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The use of multiplex-PCR method in identification of *Candida* species from vaginal candidiasis patients

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Abstract. *Susilawati, Rahadiyanto KY, Ramdja, Theodorus, Hermansyah. 2019. The use of multiplex-PCR method in identification of Candida species from vaginal candidiasis patients. Biodiversitas 20: 3063-3069*. Vaginal Candidiasis is an infection caused by a yeast called *Candida* which may be resulted in different virulences, thus leading to a varied drug of choice for therapy. The objective of this study was to determine the sensitivity and specificity of multiplex-PCR test in detecting *Candida* species in women with clinical diagnosis of vaginal candidiasis. There were 79 vaginal swab samples which fulfill inclusion criteria. Samples were cultured at ASD media for isolation of *Candida* spp. Identification of *Candida* spp. was conducted using both biochemical fermentation and multiplex-PCR methods. Primers pairs used in multiplex-PCR were universal primer ITS1 and ITS2, and specific primer CA3 and CA4. The identification test of multiplex-PCR resulted in *Candida krusei*: sensitivity: 100%, specificity:61,1%, Positive Prediction Value (PPV):63.2%, Negative Prediction Value (NPV): 100%; *C. glabrata*: sensitivity:0%, specificity: 100% PPV: 0% NPV: 76.7%; *C. tropicalis*: sensitivity:100%, spesificity:100%, PPV: 100% NPV: 100%; *C. albicans*: sensitivity: 33.3%, specificity: 100%, PPV: 100%, NPV: 93.1% and *C. stellatoidea*: sensitivity:: 100%, specificity:92.6%, PPV: 60%, NPV: 100%. These results suggested that multiplex-PCR method becomes a rapid alternative in identifying species of *Candida* due to its high sensitivity and high specificity.

Keywords: *Candida*, candidiasis, multiplex-PCR, fermentation

Abbreviations: ASD: Agar Sabouraut Dextrose; PPV: Positive Prediction Value; NPV: Negative Prediction Value; *C*: *Candida*; PCR: Polymerase Chain Reaction

INTRODUCTION

The implication of *Candida* sp examination in reproductive canal has to pay more attention due to their harmful effects in the women like getting leukorrhea and itching which caused abrasions and uncomfortable sexual intercourse. Candidiasis vaginalis can also cause *Candida* chorioamnionitis that led to abortus or an infant born with congenital candidiasis, and moreover more 75% women had endured at least one episode of candidiasis vaginalis during her lifetime, and around half of them have candidiasis vaginalis recurring (Omar 2001). The cause of candidiasis vaginalis at several locations in Jakarta is *Candida albicans* (62.3%) ,while non-*Candida albicans* is 30.4% which consists of *Candida glabrata* 18.8%, *Candida tropicalis* 8.7%, *Candida parapsilosis* 2.9% and mixed infection of total of 7.3% (Haryani et al. 2003). The isolation of fungi including *Candida* is generally undertaken by culturing the specimen on Sabouraud Dextrose Agar medium (SDA) who customarily used for isolation of various fungi. In this medium of all species of *Candida* grown as yeast colony or colonies such as yeasts that cannot be distinguished one another both a macroscopically and microscopically. Identification of species was done by test fermentation-assimilation and morphologically based conventional methods. During this method, fermentation-assimilation is regarded as raw gold and has been used widely and known to have high skill in identifying various species of *Candida* (Wahyuningsih et al 2012). An antibody-antigen specific test has been developed for identification of *Candida* spp., but proved low in sensitivity and specificity (Tarini et al. 2010). The success of treatment depends on the sensitivity of *Candida* species against anti-fungal. Species level identification is needed early in the case of candidiasis caused by *Candida* sp which is resistant to the azole so that alternative therapies can be immediately given. Conventional method (fermentation-assimilation) as the 'Gold Standard' has high sensitivity and specificity but require a long examination time (7-21 days) and troubles in terms of the provision of chemicals as well as the high costs. At this time many molecular tests that have been developed for identification *Candida* species and are being used due to their faster, more sensitive and more specific approach. One approach is molecular test multiplex-PCR which is the development, modification, and a variation of the method of analysis of the PCR in which two or more simultaneous amplified locus in the same reaction making it possible to have a high sensitivity and specification and low cost and also the fast result progress (Tarini et al. 2010). In the present study,

research was conducted to assess the multiplex PCR's sensitivity and specificity in diagnosing the species of *Candida* on participants who allegedly suffer candidiasis vaginalis. The research design was the identification of *Candida* spp. by two different methods namely, multiplex-PCR with biochemical test for fermentation as gold standard to compare their sensitivity and specificity.

MATERIALS AND METHODS

Materials

SDA medium consisted of 20 g/L dextrose, 10 g/L peptone, 17 g/L bacto agar, and 250 mg/ L chloramphenicol. Medium was sterilized in an autoclave at 121°C for 15 min. Cellulose-acetate filter with a pore diameter 0.2 µm yeast nitrogen base (YNB) medium (yeast nitrogen base), incubator, dextrose, galactose, sucrose, lactose, maltose and trehalose (all at a concentration of 6%), melibiose, cellobiose, inositol, D-xylose and starch, raffinose were used in fermentation test.

Samples of *Candida* sp.

Samples of *Candida* spp. were isolated from vaginal swabs of all patients suffering with candidiasis vaginalis clinic diagnosed by Doctor in Graha Sriwijaya Clinic, Palembang, Indonesia. Swab samples were taken using sterilized cotton stick. To determine total sample used, formula used is given as: $N = \{Z\alpha 2P (1-P)\}/d2$, where N = the sample size, Zα = the Z statistic for a 95% confidence level, P = sensitivity, and d = precision. It used sensitivity and specificity of *Candida tropicalis* which is 80% and 90.6%, respectively which obtained in total sample of 68 peoples. Topical vaginal swab specimens were streaked in a zig-zag manner on ASD media, incubated at 30°C for 24-48 hours. Then, color, shape, consistency, and size of the colonies of *Candida* spp. were observed. Positive colonies were indicated with creamy white color, rounded shape, curved, soft and smooth consistency form.

Procedure

Biochemical fermentation

Biochemical fermentation process was conducted according to (Wahyuningsih et al. 2012). Fungal suspension was prepared in vitro with 0.85% NaCl, which is derived from fungal colonies from SDA media. The suspension was homogenized with no clumps. Concentration of *Candida* spp. grown in the medium was adjusted to $5x10^6$ cell/ ml. Glucose, galactose, sucrose, lactose, maltose, and trehalose were used for filtration. Then incubation for 21 days at a temperature of 25°C. When the color changed from green to yellow or if it changed color and form gas in Durham tubes indicated positive result, while if there was no color changed indicated negative result. *Candida* spp. determination was done by matching the fermentation pattern formed by reference.

DNA preparation

DNA extraction procedure was carried out according to protocol with small modification (da Silva et al. 2012). Cells were grown for 1 hour until obtained 10^7 cells/mL of *Candida* spp., transferred to microtubes, and incubated at boiling water-bath for 3 x 5 min. During interval time, the suspension containing DNA was vigorously homogenized by vortex for 5x5 seconds and the tube was frozen on ice. The DNA sample was stored at -20°C. DNA concentration was measured using Spectrophotometer (SmartSpec Plus) at λ = 260 nm.

Multiplex-PCR

Analysis of multiplex PCR was carried out according to protocol with modification (Tarini et al. 2010). Primers pair used in this experiment were Universal primers ITS1: (5'-TCCGTAGGTGAACCTGCGG-3') and ITS2: (5'-GCTGCGTTCTTCATCGATGC-3'), specific primary CA3: (5'-GGTTTGCTTGAAAGACGGTAG-3') and CA4: (5'-AGTTTGAAGATATACGTGGTAG-3'). multiplex PCR was performed with a total volume of 25 µL consisting of 12.5 µL dream Taq Green PCR Master mixt 2x (Thermo Science), 0.4 µM each of ITS1 and ITS2 primers, 1.2 µM each of CA3 and CA4 primers, 2-6 ng/reaction of DNA template. Products were amplified using the following condition pre-denaturation temperature at 95°C for 15 second, and, then 40 cycles at 94°C (denaturation temperature) for 30 sec, annealing temperature at 60°C for 60 sec, extension temperature at 72°C for 45 sec followed by one cycle of 72°C for 5 min. For detection of amplification results, The PCR products were analyzed by electrophoresis using 2% agarose gel at 100 volts for 50 min. The DNA bands were detected by Gel doc 1000 with ultraviolet transilluminator (Biorad, USA), and visualized using Quantity One software (Biorad, USA). The length of products multiplex-PCR with primer pairs ITS1-ITS2 and CA3-CA4 detected by electrophoresis are: *C. glabrata* (482 bp 483 bp or 462 bp -463 bp), *C. guillermondii* (248 bp or 228 bp), *C. parapsilosis* (229 bp or 209 bp), *C. tropicalis* (218 bp or 199 bp), *C. albicans* (218 bp 219 bp or 198 bp -199 bp and 110 bp), *C. krusei* (182 bp or 166 bp), *C. lusitaniae* (148 bp or 128 bp), *C. dubliniensis* (198 bp), *C. stellatoidea* (190 bp) (Liguori et al. 2007, 2000).

RESULTS AND DISCUSSION

In this research, we used the total 30 samples, of which 28 peoples (93.3%) were of 18-45 year age group. The distribution data by age of 30 respondents can be seen in Table 1. The results were consistent with research conducted by Sobel et al. (2004) which showed the incidence of vaginal candidiasis is more common in young adult women (70-75%) and rarely occurs in children and postmenopausal women who are not taking hormone replacement therapy.

Table 1. Distribution of respondents by age (n = 30)

Age (Year)	Amount (person)	Percentage
18-45	28	93.3
>45	2	6.7
Total	30	100.0

Table 2. Distribution of respondents by contraception user (n = 30)

Type of contraception	Amount (person)	Percentage
Pill	18	60.0
Injection	6	20.0
No contraception	5	16.7
Intra Uterine Device	1	3.3
Total	30	100.0

Table 3. Distribution of culture test results using ASD agar plate (n = 79)

Culture	Amount (person)	Percentage
Positive	30	38,0
Negative	49	62,0
Amount	79	100,0

Table 4. Identification of *Candida* species with fermentation test and multiplex-PCR (n = 30)

No. sample	Fermentation	Multiplex-PCR
2	*C. tropicalis*	*C. tropicalis*
4	*C. tropicalis*	*C. tropicalis*
5	*C. stellatoidea*	*C. stellatoidea*
6	*C. krusei*	*C. krusei, C. glabrata*
7	*C. albicans*	*C. stellatoidea*
12	*C. glabrata*	*C. krusei*
13	*C. krusei*	*C. krusei*
18	*C. glabrata*	*C. krusei*
19	*C. krusei*	*C. krusei, C. glabrata*
20	*C. stellatoidea*	*C. stellatoidea, C. glabrata*
25	*C. krusei*	*C. krusei*
31	*C. glabrata*	*C. krusei*
32	*C. krusei*	*C. krusei*
35	*C. glabrata*	*C. krusei*
37	*C. tropicalis*	*C. tropicalis*
39	*C. krusei*	*C. krusei*
41	*C. tropicalis*	*C. tropicalis*
44	*C. albicans*	*C. albicans*
53	*C. krusei*	*C. krusei, C. glabrata*
54	*C. glabrata*	*C. krusei*
58	*C. glabrata*	*C. krusei*
60	*C. krusei*	*C. krusei*
61	*C. albicans*	*C. stellatoidea*
63	*C. krusei*	*C. krusei, C. glabrata*
65	*C. krusei*	*C. krusei*
74	*C. glabrata*	*C. krusei*
75	*C. krusei*	*C. krusei*
77	*C. tropicalis*	*C. tropicalis*
79	*C. krusei*	*C. krusei, C. glabrata*
80	*C. stellatoidea*	*C. stellatoidea, C. glabrata*
Amount	30	30

Table 5. Diagnostic test results *C. tropicalis* (n = 30)

		Fermentation test		
		Positive	Negative	Amount
Multiplex-PCR test	Positive	0	0	0
	Negative	7	23	30
	Amount	7	23	30

Users of contraceptive pills, the ranked first in this research with 18 respondents (60.0%), followed by as many as six people (20.0%) injection contraception users. Complete data on the distribution of respondents by contraception can be seen in Table 2. This result was consistent with several previous studies conducted by (Anindita 2006), which showed no significant correlation between the use of hormonal contraceptives with the incidence of vaginal candidiasis (66.7%). The high-estrogen oral contraceptive use increased colonization of *Candida* spp. in the vagina (Otasevic et al. 2002).

Culture test

Culture examination of 79 samples of vaginal secretions on SDA medium, 30 samples obtained positive with *Candida* infection (Table 3). After the culture with ASD medium, followed by germ tube test to distinguish albicans or non-albicans. Results of this study were higher than the prevalence of vaginal candidiasis research results 21%. This can be caused because of the majority of respondents were users of oral contraceptives. According to the theory that high levels of estrogen in birth control pills are one of the causes of vaginal candidiasis (Tasic et al. 2002).

Examination results fermentation and multiplex-PCR

Identification *Candida* spp. was conducted using two methods, fermentation and multiplex-PCR methods, and the results are prepared in Table 4. In this study, a discrepancy was found between the test fermentation with multiplex-PCR test for some species, for example, *C. glabrata* were found in the fermentation test, on the same sample by multiplex-PCR test identified as *C. krusei*. In addition to *C. albicans* were identified in the fermentation test, with multiplex-PCR test on the same sample identified as *C. stellatoidea*, only one sample was identified as *C. albicans* equally well with fermentation test or test-PCR multiplex. Identification of the species *C. krusei* on the same sample for the second test method had almost 100% similarity.

Comparison of *Candida tropicalis* between identification fermentation test and multiplex-PCR test can be seen in Table 5. The results showed that with multiplex-PCR test resulted 23.0% of the sample had an infection caused by more than one type of *Candida* species.

Diagnostic test results of fermentation and multiplex-PCR

Test sensitivity and specificity of multiplex-PCR to identify *Candida* species as the cause of candidiasis vaginalis on the study conducted on 30 samples were found positive by culture examination of *Candida* on SDA medium. As the gold standard test Fermentation with sweets (dextrose, maltose, sucrose, lactose, and galactose).

C. krusei diagnostic test results on 30 respondents with fermentation test showed 12 positive samples and a total of 18 samples were negative. While multiplex-PCR test on the same sample obtained 19 samples were positive and 11 negative samples. Data from the species *C. krusei* diagnostic test can be seen in Table 6. Figure 1. above shows the *C. glabrata* with size 483 bp PCR product (sample number: 6, 63,79 and 80).

In this study, *C. glabrata* infections always coincide with other *Candida* species infections (mix-infection). *C. tropicalis* diagnostic test in this study with the results obtained by fermentation test five samples was positive and 25 negative samples, on the same sample by multiplex-PCR test obtained five positive samples and 25 negative samples. Test data diagnostic *C.tropicalis* species can be seen in Table 6, while diagnostic test of *C. tropicalis* using fermentation method can be seen in Table 7.

These results are also consistent with previous studies comparing multiplex-PCR method with commercial tools (API 20C kit), known multiplex-PCR has a sensitivity and specificity values were quite high (100%) compared to API 20C Kit is its ability to identify species of 5- 10% is not perfect, for example in the study of API 20C Kit could not identify *C. glabrata* and *C. tropicalis* but with multiplex-PCR identified yeast species perfectly. In that study, the sample was identified as *C. albicans* turns with multiplex-PCR identified as *C. tropicalis*. Results of the final identification with morphological analysis of culture and chrome agar show results as *C. tropicalis*. Figure 2 shows the size of the base pairs of *C. tropicalis* (218 bp or 199 bp) that the sample number 2 and 77.

Diagnostic test of *C. albicans* against 30 respondents with fermentation test showed three positive samples and a total of 27 samples were negative. Examination by multiplex-PCR test on the same sample contained only one known positive samples and 29 negative samples. Data diagnostic test results of *C. albicans* species can be seen in table 2x2 (Table 8).

The length of the PCR product for *C. albicans* was 218 bp and 110 bp, In this study by multiplex-PCR only one sample was identified as *C. albicans*. *C. stellatoidea* with fermentation test in this study was found positive in three samples and 27 samples were negative. While the multiplex-PCR test was found five positive samples and 25 negative samples. *C. stellatoidea* diagnostic test data can be seen in Table 9.

Table 6. Diagnostic test results *Candida krusei* (n = 30)

		Fermentation test		
		Positive	**Negative**	**Amount**
Multiplex-PCR Test	Positive	12	7	19
	Negative	0	11	11
	Amount	12	18	30

Table 7. Diagnostic test of *C. tropicalis* (n=30)

		Fermentation test		
		Positive	**Negative**	**Amount**
Multiplex-PCR Test	Positive	5	0	5
	Negative	0	25	25
	Amount	5	25	30

Table 8. Diagnostic test results *C. albicans*

		Fermentation test		
		Positive	**Negative**	**Amount**
Multiplex-PCR Test	Positive	1	0	1
	Negative	2	27	29
	Amount	3	27	30

Table 9. Diagnostic test results *C. stellatoidea* (n = 30)

		Fermentation test		
		Positive	**Negative**	**Amount**
Multiplex-PCR Test	Positive	3	2	5
	Negative	0	25	25

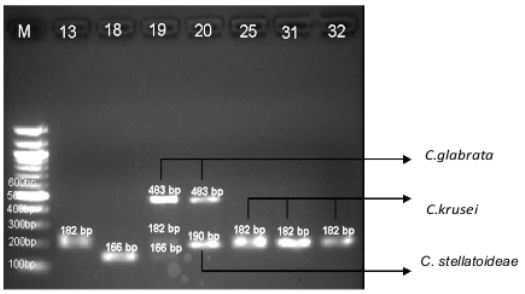

Figure 1. Samples were identified by Multiplex PCR using universal primers ITS1-ITS2 and specific primer CA3-CA4, and DNA fragment detected by agarose electrophoresis. DNA fragment bands for *C. glabrata* was 483 bp, *C. krusei* was 182 bp or 166 bp, and *C. stellatoidea* was 190 bp.

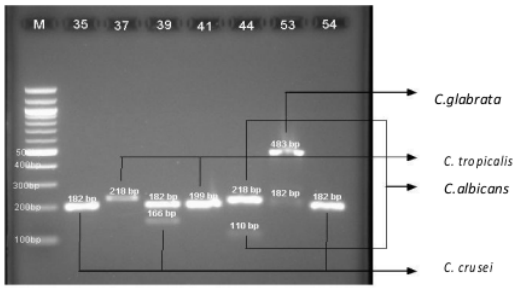

Figure 2. Samples were identified by multiplex PCR using universal primers ITS1-ITS2 and specific primer CA3-CA4, and DNA fragment detected by agarose electrophoresis. DNA fragment bands for *C. glabrata* was 483 bp, *C. tropicalis* was 218 bp or 199 bp, and *C. albicans* was 218 bp and 110 bp, *C. krusei* was 190 bp.

The test results of sensitivity and specificity in identifying by multiplex-PCR, *C. stellatoidea* have value: sensitivity 100%, specificity 92.6%, positive predictive value 60% and negative predictive value of 100%. Large base pairs to *C. stellatoidea* with the use of a combination of universal primers ITS1-ITS2 and specific primers CA3-CA4 in this study based on the literature is 190 bp (Liguori et al. 2007). Precise and rapid identification in the case of candidiasis is must, considering each *Candida* species have the virulence and sensitivity to different antifungal. Based on the study of (Chang et al. 2001), known to 30.7% of *C. glabrata* isolates turns resistant against fluconazole. While the approach to the identification of *Candida* species with conventional techniques requires a significant financial cost and a longer time. Multiplex-PCR to identify *Candida* species using universal primer pairs that amplify ITS1 and ITS2 conserve areas of 18S rDNA ITS1 which covers the border, a small part of 5.8S rDNA region. Amplification of this region produces a variety of different sizes of the species *Candida*. In addition, this study also uses a primer that allows specific to identify several species of *Candida* at the same time (Chang et al. 2001).

Discussion

As previous data (Anindita 2006), the incidence of vaginal candidiasis is commonly found in women aged 26-35 years (50%). The first diagnosis of candidiasis is commonly found at the age of 17 years and 54.7% of women aged 25 years'experience vaginal candidiasis (Geiger et al. 1995). Comparison of results between test *Candida* species identification by fermentation and multiplex-PCR test can be seen in Table 6. Results showed that with multiplex-PCR test resulted 23.3% of the sample had an infection caused by more than one type of *Candida* species, it has a higher value when compared with the results of research conducted in Iran from 2006-2008 (Rad et al. 2012), in which the study showed 10.3% of respondents experiencing vaginal candidiasis caused by more than one species of *Candida*. Similarly, research conducted by Liguori et al (2007), identification of more than one species of *Candida* on the same sample by multiplex-PCR, while the routine phenotypic identification methods only identified one species and of that study concluded that the multiplex-PCR method is a test accurate in detecting *Candida* species, allowing for the detection of more than one species of *Candida* in the absence of cross-reaction and without being influenced by bacteria or viruses. This can be clarified by the working principle multiplex-PCR that uses the target area ITS1 and ITS2, 5.8S combined with universal and specific multiplex-PCR primer allows up to seven *Candida* species identified at once in the same time. Diagnostic test for *C. krusei* species in this study had a sensitivity of 100%, in other words, the ability of multiplex-PCR method to detect a true positive result is 100%. While the value of specificity of 61.1% that it also means the ability of multiplex-PCR method in detecting true negative is 61.1%. 63.2% positive predictive value (the probability of an individual suffering from vaginal candidiasis) and negative predictive value of 100% (the probability of an individual not suffering from vaginal candidiasis). Large base pairs to *C. krusei* with the use of a combination of universal primers ITS1-ITS2 and specific primers CA3-CA4 in this study based on the literature is 182 bp or 166 bp (Liguori et al. 2007). These results are consistent with previous studies conducted by (Rad et al. 2012), multiplex-PCR has higher sensitivity and higher specificity than the API 20C in identification of *Candida* species for example in this study API 20C Kit could not identify both *C. glabrata* and *C. tropicalis*, while multiplex-PCR method identified perfectly. Diagnostic test for *C. tropicalis* species in this study had a sensitivity of 100%, in other words, the ability of multiplex-PCR method to detect a true positive result is 100%. While the value of specificity of 100%, which mean that the ability multiplex-PCR method in detecting true negative is 100%. 100% positive predictive value (the probability of an individual suffering from vaginal candidiasis) and negative predictive value of 100% (the probability of an individual not suffering from vaginal candidiasis). Large base pairs for *C. tropicalis* with the use of a combination of universal primers ITS1-ITS2 and specific primers CA3-CA4 in this study based on the literature is 218 bp or 199 bp. The value of base pairs is difficult to distinguish from *C. albicans*, but because in this study using a primer specific for *C. albicans* (CA3 and CA4), then to *C. albicans* was visible band at 110 bp in addition to the 218-219 bp or 198-199 bp (Liguori et al. 2010). Research conducted by Liguori et al. (2007) which compares a routine examination phenotype with multiplex-PCR to identify *Candida* species also showed similar results to this study, the sensitivity and specificity of multiplex-PCR in identifying *C. tropicalis* higher than the examination phenotype.

In Table 8, multiplex-PCR sensitivity in the test species *C. albicans* was 33.3% (ability of multiplex-PCR to determine true positive), while the value is 100% specificity (ability multiplex-PCR to determine the true negative). The positive predictive value (the possibility of samples experiencing candidiasis vaginalis) is 100% and the negative predictive value (likelihood samples do not experience vaginal candidiasis) is 93.1%. Results of diagnostic tests of a diagnostic method said is good if it has a value of sensitivity and high specificity. The results of diagnostic tests multiplex-PCR in previous studies have an average sensitivity and high specificity, as practiced by Rad et al. (2012) which showed that results for all species with multiplex-PCR test result by the same method of germ tube, production chlamydospore the CMA and the API 20C-AUX Kit. Test sensitivity and specificity multiplex-PCR compared with 9 kinds of test phenotype in *C. albicans* species showed multiplex-PCR method has high sensitivity and specificity (100%) (Liguori et al. 2007). API 20C AUX with the manual system, requires a long time for incubation but easy to use and not too expensive, in the study had a sensitivity of 86.9% and a specificity of 95%. While the method vitek2 been using automatic systems and allows to identify a large number of species, but in that study had the lowest sensitivity value (81.9%). Chromogenic media simple, fast and economical in

identifying species of *Candida*, but the colors are formed on the media after storage and subculture so varied that it is difficult to interpret (Momani and Qaddoomi 2005). Previous research report stated that multiplex-PCR method is accurate in identifying, simple, fast without the use of toxic chemicals and expensive. Other molecular techniques have also been developed to identify infectious disease-causing species, including *C. albicans*, but the method can not be used as a routine examination because of high cost and the need for skilled human resources. Actually, in some cases, species identification using biochemical test combined with routine examination phenotype was able to increase the sensitivity and specificity, but for specific purposes such differentiating *C. albicans* and *C. dubliniensis* still needed inspection molecular level, due to the characteristics of the same phenotype as *C. albicans*, *C. dubliniensis* also formed chlamydospore, germ tube and display the same natural resources. Identification to distinguish between two species was very important, because of the growing incidence of candidiasis caused by *C. dubliniensis* and *C. dubliniensis* known to be resistant to fluconazole (Lim and Lee 2002). The results in Table 9 are consistent with previous studies conducted on identification of *Candida* species using ITS2 genetic region, with the PCR method has high sensitivity. *C. stellatoidea* morphologically indistinguishable from *C. albicans* (McCullough et al. 1999). Test germ tube formation in the egg white and the test medium chlamydospore formation in cultured cornmeal Tween-BO (CMT test) was not able to distinguish the two species, although theoretically, the CMT test can differentiate *Candida* species based on the ability of specific pseudohyphae formation, but in some research turns pseudohyphae that form less specific. Theoretically, *C. stellatoidea* distinguished from *C. albicans* because it failed to form acid from sucrose, lighter pathogenicity, and formation chlamydospore late (Luo and Mitchell 2002). Fermentation and assimilation test can distinguish *C. albicans* and *C. stellatoidea* based on the ability of these species in the use of sugars. multiplex-PCR test in this study using specific primers for *C. albicans* allow a high sensitivity and specificity (Harmal et al. 2012). The image below shows the sample number 20 base pairs *C. stellatoidea* size (190 bp) (Figure 1). In this result, we stated that of the two types of examination *C. krusei* obtained was the most common cause of candidiasis (fermentation Test: 40%, multiplex-PCR Test: 46.7%). Using multiplex-PCR test showed that 23.3% of the sample had an infection caused by more than one species of *Candida*. multiplex-PCR diagnostic test for *C. krusei* has a sensitivity of 100%, specificity: 61.1%, NPP: 63.2% and NPN: 100%, against *C. glabrata* has a value of 0% sensitivity, specificity: 100% , NPP: 0% and NPN: 76.7%. Against *C. tropicalis* has a sensitivity of 100%, specificity: 100%, NPP: 100% and NPN: 100%, against *C. albicans* has a sensitivity of 33.3%, specificity: 100%, NPP: 100% and NPN: 93, 1%, as well as against *C. stellatoidea* had a sensitivity of 100%, specificity: 92.6%, NPP: 60% and NPN: 100%.

Based upon above results it is concluded that both fermentation and multiplex-PCR tests revealed *C. krusei* as the most common cause of candidiasis (test fermentation: 40%, multiplex-PCR Test: 46.7%). Using multiplex-PCR test showed that 23.3% of the sample had an infection caused by more than one species of *Candida*. Multiplex-PCR diagnostic test for *C. krusei* has a sensitivity of 100%, specificity: 61.1%, NPP: 63.2% and NPN: 100%, against *C. glabrata* has a value of 0% sensitivity, specificity: 100% , NPP: 0% and NPN: 76.7%. Against *C. tropicalis* has a sensitivity of 100%, specificity: 100%, NPP: 100% and NPN: 100%, against *C. albicans* has a sensitivity of 33.3%, specificity: 100%, NPP: 100% and NPN: 93 .1%, as well as against *C. stellatoidea* had a sensitivity of 100%, specificity: 92.6%, NPP: 60% and NPN: 100%.

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