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Natural Resistance Associated Macrophage Protein-1 Expression in *Salmonella typhi* Infection with Acute Recurrence State of Typhoid Fever in Endemic Areas, Indonesia

Ade Rifka Junita[1], Firdaus Hamid[1], Rosdiana Natzir[2], Rosana Agus[3], Burhanuddin Bahar[4] and Mochammad Hatta[1*]

[1]Molecular Biology and Immunology Laboratory for Infectious Diseases, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.
[2]Department of Biochemistry, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.
[3]Department of Biology, Faculty of Sciences, Hasanuddin University, Makassar, Indonesia.
[4]Department of Biostatistic, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

Authors' contributions

This work was carried out in collaboration among all authors. Authors ARJ, FH, RA and MH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ARJ, BB and MH managed the analyses of the study. Authors ARJ and FH managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aim: The molecular pathogenesis of typhoid fever is complex and the host susceptibility mechanisms such as Natural Resistance Associated Macrophages Protein-1 (NRAMP-1) are poorly understood. This study explores the expression of NRAMP-1 in the serum of Acute Recurrence State (ARS) of typhoid fever compared with typhoid fever patients and healthy persons.

**Corresponding author: E-mail: hattaram@yahoo.com;*

Methods: Thirty of ARS of typhoid fever and 30 typhoid fever patients were collect from several Primary Health Care and Hospitals in the endemic area. Diagnosis of typhoid fever based on clinical symptoms and confirmed by blood culture. Healthy persons were collect from the Blood Transfusion Unit, Makassar, Indonesia. The expression of NRAMP-1 was determined using Enzyme-linked immunosorbent assay (ELISA).
Results: The mean of NRAMP-1 expression in 30 ARS of typhoid fever and 30 typhoid fever were found 10.941,56 pg/mL and 11.027,65 pg/mL, respectively. However, the mean of NRAMP-1 expression in healthy persons was found 21.103,91 pg/mL.
Conclusion: No different the expression of NRAMP-1 in ARS of typhoid fever compared with typhoid fever patients. However, expression of NRAMP-1 in both ARS of typhoid fever and typhoid fever showed significantly lower than healthy persons. Future study is needed to explore the other molecular factors involved to become ARS.

Keywords: Acute recurrence; Salmonella typhi infection; NRAMP-1; ELISA; Endemic areas; Indonesia.

ABBREVIATIONS

NRAMP-1 : Natural Resistance Associated Macrophages Protein 1
IL-6 : Interleukin 6
TNF-α : Tumor Necrosis Factor Alpha
VDR : Vitamin D Receptor
PARK2 : Parkin 2

1. INTRODUCTION

Typhoid fever is a systemic infection in human that is caused by Salmonella enterica subsp. enterica serovar Typhi (*S. Typhi*) [1]. It is a febrile, systemic illness commonly affected human in areas where sanitation is poor [2]. *S. typhi* is responsible for 21 million infections and cause death for approximately 161,000 peoples worldwide annually [3].

In Sulawesi, Indonesia, typhoid fever is one of the most frequent infectious diseases [4]. Three to five percent of enteric fever become chronic carriers and harbour *S. typhi* throughout their lifetime [5].

Pathogenesis of typhoid fever is complex and host response is poorly understood [6,7]. When *S. typhi* infects there will be a body reaction which acts as a response that will affect the multiplication of bacteria. Furthermore, the macrophage cells have been activated and hyperactive, so when Salmonella phagocytosis occurs the release of several inflammatory mediators will then cause symptoms of systemic inflammatory reactions [8,9]. The time required for *S. typhi* bacteria in the bacteremia period to multiply between 10 - 14 days from the incubation period and *Salmonella typhi* will release lipopolysaccharide endotoxins in the local inflammatory process that attaches to capillary endothelial cell receptors [10,11]. Several study related with immune response in typhoid fever like the role of IL6, TNF alpha in inhibit of *S. typhi* in vitro dan In some cases salmonella typhi infection may affect TLR4 expression in vivo [12,13,14].

Macrophage is an integral cell that fights intracellular pathogenic microorganisms and NRAMP-1 has a significant role in the initial phase of the interaction between macrophages and pathogens. In addition, the NRAMP-1 gene functions as a metal ion transporter that regulates cellular levels which may limit intracellular pathogen replication by changing the phagolisosomal environment [15,16,17]. Recently have been reported the association of NRAMP-1 in several infectious disease such as typhoid fever, tuberculosis, leprosy, buruli ulcer [18,19,20].

There is an urgent need for adequate and efficient detection methods for the establishment of acute recurrence state of typhoid fever, perhaps based on host susceptibility of NRAMP-1 gene [18]. Due to the important role of NRAMP1 in immune response against *S. typhi* infection and the high incidence of recurrent acute in typhoid fever, research related to host susceptibility is needed. This study examined the

NRAMP-1 protein in acute recurrence and typhoid fever patients of typhoid fever and compared it with healthy people.

2. MATERIALS AND METHODS

2.1 Serum Sample of Acute Recurrence State, Typhoid Fever and Healthy Persons

Thirty serum of Acute Recurrence State of typhoid fever and 30 typhoid fever patients were select randomized from 582 typhoid fever from 2009 to 2018. In addition, 30 serum of healthy persons were collected from the Blood Transfusion Unit, Makassar, Indonesia.

2.2 Blood Culture for Identification Typhoid Fever

Diagnosis is based on clinical symptoms and confirmed by laboratory tests (blood culture, biochemical tests). The acute recurrence state was selected based on anamnesis and confirmed by medical records and positive blood cultures.

Blood is drawn aseptically by 8 ml and inserted into the bottle containing the transport medium Ox Bile Broth 9 ml (Bactec®, USA), mixed slowly until homogeneous, then incubated for 4 × 24 hours at 37°C. Furthermore, the inoculum is taken as 1 ml of inoculation in Petri dishes containing medium SSA that has been solidified, and then incubated for 18-24 hours at a temperature of 37°C. Colonies suspected of Salmonella typhi bacteria by colony growth characteristics (black colonies) Then for certainty, it was re-inoculated on the TSIA medium and the IMViC test was carried out and the sugar test was then incubated for 18-24 hours at 37°C. If Salmonella typhi is positive in culture, black colonies appear on the TSIA medium [21,22,23,24].

2.3 Widal Test

Using a quantitative method according to Tydal kit protocol, India. Take an appropriate number of sets as required; one set for each antigen suspension of the plate well and label them 1 to 8. Pipette into plate well no. 1 of all sets 1.9 ml of physiological saline and each of the remaining plate wells (2 to 8) add 1 ml of physiological saline. To well no. 1 of all sets add 0.1 ml of a serum sample to be tested and mix well and transfer 1 ml of the diluted serum sample from a plate well no 1 to plate well no. 2 and mix well. Also, transfer 1 ml of the diluted serum sample from a plate well no. 2 to plate well no. 3 and mix well. Continue this serial dilution till plate well no. 7 in each set. Furthermore, discard 1.0 ml of the diluted serum from plate well no. 7 of each set. The next step to diluted the serum sample achieved from a plate well no. 1 to 7 respectively in each set is as follows: 1:20, 1:40, 1:80, 1:160, 1: 320, 1:640, 1: 1280. Plate well no. 8 in all the sets, serves as a saline control. To all the plate wells (1 to 8) of each set add one drop of the respective well-mixed TYDAL antigen suspensions from the reagent vials and mix well and cover and incubate at 37°C overnight (approximately 18 hours). The result was observe the agglutination of each plate well [25,26,27,28].

2.4 ELISA for Detect NRAMP-1 Protein in Serum

The sandwich ELISA method were established to protein NRAMP-1 according to protocol kit of Human SLC11A1 / NRAMP-1 ELISA Kit; Sandwich ELISA, Catalog: No. Cat. No: CSB-E17597h (Cusabio Inc, USA). Serum obtained from the required sample bank is removed from the freezer -80°C and stored in ice before use. Each sample was duplicated to ensure the validity of the ELISA results. The first step was to add 100 µL of Diluent assay containing protein buffer into each well. Furthermore, 100 µL of standard fluid was added containing the recombinant human protein NRAMP-1 target from the determined KIT or dilution of the sample from the patient's serum into each well / well. Then the incubation was carried out for 2 hours at room temperature. Suction the liquid in each well / well and wash it with sterile PBS. This washing process is carried out 4 times in succession. Then 200 µL of Conjugate liquid containing streptavidin HRP was added into each well and covered with a plastic cover and incubated for 2 hours at room temperature. The liquid is sucked and then re-washed 4 times using sterile PBS. The next process was added 200 µL Substrate Solution containing TMB into each well / well and read using ELISA Reader 270 (Biomerieux, France).

2.5 Statistical Analysis

All statistical calculations were performed using the computer program EPI Info Version 6.0. The statistical data between 2 groups were tested by

student t test using SPSS Computer software. Considered significant when p value ≤0.05.

3. RESULTS

3.1 Characteristic of Acute Recurrence State and Typhoid Fever Patients

In Table 1, it can be seen that the total sample size of 30 Acute Recurrence State (ARS) and 30 typhoid fever were recorded from the several Primary Health Care and the Hospital in Eastern part of Indonesia. A random sample of 582 typhoid fever was taken to determine the NRAMP-1 protein from serum. Patient temperature from 30 each sample when entering the first examination on ARS and typhoid fever was 38.3°C and 38.3°C. The male and female sex ratios for ARS and typhoid fever were 13/17 and 15/15. The mean age of the ARS and typhoid fever patients were 21 and 26 years, respectively, and the duration of fever was found in the ARS and typhoid fever patients 5.83 and 6.33 days, respectively. From 30 samples found the average time of ARS after 21.13 days of experiencing the first typhoid fever. Clinical symptoms of apathy were found in 7 patients (23.3%) on ARS and 4 patients (13.3%) on typhoid fever. Meanwhile, the clinical symptoms of stupor were found more in typhoid fever (4 patients/ 13.3%) than in ARS (2 patients/ 6.6%). Clinical symptoms of nausea were found in 5 (16.7%) in ARS and 4 (13.3%) in typhoid fever. In ARS, there were 3 patients (10.0%) of constipation and 2 patients (6.6%) with typhoid fever. The clinical manifestations of ARS and typhoid fever, in general, were not significantly different; except that high Widal titers (1/640) were seen to be more common in ARS (6 patients/ 20.0%) compared to typhoid fever (4 patients/ 13.3%).

3.2 Comparison of the Results of NRAMP-1 Protein Levels in ARDT, DT and Healthy Persons

Table 2 shows the results of the NRAMP-1 protein levels in ARS and typhoid fever. Also, 30 serum NRAMP-1 protein levels were examined as a negative control (comparison) which were taken randomly from the sample bank of the Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia; where this serum sample was collected from the Blood Transfusion Unit, Makassar, Indonesia. Serum protein levels for patients with ARS, typhoid fever, and healthy persons were determined using the Enzyme-linked Immunosorbent Assay (ELISA) technique in units of pg/mL. The mean of ARS samples found that NRAMP-1 protein levels were 10,941.56 pg/mL; with a minimum value of 2,326.34 pg/mL and a maximum value of 15,522.19 pg/mL. Meanwhile, the mean of typhoid fever samples found that NRAMP-1 protein levels were 11,027.67 pg/mL; with a minimum value of 2,711.85 pg/mL and a maximum value of 16,757.88 pg/mL. It was found that the value of NRAMP-1 protein levels in ARS was lower than the NRAMP-1 protein levels in typhoid fever. The difference in the value of NRAMP-1 protein was not significant between ARS patients and typhoid fever patient ($p = 0.940$). As a negative control or comparison, 30 samples of healthy persons were used, taken from UTD, Makassar. Indonesia. Samples were taken randomly from serum samples in the sample bank at the Laboratory of Molecular Biology and Immunology, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

NRAMP-1 protein levels in healthy persons were found to be 21,103.91 pg/mL; with a minimum value of 11,379.26 pg/mL and a maximum value of 24,348.62 pg/mL. When compared the value of NRAMP-1 protein levels between ARS patients and healthy persons found statistically significant differences ($p = 0.00$); Likewise, a significant difference between typhoid fever and healthy persons samples was also found ($p = 0.00$).

In Fig. 1, showed that the box plot of the NRAMP-1 expression between ARS, typhoid fever and healthy persons, where the ARS patients are lower than the typhoid fever patients. Furthermore, the box plot value of NRAMP-1 protein levels was seen both in the ARs and typhoid fever patients lower than the healthy persons.

From the box plot, it can be concluded that the expression of NRAMP-1 in the ARS patient was lower than that of typhoid fever patient. Furthermore, the value of protein levels in both ARS and typhoid fever was lower than that of healthy people.

Table 1. Clinical manifestation and widal titer in acute recurrence and typhoid fever

Clinical signs		State of illness	
		Acute Recurrence (n=30)	Typhoid fever (n=30)
Temperature (mean ± SD)		38.3 ± 0.44	38,3 ± 0.42
Sex (M/F)		13 / 17	15 / 15
Age (mean ± SD) yr		21.00 ± 9.70	26.03 ± 9.51
Duration illness (mean days ± SD)		5.83 ± 1.17	6.33 ± 1.09
Time recurrence (mean days ± SD)		20.13 ± 10.47	ND
Coated tongue		29 (96.6)	29 (96.6)
Malaise		30 (100.0)	30 (100.0)
Headache		30 (100.0)	30 (100.0)
Apathy		7 (23.3)	4 (13.3)
Stupor		2 (6.6)	4 (13.3)
Nausea		5 (16.7)	4 (13.3)
Constipation		3 (10.0)	2 (6.6)
Abdominal distention		2 (6.6)	2 (6.6)
Diarrhea		2 (6.6)	1 (3.3)
Melena		1 (3.3)	1 (3.3)
Perforation		1 (3.3)	1 (3.3)
Widal titer	1/320	24 (80.0)	26 (86.7)
	1/640	6 (20.0)	4 (13.3)

Table 2. Comparison of NRAMP 1 expression among acute recurrence, typhoid fever and healthy persons

Group	NRAMP1 level Mean (min-max + SE)	Sig.
Acute recurrence	10.941,56 (2.326,34 - 15.522,19) 756,28	0 ,940
Typhoid fever	11.027,67 (2.711,85 - 1.6757,88) 852,44	0,000
Healthy persons	21.103,91 (1.8379,26 - 2.4348,62) 352,05	0,000

4. DISCUSSION

Natural Resistance-Associated Macrophage Protein 1 (NRAMP-1), which is encoded by the NRAMP-1 gene located on chromosome 2q35, encodes a divalent cation transporter protein which is involved in the control of intraphagosomal microorganism replication and macrophage activation [29]. Including the divalent cations Fe^{2+}, the which is a complement factor for various enzymes, are essential for bacterial multiplication. On the other hand, Fe^{2+} is needed to produce hydroxyl radicals which will be reacted with nitric oxide (NO) to become biocidal peroxynitrite. Therefore control of divalent transport by NRAMP-1 can play an important role in the biocidal effects of macrophages [30].

In previous studies, we have been reported on the relationship between genes NRAMP-1 and mycobacteriosis. Although several reports concluded that NRAMP-1 polymorphisms were associated with susceptibility to leprosy in Vietnam [31,32]. Other reports in Japan suggested that gene polymorphisms may have an influence on bacillary growth and development of pulmonary tuberculosis, but it is not susceptible to Mycobacterium tuberculosis [19,33]. Furthermore, it has been reported that

NRAMP-1 gene polymorphisms are associated with susceptibility to other diseases, such as rheumatoid arthritis, sarcoidosis and type I diabetes [34,35,36].

In this study showed that the protein NRAMP-1 was expressed in patients ARS and typhoid fever were significantly lower compared to healthy persons. This is consistent with the function of NRAMP-1 as activated the macrophage cells in the process of eliminating microorganisms including *S. typhi* infection. Previous study revealed that other host susceptibility gene such as Vitamin D Receptor (VDR) could inhibit the growth of *Salmonella typhi in vivo*. [37].

In this study showed that of the 30 samples, it was found that the mean time of ARS occurred after 21.13 days of experiencing the first typhoid fever. Some study revealed that 5-10% of patients treated with antibiotics experience recurrence of typhoid fever or ARS after initial recovery. Recurrence usually occurs about 1 week after treatment is stopped, but can also recur after 70 days [38,39,40]. The ARS study in Pakistan showed that 14% of children with typhoid fever were treated with antibiotics for 7 days [41] and all these children were diagnosed as culture-confirmed ARS within 4 weeks of discontinuation of therapy. Meanwhile, there is no definite incidence of ARS in Indonesia and this is what makes it difficult to diagnose ARS itself.

The study provides an overview of the role of NRAMP-1 in the incidence of acute recurrence state of typhoid fever and further research is still needed on the role of other host susceptibility genes such as Vitamin D Receptor (VDR), Nucleotide-binding Oligomerization Domain-protein 2 (NOD2) and Parkinson 2 (Park2) in ARS.

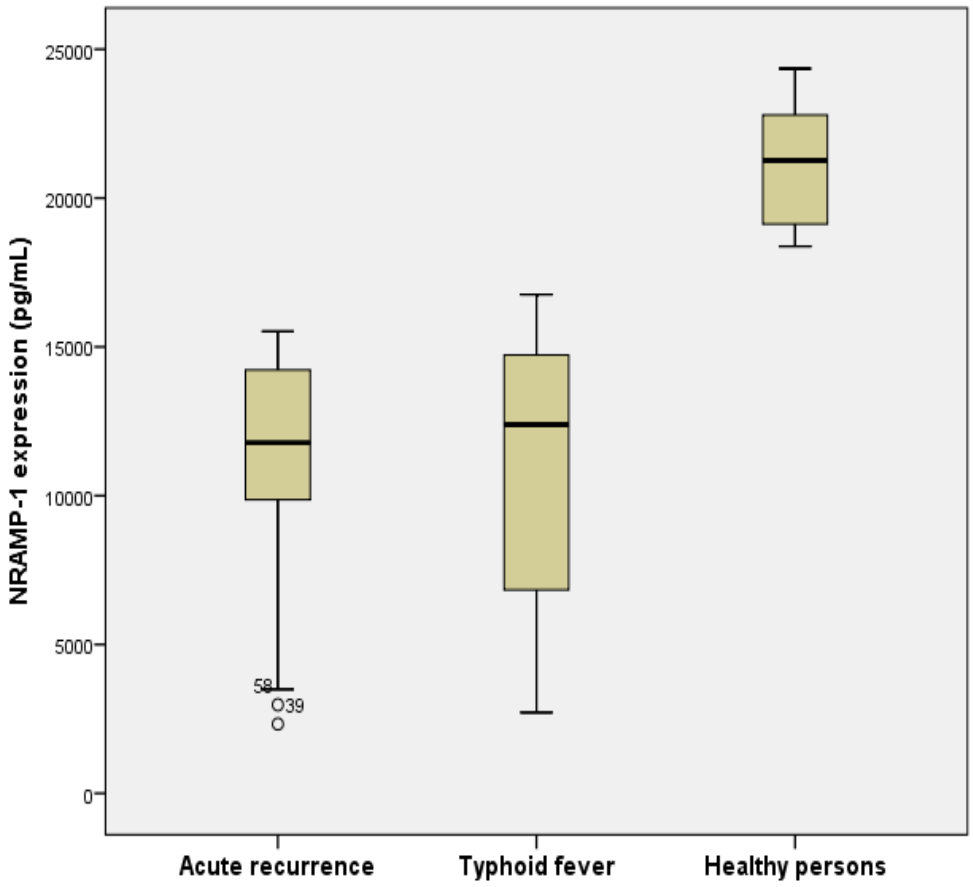

Fig. 1. NRAMP-1 expression in acute recurrence, typhoid fever and healthy persons

5. CONCLUSIONS

The expression of NRAMP-1 in ARS was lower than that of typhoid fever, but the difference was not statistically significant. In contrast, the difference NRAMP-1 expression in both acute recurrence state and typhoid fever statistically significant lower compared to healthy persons.

Low levels of NRAMP-1 protein in both acute recurrence state and typhoid fever may cause decreased NRAMP-1 function in macrophage activity to eliminate and inhibit the multiplication of *S. typhi* compared to healthy persons.

It is necessary to study a more comprehensive study in typhoid fever endemic areas with different ethnicities as a comparison of the expression of NRAMP-1 and the determination of other host susceptibility genes such as VDR, NOD2 and PARK2 to determine whether they play a role in the incidence of ARS.

ETHIC APPROVAL AND CONSENT

This study was approved by the ethical boards of the participating institutes and informed consent was obtained from all participants or from their parents/guardians.

This study was approved by the Faculty of Medicine, Hasanuddin University Makassar, Indonesia, with registration number No: 613/UN4.6.4.5.31/PP36/2020. Date: 30 September 2020.

HUMAN RIGHTS

All this study design and implementation were in accordance with the Health Medical Research Ethics Committee at the Faculty of Medicine, Hasanuddin University (Makassar, Indonesia) as the local guidelines for the care and use of patient collection samples.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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