Neutral pH and cold temperatures stabilize the protective effect of crude pili S. flexneri on the ileal mucosa of mice

Avin Ainur Fitrianingsih[1,2], Lailia Nur Rachma[1], Alvi Milliana[1]

[1]Department of Microbiology, Faculty of Medicine and Health Sciences, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia
[2]Department of Physics, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia

Background: Alternative therapies are being sought as a result of the rising concerns with inadequate and untrustworthy medical treatments for *Shigella flexneri*. The current study aimed to assess the preventive and therapeutic effects of *crude pili S. flexneri* on of neutral pH and cold temperature against *Shigella flexneri* infection in immunocompetent mice.

Methods:Twenty male Swiss albino mice were randomly assigned to one of two groups: control or experimental groups. Each group was subsequently separated into four equal subgroups. In order to infect the mice, 4 weeks before infection, the experimental subgroups were given *crude pili S. flexneri* orally in every week (three times) until the completion of the research. After following the treatment of *crude pili S. flexneri*, all group infected with *Shigella flexneri.* The small intestines of mice were processed and analyzed for the presence of the pathological lesions. Jejunal portions were measured. The findings revealed that vaccinated mice had a statistically significant increase in the quantity of health mucose as compared to non-infected group in ileal sections. *Crude pili S. flexneri* was administered to the intestinal portions of all subgroups before or after the infection, and the architecture was found to be more or less normal.

Conclusion: Our data indicate that *crude pili S. flexneri* in neutral pH and cold temperature is a useful preventative and a potentially effective treatment medication for *S. flexneri* infection.

Keyword: pH, iemperature, crude Pili *Shigella flexneri,* ileal mucosal damage

INTRODUCTION

Shigellosis is an acute intestinal infection caused by shigella. It is transmitted through feco-oral route causing severe-bloody diarrhea. Shigellosis is a major health concern in the world especially in developing countries because Shigellosis now become endemic. An epidemic happened in Latin America, Asia, and Africa since 1960s were caused by S. flexneri 2a, S. flexneri 1, and S. sonnei. In Indonesia, morbidity and mortality of this disease is still high (DG PPM & PLP, 2009).
S. flexneri is a Gram negative bacteria, grown in anaerobic and facultative anaerobic, with pH 6.4 to 7.8 acidity, and temperature of 37' C. S. flexneri was able to cross the colonic mucosa and colonize in the intestinal epithelium causing severe intestinal inflammation and necrosis of the epithelium of the colon. Mechanism of Shigella infection is mediated by pili or fimbriae (Todar, 2012) which has a cellular component that used for adhere to host cells (Jennison & Verma, 2004). The existence of specific adhesion factor on bacterial surface will stimulate the host tissues express certain receptors on their cell surface and make them attached. The bacteria then invade the intestinal mucosal epithelial cells (Tortora, 2010).
S. flexneri become resistant to many antibiotics, even the latest antibiotic. S. flexneri multidrug resistance is a serious problem in the treatment of shigellosis because it increases the risk of epidemics. Therefore, WHO prioritize the development of an effective and safe vaccine for the control of shigellosis, especially in developing countries (Cakrabarti, 2010).

The pathogenesis of diarrhea by *S. flexneri* is a complex mechanism that causes severe intestinal inflammation and necrosis of the colonic epithelium. Shigella capabilities to across the colonic mucosa, colonize in the intestinal epithelium, and downregulate antimicrobial peptide which act as natural antibiotic is a key factor that cause these bacteria extremely virulent (Sansonetti, 2004; Sperandio et al, 2008), so that the consumption of 10 -100 bacteria can cause individu suffering from severe diarrhea. (Lamps, 2009; Tortora, 2010). Due to the various capabilities of *S. flexneri* to avoid the mechanism of action of antibiotics, multidrug antibiotic resistance by S. flexneri has been widely reported. Its increasing the risk of epidemics in various countries including Indonesia. Therefore, the World Health Organization prioritize the development of an effective and safe vaccine to help Shigellosis control.

One effort to develop an effective vaccine is use crude protein. Pili S. flexneri is *crude* protein that containing adhesin molecules. It contains haemagglutinin protein (Anam, 2012). So, it is highly relevant to induce immune response memory for shigellosis by vaccination with crude Pili S. flexneri. Giving oral adhesion protein is expected to activate dendritic cells to secrete IL-22 and IL-23, which then stimulates some T cell subsets, including Th17 cells. Th17 cells then secrete IL-17 and IL-22 which induces intestinal epithelial cells to secrete antimicrobial peptides Defensins to fight off the invasion of S. flexneri (Blachitz & Raffatelu, 2010). The result is expected to support the discovery of a reliable vaccine to cope with shigellosis.

MATERIAL AND METHODS

Research design

We apply an experimental research design using 20 mice with 8-12 weeks old. The sample were randomly divided into 4 groups: (K1) control without a particular treatment, (K2) CTB (7 µg/0.3 ml PBS), (K3) adhesin protein *Crude pili S. flexneri* (250 µg/0.3 ml PBS), (K4) adhesin protein *Crude pili S. flexneri* (250 µg/0.3 ml PBS) + CTB (7 µg/0.3 ml PBS). All protein provided in neutral PH dan cold temperature. The *Crude* pili immunization treatment was given for 3 weeks orally.

Culture of Shigella spp.

The bacteria used in this research was *S. flexneri* from Reseach of Laboratory Health East Java Indonesia. The Medium used was Thiaproline Carbonate Glutamate (TCG) in order to enrich the growth of pili. This medium contains 0.02% thioproline; 0.3% NaHCO 3, 0.1% mono sodium 1-glutamate, 1% bactotryptone; 0.2% yeast extract, 0.5% NaCl, 2% bacto agar and 1 mM β amino-ethyl ether-N, N, N 'n'-tetra acid (EGTA) Ehara.

Isolation of *Shigella flexneri* pili

Isolation of *Shigella flexneri* pili was refers to the research carried out by Sumarno with modification. Bacteria pili cutting used pili bacterial cutter and was carried out for 30 seconds at a speed 5000 rpm, while the second to four cuttings used same speed. The isolation of pili fraction by centrifugation of cutting product as carried out at 12,000 rpm by using a temperature 4^{o} C. Shave repeated and stopped after the supernatant looks clear. Then, supernatants containing the bacterial pili are stored at a temperature of 40 C.

Isolation of *Shigella flexneri* protein hemeagglutinin pili crude protein Pili *Shigella flexneri*

Research method referred to Sumarno (2011). The results of pili collection was carried out electrophoretically by SDS-PAGE method. The product of electrophoresis in the form of gel was cut straight at the desired molecular weight. Then the pieces were cut perpendicularly so each piece will contain three protein bands. The resulted pieces of band above were collected and then inserted into the tube of dialysis membrane by using electrophoresis running buffer fluid. Electroellusion used a horizontal electrophoresis apparatus at 125 mV power for 25 minutes. The dialysis was performed on the product of electroellusion with PBS pH 7.4 buffer fluid as much as 2 liters during 2 X 24 hours. Dialysis fluid was replaced three times. Dialysis fluid in membrane dialysis as a result of electroellution of SDS-PAGE band was ready for hemagglutination test.

Stabilization protein on neutral Ph and cold temperature

The technique of the present invention entails adding a sufficient amount of a stabilizer to a protein preparation to decrease protein aggregation while the preparation is kept at a low pH. In one embodiment, the method includes adding a sufficient amount of one or more amino acids to achieve a final concentration of between about 1 mM and about 3 M, preferably between about 1 mM and I M, and then subjecting the solution to a low pH, preferably a pH of about 4.0 or less, more preferably between about pH 2.8 and about pH 4.0. Furthermore, stabilizers for the procedures of the present invention can be chosen from a sugar or sugar derivative such as sucrose, mannitol, and glycerol, or from inorganic salt stabilizers such as sodium EDTA, NaCl, or CaCl 2. To decrease protein aggregation at low pH, one or more sugar or salt stabilizers can be coupled with one or more amino acid stabilizers in one embodiment. All preotein were placed on 4^{o} celcius temperature.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Monitoring the molecular weight (MW) by SDS-PAGE was done by applying Laemmli methode. Protein sample was heated in 1000 C for 5 min in buffer solution containing 5 mM Tris pH 6.8, 5% 2-mercapto ethanol; 2.5% w / v sodium dodecyl sulfate, 10% v / v glycerol with bromophenol blue tracer colour. 12.5% of a mini slab gel with 4% tracking gel was selected. Electric voltage used was 120 mV. The color material used was a coomassie brilliant blue and protein markers using *sigma low range marker*.

Adhesion protein conjugation prochedur (49,8 kDa + Cholera Toxin *Crude* B)

Imunization of Pili protein of *Shigella flexneri* 49,8 kDa 100µg/100µl conjugated with CTB 12µg/25µl as adjuvant.

Immunization

We use male mice of the species mus musculus outbred Balb/C were aged 6-8 weeks, as many as 20 tails mice of the Laboratory of Pharmacology, Faculty of Medicine, University of Brawijaya. The mice

outbred Balb/C is divided into four treatment groups, each group as much as 5 mices. The experimental research had agreement about ethical clearance from The Ethical Committee Medical Research Faculty of Medicine, University of Brawijaya. Immunizations were given to Group I: Control infection, Group II: obtained CTB only, Group III: obtained immunization with adhesion protein *crude* pili 100 µg/100 µl, Group IV: obtained immunization with adhesion protein *crude* pili 100 µg/100 µl +CTB 12 µg/25 µl (under the guidance of Sigma). Immunizations were given every three days orally. On the day 35, the mice were killed and taken along the 10 cm piece of ileum to in challenge with S. Flexneri, then examined the strength of protectivity using histo PA.

Preparation of Mucosa

Preparation of mucus was carried out as follows: intestinal pieces were washed with cold PBS. Then the intestine was opened so that the visible part of the small intestine mucosa exposed. Layer of mucus was collected by scraping longitudinally with spatel and placed in tubes containing sterile PBS and protease inhibitors.

Protectivity test (Histopatological Examination)

To test the strength of protectivity, we used Mice Ligated Ileal Loop (MLIL). Protectivity were tested by using histopathological examination of small intestine of mice treated group and the control group that carried out in the pathology anatomy laboratory medical faculty of Brawijaya University. Animals were euthanized by CO2 inhalation. After had been exposed with *S. flexneri* for 4 hour, the mice ileum were removed and perfused with 10% buffered formalin phosphate (Fisher, Pittsburgh, Pa.), dehydrated, and processed in paraffin. Sections were cut at 3 mm and stained with hematoxylin and eoxin.

Statistical analysis.

For comparative analysis of immunogenicity, data were tested by one-way analysis of variance (ANOVA). This research is significant if $p < 0.05$. When significant differences were found, differences between means were determined by Tukey's multiple comparison tests. If the data does not meet the requirements of normality and homogeneity then using Kruskal Wallis test then tested further by Mann Whitney U test. All statistical analyses were carried out in GraphPad Prism (version 5.03; GraphPad Software Inc., La Jolla, CA).

RESULT

The loops were opened longitudinally to examine the mucosa of the intestine. The loops treated with *S. flexneri* strain were significantly diluted and filled with haemorrhagic thick fluid, whereas the loops treated with the *crude* pili *S. flexneri* showed minimal fluid accumulation, similar to the control loops treated with phosphate-buffered saline. Section of tissue infected with *S. flexneri* revealed a total loss of villous architecture as well adenudation of surface epithelium in some areas. Massive bleeding with neutrophilic infiltration in the lamina propria extending up to the submucosa was observed, as well as congested and dilated crypts. These observations indicate that the intake crude protein pili S. flexneri in neutral pH and cold temperature provide protection against the effects of ileal mucosa is characterized by villi in good condition and there is no edema

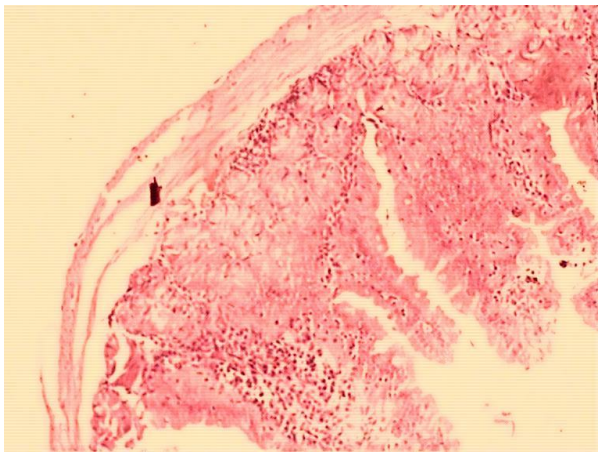

Figure 1. The result of Ileum Histopatological Examination from Control group without pH and temperature setting nor particular treatment. Histopathological appearance of mice intestinal mucosa infected by the wild-type invasive *S. flexneri* strain. The strain destroyed the architecture of the intestinal mucosa of mice by necrosis.

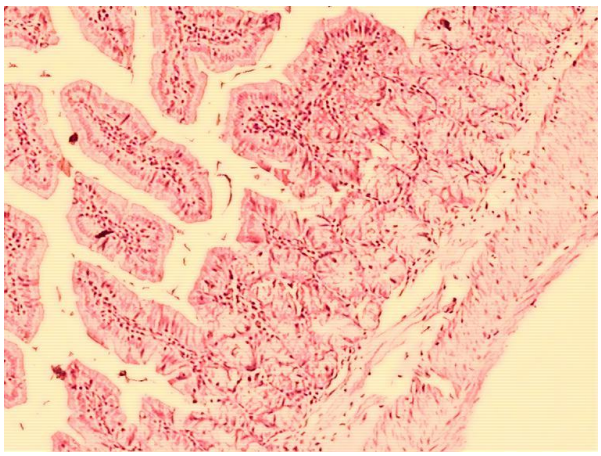

Figure 2. The Ileum Histopatological result of group CTB (7 µg/0.3 ml PBS) without pH and temperature setting. Group treated with the CTB only, revealed such alterations and retained destroyed of villous shape. Figure 2 depicts the intestinal mucosa of an infected mice.

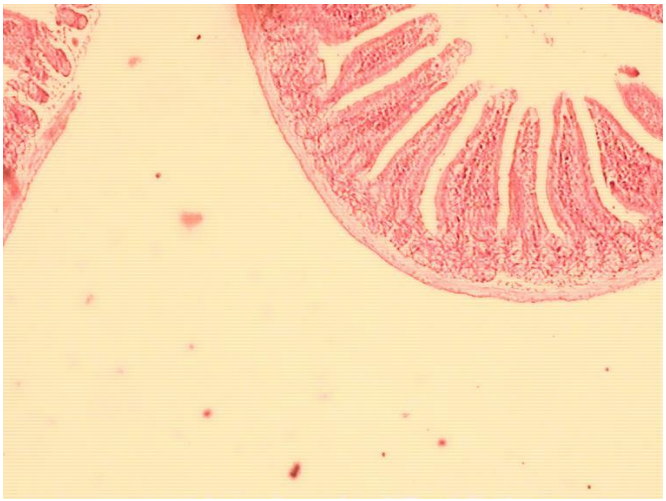

Figure 3. The result of Ileum Histopatological Examination of group protein *Crude* pili *S. flexneri* (250 µg/0.3 ml PBS) in neutral pH and cold temperature (4°celcius). The result revealed no such alterations and retained normal villous shape. Mice intestinal mucosa infected by the S. flexneri did not show lesions and the villi were conserved. Photographs were taken under low power objectives with an MPS 60 camera.

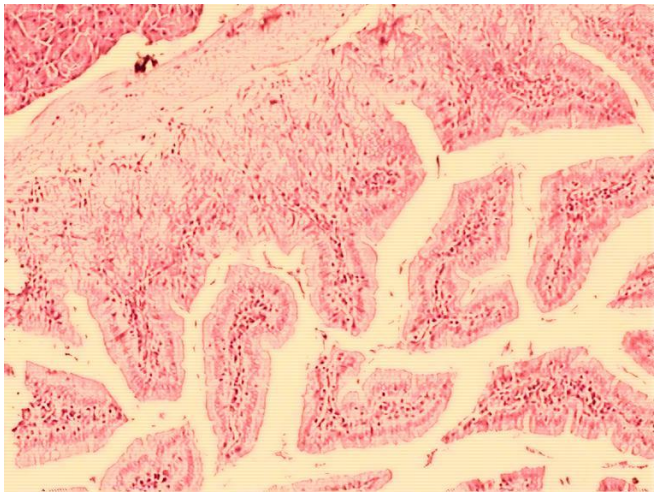

Figure 4. The result of Ileum Histopatological Examination of group protein *Crude* pili *S. flexneri* (250 µg/0.3 ml PBS) + CTB (7 µg/0.3 ml PBS) without pH and temperature setting. The result revealed destroyed villous shape. Mice intestinal mucosa infected by the S. flexneri show many lesions and the villi were destroyed.

DISCUSSION

Knowledge of the process involved in the invasion of *S. flexneri* into the gastrointestinal tract is the major theme to understand the pathogenesis of infections. The key steps in the invasion of *S. flexneri* are epithelium proliferation, invasion and movement to the tissues. The pili molecule is a major antigenic constituent of the *Shigella* cell surface. The best prophylactic measure would be to prevent the *Shigellae* from invading the mucosal lining of the intestine to limit their intracellular multiplication and spread in the tissues. A live attenuated *Shigella* strain, expressing pili and has been

suggested for vaccine formulation and should be preferred. The effect of pH and temperature toward the effectiveness *Crude* protein pili S. flexneri as a vaccine candidate never been yet understood. *Crude* protein pili S. flexneri used in the study was a *Crude* pili that has been cutted from the outer surface of the S. flexneri bacteria. This kind of protein is an adhesion protein that has local side effect and mild systemic reaction compared to a whole cell attenuated vaccine (*centers for Disease and Prevention, 2006).* Some studies have been revealed that a *Crude* vaccination could induce Th1 strongly with long term memory and immune system protection level similar to a wild type microorganism induction (Aagaard *et al,* 2011; Agger *et al,* 2008; Lindenstream *et al,* 2012).

In this study, the adhesion protein S. flexneri was given per oral as S. flexneri pathogenesis indicate that it could invade the host through feco-oral route. Beside its effectiveness, the benefits of this method include easy to administer, atraumatic and no need aseptic method. The adjuvant used in the study was *Cholera Toxin Crude-B* (CTB), a protein carrier, since it could stimulate mucosa antibody response and induce systemic cell-T in binding with antigen. The ileum tissue was examine using Hematoksilin Eosin examination after challenged by viable S. flexneri. It could be seen that the ileum histologi result in the protein pili only group described good condition characterized by long and complete mucosal villi cells. Based on the histopathology finding, it was only the protein pili group showing the presence of protective effect.

CONCLUSION

It could be concluded that *crude* pili *S. flexneri* immunization in neutral pH and cold temperature providing a protection against ileal mucosal damage caused by *S. flexneri*. The adhesion protein used in the study has a great potentiality to be a vaccine candidate for shigellosis. For further investigation, it is necessary to explore specifically the sub unit pili *S. flexneri*. It also essential to investigate cytokines level for complementing the effect of *crude* pili *S. flexneri* toward patahogenesis pathway of S. flexneri infection.

ETHICAL CONSIDERATION

The study applied the ethical principles for animal model as the reseach subject including the implementation of replacement, reduction, and refinement principles. The study gained an ethical approval from the Health Research Ethics Committee Faculty of medicine, University of Brawijaya, Malang.

Acknowledgement

The research project was supported by Lembaga Penelitian dan Pengabdian Masyarakat UIN Maulana Malik Ibrahim Malang (LPPM, Ministry of Religious Affair - Republic of Indonesia) through BOPTN Scheme 2019.

REFERENCES

Aagaard C, Hoang T, Dietrich J, cardona P, Izzo A, Dolganov G, *et al*. 2011. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat. Med. 17:189 –194.

Agger EM, Rosennkrands I, Hansen J, Brahimi K, vandahl B, Aagaard C, *et al*. 2008. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. PLoS One 3:e3116. doi:10.1371/journal.pone.0003116.

Amy V, Jennison and Naresh K. 2004. *S. flexneri* Infection: Pathogenesis and Vaccine Development. FEMS Microbiology Review. 28. 43-58

An YH., Friedman RJ. 2000. Handbook of bacterial adhesion : principle methods, and application. Humana Press Inc, Totowa, New Jersey

Anam K., Sumarno., Suyuti H. 2012. Identifikasi Protein Hemagglutinin Sub-unit Pili 49,8 kDa dan Anti Hemaglutinin 7,9 kDa serta Uji Respon Imun Reaksi Silang *Shigella* spp. Thesis Program Pasca Sarjana Univ. Brawijaya Malang

Ashida H. Mimuro H., Sasakawa C. 2015, *Shigella* Manipulates Host Immune Responses by Delivering Effector Proteins with Specific Roles. Front Immunol. 2015; 6: 219. doi: 10.3389/fimmu.2015.00219

Bardhan, P. A.S.G. Faruque, Aliya Naheed, and David A. Sack. 2010. Decreasing Shigellosis-related Deaths without *Shigella* spp.–specific Interventions, Asia. Emerg Infect; 16 (11): 1718–1723. doi: 10.3201/eid1611.090934

Behrman, Kliegman, Arvin. 2000. Ilmu Kesehatan Anak (Nelson Textbook of Pediatrics). Alih bahasa oleh Wahab Samik. Vol 2. ECG. 974-976

Blaschitz C & Raffatelu M. 2010. Th 17 cytokines and the gut mucosal barrier. J Clin Immunol. 30. 196-203

Bodera, P. & Chcialowski, A. 2009. Immunomodulatory Effect of Probiotic Bacteria. Recent Patents on Inflammation & Allergy Drug Discovery 3, 58-64.

Brogden , K.A. 2005 . Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 : 238 – 250 .

Bowdish , D. M. E. Davidson, D. J. 2006. Immunomodulatory Properties of Defensins and Cathelicidins. ・ R. E.W. Hancock Centre for Microbial Diseases and Immunity Research, University of British Columbia, 232 Lower Mall Research Station, Vancouver BC,V6T 1Z4, Canada, P 9, 39

Burgey C., Kern WV., Winfried R. 2015. The innate defense antimicrobial peptides hBD3 and RNase7 are induced in human umbilical vein endothelial cells by classical inflammatory cytokines but not Th17 cytokines. Microbes and Infection. DOI: 10.1016/j.micinf.2015.01.005

Carneiro L.A.M., Travassos L.H., Soares F., Tattoli I., Magalhaes J.G., Bozza M.T., Plotkowski M.C., Sansonetti P.J., Molkentin J.D., Philpott D.J., Girardin S.E. 2009. *Shigella* induces mitochondrial dysfunction and cell death in nonmyleoid cells. Cell Host Microbe. 5:123–136 10.1016

Center For Disease Control and Prevention. 2006. General Recommendation on Immunization. Recommendation of the advisory Committee on Immunization Practice. MMWR. 55: 1 – 48

Center For Disease Control and Prevention. 2013. Shigellosis : Infectious Diseases Related To Travel. Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Global Migration and Quarantine (DGMQ). Atlanta

Cerda JP., Cossart P. 2006. Bacterial Adhesion and Entry into Host Cells. Cell 124, 715-727

Cherla, R. P., S. Y. Lee, and V. L. Tesh. 2003. Shiga toxins and apoptosis. FEMS Microbiol. Lett. 228:159-166.Cross Ref Medline

Corr SC., Cormac CGM., Gahan., Hill C.. 2007. M-cells: Origin, Morphology and Role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol 52: 2-12

De Haan L, Hirst TR. 2004. Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms. Mol Membr Biol. 21(2):77-92.

Dekker CL., Gordon L., Klein J. 2008. Dose optimization strategies for vaccines: The Role of adjuvants and New Technologies. The NVAC Subcommitee on vaccine Development and Supply

Direktorat Jenderal Pemberantasan Penyakit Menular dan Penyehatan Lingkungan Pemukiman (Ditjen PPM & PLP). 1999. Tatalaksana Kasus Diare Bermasalah. Departemen Kesehatan RI; Badan Koordinasi Gastrointerologi Anak Indonesia

Elson, C. O., M. T. Dertzbaugh. 1999*. Mucosal adjuvants. P. L. Ogra, and J. Mestecky, and M. E. Lamm, and W. Strober, and J. Bienenstock, and J. R. McGhee, eds.* Mucosal Immunology, 2nd Ed 817 *Academic, San Diego.*

Erben U., Loddenkemper C., Doerfel K, Spieckermann S, Haller D, Heimesaat MM, Zeitz M, Siegmund B, Kühl AA,. 2014. A guide to histomorphological evaluation of intestinal inflammation in mouse models. Int J Clin Exp Pathol 7(8):4557-4576

Ericson Charles D, Dupont Herbert L, Steffen Robert. 2003. Travelers Diarrhea. Hamilton. 17 - 183.

Faisal, Sumarno, Handono K. 2013. Susu Kuda Sumbawa Terfermentasi sebagai Immunostimulant untuk 37.8 kda V. cholerae Vaccine. Jurnal Kedokteran Brawijaya

Fritz , J.H. , R.L. Ferrero , D.J. Philpott , and S.E. Girardin . 2006 . Nodlike proteins in immunity, infl ammation and disease. Nat. Immunol. 7 : 1250 – 1257

Funderburg N, Lederman MM, Feng Z, Drage MG, Jadlowsky J, Harding CV, *et al*. 2007. Human Beta-defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. Proc Natl Acad Sci 104:18631–18635

Gagliardi MC, Sallusto F, Marinaro M, Langenkamp A, Lanzavecchia A, De Magistris MT. 2000. Cholera toxin induces maturation of human dendritic cells and licences them for Th2 priming. Eur J Immunol 30(8):2394-2403.

Ganz , T. 2003 . Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. 3 : 710 – 720

Gerstel U, Czapp M, Bartels J, Schroeder JM. 2009. Rhamnolipid-induced shedding of flagellin from Pseudomonas aeruginosa provokes hBD-2 and IL-8 response in human keratinocytes. Cell Microbiol 11:842–853

Hamada S, Umemura M, Shiono T, Tanaka K, Yahagi A, Begum MD, *et al*. 2008. IL-17A produced by gammadelta T cells plays a critical role in innate immunity against Listeria monocytogenes infection in the liver. J Immunol. 181: 3456–3463.

Hamrick T S, Harris S L, Spears P A Havell E A, Horton J R, Russell P W, Orndorff P E. 2000. Genetic Characterization of Eschericia Colli type I pilus Adhesin Mutants and Identification of a Novel Binding Phenotype. J Bacteriol 182:4012–4021.

Hanh E., P. Wild, U. Hermans, P. Sebbel, R. Glockshuber, M. Haner, N. Taschner, P. Burkhard, U. Aebi and S.A. Muller. 2002. Exploring the 3D moleculerarchitechture of Escherichiae coli type 1 pili. J MolBiol 323 (5): 845-57.

Harrington AT, Hearn PD, Picking WL, Barker JR, Wessel A, Picking WD. 2003. Structural characterization of the N terminus of IpaC from *S. flexneri*.. Infect Immun. 71(3):1255-64.

He Y, Zhang J, Mi Z, Robbins P, Falo LD. 2006 Skin-derived dendritic cells induce potent CD8+ T cell immunity in recombinant lentivector-mediated genetic immunization. Immunity 24:643–656

Hernawa E., Surjawidjaja JE., Salim OC., Indriani N., Bukitwetan P., Lesmana M. 2010. *Shigella*-Associated Diarrhea in children in South Jakarta-Indonesia. Southeast Asian J Trop Med Public Health. 41(2). 418-425

Hill T, **Krougly O**, **Nikoopour**, **Bellemore S**, **Chan EL**, **Fouser LA**, **Hil DJ, Singh B. 2013.** The involvement of interleukin-22 in the expression of pancreatic beta cell regenerative *Reg* genes. Cell Regeneration Journal. doi:10.1186/2045-9769-2-2

Iqbal M S., Rahman M, Islam R, Banik A, M. Amin B., Akter F., Talukder KA. 2014. Plasmid-Mediated Sulfamethoxazole Resistance Encoded by the sul2 Gene in the Multidrug-Resistant *S. flexneri* 2a Isolated from Patients with Acute Diarrhea in Dhaka, Bangladesh. Plos one journal, DOI: 10.1371/journal.pone.0085338

Islam , D. , L. Bandholtz , J. Nilsson , H. Wigzell , B. Christensson , B. Agerberth , and G. Gudmundsson. 2001 . Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat. Med. 7 : 180 – 185

Iwalokun BA., Gbeni GO., Ogunledun A. 2003. Patterns and Properties of Haemagglutinins Expressed by *Shigella* Serogroups in Lagos, Nigeria. J Health Popul Nutr. 21.20: 90 - 95

Jennison & Verma. 2004. *S. flexneri* Infection: Pathogenesis and Vaccine Development. FEMS Microbiology Review. 28. 43-58

Jones, B.C., Logsdon, N.J., and Walter, M.R.2008. Crystallization and preliminary X-ray diffraction analysis of human IL-22 bound to the extracellular IL-22R1 chain. PubMed Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 64: 266–269

Joly , S. , C.C. Organ , G.K. Johnson , P.B. McCray Jr ., and J.M. Guthmiller . 2005 . Correlation between beta-defensin expression and induction profi les in gingival keratinocytes. Mol. Immunol. 42 : 1073 – 1084

Khan,WA[1,*] Jeffrey K. Griffiths,[2] and Michael L. Bennish· 2013. Gastrointestinal and Extra-Intestinal Manifestations of Childhood Shigellosis in a Region Where All Four Species of *Shigella* Are Endemic. PLoS One. doi: 10.1371/journal.pone.0064097

Kiyono, H. & Fukuyama, S. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. Nat Rev Immunol, 4, 699-710

Kramer , R.W. , N.L. Slagowski , N.A. Eze , K.S. Giddings , M.F. Morrison , K.A. Siggers , M.N. Starnbach , and C.F. Lesser . 2007 . Yeast functional genomic screens lead to identifi cation of a role for a bacterial eff ector in innate immunity regulation. PLoS Pathog : e21

Lamps Laura W. 2009. Surgical Pathology of the Gastrointestinal system : Bacterial, Fungal, Viral, and Parasitic Infections. Springer. 33 – 36

Levine MM1, Kotloff KL, Barry EM, Pasetti MF, Sztein MB. 2007. Clinical trials of *Shigella* vaccines: two steps forward and one step back on a long, hard road.Nat Rev Microbiol. 5(7):540-53.

Li , H. , H. Xu , Y. Zhou , J. Zhang , C. Long , S. Li , S. Chen , J.M. Zhou , and F. Shao . 2007 . The phosphothreonine lyase activity of a bacterial type III eff ector family. Science . 315 : 1000 – 1003

Lindenstram T, Woodworth J, Dietrich J, Aagaard C, Andersen P, Agger EM. 2012. .Vaccine-Induced Th17 Cells Are Maintained Long-Term Postvaccination as a Distinct and Phenotypically Stable Memory Subset. Journal ASM.*Volume* 80(10) : 3533–3544

Liu AY, Destoumieux D, Wong AV, Park CH, Valore EV, Liu L, *et al*. 2002. Human [bgr]-Defensin-2 production in keratinocytes is regulated by Interleukin-1, bacteria, and the state of differentiation. J Invest Dermatol 118:275

Liu Y, Edward G. Shepherd & Leif D. Nelin. 2007. MAPK phosphatases — regulating the immune response. Nature Reviews Immunology. doi:10.1038/nri2035

Lockhart E, Green AM, Flynn JL. 2006.. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells duringMycobacterium tuberculosis infection. J Immunol ; 177: 4662–4669.

Meeks KD, Sieve AN, Kolls JK, Ghilardi N, Berg RE. 2009. IL-23 is required for protection against systemic infection with Listeria monocytogenes. J Immunol. 183: 8026–8034.

Moore, K.W., de Waal Malefyt, R., Coffman, R.L., and O'Garra, A. 2002. Interleukin-10 and the interleukin-10 receptor.PubMed. Annu. Rev. Immunol. 19: 683–765

Moralez E, Lofland D. 2011. Shigellosis with resultant septic shock and renal failure.. Clin Lab Sci. 2011 Summer. 24(3):147-52.

Taneja N, Mewara A, Kumar A, Verma G, Sharma M. 2012. Cephalosporin-resistant *S. flexneri* over 9 years (2001–09) in India. J Antimicrob Chemother doi:10.1093/jac/dks061

Niebuhr, K., Jouihri, N., Allaoui, A., Gounon, P., Sansonetti, P., and Parsot, C. 2000. IpgD, a protein secreted by the type III secretion machinery of *S. flexneri*, is chaperoned by IpgE and implicated in entry focus formation. Mol Microbiol 38: 8–19.

Niyogi, S.K .2005. Shigellosis. J. Microbiol 43(2):m133-43.

O'Neal C, Jobling M, Holmes R, Hol W. 2005.. Structural basis for the activation of cholera toxin by human ARF6-GTP. Science 309 (5737): 1093–6.

Oppenheim JJ, Biragyn A, Kwak LW, Yang D. 2003. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Ann Rheum Dis 62 : ii17–ii21

Pandey RS, Dixit VK. 2010. Evaluation of ISCOM vaccine for Mucosal Immunization against hepatitis B. Pubmed, 18 (4): 282-91

Page, A.L., Sansonetti, P, Parsot, C. 2002. Spa15 of *S. flexneri*, a 3rd type of chaperone in the type III secretion pathway. Mol Microbiol 43: 1533–1542.

Parker JN, Parker PM. 2002.The 2002 Official patients Sourcebook on Shigellosis. ICON Health Publication. 9-15

Passwell, J.H., Harlev, E., Ashkenazi, S., Chu, C., Miron, D., Ramon, R., Farzan, N., Shiloach, J., Bryla, D.A., Majadly, F., Roberson, R., Robbins, J.R. and Schneerson, R. 2001. Safety and immunogenicity of improved *Shigella* O-specific polysaccharide- protein conjugate vaccines in adults in Israel. Infect Immun. 69.1351–1357

Pendaries, C., H. Tronchere, L. Arbibe, J. Mounier, O. Gozani, L. Cantley, M. J. Fry, F. Gaits-Iacovoni, P. J. Sansonetti, and B. Payrastre. 2006. PtdIns5P activates the host cell PI3-kinase/Akt pathway during *S. flexneri* infection. EMBO J. 25:1024-1034.

Prabowo A., E.H. Tinny and R.P. Sumarno. 2011. Partial characterization of adhesions pili on *Shigella* dysentriae.Thesis Program Pasca Sarjana Univ. Brawijaya Malang

Remaut, H*.,* Rose, R.J*.,* Hannan, T.J*.,* Hultgren, S.J*.,* Radford, S.E*.,* Ashcroft, A.E*., and* Waksman, G. 2006. Donor-strand exchange in chaperone-assisted pilus assembly proceeds through a concerted beta strand displacement mechanism*.* Mol Cell 22*:* 831–842*.*

Ryanna, K., Stratigou, V., Safinia, N., Hawrylowicz, C. 2009. Regulatory T cells in bronchial asthma. Allergy 64, 335–347.

Röhrl J, Yang D, Oppenheim JJ, Hehlgans T. 2008. Identification and Biological Characterization of Mouse beta-defensin 14, the orthologue of human beta-defensin 3. J Biol Chem. 283(9):5414-9. doi: 10.1074/jbc.M709103200.

Rui XL., Xu YQ., Wu XD., *et al*.1997. Construction of a trivalent candidate *Shigella* vaccine strain with host vector balanced-lethal system . science in China 40 : 52-59

Sanchez J, Holmgren J. 2011. Cholera Toxin. IndianJourMed doi 133(2):153–163

Santoso S. 2002. Protein Adhesi Salmonella typhi sebagai virulensi berpotensi imunogenik terhadap produksi sIgA protektif. Disertasi Program Pascasarjana Univ. Airlangga Surabaya

Sansonetti P.J., C. Egile .1998. Moleculer basic of epithelial cell invasion by *Shigella*flexneri.Antonie Van Leeuwenhoek 74 (4): 191-7.

Sansonetti, P. J. 2004. War and peace at mucosal surfaces. Nat. Rev. Immunol. 4:953–964.

Sasakawa C. 2010. A new paradigm of bacteria-gut interplay brought through the study of *Shigella*.Proc Jpn Acad Ser B PhysBiol Sci. 86 (3): 229-43.

Schroeder ,GN. Hilbi Hubert. 2008. Molecular Pathogenesis of *Shigella* Spp.: Controlling hosct cell signaling, invasion and death by type 3 secretion. Clinical Microbiology reviews. 21(1). 134-149

Sebastian F., Stabat D., Hodgkinson JL., Veenendaal AK., Johnson S., Blocker AJ. 2007. Identification of minor inner-membrane components of the *Shigella* type III secretion system 'needle complex'. Microbiology. doi 10.1099/mic.0.2007/007781-0

Seidlein LV., Kim DR., Ali M., Lee H., Wang XY. 2006. A Multicentre Study of *Shigella* Diarrhoea in Six Asian Countries: Disease Burden, Clinical Manifestations, and Microbiology..PLlosonejournal. doi: 10.1371/journal.pmed.0030353

Sellge G , Phalipon A., Bandeira, James P. Di Santo, Phillippe J. Sansonetti, Fritz, Pabon WS, Antonio GE, Magalhaes JG. 2010. Th17 Cells Are the Dominant T Cell Subtype Primed by *S. flexneri* Mediating Protective Immunity. J Immunol. 184 : 2076-2085

Shin JS, Abraham SN. 2001. Cell biology. Caveolae--not just craters in the cellular landscape. Science 293(5534):1447-1448.

Sorensen , O.E. , D.R. Thapa , A. Rosenthal , L. Liu , A.A. Roberts , and T. Ganz . 2005 . Differential regulation of beta-defensin expression in human skin by microbial stimuli. J. Immunol. 174 : 4870 – 4879

Sumarno, Noorhamdani A, Samsul I, Sjoeker M and Ichinose Y, 1991. Purifikasi Protein hambatan aglutinasi Vibrio cholerae El Tor T79-6. Majalah Kedokteran Univ. Brawijaya Malang. p. 11-14.

Sperandio B., Regnaulthttp://jem.rupress.org/content/205/5/1121.full - target-3 B., Guo—J , Zhanghttp://jem.rupress.org/content/205/5/1121.full - aff-2 Z, Samuel L. Stanley Jr. Sansonetti PJ., Pédron T. 2008. Virulent *S. flexneri* subverts the host innate immune response through manipulation of antimicrobial peptide gene expression. *JEM* vol. 205 no. 5 1121-1132 The Rockefeller University Press, doi: 10.1084/jem.20071698

Strockbine NA, Maurelli AT. 2005. Genus *Shigella*. In Bergey's Manual of Systematic Bacteriology, 2nd ed. Brenner, D.J., Krieg, N.R., Staley, J.T., Eds Springer: New York, USA 2B, 811-823.

Thomas S, Preda-Pais A, Casares S, Brumeanu TD. 2004. Analysis of lipid rafts in T cells. Mol Immunol 41(4):399-409.

Todar K. 2012. *Shigella* and Shigellosis. www.textbookofbacteriology.net. Diakses Oktober 2012

Tokuhara D, Yuki Y, Nochi T, Kodama T, Mejima M, Kurokawa S, Takahashi Y, Nanno M, Takaiwa F, Honda T, Kiyono H. 2010. Secretory IgA responses induced by rice-based oral CTB *crude* vaccine are solely responsible for antibody-mediated long-standing cross-protection against *Vibrio cholerae* - and enterotoxigenic *E. coli* -induced diarrhea. *The Journal of* Immunology. 184: 46.3

Tortora Gerard J, Funke Berdell R, Case Christine L. 2010. Microbiology an Introduction. Tenth edition. Benjamin Cummings

Torres A.G. 2004.Currents aspects of *Shigella* pathogenesis. Rev LatinoamMicrobiol. 46 (34): 89-97.

Umemura M, Yahagi A, Hamada S, Begum MD, Watanabe H, *et al*. 2007. IL-17-mediated regulation of innate and acquired immune response against pulmonary Mycobacterium bovis bacille Calmette-Guerin infection. J Immunol. 178: 3786

Uni, Z., Y. Noy and D. Sklan. 1999. Posthatch development of small intestinal function in the poult. Int. J. Poult. Sci., 78: 215-222.

UNICEF , Kearney, A. 2013.. Semakin banyak anak bertahan hidup melewati usia lima tahun. http://www.unicef.org/indonesia/id/media_23111.html

Utami WU., Setyorini D., Agustina W., Sumarno. 2012. Respon Imun s-IgA molekul adhesin *S. flexneri* 7,9 kDa dan 49,8 kDa terhadap sekresi cairan usus mencit. RISBIN IPTEKDOK 2012

Wang L, Cao B, Liu B, Li Y, Liu D, Gao Q, *et al*. 2009. Molecular detection of all 34 distinct O-antigen forms of *Shigella*.Jour.of Medical Microbiology. 58: 69–81

Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. 2004. IL-22 increases the innate immunity of tissues. Immunity 21:241–254

Xu, B., Wagner, N., Pham, L. N., Magno, V., Shan, Z., Butcher, E. C. & Michie, S. A. 2003. Lymphocyte Homing to Bronchus-associated Lymphoid Tissue (BALT) Is Mediated by L-selectin/PNAd, α4β-integrin/VCAM-1, and LFA-1 Adhesion Pathways. J. Exp. Med. 197: 1255–1267

Yang , D. , A. Biragyn , D.M. Hoover , J. Lubkowski , and J.J. Oppenheim . 2004 . Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. Annu. Rev. Immunol. 22 : 181 – 215

Yang , D. , O. Chertov , S.N. Bykovskaia , Q. Chen , M.J. Buff o , J. Shogan , M. Anderson , J.M. Schroder , J.M. Wang , O.M. Howard , and J.J. Oppenheim . 1999 . Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science . 286 : 525 – 528

Yang, D., A. Biragyn, L.W. Kwak, and J.J. Oppenheim. 2002. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol 23:291-296.

Yang , D. , Q. Chen , D.M. Hoover , P. Staley , K.D. Tucker , J. Lubkowski , and J.J. Oppenheim . 2003 . Many chemokines including CCL20/MIP- 3alpha display antimicrobial activity. J. Leukoc. Biol. 74 : 448 – 455

Zasloff , M. 2002 . Antimicrobial peptides of multicellular organisms. Nature .Microbes Infect. 415 : 389 – 395.

Ziegler SF and Buckner JH. 2009. FOXP3 and the Regulation of Treg/Th17 Differentiation. Microbes Infect doi: 10.1016/j.micinf.2009.04.002