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# The Analysis of Vitamin D Receptor Protein on *Salmonella typhi* infection in acute recurrent cases in endemic area in Eastern Indonesia

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**ABSTRACT**

The host susceptibility mechanisms such as Vitamin D Receptor (VDR) is involved in the modulation of macrophage function and may possibly correlate with immunity disease including the severity of typhoid fever symptoms. The study aimed to assess the VDR Protein expression in the serums of recurrent acute typhoid fever (RATF) patients and compares it with typhoid fever (TF) patients, and healthy persons (HP). The study employed 30 RATF patients and 30 TF patients selected from primary health centres and hospitals in Eastern Indonesia as the endemic area. All the samples were obtained from several health centers in South Sulawesi, Southeast Sulawesi, Central Sulawesi, East Kalimantan and Papua and then collected in the sample bank Biology Laboratory of Molecular Immunology, Faculty of Medicine, Hasanuddin University. As a comparison, 30 samples of healthy persons were also selected from the Blood Transfusion Unit in Makassar, South Sulwesi Indonesia. The profile of VDR Protein was analyzed with *Enzyme-linked Immunosorbent Assay* (ELISA). VDR protein content data on RATF and TF were designed according to completely randomized design T test. Subsequently, it correlated to Pearson correlation to determine the interaction between Widal titre and VDR protein levels. A comparison between Widal titre and VDR Protein level was also made to identify the correlation. It was found that the mean of VDR protein expression of RATF was 13,44 ng/mL, the mean of VDR protein expression of TF was 24,88 ng/mL, and the mean of VDR protein expression of HP was 43,49 ng/mL. The correlation results between RATF-TF Widal titre and VDR protein level indicated a negative correlation with p-value of 0,004. There were significant differences in the VDR expression in the RATF, TF, and HP. RATF VDR expression lower than TF and HP and there was also a correlation between Widal titre with VDR Protein expression.

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## INTRODUCTION

Typhoid fever is an acute disease of the gastrointestinal tract caused by *Salmonella typhi*, with symptoms of headache, dry cough, sore throat, hepatomegaly, splenomegaly, and bradycardia (Gillespie, 2014; Hamer, 2010). Typhoid fever remains a health issue over the world especially in developing countries including Indonesia (Hatta and Smits, 2007a).

Typhoid fever is an endemic infectious disease in Indonesia and rated as the fourth most frequent infectious disease in a number of Indonesian regions. In addition, typhoid fever is also a major cause of septicemia obtained from the public, with reported incidence rates exceeding 250/100,000 population (Hatta et al., 2009; Hatta and Ratnawati, 2008) In the endemic areas, approximately 1-4% of infected people become chronic asymptomatic carriers which may threaten the local community (Di Domenico et al., 2017) (Sutiono et al., 2010) (Buckle et al., 2012; Im et al., 2020).

Macrophage is one of the *Salmonella* lipopolysaccharide (LPS) that stimulates endotoxin to produce lymphokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF), which serves to decrease the number of organisms required to initiate infection by preventing phagocytosis (Di Domenico et al., 2017; Gillespie, 2014).

The immune system also affects the carrier and recurrent cases especially that correlates with the macrophage cell activity as the phagocytosis system (Esmailnia et al., 2020),(Dougan and Baker, 2014; Hatta, 2011),(Dwiyanti et al., 2015). Macrophage function affects the severity development of the fever (Dwiyanti et al., 2017),(Junita et al., 2020).

Vitamin D binds or VDR as the autophagy regulatory effect and a variety of immune cells including the macrophage in reducing the proinflammation cytokine expression (Efendi et al., 2019),(Kongsbak et al., 2013). When the body is infected especially if caused by pathogens, the body will perform the immune response by transforming inactive Vitamin D (25-hydroxy vitamin $D_3$) to active Vitamin D (1.25-dihydroxy vitamin $D_3$) and the increasing concentration of 25-dihydroxy vitamin $D_3$ within the cellular will activate VDR gene. The interaction between 1,25-dihydroxy vitamin $D_3$ with VDR will lead to an increase in LL-37 cellular which encodes intracellular pathogen eliminating protein by producing *antimicrobial peptide*(CAMP) and β-defensin-4 (DEFB4) and anti-microbial activity will be completed by the phagocyte (MPKB, 2012; Zasloff, 2006).

The state of acute and recurrent typhoid fever infection, there is a decrease in the function of the VDR in the process of eliminating microorganisms. It is indicated that if there are repeated infections, the VDR level also decreases compared to the first *S. typhi* infection. When VDR levels are low, it can be used as a diagnostic determination of the incidence recurrent acute typhoid fever (RATF).

Due to the important roles of VDR in the immune system including in the anti-microbial process, it is necessary to perform accurate detection methods in the case of recurrent typhoid fever depending on the host susceptibility of VDR genes. This study focuses on identifying the VDR protein level in patients with recurrent acute typhoid fever (RATF) and typhoid fever (TF). This study is a cross-sectional study that identifies the VDR level in the serums of RATF patients and TF patients.

## MATERIALS AND METHODS

### Sampling and Sample Criteria

The samples of the study were serums which were randomly collected from 30 RATF patients and 30 TF patients. The Diagnosis is based on clinical symptoms and confirmed by laboratory tests (blood culture and biochemical tests).

All employed samples were obtained from several health centers in Eastern Indonesia such

as South Sulawesi, Southeast Sulawesi, Central Sulawesi, East Kalimantan and Papua and then collected in the sample bank Biology Laboratory of Molecular Immunology, Faculty of Medicine, Hasanuddin University. As a negative control, 30 samples of healthy persons were randomly selected from the sample bank of Blood Transfusion Unit of Makassar, South Sulawesi, Indonesia.

**Research Procedure**

*Identification and Culture of S. typhi*

Venous blood was sampled as much as 9 cc. And then 1 cc of the sample was injected into micro cups as and 8 cc into Transport Botle containing 9 ml Ox Bile Broth (Bactec®) medium. Micro cups filled with the samples were centrifuged to acquire serums for the serology Widal test. The sample contained in the Bactec bottle was incubated for 4 x 24 hours at a temperature of 37°C (Flayhart et al., 2007; McDonald et al., 1996, 2001).

*Sample Preparation*

There were 8 ml bloods collected aseptically and injected into transport bottle containing 9 ml Ox Bile Broth (Bactec®) medium and diluted gradually until it was homogenized. After that, the medium was incubated at a temperature of 37°C for 4 x 24 hours.. As much as 1 ml inoculum was taken from the inoculation into a petri dish filled with SSA medium and was incubated for 18-24 hours at a temperature of 37°C. *Salmonella typhi* colonies can be identified based on the colony growth characteristics (black colour). To further confirm this, Inoculation was repeated again on TSI agar medium for 18-24 hours at 37°C followed by IMViC and glucose fermentation tests. Positive indication of *Salmonella typhi* on the culture will show black bacterial colony on TSI agar medium (Hatta, 2011; Hatta et al., 2011; Hatta and Smits, 2007b).

*Widal test*

The test was performed by using agglutination slide tools. 4 slides of Widal test were prepared. In each slides circles were made and the slides were given the labels of H,O negative control (-) and positive control (+). With Pasteur pipette, undiluted serum was dropped at 20µL on both first circles. On the third and fourth circles, drop the positive control serum (+) and negative control serum (-). *Salmonella enterica serotype typhi* antigen H was dropped on the first circle and the antigen O was dropped on the second circle. Antigen H was dropped on the third and fourth circle. After that, the mixture of serum and antigen was obtained. The serum and antigen were diluted with a separate applicator stick equally until they filled the overall surface of the circle. The slide was rocked for one minute using a Rotator and the presence of agglutination was observed. Widal Titre was 1:80 or at the dilution of 20 µL serum and one drop of antigen, agglutination occurred. If no visible agglutination was observed, dilution was done with a mixture of 10 µL serums and one drop of antigen. Titre was determined at the final agglutination. If agglutination was visible, the result will be considered as positive, while in the case of no visible agglutination, the result will be considered as negative (Nakamura-Uchiyama and Ohnishi, 2010; Olsen et al., 2004).

*The Method of VDR Elisa Kit*

The acquired serum obtained from the sample bank was taken from the freezer at -20°C and stored in the ice before use. Each sample was tested in duplicate to ensure the validity of ELISA results. At the first stage, 50 µL PBS (0.02mol/L pH 7.0-7.2) was added in each empty well. 50 µL balance solution was added to each sample of the supernatant and diluted. 50 µL Standard solution containing the determined amount of target recombinant VDR KIT or sample dilution of patient's serums were added into each well. 100 µL conjugated HRP was added to each well except the empty well. After that, it was covered with plastic and incubated for 1 hour at 37°C. The solution in each well is sucked and washed. The washing process was repeated 5 times successively by adding 350 µL washing

solution using a sprayer, multichannel pipette, dispenser, or automatic washer. In each wash, the well plate was left for 10 seconds before fully sucked. After the last wash, the remaining washing solution was sucked. The plate was flipped over and tapped on a clean absorbent paper. 50 µL of Substrate A and B was added to each well including the empty well and incubated at 37°C in a dark room for 15-20 minutes. 50 µL of Stop solution was added into each well and was measured with ELISA Reader 270 (Biomerieux, France)

*Statistical Analysis*

The data analysis of VDR protein level on RATF and TF was made in univariate statistics including the detail and the differences of VDR level on RATF and TF cases using Completely Randomized Design with Student T-Test and level of significance $p$ <0,05. Pearson Correlation was applied to identify the correlation between Widal titre and VDR protein level through SPSS Software.

## RESULTS AND DISCUSSION

### Widal test results on Recurrent Acute Typhoid Fever (RATF) and Typhoid Fever (TF)

The total number of RATF samples was 30 patients and TF patients were 30. The samples were collected randomly and recorded from 582 typhoid fever patients at the primary health centres and hospitals. Widal titre results on RATF patients (Table 1) showed that there were 24 RATF patients from the 1/320 Widal titre results and 6 RATF patients from the 1/640 Widal titre results. There were 26 TF patients from the 1/320 Widal titre results and 4 TF patients from the 1/640 Widal titre results.

**Table 1**. Widal Titre on RATF and TF

| Widal Titre | RATF (n=30) | TF (n=30) |
|---|---|---|
| 1/320 | 24 | 26 |
| 1/640 | 6 | 4 |

### The result of VDR Protein serum level on the patients of Recurrent Acute Typhoid Fever (RATF), Typhoid Fever (TF) and Healthy Individuals (HI)

Table 2 indicated the mean of RATF VDR protein level was 13,44 ng/mL; with a standard deviation of 3,99 ng/mL. Meanwhile the mean of TF VDR Protein level was 24,88 ng/mL; with a standard deviation of 2,99 ng/mL. It was found that RATF VDR Protein Level was lower compared to TF VDR Protein Level and the comparison was statistically significant (P<0,001).

From figure 1, Box plots illustrate the VDR expression among RATF, TF, and HP with the result of VDR level in RATF patients was lower compared to TF patients and VDR level in TF patients was lower than healthy persons (HP). From the box plot, it can be concluded that the VDR expression in RATF patients was lower compared to the VDR expression in TF patients. In addition, the VDR expression in TF patients was lower than in healthy persons (HP).

In addition, negative control results taken from HP VDR Protein Level and the mean results of HP VDR Protein Level was 43,49 ng/mL; with a standard deviation of 4,40 ng/mL. the mean of TF VDR Protein Level was lower compared to HP VDR level with an indication of significant difference (P<0,001). Similarly, from the difference between RATF VDR Protein Level and HP VDR Protein Level, a significant difference was identified (P<0,001).

In addition, negative control results taken from HP VDR Protein Level and the mean results of HP VDR Protein Level was 43,49 ng/mL; with a standard deviation of 4,40 ng/mL. the mean of TF VDR Protein Level was lower compared to HP VDR level with an indication of significant difference (P<0,001). Similarly, from the difference between RATF VDR Protein Level and HP VDR Protein Level, a significant difference was identified (P<0,001).

From figure 1, Box plots illustrate the VDR expression among RATF, TF, and HP

with the result of VDR level in RATF patients was lower compared to TF patients and VDR level in TF patients was lower than healthy persons (HP). From the box plot, it can be concluded that the VDR expression in RATF patients was lower compared to the VDR expression in TF patients. In addition, the VDR expression in TF patients was lower than in healthy persons (HP).

**Table 2.** VDR Protein Level Comparison in RATF, TF and HP

| Group | VDR Protein Level Mean + SD (ng/mL) | Sig. |
|---|---|---|
| Recurrent Acute Typhoid Fever (RATF) | 13,44 ± 3,99 ng/mL | 0.001 |
| Typhoid Fever (TF) | 24,88 ± 2,99 ng/mL | 0.001 |
| Healthy Persons (HP) | 43,49 ± 4,40 ng/mL | 0.001 |

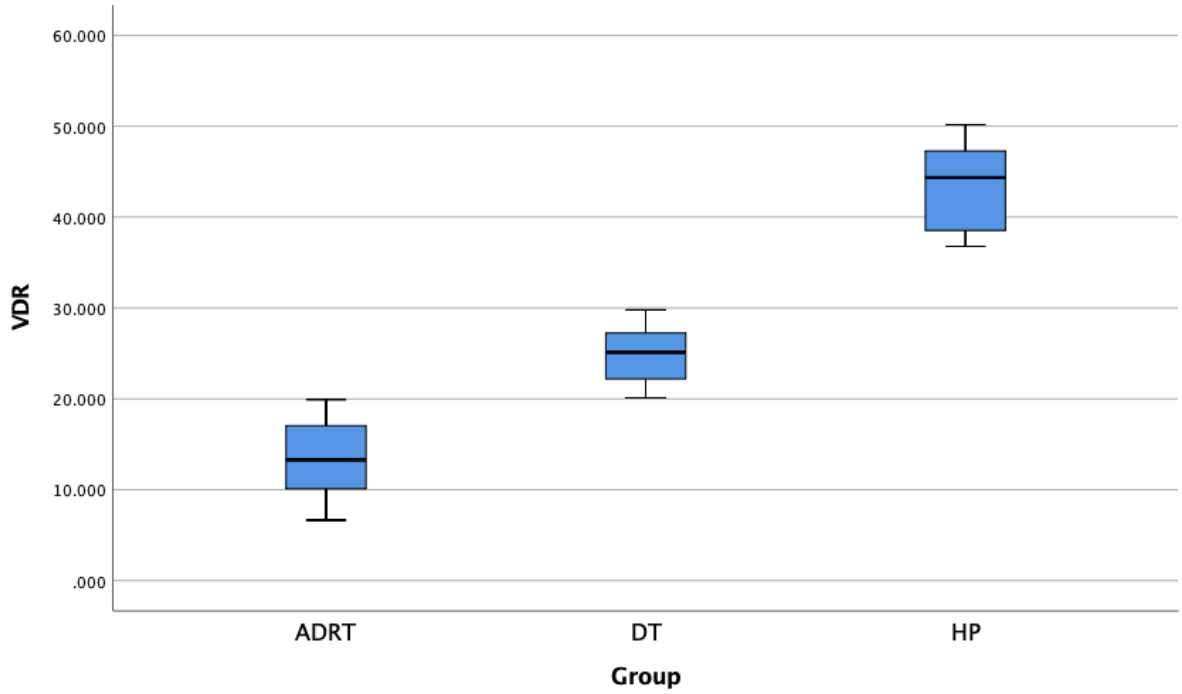


**Figure 1.** Box Plot of the VDR Protein Level in RATF, TF and HP

**Correlation of Widal Titre and VDR Protein Levels In RATF and TF**

To identify the correlation between the Widal titre and VDR Protein level, a pearson correlation test was applied (table 3). Based on the result of the study, a statistically significant negative correlation between Widal titre and VDR Protein level was identified with r correlation of -0,302 and *p value* of 0,004. This implied the existing correlation between Widal titre with VDR Protein level, the higher the value of Widal titre, the lower the VDR Protein level and vice versa.

**Table 3.** Correlation of Widal Titre and VDR Protein Levels In RATF and TF

| Variable | r-value | P value* |
|---|---|---|
| VDR (ng/mL) | -0.302 | < 0,004 |

*Data categorized as correlation coefficient (r-value) and p-value, p<0,05 considered as significant

## DISCUSSION

VDR Protein located in chromosome 12q12-g14 and has more than 479 *single nucleotide polymorphisms* (SNP). Some of them could modulate the *uptake* 1,24 $(OH)_2D$ has the autophagy regulatory effect and some immune cells including T cells, B cells, macrophage, dendritic cells, and epithelial cells(Kongsbak et al., 2013). VDR is distributed to the overall body cell although it is not universal such as brain, liver, pancreas, skin, lymphatic, immune cells, gonad and prsotate (Bikle, 2017; Wang et al., 2012). Immune cells that express VDR could respond to the circulation of active Vitamin D similarly to the classic vitamin D target cell such as intestine, kidney, and bone with three immune mechanism channels of 1,25-$(OH)_2$ D-VDR including intracrine, paracrine, and endocrine (Chun et al., 2014; Hewison, 2012).

There were studies performed to identify the correlation between host susceptibility genes and pathogenesis of various infectious diseases including the infection caused by microorganisms (Dwiyanti et al., 2017; Junita et al., 2020; Paschoal, 2014). In this study, it appears that the protein VDR, which is expressed in patients RATF and TF are significantly lower than the HP. This implied a present decrease in the VDR expression of RATF patients compared to TF patients and HP. Such indication was relevant to the function of VDR as an anti-microbial agent against *Salmonella typhi*. Previous studies confirmed the correlation between the expression of VDR genes and its relationship to the bacterial load of *Salmonella typhi* and VDR role in inhibiting their growth (Efendi et al., 2019)(Febriza et al., 2020).

Some studies stated that the polymorphism of the VDR gene affects a number of diseases such as tuberculosis, hepatitis B, HIV and leprosy (Paschoal, 2014). Another report indicated that polymorphisms of the VDR may be associated with the selected genes in the pathway of vitamin D channel in the cases of colon, breast, and prostate cancer (Çiçek et al., 2017; Glocke et al., 2013).

This study indicated a correlation between Widal titre and the VDR protein level. Widal test is used to detect agglutination antibody against the antigen O and H of *Salmonella typhi*. This test has a sensitivity of 70 – 80% and specificity of 80 – 95% (Sattar et al., 2014) but for such result can be false-positive because *S. enterica serotypes typhi* share antigens with other serotypes of *Salmonella* and share epitope that intersects with other *Enterobacteriaceae* (Paul and Bandyopadhyay, 2017). For better accuracy, sensitivity, and specificity, igG/igM ELISA test was more prioritized compared to Widal test (Retnosari and Tumbelaka, 2016).

The incidence of recurrent typhoid fever is related to high carrier cases after 1-year recovery and approximately 2-5% of typhoid fever patients failed to cleanly eliminate the *Salmonella typhi* which persistently live in the gallbladder (Ryan, 2014). A number of studies in Pakistan indicated the recurrent of typhoid fever in children below 15 treated with antibiotics with 18% incidence in children at the age of 2. Such

finding was confirmed with the culture examination within 4 weeks after the medication is stopped (Cohen, 2018; Hanif et al., 2021; Qamar et al., 2014),(Rasheed et al., 2019). However, research concerning the recurrent acute typhoid fever in Indonesia is still considered very little and consequently, this prevents the correct diagnosis for RATF cases. VDR, NOD2, NRAMP-1 are host susceptibility genes that affect typhoid fever and typhoid fever recurrence. Previous study also stated that it is known that there is a NOD2 gene polymorphism of 6.7% of suspected typhoid fever patient (Dwiyanti et al., 2017; Mukherjee et al., 2019). When the NOD2 protein level is lower, the risk of ARDT will be higher (Wahyuni et al., 2021). According to a study performed by Junita et al (2020), when compared to healthy people, low levels of NRAMP-1 protein in both acute recurrence and typhoid fever may cause reduced NRAMP-1 function in macrophage activity to eliminate and prevent the growth of *S. typhi*. This study provides insight into the role of VDR as one host susceptibility gene in the cases of RATF.

## CONCLUSION

There was a significant difference in VDR expression of RATF, TF, and HP. With the finding of lower VDR expression in RATF compared to TF and lower VDR expression in TF compared to HP, this study confirmed the decreasing expression of VDR protein in RATF patients compared to the TF patients and healthy persons. Therefore, low VDR level in the case of typhoid fever infection and acute recurrence may possibly reduce the VDR function on macrophages as an antimicrobial agent in inhibiting disease multiplication. It is necessary to do the same study in endemic areas of typhoid fever recurrence as a comparison of the expression of VDR and the incidence of typhoid fever recurrence.

## CONSENT AND ETHICS

This study was approved by the research ethic boards of the participating institutions and approval has been obtained from all participants or from their parents or their representatives. This study has been approved by the Commission on Health Research Ethics FK UNHAS No: 834/UN4.6.4.5.31/PP36/2020 dated on December 30, 2020.

## CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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