INA RESPOND
INDONESIA RESEARCH PARTNERSHIP ON INFECTIOUS DISEASE

INA-RESPOND Secretariat

Badan Litbangkes, Kemenkes RI,
Building 4, 5th Floor,
Jl. Percetakan Negara No. 29,
Jakarta, 10560.
Phone: +62 21 42879189
Email: INA.Secretariat@ina-respond.net
Website: www.ina-respond.net

Newsletter
May 2016

In This Issue

Typhus Fever (Rickettsial Diseases): Lab Detection of Rickettsiae

What is Typhus Fever? Is it the same as what we call *Tifus* or *Tipes* (Indonesian)? If you are not used to medical world, you will probably have mistaken Typhus with Typhoid fever or even feel confused what they mean altogether.

The disease is transmitted by the human body louse, which becomes infected by feeding on the blood of patients with acute typhus fever. Infected lice excrete rickettsia onto the skin while feeding on a second host, who becomes infected by rubbing louse faecal matter or crushed lice into the bite wound.

As reported by WHO, Typhus fever occurs in colder (i.e. mountainous) regions of central and eastern Africa, central and South America, and Asia. In recent years, most outbreaks have taken place in Burundi, Ethiopia and Rwanda. Typhus fever occurs in conditions of overcrowding and poor hygiene, such as in prisons and refugee camps.

Find out more about this disease in this edition.

Page 5

Sample… How important is it?

As researchers, you are likely to have had some experiences dealing with samples. How important is sample in research? Find out here on

Page 4

Save The Date

We have been working on a new network's website design. Our IT Specialist is working really hard, and hopefully, by the end of this month, the new design will be ready and we can enjoy a better, more sophisticated website.

Look out for the launch date!

May Birthday

2 May	dr. Dewi Murniati	Site PI Site 540
5 May	Ms. Ni Wayan Nilawati	Lab Tech Site 520
13 May	dr. Titik Nuryastuti	Co-PI TRIPOD Site 580
17 May	dr. Risna Halim Mubin	Co-PI INA101 Site 550
27 May	dr. Siswanto	Governing Board NIHRD

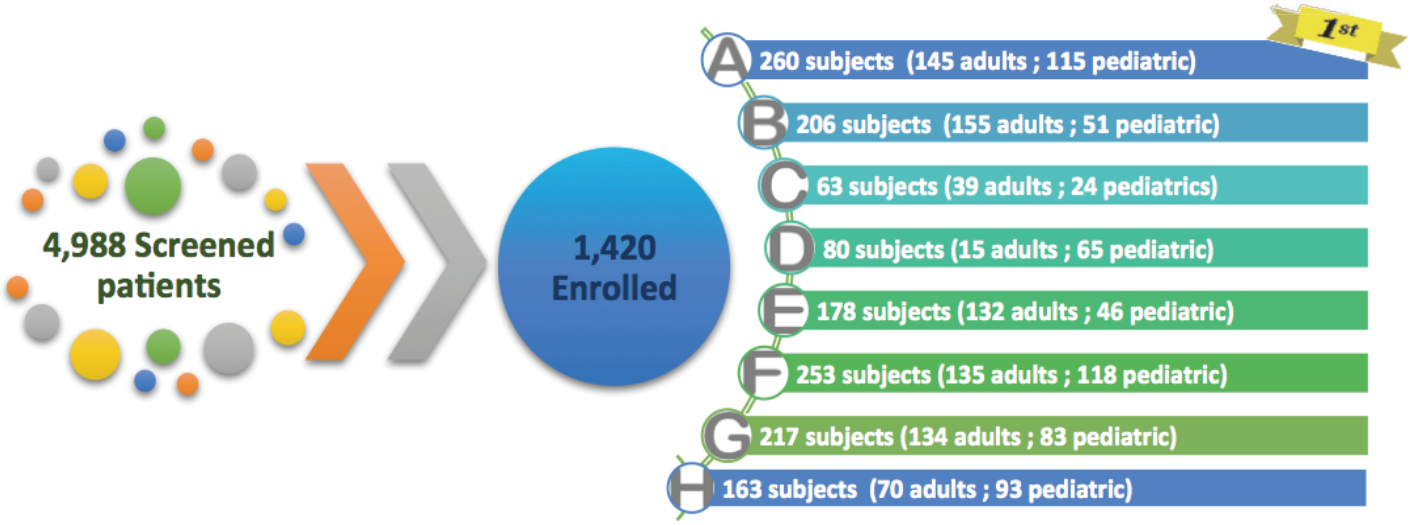
Congratulations to new proud moms! Nasreen Jahed's baby boy, **Gavin Liam**, was born on 4 April 2016, and Anandika Pawitri's baby girl, **Wilona Hirananda**, was born on 21 April 2016. Happy Birthday to your little ones! We know this is an exciting time for your families, and we hope you are blessed with years of happiness.

INA-RESPOND Study Updates

By dr. Anandika Pawitri,
dr. Nurhayati,
Ms. Novitasari

AFIRE Study (INA101) Updates

Updated 30 April 2016



A – Site 510 – RSUP dr Hasan Sadikin, Bandung
B – Site 520 – RSUP Sanglah, Denpasar
C – Site 530 – RSUPN dr Cipto Mangunkusumo, Jakarta
D – Site 540 – RSPI Prof Dr Sulianti Saroso, Jakarta
E – Site 550 – RSUP dr Wahidin Sudirohusodo, Makassar
F – Site 560 – RSUP dr Kariadi, Semarang
G – Site 570 – RSUD dr Soetomo, Surabaya
H – Site 580 – RSUP dr Sardjito, Yogyakarta

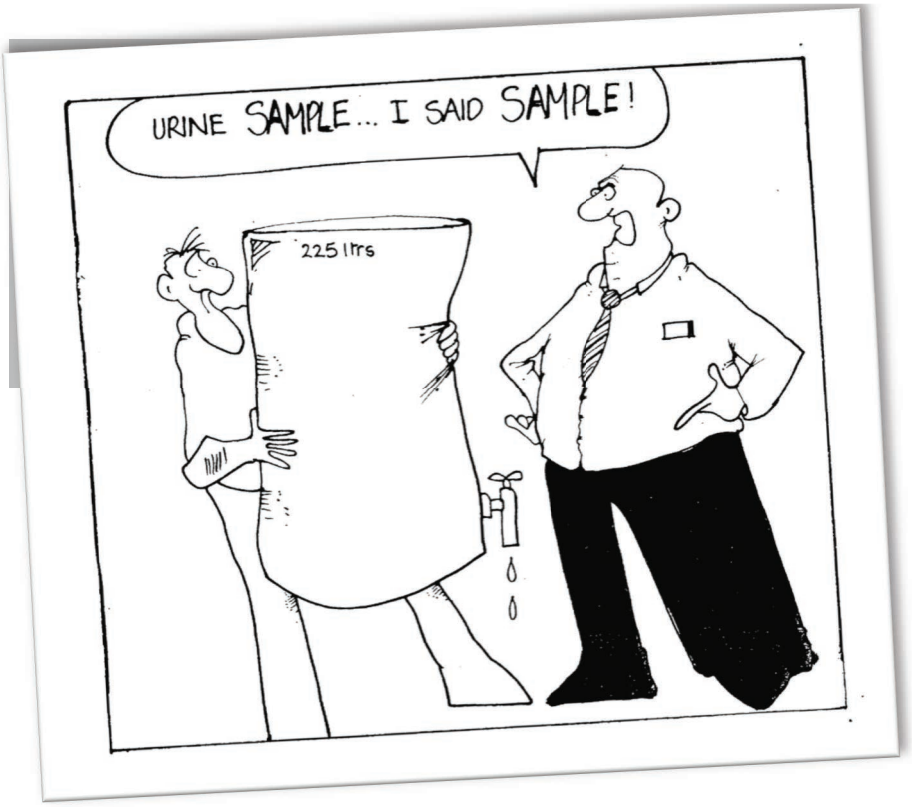
Detailed screening and enrollment progress is available in portal folder: Studies\INA101\Screening progress.pdf or go to the following link: https://ina-respond.s-3.com/EdmFile/getfile/797233

Sepsis Study (SEA050) Updates

It has been 5 months since the enrollment of Sepsis study ended at all sites on 31 December 2015, where 79 subjects (65 adults and 14 pediatric) had been enrolled. After weeks of data cleaning process at sites, we now have the clean data, and they are ready to be used for manuscript writing.

In March 2016, 2 meetings were held to discuss laboratory issues and research results, and a SEAICRN Protocol Committee meeting was held on 20-21 April in Bangkok to talk about data sharing and manuscript writing. Prof. Mansyur Arief, dr. Abu Tholib, and dr. Armaji Kamaludi attended the meeting.

For further information on this study, please go to: www.ina-respond.net/sepsis-study/



Sample… I Said Sample! Yes, SAMPLE!!

By:

dr. Aly Diana

As researchers, we hear the word "sample" almost every day. The next question after the main objective of the study was confirmed is most likely about sample: target population, sampling method, and sample size. When we were making the research protocol, we also had to deal with the "sample". As if we haven't got enough, Ethical Committee's favorite question is usually about sample too.

"Why don't we use total sampling?" is the question that keeps popping up in my mind, as having no consideration about sample means an easier research life. However, collecting total sampling of the target population is almost impossible as it may take forever to conduct, needs a large number of resources, and is ridiculously expensive. To top them all, it is not ethical to take other people's time or body fluids (blood, urine, saliva, etc.) or to kill animals more than we actually need.

Since it is not always realistic to use total sampling, what we can do is to take enough samples that can represent the target population, so we can make an inference about the target population through the results we obtained from the samples.

The next question is then, "how can we get a representative sample?" The best choice is to do a simple random sampling. However, this is not always possible. For example, we cannot do a simple random sampling of HIV-positive people in Jakarta if we have never done any screening for HIV in that specific population.

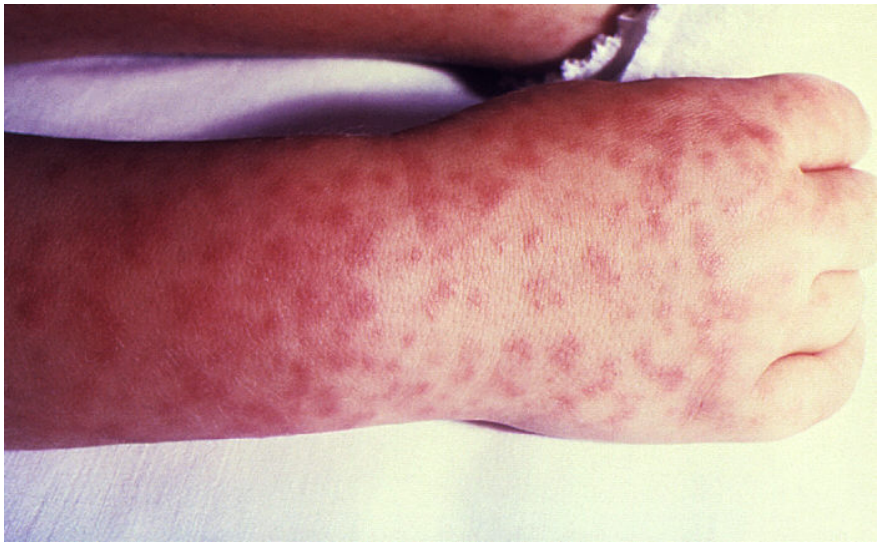
Then, what is the second best method? We may choose one from available probability sampling methods: stratified random sampling, cluster sampling, systematic sampling, or multi-stage sampling. Probability sampling gives an equal opportunity/ chance for each respondent of being selected. This equal chance reduces the potential of selection bias, which is the enemy of most studies. Only when probability sampling is impossible to conduct, we can start to consider non-probability sampling methods: convenience sampling, volunteer sampling, judgment sampling, or quota sampling.

The next difficult question is how many respondents are actually needed. This actually depends on the study objectives and the sampling methods. Different sampling methods have different formula to calculate sampling size. For example, sample size for a study using cluster random sampling is bigger than a study using simple random sampling. Please avoid the common mistake of deciding the sample size first and adjusting the formula's variables to meet the "desired" sample size.

Moral of the comic corner:

Please find the best sampling method to meet the study objectives. In the selection process, we must always bear in mind the limitations of every sampling method. Once we decide, we need to ask ourselves again whether it is really the best (possible) method. If yes, we can start to calculate the sample size.

Machin D, Campbell MJ, Walters SJ, 2007. Medical Statistics Fourth Edition. John Wiley & Sons, Ltd.



Typhus Fever (Rickettsial Diseases): Lab Detection of Rickettsiae

By

Wahyu Nawang Wulan
Deni Pepy R. Butarbutar

Typhus fever. What disease is that? Confusions often arise with the presence of another term, "typhoid fever", which in Indonesia is known as "*tifus*" or "*tipes*". Both diseases are generally characterized with fever, headache, myalgia, and malaise. The mix up started in the past when diagnosis relied mostly on prominent clinical presentations, and the fact that both diseases were associated with poor hygiene. It was eventually clarified in the 19th century after patients of typhoid fever were also known to develop gastroenteritis. Thus, typhoid is a term used to describe a disease with general symptoms undifferentiated to typhus fever ("oid" = resemble, typhoid = resemble typhus). The differentiation was further clarified with the discovery of the causative agents, i.e. the enterobacter *Salmonella typhi* as the cause of typhoid fever and *Rickettsia* sp. as the cause of typhus fever. In Indonesia, rickettsial infection generally receives minor attention due to its mild and undifferentiating symptoms, as well as challenges in routine diagnosis. This article presents an overview of rickettsial disease, particularly from the viewpoint of diagnosis.

To begin with, the taxonomical order of *Rickettsia* sp. is:

Phylum : Proteobacteria
Class : Alphaproteobacteria
Order : Rickettsiales
Family : Rickettsiaceae
Genus : *Rickettsia*, *Orientia*
Species : to name a few: *R rickettsii*, *R conorii*, *R typhi*, *O tsutsugamushi*.

The prototype species is *Rickettsia rickettsii*, named after the discoverer, Howard T Ricketts, who ironically died of typhus fever.

Rickettsial disease is zoonotic (a disease that is transmissible directly or indirectly from animals or humans). As such, the bacterial

(continued)

TAKE ACTION:

GOVERNMENT

EDUCATE HEALTHCARE PROVIDER TO DISTINGUISH RICKETTSIAL DISEASES FROM OTHER TROPICAL INFECTIOUS DISEASES.

EVERYONE

USE INSECT REPELLENT ON EXPOSED SKIN TO PREVENT POSSIBLE TICK BITES DURING OUTDOOR ACTIVITIES

ALWAYS KEEP OUR HOME CLEAN OF RATS, AND OUR PETS CLEAN OF FLEAS.

circulation is maintained within animal reservoir that are generally asymptomatic. The bacteria are transmitted to humans through the bites of arthropod vectors that infest those animal hosts. There are three types of rickettsial diseases, with regards to the three biotypes of rickettsiae grouped on the basis of immunological cross-reactivity and vector. The three rickettsial diseases are spotted fever, murine typhus, and scrub typhus [2]. Spotted fever is caused by infection with the "spotted fever group (SFG)", such as *R rickettsii*, *R conorii*, *R parkeri*, *R sibirica*, *R australis*, *R japonica*, to mention a few. Murine typhus is caused by *R typhi* or *R prowazekii*. Scrub typhus is caused by *Orientia tsutsugamushi* (previously described as *Rickettsia tsutsugamushi*). Members of the spotted fever group can infect various hosts, including rats, cats, dogs, as well as lizards and ruminants, and are generally transmitted by the hard-body ticks of the family Ixodidae. The causative agents of murine typhus, *R typhi* and *R prowazekii*, are maintained within rodent population and transmitted to humans by fleas (*Xenopsylla cheopis*). *O tsutsugamushi* is also maintained in rodents, but transmitted to humans via the bite of the chigger (larval stage) of trombiculid mites. The bite site often develops into an eschar.

Rickettsiae are gram-negative, obligate intracellular bacteria (Figure 1). In humans, they infect endothelial cells. The infected blood vessel-lining cells cause vascular damage that manifests as rash, interstitial pneumonia, encephalitis, interstitial nephritis, interstitial myocarditis, as well as lesions in the liver, gastrointestinal wall, pancreas, and potentially any vascularized tissue of the body [7]. The most important effect is increased vascular permeability, which can lead to edema, loss of blood volume, hypoalbuminemia, decreased osmotic pressure, and hypotension, and thus can be life threatening.

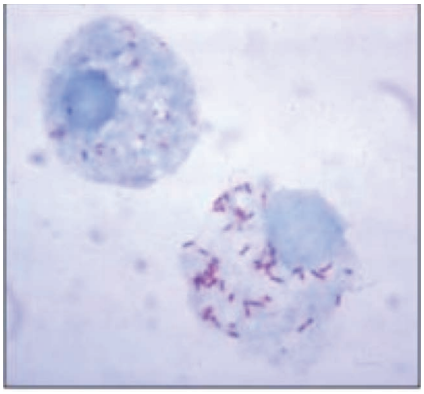


Figure 2 Gimenez stain of rickettsial infection in tick hemolymph cells. *R rickettsii* are shown as dark pink bodies. (Source: Wikipedia)

In general, the symptoms of rickettsial infection are indistinguishable and usually self-limiting, such as fever, headache, chills. However, in the absence of treatment and/or in high risk individuals, multiple organ failure (liver, heart, kidney) and neurological complications can take place, and fatality rates might span between 20-80% [6]. Chloramphenicol and tetracycline were used to treat rickettsial disease. Nowadays, doxycycline is the antibiotics of choice.

Rickettsial disease is endemic all over the world. In Indonesia, the endemicity of the three biotypes has been reported. The circulation of *R felis* and other member of SFG, as well as the causative agent of murine typhus *R typhi*, have been observed within the *X cheopis* fleas in East Java, West Java, East Kalimantan, and North Sulawesi [5, 8]. Seroreactivity either to murine typhus (*R typhi*), SFG (*R rickettsii* and *R conorii*), or scrub typhus (*O tsutsugamushi*), was shown in rodent population of Jayapura (Papua) as well as in human population of Malang (East Java) and Gag Island (Papua) [1, 3, 4], although detection of rickettsial genome in human specimen has never been described. These records suggest that rickettsial disease is a threat to human health across Indonesia.

Accurate lab diagnosis of rickettsial infection is important because main visible symptoms (fever, headache, chill, myalgia, malaise) are not unique to another prominent tropical disease, dengue fever. Distinguishing symptoms like eschar are not

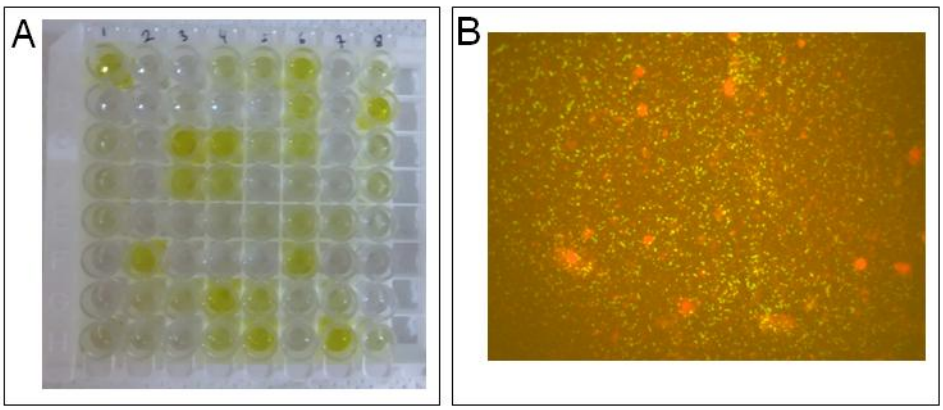


Figure 2
A. Rickettsia ELISA plate, deeper color corresponds with higher antibody titer.
B. The murine typhus IgM detected via IFA (400X enlargement).

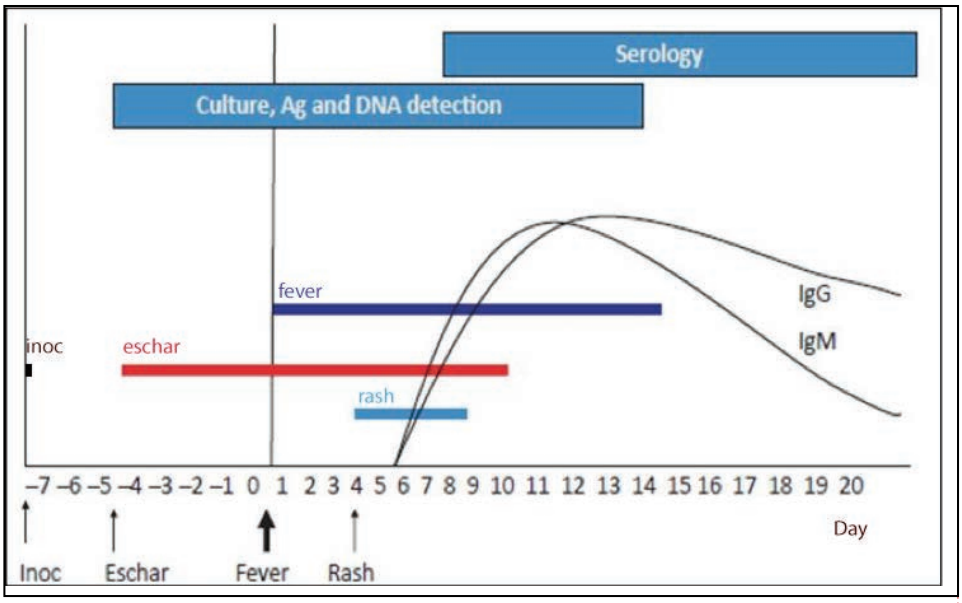


Figure 3 Time points of rickettsial infection in relationship with detection and diagnosis [9].

painful and often not recognized by patients or healthcare provider, while rash generally appears 3-5 days after fever presentation and might not be easily observed in dark-skinned person [6, 9]. Diagnosis is done either by detecting antibody expressed by infected individuals or detecting the bacteria (the antigen) from patient's blood. Antibody-based detection (serology) include enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescent assay (IFA). Antigen-based detection can be done by culturing the bacteria in lab animals, embryonated chicken eggs, and cell lines (Vero, C6/36, L929, S2), or by detecting the rickettsia-specific gene in polymerase chain reaction (PCR).

ELISA measures the expression of immunoglobulin G (IgG) and immunoglobulin M (IgM) against rickettsiae. The rickettsia-specific IgG or IgM binds to a surface-coating antigen, an enzyme is linked to the antibody, then enzyme-specific substrate added such that a certain fluorescent color developed. An ELISA plate reader measures the fluorescence intensity, where higher fluorescent level (visible deeper color, Figure 2A) corresponds to higher antibody titer. Rickettsial infection is confirmed when fourfold rise of IgG between sample taken at the acute period of infection and during the convalescent time points (at least 14 days apart) is observed; or, if there is a significant increase in IgM titer. However, the current 'gold standard' of rickettsial detection is IFA. In IFA, the rickettsia antibody firstly binds to a surface-coating antigen. A secondary, fluorophore-labeled antibody, is added to the complex, and fluorescence observed using a fluorescent microscope. The presence of rickettsia-specific antibody is shown as green fluorescent bodies (Figure 2B).

Since antibody develops after symptoms appear, confirmation of rickettsial infection by antibody detection does not allow ELISA and IFA to guide treatment (clinical information is required at the time of disease onset). Antigen-based detection (PCR) fills in this gap because bacterial multiplication has started even before symptoms appear (Figure 3).

Polymerase chain reaction (PCR) detects rickettsiae in clinical specimens by amplifying a portion of the bacterial DNA multiple times. A modified technique, known as realtime PCR, makes use of a fluorescent probe to mark the 'amplicons'. Detection of fluorescence during PCR cycles confirms the presence of rickettsial DNA. More copies of DNA (more bacteria) correspond with lesser values of Ct (cycle threshold). Successful detection is more likely when using samples containing more cells (e.g buffy coat) since rickettsiae are intracellular parasite. However, we have also demonstrated successful detection from plasma.

PCR is advantageous compared to serological assay in terms to confirm rickettsial infection in the early stages of disease, such that patients can obtain appropriate treatment. It is also highly sensitive (theoretically it can detect down to 1 DNA copy), which unfortunately makes it prone to cross-contamination leading to false positive result. Bacterial culture is the most definitive means of antigen detection. However, the procedure is laborious, takes longer time (days, can be weeks), and requires the high safety containment (biosafety level 3). The delicate nature of PCR, high biosafety level space requirement for culturing, costly diagnosis materials/instruments, added with the needs for high-skilled technician, have prevented routine diagnosis of rickettsiae in facility-limited settings, including hospitals in developing countries, and

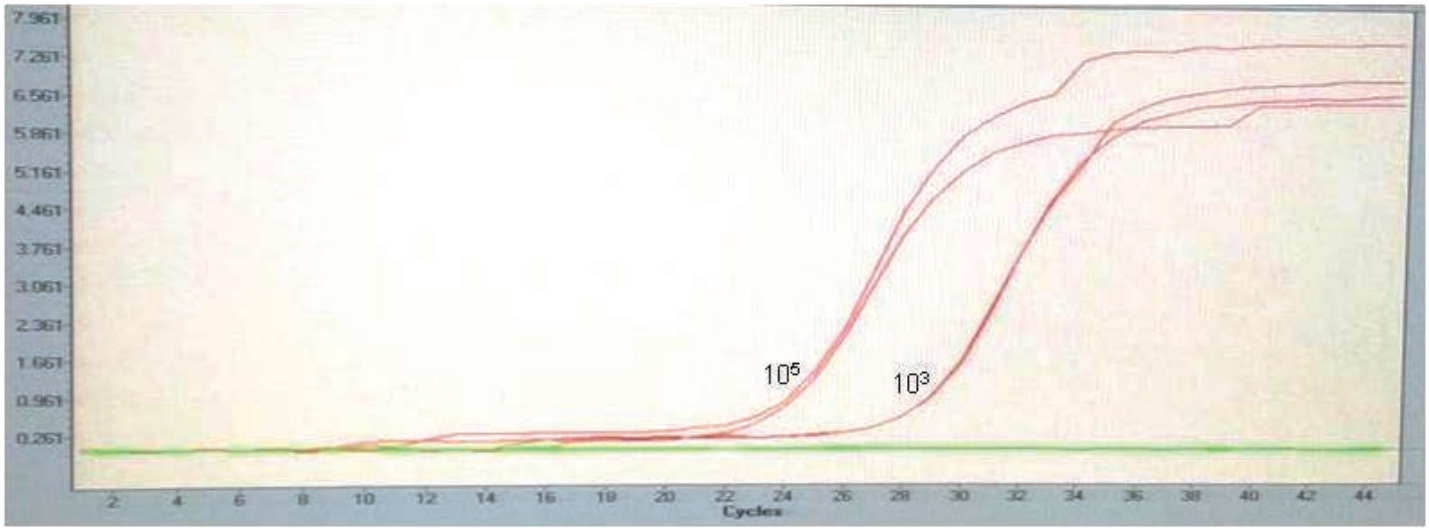


Figure 4 Realtime PCR detection of *O tsutsugamushi*. Earlier Ct detection means there are more DNA (bacteria) within the sample, 4 X 10^5 DNA copies were detected at Ct 23 while 4 X 10^3 DNA copies were detected at Ct 28.

contributed to rickettsial diseases grouping into one of the neglected tropical diseases.

To conclude, rickettsial infection is a mild disease, but can turn out to become severe in high-risk individuals and if left untreated. To facilitate proper treatment, accurate diagnosis is important. PCR or realtime PCR is a suitable means to specifically detect rickettsiae in suspected individuals; while ELISA or IFA is a good option to study rickettsial prevalence in a population. With regard to the fact that rickettisal disease is one of the neglected, the outcome of AFIRE study is expected to provide a clear description of rickettsial epidemiology, risks, and burden in Indonesia, in order to assist clinicians to better manage infectious diseases.

Further Reading:

1. Richards, A.L., et al. 1997. *Am J Trop Med Hyg* **57**(1)
2. Andersson, S.G.E. et al. 1999. *Mol Biol Evol* **16**(7)
3. Richards, A.L. et al. 2002. *Am J Trop Med Hyg* **66**(4)
4. Richards, A.L. et al. 2003. *Am J Trop Med Hyg* **68**(4)
5. Jiang, J. et al. 2006. *Emerging Infectious Diseases* **12**(8)
6. Eremeeva, M & Dasch, G. 2008. *In* Heyman, D.L. Control of Communicable Diseases Manual, 19th ed.
7. Todar,K. http://textbookofbacteriology.net/Rickettsia_2.html.
8. Barbara, K.A. et al. 2010. *J Med Entomol* **47**(6)
9. Richards, A.L. 2012. *FEMS Immunol Med Microbiol* **64**

INA-RESPOND Newsletter

Advisors : dr. M. Karyana, M.Kes, dr. Herman Kosasih
Art & Language : Dedy Hidayat S, S.Kom, Dona Arlinda, MD
Columnists : dr. Anandika Pawitri, dr. Nurhayati, Ms. Novitasari, dr. Aly Diana, Ms. Nawang
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