Candida Albicans CT Value in Asthmatics with Prolonged Corticosteroid Inhaler Uses

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ABSTRACT Corticosteroid inhaler is one of the first-line drugs given for the control and prevention of asthma attacks in the long term and continuously. The use of corticosteroid inhalers for a long time has systemic side effects in the oral cavity which can be a predisposing factor for normal fungal infection of Candida albicans microflora, an increase of Candida albicans’s amount in the oral cavity can cause oral candidiasis. Candida albicans detection using q-PCR to detect specific genes in oral swab specimens can provide an indirect picture of the amount of Candida albicans in the samples taken. The purpose of this study was to determine the relationship between the length of use of corticosteroid inhalers and the cycle threshold value in oral swab samples of asthma patient. This type of research is correlational research with data collection techniques using a purposive sampling technique on 30 respondents at the Lung Polyclinic at Bhayangkara Hospital H.S Samsoeri Mertojoso Surabaya who fit the inclusion criteria. Samples were examined using q-PCR to detect specific genes in the ITS-2 region to detect Candida albicans. This research was conducted at the Molecular Biology Laboratory of the Ministry of Health Surabaya Polytechnic during the period April – May 2023. The results showed that there were 26 samples (86.7%) positive for the ITS-2 gene and 4 samples (13.3%) negative for the ITS-2 gene. with sig. 0.307 so that it can be concluded that there is no relationship between the length of use of corticosteroid inhalers and the cycle threshold value of C. albicans in asthma’s oral swab sample.

INDEX TERMS Corticosteroid inhaler, Candida albicans, q-PCR

I. INTRODUCTION

Each year the number of asthma deaths is recorded at around 180,000 cases with wide variations across ages, economic groups, continents, and regions [1]. According to the Indonesian Ministry of Health (2019) from 2007 to 2018 there was an increase in asthma prevalence nationally by 0.5% with the highest number of hospitalized cases in East Java province at 7,942 cases [2]. Inhaler corticosteroid (ICS) has been suggested as a therapy in all patients once they are confirmed by asthma and it becomes a universal treatment replacing SABA (short acting β-agonist). ICS should be used regularly to provide maximum benefit; other types of steroid medications such as Beclomethasone dipropionate, Budesonid, Fluctica propionate, and Momethasone furoate have similar effectiveness in asthma therapy [3].

Inhalation corticosteroids have milder systemic effects than oral corticosteroids, but some cases of side effects have been reported. One of the side effects of using Corticosteroid Inhalers is a change in the condition of the oral cavity, partial deposition of drugs in the oropharynx causes side effects such as localized such as dysphagia and oral candidiasis [4]. Ideally, corticosteroid inhalers have minimum drug deposition in the oropharynx and maximal drug deposition in the lungs.

Deposition of corticosteroid inhalers in the oropharyngeal cavity can cause oral candidiasis, this occurs due to a decrease in local immunity involving inhibition of the defense function of normal immune cells (neutrophils, macrophages, and T-lymphocyte cells) on the surface of the oral mucosa or caused by increased glucose levels in saliva that can stimulate the growth of Candida albicans[5].

A study conducted by Erdogan, (2019) [5] said that the incidence of oral candidiasis in adult asthmatics is quite high, the incidence of oral candidiasis in asthmatics is 1-70%, depending on the diagnostic criteria used, variations in the magnitude of incidence occur considering that oral candidiasis does not always cause symptoms, only 33% of patients with cases of oral candidiasis complain of pain in the mouth. The strain of Candida albicans species was the highest strain isolated from asthmatics who used corticosteroid inhalers for long periods, C. albicans from asthma patients who used corticosteroid inhalers for long periods had significantly higher amounts than the amount of C. albicans taken from samples of healthy people[6].

Along with the development of the era, identification of infection of a microbe can be done using the Polymerase
Chain Reaction (PCR) method to detect specific genes with low concentrations in the sample. Molecular identification of Candida can be done using gene sequence data, usually, the gene used in the identification of yeast accurately and quickly is a gene found in ribosomal DNA found in all living things so the primer used is universal and easy to sequence. The genes that code for rDNA consist of non-coding and coding regions. The non-coding region consists of an intergenic spacer (IGS) and internal transcribed spacer (ITS1 and ITS2), while the coding region consists of large sub-units (LSU rDNA 28s), small subunits (SSU rDNA 18s), 5.8s genes, and 5S genes.[7]

This method will produce the Ct value which is the cycle value that the fluorescent signal needs to cross the threshold, the Ct value is inversely proportional to the initial nucleic acid amount in the sample. The lower of Ct value indicates that the amount of C. albicans nucleic acid in the sample is increasing.[8] A low Ct value indicates fewer amplification cycles required to reach the threshold because the nucleic acids contained in the sample are high[9].

Most previous study provides the oral candidiasis incident or colony counting of oral sample result using a conventional or qualitative method that might give less objective or less sensitive result. Research on the length of use of corticosteroid inhalers with CT values in asthmatics can be used as a raw interpretation of the number of fungi in the sample considering that oral candidiasis is usually asymptomatic. This study aimed to discover whether the Cycle threshold value have significant relation in oral swab sample of asthmatics with prolonged corticosteroid inhaler uses.

II. METHODS

A. STUDY DESIGN

This research is quantitative correlational research (correlation research) that involves collecting data to determine whether there is a relationship between two variables. The data used in this study are primary data with sampling techniques that use purposive sampling techniques following the inclusion criteria.

B. POPULATION AND SAMPLING TECHNIQUE

The population in this study was 30 outpatient asthma patients at the Lung Poly of Bhayangkara Hospital Surabaya. This study uses purposive sampling technique with inclusion criteria, such as using corticosteroid inhalers for at least one year, agreeing to sign informed consent, not having a history of immune disorders (Autoimmune / HIV), Diabetes, Cancer, and Respondents were not taking oral steroid drugs or antibiotics. It is aimed introduce selection bias and reduce the generalizability of result.

C. STUDY SETTING

This study was conducted at the Outpatient of Lung Poly of Bhayangkara Hospital Surabaya for oral swab collection. Inoculation on Sobaraud Dextrod Agar (SDA) media as well as macroscopic and microscopic observations were carried out at the Mycology Laboratory of the Poltekkes of the Ministry of Health Surabaya, while further examination using q-PCR was carried out at the Molecular Biology Laboratory of the Poltekkes of the Ministry of Health Surabaya.

D. DATA COLLECTION PROCEDURE

1. MEDICAL HISTORY DATA

The respondent's medical history to fill the inclusion criteria is obtained by direct interviews with respondents.

2. SWAB ORAL SAMPLING

The equipment used for oral swab sampling is Cotton Swab, sample pot, and test tube. Samples are taken using the oral swab technique using a cotton swab by wiping the lesion area if there are lesions or on the dorsal part of the tongue three strokes and then dissolved into Phosphate Buffer Saline (PBS) solution.

3. Candida albicans IDENTIFICATION

The materials needed include Phosphate Buffer Saline (PBS) to take concentrated oral swab samples, Saboroud Dextrose Agar (SDA) media, chloramphenicol antibiotics, aquades, spiritus, Lactophenol Cotton Blue (LCB), pure isolate of Candida albicans ATCC 100231, DNA extraction kit, 5'-GGGTGGGTTGCTTGAAGACGGTA-3' forward primer and 5'-TTGAAGATATACGTGGTGTTGAGCTTA3' reverse primer, 12.5 μL Mastermix (0.5 U Taq polymerase, 0.2 mM dNTP, 1.5 mM MgCl2, and buffer 1x) [10]. Samples from PBS were inoculated on SDA media and viewed colony growth and morphology under a microscope with LCB staining. Colonies grown on SDA media were then continued to carbohydrate fermentation tests for species-level identification. After the preliminary test, the PBS solution contains a molecularly identified sample using the RT-PCR method which will give results Ct values form.

E. DATA ANALYSIS

Data analysis techniques used in research include the Shapiro-Wilk test to test data normality and continued using the Pearson / Spearman correlation parametric test to determine whether there is a relationship between two variables.

F. ETHICAL CONSIDERATION

The study was given ethical clearance approval by Health Research Ethics Committee Poltekkes Kemenkes Surabaya to conduct the study and collect data.

III. RESULT

The results of interviews with respondents obtained the following data:
Complaints regarding the symptoms of oral candidiasis were known through interviews with 30 respondents:

<table>
<thead>
<tr>
<th>Oral Candidiasis Symptoms</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Redness or soreness on inner cheeks, tongue, roof of the mouth, and throat</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>dysphonia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pain while eating or swallowing</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Loss of taste</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>White tongue</td>
<td>11</td>
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</table>

30 samples that have been obtained, inoculated on Sabouraud Dextrose Agar (SDA) media to see colony growth, colonies that grow after the inoculation and incubation process and then observed microscopically using Lactophenol Cutton Blue (LCB) stain at 400x magnification.

There were 12 samples with colony growth that had colony characteristics of Candida sp. Such as white, has a smooth surface, and smells of yeast. 12 samples that had colony growth with characteristics such as Candida sp. then observed microscopically using Lactophenol Cutton Blue (LCB) dye and showed microscopic images in the form of yeast cells and hyphae typical of Candida sp. The sample identified as Candida sp. Followed by the examination of carbohydrate fermentation tests to determine the species of candida fungi in the sample. In the carbohydrate fermentation test, Candida albicans will give positive results on fermenting Glucose, Sucrose, and Maltose and give negative results on fermenting Lactose. From 12 samples, it was found that all samples had carbohydrate fermentation test results indicating that the sample was a species of Candida albicans. To determine the relationship between the length of use of corticosteroid inhalers and the Cycle threshold value, all samples dissolved in the Phosphate Buffer Saline solution are extracted first before being examined on the rRT-PCR device. After the extraction process, the sample is tested for purity and DNA concentration using a nano-drop spectrophotometer. The results of DNA purity tests and the concentration of 30 samples at λ260/absorbance from all samples are in a good category, namely in the range of 1.8 – 2.0. After the sample meets the requirements for PCR examination, the sample is run in an rRT-PCR tool to detect the specific gene Candida albicans in the ITS-2 region and find out the Ct value of each sample. From research conducted using the RT-PCR method, it was found that:

<table>
<thead>
<tr>
<th></th>
<th>Albicans</th>
<th>Non albicans</th>
<th>Total</th>
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<tbody>
<tr>
<td>Media: Inoculation on SDA Media</td>
<td>12</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>Microscopic examination with LCB Stain</td>
<td>12</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>Carbohydrate fermentation test</td>
<td>12</td>
<td>18</td>
<td>30</td>
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</table>
Examining all samples on the rRT-PCR device obtained results, namely 26 oral swab samples from asthma patients using positive corticosteroid inhalers containing Candida albicans specific genes from the ITS-2 region marked with Ct < 39 values and there were 4 oral swab samples showing negative results containing Candida albicans specific genes marked with N/A results and Ct > 39 values. The results of the Spearman correlation test showed the efficacy value of the duration of corticosteroid inhaler use against a Ct value of 0.307 which is greater than 0.05. The value of the Correlation coefficient of the duration of use of corticosteroid inhalers with a Ct value of 0.193 which means that between the two variables has a weak relationship.

### IV. DISCUSSION
Research conducted on 30 respondents showed that the frequency of asthma patients based on age has the highest percentage in the age range of 60 – 79 years at 60%, asthmatics with older age have an odds ratio (OR) of 3.83 in the severity of asthma compared to younger patients, this can be because older asthmatics have a more severe rate of airflow obstruction and lower quality of life than younger sufferers. In addition, other factors such as the presence of comorbidities that aggravate asthma symptoms experienced by older asthma patients contribute to aggravating the severity of asthma[11].

From the results of interviews with all respondents who use corticosteroid inhalers regarding the symptoms of oral candidiasis experienced, it was found that all patients did not experience symptoms of oral candidiasis. However, 37% of respondents had a white coating on the surface of the tongue. This can also occur due to the aggregation between normal microflora in the mouth with epithelial cells, food waste, saliva, and serum components that can form a layer on the surface of the tongue. White plaque on the surface layer of the tongue can be affected by oral hygiene, increasing age which affects changes in salivary production, smoking habits, and other factors[12].

The results of observations on LCB staining showed that colonies grown from 12 samples had morphology of single yeast cells and found hyphae / pseudohyphae in the preparation. The yeast cell shape makes C. albicans easier to spread than the hyphal form, while the hyphal form makes it easier for C. albicans to penetrate the host body. Therefore, in the process of tissue invasion by C. albicans hyphal forms are more often detected[13]. Colonies identified as Candida were continued on carbohydrate fermentation tests to determine species of the genus Candida sp. From 12 colonies, C. albicans were obtained with positive fermentation of glucose, sucrose, and maltose yet it shows negative results of fermenting lactose. Nevertheless, this result might be shown too in C. dubliensis which shares a high grade of phenotypic similarities with C.albicans. Moreover, carbohydrate fermentation test is considered less sensitive that it can give a less specific result of Candida sp genus[14].

Screening examination for species identification using convensional methods have a low sensitivy and specificty that lead to misinterpretation or false result compared to molecular method. Molecular method which uses the genotype identification principle provides more sensitive and specific result in spieces examination.

Extraction is performed on the entire sample first to separate the DNA from other materials such as proteins, fats, and carbohydrates. The extraction results that have been incubated for 1x24 hours are checked for purity and concentration, the results obtained from purity measurements at absorbance \(\lambda\) 260/280 nm have an average value of 1,908 and an average concentration value of 15.75 ng / UL. These results are in accordance with the criteria required by the sample for PCR method examination, good purity is in the range of 1.8 – 2.0 values while the minimum DNA concentration in the sample is 5 ng / UL to be detected by PCR[15].

Before running the sample on the PCR tool, temperature optimization is carried out first with the aim of determining the best temperature in the annealing process so that primary attachment to the DNA template occurs optimally. From the results of temperature optimization, the optimal temperature for the annealing process is 58.00C.
Detection of specific genes Candida albicans is carried out especially in the ITS-2 region. Candida DNA detection is carried out by amplification of small rRNA subunits in the non-coding region Internal transcribed spacer (ITS) which is divided into two spacers, namely ITS-1 and ITS-2. ITS-2 regions with secondary structure are more conserved than the DNA sequences alone. Which could provide information that is useful for cladistic inference of relationship [16].

From the amplification carried out on 30 samples, 26 oral swab samples from asthmatics using corticosteroid inhalers (86.6%) had a Ct value of < 39 which indicated a positive sample containing target DNA from Candida albicans and there were 4 oral swab samples (13.3%) which showed negative results containing target DNA from Candida albicans marked with N/A results.

From all stages carried out, there were two samples, namely S25 and S27 samples which had negative results containing target DNA from Candida albicans on rRT-PCR examination but there was colony growth and morphology on the preparation that was similar to Candida spp species, the sample also had the same fermentation test results as Candida albicans species. This can happen with the possibility that in the sample there is another species, namely Candida dubliniensis, the species C. dubliniensis has the same morphology both in the SDA media and in LCB staining.

C. dubliniensis also has the same fermentation test results as Candida albicans species. C. dubliniensis has almost the same phenotypic characteristics as C. albicans so these two species cannot be distinguished using conventional methods alone. The molecular method is the most effective method of distinguishing these two species based on their differences genotypically[17]. The genotypic difference between Candida albicans and Candida dubliniensis on real-time PCR examination is found in melting temperatures (Tm) difference values in the gene amplification of the ITS-2 region, C. albicans has a Tm value of 86.55°C while C. dubliniensis has a Tm value of 82.75°C, C. albicans has a higher Tm value than C. dubliniensis [18].

In 26 samples detected positive containing Candida albicans target DNA with molecular examination there were 16 samples that did not have colony growth on SDA media but had positive results on examination at the rRT-PCR tool, this can be caused by lack of homogeneity in PBS solution before the inoculation process on SDA media or the amount of Candida albicans in the sample is too small to be able to grow in SDA. Meanwhile, target DNA identification using molecular methods on the rRT-PCR tool can detect specific genes with a minimum concentration of 5 ng / UL.

Positive results that show a higher percentage than negative results on molecular method examination are because basically Candida albicans is a normal flora that can be found in the mouth, especially in the tongue where the area is the area where oral swabs are taken in this study. Distribution of Candida is throughout the mouth with the most common site of isolation is in the dorsum of tongue [19].

In this study, the Cycle threshold value was obtained in the sample that was relatively the same between patients who used corticosteroid inhalers for a short period of time with patients who used corticosteroid inhalers for a longer period of time.

The results of Ct values in all samples did not show a significant difference between patient samples with the use of corticosteroid inhalers in a short time with a longer time. These results have similarities with research that states that there is no significant relationship between the duration of asthma medication use and the amount of Candida albicans CFU/ml[20].

The results of higher Ct values in patients with longer use of corticosteroid inhalers can be associated with the results of studies that state that the risk of oral candidiasis in asthma patients occurs in the first month of corticosteroid inhaler use and decreases in the period afterwards. This can be due to improved inhaler use techniques by patients so that drugs can be dispositioned more precisely to the lungs and reduce the risk of local immunosuppression in the oral area due to steroid drugs [21]. Moreover, all of patients have been given a socialization to rinse their mouth after usage. Mouth rinsing after using inhalers can prevent local immunosupression that lead to oral candidiasis [22].

Other factors that can also affect the results are the source and method of sample collection. Concentrated sample types, oral swabs and imprint cultures are recommended for quantitative research rather than swab methods [23]. The sampling method using concentrated swab rinse also has better sensitvity than the oral swab method in describing the condition of Candida albicans in the oral cavity [24]. In addition, the Ct value has a limitation that it cannot be used as a quantitative description of the amount of Candida albicans in the sample, to get an idea of the specific amount of Candida albicans in the sample used molecular methods with stratified dilution from a known standard and made in a regression curve [25].

Analysis of the relationship between the duration of use of corticosteroid inhalers and the Ct value of Candida albicans in oral swab samples of asthmatics was tested using non-parametric statistical analysis, namely the Spearman correlation test. In this study, the sig value was obtained in the Spearman correlation test of 0.307 > α 0.05 so that the meaning that H0 is accepted which means there is no relationship between the length of use of corticosteroid inhalers and the Ct value of Candida albicans from oral swab samples in asthmatics. The correlation coefficient value of 0.193 means that between two variables there is a weak relationship. So it can be said that The Ct value from rRT-PCR was not correlated with the actual amount of Candida albicans in the oral swab samples as it did not use a standart curve or stratified dilution method.

It is required to take a note that the result of this research does not represent the general population of asthmatic patient who use corticosteroid inhaler due to the small of sample size in research. In addition, this research
also doesn’t control other factor that affect the occur of oral candidiasis such as oral hygiene, comorbid, smoking habits, etc.

V. CONCLUSION

Examination of the target gene Candida albicans in the ITS-2 region using the rRT-PCR tool showed the results of 26 samples (86.6%) identified positive for the ITS-2 gene and there were 4 samples (13.3%) identified negative for the ITS-2 gene. There is no relationship between the duration of use of corticosteroid inhalers with Ct values from oral swab samples of asthmatics at the Lung Poly of Bhayangkara Hospital Surabaya. We suggest the future study to use the stratified standart dilution to get real amount of Candida albicans on sample and take a larger sample size with non-corticosteroid user as a control group to be compared.

REFERENCES