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Research Article

The Fermented Horse Milk Can As an Immune Stimulant Pili Sub Units of *S. flexneri* Protein Conjugated With Ctb in Mice

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Abstract

Sumbawa Horse Milk (SHM) had been known can enhance the immune response. Mice Legated Ilea Loop (MLIL) method may indicate the occurrence of diarrhea by bacteria. This study would like to clarify whether hem agglutinin pili subunit S. flexneri protein as an adhesion molecules and if combined with CT and SHM are having inhibitory discharge water towards the lumen of the intestine organ. Isolation of pili subunits of S. flexneri protein use bacteria pili cutter. Confirmation of morphological protein did SAM method and profile protein was detected by SDS-PAGE. Hem agglutinin subunits of S. flexneri protein as adhesion molecules applied immune cyto chemistry. Prevention of discharge water towards the lumen of the intestine organ used MLIL. Four groups mice, first group as a control, second received SHM, third SHM and CTB and the last SHM, CTB and adhesion molecule. The immune cyto chemistry method had confirmed that a protein with MW 49.8 kDa was an adhesion molecule. The best result was found in the combination SHM, CTB and adhesion molecule. The conclusion of this study is the protein with MW 49.8 kDa pili S. flexneri which was the adhesion molecules and its immune response can be improved by adding SHM and CTB.

Keywords: Pili; S. flexneri; immune stimulant; SHM

Introduction

At least 80 million bloody diarrhea cases in the world are found and estimated to cause 700,000 deaths and mostly found in 60% of children under five years [1,2]. In Indonesia, diarrhea is the number three cause of death for newborns. During 2005-2007 shigellosis was found in 612 children aged 0-12 years. *S. flexneri* is the leading cause of shigellosis and the incidence is of approximately 63.2% (36/57) [1,3,4]. To handle for shigellosis can be done with the use of antibiotics, early diagnosis, sanitation, hygiene and vaccination. Until now there is no vaccine currently used. Pili subunits hem agglutinin with MW 49.8 kDa *S. dysenteriae* protein are adhesion molecules and can be a vaccine candidate shigellosis [5]. An example of the vaccine to prevent pertussis is (invarynx) which contain adhesion molecules [6].

Kumis is a fermented dairy product traditionally made from mare's milk. The drink remains important to the peoples of the Central Asian steppes [7]. Sumbawa island in Indonesia is the center of horse milk producer called Sumbawa Horse Milk (SHM). Pasteurization and fermentation of horse milk will produce of α-lactalbumin, lactoglobulin β-, κ casein and β-lactoglobulin [8]. These substances as an immune stimulant can increase a response of Balb/C mice to produce s-IgA and prevent moving solution into mucous intestine in Mice Ilea Legated Loop model [9].

Our research by doing a combination of pili 37.8 kDa subunit protein of *V. cholerae* with cholera toxin subunit B was found to perform protection discharge into the lumen of the intestine of mice [10]. Furthermore pili subunit hem agglutinin with MW 49.8 kDa *S. dysenteriae* protein and pili subunit anti hem agglutinin with MW 7.9 kDa *S. dysenteriae* protein were an adhesion molecule on Bulb/C enterocytes. 5 After that have proven also there is an identical immunological cross-reaction between the molecules pili adhesin subunit protein hemagglutinin 49.8 kDa and anti molecules pili adhesin subunit protein 7.9 kDa S. boydii [11]. Recent study found that pili subunits hem agglutinin with MW 49.8 kDa S. dysenteriae protein was an adhesion molecule Bulb/C enterocytes. In giving oral administration pili subunit hem agglutinin with MW 49.8 kDa *S. dysenteriae* protein that conjugated with ISCOM show that protein can prevent the destruction of Balb/C colon epithelium [12]. Sumbawa Horse Milk can as an immune stimulant of sub unit pili hem agglutinin with MW 49.8 kDa S. flexneri protein and conjugated with CTB. The results show that increasing s-IgA and prevent movement of solution to lumen intestine demonstrated using the model MILL.

Materials and Methods

Sampling of milk (Sumbawa horse)

The study refers to the Faisal research, and horse milk samples obtained by milking directly on Sumbawa horse milk farmers in Bima district [9]. Samples from a mother milked horse 1 month after delivery with a milk volume of 350 ml and placed in a sterile container and stored in a cool box. Milk measured the degree of acidity (pH) shortly after the milking and for granted in mice.

Method for giving horse milk drink in mice

Balb/c mice 8-12 weeks old males are conditioned for 7 days, to adapt to the environment, weight control and uniformity of the food. After 7 days, mice divided into 4 groups: control group giving distilled water and the three groups test was supplied fermented horse milk Sumbawa with t a dose 2.6g by means of the sonde for 14 days. For giving of Sumbawa horse milk done in the morning from 9:00-10:00.

Culture method *S. flexneri*

The bacteria used for this research is *S. flexneri* which no process for typing obtained from Surabaya Referral Laboratory East Java Indonesia. Culture method referred according to our previous study [12]. TCG medium used is to enrich the growth of *S. flexneri* pili. *S. flexneri* were selected on culture in a petri dish containing media Salmonella Shigella agar and result cultured was poured with sterile PBS at pH 7.4, 10 ml. Bacterial suspension was collected by scraping. Furthermore, the bottle shaken gently for 30 minutes in a water bath at a temperature of 37°C and from the bottle 10 ml bacterial suspension poured onto TCG and the incubation was carried out at a temperature of 37°C for 2 x 24 hours.

Isolation methods pili *S. flexneri*

Isolation pili fraction refers to our previous study. Rich bacterial pili will be collected after harvest to bottle TCG media. The bacterial suspension was cut by using pili bacteria cutter. Rich supernatant pili was carried out from first until third cut by doing centrifugation 10000 rpm for 30 seconds, with the temperature 4°C. While isolating pili from cutting the fourth stage to the sixth stage is done by centrifugation with a speed of 10,000 rpm for 1 min with temperature 4°C. Subsequently all of the rich supernatant pili stored at temperature 4°C after having done samples centrifugation at 12,000 rpm for 30 minute with temperature of 4°C [10].

Scanning electrone microscope (SEM) determination

The instruction method was done according to protocol [13]. The bacterial pellet was isolated from bacteria culture grown in TCG media using centrifugation. The dilution sample had 1:100 ratio with physiological saline solution in order to separate the bacteria. Preparations were made on the cover glass then fixed by using methanol. Furthermore, the sample was put in the instrument sputter coater for coating with gold-palladium coating to turn it into a seemingly purple color. Samples were removed from the engine sputter coater, and then inserted into the SEM.

Sodium dodecyl sulfate polyacrylamid gel electrophorosis (SDS-PAGE)

Monitoring of the molecular weight is done by SDS-PAGE method [14]. Protein sample was heated for 5 min in a buffer solution containing 5 mM Tris pH 6.8; 5% 2-mercapto ethanol; 2.5% w/v sodium dodecyl sulfate, 10% v/v glycerol using bromophenol blue tracking color. 12.5 Selected mini slab gels with 4% gel tracking. Voltage electricity used is 120 mV. As a color material used is coomassie brilliant blue and sigma standard low range molecular markers. After calculation of the molecular weight to propagation protein with a molecular weight of 49.8 kDa, and then carried out the protein purification using electro elution.

Metode isolasi protein pili *S. flexneri*

The method referred to Agustina 2012 and would be described briefly [5]. The six pieces of formed gel electrophoresis cut perpendicular to each intersection is going to contain three protein bands. The results of the above pieces of tape were collected and than was inserted into the membrane tube which contained electrophoresis fluid running buffer. Furthermore protein was eluted using horizontal electrophoresis apparatus. Electric current used 120 mV for 90 minutes. The results of electro elution then dialysis with PBS pH 7.4 buffer fluid as much as 2 liters for 2 x 24 hours.

Hem agglutination test method

Hem agglutination assay was done according to the instructions of Hanne and Finkelstein's [15]. Sample dilutions were made in half concentration on micro plate V where each well volume sample was 50 µl. In every well, red blood suspension of mice with a concentration of 0.5% was added in the same volume of 50 µl. Then it was shaken using a rotator plate for 1 minute. Subsequently, it was placed at room temperature for 1 hour. The titer was determined by observing the agglutination of red blood at the lowest dilution.

Coupling of 37.8 kDa protein with CTB

Cholera Toxin sub unit B conjugated protein vaccine manufacturing procedures used a method modified from [16]. Result of collection 49.8 kDa *S. flexneri* protein adhesion 8 mg and 0. 230 CTB protein dissolved in 1.5 ml of PBS. Then 2% glutaraldehyde was added 3 ml, stirred in a fume hood and carried out for 1 hour by stirring slowly. After that added glycine pH 7.2 as much as 2:24 ml (MW = 75.07), the incubation was done for 1 hour RT with slow stirring. Further more the result was dialyzed overnight with PBS 4 times. Then the product was stored in -20°C.

Protectively test of immunized mice used adhesion molecule *S. flexneri* combined with horse milk

This method referred to our previous study which we name Mice Ilea Legated Loop (MILL). Protectively test using experiments with the small intestine of mice. We used five groups mice, first for control, second giving horse milk, third adhesion molecule and CTB, the forth adhesion molecule, CTB and horse milk and the lastly giving adhesion molecule and CTB for isolation s-IgA. Schedule immunization have 3 times, the first immunization on day 1, second on day 7 and the last schedule on day 21. Day 28 mice were killed by dislocation of the cervical vertebrae. Intestinal part along of the end the stomach until the end of the large intestine four groups was cut. After that the intestine cut and in every piece had 10 cm long, then the two ends of every pieces tied with thread. Subsequently all piece of intestine is inserted with *S. flexneri* 100 ul (10^5/ml). Then included in the flash media containing RPMI and placed in the rotated incubator at 37°C for 4 hours. Than every pieces of intestine was observed by weighing [10].

Collection of mucous and isolation of s-IgA

Mucus preparation is as follows: intestinal pieces of fifth group were washed with cold PBS containing protease inhibitors (25 ug/ml inhibitor cocktail) and 1.0 mM EDTA. Then the intestinal organs opened to expose the surface of the intestinal mucosa. Layer of mucus collected by scraping longitudinally using a spatula and placed in tubes containing sterile PBS and protease inhibitors. The suspension was shaken and then centrifuged at 12,000 rpm for 10 min 4°C. Supernatant was taken conducted purification, was suspended with PBS and dialysis performed using PBS and used as samples for examination of s-IgA by ELISA [10].

Detection of hem agglutinin subunit pili 48.9 kDa *S. flexneri* protein as an adhesion molecule using immune cyto chemistry method.

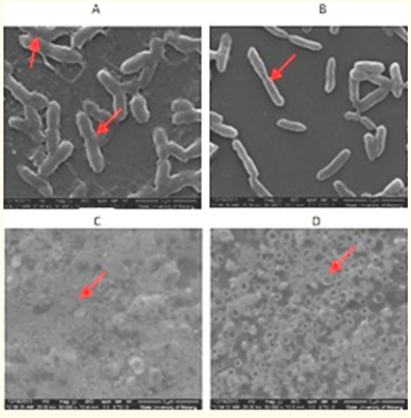
Methods used to refer study done by Agustina [5]. Briefly done as follows, first we must isolation rat enterocyte balb/C intestine by Weisler and quoted from Nagayama [17]. Secondly isolation of antibody anti hem agglutinin subunit pili 48.9 kDa *S. flexneri* protein referred to Harlow and Lane [18]. While the lastly is the detection of hem agglutinin pili subunits of 48.9 kDa protein *S. flexneri* as adhesion molecules by using immune cyto chemistry method. The method did according to Sumarno [18]. For sample preparation protein adhesion subunit pili with MW 49.8 kDa *S. dysenteriae* in cells of mice enterocytes had been fixed with methanol, and then the process was continued by washing with PBS pH 7.4, inducing with hydrogen peroxide (H_2O_2) 3%, and finally, washing with PBS. After that blocking was done with triton (triton X-100 (0.25%) in BSA blocking buffer) for 1h at room temperature, then washed with PBS pH 7.4 for 3 x 5 minutes. Subsequently incubation with primary antibodies (s-IgA polyclonal antibody protein adhesion subunit pili with MW 49.8 kDa *S. flexneri*) was performed. For the next stage incubation with secondary antibody anti-mouse IgG. After that SA-HRP was poured for each slide and followed to incubate with DAB (diaminobenzidine). HE (hematoxylin meyer) was poured directly. Once dried, the samples was ready to be examined under a microscope.

Data analysis

Methods of analysis of data on levels of s-IgA examination and heavy intestine of mice using ANOVA statistical analysis using SPSS for Windows 13 was conducted to determine whether there are differences among the treatments. Followed by Tukey's test (honest significant difference test).

Results

The results of *S.flexneri* pili isolation using bacteria pili cutter was depicted on Figure 1.



A. Shigella flexneri was picked up from TCG medium
B. Shigella flexneri was picked up from the last cutting sediment (4th cutting)
C. Shigella flexneri pili from supernatant first cutting
D. Shigella flexneri pili from supernatant third cutting.

Figure 1: Morphology of S. flexneri bacteria and pili, before and after cutting using Pili Bacteria Cutter.

Figure 1A show rod shape of *S. flexneri* bacteria. The pili take place onto surface bacteria there for the surface of bacteria appear rough. It is different if compare with Figure 1B where the bacteria surface is smooth. No pili appear because was shared by pili bacteria cutter. Figure 1C show the result from first cutting supernatant and we cannot see the morphology of bacteria. We can look pili fraction of the existing clusters. After that if we examine Figure 1D (from the fourth cutting) we can observe the morphology of pili *S. flexneri* protein almost same with Figure 1C, but there is a difference in the appearance of their shape like a donut.

Profile of pili *S. flexneri* protein can observe from SDS-PAGE as we can find in Figure 2.

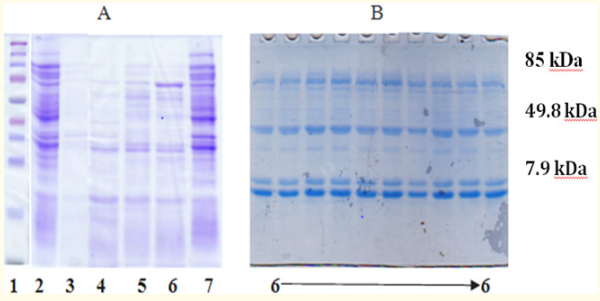


Figure 2: A. Profil of MW of S flexneri pili protein which isolated by pili bacteria cutter.

1. Marker.
2. Whole cell bacteria. 3-6. First-fouth cutting pili bacteria. 7. Sediment bacteria last cutting

B. Profil of MW sub unit pili *S flexneri* proteins 4th cutting.

The most prominent pili proteins were found in the third and fourth cutting (Figure 1). In Fig B has 3 prominent proteins bands were apparent with each MW is: 85 kDa, 49.8 kDa and 7.9 kDa. Using electro elution this protein band derived from pili fourth cutting (Figure 2B, 6) did hem agglutination test using mice erythrocyte. The result was depicted in Figure 3.

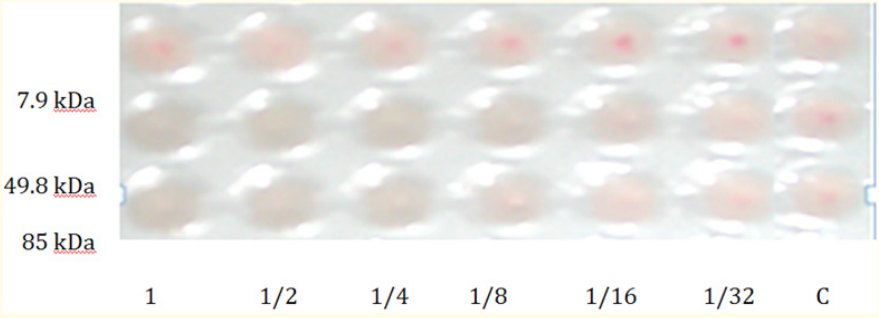


Figure 3: Results of tests HA 7.9, 49.8 and 85 kDa sub unit pili S. flexneri proteins.
By using electro elution method it will get the protein subunit of pili S flexneri MW 49.8 kDa that is ready to be used purely as an antigen for experimental studies in stage.

The results of show Figure 4A The brown color of the cells means positif result due to the attachment of protein and perfect anti body. Different circumstances can be seen in Figure 4C looks cells with blue color is caused not hem agglutinin protein adhesion to enterocytes. To determine the ability of prevention of discharge water towards the lumen of the intestine organ which has been immunized mice by adhesion molecule protein subunits of *S. flexneri* did MLIL method.

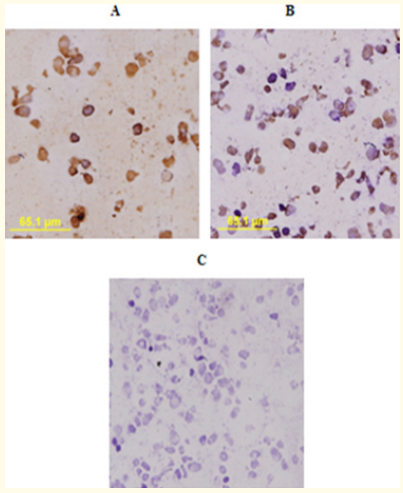


Figure 4: Immune cyto chemistry hem agglutinin subunit pili 48.9 kDa *S. flexneri* protein with various doses of dilution.
1/1000 dilution. B. 1/4000 dilution. C. 1/16000 dilution

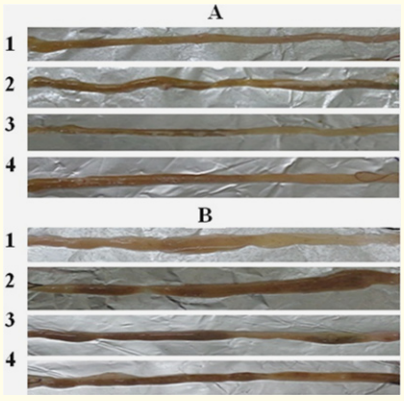


Figure 5: The results of protectively intestine exposed with *S. flexneri* with various immunization used MLIL model
A. MLIL before exposed with *S. flexneri*
B. MLIL after exposed with *S. flexneri*

1. MLIL control
2. MLIL immunized with protein SHM
3. MLIL immunized with protein 48.9 kDa+CTB
4. MLIL combine immunized protein 48.9 kDa+CTB+SHM

Mean weight difference MLIL due various treatments can be seen in the following TABLE.
Table the results of weighing intestinal tied in four groups.

No	Group	Mean ± SE	Siq
1	Control	192,60 ± 16,207	a
2	SHM	137,60 ± 16,360	ab
3	CTB + Sub unit pili protein 7.9 kDA	57,80 ± 11,382	c
4	CTB + Sub unit pili protein 7.9 kDa + HM	21,20 ± 2,035	d

The same letter in the column Siq show a significant difference, where as different letters indicate statistically significant differences ($p \leq 0.05$).

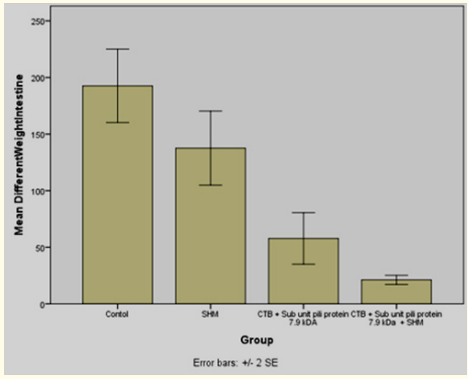


Figure 6: The relationship between the four treatment groups weight difference MLIL before being exposed with after being exposed to *S. flexneri*.

Figure 4,5 and Table show significant differences between not immunized group with immunized pili subunits of 49.8 kDa *S. flexneri* protein combined with CTB group and the immunized pili subunits of 49.8 kDa protein *S. flexneri* combined with CTB + SHM group.

The same result found between SHM group with immunized pili subunits 49.8 kDa protein *S. flexneri* combined with CTB group and with immunized pili subunits 49.8 kDa protein *S. flexneri* combined with CTB+SHM group.

Discussion

The role of pili bacteria are, transfer of material gene mediated by transduction, conjugation or transduction, twitching motility and as an adhesion molecule which have been bound to the eukaryotic cell [20-23]. Bacterial pili consists of proteins that make up the composition of the polymer [24]. Figure 1 B show 3 bands pili Shigella protein which have MW 85 kDa, 49.8 kDa and 7.9 kDa. After doing the hem agglutination assay all protein bands have hem agglutination activity. The same hem agglutination titer (1/32) found in protein bands 85 and 49.8 kDa while titer (1/16) in protein band with MW 7.9 kDa. Setyorini found that pili 49,8 kDa *S. dysenteriae* can protect against bacterial colonization and colonic epithelial cells damage in Balb/C. Referring to the results of these studies, the sub-unit further pili 49.8 kDa *S. flexneri* protein used in this study [12].

Pili other than as an adhesion molecule bacteria also can be used to inject Ipad proteins. It is called needle complex (NC) or T3SS apparatus (T3SSA) [25,26]. These proteins will lead to lysis the cell membrane which is accompanied by the spread, causing diarrhea [27,28]. Figure 1 C and D are the result pili sheared of *S. flexneri*. Figure 1 D shows with have appearance difference there any morphology cake donuts look like. We think that the picture express some a part of a cross-sectional illustration of needle complex.

Recently Mitra, have found that hem agglutination activity is directly correlated with colonization ability of *Shigella spp* in the suckling mouse model [29]. Hem agglutinin is a virulence factor of *Shigella spp*. Hem agglutinin molecule can serve as adhesion molecule [5,30]. Hem agglutination reaction can be as a screening tool to prove that proteins are adhesion molecules. These results have found that sub unit pili 48.9 kDa *S. flexneri* protein have been confirmed as hem agglutinin protein (Figure 3 and Figure 4). The isolated adhesins or adhesin analogs will bind to the eucaryotic cell surface [23]. Adhesion molecules that have been attached to the eukaryotic cell can be determined indirectly by using the immune cyto chemical method [5]. With reference to this study using immune cyto chemistry, the study found that the hem agglutinin sub unit pili 48.9 kDa protein S. flexneri can be demonstrated this protein is adhesion molecule (Figure 4).

Research results in Figure 5,6 and Table, show that MLIL method can be used in research diarrhea caused by enteric pathogens such as bacteria *S. flexneri*. The best results are found in the 3 groups, namely the combined pili subunits of *S. flexneri* 49.8 kDa protein combined with CTB + SHM. Our results do not differ from the study by Faisal, 2010 who studied the SHM as adjuvant adhesion molecules with MW 36.8 kDa pili *V. cholerae* protein [9].

Two kinds of in vivo methods which often used to examine the diarrhea caused by bacteria there are Rabbit Ilea Loop (RIL) test and suckling mouse assay (SMA) [31,32]. For example to show the accumulation of fluid in the lumen of the intestine and damage of enterocyte if exposure with *V. cholerae* can be shown by (RIL) model [33]. Subsequently Dean use the SMA method for application in a study of diarrhea in children which caused by *Escherichia coli enterotoxin* [32]. After that refer to the previous our study we apply MLIL ex vivo model. This model can also to examine, damage of enterocyte, bacterial colonization of *S. dysenteriae*, the accumulation of fluid and s-IgA in the lumen of the intestine [9,12].

Figure 6 show that SHM can protect movement solution from intestine toward lumen intestine. The difference between the control group and SHM group is found no significant. The best results are found in the group of pili subunits of 49.8 kDa protein *S. flexneri* combined with CTB + SHM group. Ganglion monoside (GM1) on the surface of intestine is the receptor for binding cholera toxin sub unit B (CTB) [34]. CTB can attaches on surface of enterocyte and carries pili subunits of 49.8 kDa *S . flexneri* protein and subsequently enhanced the immune response to produce s-IgA. Our previous study show the same result by using pili subunits of 37.8 kDa protein *V. cholera* [9].

Horse milk as an immune stimulant containing at least has two important components. These components are lactoferrin and probiotics. Lactoferrin has a bactericidal effect and an iron-binding. Mean while probiotic substances can increase the production of s-IgA or IgG and can be exploited on the specific antimicrobial activity against a variety of Gram-positive bacteria, Gram-negative bacteria, fungi, yeast, viruses and parasites [8].

The conclusion of this study is the SHM can enhance of mucosal immunity of the intestine to protect the discharge of moving solution to ward lumen intestine mice. Further research is needed to determine which parts are contained in SHM that can help improve the mucosal immune response. This research has been approved by the Ethical Commitee of Medical Faculty University Brawijaya Malang Indonesia and the authors declare that there is no conflict of interest regarding the publication of this article.

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