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Diagnosis of some Common Uropathogens from Patients with Urinary Tract Infection Symptom in Vietnam by PCR Method

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ABSTRACT: Urinary tract infections (UTIs) are frequent disease in Vietnam. Timely, sensitive and accurate identification of uropathogens is great value for UTI management. In this study, 66 urine specimens were collected from patients with UTI symptoms in Saint Paul Hospital, Vietnam. By PCR, specific genes of *E. coli, E. faecalis, P. aeruginosa, P. mirabilis, K. pneumoniae* were amplified from 46 samples (71.2%) and 71.7% were monopathogens. The dominant uropathogens were *E. coli* (36.4%), *E. faecalis* (34.8%), *P. aeruginosa* (18.2%). *E. coli* was important agent in multi-bacterial infection. Conventional culture detected bacteria in 30.3% samples but had limitation in the detection of *Enterococcus, P. aeruginosa.* Moreover, of the 35 negative cultured-samples, we also found the bacteria in 25 samples. This finding suggests that PCR is a good method for *E. feacalis, P. aeruginosa* detection and is the fast, sensitive method for supplementing conventional urine culture to identify uropathogens.

Keywords: *E. coli*, *E. faecalis*, *K. pneumoniae*, PCR, *S. saprophyticus*, *P. aeruginosa*, *P. mirabilis*, uropathogens.

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INTRODUCTION

Bacterial urinary tract infection (UTI) is one of the most commonly occurring community-acquired infections and mainly caused by *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Staphylococcus saprophyticus* (Flores-Mireles *et al.*, 2015; Nguyen, 2011). Timely and accurate identification of uropathogens plays an important role for the personalized treatment to increase treatment quality, to reduce cost, drug side effect, uncomfortable feelings, and also restrict the development of antidrug resistant bacteria.

Since the 1950s, Kass established a guideline for diagnosis of urinary tract infections (UTIs) using standard clinical urine culture protocol. Accordingly, patients will be diagnosed with UTI if bacterial counts in urine of $\geq 10^5$ CFU/ml (Kass 1957). This method is widely used in hospitals over the world and particularly in Vietnam and seemed as a gold standard for pathogen identification. However recent researches aware that number of 10^2-10^3 CFU/ml urine is afford to cause UTI (Le *et al.*, 2004; Krcmery *et al.*, 2001). However, standard urine culture protocol only detected 33% of all detected uropathogens, 50% of those detected in the UTI cohort, and only 7% of those detected the non-UTI cohort (Price *et al.*, 2016). Recent evidence reports bacteria in ~90% of "no growth" standard urine cultures (Hilt *et al.*, 2014; Pearce *et al.*, 2014).

Although some improved culture methods are being investigated (Price *et al.*, 2016; Hilt *et al.*, 2014), bacterial culture protocols implemented in clinical practice remain limitation (Price *et al.*, 2016). The first is the time-consuming (18 h for culturing results, and 48h-62h for culture report with antimicrobial susceptibility testing (Davenport *et al.*, 2017; Palmqvist *et al.*, 2008)) leading to the therapeutic regimen is often not culture-based. The disadvantages of the delayed or inappropriate antimicrobial therapy give an unfavorable condition for patients and develop pathogen resistances (Lehmann *et al.*, 2011; Kuti *et al.*, 2008; Kumar *et al.*, 2006; Elhanan *et al.*, 1997). The second is the sensitivity (only ~50% UTI samples are culture positive) (Heytens *et al.*, 2017; Price *et al.*, 2016).

Compared with culture-base methods, polymerase chain reaction (PCR) is faster, more sensitive and more specific to detect bacterial strains (Lehmann *et al.*, 2008). The development of multiple PCR to simultaneously detect some common uronpathogens from UTI patients are interested by many research groups over the world (Zee *et al.*, 2016; García *et al.*, 2015; Tajbakhsh *et al.*, 2015).

This study evaluates the feasibility for indentifying common uropathogens (*Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus mirabilis*, and *Staphylococcus saprophyticus*) in urine from Vietnamese UTI patients using polymerase chain reaction (PCR) method with primers designed by Garcia *et al.*, (2015) (García *et al.*, 2015) and to compare the results with the conventional urine culturing.

MATERIALS AND METHODS

A. Research conducted

Research conducted at Genetic Engineering Lab, Institute of Biotechnology, VAST, 18, Hoang Quoc Viet, Hanoi, Vietnam.

B. Urine sample preparation and conventional urine culture

The inclusion criteria were urological patients with age ≥30 years having at least one of the symptoms: strong, persistent urge to urinate; burning sensation when urinating; passing frequent, small amounts of urine; urine appears cloudy; strong-smelling urine or pelvic pain. The patients (66 patients) were requested for urine sampling according to St. Paul hospital guideline for preventing artificial contamination. Every patient was requested to take at least 10 ml of midstream urine sample then the samples were analyzed directly by electron microscope. A sample was regarded as a positive infection (total 55 urine samples with CFU of bacteria is not lower than 10^5/ml urine, number of leukocyte is not lower than 10 cells/ml urine) was cultured for bacterial pathogen detection; whereas, negative samples (11 urine samples) were not cultured. The urine specimens were cultured and analyzed at Microorganism department of St. Paul hospital according to standards of local hospital guidelines.

C. Bacterial DNA extraction

All urine specimens (66 samples) were stored at 4°C for up to 6 h after collection and transferred to National Key Laboratory of Gene Technology, Genetic Engineering Laboratory, Vietnam Academy of Science and Technology for analysis by PCR. The samples was centrifuged at 600 rpm (approximately 150 -200 X*g*) to separate bacteria from other cells. A 10 ml of the supernatant containing bacteria was centrifuged at 3,000 X*g* for 15 minutes at 10°C to remove cell-free DNA. Urinary pellets were washed twice with a 10-fold volume of PBS (137 mM NaCl, 2.0 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2.7 mM KCl, pH 7.4). The pellets were suspended in 300 µl of 10% Chelex® solution (TaKaRa, Japan), heated at 100^{o}C for 5 minutes, then quickly cool by placing on ice for 1 minute or more. The samples were centrifuged at 12 000 rpm (approx. 13 000 X*g*) for 1 minute. The supernatants were transferred into new Eppendorf tubes, and the quality and quantity of the DNA in supernatants were determined using a NanoDrop ND-2000C spectrophotometer (Isogen, The Netherlands).

D. PCR for amplification of specific genes of E. coli, S. saprophyticus, E. feacalis, P. aeruginosa, P. mirabilis, K. pneumoniae

Based on the multiplex-PCR primers designed by Garcia *et al.*, (2015) for amplification of specific fragments of *FimH* (235 bps) from *E. coli*, rRNA 16S (741 bps) from *S. saprophyticus*, rRNA 16S (440 bps) of *E. feacalis*, *ETA* (505 bps) of *P. aeruginosa*, *ZapA* (571 bps) from *P. mirabilis* and *FimK* (315 bps) from *K. pneumoniae* (García *et al.*, 2015), all the fragments were amplified from the DNA samples extracted from urine specimens by PCR.

The touchdown and touchup reaction was performed in 25 µl reaction mixture containing 0.25 µl of each forward and reverse primers (10 µM), 2.5 µl of 10× solution buffer (Thermo Scientific), 2 µl of four mixed dNTPs (2 mM, Thermo Scientific), 0.25 µl of 5 U/µl Dream Taq DNA polymerase (Thermo Scientific), 1.25 µl (60 ng/µl) of template DNA. The touchdown and touchup PCR programes are described in Table 1. The PCR products were run on 1% agarose gel and visualized by ethidium bromide staining on UV light. Some PCR products were purified by QIA quick Gel Extraction kit (QIAGEN) and followed by direct sequencing using a ABI3100 sequencer (AB Applied Biosystems, USA).

Table 1: Touchdown and touchup PCR programs for amplification of the fragments of *fimH* from *E. coli*, rRNA 16S from *S. saprophyticus*, rRNA 16S of *E. feacalis*, *ETA* of *P. aeruginosa*, *ZapA* from *P. mirabilis* and *FimK* from *K. pneumonia.*

Specific genes' fragments from:	PCR programs				
	Step 1	Step 2 (30-35 cycles), reduce or increase of anealling temperature in 2^{o}C every 5 cycles			Step 3
E. coli, S. saprophyticus	94°C, 4 min	94°C, 1 min	65→53^{o}C, 1 min	72°C, 1 min	72°C, 10 min
E. faecalis, P. aeruginosa			60→48^{o}C, 1 min		
P. mirabilis, K. pneumoniae			50→62^{o}C, 50 sec		

RESULTS AND DISCUSSION

A. Capacity of PCR for detection of E. coli, S. saprophyticus, E. feacalis, P. aeruginosa, P. mirabilis, K. pneumoniae in urine samples

The overview picture of pathogen detection by PCR and by microbiological culture method was described in Table 2.

Of the 66 received urine specimens, by directly urine visualization by electron microscope 11 patients without UTI and 55 patients (83.3%) had a suspected UTI. Among the 55 specimens from suspected patients, microbiological culture method detected pathogens in 20 specimens (accounted for 36.4% cultured samples or 30.3% samples from 66 patients). However, by PCR, the specific genes of *E. coli*, *E. faecalis*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae* were amplified from 40 of 55 positive samples (accounted for 72.7%) and 46 of 66 samples from patients (accounted for 71.2%) (Fig. 1, 2, Table 1). The DNA of *S. saprophyticus* was diagnosed in 14 urine samples (Fig. 1). Thus PCR is higher sensitivity than culture for detection of the bacterial pathogens in urine samples.

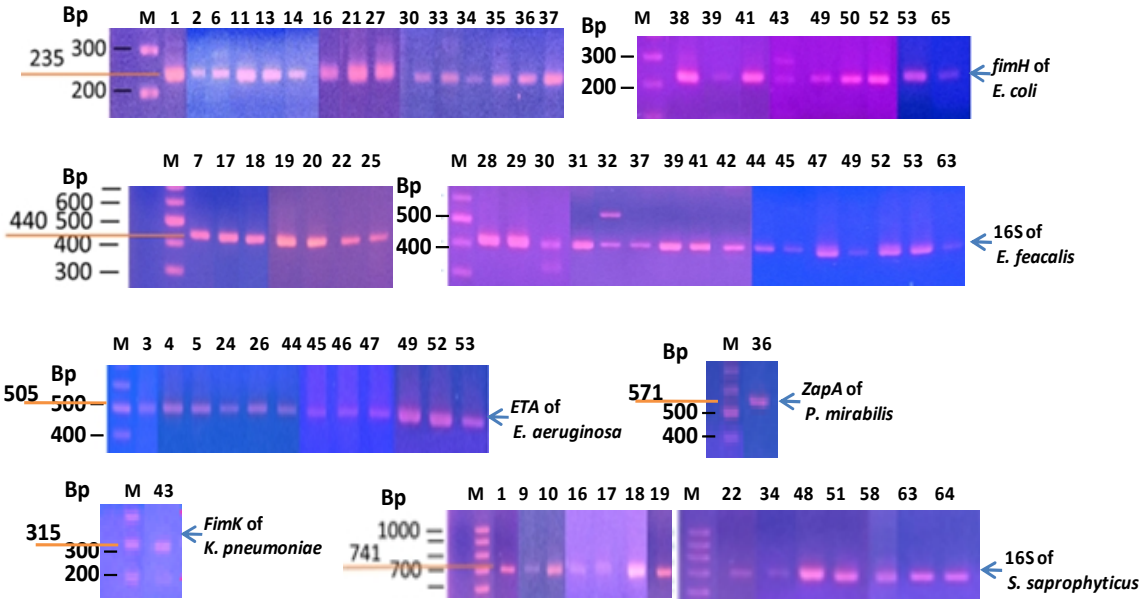

Fig. 1. Agarose gel electrophoresis (2%) for analysis of PCR products amplified specific genes of *E. coli, S. saprophyticus, E. feacalis, P. mirabilis, P. aeruginosa, K. pneumoniae from 66 urine specimens. (*M. 100 bp DNA ladder (Fermentas); the numbers indicate corresponding numbers of urine samples).

For sure of the amplified genes derivation, we randomly chose 2 amplified DNA from each primer pair for sequencing. The results showed that two sequences of *fimH* fragments amplified from DNA samples number 53 and 65 were 100% identity with the corresponding gene of *E. coli* NCBI code KJ190226, FJ865803. Two sequences of *S. faecalis* 16S fragments amplified from samples 53 and 63 were 99% identify with the corresponding genes of *S. faecalis* in NCBI code HQ717186, KR809380, MK208703. Two sequences of PCR products amplified from *ETA* of *P. aeruginosa* samples 48 and 50 were 99% identity with the corresponding genes code CP030910, CP015650, CP15877 from *P. aeruginosa* in NCBI. Sequence of 16S rRNA fragment of *S. saprophyticus* that were amplified from sample 77 was 99% identity with the gene of *S. epidermidis* (code MH118521, CP034111) and from sample 78 was 95% identity with the gene from *S. haemolyticus* (code MG792305, CP025031). Thus the primers for *S. saprophyticus* are not specific. So the result for detection of *S. saprophyticus* is not accuracy, should not analyze any more.

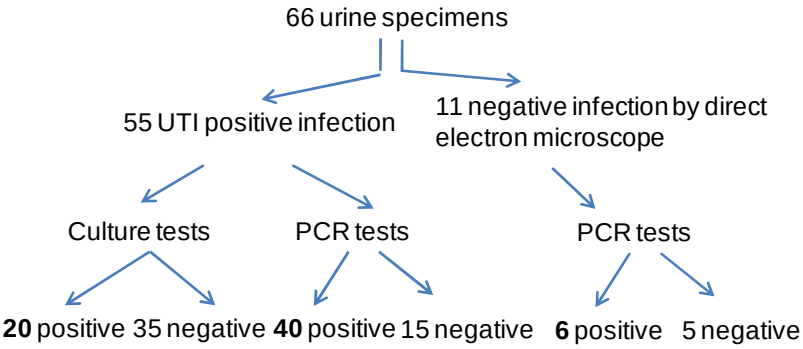

Fig 2. The distribution of the 66 urine specimens to the different tests and overview results.

Of the 20 positive cultured samples, 15 urine samples were positive with PCR. The five urine samples were negative with PCR because of the specific genes used, and will be explained later. Among the 35 negative cultured samples, 25 samples were positive with PCR. Even in the samples (11 samples) did not found bacteria by directly visualization in electron microscope, but PCR also found six samples containing the pathogens (Table 1). These results confirmed PCR is a much more sensitive than culture method.

B. The infection of E. coli, E. feacalis, P. aeruginosa, P. mirabilis, K. pneumoniae in urine samples

The PCR detected the bacterial pathogens in 46 samples but only 34 infected samples were monopathogen (accounted for 71.7%). Other research gave the same results, in which 33% of urine cultures are polymirobial infection (Cove-Smith and Almond 2007).

In the multimicrobial infection, PCR detected 3 urine samples infected 3 pathogenic bacteria (*E. coli*, *E. feacalis*, *P. aeruginosa*), nine urine samples containing 2 pathogenic bacteria (four samples infected with *E. coli* and *E. feacalis*, three samples infected with *E. feacalis*, *P. aeruginosa* and one sample infected with *E. coli* and *P. mirabilis*, one sample infected with *E. coli* and *K. pneumoniae*) (Figure 1). This result revealed that *E. coli* is important agent in multi-bacterial infection.

In the other hand, *E. coli* present in polymicrobial UTI was known to be more invasive into epithelial cell to develop the UTI (Croxall *et al.*, 2011). The infection of *E. feacalis* may accompany with *E. coli* or *P. aeruginosa* infection. In culture result, there were 4 urine samples infected with 3 pathogens but culture could not identify what were the pathogens. In agreement with culture results, 8 were positive with *E. coli*, 3 samples was positive with *E. feacalis*, 1 was positive with *K. pneumoniae* by PCR (Table 1). The reason to explain for 4 samples positive with *E. coli* by culture but negative by PCR lies in the specific gene amplified. The gene amplified from *E. coli* associated with UTI in this study was *fimH*. This gene coding for type 1 fimbriae plays important role for *E. coli* adhesion into host bladder, so it is seemed as virulent gene of *E. coli* (Jalali *et al.*, 2015). The *fimH* is present in 73% *E. coli* isolated from urine samples (Jalali *et al.*, 2015). Thus, in this study, 8 patient infected with virulent *E. coli* of 12 patients infected with *E. coli* which were detected by culture (accounted for 66.6%).

By culture, all the samples were negative with *P. mirabilis* and *P. aeruginosa*. However, by PCR, we found 12 urine samples were positive with *P. aeruginosa* and 1 sample was positive with *P. mirabilis*. Especially, the samples were negative with bacterial culture (35 samples), we also found 10 samples infected with *E. coli*, 13 samples infected with *E. feacalis*, and 10 samples infected with *P. aeruginosa* by PCR method (Table 2). In theory, culture detects live bacteria, PCR detects all a live bacteria or deadly bacteria or even DNA fragment of bacterial lysate. In this study, we intended to PCR only DNA isolated from bacteria by separating the large cells and free-cell DNA in urine before DNA extraction. And we used 75 ng of metagenomic DNA for a PCR reaction, that according to DNA from approximately 1.5×10^7 bacterial cells. Thus we supposed that PCR is much more sensitive than culture, or culture method is difficult to culture of *E. coli*, *E. feacalis* and *P. aeruginosa* if the urine samples contain multiple bacteria and the number of these bacteria was not dominant. Presently, it is found that urine is not sterile. Urine also contain many bacteria, but bacterial community in urine of healthy people is absolutely different from the bacterial community in urine of uncomplicated UTI people and different to the community of complicated UTI people (Moustafa *et al.*, 2018). This fact may reduce the sensitivity of culture method for detection of pathogenic bacteria in urine samples.

If only considering to 20 positive samples by culture test, the detected *E. coli* accounted for 60.0%, *Enterococcus sp*. corresponded for 15.0% and *K. pneumoniae* was 5.0%. This result is in agreement with the result described by Le Quang Phuong that in Vietnam, of 40 children diagnosed urinary tract infection in National Children's Hospital, 19 urine samples were positive with bacterial culture. In which, *E. coli* presents in 63.2% positive samples, *Klebsiella* accounts 15.8% cases (Le *et al.*, 2016). In general, *E. coli* is main pathogen, present in 47-54% of positive culture tested samples from UTI children in Vietnam (Nguyen *et al.*, 2010). By PCR, from 46 positive samples, 52.2% samples contained DNA of *E. coli fimH*, and 50.0% samples were positive with *E. feacalis*, 2.2% presented with *K. pneumoniae*, 26.1% infected with *P. aeruginosa* (Table 1).

Table 2: Detection of *E. coli, E. feacalis, P. mirabilis, P. aeruginosa, K. pneumoniae* in urine samples with PCR and culturing.

Culture	PCR				
	E. coli	*E. feacalis*	*P. mirabilis*	*P. aeruginosa*	*K. pneumoniae*
Trimicrobial infection (4)	2	1	0	0	0
E. coli (12)	8	3	1	0	0
Enterococus sp. (3)	1	3	0	0	0
K. pneumoniae (1)	1	0	0	0	1
No identification (35)	10	13	0	10	0
No culture (11)	2	3	0	2	0
Total (66)	**24**	**23**	**1**	**12**	**1**

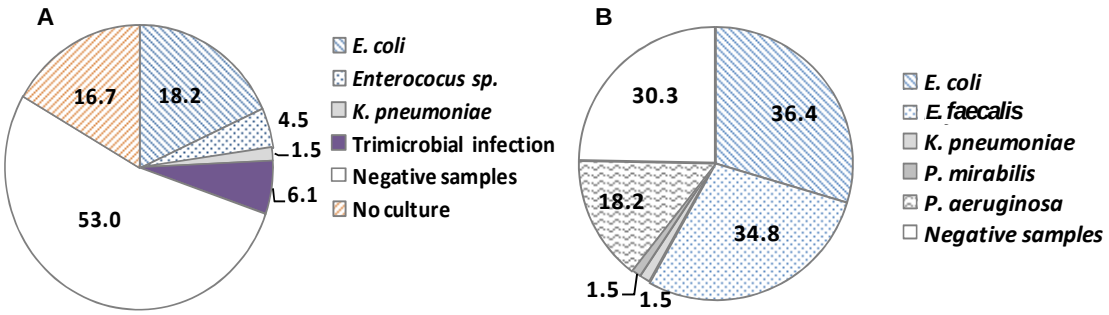

Fig 3. Percentage of the *E. coli, S. saprophyticus, E. feacalis, P. mirabilis, P. aeruginosa, K. pneumoniae* detection in urine samples from 66 symptomatic patients by culture (A) and PCR (B).

This result reveals that PCR method is better than culture method for *E. feacalis* and *P. aeruginosa* detection. In the overall view of the bacterial detection from all 66 urine samples of 66 symptomatic patients, by only culture method at the St. Paul hospital, combination of direct visualization by electron microscope and culture of urine specimens, only 30.3% samples were detected to be infected with bacteria (Fig. 2), that mean only 30.3% patients can get a targeting treatment based on bacteria infected. About 16.7% patients will not be treated because of no bacteria visualized in urine by electron microscope and 53.0% patients will be treated by general treatment regimen for unidentification of pathogen. However, by PCR, 69.7% samples were detected to be infected with at least one of these bacteria *E. coli*, *E. feacalis*, *P. mirabilis*, *P. aeruginosa*, *K. pneumoniae*. In which, 34.8% samples infected with *E. feacalis* and 18.2% infected with *P. aeruginosa* was detected by PCR and negative with culture (Fig. 3). Thus, if PCR method was used as a complimentary method with culture, number of symptomatic patients could get targeting treatment is higher (from 30.3% to 69.7%). This can help to reduce treatment period, reduce side effect by drug, economy and uncomfortable feeling for the patients. In the other hand, the time for bacterial identification by PCR was ~4 h. Thus the final results from PCR were available at least 14 h before the culturing results and 38 h before the culture report.

CONCLUSION

These findings suggest that PCR method is powerful for detection of *E. feacalis*, *P. aeruginosa* than culturing method, and almost negative-cultured samples from patients with typical urinary complaints still have an infection. PCR is the fast, sensitive and accurate method maybe supplementing urine culture for identifications of uropathogens.

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