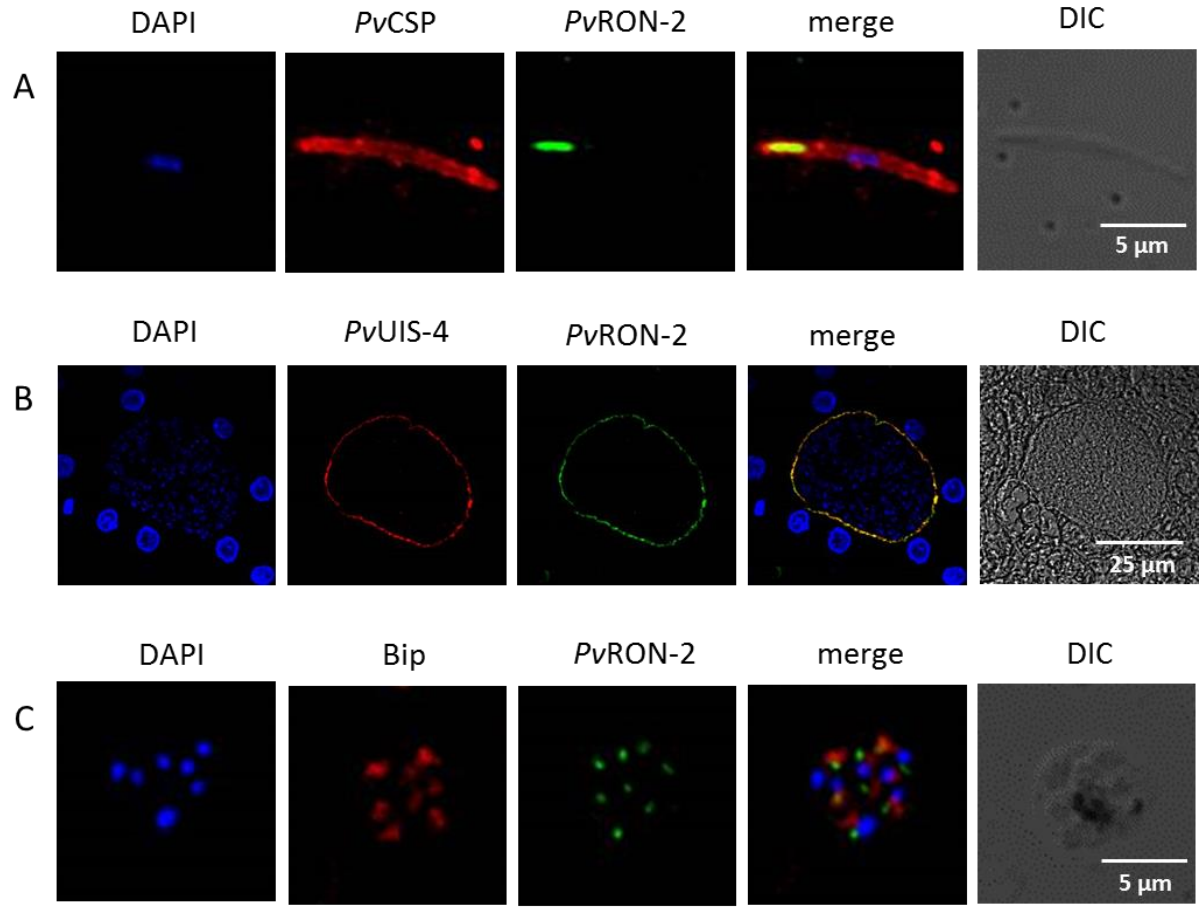

Figure 1 The immunofluorescence image of *Pv*RON-2 expression in sporozoite (A), liver stage schizont; day8 after *P. vivax* sporozoite inoculation (B) and blood stage schizont (C). Each stage was stained with fluorescent stain for DNA (DAPI; blue), *Pv*RON-2 (*Pv*RON-2 primary antibody plus goat anti-rabbit conjugated with Alexa 488; green) and counterstained with the well-known markers. *Pv*CSP was counterstained for sporozoite surface (*Pv*CSP primary-antibody plus goat anti mouse conjugated with Alexa 568; red). *Pv*UIS-4 was counterstained for the PVM of liver

stage schizont (*Pv*UIS-4 primary antibody plus goat anti-mouse conjugated with Alexa 568; red). Bip was counterstained for the endoplasmic reticulum of the parasite in blood stage schizont (Bip primary antibody plus goat anti-mouse conjugated with Alexa 568; red). The structure of each stage was represented by DIC image. The micron bar was indicated on DIC image of each parasite stage.

Expression of RON-2 in *P. vivax* liver stage and hypnozoite

In the present, UIS-4 is an only well-known marker for liver stage parasite. Our study showed that RON-2 was also detected at the liver stage schizont on day8 after *P. vivax* sporozoite inoculation and co-localized with UIS-4 (Figure 1B). Therefore, in this experiment, the expression of RON-2 was evaluated in *P. vivax* hypnozoite, which known as the existence of small and non-replication form. Normally, the period of liver stage growth and development to the mature schizont in the hepatocyte is around 8-9 days before the merozoites transitioned into the blood stream and infected the red blood cells (Mikolajczak et al., 2015). Thus, the parasite with a single fluorescent nuclear stain (DAPI) and the PVM marker (UIS-4) positive could be identified as a hypnozoite. Interestingly, *Pv*RON-2 was detected as a circumferential pattern at the peripheral of parasite and co-localized with UIS-4 in both the liver stage schizont (Figure 2A) and hypnozoite (Figure 2B). The results revealed that *Pv*RON-2 could be a novel marker of *P. vivax* liver stage schizont and hypnozoite.

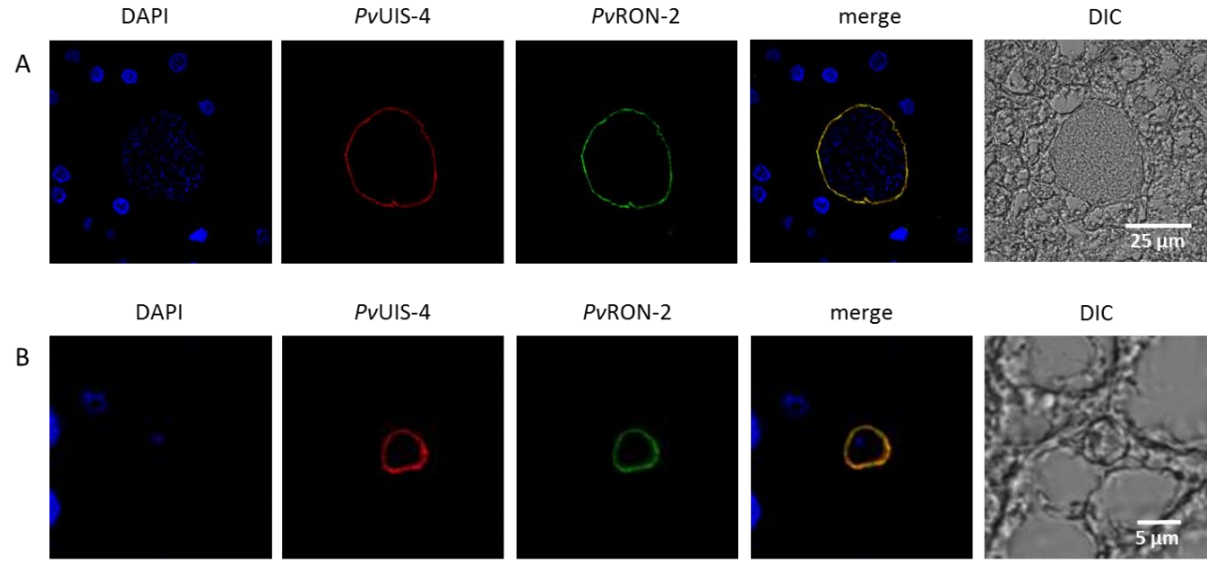

Figure 2 Immunofluorescence image of *Pv*RON-2 expression in liver stage schizont (A) and hypnozoite (B). The liver slide sample from day8 after *P. vivax* sporozoite inoculation was stained by fluorescent nuclear stain (DAPI; blue). *Pv*RON-2 (*Pv*RON-2 primary antibody plus goat anti-rabbit conjugated with Alexa 488; green) and *Pv*UIS-4 was counterstained for the PVM marker (*Pv*UIS-4 primary antibody plus goat anti-mouse conjugated with Alexa 568; red). The structure of each stage was represented by DIC image. The micron bar was indicated on DIC image.

Conclusion and Discussion

RON-2 was claimed as the critical protein for the parasite invasion. It was secreted by parasite and inserted into the host cell membrane to form the MJ during invasion. (Lamarque et al., 2011). Similarly, the Bioinformatics analysis of *Pv*RON-2 in our results also showed 22 amino acids of transmembrane protein which revealed the

function of *Pv*RON-2 as the membrane portion. The previous study suggested that RON-2 was the well-known rhoptry marker of sporozoite and merozoite (Arévalo-Pinzón et al., 2011). Likewise, our study showed that *Pv*RON-2 was expressed at the apical end of the *P. vivax* sporozoite and also detected as the punctate pattern at the apical end of merozoite in *P. vivax* blood stage schizont. Moreover, our results also showed the expression of *Pv*RON-2 in both liver stage schizont and hypnozoite as a circumferential pattern at the peripheral of parasite and co-localized with UIS-4. It can be realized that after Thus, RON-2 can be the novel marker for the liver stage schizont and hypnozoite which would be benefit for identification of malaria parasite in further research on liver stage schizont and hypnozoite, especially for vivax malaria biology. In addition, the evidence of *Pv*RON-2 expression in hypnozoite would benefit for research on the drug discovery which aims to treatment and control of relapse of disease.

Acknowledgements

This research was supported by Department of Pathobiology (Faculty of Science, Mahidol University, Thailand), Mahidol Vivax Research Unit (Faculty of Tropical Medicine, Mahidol University, Thailand) and Science Achievement Scholarship of Thailand. We would like to thanks Dr.Sebastian Mikolajczak from Novartis, Emeryville, California, United State of America, and Dr. Chise Tateno-Mukaidani from PhoenixBio Co., Ltd., Higashihiroshima, Japan for their support and suggestion on animal model. Besides, we would like to thanks Dr. Tomoko Ishino form Department of Molecular Parasitology, Ehime University, Japan for support on antibodies.

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