

Peran ZFH-04269, Pilicides ec240, dan Mannosides sebagai Inhibitor FimH Adhesins Uropathogenic *Escherichia coli* (UPEC) dan Multiplex Real-Time PCR sebagai Metode Potensial untuk Diagnosis Infeksi Saluran Kemih

*The Role of ZFH-04269, Pilicides ec240, and Mannosides as FimH Adhesins Inhibitor of Uropathogenic *Escherichia coli* (UPEC) and Multiplex Real-Time PCR as a Potential Method in Diagnosing Urinary Tract Infections*

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ABSTRAK Infeksi saluran kemih (ISK) mungkin sulit untuk didiagnosis dan oleh karena itu dibutuhkan metode yang lebih cepat dan lebih murah tetapi masih memiliki hasil kehandalan yang tinggi. Multiplex real-time PCR dapat mendiagnosis ISK dengan lebih baik, karena memiliki akurasi dan efisiensi waktu yang tinggi. Di sisi lain, karena banyak infeksi disebabkan oleh perlekatan bakteri pada sel, terapi anti-adhesi dapat menjadi pilihan untuk metode pengobatan. Tujuan kami adalah untuk menganalisis apakah PCR multipleks waktu-nyata dan terapi anti-perekat dapat menjadi metode terbaik untuk masing-masing pilihan diagnosis dan perawatan. Hasil menunjukkan bahwa multiplex real-time PCR layak digunakan untuk mengidentifikasi uropatogen dengan akurasi lebih dari 90%; sementara isoquinolone-mannoside yang dimodifikasi terbukti memiliki afinitas FimH tertinggi dan potensi antagonis yang dilaporkan hingga saat ini. Akhirnya, kami menyimpulkan bahwa PCR real-time multipleks dan penggunaan ZFH-04269, pilicides ec240, dan mannosides sebagai inhibitor adhesin FimH mungkin dapat

ABSTRACT

diterapkan sebagai metode diagnostik baru dan pengobatan untuk ISK.

Urinary tract infections (UTIs) can be difficult to diagnose and therefore a quicker and cheaper method but still having a high reliability result is needed. Multiplex real-time PCR can diagnose UTI better, as it has a high accuracy and time-efficiency. In other hand, as many infection is caused by bacterial attachment to cells, anti-adhesion therapeutics can be an option for treatment methods. Our objective is to analyse whether real-time multiplex PCR and anti-adhesive therapeutics can become the best methods for diagnosis and treatment options respectively. Results showed that it is feasible to identify uropathogens with more than 90% accuracy by using multiplex real-time PCR; while modified isoquinolone-mannoside is proved to have the highest FimH affinity and potency of any antagonists reported to date. Finally, we conclude that multiplex real-time PCR and the use of ZFH-04269, pilicides ec240, and mannoses as FimH adhesins inhibitor might be applicable as novel diagnostic method and treatment for UTIs.

INTRODUCTION

Urinary tract infections (UTIs) caused by Uropathogenic Escherichia coli (UPEC) affect 150 million people every year worldwide. More than 80% of Urinary Tract Infections are caused by E. coli (Flores et al., 2015). E. coli is a type of bacteria commonly found in gastrointestinal (GI) tract. This bacterium could also spread to the urinary tract, thus, causing problems such as infection of the upper urinary tract (pyelonephritis) and infection of the lower urinary tract (cystitis) (Schmiemann et al., 2010).

UTI can be difficult to diagnose. Often, patients are treated on symptoms alone and the diagnosis of UTI by clinical criteria alone has an error of 33% (Zee et al., 2016; Mambatta et al., 2015). The gold standard for the diagnosis of Urinary Tract Infection is the detection of pathogen in the

presence of clinical symptoms. The pathogens is detected and identified by urine culture. But this method is time consuming, showing a low sensitivity in the primary care setting and the minimum level of bacteria demonstrating an infection has yet to be cleared (Greene et al., 2014).

In this case, surely, a quicker and cheaper method but still having a high reliability result is needed. An example is done by using urine dipstick analysis that can check urine's property like gravity, pH, urobilinogen, glucose, ketone, blood, leukocyte esterase and nitrite for UTI screening. This method unfortunately is not quite reliable as the error in the interpretation of dipstick itself is 15-30% (Aspevall et al., 2000). Therefore, another novel method is needed (Greene et al., 2014).

Another way to diagnose UTI that has proven a high accuracy, high

sensitivity, and could identify the cause of UTI within hours is the real-time multiplex PCR. The idea is, by targeting a single copy gene to allow for identification and semi quantification of bacteria present in the urine sample. By using the real-time multiplex PCR, it is not limited to detection of only a single pathogen or detect only Gram-positive and Gram-negative bacteria, but it is capable on detecting a large number of pathogens by using a large number of genus/species specific probe (Mambatta *et al.*, 2015).

UTI commonly is treated by antibiotics. However, we are now facing a problem with antibiotic usage as many microorganisms has developed some drug-resistant gene and therefore alternatives ways of fighting UTI is needed (Greene *et al.*, 2014; Jarvis *et al.*, 2016). As many infections are caused initially by bacterial attachment to cells, anti-adhesion therapeutics can be an option for a prevention and treatment methods. Bacteria use their pili to attach their cell to human healthy cell for beginning the colonization and biofilm formation. These pili is assembled by using chaperone-usher pathway and this is found in bacteria including *Escherichia coli*, *Klebsiella*, *Pseudomonas*, *Haemophilus*, *Salmonella*, and *Yersinia*, where uropathogenic *E. coli* (UPEC) is the most dominant bacteria causing UTI (Jarvis *et al.*, 2016). Mostly UPEC has type 1 fimbriae mediate-host pathogen interaction which play an important role in pathogenesis (Totsika *et al.*, 2013). The FimH adhesins, located at the tip of type 1 fimbriae binds to mannosylated glycoproteins on human and mouse

bladder epithelial cell, so the UPEC cells can invade. After the invasion, UPEC can penetrate through the endocytic vesicle and then replicate itself within the urothelial cytoplasm, forming intracellular bacterial communities (IBCs) that resembles biofilm (Costa *et al.*, 2015).

Recently, the emersion of a multidrug-resistant phylogenetic group of *E. coli* which causes major pathogenic UTI has led the scientist to seek further explanation about what factors contribute to the successful of *E. coli* dissemination. Generally, the combination between antibiotic resistance and virulence characteristics may strengthen the colonization of bacteria inside a cell and therefore provide a suitable environment for the replication process to occur (Neamati *et al.*, 2017).

At least there were three potential virulence factors, such as numerous adhesins, autotransporters, and siderophore receptors (Totsika *et al.*, 2013). For example, mostly *E. coli* ST131 coding several types of beta-lactamases, particularly of the CTX-M family of extended-spectrum beta-lactamases (ESBLs). In the other hand, this strain of *E. coli* is resistant to 8 classes of antibiotics, including cephalosporins, fluoroquinolones, and aminoglycosides (Tal *et al.*, 2015). Unfortunately, the virulence mechanism and widespread remain poorly defined.

In attempt to prevent and treat the infection of multidrug-resistant *E. coli*, which means antibiotic has no longer effect on it, a novel mannosides FimH inhibitors including compound ZFH-04269 (4'-[a-D-

Mannopyranosyloxy] - N,3' - dimethylbiphenyl - 3 - carboxamide) (Neamati *et al.*, 2015) were introduced to weaken UPEC virulence in mice by interfering FimH binding to the bladder epithelium. Within a few hours, the bladder bacterial titers will decrease significantly during both acute and chronic cystitis. This novel method can become alternatives for antibiotic usage, as well as treat prolong biofilm formation (Greene *et al.*, 2014; Jarvis *et al.*, 2016).

MATERIAL AND METHODS

Source of reference is from Google Scholar and ProQuest. Keywords to find reference are *Escherichia coli*, UTI, Anti-adhesive, and PCR and only include a journal in the past 6 years. English journal is the only language that included during reference searching.

RESULTS AND DISCUSSION

An examination aims to know whether *E. coli* EC958 could induce acute infection of urinary tract was done using eight-weeks old C3H/HeN female mice. The mice were inoculated with 2×10^7 CFU of *E. coli* EC958, a multidrug-resistant phylogenetic group B2 strain of serotype O25b:H4 and sequence type 131, using transurethral catheter. The colonization ability of *E. coli* EC958 was examined by determining bacterial CFU in the mouse bladder and kidneys at 6, 16, and 24 hours after infection (Hannan *et al.*, 2010).

At 6 hours after infection, the IBC formation appears in all infected bladders, indicates that *E. coli* EC958 has an ability to invade urothelial cells and proliferate inside the cell to establish a strong colony of IBC. Large IBC are shown by confocal microscopy using immunofluorescence labelling of *E. coli* EC958-infected bladders (Figure 1) (Hannan *et al.*, 2010).

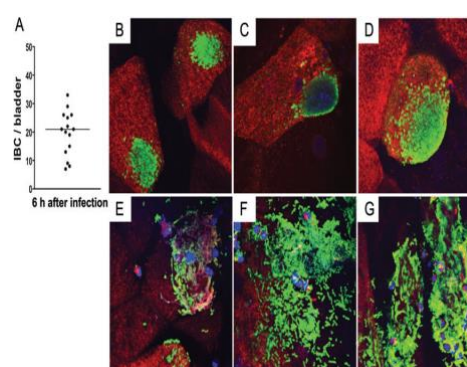


Figure 1. *Escherichia coli* ST131 pathway through IBC during acute bladder infection in female C3H/HeN mice.

By 16 hours after infection, most IBC-containing urothelial cells will burst and secrete *E. coli* EC958 cells in rod or long filamentous-shaped bacteria (Hannan *et al.*, 2010).

There is also a straight correlation between the number of bacterial titers with the possibilities of *E. coli* EC958 to develop chronic cystitis. This might be caused by inflammation process which occur during the invasion, and furthermore causes a grossly enlarged bladder from mice with persistent bacteriuria (Najeeb *et al.*, 2015). Research done by Mambatta, et.al. proved that usage of dipstick assay for screening UTI may not be very

sensitive if only using one parameter (Mambatta *et al.*, 2015). When compared to urine culture method which is the standard method to diagnose UTI (Jarvis *et al.*, 2016; Najeeb *et al.*, 2015), sensitivity achieved by using only nitrite can reach just 23.31% accuracy. Using only leukocyte esterase parameter increase sensitivity to 48.5% while the highest sensitivity using only one parameter is proved by using the blood trace parameter which is 63.94%.

Combination of two parameters can give a higher sensitivity. The highest sensitivity using two parameters proved by using both leukocyte esterase and blood parameter that showed up to 72.28%. When all the parameters in this study is combined, the result is much higher that is 74.02% (Jarvis *et al.*, 2016). Judging by the fact that the sensitivity of using dipstick assay cannot be very reliable especially if the urine tested has only nitrite as a sign of UTI, this may result into a false-negative for UTI. Person with symptoms of UTI but do not get a positive result from using dipstick is suggested to undergo for a urine culture check (Jarvis *et al.*, 2016; Demilie *et al.*, 2014).

Eight out of ten of UTI are caused by *E. coli*. A study has been conducted using the PCR to specifically detect this uropathogenic bacteria *E. coli* by targeting gene LacZ that encode for the enzyme Beta-galactosidase, also known as lactase which responsible for breaking down disaccharide into glucose and galactose, thus producing energy needed by the bacteria. Urine samples were collected from UTI's patients that were diagnosed according to the presence of pyuria. A classical

culture method for detection of *E. coli* was recorded in the presence of *E. coli* in 20 out of 30 UTI samples (66%) and in the other hand by using PCR and targeting a specific LacZ gene, *E. coli* was found only in 18 out of 30 UTI samples (60%). However, this difference between classical culture and the PCR method for the detection of *E. coli* was non-significant ($p=0.05$) and this may due to the presence of an evolved Beta-Galactosidase LacY in some strain of the *E. coli* which has the same activity or expression with LacZ gene. This experiment strongly encouraged using PCR test that is faster and perfect for *E. coli* diagnosis on molecular level (Ibraheam *et al.*, 2016).

On the other hand, A study done by Zee, et al. shows that it is feasible to detect and identify several uropathogens, not only *E. coli*, with multiplex real-time PCR assay. To prove his hypothesis, they did an experiment with urine specimens collected from 211 hospitalized or outpatients, which 57% of them were female with mean age of 54 years and 43% were male with mean age of 60 years, and all of them were suspected for having an UTI. The experiment was done by comparing multiplex real-time PCR, culture method, and the commercial kit of Seegene for detection of uropathogen (Ibraheam *et al.*, 2016).

First, the PCR and culture method are compared. Urine specimens of 211 patients were analyzed in parallel with a cut-off value of Cq 33 was applied to PCR since the value sets the limit for approximately 10^3 bacteria per ml. The result of this comparison with 211 specimens are: 86 patients were negative both by culture (CFU<103),

and by PCR ($C_q > 33$). 62 patients were positive in PCR but only 44 patients gave a positive result in culture method. 18 patients were PCR positive but unidentified with culture, and there are also 10 patients with positive result in culture but could not be confirmed by species specific PCR, but some are weakly supported by 16S based PCR. 53 patients had inconclusive result both in PCR and in culture method. Of 44 PCR positive specimens, 2 specimens were positive for 3 uropathogens but only one was confirmed by culture. Seven specimens were PCR positive for 2 uropathogens, 4 of which were culture positive for one, 2 were culture confirmed for both, and one specimen was culture negative for the PCR detected pathogens but positive for *Streptococcus spp.*, which is not detected by PCRs. PCR detected single pathogens in the remaining 35 specimens, and all were confirmed by culture. In 5 cases an additional potential pathogen was cultured. Of 18 PCR positive specimens, 4 were positive for 2 pathogens, but culture results were inconclusive. The mean C_q value of these PCRs was 29.0, and the mean C_q value of culture confirmed PCRs was 26.9. Among the specimens, the highest rate of culture positivity was found when C_q values were lowest. This concluded that the concordance of PCR and culture is 40% due to the higher sensitivity of PCR. The concordance is 98% (43/44), when culture results are available (Zee *et al.*, 2016). An experiment done by Lutz *et al.*, also showed a similar result with the concordance of 97,5% (81/83) between PCR and culture method (Lehmann *et al.*, 2010).

The second experiment done by Zee *et al.*, is aimed to make a comparison between the PCR and Seegene kit. The PCR result of urine specimens of 83 patients (subgroup) were compared with the Seeplex UTI ACE Detection Kit. The detection of *Citrobacter spp.* and *Enterobacter spp.* was omitted because they could not be detected using the Seegene kit. *S. saprophyticus* was not detected at all, and was omitted as well. The concordance between the two methods is high ($p = 0.0433$). Most notable is that of all PCR positive *E. faecalis*, Seegene Kit also gave positive results. Of 47 Seegene negatives, 12 were culture positive for *E. coli*, one for *Enterococcus spp.*, one for *K. pneumoniae*, and one for *P. mirabilis*. In four specimens, inhibition of PCR occurred. All Seegene positives were also identified with real-time multiplex PCRs. This concluded that of all Seegene positive specimens 48% (15/31) were concordant with the results of the culture.

Another experiment related to the effectiveness of a commercially available SeptiFast multiplex real-time PCR compared to dip-slide and microbiological culture was done by Lutz *et al.* A total of 82 urine samples were collected from 81 patients suspected with UTI. The result was out of 82 samples, 61 samples were Septifast positive but 21 samples were negative, whereas 67 samples were positive and only 15 samples were negative with dipslide culture method.

Comparison of Gram-positive pathogens showed a result of 65/82 (79%) concordance of Coagulase-negative staphylococci as the lowest concordance level whereas a result of

81/82 (99%) concordance of *Streptococcus pneumoniae* as the highest concordance level. Overall concordance of Gram-positive group was 371/410 (90%). In the Gram-negative group, the pathogen concordance ranged between 77/82 (94%) and 81/82 (99%) with the lowest concordance for *E. coli*. Overall concordance of Gram-negative group was 477/492 (97%). Finally, in the fungi group, the pathogen concordance ranged between 75/82 (91%) and 82/82 (100%) with an 238/246 (97%) of overall concordance for the fungi group. This experiment proved that the overall concordance between the 4 hours Septifast multiplex real-time PCR and the 48 hours culture method is more than 90%, thus, it might be applicable in principle for the qualitative identification of urinary tract pathogens (Lehmann *et al.*, 2011).

After we know how to diagnose the emergence of UPEC strain in most cases of fatal infection, the further step is to give the best treatment.

The FimH adhesins inhibitor, as previously been explained, are tested to impede *E. coli* EC958 attachment to the adhesin surface of urothelial bladder. A research done by using two groups of female mice, where one group are given 1 oral dose of compound ZFH-04269 (50 mg/kg) and the other group are given PBS (Phosphate Buffer Saline). The delivery of these treatments came in two different times, and therefore need the two groups for each treatment: First treatment is done by 30 minutes before the intraurethral inoculation with *E. coli* EC958 (10^7 CFU), and the second one is done at day 14 after infection (Naveen *et al.*, 2015).

As a result, the mice treated with compound ZFH-04269 has a significant bacterial clearance in its bladder within 6 hours. This remarkable result not only suitable for *E. coli* EC958, but also applicable to other types of UPEC strains that have different virulence profiles but express type 1 fimbriae, which has a similar pathway that result in a common symptom of cystitis (Naveen *et al.*, 2005).

In contradiction, there was no effect obtained from the treatment of ZFH-04269 on kidney's bacterial colonization. This phenomenon is caused by the susceptible to vesicoureteral reflux, where kidney colonization is typically not dependent in type 1 fimbriae (Soylu *et al.*, 2016).

On the other hand, Greene *et al.*, year? stated that pilicides ec240 can be potential to inhibit the adhesion of type 1 pili of Uropathogenic *Escherichia coli* strain UTI89. This can be done by disrupting the production of the pili and inhibit its biofilm production if the treatment is done even after the adhesion has persisted (Chorell *et al.*, 2012). Result showed that pilicides ec240 can reduce HA titer from 2^8 to 2^3 which means that it can successfully minimize the adhesion of UPEC to the red blood cell, hence the reduction of the HA titer (Piatek *et al.*, 2013). RNA seq showed that *pap* and *sfa* gene that encodes for P and S pili is upregulated 49-folds and 25-folds respectively. Although this gene is upregulated, results from immunoblot assay for *sfa*A subunit showed a degradation for its band. This means that pilicides ec240 can somehow inhibit the translation or blocked S pilus assembly by inhibiting chaperone-subunit interaction, a

process where UPEC's 9 pili is constructed before adhere to the epithelial cell in the urinary tract (Chorell *et al.*, 2012; Piatek *et al.*, 2013). As for *pap* gene of UTI89 encodes PapGIII that cannot bind to human red blood cell, plasmid pFJ29 is used to encodes PapGI to test the potential of pilicides ec240. HA titers analysis proved that there is a reduction of P piliation up to 2-folds. RNA Seq also showed that there is a downregulation for *fim* gene that encodes type 1 pili up to 17-folds. These results suggest that using pilicides ec240 can disrupt the process of adhesion from UPEC's pili to epithelial cells in urinary tract and therefore has the potential to prevent and treat UTI (Piatek *et al.*, 2013).

Another chemical substance can also become an anti-adhesive agent like pilicides. Mannosides can be used to prevent adhesion of pili type-1 from UPEC to epithelial cells of urinary tract (Guiton *et al.*, 2012; Cusumano *et al.*, 2011). As the structure of molecules is different from pilicides, further modification of mannosides can achieve the highest FimH affinity and potency of any antagonist reported to date with HAI can resulted up to 1 nM. This mannosides is modified by introducing an *ortho*-methyl group on the A-ring of the biaryl glycone and isoquinolone on the B-ring of mannosides (A-ring *o*-methyl B-ring isoquinolonemannoside). At this time, it will reduce HAI amount from 0.5 μ M achieved by the preliminary biphenyl mannosides to 0.031 μ M and reduce biofilm Inhibitor Concentration (IC) from 1.35 μ M to 0.13 μ M. Bioavailability is also increased by 7%, CFU is reduced 10-fold at 6 hours post dosing compared to the mannoside

which only receive *ortho*-methyl group. Modification by isoquinolone substitution with alkyl bromides, acetyl deprotection, saponification and followed by standard peptide coupling to various amines, will then reduce HAI from 0.031 μ M to 1 nM, which is the highest FimH affinity and potency of any antagonists reported to date. In addition to that, it can also reduce biofilm IC to 20 nM.¹⁶ Furthermore, multivalent-mannoside modification strategy, in which mannosides are grafted in multiple copies onto a common scaffold, conducted by Chalopin *et al.* did not result in a better inhibition effectivity toward *E. coli* UTI89. This suggest that monovalent mannoside still a better choice for UTI treatment (Chorell *et al.*, 2012).

CONCLUSION

The use of multiplex real-time PCR are proved 12 times faster than the conventional culture method and 4.5 times faster than the commercial kit of Seegene for detection of uropathogen, with sufficient accuracy and specificity mainly focused to identify Uropathogenic *Escherichia coli* (UPEC) strains. In other hand, due to the increase of antimicrobial resistance among UPEC strains, the use of ZFH-04269, pilicides ec240, and mannosides as FimH Adhesins Inhibitor might be applicable as novel treatment for common urinary tract infections.

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