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Original Article

Examining convalescent plasma transfusion in severe COVID-19 patients, recent research highlights the significance of S-RBD antibodies and IL-10 levels

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ABSTRACT

Convalescent plasma (CP) may be an option for people with severe COVID-19. However, its efficiency remains unknown. A study was done to determine whether the levels of Anti-SARS-CoV-2 Spike Receptor-Binding Domain (S-RBD) antibodies and IL-10 in COVID-19 patients who had CP transfusion were related to their survival status. The observational cohort study included 40 patients with severe COVID-19 who were followed for 28 days after receiving a CP transfusion. Antibody and IL-10 levels were assessed on Day 1 before to CP transfusion and on Days 1, 2, and 7 following CP transfusion. Twenty six (65%) of the 40 patients survived. Anti-SARS-CoV-2 S-RBD antibody levels were observed to be significantly higher on Days 1, 2, and 7 following CP transfusion (p-value 0.05). Furthermore, IL-10 levels dropped significantly on Days 2 and 7 (p-value 0.05). However, neither the CT value nor the patients' survival status were linked to greater antibody levels or changes in IL-10 levels. According to the findings, CP transfusion can greatly enhance anti-SARS-CoV-2 S-RBD antibody levels while drastically decreasing IL-10 levels. These findings may have therapeutic implications for the use of CP as a COVID-19 therapy option. More research is needed to determine its efficacy in enhancing the survival rate of COVID-19 patients with severe symptoms.

Keywords: Anti-SARS-CoV-2 S-RBD antibody, IL-10, infectious disease, convalescent plasma, and mortality.

Highlights: This study showed that there was a decreasing trend of IL-10 after CP transfusion, which suggest that the immunomodulating effect from CP transfusion had successfully reduced IL-10 level in severe COVID-19 patients.

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INTRODUCTION

The coronavirus sickness 2019 (COVID-19) is caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). It affects roughly 100 million people worldwide and poses a major threat to public health.[1] According to the World Health Organization (WHO), there were more than 21 million new cases in six regions during the most recent reporting period, reaching the highest weekly case total since the pandemic began. There were also approximately 50,000 additional fatalities reported. COVID-19 had been related to about 346 million verified illnesses and 5.5 million fatalities worldwide by January 23, 2022.[2]

The pathogenesis of COVID-19 is mediated by the host's immunological response.[3] Acute respiratory distress syndrome (ARDS) and systemic inflammatory response syndrome (SIRS) are symptoms of immunological dysregulation in severe cases.[4] The invasion of inflammatory cells and the massive production of proinflammatory cytokines or chemokines are the characteristics of this, which cause organ damage and patient death.

SARS-CoV-2 is not yet a definite therapeutic approved for the treatment of COVID-19, while a number of antiviral medications have been employed in different clinical trials.[5] Convalescent plasma (CP), however, is making a comeback as a potentially effective COVID-19 therapeutic option. Because it contains a variety of proteins, including albumin, cytokines, antibodies, and anti-coagulation proteins, CP has been used to treat coronavirus infections in the past with encouraging outcomes. Additionally, CP has antithrombotic capabilities, which can re-establish hemostasis and reverse hypercoagulable states in COVID-19 patients.[6,7,8] By neutralizing antibodies that bind to the receptor-binding domain of the S1 spike antigen (sRBD) of the SARs-CoV-2 virus, it provides passive protection by preventing virus entry and restricting its amplification. Because CP contains antibodies and anti-inflammatory cytokines that block complement, inflammatory cytokines, and autoantibodies, it also has immunomodulatory effects.[6,7]

Cytokine IL-10 (interleukin10) has anti-inflammatory properties and is thought to function as a negative feedback mechanism to reduce inflammation.[9] On the other hand, it has been demonstrated that elevated IL-10 plays a pathogenic part in the severity of COVID-19. Because of its anti-SARS-CoV-2 S-RBD concentration, CP transfusion has been recommended for the treatment of severely and critically ill COVID-19 patients.[10] Despite its advantages, CP administration is still debatable, and opinions differ. Furthermore, no research has been done to link the survival status following CP transfusion to the levels of IL-10 and Anti-SARS-CoV-2 S-RBD antibody.[11] As a result, numerous laboratory markers are being studied to determine the utility of CP transfusion, and numerous clinical trials are still being conducted to determine its effectiveness. The purpose of this study is to assess the anti-SARS-CoV-2 S-RBD antibody and IL-10 levels, as well as their relationship to the survival status of patients with severe COVID-19 after CP transfusion.

MATERIALS AND METHODS

Study Design

This study used a prospective cohort design and was an observational analytical investigation. Patients with COVID-19 who were hospitalized in the isolation ward of the Dr. Soetomo Hospital in Surabaya, Indonesia between June and December 2020 comprised the study population. This hospital treats severe COVID-19 cases and serves as a teaching hospital as well as one of East Indonesia's referral hospitals. The Dr. Soetomo Hospital's health research ethics committee granted ethical permission. Using

a successive sampling strategy, 40 patients were recruited who fulfilled the inclusion criteria. The recipients' positive COVID-19 status was confirmed by a polymerase chain reaction (PCR), analysis of the nasopharyngeal swab, and they were also required to receive therapy at the Special Inpatient Installation (Isolation Ward) and had severe or critical disease. Participants in this study were split into two groups: those who survived and those who succumbed, based on mortality rates.

COVID-19 severity can be determined using the following criteria:[12]

a. Breathing difficulty accompanied by a respiration rate of 30 breaths per minute.
b. A saturation of oxygen (SpO2) of 93% or below while breathing room air.
c. A PaO2/FiO2 ratio of no more than 300 mmHg.
d. Chest X-ray lesions that have worsened by more than 50% in the last 24-48 hours.

The following criteria are utilized to identify critically ill COVID-19 patients at Dr. Soetomo Hospital:[12]

a. Severe pneumonia that advances swiftly and has frequent increases in viral load while receiving hospital-prescribed COVID-19 medication on a regular basis.
b. Acute Respiratory Distress Syndrome (ARDS) with a PaO2/FiO2 ratio less than 300.
c. Requiring or receiving mechanical ventilation therapy

Procurement of Convalescent Plasma (CP)

This study's convalescent plasma was acquired through apheresis donation utilizing Haemonetics MCS+ machine technology, in accordance with National Standards for Blood Transfusion Service and blood bank protocols. Each donor donated 200-400 mL of convalescent plasma, which was then transfused to each recipient in 200 mL increments over two days.

To be eligible for donating convalescent plasma, the following criteria must be met:

a. Age must be between 17 and 60.
b. The donor must have recovered from COVID-19 and provide two consecutive negative PCR test results for nasopharyngeal swabs.
c. The donor must not have any symptoms of COVID-19 or must have completed a 14-day symptom-free period before donating plasma.
d. Patients who have tested negative on re-PCR results of nasopharyngeal swabs 24 hours before plasma donation will not be eligible for donation if they are outpatients or self-isolation patients.
e. The donor must not have any comorbidities such as diabetes, hypertension with target organ damage (stroke, coronary heart disease, and renal disease), CKD, and inadequate vascular access.
f. Negative test results for hepatitis B, hepatitis C, human immunodeficiency virus, and syphilis are required.
g. The donor must have a blood antibody titer to SARS-CoV-2 more than 1:320, as measured by a rapid test assay using the PANBIO COVID-19 IgG rapid test equipment.

Exclusion criteria for convalescent plasma (CP) donors:

a. Donors who are getting COVID-19 treatment but have insufficient clinical information
b. Positive antibody screening test donor

Laboratory parameters

On Day 1 before CP transfusion, we tested for Anti-SARS-CoV-2 S-RBD antibody, IL-10, and rt-PCR (real time-

Polymerase Chain Reaction), as well as on Day 1, Day 2, and Day 7 following CP transfusion. Blood was taken using BD Vacutainer® SSTTM Tubes to determine antibody and IL-10 levels. The tests were performed using the sCOVG ADVIA Centaur for anti-SARS-CoV-2 antibody levels, the Cytometric Beads Array (CBA) method with the BD FACS Calibur TM flow cytometry for IL-10 levels, and the PCR SARS-CoV-2 by Abbott m2000 for rt-PCR analysis of nasopharyngeal swab. Prior to testing, serum antibody and IL-10 analysis samples were maintained at -80°C. After collecting all of the samples, the tests were carried out.

Patient monitoring and evaluation

On Day1, we assessed the demographics and clinical features of 40 COVID-19 patients before transfusing convalescent plasma. The patients' progress was tracked for 28 days after the transfusion.

Statistical analysis

The frequency of categorical variables was calculated, while quantitative data was provided as mean and standard deviation (SD) or median and interquartile range (IQR). To compare dependent samples, the Wilcoxon Sign Rank test was utilized, and the Mann-Whitney U test was used to compare independent samples. The Spearman correlation test was employed to assess the link between anti-SARS CoV-2 S-RBD antibody levels, IL-10 levels, CT value, and survival status. The significance level was set at 0.05.

RESULTS

The inclusion criteria were met by 40 COVID-19 patients who were severely to critically ill. The patients were primarily male (75%), had comorbidities (72.5%), were ventilated (50%), and had COVID-19 problems (95%) as shown in Table 1.

Table 1. Details of 40 COVID-19 patients' demographics and clinical characteristics.

Characteristics	n (%)
Age* (years)	49.83 ± 9.159
BMI* (kg/m^2)	25.90 ± 3.970
Gender	
Male	30 (75%)
Female	10 (25%)
Severity of infection	
Non-ventilated	20 (50%)
Ventilated	20 (50%)
Comorbid	
Yes	29 (72.5%)
No	11 (27.5%)
Comorbid Type	
DM	14 (48.3%)
Hypertension	14 (48.3%)
Overweight - Obesity	7 (14.9%)
CHD	2 (4.3%)
Hypothyroid	2 (4.3%)
Others	8 (17%)
Complications	
Yes	38 (95%)
No	2 (5%)
Complication type	
Respiratory failure	37 (97.4%)

Septic shock	10 (26.3%)
Non-specific hepatitis	6 (15.8%)
AKI	6 (15.8%)
Hypoalbuminemia	5 (10.5%)
Others	4 (7.4%)

BMI = Body Mass Index, DM = Diabetes Mellitus, CHD = Chronic Heart Disease, AKI = Acute Kidney Injury(*)Mean ± Standard deviation

From Day 1 to Day 7, the anti-SARS-CoV-2 S-RBD antibody considerably increased (p<0.001) (Table 2). The highest antibody level (723.67 U/mL) was recorded on Day 7 following CP transfusion. Table 3 revealed that IL-10 levels differed significantly on Day 2 and Day 7 (p<0.05). Pre-CP transfusion, the median baseline concentration of IL-10 was 5.64 pg/mL, and it gradually declined until Day 7.

Table 2. Differences in anti-SARS-CoV-2 S-RBD antibody levels before and after receiving CP tranfusion on Days 1, 2, and 7 in severe COVID-19 patients.

anti SARS CoV-2 S-RBD antibody levels	Median (min – max)	Differences of antibody level from baseline Median (min – max)	p-*value**
Day-1	4.96 (0.05 – 362.44)		
Day 1	16.78 (0.26 – 557.18)	8.51 (-13.05 – 411.93)	< 0.001
Day 2	31.99 (0.38 – 709.01)	16.13 (-37.49 – 632.83)	< 0.001
Day 7	75.31 (0.59 – 723.67)	37.81 (-90.65 – 497.50)	< 0.001

* Results showed statistical analysis were significant, with P < 0.05 using Wilcoxon Sign Rank test

Table 3. Differences in IL-10 levels before and after receiving CP tranfusion on Days 1, 2, and 7 in severe COVID-19 patients

IL-10 levels	Median (min – max)	Differences of IL-10 level from baseline Median (min – max)	p *value**
Day-1	5.64 (2.92 – 25.48)		
Day 1	4.64 (2.82 – 33.11)	0.95 (17.92 – 26.05)	0.090
Day 2	4.24 (2.24 – 25.48)	1.56 (20.68 – 19.84)	0.003
Day 7	4.09 (1.00 – 36.23)	1.49 (19.50 – 30.59)	0.003

* Results showed statistical analysis were significant at Day 2 and Day 7 , with p < 0.05 using Wilcoxon Sign Rank test

On Day 2, our investigation found median changes in antibody levels in both the recovered and succumbed groups (Figure 1a), as well as median changes in IL-10 levels (Figure 1b).

Further statistical analysis, however, revealed a statistically negligible connection between antibody or IL-10 levels and survival state, with p = 0.411 and p = 0.734, respectively.

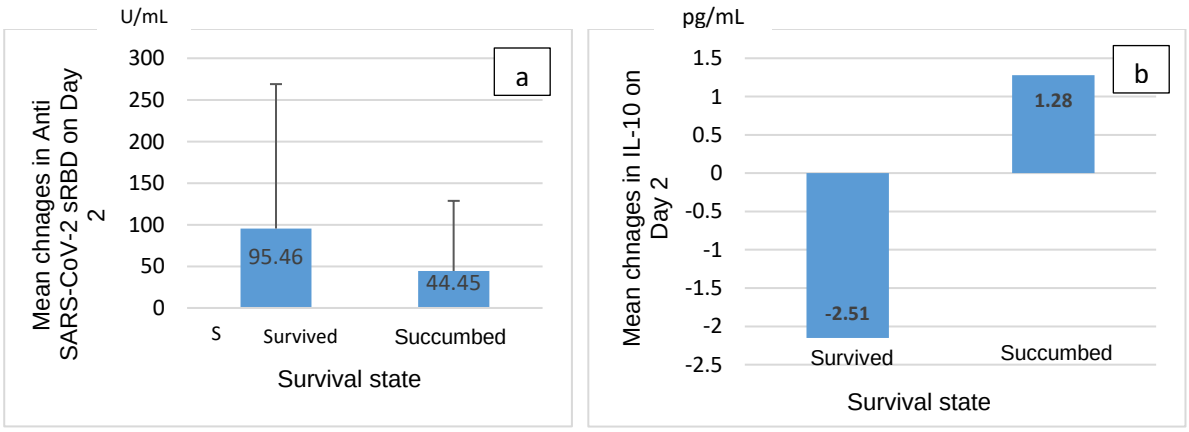

Figure 1. (a) Mean changes in anti-SARS-CoV-2 S-RBD antibody levels after receiving CP transfusion on Day 2 and survival state (b) Mean changes in IL-10 levels after receiving CP transfusion on Day 2 and survival state. Results of the Mann Whitney U test showed that there was no significant difference in antibody and IL-10 levels before and after receiving CP transfusion on Day 2 between those who recovered and deceased ($p > 0.05$).

Table 4 demonstrates a significant relationship ($p<0.05$) between anti-SARS-CoV-2 S-RBD antibody and IL-10 levels following CP transfusion on Days 2 and 7. Figure 2 shows a small but significant positive relationship between the two variables ($r = 0.31$, $r = 0.27$).

The Mann Whitney U test demonstrated that there was no significant connection between changes in anti-SARS-CoV-2 S-RBD antibody and IL-10 levels after receiving CP transfusion on Day 2 for both survivors and non-survivors ($p > 0.05$). Furthermore, Table 5 reveals that there was no significant association between changes in anti-SARS-CoV-2 S-RBD antibody on Day 7 and changes in CT value prior to receiving CP transfusion on Day 7 ($p > 0.05$).

Table 4. Correlation between anti-SARS-CoV-2 S-RBD antibody with IL-10 levels following CP transfusion on Days 1, 2, and 7 in severe COVID-19 patients.

Variable	*Spearman Correlation*	
	r_s	p-value*
Delta* anti SARS CoV-2 S-RBD Day 1 – Delta IL-10 Day 1	0.244	0.064
Delta anti SARS CoV-2 S-RBD Day 2 – Delta IL-10 Day 2	0.311	0.025
Delta anti SARS CoV-2 S-RBD Day 7 – Delta IL-10 Day 7	0.273	0.044

* Delta means changes in anti-SARS-CoV-2 S-RBD antibody and IL-10

* Results showed statistical analysis were significant, with $P < 0.05$ using *Spearman Correlation test*

Table 5. Correlation of Delta sCOVG changes with CT values

Variable	*Spearman Correlation*	
	r	p-value
Delta sCOVG Day 7 – CT Day 1	-0.131	0.210
Delta sCOVG Day 7 – CT Day 7	0.049	0.383

* Delta means changes in anti-SARS-CoV-2 S-RBD antibody and Cycle Threshold on rt-PCR

* Results showed statistical analysis were significant, with P < 0.05 using *Spearman Correlation test*

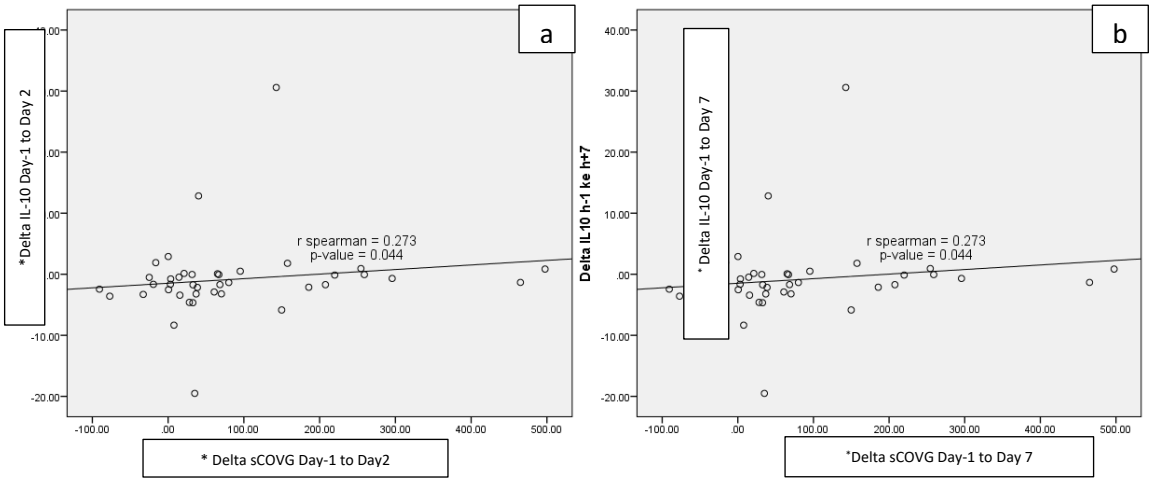

Figure 2. (a) Scatter Plot correlation of anti-SARS-CoV-2 S-RBD antibody with IL-10 on Day 2. (b) Scatter Plot correlation of anti-SARS-CoV-2 S-RBD antibody with IL-10 on Day 7. Both figures showed significant correlation using the Spearman correlation test with p<0.05. * Delta means changes in anti-SARS-CoV-2 S-RBD antibody and IL-10.

DISCUSSION

In this study, anti-SARS-CoV-2 (S-RBD) antibody and IL-10 levels were connected to survival status following CP transfusion in severe COVID-19 patients. The patients in our study were almost mostly male (75%). This is consistent with a previous study, which discovered that men were more likely than women to acquire substantial COVID-19 (69.8%).[13] There were two non-identical routes for viral entrance in gender susceptibility to COVID-19 via sex hormonal stabilization of angiotensin-converting enzyme 2 (ACE2). On the one hand, estrogens or X chromosomal inactivation escape can enhance ACE2 expression in women, allowing for a larger supply of ACE2 to maintain the elemental balancing axis of the Renin-Angiotensin System (RAS). Nonetheless, because the X regulatory genes were activated, women had not only lower viral loads but also greater CD4 T-cell counts and higher expression of Toll-like receptor 7 (TLR7), making them more resistant to severe COVID-19 than men.[14]

According to our findings, the majority of patients who received CP infusions had underlying comorbidities such as diabetes, hypertension, or obesity. In prior research, diabetes and hyperglycemia were linked to COVID-19 severity and death. Furthermore, COVID-19 patients comorbidities can lengthen their stay in the ICU and increase patient mortality.[15] COVID-19 patients with cardiovascular comorbidities are also more likely to suffer from sudden cardiac death and heart failure.[17] This is because viruses that reach the lungs may have direct or indirect consequences, such as cytokine storms.[18]

Antibody levels increased after Convalescent Plasma (CP) transfusion, according to our findings. This is in keeping with the notion that CP is a type of passive vaccination containing antibodies, cytokines, and coagulation factors.[10,11,19] The antibodies in CP neutralize infections before eradicating the virus, leading in a decrease in clinical symptoms. Another study discovered a decreasing trend in IL-10 levels after CP transfusion, demonstrating that the immunomodulatory effect of CP transfusion

successfully reduced IL-10 levels in severe COVID-19 patients. However, a previous study discovered that IL-10 levels increased after CP transfusion. High IL-10 expression in COVID-19 patients was found to predict poor outcomes. In addition to IL-10, patients with severe COVID-19 had increased levels of IL-2, IL-6, IL-7, granulocyte colony-stimulating factor, monocyte chemoattractant protein-1, tumor necrosis factor, macrophage inflammatory protein 1 alpha, and C-reactive protein.[21]

Although earlier administration of CP may reduce severity and mortality, high anti-SARS-CoV-2 S-RBD titers had no effect on viral load kinetics. This occurrence does not appear to be unique.[22] The period of delivery influences the efficacy of CP, and it is recommended that CP be provided within 72 hours after the onset of symptoms. A prior study found that CP infusions could decrease the mortality of ICU patients, and their success was impacted by characteristics such as disease severity, comorbidities, complications, and duration of stay.[23,24] Patients with underlying comorbidities showed inappropriate and inadequate immune responses, which may encourage viral replication and heighten the consequences associated with SARS-CoV-2 infection.[23]

The researchers discovered no link between changes in anti-SARS-CoV-2 S-RBD antibody levels and Ct values on Day 7 after receiving convalescent plasma (CP).[19] The mean Ct value before the CP transfusion was 18.61, but it jumped to 24.62 after the transfusion. This implies that antibody levels have no effect on viral clearance and that Ct value is unrelated to a patient's clinical status. Aranha et al. revealed in a prior study that COVID-19 positive cases with Ct values of 31 or above were later tested negative for SARS-CoV-2 RNA within 7 days of initial identification. However, the clinical conditions of the individuals were not disclosed.[25]

STRENGTH AND LIMITATION

Our study highlights the impact of CP transfusion as passive immunization involving antibodies, cytokines, and coagulation factors. This antibody level in CP neutralizes pathogens, which subsequently eliminates the virus resulting in improvement in clinical manifestations.

The study has some limitations, including insufficient data on the date of infection, which makes assessing the onset of symptoms difficult and may affect anti-SARS-CoV-2 S-RBD antibody, IL-10, and Ct value levels. Furthermore, because there was no control group (severe COVID-19 patients who did not get CP transfusion), no comparison between anti-SARS-CoV-2 S-RBD antibody, IL-10, and Ct value could be made. The study's small sample size is also an issue. Future research should include larger sample sizes, multi-center investigations, and more biological markers.

CONCLUSIONS

Transfusion with CP significantly increased the level of anti-SARS-CoV-2 S-RBD antibody and significantly reduced the level of IL-10. However, these parameters are not significant in predicting the survival state among severe COVID-19 patients transfused with CP.

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All authors approved the final manuscript and Siemens Healthiness Indonesia provided reagents without influencing study design or analysis.

ETCHICAL CLEARANCE

This study was approved by the Health Research Ethics Committee of Dr. Soetomo Hospital, Surabaya, Indonesia (approval number: 0001/KEPK/V/2020).

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There is no funding for this research.

CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

AUTHOR CONTRIBUTION

AEP, BAT and BPS contributed in the study conceptualization, methodology, data curation, writing - review & editing. NMY and SSN contributed to the writing - review & editing. All authors read and approved the final version of the manuscript.

REFERENCES

1. WHO Indonesia. Coronavirus Disease Coronavirus Disease Coronavirus Disease World Health World Health Organization Organization. World Heal Organ [Internet]. 2020;19(May):2. Available from: https://covid19.who.int/
2. Asia S. Weekly epidemiological update on COVID-19 - 25 January 2022 Edition 76. 25 January 2022. 2022;(January):22. Available from: https://www.who.int/publications/m/item/weekly-epidemiological-update-on-covid-19---25-january-2022
3. Hojyo S, Uchida M, Tanaka K, Hasebe R, Tanaka Y, Murakami M, et al. How COVID-19 induces cytokine storm with high mortality. Inflamm Regen. 2020;40(1).
4. Mahdi BA, Suryantoro SD, Romadhon PZ, Windradi C, Widiyastuti KN, Novendrianto D, Widiasi ED, Agustin ED, Firdausa S. Convalescent plasma therapy: The early use in moderate to severe COVID-19 patients in hospitals with limited resources. Journal of Tropical and Infectious Disease. 2021;9:3.
5. Natarajan H, Crowley AR, Butler SE, Xu S, Weiner JA, Bloch EM, et al. SARS-CoV-2 antibody signatures robustly predict diverse antiviral functions relevant for convalescent plasma therapy. medRxiv Prepr Serv Heal Sci [Internet]. 2020;9922:1–27. Available from: http://www.ncbi.nlm.nih.gov/pubmed/32995801%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC7523138
6. Sun M, Xu Y, He H, Zhang L, Wang X, Qiu Q, et al. A potentially effective treatment for COVID-19: A systematic review and meta-analysis of convalescent plasma therapy in treating severe infectious disease. Int J Infect Dis [Internet]. 2020;98:334–46. Available from: https://doi.org/10.1016/j.ijid.2020.06.107
7. Langhi DM, Santis GC De, Bordin JO. COVID-19 convalescent plasma transfusion. Hematol Transfus Cell Ther [Internet]. 2020;42(2):113–5. Available from: https://doi.org/10.1016/j.htct.2020.04.003
8. Franchini M, Glingani C, Liumbruno GM. Potential mechanisms of action of convalescent plasma in COVID-19. Diagnosis. 2021;8(4):413–20.
9. Riwu1a AG, Nugaraha J, Dachlan YP. Differences of Interleukin-18 and Interleukin-10 Levels in Pulmonary Rifampicin Resistant dan Rifampicin Sensitive Tuberculosis Patients in Dr. Soetomo Hospital Surabaya
10. Lu L, Zhang H, Dauphars DJ, He YW. A Potential Role of Interleukin 10 in COVID-19 Pathogenesis. Trends Immunol [Internet]. 2021;42(1):3–5. Available from: https://doi.org/10.1016/j.it.2020.10.012

11. Harvala H, Robb ML, Watkins N, Ijaz S, Dicks S, Patel M, et al. Convalescent plasma therapy for the treatment of patients with COVID-19: Assessment of methods available for antibody detection and their correlation with neutralising antibody levels. medRxiv. 2020;
12. Wu Z, McGoogan JM. Characteristics of and Important Lessons from the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72314 Cases from the Chinese Center for Disease Control and Prevention. JAMA - J Am Med Assoc. 2020;323(13):1239–42.
13. Jin JM, Bai P, He W, Wu F, Liu XF, Han DM, et al. Gender Differences in Patients With COVID-19: Focus on Severity and Mortality. Front Public Heal. 2020;8(April):1–6.
14. Lakbar I, Luque-Paz D, Mege JL, Einav S, Leone M. COVID-19 gender susceptibility and outcomes: A systematic review. PLoS One [Internet]. 2020;15(11 November):1–15. Available from: http://dx.doi.org/10.1371/journal.pone.0241827
15. Holman N, Knighton P, Kar P, O'Keefe J, Curley M, Weaver A, et al. Risk factors for COVID-19-related mortality in people with type 1 and type 2 diabetes in England: a population-based cohort study. Lancet Diabetes Endocrinol [Internet]. 2020;8(10):823–33. Available from: http://dx.doi.org/10.1016/S2213-8587(20)30271-0
16. Parasher A. COVID-19: Current understanding of its Pathophysiology, Clinical presentation and Treatment. Postgrad Med J. 2021;97(1147):312–20.
17. Herman H, Gharini PPR, Dinarti LK. Clinical cardiac manifestations in coronavirus disease 2019 (COVID-19) patients with. 2020;52(3):120–37.
18. Bhaskar S, Sinha A, Banach M, Mittoo S, Weissert R, Kass JS, et al. Cytokine Storm in COVID-19—Immunopathological Mechanisms, Clinical Considerations, and Therapeutic Approaches: The REPROGRAM Consortium Position Paper. Front Immunol. 2020;11(July).
19. Acosta-Ampudia Y, Monsalve DM, Rojas M, Rodríguez Y, Gallo JE, Salazar-Uribe JC, et al. COVID-19 convalescent plasma composition and immunological effects in severe patients. J Autoimmun. 2021;118.
20. Han H, Ma Q, Li C, Liu R, Zhao L, Wang W, et al. Profiling serum cytokines in COVID-19 patients reveals IL-6 and IL-10 are disease severity predictors. Emerg Microbes Infect. 2020;9(1):1123–30.
21. Ye Q, Wang B, Mao J. The pathogenesis and treatment of the 'Cytokine Storm'' in COVID-19.' J Infect [Internet]. 2020;80(6):607–13. Available from: https://doi.org/10.1016/j.jinf.2020.03.037
22. Sewell HF, Agius RM, Kendrick D, Stewart M. Vaccines, convalescent plasma, and monoclonal antibodies for covid-19. BMJ. 2020;370:1–2.
23. French MA, Moodley Y. The role of SARS-CoV-2 antibodies in COVID-19: Healing in most, harm at times. Vol. 25, Respirology. 2020. p. 680–2.
24. Duan K, Liu B, Li C, Zhang H, Yu T, Qu J, et al. Effectiveness of convalescent plasma therapy in severe COVID-19 patients. Proc Natl Acad Sci U S A. 2020;117(17):9490–6.
25. Aranha C, Patel V, Bhor V, Gogoi D. Cycle threshold values in RT-PCR to determine dynamics of SARS-CoV-2 viral load: An approach to reduce the isolation period for COVID-19 patients. J Med Virol. 2021;93(12):6794–

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Original Article

Risk Factors Associated with Suspected *Clostridium difficile* Infection (CDI) in Elderly Diarrhea Patients at Prof. Dr. I.G.N.G. Ngoerah Hospital

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ABSTRACT

Clostridium difficile infection (CDI) is a typical healthcare-associated infection that contributes to a significant proportion of morbidity and mortality among hospitalized patients. Anaerobe microbiological laboratory examinations cannot be performed at Prof. Dr. I.G.N.G. Ngoerah Hospital, leading to many undetected cases; therefore, it is crucial to determine the risk factors. Identification of cases may help to prevent, recognize, and appropriately treat CDI, reducing morbidity and mortality. This study aimed to determine the risk factors associated with suspected CDI in elderly patients with diarrhea due to their vulnerability to immunosenescence. This research used medical records as secondary data to determine the risk factors associated with suspected CDI in elderly diarrhea patients at Prof. Dr. I.G.N.G. Ngoerah Hospital during the year period of 2017 to 2021 with total of 70 samples who met inclusion criteria by purposive sampling technique. One hundred percent of all samples had a history of hospitalization for ≥ 48 h. There were 53 patients (77.1%) had a history of antibiotic use, 26 (37.1%) proton pump inhibitors (PPIs), 66 (94.3%) chemotherapy, 66 (94.3%) urinary tract infections, 6 (8.6%) kidney disease, and 7 (10%) myocardial infarction. Seven (10%) patients had vascular disease, and 11 (15.7%) had diabetes mellitus. Risk factors associated with suspected CDI in elderly patients with diarrhea at Prof. Dr. I.G.N.G. Ngoerah Hospital include hospitalization ≥ for 48 hours, use of antibiotics in the last few months, proton pump inhibitors, decreased immune system, urinary tract infection, kidney disease, myocardial infarction, vascular disease, and diabetes mellitus.

Keywords*:* *Clostridium difficile* infection (CDI), risk factors, elderly, diarrhea, and immunosenescence.

Highlights: This is a preliminary descriptive study to determine risk factors of CDI among elderly patients that could be reference for further research in Indonesia.

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INTRODUCTION

Clostridium difficile is an anaerobic Gram-positive bacillus capable of forming spores and toxins that are transmitted to humans via the fecal–oral route. In the United States, *C. difficile* is the most commonly reported pathogen of HAIs, with a case fatality rate of 14% within 30 days of diagnosis.[1] In addition, the high health costs associated with *Clostridium difficile* infection (CDI) increase the government's financial burden on health spending. It was noted that half a million infections were associated with CDI in the United States in 2011, with an incidence rate of 8.75 cases/1,000 adults treated in 2009.[2,3] A literature study by Collins et al. obtained CDI case data. The study found that The Japanese study only reported *C. difficile* ribotyping results without information on the prevalence or incidence of CDI in Japan; the incidence of CDI increased from 1.7/1,000 to 2.7/1,000 adults in Korea, and 17.1/10,000 hospitalized patients in Shanghai were associated with CDI. Meanwhile, approximately 44% and 14% of colitis patients were diagnosed as positive for *C. difficile* toxin in the Philippines and Malaysia.[4] A more recent study showed that the prevalence of CDI was 9.2% in Thailand.[5] There are few reports on the incidence or prevalence of CDI in Indonesia. One study reported that eight strains of *C. difficile* appeared in healthy people, while another study showed that the prevalence of *C. difficile* (toxin A) was 1.3% in communities and hospitals in Jakarta.[6,7] The latest report from Central Java showed a CDI prevalence of 20.6% in 2017.[8]

Several risk factors, including advanced age, exposure to antibiotics, and hospitalization, are strongly associated with CDI.[9] The ability of the elderly group's immunity to decrease with increasing age, including the speed of the immune response against infections. Elderly individuals with recent antibiotic treatment are at the highest risk for CDI because they lack beneficial gut microbiota and have low immunity due to age and other comorbidities.[10-13] This group is severely affected and has the highest mortality from CDI, with a 2% increase in risk each year after age 18 years. A report described that around one in ten deaths due to CDI in the elderly in the United States in 2010.[14] There are no data on CDI in the elderly in Bali, Indonesia due to lack of surveillance of CDI cases followed by limited laboratory facilities in hospitals capable of diagnosing CDI. In addition, cases of relapse (relapse/reinfection) and death from CDI in the elderly will be higher due to inappropriate treatment.

One cohort study estimated that about 40% of CDI cases were community-acquired (CA-CDI).[15] CA-CDI occurs in younger people, symptoms are less severe, hospital stays are shorter, relapse rates are lower, but no deaths have been reported due to CA-CDI.[16] In addition, CDI was exacerbated by the discovery of hypervirulent strains and antibiotics that were resistant to quinolones, gatifloxacin, and not levofloxacin.[17] The appearance of CA-CDI is a risk factor for domestic and foreign tourists visiting Bali. The identification of cases and appropriate treatment will reduce morbidity and mortality due to CA-CDI. Therefore, increasing the laboratory capacity to detect CDI and clinical awareness of the presence of CA-CDI is very important. Microbiological CDI diagnosis was not performed at Prof. Dr. I.G.N.G. Ngoerah Hospital, even though there are likely to be many undetected cases. Furthermore, if there is an underdiagnosis of CDI, the death rate of the elderly due to CDI will increase. Therefore, the modality of CDI examination is crucial. Owing to geriatric vulnerability to immunosenescence, this study aimed to determine the risk factors associated with *Clostridium difficile* infection (CDI) in elderly patients with diarrhea at Prof. Dr. I.G.N.G. Ngoerah Hospital.

METHODS

This type of research was a retrospective study using hospital medical records that aimed to determine the risk factors associated with suspected CDI in elderly patients with diarrhea at Prof. Dr. I.G.N.G. Ngoerah Hospital, Bali, Indonesia. The target population of this study included all elderly inpatients with suspected CDI. Based on the study of Collin *et al.* , the sample used in this study were all elderly inpatients (age 65 years and over) with suspected CDI at Prof. Dr. I.G.N.G. Ngoerah Hospital for the last 5 years (2017 to 2021), which included diarrhea patients with one or several risk factors: (i) pharmacotherapy: history of use of antibiotics (clindamycin, fluoroquinolones, second-generation cephalosporins or higher), chemotherapy, proton-pump inhibitors, and histamine type 2 blockers for at least two weeks; (ii) decreased host immunity: presence of comorbid diseases such as diabetes mellitus, chronic kidney disease, and human immunodeficiency virus; (iii) have experienced CDI previously.[4] CDI was diagnosed based on the Infectious Diseases Society of America (IDSA) 2011 criteria.[2,4] The sampling technique used was purposive sampling. The data-collection process began during the preparation stage. The processes carried out from the preparation stage to the data collection process included: (i) administration of research ethics and permits at the ethics committee of the Faculty of Medicine, Universitas Udayana, Prof. Dr. I.G.N.G. Ngoerah Hospital; (ii) arrangement of permits for conducting research from the Education and Training Section of Prof. Dr. I.G.N.G. Ngoerah Hospital; and (iii) data collection was carried out from medical records.

RESULTS

The research sample totaled 70 participants for the last five years (2017–2021) who met the inclusion criteria. One hundred% of all samples had a history of hospitalization ≥ for 48 h are showed on Table 1. Antibiotic use is frequently reported as a risk factor are showed on Table 2. A total of 53 patients (77.1%) had a history of antibiotic use. A total of 26 patients (37.1%) had a history of using proton pump inhibitors (PPIs), 66 samples (94.3%) had a history of chemotherapy, 66 samples (94.3%) had a history of urinary tract infections, 6 samples (8.6%) had a history of chronic kidney disease, 7 samples (10%) had a history of myocardial infarction, 7 samples (10%) had a history of vascular disease, and 11 samples (15.7%) had a history of diabetes mellitus.

Table 1. Risk Factors Associated with Suspected CDI in Elderly Diarrhea Patients at Prof. Dr. I.G.N.G. Ngoerah Hospital.

Variable	Percentage (%) (n=70)
History of hospitalization	
< 48 hours	0 (0)
≥ 48 hours	70 (100)
History of antibiotic usage	
Yes	53 (77.1)
No	16 (22.9)

History of *proton pumps inhibitor* usage	
Yes	26 (37.1)
No	44 (62.9)
History of chemotherapy	
Yes	4 (5.7)
No	66 (94.3)
History of human immunodeficiency virus	
Yes	11 (15.7)
No	59 (84.3)
History of urinary tract infection	
Yes	4 (5.7)
No	66 (94.3)
History of chronic kidney disease	
Yes	6 (8.6)
No	64 (91.4)
History of *myocardial infarction*	
Yes	7 (10)
No	63 (90)
History of vascular disease	
Yes	7 (10)
No	63 (90)
History of diabetic mellitus	
Yes	11 (15.7)
No	59 (84.3)

Table 2. Antibiotic Used in Elderly Diarrhea Patients at Prof. Dr. I.G.N.G. Ngoerah Hospital.

Antibiotic	**Percentage (%) (n= 53)**
Azithromycin	2 (3,8)
Doxycycline	1 (1,9)
Levofloxacin	7 (13,2)
Metronidazole	5 (9,4)

Cefoperazone	8 (15,1)
Ciprofloxacin	30 (56,6)

Eleven patients (15.7%) experienced diarrhea accompanied by blood and/or pus, 48 patients (68.6%) experienced cramps and abdominal pain, 16 patients (22.9%) experienced flatulence, 23 patients (32.9%) %) had fever, 16 patients (22.9%) experienced nausea, 23 patients (32.9%) experienced dehydration, 13 patients (18.6%) experienced decreased appetite, and 11 patients (15.7%) experienced decreased weight. Symptoms of suspected CDI in elderly diarrhea patients are showed on Table 3.

Table 3. Symptoms of Suspected CDI in Elderly Diarrhea Patients at Prof. Dr. I.G.N.G. Ngoerah Hospital.

Variable	Percentage (%) (n=70)
History of diarrhea	
With blood and/or pus	11 (15.7)
Without blood and/or pus	59 (84.3)
History of abdominal cramp and pain	
Yes	48 (68.6)
No	22 (31.4)
History of bloated stomach	
Yes	16 (22.9)
No	54 (77.1)
History of fever	
Yes	23 (32.9)
No	47 (67.1)
History of nausea	
Yes	16 (22.9)
No	54 (77.1)
History of dehydration	
Yes	23 (32.9)

No	47 (67.1)
History in decreased of appetite	
Yes	13 (18.6)
No	57 (81.4)
History in decreased of body weight	
Yes	11 (15.7)
No	59 (84.3)

DISCUSSION

Risk factors associated with suspected *Clostridium difficile* infection (CDI) in elderly diarrhea patients at Prof. Dr. I.G.N.G. Ngoerah Hospital

Previous studies have shown that most CDI cases are related to contact with healthcare facilities, and previous hospital admission has been widely described as a risk factor for CDI. *C. difficile* spores can survive for a long time on inanimate objects (resistant to heat, acids, and antibiotics), which is the main reason why these bacteria can cause infection in patients with long hospitalizations in health care facilities.[3,5,7,9] *C. difficile* spreads via the fecal-oral route and causes disease in humans through the production of two protein exotoxins (toxin A and toxin B) that are cytotoxic to colonic epithelial cells.[11,12]

Almost any antibiotic can increase susceptibility to CDI infection, but cephalosporins, fluoroquinolones, clindamycin and certain penicillins (eg co-amoxiclav) increase the risk to a greater extent.[9,11,15,16] The impact of antibiotic on the gut microbiome remains the most important risk factor. Antibiotic-associated diarrhea is one of the most common side effects of antibiotic use. Most cases are mild, but *C. difficile* infection causes a spectrum of illnesses, ranging from diarrhea to colitis, toxic megacolon, and potentially death.[17-19] Intestinal microbiota biology has been considered to play a role in the pathogenesis of this condition as well as the role of gut microbiota manipulation as a new therapeutic approach. Antibiotic use can cause diarrhea through a variety of mechanisms, including osmotic diarrhea (through the loss of gut bacteria that absorb short-chain fatty acids) and colonization and overgrowth of toxin-secreting *C. difficile.*[18,19]

Proton pump inhibitors (PPIs) are among the most prescribed outpatient and inpatient agents worldwide, with sales reaching billions of dollars worldwide. PPI has been shown to be effective in the treatment of stomach ulcers (including bleeding gastric ulcers), gastroesophageal reflux disease, *Helicobacter pylori* (in combination with antibiotic), Zollinger-Ellison syndrome, in prophylaxis of upper gastrointestinal complications with non-steroidal anti-inflammatory drugs (NSAIDs), ulcer prophylaxis stress in intensive care unit (ICU) patients, and functional dyspepsia.[11-17] The widespread use of PPIs over the last 25 years in clinical practice is a result not only of their high efficacy but also their excellent safety profile, proving to be one of the safest drug classes used in gastroenterology.[18-20] The relationship between the use of PPI and CDI is, at least theoretically, rational. Intestinal homeostasis is maintained by host defense mechanisms in which gastric acid plays an important role as a barrier to ingested bacteria and bacterial overgrowth. PPI therapy severely inhibits gastric acid production, leading to spore proliferation and transformation into the vegetative form of *C. difficile*. In addition, PPI impair leukocyte

function by inhibiting phagocytosis and acidification of phagolysosomes.[21,22]

Chemotherapy that disrupts the gut microbiome and causes mucositis may make germination of *C. difficile* spores more common, leading to greater virulence.[1-7] While CDI rates in patients with cancer vary between studies, that is, 10% during chemotherapy and up to 20% risk overall.[11-15] A total of 59 patients (84.3%) had a history of decreased immune system. Immunocompromised patients have an increased incidence and poor prognosis when accompanied by CDI. Several studies have demonstrated clinical outcomes and risk factors for CDI and CDI relapse in haemato-oncological and haematological stem cell transplanted (HSCT) patients.[15-19] The etiology for high CDI and rCDI rates in the immunocompromised population is multifactorial, consisting of repeated and prolonged exposure to broad-spectrum antibiotics, high-dose chemotherapy, acute graft-versus-host disease on HSCT, history of hospitalization, exposure to antibiotics, and repeated hospitalizations.[15,17] In contrast to antimicrobials that disrupt the normal gut microbiome, facilitating *C. difficile* proliferation, and in contrast to PPIs that allow survival of the vegetative forms of *C. difficile*, the biological mechanism is the negative impact of corticosteroids on the integrity of the gastrointestinal mucosa.[19-22]

Most comorbidities of CDI require polypharmacy and prolonged hospitalization, which directly influence the shift from *C. difficile* colonization to subsequent CDI.[15,17] Previous studies comparing patients with and without underlying chronic kidney disease found that patients with chronic kidney disease had a higher risk for both initial and recurrent episodes of CDI. Similarly, this finding was supported by a recent study that observed a nearly four-fold increased risk (OR:3.68, CI:1.63-8.31, p=0.002) of developing CDI in patients with underlying chronic kidney disease.[18,19] Reduced kidney function not only impairs the elimination of toxins from the body but also alters the functioning of the gut microbiota and activates systemic inflammation. Hypertension affects gut microbiota dysbiosis. In contrast, antihypertensive drugs have been shown to improve or harm the gut microbiota. For example, verapamil protects cells from *C. difficile* toxicity.[20,21]

Furthermore, several studies have shown that patients with diabetes are three times more likely to be at risk of CDI than non-diabetic patients.[13-19] The relationship between CDI and diabetes has been extensively studied. Diabetes is a possible independent risk factor for primary and recurrent CDI. Diabetes causes structural remodeling of the colon, which affects various functions of the digestive tract, leading to impaired motility and changes in the composition of the gut microbiota, which can lead to *C. difficile* diarrhea.[17-21] Similarly, an intervention study observed that metformin-treated diabetic patients experienced higher levels of *Clostridium* spp., which can significantly impact *C. difficile* colonization. The potential mechanism that has been investigated is that metformin alters secondary bile acid reabsorption and consequently inhibits spore development, vegetative growth, and toxin activity in *C. difficile* strains. Structural and functional changes in the colon caused by diabetes or diabetes treatment are likely to change the composition of the gut microbiota, consequently increasing or decreasing the risk of CDI; therefore, various measures have been implemented to limit potential exposure.[11,12]

Symptoms of suspected CDI in elderly diarrhea patients at Prof. Dr. I.G.N.G. Ngoerah Hospital

The clinical manifestations of CDI are very heterogeneous, ranging from an asymptomatic carrier state, mild or moderate diarrhea, to life-threatening fulminant colitis.[15-19] Although the incubation period is

not precisely defined, and according to some studies is 2-3 days, more recent studies have shown that the incubation period may be longer than 3 days and is highly individual-dependent. CDI can affect any part of the large intestine; however, the distal segment is the most frequently infected site.[13,15] Most patients with CDI have mild diarrhea and recover spontaneously after 5-10 days of discontinuation of antibiotic therapy discontinuation. Diarrhea occurs in most cases during or immediately after antimicrobial therapy, although CDI onset may also occur several weeks later.[17,19]

Clinical manifestations of CDI, apart from diarrhea, include abdominal pain, fever, nausea and vomiting, weakness, and appetite loss. Fecal occult blood tests are often positive, although active bleeding is rare. In the most severe clinical presentation of CDI, symptoms are life-threatening, and include significant dehydration, abdominal distention, hypoalbuminemia with peripheral edema, and subsequent circulatory shock, renal failure, systemic inflammatory response syndrome, septicemia, and death.[23-25] Extracolonal manifestations of CDI are rare, and most often involve small intestinal infiltration, reactive arthritis, and bacteremia.[25,27] The direct mortality rate due to CDI is estimated at 5%, while mortality due to complications of CDI reaches 15-25%, and reaches 34% in intensive care. units (ICU). Mortality doubled in ICU patients with CDI compared to ICU patients without CDI. A poor prognosis is associated with older age, high leukocytosis, hypoalbuminemia, and high creatinine levels. The first episode of CDI also increases the overall risk of death.[28-30]

Relapses of CDI symptoms most often occur during the first week after the initial episode when treatment is completed.[25]After effective treatment of the first CDI episode, at least one new recurrent episode occurs in 10–25% of patients, and up to 65% in patients who have already had > 1 episode of recurrent CDI.[26,27] Some studies have shown that half of recurrent CDI cases are due to reinfection with the original strain, whereas the other half are due to reinfection with a different strain. Impaired immune responses to *C. difficile* toxins, as well as recent exposure to its spores, are thought to contribute to relapses.[29] However, antibiotic resistance does not appear to affect the risk of recurrence. Complications of *C. difficile* include electrolyte imbalance, renal failure due to severe dehydration, systemic inflammatory response syndrome, and sepsis.[25-29] Bacteremia is rare, with few case reports of *C. difficile* bacteremia. Diagnosis is based on signs and symptoms of CDI, with confirmed microbiological evidence of toxin-producing *C. difficile* in the stool, or colonoscopic or histopathologic findings of pseudomembranous colitis, especially with the exclusion of other causes.[27] However, not all patients with CDI have pseudomembranes, especially those with mild or partially treated infections.[29]

STRENGTH AND LIMITATION

The strength of this study is its representativeness, as the data were collected for five years. A limitation of this study is that it was conducted in only one hospital. This needs to be developed as a pilot study in more hospitals, especially referral center hospitals in Indonesia.

CONCLUSIONS

Several risk factors associated with suspected CDI in elderly diarrhea patients at Prof. Dr. I.G.N.G. Ngoerah, namely hospitalization ≥ for 48 h, use of antibiotics in the last few months, use of proton pump inhibitors, decreased immune system, history of urinary tract infection, history of chronic kidney disease, history of myocardial infarction, history of vascular disease, and history of diabetes mellitus.

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ETHICAL CLEARANCE

This study was approved by the Research Ethics Committee of the Faculty of Medicine, Universitas Udayana, Prof. Dr. I.G.N.G. Ngoerah Hospital (reference letter number 1243/UN14.2.2VII.14/LT/2022).

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CONFLICT OF INTEREST

The authors affirmed that there were no conflicts of interest in this study.

AUTHOR CONTRIBUTION

Aryana IGPS determined the idea, provided guidance, conducted research, and was the guarantor and conceptor of the manuscript. Budayanti NNS conducted the research, reviewed drafts, and final contents of the manuscript. Wedari NLPH conducted the study, searched the literature, and wrote the manuscript.

REFERENCES

1. Kotila SM, Mentula S, Ollgren J, Virolainen-Julkunen A, Lyytikäinen O. Community- and Healthcare-Associated *Clostridium difficile* Infections, Finland, 2008−2013. Emerging Infectious Disease Journal. 2016;22(10):1747.
2. Banaei N, Anikst V, Schroeder LF. Burden of *Clostridium difficile* infection in the United States. The New England Journal of Medicine. 2015;2368–2369.
3. Czepiel J, Dróżdż M, Pituch H, et al. *Clostridium difficile* infection: review. European Journal of Clinical Microbiology and Infectious Diseases. 2019;38(7):1211-1221.
4. Collins DA, Hawkey PM, Riley T V. Epidemiology of *Clostridium difficile* infection in Asia. Antimicrobial Resistance and Infection Control. 2013;2(1):21.
5. Putsathit P, Maneerattanaporn M, Piewngam P, Kiratisin P, Riley T V. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Thailand. New Microbes and New Infections. 2017;15:27–32.
6. Diniz AN, Moura LNF, Cruz DSG, et al. Characterization of the virulence of three novel clade 2 *Clostridioides (Clostridium) difficile* strains and a two-year screening in animals and humans in Brazil. PLoS One. 2022;17(8):e0273013.
7. Nana T, Moore C, Boyles T, et al. South African Society of Clinical Microbiology *Clostridioides difficile* infection diagnosis, management and infection prevention and control guideline. *S Afr J Infect Dis*. 2020;35(1):219.
8. Collins DA, Gasem MH, Habibie TH, Arinton IG, Hendriyanto P, Hartana AP, et al. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Indonesia. New Microbes and New Infections. 2017;18:34–37.
9. Rupnik M. *Clostridium difficile* : (Re)emergence of Zoonotic Potential. Clinical Infectious Diseases. 2010;51(5):583–584.
10. Azimirad M, Krutova M, Yadegar A, et al. *Clostridioides difficile* ribotypes 001 and 126 were predominant in Tehran healthcare settings from 2004 to 2018: a 14-year-long cross-sectional

study. Emerg Microbes Infect. 2020;9(1):1432-1443.
11. Nibbering B, Gerding DN, Kuijper EJ, Zwittink RD, Smits WK. Host Immune Responses to *Clostridioides difficile*: Toxins and Beyond. Front Microbiol. 2021;12:804949.
12. Engevik MA, Danhof HA, Chang-Graham AL, et al. Human intestinal enteroids as a model of *Clostridioides difficile*-induced enteritis. Am J Physiol Gastrointest Liver Physiol. 2020;318(5):G870-G888.
13. Engevik MA, Danhof HA, Chang-Graham AL, et al. Human intestinal enteroids as a model of *Clostridioides difficile*-induced enteritis. Am J Physiol Gastrointest Liver Physiol. 2020;318(5):G870-G888.
14. Bagdasarian N, Rao K, Malani PN. Diagnosis and treatment of *Clostridium difficile* in adults: a systematic review. JAMA. 2015;313(4):398-408.
15. Vázquez-Cuesta S, Villar L, García NL, et al. Characterization of the gut microbiome of patients with *Clostridioides difficile* infection, patients with non-*C. difficile* diarrhea, and *C. difficile*-colonized patients. Front Cell Infect Microbiol. 2023;13:1130701.
16. Ventura MT, Cascario M, Gangemi S, Buquicchio R. Immunosenescence in aging: between immune cells depletion and cytokines up-regulation. Clinical and Molecular Allergy. 2017;15(21):1-8.
17. Fuentes E, Fuentes M, Alarcon M, Palomo I. Immune system dysfunction in the elderly. Annals of the Brazilian Academy of Science. 2017;89(1):285-299.
18. Magnusson C, Mernelius S, Bengnér M, et al. Characterization of a *Clostridioides difficile* outbreak caused by PCR ribotype 046, associated with increased mortality. Emerg Microbes Infect. 2022;11(1):850-859.
19. Magnusson C, Mernelius S, Bengnér M, et al. Characterization of a *Clostridioides difficile* outbreak caused by PCR ribotype 046, associated with increased mortality. Emerg Microbes Infect. 2022;11(1):850-859.
20. Ho J, Dai RZW, Kwong TNY, Wang X, Zhang L, Ip M, et al. Disease Burden of *Clostridium difficile* Infections in Adults, Hong Kong, China, 2006-2014. Emerging Infectious Diseases. 2017;23(10):1671–1679.
21. Kim YS, Han DS, Kim YH, Kim WH, Kim JS, Kim HS, et al. Incidence and clinical features of *Clostridium difficile* infection in Korea: a nationwide study. Epidemiology and Infection. 2013;141(1):189–194.
22. Sartelli M, Di Bella S, McFarland LV, et al. 2019 update of the WSES guidelines for management of *Clostridioides* (*Clostridium*) *difficil e* infection in surgical patients. *World J Emerg Surg*. 2019;14:8.
23. Hain-Saunders NMR, Knight DR, Bruce M, Riley TV. *Clostridioides difficile* infection and One Health: an equine perspective. Environ Microbiol. 2022;24(3):985-997.
24. Jones AM, Kuijper EJ, Wilcox MH. *Clostridium difficile*: a European perspective. The Journal of Infection. 2013;66(2):115–128.
25. Cheng JKJ, Unnikrishnan M. *Clostridioides difficile* infection: traversing host-pathogen interactions in the gut. Microbiology (Reading). 2023;169(2):001306.
25. Guerrero DM, Nerandzic MM, Jury LA, Jinno S, Chang S, Donskey CJ. Acquisition of spores on gloved hands after contact with the skin of patients with *Clostridium difficile* infection and with environmental surfaces in their

rooms. American Journal of Infection Control. 2012;40(6):556–558.
26. Asempa TE, Nicolau DP. *Clostridium difficile* infection in the elderly: an update on management. Clinical Interventions in Aging. 2017;12:1799–1809.
27. Raeisi H, Azimirad M, Nabavi-Rad A, Asadzadeh Aghdaei H, Yadegar A, Zali MR. Application of recombinant antibodies for treatment of *Clostridioides difficile* infection: Current status and future perspective. Front Immunol. 2022;13:972930.
28. Leffler DA, Lamont JT. *Clostridium difficile* Infection. New England Journal of Medicine. 2015;372(16):1539–1548.
29. De Roo AC, Regenbogen SE. *Clostridium difficile* Infection: An Epidemiology Update. Clinics in Colon and Rectal Surgery. 2020;33(2):49-57.
30. McDonald LC, Gerding DN, Johnson S, et al. Clinical Practice Guidelines for *Clostridium difficile* Infection in Adults and Children: 2017 Update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin Infect Dis. 2018;66(7):e1-e48.

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Original Article

Polysaccharide Capsule Serotype and Antibiotic Susceptibility Pattern of *Streptococcus pneumoniae* Clinical Isolates in Bali

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ABSTRACT

Streptococcus pneumoniae (S. pneumoniae) is a causative agent of pneumonia that can spread progressively, cause invasive disease, and increase mortality in humans. Pneumococcal or polysaccharide conjugate vaccination reduces pneumonia rates by vaccine-covered serotypes, but increases infection by non-vaccine serotypes. To determine the polysaccharide capsule serotype of *S. pneumoniae* isolates that cause infection at Prof. Dr. I.G.N.G. Ngoerah General Hospital and patterns of *S. pneumoniae* susceptibility to antibiotics from April 2017 to March 2022. All *S. pneumoniae* isolates from April 2017 and April 2022 were stored in STGG media in a freezer at -80 ℃ then subcultured on sheep blood agar. Polymerase Chain Reaction (PCR) was performed to determine pneumolysin and capsular polysaccharide serotypes of *S. pneumoniae*. Of the 22 isolates studied, the order of the number of serotypes from the highest was serotype 19F, 3, 6A/B, 33F, 15B/C, 4, and 6V. Seven isolates were untypeable. Antibiotic sensitivity pattern *S. pneumoniae* was found to be sensitive to linezolid 91%, vancomycin 86%, levofloxacin and benzylpenicillin 82%, ceftriaxone and clindamycin 73%, erythromycin 55%, and chloramphenicol 45%. Serotype 19F was identified as the most dominant capsular serotype; however, serotypes 33F and 15B/C were also found. Interestingly, the 33F serotype is not covered in the 13-valent pneumococcal conjugate vaccine (PCV13) but is covered in pneumococcal polysaccharide vaccine 23 (PPSV23), and the 15B/C serotype is not included in either PCV13 or PPSV23. The antimicrobial susceptibility patterns revealed that *S. pneumoniae* was susceptible to linezolid, vancomycin, benzylpenicillin, and levofloxacin.

Keywords: : Antibiotic Susceptibility, Polysaccharide Capsule Serotype, Pneumococcal Vaccine, Polymerase Chain Reaction, dan *Streptococcus pneumoniae*.

Highlights: A serotype of *S. pneumoniae* was found outside the vaccine candidate and is helpful for the development of a vaccine for *S. pneumoniae* infection in the future.

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INTRODUCTION

Pneumonia, meningitis, sepsis, and otitis media are among the most prevalent invasive and non-invasive illnesses caused by *Streptococcus pneumonia*, especially in younger and older individuals.[1,2] The invasive disease of *S. pneumoniae* is caused by colonization of these bacteria in the respiratory tract, which progressively infect and spread to other cells.[3] Approximately one million children under five die annually because of these bacteria.[2]

A polysaccharide capsule on the surface of *S. pneumoniae* is considered a significant virulence factor. It was discovered and published by Pasteur in 1880. A study carried out during the first three decades of the 20th century showed the presence of several capsule serotypes of *S. pneumoniae*. The polysaccharide capsule of *S. pneumoniae* was first isolated by Dochez and Avery in 1917. They found that the capsule of *S. pneumoniae* is a soluble pneumococcal substance consisting of proteins. In 1925, Avery et al. discovered that the capsule of *S. pneumoniae* consisted of polysaccharides, the first known non-protein antigen. The thickness of the capsule was approximately 400 nm, accounting for more than half of the volume of *S. pneumoniae.*[4] These non-protein antigens protect bacteria from phagocytosis. Capsules are powerful antioxidants against oxidative stress. They can evade immune responses, such as protection from endosome killing by the host and increasing the translocation rate into organs during bacteremia.[1] There are 100 types of *S. pneumoniae* capsules, yet only 20–30 types of capsules are capable of causing invasive disease. This serotype indicates that each capsule has different specific properties.[1] Various capsule types are linked to the disease, with serotypes 14 and 1 causing most childhood pneumonia cases, and serotypes 3, 6A, 6 B, 9N, and 19F, which exhibit higher mortality rates.[3] A study in Central Lombok revealed that the predominant capsule types of *S. pneumoniae* as 6A/B, 19F, 23F, and 15B/C.[5] Meanwhile, in a study in Jakarta, the capsule serotypes found were 3,6A/B, 15B/C and 35F.[6] Research conducted in Semarang found that capsular serotypes in children were 6A/B, 15B/C, 11A, 23F, 19F, and 23A, and 6A/B, 15B/C, and 15A in adults.[7] According to a prior investigation carried out at Prof. Dr. I.G.N.G. Ngoerah General Hospital, the primary serotypes identified were 19F (7 isolates), 23F (2 isolates), 6A/B (2 isolates), 7F (1 isolate), and 15B/C (1 isolate).[8]

Bacterial pneumonia can be prevented using polysaccharide and pneumococcal conjugate vaccines. Four types of conjugate vaccines for pneumonia have been developed: PCV7, PCV9, PCV10, and PCV13. Each type of PCV is associated with a different serotype. Consequently, the efficacy of a given vaccine is contingent on its ability to effectively target its serotypes.[9] PCV7 has been granted an official licensure by the United States and European Union governments. This vaccine is composed of serotypes 23F, 19F, 18C, 14, 9V, 6 B, and 4. PCV13 and PCV10 vaccines were released in 2000 and 2001, respectively. PCV7 vaccination was improved by adding serotypes 7F, 1, and 5, which were already in use for PCV10, and by adding serotypes 19A, 7F, 6A, 5, 3, and 1 for PCV13.[10,11] The UK has sold PPSV23 since 2003. This vaccine contains PCV13 serotypes, except 6A and 11 others:9N, 10A, 2, 8, 11A, 17F, 20, 22F, 12F, 15 B, and 33F. The advice is for those aged ≥ 65 years and in clinical risk categories.[12,13]

S. pneumoniae has shown increased resistance to antimicrobials that were once efficacious. Therefore, the antimicrobial susceptibility pattern of *S. pneumoniae* helps determine empirical therapy while waiting for culture results to establish definitive therapy.[3] In a retrospective, multicenter study conducted in 14 institutions from 13 provinces in China, Chen et al. discovered that antibiotic regimens for the definitive treatment of invasive pneumococcal disease

were frequently inappropriate, with excessive prescriptions of carbapenems, vancomycin, and linezolid.[14] Additionally, a descriptive study conducted by Mohanty et al. in the United States demonstrated increased levels of antimicrobial resistance (AMR) in *S. pneumoniae* isolates acquired from adults diagnosed with either invasive or noninvasive pneumococcal disease. Furthermore, this research revealed a tendency toward heightened macrolide resistance.[15]

The researchers aimed to analyze the distribution of serotype polysaccharide capsules of *S. pneumoniae* isolates and their antibiotic susceptibility profile at Prof. Dr. I.G.N.G. Ngoerah General Hospital between April 2017 and March 2022 based on the provided data.

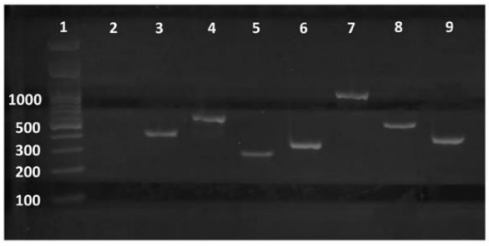

Figure 1. Gel Electrophoresis Results of PCR Serotype Streptococcus Pneumoniae. Line (1) Marker, (2) Negative Control, (3) Serotype 3 (371bp), (4) Serotype 15B/C (496bp), (5) Serotype 6A/B (250bp), (6) Serotype 19F (304bp), (7) Serotype 9V (753bp), (8) Serotype 4 (430bp), (9) Serotype 33F (338bp).

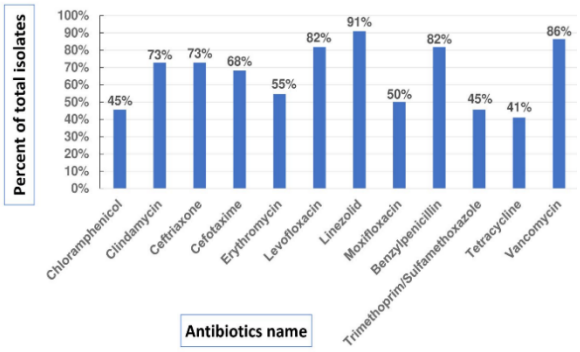

Figure 2. Antibiotic Susceptibility Pattern 22 Isolates of *Streptococcus pneumoniae*.

MATERIALS AND METHODS

Bacterial strain, medium, and growth conditions

A total of 22 isolates of *S. pneumoniae* were stored in STGG media at a temperature of -80 °C and were subjected to cultivation on 5% sheep blood agar. The process of incubation was incubated in an aerobic environment at a temperature of 37 °C for a duration of 24 h. Microbial isolates were obtained from blood (18%, n=4), sputum (36%, n=8), and other clinical specimens (45%, n=10) between April 2017 and April 2022. Identification of *S. pneumoniae* was carried out presumptively by evaluating colony characteristics, type of hemolysis, and susceptibility using 5 g optochin discs (Oxoid, Thermo Fisher Diagnostics B.V., The Netherlands). If the Opthocin test results were sensitive, the bacterial colonies were subjected to identification tests (GP ID card) and AST (AST-ST03 card) using the VITEK-2 Compact machine (bioMérieux®). Antibiotics tested for sensitivity were linezolid, vancomycin, benzylpenicillin, levofloxacin, ceftriaxone, clindamycin, cefotaxime, erythromycin, moxifloxacin, chloramphenicol, trimethoprim/sulfamethoxazole, and tetracycline.

Bacterial DNA Isolation

S. pneumoniae was extracted using a Roche High Pure PCR Isolation Kit Template (Roche Life Science, Indianapolis, USA). *S. pneumoniae* colonies were suspended in 200 µL of pH 7.3 phosphate-buffered saline. DNA isolation from the bacterial suspensions was performed according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR) for the pneumolysin gene (ply)

PCR was used to detect the presence of the pneumolysin gene (ply). Amplification of the PCR mixture was performed using GoTaq Green Master Mix (Promega, Madison, WI, USA). The present study employed a specific primer pair targeting ply, with the forward primer sequence being 5'-

ATTTCTGTAACAGCTACCAACGA-3' and the reverse primer sequence being 5'-GAATTCCCTGTCTTTTCAAAGTC-3. '[16]

The PCR process consisted of an initial pre-denaturation step at a temperature of 94 °C for 2 min. This was followed by 35 cycles of denaturation at 94 °C for 30 seconds, primary annealing at 54 °C for 30 seconds, and extension at 72 °C for 1 min. The final step involved a final extension at 72 °C for 5 min. An iCycler, a Bio-Rad thermal cycler, was used for this process. The amplicons were subjected to electrophoresis on 1.5% agarose gel in TBE buffer at 100 volts for 35 min. DNA was visualized using GelRed Nucleic Acid Gel Stain (Biotium, Hayward, CA 94545) and subsequently captured using Gel Doc (Bio-Rad). Amplification of a 348 bp band size has yielded positive results.

Polymerase Chain Reaction (PCR) for Serotyping Capsular Polysaccharides (CPS)

PCR used serotype primers 4, 5, 18, 19A, 7C, 15A, 9V, 17F, 1, 14, 23A, 3, 19F, 12, 33F, 15B/C, 23F, 6A/B, and 7F for capsular serotyping. PCR was performed using two distinct techniques. First, multiplex PCR was used to simultaneously determine CPS type. Multiplex PCR was performed using the Kapa 2G Fast Ready Mix PCR Kit with Dye (Kapa Biosystems, Promega, Madison, WI, USA) as its master mix. Modifications to the multiplex PCR protocol were described in a previous study.[17] The cycle consisted of 2 min of pre-denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing primer at 54 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min (iCycler, Bio-Rad thermal cycler). PCR for CPS serotyping was performed using primers detailed in previous studies.[8,17] Uniplex PCR was performed with Go Taq® Green Master Mix (Promega, Madison, USA) to ascertain the type of CPS that multiplex PCR had not typed. For CPS serotyping using uniplex PCR, primer concentrations of 0.3 M were utilized. The PCR cycle was initiated with a 2-minute pre-denaturation at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, primary annealing at 54 °C for 1 min, extension at 72 °C for 1 min, and a 5-minute final extension at 72 °C (iCycler, Bio-Rad thermal cycler). The amplicons were then electrophoresed for 35 min on a 1% agarose gel in TBE buffer at 50 volts. DNA was visualized using GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA 94545) and then documented using Gel Doc (Bio-Rad).

The positive control used in this study was obtained from a previous study conducted by Fatmawati et al. ; however[8], the positive controls for newly discovered serotypes 3, 4, 6V, and 33F were not included.

Examination of the sensitivity of *S. pneumoniae* to antibiotics

Antibiotic sensitivity information was obtained from the VITEK-2 Compact (bioMérieux®) results using a VITEK® 2 AST-ST03 card. Then, using Microsoft Excel 2019, the antibiotic susceptibility patterns of the clinical isolates of *S. pneumoniae* in Bali were determined.

RESULTS AND DISCUSSION

All isolates were found to be *S. pneumoniae* based on sensitivity to optochin, presence of a pneumolysin-specific gene, and phenotypic matching to the colony description. Of the 22 isolates studied, the most common serotype was 19F (32%; n=7), followed by serotype 3 (9%; n=2) and serotype 6A/B (9%; n=2), followed by serotype 33F, 15B/C, 4, and 6V which were obtained for each serotype. The positive controls for capsule *S. pneumoniae* serotype detection used in this study were obtained from a previous study conducted by Fatmawati et al. ; however[8], the positive controls for newly discovered serotypes 3, 4, 6V and 33F were not included. The remaining

isolates were confirmed to be untypable strains (32%; n=7). The types of serotypes found can be seen in the PCR results shown in Figure 1. In this study, 15 isolates were serotyped, accounting for 87% (n=13/15) of the total serotyped isolates covered by PCV 13. Interestingly, in this study, two isolates were not included in PCV 13, namely serotypes 33F and 15B/C.

This study obtained seven untypable isolates because not all capsule pneumonia serotypes were designed for primary sequence detection by PCR. Therefore, an untypable isolate may represent another serotype outside the designed primary sequence.

The antibiotic susceptibility pattern from these 22 isolates is sensitive to linezolid (91%; n=20), vancomycin (86%; n=19), benzylpenicillin (82%; n=18), levofloxacin (82%; n=18), ceftriaxone (73%; n=16), clindamycin (73%; n=16), cefotaxime (68%; n=15), erythromycin (55%; n=12), moxifloxacin (50%; n=11), chloramphenicol (45%; n=10), trimethoprim/sulfamethoxazole (45%; n=10), and tetracycline (41%; n=9), as shown in Figure 2.

In this study, serotype 19F was dominant among the isolates, followed by serotype 3, 6A/B, 33F, 15B/C, 4, and 6V. This result supports the findings of Zhao et al. , who discovered that serotype 19F was the largest five-serotype capsule of *S. pneumoniae* found in China.[18]

The five serotypes (19F, 6A/B, 3, 4, and 6V) found in our study included the PCV13 serotype, two serotypes non-PCV13 were serotype 33F and 15B/C, and one serotype that excluded both PCV13 and PPSV23 was 15B/C.

Although the efficacy of the current pneumococcal conjugate vaccination against the targeted serotype has significantly decreased the disease burden produced by this serotype, several countries have reported an increase in infectious pneumococcal illnesses caused by non-vaccine strains. The increased prevalence of infection with serotypes not covered by PCV13 in children and adults, specifically due to serotypes 22F and 33F, has been documented in multiple nations.[19] Several studies have also identified non-vaccine strains, namely serotype 15B/C, in France, Colombia, Thailand, and Indonesia (Lombok, Jakarta, and Semarang).[5–7,20–22] Interestingly, serotype 15B/C is not covered by PCV13 or PPSV23. Serotype 15B/C has potential as a vaccine candidate, considering that cases of invasive pneumonia caused by this serotype, including serotype 15B/C, are increasing in several countries such as France, Colombia, Thailand, and Indonesia. [5–7,20–22]

The introduction and use of unconjugated versus conjugated pneumococcal polysaccharide vaccines have reduced the proportion of pneumococcal infections caused by *S. pneumoniae*. Those two years of age or older are protected against invasive illness caused by the 23 capsular serotypes included in the PPSV23 vaccination because of the development of a vaccine called PPSV23. Children under two years old do not have an immune response that may be considered protective after receiving the PPSV23 vaccination. The PCV7 vaccine was developed to target seven PPSV23 serotypes responsible for causing invasive pediatric diseases. It was specifically designed and recommended for administration to children under two years of age. In addition, the development of vaccines aligns with the *S. pneumoniae* serotype responsible for pneumonia in humans, specifically PCV10, with the most recent iteration being PCV13. The PCV13 vaccine comprises seven PCV7 serotypes, five supplementary PPSV23 serotypes, and an additional serotype. Novel pneumococcal serotypes that are absent in PPSV23 and PCV7 have been identified.[23] Multiple systematic reviews and meta-analyses have presented empirical data supporting the efficacy and safety of PCV13 and PPSV23 vaccines in preventing pneumococcal

disease. Studies have shown that these vaccines are safe for both children and older people.[24] Thromp et al. showed that PCV13 protects fewer serotypes than PPSV23; however, PCV13 is recommended for pneumococcal vaccines because of its higher immunogenic potential than PPSV23. This study also demonstrated that PCV13 could be administered before PPSV23 to achieve a more robust response. To date, no studies have compared the optimal interval between PCV13 and PPSV23 vaccinations.[25]

Currently, PCV is effective in reducing the incidence of IPD caused by vaccine-covered serotypes. However, difficulties remain, primarily related to the emergence of non-vaccine serotypes as a cause of IPD. A pneumococcal protein ubiquitously expressed in all serotypes is an intriguing alternative for the development of pneumococcal vaccines, independent of serotypes. However, additional assessment of this approach is necessary. Preclinical studies have assessed protein fragments and peptides with antigenic properties as potential vaccine antigens. These antigens have been studied independently and in conjunction with complete pneumococcal proteins as vaccine candidates, and have shown potential as viable alternatives. The antigens above possess the beneficial characteristics of protein antigens, such as the ability to elicit an immune response in neonates, the capacity to establish immune memory, and the capability of "priming/boosting" independent of serotype. Peptide-based vaccines, which are both low-cost and stable, can combine multiple antigens that are highly conserved. These vaccines can potentially activate both cellular and humoral immune responses, thereby preventing various stages of pneumococcal disease. In addition, they provide a more comprehensive range of serotype coverage and offer improved protection. The vaccines in question will utilize innovative delivery system methodologies, including conjugation to Toll-like receptors (TLRs) and the encapsulation of peptide antigens within nanoparticles, to substantially enhance their immunogenicity.[26]

In this study, most *S. pneumoniae* isolates remained sensitive (> 80%) to benzylpenicillin, levofloxacin, vancomycin, and linezolid. The sensitivity to chloramphenicol, clindamycin, ceftriaxone, cefotaxime, erythromycin, moxifloxacin, trimethoprim, sulfamethoxazole, and tetracycline started to decrease below 80%. This resembled the findings of Sander et al. However, in a study by Sander et al., the MDR and XDR sensitivity patterns of *S. pneumoniae* were specifically explained.[27] In contrast to a study by Assefa et al., the sensitivity of *S. pneumoniae* to chloramphenicol was 45%. In contrast, the study by Assefa et al. in Northwest Ethiopia found 100% of isolates sensitive to chloramphenicol, with all populations from the study being adults. [28] In the present study, the population of stored isolates varied with age. In addition, it is different from the research of Temesgen et al., who found that the sensitivity of *S. pneumoniae* to erythromycin was 96.7%, whereas the sensitivity of isolates in this study was 55%.[29] In most outpatient cases of CAP, the Infectious Disease Society of America (IDSA) recommends the use of doxycycline, macrolides, or fluoroquinolones as first-line antibiotic treatment. In this study, *S. pneumoniae* was 82% sensitive to levofloxacin.[30]

Serotype 19F had the highest antibiotic resistance, which was resistant to trimethoprim/sulfamethoxazole (86%; n=6/7), followed by erythromycin (71%, n=5/7). Apart from being resistant to trimethoprim/sulfamethoxazole and erythromycin, the possibility of resistance to other antibiotics cannot be ruled out. These results are similar to those of a study by Nagaraj et al. , who showed 100% serotype 19F resistance to erythromycin (n=2/2).[31]

STRENGTH AND LIMITATION

The limitation of this study pertains to the restricted number of isolates that were subjected to testing, as only 22 isolates were retained. However, positive controls for serotypes 3, 4, 6V and 33F were not included in this study because sequencing was planned for further studies. In addition, future studies are necessary to improve the serotype test by designing primer sequences to test other serotypes that were not present in this study, as seven isolates remained non-typeable.

CONCLUSIONS

Serotype 19F is the predominant capsule serotype of *S. pneumoniae*. Serotype 33F, which is not part of PCV13 but part of PPSV23, was also found. Serotypes 15B/C were also identified in this study. This serotype is not a part of PCV13 or PPSV23.

S. pneumoniae was found to be sensitive to linezolid in 91% of the cases, vancomycin in 86% of the cases, levofloxacin and benzylpenicillin in 82%, ceftriaxone and clindamycin in 73%, erythromycin in 55%, and chloramphenicol in 45%.

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ETHICAL CLEARANCE

The research protocol was approved by Faculty of Medicine Research Ethics Commission Udayana University, ETHICAL CLEARANCE No: 1078/UN 14.2.2.Vll.14/LT/2022.

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CONFLICT OF INTEREST

All authors who contributed to this work confirmed that there were no conflicts of interest.

AUTHOR CONTRIBUTION

INMM performed the writing, original draft, investigation, and visualization. IPBM performed the writing – review and editing, methodology, software, and data curation. NMAT performed the writing – review and editing, supervision, validation, and conceptualization.

REFERENCES

1. Orihuela C. Capsule protects against intracellular killing and enables vascular endothelial cell translocation during invasive pneumococcal disease. 2021;
2. Gonzales-Siles L, Salvà-Serra F, Degerman A, Nordén R, Lindh M, Skovbjerg S, et al. Identification and capsular serotype sequetyping of *Streptococcus pneumoniae* strains. J Med Microbiol. 2019;68(8):1173–88.
3. Abdallah FE, Fathy M, Fouda E, Fahmy H, Abdallah N. Antibiotic susceptibility and capsular genes of *Streptococcus pneumoniae* colonizing children with chronic respiratory diseases. Microbes and Infectious Diseases. 2021;0(0):0–0.
4. Paton JC, Trappetti C. *Streptococcus pneumoniae* Capsular Polysaccharide . Microbiol Spectr. 2019;7(2):304–15.
5. Hadinegoro SR, Prayitno A, Khoeri MM, et al. Nasopharyngeal carriage of *Streptococcus pneumoniae* in healthy children under five years old in Central Lombok Regency, Indonesia. Southeast Asian J Trop Med Public Health. 2016;47:485–93.

6. Safari D, Harimurti K, Khoeri MM, Waslia L, Mudaliana S, A'yun HQ, et al. *Staphylococcus aureus* and *Streptococcus pneumoniae* prevalence among elderly adults in Jakarta, Indonesia. Southeast Asian Journal of Tropical Medicine and Public Health. 2015;46(3):465–71.
7. Farida H, Severin JA, Gasem MH, Keuter M, Wahyono H, Van Den Broek P, et al. Nasopharyngeal carriage of *Streptococcus pneumoniae* in pneumonia-prone age groups in Semarang, Java Island, Indonesia. PLoS One. 2014;9(1):16–8.
8. Fatmawati NND, Tarini NMA, Mayura IPB. Multiplex Pcr For Detection Of Capsular Polysaccharides Types Of *Streptococcus pneumoniae* Clinical Isolates In Bali. International Journal of Bioscience and Biotechology. 2015;II(2):93–9.
9. Seftiani F, Saputri D, Wathoni N. Analisis Efektivitas Biaya Penggunaan Vaksin Pneumonia Di Berbagai Negara. Vol. 18. 2020. Available from: https://jurnal.unpad.ac.id/farmaka/article/view/22281/pdf
10. Amari S, Warda K, Elkamouni Y, Arsalane L, Bouskraoui M. Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* among children with acute otitis media in Marrakech, Morocco [Internet]. Vol. 14. 2022. Available from: http://ijm.tums.ac.ir
11. Sings HL, De Wals P, Gessner BD, Isturiz R, Laferriere C, Mclaughlin JM, et al. Effectiveness of 13-Valent Pneumococcal Conjugate Vaccine against Invasive Disease Caused by Serotype 3 in Children: A Systematic Review and Meta-analysis of Observational Studies. Vol. 68, Clinical Infectious Diseases. Oxford University Press; 2019. p. 2135–43.
12. Lawrence H, Pick H, Baskaran V, Daniel P, Rodrigo C, Ashton D, et al. Effectiveness of the 23-valent pneumococcal polysaccharide vaccine against vaccine serotype pneumococcal pneumonia in adults: A case-control test-negative design study. PLoS Med. 2020 Oct 23;17(10).
13. Matanock A, Lee ; Grace, Gierke R, Kobayashi M, Leidner A, Pilishvili T. Morbidity and Mortality Weekly Report Use of 13-Valent Pneumococcal Conjugate Vaccine and 23-Valent Pneumococcal Polysaccharide Vaccine Among Adults Aged ≥65 Years: Updated Recommendations of the Advisory Committee on Immunization Practices [Internet]. 2019. Available from: https://www.cdc.gov/mmwr/volumes/68/wr/pdfs/mm6846a5-H.pdf
14. Chen T ming, Li W hui, Wang F, Tan K, Zhu Q xiong, Zhou K, et al. Antibiotics prescription for targeted therapy of pediatric invasive pneumococcal diseases in China: a multicenter retrospective study. BMC Infect Dis. 2021 Dec 1;21(1).
15. Mohanty S, Johnson KD, Yu KC, Watts JA, Gupta V. A Multicenter Evaluation of Trends in Antimicrobial Resistance Among *Streptococcus pneumoniae* Isolates From Adults in the United States. Open Forum Infect Dis [Internet]. 2022;9(9):1–9. Available from: https://doi.org/10.1093/ofid/ofac420
16. Muneer B. Screening of Pneumococcal Pneumonia by Amplification of Pneumolysin Gene in Children Visiting Hospitals in Lahore, Pakistan [Internet]. 2012. Available from: https://www.researchgate.net/publication/235691319
17. Pai R, Gertz RE, Beall B. Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J Clin Microbiol. 2006;44(1):124–31.
18. Zhao W, Pan F, Wang B, Wang C, Sun Y, Zhang T, et al. Epidemiology

characteristics of *Streptococcus pneumoniae* from children with pneumonia in shanghai: A retrospective study. Front Cell Infect Microbiol. 2019;9(JUL).
19. Sempere J, de Miguel S, González-Camacho F, Yuste J, Domenech M. Clinical Relevance and Molecular Pathogenesis of the Emerging Serotypes 22F and 33F of *Streptococcus pneumoniae* in Spain. Front Microbiol. 2020 Feb 27;11.
20. Phongsamart W, Srifeungfung S, Chatsuwan T, Rungnobhakhun P, Maleesatharn A, Chokephaibulkit K. *Streptococcus pneumoniae* Causing Invasive Diseases in Children and Adults in Central Thailand, 2012–2016. Vaccines (Basel). 2022 Aug 1;10(8).
21. Varon E, Cohen R, Béchet S, Doit C, Levy C. Invasive disease potential of pneumococci before and after the 13-valent pneumococcal conjugate vaccine implementation in children. Vaccine [Internet]. 2015;33(46):6178–85. Available from: http://dx.doi.org/10.1016/j.vaccine.2020.10.015
22. Andrea Palacios P, Duarte C, Sanabria O, Moreno J. Molecular characterization of non-vaccine *Streptococcus pneumoniae* serotypes 11A, 15 B/C and 23A recovered from invasive isolates in Colombia. Biomédica. 2017;37:390–6
23. Daniels CC, Rogers PD, Shelton CM. A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens [Internet]. Vol. 21, J Pediatr Pharmacol Ther. 2016. Available from: www.jppt.org
24. Asai N, Mikamo H. Recent topics of pneumococcal vaccination: Indication of pneumococcal vaccine for individuals at a risk of pneumococcal disease in adults. Vol. 9, Microorganisms. MDPI; 2021.
25. Tromp KM, Campbell MW, Vazquez A. Recent Developments and Future Directions of Pneumococcal Vaccine Recommendations. Vol. 37, Clinical Therapeutics. Excerpta Medica Inc.; 2015. p. 928–34.
26. Lagousi T, Basdeki P, Routsias J, Spoulou V. Novel protein-based pneumococcal vaccines: Assessing the use of distinct protein fragments instead of full-length proteins as vaccine antigens. Vaccines (Basel). 2019;7(1).
27. Sader HS, Mendes RE, Le J, Denys G, Flamm RK, Jones RN. Antimicrobial susceptibility of *Streptococcus pneumoniae* from North America, Europe, Latin America, and the Asia-Pacific Region: Results from 20 years of the SENTRY Antimicrobial Surveillance Program (1997-2016). Open Forum Infect Dis. 2019 Mar 15;6:S14–22.
28. Assefa M, Tigabu A, Belachew T, Tessema B. Bacterial profile, antimicrobial susceptibility patterns, and associated factors of community-acquired pneumonia among adult patients in Gondar, Northwest Ethiopia: A cross-sectional study. PLoS One. 2022 Feb 1;17(2 February).
29. Temesgen D, Bereded F, Derbie A, Biadglegne F. Bacteriology of community acquired pneumonia in adult patients at Felege Hiwot Referral Hospital, Northwest Ethiopia: A cross-sectional study. Antimicrob Resist Infect Control. 2019 Jun 14;8(1).
30. Yadegarynia D, Tehrani S, Nejad FM, Shojaeian F, Keyvanfar A. Levofloxacin versus ceftriaxone and azithromycin for treating community-acquired pneumonia: a randomized clinical trial study [Internet]. Vol. 14. 2022. Available from: http://ijm.tums.ac.ir
31. Nagaraj S, Kalal BS, Manoharan A, Shet A. *Streptococcus pneumoniae* serotype prevalence and antibiotic resistance among young children with invasive pneumococcal disease: Experience from a tertiary care center in South India. Germs. 2017;7(2):78–85.

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Case Report

A Peculiar Manifestation of Urinary Tract Infection in a 76-year-old Female

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ABSTRACT

Purple Urine Bag Syndrome (PUBS) is a rare and benign clinical phenomenon associated with urinary tract infections, characterized by the distinctive purple discoloration of a urinary catheter bag. The underlying cause of PUBS is related to bacterial activity involved in the breakdown of tryptophan, mainly in individuals with predisposing factors such as long-term catheterization, constipation, alkaline urine, and being female. We present the case of a 76-year-old female patient with a history of tuberculous spondylitis who developed PUBS during her hospital stay. The patient had been bedridden for three years and had been given a urinary catheter, which was routinely replaced by a non-medical family member and without a proper antiseptic procedure. A urine culture revealed the presence of Proteus mirabilis, and the patient was treated with intravenous antibiotics while also replacing the urinary catheter and bag. After five days of treatment, the urine bag and catheter remained clear, and the patient was discharged. PUBS is a significant indicator of urinary tract infection, necessitating prompt medical intervention to prevent complications. Healthcare professionals should be aware of this syndrome to enable early detection and appropriate management.

Keywords: Urinary tract infection, CAUTI, purple urine bag syndrome, Proteus mirabilis, and tryptophan.

Highlights: Purple urine bag syndrome is a rare clinical sign of urinary tract infection, manifested as purple discoloration of urinary catheter bag. Healthcare professionals should be aware of this syndrome in order to provide early medical intervention and to prevent complications.

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INTRODUCTION

Purple urine bag syndrome (PUBS) is a rare condition that was first reported in 1978.[1] It is a purplish discoloration of the urinary bag, and a rare manifestation of urinary tract infection (UTI).[2,3] It is caused by bacteria involved in the tryptophan metabolism in patients with predisposing factors such as long-term use of a urinary catheter, constipation, and female gender.[4,5] Notably, while the vivid discoloration is benign, it acts as a visual cue for clinicians as the most common clinical presentation.[6]

Although the appearance of PUBS is benign, it can serve as a disconcerting sight for family members and caregivers alike. For medical professionals, the emergence of PUBS indicates an underlying red flag, often pointing toward a UTI that may have arisen due to lapses in the maintenance or hygiene of the urinary catheter. Such overlooked infections could escalate, ushering in considerable morbidity and even life-threatening consequences.[7,8] We present a rare purple urine bag syndrome in a 76-year-old woman with a history of long-term use of a urinary catheter.

CASE REPORT

A 76-year-old Indonesian woman was admitted to the hospital due to nausea and vomiting that had worsened for two hours before admission. She had a history of tuberculous spondylitis two years before admission and was treated with anti-tuberculosis drugs for one year. A spine MRI detected a Th10-Th12 Spondylitis with paravertebral abscess due to suspect Tuberculosis, Spondylarthroses L3-L4, L4-S1 Spondylarthroses, and Herniated Disc L2-L3, L3-L4, L4-L5. She was then immobilized and bedridden for most of her days. Her lower limb muscles are atrophic, and there was no decubitus ulcer. She was fitted with an indwelling urinary catheter from 2019 with a one-month interval of self-changing. The patient also had a history of epilepsy, with her first seizure in 2019, and is currently on maintenance therapy.

The patient also suffered from chronic constipation since she was a teenager, and it became severe since she was bedridden. She denied complaints and manifestations of urinary symptoms.

Figure 1. The Urinary Catheter Bag Showing Purple Staining.

During hospitalization, her vital signs were within normal limits, and her signs of dehydration were improving. She had normal urine output and creatinine with eGFR 88.82 mL/min/1.73 m^2. On the fifth day of admission, her urinary catheter and bag showed purplish discoloration as shown in Figure 1. However, when collected, the urine was yellowish (Figure 2).

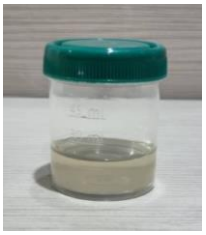

Figure 2. Urine Collected from Urine Bag.

Further history-taking revealed that her urinary catheter was inserted every month by a non-medic without any antiseptic procedure. Her urinalysis revealed a pH of 8.0 (6.5-8.5), leukocyte esterase +3 (negative), positive bacteria (negative), and triple phosphate crystal (negative). A urine culture sample was collected and later revealed $>10^5$ CFU of *Proteus mirabilis,*

which was resistant to nitrofurantoin and trimethoprim/sulfamethoxazole. She has subsequently managed with intravenous ciprofloxacin 400 mg twice daily with a urinary catheter and bag replacement.

After five days of antibiotic therapy, the urine bag and catheter remained clear and normal, with no purple discoloration (Figure 3). Additional urinalysis and urine culture showed no bacteria present. The patient was discharged and scheduled for a follow-up seven days post-discharge.

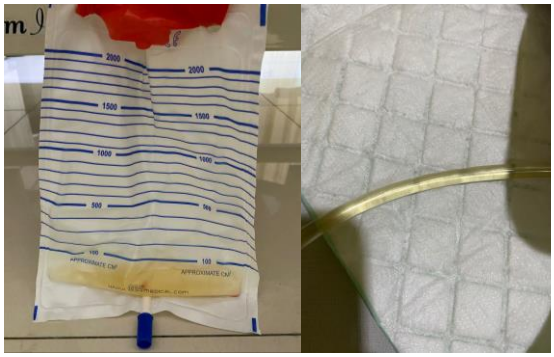

Figure 3. Normal Urine After Treatment with Antibiotics and Replacement of A Urine Catheter and Urine Bag.

DISCUSSION

Purple urine bag syndrome (PUBS) is a rare presentation of urinary tract infection that involves a series of processes beginning with the intake of tryptophan.[9] Tryptophan metabolite is believed to have a role in purple urine discoloration.[2] The gut bacteria in the digestive system break down tryptophan to produce indole. As indole enters the liver, it is converted into indoxyl sulphate through the portal circulation.[10] Indoxyl sulphate is excreted mostly into the urine. Some bacteria may have an enzyme called indoxyl sulphatase and phosphatase, which converts the indoxyl sulphate into indoxyl.[2,11,12] The indoxyl, in the alkaline urine, will be oxidized into indigo (blue pigment) and indirubin (red pigment), which will mix and react with the urinary catheter bag, producing purple colour.[13] However, PUBS has also been encountered in patients with acidic urine. A high bacterial load is needed to precipitate PUBS. Aside from the sulphatase and phosphatase-producing bacteria, some predisposing factors must be present to develop PUBS.[6,13]

Patients with long-term urinary catheter usage, constipation, dementia, alkaline urine, dehydration, and female gender are at risk of developing PUBS.[4,13,14] A high bacterial load in the urine combined with these factors facilitates the development of PUBS and increases the availability of bacterial sulphatases and phosphatases. A previous study reported PUBS prevalence was 8.3-16.7% in patients using a long-term indwelling catheter. Colonic bacterial overgrowth occurs in chronic constipation, intussusception, and ileal diversion. The overgrowth bacteria increase the determination of tryptophan, and also increases the production of indole. Indoxyl production is increased in catheter-associated urinary tract infections (CAUTI). Some bacteria have been reported in PUBS, including *Proteus species, Escherichia coli, Providencia rettgeri*, *Providencia stuartii, Klebsiella pneumoniae, Enterococcus species, Pseudomonas aeruginosa,* and *Morganella morganii*.[8,15,16] Our patient is a 76-year-old female with a history of immobilization in the past three years and was on chronic urinary catheterization, which was applied by a non-medic staff with no sterile procedure. She often experienced constipation. A purplish hue was observed exclusively in the urinary catheter and bag during the hospital stay. However, the urine itself remained clear when collected. The reason behind this clear urine remains uncertain. However, these observations are consistent with another documented report.[17,18] Her urinalysis showed a pH of 8.0, the presence of +3 leukocyte esterase, triple phosphate crystal, and positive bacteria, which was later revealed as *Proteus mirabilis* from the urine culture. *Proteus mirabilis* is a gram-negative rod-shaped bacterium that frequently causes CAUTI, either as a single

or polymicrobial agent. The bacterium has urease, so it can increase the hydrolysis of urea and later causes the alkalinization of urine. Urinary tract infection which involves *P. mirabilis* may be complicated by the formation of urolithiasis, permanent renal damage, and may further progress into urosepsis.[19]

The most common manifestation present is fever and hypotension. Most live in long-term care units, and the most common comorbidity is diabetes. The pH of the urine ranged between 8.0 and 8.1 across each decade, which is consistent with the documented finding from PUBS research that PUBS occurs more frequently in alkaline than acidic urine.[20,21]

Although PUBS is usually benign and asymptomatic in most cases, the presence of PUBS should alert the clinician of underlying UTI, which should be the center of concern in patient management.[17,22] Patients on prolonged catheterization, multimorbid, and geriatric patients may not have classic signs of UTI.[23] Replacing the urinary catheter and the urinary bag should be done, and the antibiotic for UTI should be administered.[17,24,25] In our case, we administered intravenous ciprofloxacin 400 mg twice daily for empirical antibiotics. The urine culture later revealed that the bacteria were sensitive to the antibiotic. Specific measures like increasing mobility, implementing safe and hygienic methods during catheterization, and treating other risk factors like constipation can decrease the chance of developing PUBS.

STRENGTH AND LIMITATION

In our case study, we rigorously cataloged the patient's medical background and performed in-depth laboratory tests. We attributed UTI as the causative element for PUBS even without pronounced clinical indications. Appropriate treatment of the UTI not only facilitated the rapid mitigation of PUBS but also underscored the efficacy of timely intervention in promptly addressing PUBS manifestations. However, it is essential to note that this report is based on a singular case.

CONCLUSIONS

Purple urine bag syndrome is mostly a benign condition. Families and healthcare professionals who are unaware of this phenomenon, however, could concern with the sudden unexplainable changes in the urine bag color. However, physicians should be aware that this condition indicates underlying recurrent UTIs brought on by improper placement and management of urinary catheters. While being largely harmless and readily cured, it can be linked to significant morbidity and death.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

There is no conflict of interest in this study.

AUTHOR CONTRIBUTION

Concept - AC, AY, NA, PM; Design - AC, AY, NA, PM; Supervision - AC; Resources - XX; Materials - XX; Data Collection and/or Processing - AC, AY, NA, PM; Analysis and/or Interpretation - AC, AY, NA, PM; Literature Search - AC, AY, NA, PM; Writing Manuscript - AC, AY, NA, PM; Critical Review - AC.

REFERENCES

1. Rooney H, Mokool L, Ramsay A, Nalagatla S. Purple urine bag syndrome: a truly harmless sign? Scott Med J. 2018; 63(3):99–101.

2. Al Montasir A, Al Mustaque A. Purple urine bag syndrome. J Family Med Prim Care. 2013; 2(1):104.
3. Wattanapisit S, Wattanapisit A, Meepuakmak A, Rakkapan P. Purple urine bag syndrome in palliative care. BMJ Support Palliat Care. 2019 Jun; 9(2):155–7.
4. Kannan L, Bauman AA. Purple urine in a patient with UTI and constipation. Oxford Oxf. Medical Med. Case Reports. 2023 Feb 1;2023(2):omad013.
5. Mohib O, Roland T, Fontaine M, Laurent F, Rossi C. Purple urine bag syndrome is a rare manifestation of bacteriuria on bladder catheter: case report. Afr J Urol. 2021; 27(1):81.
6. Faridi MS. Purple Urine Bag Syndrome-An Alarming Situation. JCDR [Internet]. 2016 [cited 2023 Feb 12]; Available from: http://jcdr.net/article_fulltext.asp?issn=0973-709x&year=2016&volume=10&issue=2&page=PD05&issn=0973-709x&id=7209
7. Su YJ, Yang HW. Risk factors of mortality in patients with purple urine bag syndrome. J.ournal of Drug Assessment. 2019; 8(1):21–4.
8. Abubacker NRT. Purple Urine Bag Syndrome. JCDR [Internet]. 2015 [cited 2023 Mar 12]; Available from: http://jcdr.net/article_fulltext.asp?issn=0973-709x&year=2015&volume=9&issue=8&page=OD01&issn=0973-709x&id=6304
9. Kaur H, Bose C, Mande SS. Tryptophan Metabolism by Gut Microbiome and Gut-Brain-Axis: An in silico Analysis. Front Neurosci. 2019 Dec 18; 13:1365.
10. Kumar U, Singh A, Thami G, Agrawal N. Purple urine bag syndrome:Asyndrome: A simple and rare spot diagnosis in Uroscopic rainbow. Urology Urol. Case Reports. 2021 Mar; 35:101533.
11. Sabanis N, Paschou E, Papanikolaou P, Zagkotsis G. Purple Urine Bag Syndrome: More Than Eyes Can See. Curr.ent Urology. 2019; 13(3):125–32.
12. Kalsi DS, Ward J, Lee R, Handa A. Purple Urine Bag Syndrome: A Rare Spot Diagnosis. Disease Markers. 2017; 2017:1–6.
13. Jappi Y, Hadi U. Purple Urine Bag Syndrome in Urinary Tract Infection. Journal of .Glob.al Infect.ious Diseases. 2023; 15(2):84–5.
14. Tirtayasa PMW, Sugianto R, Valentina I, Samuel AG. Purple urine bag syndrome in neurological deficit patient: A case report. Int. J. Surg. Case RepInternational Journal of Surgery Case Reports. 2023;104:107953.
15. Katyal A, Menon AS. Purple Urine Bag Syndrome in Acidic Urine! Amrita Journal of . Medicine. 2021; 17(4):146–8.
16. DeRon N, Legan C. Purple Urine Bag Syndrome: A Rare and Surprising Clinical Presentation. Cureus [Internet]. 2023 [cited 2023 Sep 28]; Available from: https://www.cureus.com/articles/131911-purple-urine-bag-syndrome-a-rare-and-surprising-clinical-presentation
17. Pandey S, Pandey T, Sharma A, Sankhwar S. Purple urinary bag syndrome: what every primary healthcare provider should know. BMJ Case Reports. 2018 Jul 18; bcr-2018-226395.
18. Popoola M, Hilier M. Purple Urine Bag Syndrome as the Primary Presenting Feature of a Urinary Tract Infection. Cureus. 2022; 14(4):e23970.
19. Armbruster CE, Mobley HLT, Pearson MM. Pathogenesis of *Proteus mirabilis* Infection. Donnenberg MS, editor. EcoSal Plus. 2018 Feb 8; 8(1):ecosalplus.ESP-0009-2017.
20. Yang HW, Su YJ. Trends in the epidemiology of purple urine bag syndrome: A systematic review. Biomed Rep. 2018; 8(3):249–56.

21. Alex R, Manjunath K, Srinivasan R, Basu G. Purple urine bag syndrome: Time for awareness. J Family Med Prim Care. 2015; 4(1):130.
22. Neniwal VK, Swain S, Rulaniya SK, Hota D, Agarwal P, Yadav PK. Purple urine bag syndrome: An unusual manifestation of urinary tract infection, our experience at a tertiary care center. Curr Urol. 2023; 17(2):125–9.
23. Worku DA. Purple urine bag syndrome: An unusual but important manifestation of urinary tract infection. Case report and literature review. SAGE Open Medical Med. Case Reports. 2019; 7:2050313X1882310.
24. Le Mouel JP, Fumery M. Purple urine bag syndrome. European Eur. Journal of .Internal Intern. Medicine. 2018 Sep; 55:e1–2.
25. Ahmed SI, Waheed MA, Shah S, Muhammad Shah SY, Mumtaz H. Purple urine bag syndrome: A case report. International Int. J. ournal of Surgery Surg. Case Reports. 2022; 99:107721.

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Original Article

Screening of *Legionella pneumophila* from Well Water in Magetan Regency, Indonesia

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ABSTRACT

Legionellosis is a respiratory infection caused by *Legionella pneumophila*, a bacterium that can infect protozoa and human lung cells. The disease can be mild or severe, and sometimes fatal, especially in immunocompromised people. Some types of antibiotics that can be used to treat *Legionella* disease are macrolides such as rifampicin, azithromycin and clarithromycin, as well as fluoroquinolones such as levofloxacin and moxifloxacin. The aim was to investigate the distribution and prevalence of *Legionella* in well water. This study collected and tested water samples from a different well water in Magetan Regency, East Java, Indonesia using DNA extraction, two-step PCR methods, and visualization with 1.5% agarose gel in UV transluminator. The results were visualized with QGIS 3.28.6 and compared with other tests. The results showed that none of the eight water samples were contaminated with *L. pneumophila* compared with positive control (403 bp).

Keywords: *Legionella pneumophila*, well water, Magetan Regency, DNA extraction, and PCR.

Highlights: This Study used DNA extraction and two-step PCR methods to detect *Legionella* in water sources in Magetan Regency, East Java, Indonesia, and can provide useful information for public health authorities to prevent and control *Legionella* outbreaks and to improve the quality and safety of water sources in the area.

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INTRODUCTION

The half of the 20th century saw widespread infection with a type of legionellosis caused by the bacterium *Legionella pneumophila*.[1,2] This type of bacteria has a morphology resembling the stem of γ-proteobacteria and can be found in freshwater habitats, moist soil, and compost material.[3] There are several grades of *Legionella* infection ranging from low-grade fever (Pontiac fever) to a potentially fatal form of pneumonia (Legionnaires' disease) that can affect anyone, particularly vulnerable hosts due to age, disease, immune suppression or other risk factors, such as smoking.[4-7]

L. pneumophila was the first bacteria described to multiply inside protozoan hosts, primarily aquatic amoeba, which had the idea that the bacteria's capacity to infect protozoa also allowed them to replicate inside human lung macrophages, a finding confirmed later through numerous studies.[3] Legionnaires' disease is an important but relatively uncommon respiratory infection that can cause great morbidity and mortality.[8,9] First recognized as a fatal cause of pneumonia more than three decades ago, little progress has been made in investigations, clinical and incident management, and public health responses to cases and outbreaks.[10] *Legionella* disease has spread to almost various countries around the world. In Indonesia, cases of this disease have been reported from 2010-2019 from foreign tourists traveling to Bali and West Java based on the discovery of cases from the country of origin. In addition, on May 30, 2023, the first 2 confirmed cases of Legionellosis (Indonesian citizen) have been reported in Bandung, West Java.[11]

Research conducted by Yasmon et al. from 9 cooling tower water samples taken from buildings in Jakarta, Indonesia from November 2007 to June 2008 tested with duplex PCR (dPCR) tests, 6 of them were positive for *Legionella sp.*, 1 positive for *L. pneumophila*, and 2 negative for both.[12] Another study also examined *Legionella* bacteria where as many as 17 water samples were taken from 17 different sources, of which 10 samples were obtained from hospitals in North Jakarta, and 7 samples from West Jakarta. Water samples were all cultivated in all four media variants Culture media removed from the incubator on days 3, 7 and 10 to observe growth and presence of colonies corresponding to the characteristics of *L. pneumophila*. Eight of the 17 water samples showed colony morphology of bacteria with traits such as *L. pneumophila*. Sixty percent (6/10) of water samples from North Jakarta Hospital showed *L. pneumophila* colony suspects, while those from West Jakarta hospitals only showed 29% (2/7). Colony growth is suspected as *L. pneumophila* in media variant 1.[13]

The initial symptoms of Legionellosis disease are very similar to those of the flu (low-grade fever, headache, fatigue, joint and muscle pain, and loss of appetite). After a few days (1 or 2 days) more severe pneumonia symptoms may appear (high fever with a temperature of 39°C - 41°C, cough, dyspnea or difficulty breathing, chills, and chest pain). Gastrointestinal symptoms such as diarrhea and nausea may appear. In many cases severe pneumonia requires hospitalization and in some cases Legionellosis can lead to death. The probability of an immunocompromised person dying from Legionellosis is 40-80%. However, this figure can decrease by 5-30% if treatment or case management is carried out properly.[14]

Currently, treatment for *Legionella* disease usually involves administering antibiotics. Some types of antibiotics that can be used to treat *Legionella* disease are macrolides such as azithromycin and clarithromycin, as well as fluoroquinolones such as levofloxacin and moxifloxacin. Rifampicin may also be used to treat Legionella's disease. Fluoroquinolones should be used for 7-14 days, among macrolides azithromycin may be preferred

for 5-10 days. A potentially useful drug is also doxycycline (in the form of uncomplicated immunocompetent patients.[15,16]

Legionella detection can be done in several ways, such as traditional culture on buffered charcoal yeast extract agar, bronchoscopy to see signs of legionella bacteria in the lungs, thoracentesis to see signs of bacterial infection outside the lungs, and CT scan to get a picture of the condition of the lungs. For this reason, it is important to detect the spread of these bacteria in water samples in various sampling sites because bacterial detection is an important step for handling the spread of a disease because by detecting the location and characteristics of the bacteria spread, we can anticipate the source of infection and immediately plan further steps.[17,18]

An assay used for detecting *L. pneumophila* bacteria is two-step PCR. In two different tubes, for reverse transcription and amplification, have been prepared. That is why this variation is known as two-step PCR. Notably, both reactions have different conditions and materials are used. The first reaction uses reverse transcriptase enzymes, dNTPs, reaction buffers, oligo-primers (dT), and random primers to construct cDNA. After completing the reaction, a stock of cDNA is obtained. The stock of cDNA can be stored at adequate conditions or it can be used directly for gene expression studies. The second step is using cDNA as a template for measuring mRNA. In the second reaction, along with all PCR reagents, Taq DNA polymerase completes the reaction and is quantified using dyes or probes. At this stage, no reverse transcriptase reaction is required. The second reaction quantifies cDNA (formed from the first step). One-step PCR does have an easier and more efficient procedure, but Two-step RT-PCR switches quickly from RNA to more stable cDNA without knowing exactly which genes will be targeted downstream. It can choose various targets after seeing the initial qPCR results or even store the cDNA samples indefinitely, and revisit them later to look for gene expression that might not have been thought of at first. One of the biggest advantages of the two-step method is that it can store reverse cDNA transcribed from RNA. In two-step PCR, three sets of primers are used, one set of oligo primers (dT), a set of random primers, and sequence-specific primers to subsequently have high specificity compared to the one-step method. This method performs very well for a small number of samples, but the use of additional steps makes it more susceptible to reaction failure and contamination.[19]

Various countries have recommended strategies to prevent the growth of *L. pneumophila*. Important differences can be seen in the way they measure hazardous concentrations, use water sampling frequency and *L. pneumophila* alertness levels, and institute necessary measures. The recommended strategy depends on local regulations, although this problem occurs worldwide because *Legionella* naturally exists in water. Geographic differences are not significant enough to require adjusting infection control practices by region.[20] The hope is that this detection can find out how the characteristics of bacteria live and multiply so that they can further develop drugs and vaccines that can reduce the severe impact of *Legionella* infection.

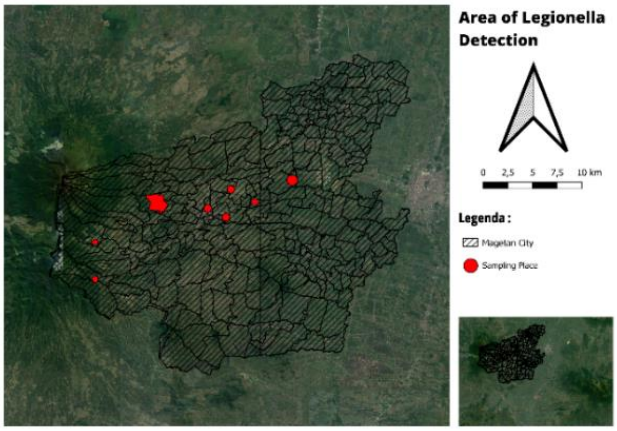
MATERIALS AND METHODS

Samples Collection

Water samples were collected in December 2021 from a variety of well that focus on the scope area of Magetan Regency, East Java, Indonesia. The location of the sample distribution was visualized with QGIS 3.28.6 by focusing on potential places for *Legionella* growth, namely tap water and also river water which is the main source of disease spread by *Legionella* bacteria in humans by obtaining 8 water samples from different places in Magetan Regency. Water

is transferred to a sterile container then tightly closed and taken to the laboratory for testing. To find out whether there are *Legionella* bacteria or not can be done by extracting bacterial DNA first which is then carried out PCR tests. This test can detect *Legionella* DNA in water samples or other environments. In addition, other tests such as culture tests and serology tests can be used to detect the presence of *Legionella*.[21]

Figure 1. Sample Collection Area for *Legionella* Detection



The research location was visualized with QGIS desktop ver 3.6.3 where water samples were collected at several points in Magetan Regency (Figure 1) with the following details in the Table 1.

Table 1. Location Details of Samples Collection

No.	Sampling location
1.	Singget Hamlet, Sukomoro District, Magetan Regency, East Java
2.	99CF+QR5, Jl. Raya Maospati, Kem, Sukomoro, Sukomoro District, Magetan Regency, East Java 63391 (Warung pecel pincuk marsel)
3.	Sumber Sawit Village, Sidorejo District, Magetan Regency, East Java
4.	Telaga sarangan, Plaosan District, Magetan Regency, East Java
5.	Jl. Mayjen Sukowati No.52, Bangunsari, Sukowinangun,Magetan District, Magetan Regency, East Java 63312
6.	Warung Lesehan Suminar Jl. Panglima Sudirman No.2 Tambran, Magetan 63318 Indonesia
7.	Jl. Sawo, Magetan Regency, East Java
8.	Geni Langit Tourism, Genilangit, Poncol District, Magetan Regency

DNA Extraction

DNA extraction using Zymo Kit™ by Zymo Research, USA, according to its label instructions. Preparing DNA / RNA Shield™ as much as 300 µL, PK Digestion Buffer 30 µL and Proteinase K 15 µL added to each water sample, then homogenized with vortex and incubated at 55°C for 5 hours to obtain maximum extraction results. The tissue sample dissolved in the liquid was then centrifuged at 15,000 g for 1 min. The supernatant (300 µl) is transferred to an DNAse-free tube, added to an DNA lysis buffer of equal volume and vortexed. For DNA purification, the sample is placed on a yellow Spin-Away™ filter already attached to the collection tube and then centrifuged. The passing filter is transferred to a new collection tube, to which ethanol is added in a ratio of 1:1 to the sample volume and stirred. The mixture is then transferred to a green CG Zymo-Spin™ III column which is attached to a collection tube and centrifuged. Save the filtered sample, dispose of the solution on the collection tube and reinstall the collection tube. Then add 400 µL DNA Prep Buffer and centrifuge, store the filtered sample, dispose of the solution in the collection tube and reinstall the collection tube. The filtrate is discarded and the column is washed twice with 700 µl and 400 µl of DNA washing buffer. The column is then transferred to a DNase-free tube and centrifuged at maximum speed for 2 minutes to ensure the purity of the DNA sample. DNA samples were eluted with 100 µl of DNase/RNase-free water and centrifuged.

Polymerase Chain Reaction

The analysis method uses the principle of thermal-based polymerization to

multiply DNA from bacteria contained in water samples so that it can be detected with gel documentation, first and second step PCR using GoTaq® DNA polymerase by Promega, USA, (contains taq DNA polymerase, dNTPs, $MgCl_2$), *Legionella* primers (forward & reverse)[22], nuclease free water, and RNA from samples. PCR was run with a pre-denaturation initial temperature of 95°C for 5 minutes and denaturation of 1 minute, annealing 55°C for 1 minute, extension of 72°C for 1 minute, final extension of 72°C for 10 minutes. After the second PCR product sample has been propagated then electrophoresis is performed to measure the bp length of the DNA.

Electrophoresis

Prior to electrophoresis the sample was inserted into 1.5% agarose gel that had been perforated on one side. Electrophoresis is carried out by preparing 3 µl of 100 bp markers as a comparison or bp measuring target which was inserted into 1.5% agarose gel, then 3 µl of multiplied samples are also added to another hole, then put agarose gel into the electrophoresis instrument, then pour TAE buffer until the agarose gel is closed. Electrophoresis instruments allow DNA to move through these pores. The molecules in the sample will move towards the pole of the electrode opposite to its electric charge. Smaller, negatively charged molecules will move faster and farther away than larger, positively charged molecules. After running on electrophoresis with a power of 100 volt for 30 minutes then continue the staining stage (staining).

Staining

The electrophoretic agarose gel is placed in a container filled with Ethidium bromide and shaken using a shaker for 30 minutes. The staining process is carried out for the staining process so that the sample molecules that have been separated can be seen.

Gel Documentation

The agarose gel is then read using documentation gel with UV light radiation. The DNA bands on agarose gel appear according to their size, which can be measured by comparing DNA markers to known sizes. The readings are printed for analysis.

Data Analysis

Readings with gel documentation were analyzed based on the molecular weight (bp) that appeared and then compared with the molecular weight (bp) of the target gene for *Legionella* bacteria, namely 403 bp. This is used to determine whether *Legionella* bacteria are present in the sample.

RESULTS AND DISCUSSION

PCR is carried out in two stages where the first stage is carried out with the code L1A-L8A, then after PCR is complete, PCR is carried out with the same stage for the second time (L1B-L8B). The PCR results in electrophoresis are then visualized with gel documentation with the following results in the Figure 2.

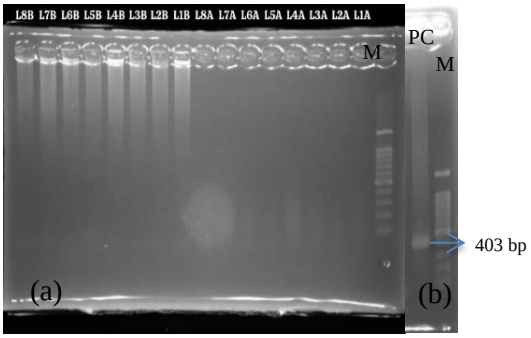


Figure 2. (a) DNA Visualization of *Legionella pneumophila* from Magetan Regency (well water samples), (b) Positive Control (PC) of *Legionella pneumophila*, and DNA ladder (M) used 100 bp.

All of 8 water samples showed negative results for *L. pneumophila*. Warm pools and spas are the main routes of transmission of *Legionella*, which are optimal conditions for breeding as well as

containing nutrients for growth. The visualized in Figure 2a all amplified water not showing any bands. For positive control we can see at Figure 2b. Below which gave rise to the band at 403 bp that indicated contamination of *L. pneumophila* bacteria.

There are several factors that causes of bacterial detection to show negative results. Firstly, *Legionella* needs the amoeba's intracellular environment to reproduce. In aquatic habitats the amoeba itself acts as a host.[22] Thus, by the time *Legionella* bacteria leave the intracellular environment, they experience stress caused by changes in diet, pH, temperature, salinity, and oxygen. To adapt to the changing environment, *Legionella* enters a living state but cannot be cultured, meaning the bacteria are alive but not evolving.[23,24] When collecting water samples in Magetan, samples are stored using non-sterile bottles and long delivery to the laboratory, this causes a changes in pH, temperature, salinity, and oxygen so that *Legionella* bacteria in water samples die. This often leads to failure of *Legionella* cultures from environmental samples.[25] The suggestion for further research is to pay attention to storage of the sample to retain the bacteria before extraction.

Then according to the Indonesian Ministry of Health No: 416/MenKes/Per/IX/1990, these bacteria are increasingly active in water, especially in warm or hot temperature conditions. These bacteria can live at temperatures between 57 °C to 63 °C and thrive at temperatures of 30°C - 45°C. So that for storage of *Legionella* samples can maintain temperatures in the range of 30°C - 45°C. The results of this detection indicate the need for wider detection and attention to how to store samples from the point of collection to testing on a laboratory scale.[26]

Currently, steps that can be a recommendation to minimize the spread of *Legionella* disease is to pay attention to the source of water consumed and treat well water that will be used first like cook water until cooked, namely at boiling temperatures (100°C) in order to ensure there are no bacteria contaminated in drinking water. In addition, maintenance and cleaning of air conditioning towers and condensers to prevent the development of Legionella at least 2 times a year cleaning and use of chlorine.

STRENGTH AND LIMITATION

This research has advantages in supporting early detection of the spread of legionella bacteria which is the source of pneumonia, where early detection can be a benchmark for areas that must be of special concern for handling *Legionella* bacteria and characterizing the habitat of *Legionella* bacteria so as to reduce the risk of more severe spread, but this study has shortcomings where sampling from water samples is less widespread so that there are no bacteria which is detected from testing so that wider sampling is needed to determine the spread of the *Legionella* bacteria.

CONCLUSIONS

Of the 8 samples taken from different places, all samples were negative for legionella bacteria.

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ETHICAL CLEARANCE

This study does not require an ethical review.

FUNDING

There is no funding for the research.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in writing this manuscript.

AUTHOR CONTRIBUTION

Each author has contributed in the preparation of this study. Author THS, SF, and FA in this study is in charge of collecting of samples, NSF and AMW analyzing data, then NNH and MRHK writing article reviews. Meanwhile, EBAH is a principle investigator who provides study ideas and validates data.

REFERENCES

1. Farnham A, Alleyne L, Cimini D, Balter S. Legionnaires' Disease Incidence and Risk Factors, New York, New York, USA, 2002-2011. Emerg Infect Dis. 2014;20(11):1795-1802. doi: 10.3201/eid2011.131872
2. Garin N, Marti C, Lami AS, Prendki V. Atypical Pathogens in Adult Community-Acquired Pneumonia and Implications for Empiric Antibiotic Treatment: A Narrative Review. Microorganisms. 2022;10(12):2326 doi: 10.3390/microorganisms10122326
3. Mondino S, Schmidt S, Rolando M, Escoll P, Gomez-Valero L, Buchrieser C. Legionnaires' Disease: State of the Art Knowledge of Pathogenesis Mechanisms of Legionella. Annu Rev Pathol. 2020;24(15):439-466. doi: 10.1146/annurev-pathmechdis-012419-032742
4. Lama A, Drennan SL, Johnson RC, Rubenstein GL, Cambronne ED. Identification of conserved ABC importers necessary for intracellular survival of Legionella pneumophila in multiple hosts. Front Cell Infect Microbiol. 2017;7(485):1-14. doi: 10.3389/fcimb.2017.00485
5. Sherwood RK, Roy CR. Autophagy evasion and endoplasmic reticulum subversion: The Yin and Yang of Legionella intracellullar infection. Annu Rev Microbiol. 2016;70(1):413-433. doi: 10.1146/annurev-micro-102215-095557
6. Du J, von Wrisberg MK, Gulen B, Stahl M, Pett C, Hadberg C, Lang K, Schneider S, Itzen A. Rab1-AMPylation by Legionella DrrA is allosterically activated by Rab1. Nat Commun. 2021;12(460):1-16. doi: 10.1038/s41467-020-20702-2
7. Alrahimi J, Aldahlawi A, Hassoubah S, Al-Jadani S, Alyamani W, Alotaibi N. AnInsight into the Microbiology, Epidemiology, and Host Cell Biology of Legionella Pneumophila: A Review of Literature. Biosci Biotechnol Res Asia. 2022;19(3):561-577. doi: 10.13005/bbra/3010
8. Gea-Izquierdo E, Gil-Prieto R, Hernández-Barrera V, Rodrígues-Caravaca G, Gil-de-Miguel Á. Legionellosis-Associated Hospitalization in Spain from 2002 to 2021. Microorganisms. 2023;11(7):1693. doi: 10.3390/microorganisms11071693
9. Barimani, MJ. Legionella: An uncommon cause of community-acquired pneumonia. JAAPA. 2022;35(10):38-42. doi: 10.1097/01.JAA.0000873792.00538.78
10. Phin N, Parry-Ford F, Harrison T, Stagg HR, Zhang N, Kumar K, Lortholary O, Zumla A, Abubakar I.

Epidemiology and clinical management of Legionnaires' disease. Lancet Infec Dis. 2014;14(10):1011–21. doi:10.1016/S1473-3099(14)70713-3.
11. Menistry of Health of Indonesian Republic. Frequently Asked Question (FAQ) LEGIONELLOSIS. 2023. Available from: https://infeksiemerging.kemkes.go.id/penyakit-virus/frequently-asked-questions-faq-legionellosis
12. Yasmon A, Yusmaniar Y, Anis A, Bela B. Simultaneous detection of Legionella species and Legionella pneumophila by duplex PCR (dPCR) assay in cooling tower water samples from Jakarta, Indonesia. Med J Indones. 2010;19(4):223–227. doi:10.13181/mji.v19i4.408
13. Moehario L, Robertus T, Grace Y, Tjoa E. Screening of Legionella pneumophila from water sources in the hospitals in Jakarta. Health Sci J Indones. 2019;10(1):21–26. doi: 10.22435/hsji.v10i1.1852
14. Brady MF, Sundareshan V. Legionnaires' Disease. [Updated 2022 Jul 4]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK430807/
15. Viasus D, Gaia V, Manzur-Barbur C, Carratalà. Legionnaires' Disease: Update on Diagnosis and Treatment. Infect Dis Ther. 2022;11(3):973-986. doi: 10.1007/s40121-022-00635-7
16. Arget M, Kosar J, Suen B, Peermohamed S. Successful Treatment of Legionnaires' Disease with Tigecycline in an Immunocompromised Man with a Legion of Antibiotic Allergies. Cureus. 2019;11(4):e4577. doi: 10.7759/cureus.4577
17. Boczek LA, Tang M, Formal C, Lytle D, Ryu H. Comparison of two culture methods for the enumeration of Legionella pneumophila from potable water samples. J Water Health. 2021;19(3):468-477. doi: 10.2166/wh.2021.051
18. Torres A, Menéndez R, Wunderink RG. Bacterial Pneumonia and Lung Abscess. Murray and Nadel's Textbook of Respiratory Medicine. 2016:557-582.e22. doi: 10.1016/B978-1-4557-3383-5.00033-6
19. Eble D, Gehrig V, Schubert-Ullrich P, Köppel R, Füchslin HP. Comparison of the culture method with multiplex PCR for the confirmation of Legionella spp. and Legionella pneumophila. J Appl Microbiol. 2021;131:2600-2609. doi: 10.1111/jam.15103
20. Kenhove EV, Dinne K, Janssens A, Laverge J. Overview and comparison of Legionella regulations worldwide. Am J Infect Control. 2019;47(8):968–978. doi: 10.1016/j.ajic.2018.10.006.
21. Boss R, Baumgartner A, Kroos S, Blattner M, Fretz R, Moor D. Rapid detection of viable Legionella pneumophila in tap water by a qPCR and RT-PCR-based method. J Appl Microbiol. 2018;125(4):1216-1225. doi: 10.1111/jam.13932
22. Aksono EB, Farahdiba AA, Hestianah EP. Bakteri Legionella pneumophila Terdeteksi pada Air Kolam Renang di Kota Surabaya dengan Nested Polymerase Chain Reaction. Jurnal Veteriner. 2017;18 (2):221–225. doi:10.19087/jveteriner.2017.18.2.221.
23. van der Kooij D, Bakker GL, Italiaander R, Veenendaal HR, Wullings BA. Biofilm composition and threshold concentration for growth of Legionella pneumophila on surfaces exposed to flowing warm tap water without disinfectant. Appl Environ Microbiol. 2017;83(5):e02737-16

Ohno A, Kato N, Yamada K, Yamaguchi K. Factors Influencing Survival of Legionella pneumophila Serotype 1 in Hot Spring Water and Tap Water. Appl Environ Microbiol. 2003;69(5):2540-2547. doi: 10.1128/AEM.69.5.2540-2547.2003

24. Olivia G, Sahr T, Buchrieser C. The Life Cycle of L. pneumophila: Cellular Differentiation Is Linked to Virulence and Metabolism. Front Cell Infect Microbiol. 2018;8(3):1-12. doi: 10.3389/fcimb.2018.00003
25. Verayani E. Identifikasi Legionella, Kualitas Udara Ruang dan Keluhan Sick Building Syndrome Pada Petugas Instalasi Transfusi Darah RSUD Dr. Soetomo. Jurnal Kesehatan Lingkungan. 2018;10(3):299–305.
26. Menistry of Health. Peraturan Menteri Kesehatan No. 416 Tahun 1990 Tentang : Syarat-syarat dan Pengawasan Kualitas Air. 2023. Available from: https://katigaku.top/wp-content/uploads/2018/12/1.-PerMenKes4161990-SyaratPengawasan_Kualitas_Air.pdf

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Original Article

Risk Factors Influencing the Degree of Tuberculous Spondylitis Based on MRI Modality

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ABSTRACT

The high prevalence and mortality of tuberculosis (TB) is serious threats to the world. Tuberculous spondylitis accounts for 50% of all bone and joint TB cases. The accuracy of diagnosis to detect disease severity using Magnetic Resonance Imaging (MRI) modalities is important when considering potential risk factors. This study aimed to analyze the association of risk factors, including age, sex, spinal lesion location, and abscess location, with the severity of tuberculous spondylitis based on MRI modality using the Gulhane Askari Tip Akademisi (GATA) classification. This study had a cross-sectional design. The study sample consisted of 50 patients who met the inclusion criteria and underwent MRI. The statistical analysis performed in this study was multivariate analysis using multiple linear regression. The results showed that 84% (n = 42) of tuberculous spondylitis affected patients aged 18-65 years, and 70% (n = 35) of cases were found in female patients. In addition, 42% (n = 21) of cases were classified as tuberculous spondylitis grade III according to the GATA classification and were the most common cases in this study. Statistical tests showed no association between age, the location of spinal lesions, and the degree of tuberculous spondylitis. However, there was an association between sex, spinal abscess location, and degree of tuberculous spondylitis. There was no significant association between age and spinal lesion location or degree of tuberculous spondylitis. However, there was a significant association between sex, spinal abscess location, and the degree of tuberculous spondylitis.

Keywords: risk factors, tuberculous, spondylitis, MRI, and Indonesia.

Highlights: Sex and spinal abscess location was found as the risk factors that affect the severity of tuberculous spondylitis based on MRI modality using the GATA classification.

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INTRODUCTION

Tuberculosis (TB) is commonly known as a deadly infectious disease that occurs worldwide.[1,2] The prevalence of TB reached 5.8 million in 2019, with India, Indonesia, and the Philippines being the most affected countries in the world. In 2013 – 2014, there were 1.6 million TB cases in Indonesia[3], and the global mortality rate of this disease has increased from 1.4 million in 2019 to 1.5 million in 2020.[1] TB exposure is a serious threat, especially when it affects other organ systems such as the musculoskeletal system.[4,5] Tuberculous spondylitis accounts for 50% of bone and joint TB cases in the musculoskeletal system.[5–8] Moreover, late diagnosis of musculoskeletal TB, especially tuberculous spondylitis, might lead to paralysis of the extremities.[9] Therefore, early diagnosis and monitoring are crucial to reduce disease severity. Moreover, the accuracy of diagnosis using Magnetic Resonance Imaging (MRI) is important when considering potential risk factors.

A study from China found that men and aged 18-45 years were at higher risk of developing this disease.[6] In contrast, other studies reported that female patients were more likely to experience tuberculous spondylitis.[10] Tuberculous spondylitis is more frequently reported in children than in adults.[11,12] A previous study reported that the thoracic segment (48.03%) was the most common location of the spinal lesions.[13] However, another study found that the lumbar segment (38.2%) was the most common location.[14] Moreover, it has been reported that the cervical and upper thoracic segments (10%) are the rarest sites for spinal abscess formation.[15] Other studies also mention that the lumbosacral segment has the fewest abscesses.[16]

Imaging modalities are the most accurate diagnostic tools for tuberculous spondylitis, considering that *Mycobacterium tuberculosis* culture is difficult and takes approximately 4-6 weeks to obtain the results.[5] A previous study compared several diagnostic modalities, which involved 40 patients: 29 patients were assessed with a CT Scan, 11 were assessed with an MRI, and 10 were assessed with a CT Scan and MRI, which included the type and level of spinal and soft tissue involvement. This study found that MRI was superior in terms of accuracy, especially for soft tissue assessment due to tuberculous spondylitis.[17] CT-Scan is more useful in assessing bone damage, such as lytic lesions and disc collapse, but has disadvantages in soft tissue assessment.[9,17]

Tuberculous spondylitis has four degrees of disease severity according to the *Gülhane Askeri Tıp Akademisi* (GATA) classification: IA, IB, II, and III. This GATA classification is based on a retrospective study of 78 tuberculous spondylitis cases followed up for the past two years. This study conducted radiological assessment using CT and MRI modalities, and the assessment was based on seven criteria. This study found that 11 patients were categorized as grade I, 48 as grade II, and 17 as grade III.[18]

Based on this description, the classification of the tuberculous spondylitis degree was assessed based on the accuracy of the MRI modality and by examining the risk factors from previous studies, such as the association of age, sex, location of the abscess, and spinal lesions with the incidence of tuberculous spondylitis, which is still under debate. Therefore, this study aimed to analyze the association of risk factors (age, sex, spinal lesion location, and abscess location) with the severity of tuberculous spondylitis based on MRI modality using the GATA classification. The severity of tuberculous spondylitis can be minimized via a comprehensive assessment of existing risk factors.

MATERIALS AND METHODS

This was an analytical, observational study with a cross-sectional design. The data

collected in this study were secondary data in the form of electronic medical records and imaging results in the form of MRI images. Data collection was conducted from June to August 2022. Fifty patients with tuberculous spondylitis who met the inclusion and exclusion criteria were included in this study at Dr. Kariadi Hospital, Semarang. The inclusion criteria in this study were patients clinically diagnosed with tuberculous spondylitis or by radiological examination obtained from medical record data in the form of MRI examination photos of the spine. Exclusion criteria in this study were Patients with spinal fractures other than those caused by tuberculous spondylitis, patients with a history of spinal tumors, and patients with a history of spinal surgery were excluded. Sample selection was performed using a purposive sampling method based on medical records, with inclusion and exclusion criteria as a reference.

The data obtained were analyzed using IBM SPSS™ 25 statistical program. Data analysis was performed using univariate, bivariate, and multivariate analyses using post-hoc follow-up tests. The characteristics of the respondents were analyzed using univariate analysis (including age, sex, location of lesions, and spinal abscesses), which are then presented in a table containing frequencies and percentages. Bivariate hypothesis testing was performed using Mann-Whitney and Kruskal-Wallis tests. Differences were considered statistically significant when the p-value was less than 0.05. A multiple linear regression test was used for multivariate analysis.

RESULTS AND DISCUSSION

The respondents' characteristics are listed in Table 1. Table 1 shows that 15 (30%) males and 35 (70%) females were included in this study. The age variable data showed that the age range of 18-65 years had the highest frequency (n= 42, 84%). The most common locations for spinal lesions were the thoracic segment in 24 patients (48%), followed by the lumbar segment in 17 patients (34%). The most frequent location of spinal abscesses was the thoracic segment in 19 patients (38%), followed by the lumbar segment in 13 patients (26%). The percentage of tuberculous spondylitis degrees found in this study was 18% for grade IA, 28% for grade IB, 12% for grade II, and 42% for grade III—which hold the highest percentage.

Table 1. Respondent's Characteristics Data.

Variable	Frequency	%
Age (year)		
0-17	2	4.0
18-65	42	84.0
> 65	6	12.0
Sex		
Male	15	30.0
Female	35	70.0
Spinal lesion location		
Cervical	4	8.0
Thoracic	24	48.0
Lumbar	17	34.0
Sacral	0	0.0
Multiple	5	10.0
Spinal abscess location		
None	8	16.0
Cervical	2	4.0
Thoracic	19	38.0
Lumbar	13	26.0
Sacral	0	0.0
Multiple	8	16.0
Tuberculous spondylitis degree		
IA	9	18.0
IB	14	28.0
II	6	12.0
III	21	42.0

Age data (Table 2) were categorized into three categories according to the WHO; 0-17 years (children), 18-65 years (adults), and > 65 years (elderly). Age data were analyzed using an alternative test: the Kruskal-Wallis test. The results of the bivariate statistical test showed a p-value > 0.05, which showed no significant association between the subject's age and the degree of tuberculosis spondylitis as a disease severity indicator.

Table 2. Association Between Age and the Degree of Tuberculous Spondylitis Statistical Test Result.

Variable	Tuberculous spondylitis degree				*p*
	IA	IB	II	III	
Age (year)					
0-17 (children)	1 (50.0)	0 (0.0)	0 (0.0)	1 (50.0)	0.834
18-65 (adult)	6 (14.3)	14 (33.3)	6 (14.3)	16 (38.1)	
>65 (elderly)	2 (33.3)	0 (0.0)	0 (0.0)	4 (66.7)	

There were two categories of sex variables, male and female. Table 3 shows the association between sex and the degree of tuberculous spondylitis with a p-value of 0.007 ($p<0.005$), which indicated that there was a significant association between sex and the degree of tuberculous spondylitis.

Table 3. Association Between Sex with The Degree of Tuberculous Spondylitis Statistical Test Result.

Variable	Tuberculous spondylitis degree				*p*
	IA	IB	II	III	
Sex					
Male	2 (13.3)	0 (0.0)	2 (13.3)	11 (73.3)	0.007
Female	7 (20.0)	14 (40.0)	4 (11.4)	10 (28.6)	

Table 4 shows that the location of spinal lesions in tuberculous spondylitis patients was categorized into five groups. The Kruskal-Wallis statistical alternative test was used to find an association between the location of spinal lesions and the severity of tuberculous spondylitis. The location of spinal lesions associated with the severity of tuberculous spondylitis showed a $p>0.05$, meaning there was no significant association between the location of spinal lesions and the severity of tuberculous spondylitis.

Table 4. Association of Bone Lesion Location with Tuberculous Spondylitis Degree Statistical Test Result.

Variable	Tuberculous spondylitis degree				*p*
	IA	IB	II	III	
Lesion location					
Cervical	0 (0.0)	2 (50.0)	0 (0.0)	2 (50.0)	0.199
Thoracic	5 (20.8)	6 (25.0)	1 (4.2)	12 (50.0)	
Lumbar	4 (23.5)	5 (29.4)	5 (29.4)	3 (17.6)	
Sacral	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Multiple	0 (0.0)	1 (20.0)	0 (0.0)	4 (80.0)	

The final factor associated with the degree of tuberculous spondylitis was the location of spinal abscess. Spinal abscess locations were categorized into five groups and analyzed using the Kruskal–Wallis alternative statistical test. Table 5 shows a p-value < 0.05, indicating a significant association between the location of the spinal abscess and the severity of tuberculous spondylitis. Moreover, a follow-up Post Hoc test using the Mann-Whitney test was performed to discover more about the association between each data point in the spinal abscess location variable.

Table 5. Association of Abscess Location with Tuberculous Spondylitis Degree Statistical Test Results.

Variable	Tuberculous spondylitis degree				*p*
	IA	IB	II	III	
Abscess location					
None	8 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	<0.001
Cervical	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	

Thoracic	1 (5.3)	5 (26.3)	1 (5.3)	12 (63.2)
Lumbar	0 (0.0)	5 (38.5)	5 (38.5)	3 (23.1)
Sacral	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Multiple	0 (0.0)	2 (25.0)	0 (0.0)	6 (75.0)

The results of the Post Hoc follow-up test in Table 6 show the asterisks, indicating a significant difference between the group without abscesses and the other groups — cervical, thoracic, lumbar, and multiple abscesses–in the spinal abscess location variable. Therefore, it can be assumed that spinal abscess location affects the severity of tuberculous spondylitis.

Table 6. The Results of the Follow-Up Post Hoc Test with the Mann-Whitney Test.

Abscess location		*p*	Note
I	**II**		
None	Cervical	0.003*	Significant
	Thoracic	<0.001*	Significant
	Lumbar	<0.001*	Significant
	Multiple	<0.001*	Significant
Cervical	Thoracic	0.103	Not significant
	Lumbar	0.142	Not significant
	Multiple	0.066	Not significant
Thoracic	Lumbar	0.154	Not significant
	Multiple	0.564	Not significant
Lumbar	Multiple	0.088	Not significant

Multivariate analysis (Table 7) was performed on sex, spinal lesion location, and spinal abscess location variables because multivariate analysis requires a p-value of < 0.25. Multiple linear regression analysis showed that sex and spinal abscess location had p-values <0.05. Moreover, the female sex and thoracic segments were found to be the predominant locations of spinal abscesses. Overall, among the three risk factors for tuberculous spondylitis, sex variables and location of spinal abscesses were risk factors that predominantly influenced the degree of tuberculous spondylitis. In addition, the location of the spinal abscess was the most influential risk factor for tuberculous spondylitis severity, as evidenced by a p-value of <0.001.

Table 7. Multivariate Analysis Results.

Variable	Beta	*p*	Note
Sex	-0.306	0.011	Significant
Spinal lesion location	-0.138	0.267	Not significant
Spinal abscess location	0.503	<0.001	Significant

In this study, sex and spinal abscess location variables were significantly associated with the severity of tuberculous spondylitis according to the GATA classification and assessed using MRI. This study found that there were 15 male patients with tuberculous spondylitis, which was lower than that of female patients, accounting for 35 cases. This result was in line with a previous study that found more tuberculous spondylitis cases in female patients (1378 cases) than in males (972 cases).[10] In addition, the thoracic spine was the most common spinal abscess location variable, with a total of 19 cases (38%). According to a previous study, approximately 40–50% of abscesses were found in the thoracic segment.[19] Patients with tuberculous spondylitis aged 18-65 years (84%), which were included in the adult age category, tended to be more common than those in other age categories, with a mean age of the patients was 40.48 years. This finding was in line with that of a study by Sianaturi et al. which found that adults with tuberculous spondylitis had a mean age of 39.5 years.[20]

Other characteristics, such as the location of the spinal lesion, had the thoracic spine being the most common location, accounting

for 24 cases (48%). Several previous studies also mentioned that the thoracic segment had the highest resistance and was the most common location of tuberculous spondylitis lesions.[9,13] Moreover, the most common degree of tuberculous spondylitis was grade III, which accounted for 21 cases (42%), followed by grade IB in 14 cases (28%), grade IA in nine cases (18%), and grade II in six cases (12%).

In addition, untreated tuberculous spondylitis is a serious threat because it may lead to spinal deformities, resulting in paralysis of the extremities.[9] Progressive destructive events in the spine that can cause spinal cord compression require cautious attention in both men and women.[11] A previous study reported that tuberculous spondylitis was mostly found in women.[10]

A fundamental theory regarding the influence of estrogenic hormones on bone metabolism supports this finding. A drastic decrease in estrogen levels, particularly in postmenopausal women, increases bone resorption, resulting in an imbalance between bone resorption and formation.[21] An imbalance between bone resorption and formation in patients with tuberculous spondylitis exacerbates the progression of spinal destruction and increases the severity of tuberculous spondylitis.

Cold abscess formation is a common sign in patients with tuberculous spondylitis.[22] Abscess formation involves TB bacteria and is a complex process.[23] Abscess can occur in any spinal segment, with the thoracic segment being the most common location.[19] The degree of severity of abscess formation is due to pressure and the spread of cold abscesses to the surrounding environment.[11]

In this study, a significant association was found between the location of the spinal abscess and the severity of tuberculous spondylitis. Severe tuberculous spondylitis, characterized by the formation of widespread abscesses and an increase in size, is the criterion for performing spinal surgery. However, an abscess located in the thoracolumbar segment can cause severe conditions if a psoas abscess is present. This abscess can cause complications such as paralysis of the extremities.[9]

Tuberculous spondylitis is a disease that becomes a common cause of neurological deficits in the spine following injury or fracture.[24] Severe tuberculous spondylitis cases are often found due to delays in diagnosis because most patients seek treatment after experiencing severe pain.[5] This disease can affect all age ranges owing to its morbidity, which can be preceded by a highly contagious TB infection.[13,14] In Western countries, tuberculous spondylitis mostly affects people of older age due to reactivation.[5] In contrast, tuberculous spondylitis affects many young adults and children in TB endemic areas.[8]

Tuberculous spondylitis can affect people of all ages, with varying degrees of severity.[25] The current study found no significant association between age and tuberculous spondylitis severity. The reasons for the influence of age on the severity of tuberculous spondylitis are not well understood. Various theories have been proposed, such as tuberculous spondylitis in children, which tends to be much more severe owing to the immature condition of the spine. In addition, children's bone condition is more vascular, which induces the disease to spread and progress even faster and sometimes involves severe spinal collapse.[19]

Any spinal segment can be the site of a tuberculous spondylitis lesion.[13,14] Spinal damage due to tuberculous spondylitis infection results from the hematogenous spread, and early diagnosis is often difficult because of the indolent nature of the infection.[17] The best choice for the early detection of tuberculous spondylitis to determine the location of damage and spinal abscess formation can be performed by radiological examination using MRI.[19] MRI can determine the specific location of lesions and abscesses in the spine. The most

commonly reported lesion locations are the thoracic region, followed by the lumbar and cervical regions.[19]

This study reported no significant association between the location of spinal lesions and the severity of tuberculous spondylitis. In this study, the thoracic segment was the most common location of the lesion. However, the theory states that the spinal segments with the greatest risk of being the site of a lesion are those that bear heavy loads and have great mobility, especially the lower thoracic segment. This is because the lower thoracic segment is the area with the maximum movement and the focus area of body pressure.[11] If the damage to this segment becomes more extensive, the biggest complication in the form of paraplegia can occur and increase the severity of tuberculous spondylitis.[9]

STRENGTH AND LIMITATION

The strength of this study is that it focused on risk factors for age, sex, location of lesions, and location of abscesses, which are related to the severity of tuberculous spondylitis assessed based on MRI modality. The limitations of this research are the other factors that can affect the degree of tuberculous spondylitis, such as the pattern of physical activity, length of time the subject has suffered from tuberculous spondylitis, history of pulmonary TB, and abscess size, which may affect the severity of the tuberculous spondylitis degree. Some subjects were confirmed to have tuberculous spondylitis based on clinical findings and not on the results of the histopathological examination.

CONCLUSIONS

Sex and spinal abscess location are the risk factors that affect the severity of tuberculous spondylitis. Meanwhile, age and spinal lesion location have no significant risk to contribute in severity of tuberculous spondylitis disease based on GATA classification.

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ETHICAL CLEARANCE

The research protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Diponegoro (reference number: 127/EC/KEPK/FK-UNDIP/V/2022).

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CONFLICT OF INTEREST

The authors declare that this manuscript was approved by all authors, and that no competing interests exist.

AUTHOR CONTRIBUTION

Conception, design, and/or analysis and interpretation of data: MS. Drafting the article, discussion, and critical revision for important intellectual content: MS and HS. Review, supervision, and final approval: AP and CHNP.

REFERENCES

1. World Health Organization. Global tuberculosis report 2021 [Internet]. Geneva: World Health Organization;

2021. Available from: http://apps.who.int/bookorders.
2. Floyd K, Glaziou P, Zumla A, Raviglione M. The global tuberculosis epidemic and progress in care, prevention, and research: an overview in year 3 of the End TB era. Vol. 6, The Lancet Respiratory Medicine. Lancet Publishing Group; 2018. p. 299–314.
3. World Health Organization. Tuberkulosis [Internet]. World Health Organization. 2022 [cited 2022 Dec 1]. Available from: https://www.who.int/indonesia/news/campaign/tb-day-2022/fact-sheets
4. Vielgut I, Putzl L, Thomüller I, Igrec J, Brcic I, Valentin T, et al. Musculoskeletal tuberculosis revisited: bone and joint tuberculosis in Austria. Arch Orthop Trauma Surg. 2023 Jul 1;143(7):3845–55.
5. Small J, Noujaim D, Ginat D, Kelly H, Schaefer P. Neuroradiology: Spectrum and Evolution of Disease. 1st ed. Elsevier Inc; 2018. 222–226 p.
6. Zeng H, Liang Y, He J, Chen L, Su H, Liao S, et al. Analysis of Clinical Characteristics of 556 Spinal Tuberculosis Patients in Two Tertiary Teaching Hospitals in Guangxi Province. Biomed Res Int. 2021;2021(1344496):1–11.
7. Aslan G, Kurnaz N, Ülger M, Ayan İ, Tok Umay S, Gürses İ. Retrospective Evaluation of 14 Patients with Bone and Joint Tuberculosis in Mersin, Turkey. Flora the Journal of Infectious Diseases and Clinical Microbiology. 2020 Jun 15;25(2):197–205.
8. Putu Indira Paramitha Maharani, Baiq Auliya Cahya Putri, Siti Annisa Rahmasita. Musculoskeletal tuberculosis. International Journal of Health and Science. 2021 Sep;1(3):295–303.
9. Chen CH, Chen YM, Lee CW, Chang YJ, Cheng CY, Hung JK. Early diagnosis of spinal tuberculosis. Journal of the Formosan Medical Association. 2016 Oct 1;115(10):825–36.
10. Kim YJ, Hong JB, Kim YS, Yi J, Choi JM, Sohn S. Change of pyogenic and tuberculous spondylitis between 2007 and 2016 year: A nationwide study. J Korean Neurosurg Soc. 2020 Nov 1;63(6):784–93.
11. Rahyussalim. Spondilitis Tuberkulosis: Diagnosis, Penatalaksanaan, Dan Rehabilitasi. Indonesia; EC00201931054, 2019.
12. Leos-Leija AK, Padilla-Medina JR, Reyes-Fernández PM, Peña-Martínez VM, Montes-Tapia FF, Castillo-Bejarano JI. Vertebral destruction in an 11-month-old child with spinal tuberculosis: a case report and review of literature. Annals of Pediatric Surgery. 2022 Dec 1;18(1).
13. Shi T, Zhang Z, Dai F, Zhou Q, He Q, Luo F, et al. Retrospective study of 967 patients with spinal tuberculosis. Orthopedics. 2016 Sep 1;39(5):e838–43.
14. Liu Z, Wang J, Chen GZ, Li WW, Wu YQ, Xiao X, et al. Clinical Characteristics of 1378 Inpatients with Spinal Tuberculosis in General Hospitals in South-Central China. Biomed Res Int. 2019;2019(9765253):1–12.
15. Khanna K, Sabharwal S. Spinal tuberculosis: a comprehensive review for the modern spine surgeon. Spine Journal. 2019 Nov 1;19(11):1858–70.
16. Chandra SP, Singh A, Goyal N, Laythalling RK, Singh M, Kale SS, et al. Analysis of changing paradigms of management in 179 patients with spinal tuberculosis over a 12-year period and proposal of a new management algorithm. World Neurosurg. 2013 Jul;80(1–2):190–203.

17. Sinan T, Al-Khawari H. Spinal tuberculosis: CT and MRI features [Internet]. 2004. Available from: www.kfshrc.edu.sa/annals
18. Oguz E, Sehirlioglu A, Altinmakas M, Ozturk C, Komurcu M, Solakoglu C, et al. A new classification and guide for surgical treatment of spinal tuberculosis. Int Orthop. 2008 Feb;32(1):127–33.
19. Rajasekaran S, Soundararajan DCR, Shetty AP, Kanna RM. Spinal Tuberculosis: Current Concepts. Global Spine J. 2018 Dec 1;8(4_suppl):96S-108S.
20. Sianturi RN, Muslim Z, Leonas R, Uli HM, Farida A, Saleh I. Compatibility between MRI Examination and Histopathological Findings in Tuberculous Spondylitis Patients at Dr. Mohammad Hoesin Palembang. Sriwijaya Journal of Surgery [Internet]. 2022;5(1):472–7. Available from: https://doi.org/10.37275/OAIJSS.v5i1.71
21. Bollerslev J, Pretorius M, Heck A. Parathyroid hormone independent hypercalcemia in adults. Vol. 32, Best Practice and Research: Clinical Endocrinology and Metabolism. Bailliere Tindall Ltd; 2018. p. 621–38.
22. Na S, Lyu ZS, Zhang S. Diagnosis and Treatment of Skipped Multifocal Spinal Tuberculosis Lesions. Orthop Surg. 2023 Jun 1;15(6):1454–67.
23. Rajasekaran S, Kanna RM, Shetty AP. Pathophysiology and Treatment of Spinal Tuberculosis. JBJS Rev. 2014 Sep 23;2(9).
24. Vidya Ardiyati A. Factors Related to Treatment Seeking Behavior among Spondylitis Tuberculosis Patient: A Systematic Review. Vol. 2, International Journal of Nursing and Health Services (IJNHS). 2019.
25. Broekx S, Buelens E. Tuberculous spondylitis following intravesical bcg-instillation in the treatment of transitional cell carcinoma: Case report and systematic review. Clin Neurol Neurosurg. 2020 Jul 1;194(105944):1–8.

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Review Article

Molecular Diagnostic Tools for *Treponema pallidum*

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ABSTRACT

Syphilis, a common sexually transmitted disease, is caused by *Treponema pallidum subsp. pallidum*. Owing to the chameleonic behavior of syphilis, ocular involvement still presents a therapeutic problem. Direct detection of *Treponema pallidum* in the vitreous offers a potential diagnostic method because serodiagnosis has considerable limitations. The worldwide identification of *T. pallidum* substypes has occurred since the advent of molecular typing approaches. The purpose of this article is to provide more information on the development of a molecular approach for *Treponema pallidum* detection. A body of literature was gathered using automated database searches in Google Scholar, PubMed, and ScienceDirect. Although prior studies have focused on other genes, such as polA, 16S RNA, and the whole genome, there are still some that use the study of the arp and *T. pallidum* repeat (tpr) genes to subtype. Whole blood, vaginal ulcers, skin biopsies, and other samples can be used in molecular methods. Comparing quantitative reverse trascription-polymerase chain reaction (qRT-PCR) to traditional methods, such as reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFA) assay, and virus isolation, qRT-PCR has the advantage of being faster and more sensitive. Quick molecular methods, particularly polymerase chain reaction (PCR) results, will enable early detection of primary, secondary, and latent syphilis, which will lead to prompt treatment and prevention of disease progression as well as a reduction in the amount of time that the patient's sexual partners are exposed to the illness.

Keywords: *Treponema pallidum*, syphilis, detection tool, molecular method, and gene.

Highlights: This review describes the development of molecular methods for the detection of *Treponema pallidum* with various target genes. The benefit of this study is that it can be used as a reference for developing molecular methods for future research on *Treponema pallidum* detection.

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INTRODUCTION

Syphilis is a condition caused by infection with *Treponema pallidum subsp. pallidum* (*T. pallidum*) which cannot be grown in a dish. Multistage sexual transmission is still a public health issue, with an estimated 12 million new cases annually.[1] Syphilis first appeared in the 15th century, and by the 20th century, its frequency had decreased. However, the predominance has risen once more during the past few decades in America, Europe, and Asia.[2] Syphilis and an elevated risk of Human Immunodeficiency Virus (HIV) transmission in homosexuals have been significantly correlated in Makassar, Indonesia. Syphilis should be regarded as a major risk factor due to the increased prevalence of syphilis and HIV double infection among homosexuals, which can promote the spread of both illnesses.[3]
Treponema pallidum is one of the small main bacterial infections of humans that cannot be cultured on synthetic medium, making the diagnosis of syphilis challenging.[4] *Treponema pallidum* may currently only be detected in clinical samples using technologies that are either insensitive, such as dark-field microscopy.[5] The cornerstones of laboratory diagnosis for *T. pallidum* are serologic testing. However, for some forms of disease, they are either too sensitive, specific, or both.

Polymerase chain reaction (PCR) can selectively amplify the copy number of a target gene by more than 106-fold. The use of PCR to identify numerous fastidious or slowly proliferating bacterial pathogens has already been demonstrated, and has the potential to significantly improve our ability to identify infectious illnesses caused by these microbes. Therefore, the goal of this study is to learn more about how the molecular method for *T. pallidum* detection has been developed.

MATERIALS AND METHODS

Using automated database searches in Google Scholar, PubMed, and ScienceDirect, a body of literature was gathered. The goal was to compile data on genetic diagnostics for *T. pallidum* that had been published in the last 22 years, from 2001 to the most recent year. This is because there has not been much research on *T. pallidum* molecular diagnostics. An extensive literature search was conducted based on this idea, and references to pertinent publications were examined.

RESULTS AND DISCUSSION

Treponema pallidum linier gene

In addition, 3 *T. pallidum* repeat (*tpr)* genes had many several single nucleotide polymorphisms (SNPs) and single nucleotide indels that caused frameshift gene alterations, and eight genes had tiny indels and SNPs.[6] In the prior study, A molecular subtyping method for *T. pallidum* was developed by Pillay et al. based on the analysis of the *arp* and *tpr* repeat genes.[7] To identify these genes, though, they must either be directly amplified from clinical specimens or propagated in rabbit testis. *tpr* gene's restriction fragment length polymorphism analysis enables strains to be categorized and given letter names based on a combination of fragment sizes.[8] Genome of *T. pallidum* showed in Figure 1.

Molecular techniques for *Treponema pallidum* detection

Certain *T. pallidum* genes have been identified using molecular techniques such as PCR amplification, with varying degrees of sensitivity. The PCR detection method appears to be more precise. It was possible to confirm that the amplification product was unique to the *tp47* gene using the *tp47* hybridization probe. Additionally, it was demonstrated that *tp47* is extremely specific to *T. pallidum* and distinct from other saprophyte spirochetes and microorganisms often detected in sexual diseases, and that it

shares no homology with other bacterial or eucaryotal proteins.[9] Sensitivity of PCR method ranged from 42 to 100%.

A final diagnosis should be made using a combination of serological findings and clinical symptoms along with epidemiological, geographic, and anamnestic information because of the significant epidemiological overlap between venereal syphilis and endemic treponemal diseases in Cuba, and the inability of serology to differentiate between syphilis and other endemic treponematoses (including yaws and bejel). At research laboratories in wealthy countries, PCR that can distinguish between syphilis and treponematosis in clinical samples is now accessible. Routine access to these technologies is now possible.[10]

The platform should be considered when interpreting Ct values, and quantification should only be attempted after direct comparison with established standards. Most likely, the samples from the analytical panel that yielded Rotor-Gene false-negative results had little DNA. Carry-over contamination may account for these inconsistencies, because the false-positive samples in both panels were positioned after a high-load positive sample. Less than one working day was required to complete the assay from the time the sample was received until the findings were reported. Hence, this assay is excellent for use in ordinary diagnostic laboratories.[11]

PCR amplification of target DNA from *T. pallidum* is a sensitive method that can detect a single copy of the treponemal chromosome. In actuality, nested PCR procedures showed a sensitivity that was very close to the theoretical maximum. Despite this excellent sensitivity, the minimal number of clinical samples analyzed by PCR is limited on PCR detection. This restriction is crucial for biological samples containing a few treponemes, such as blood and cerebrospinal fluid.[12]

Confirm the previous study's finding that it might be appropriate to use PCR as the standard diagnostic assay for syphilis with an early phase stadium. There are several reasons why *T. pallidum* PCR (Tp-PCR) is preferred. First, compared to our study's expanded definition, Tp-PCR was more accurate than dark-field microscopy (DFM). Second, it might be challenging to obtain high-quality DFM results, particularly when the test is not conducted consistently. Finally, Tp-PCR results may be more consistent and testing less expensive if conducted on a regular basis because it is less dependent on human judgment than DFM.[13]

The programmatic priority is the discovery of *T. pallidum*, although the clinical supervision of individuals with yaw suspension benefits from the detection of *Haemophilus ducreyi*. The loop-mediated isothermal amplification (LAMP) assay might offer quick, molecular detection of the presence of *T. pallidum* and *H. ducreyi* because the median time to amplification was less than 15 min for both *T. pallidum* and *H. ducreyi*. To ensure that cases of yaws are not overlooked, the performance of the *T. pallidum* component must be improved by assay optimization, especially in the case of co-infection. Because qPCR requires very expensive equipment, particularly thermal cyclers, it can be up to ten times as expensive as a tube scanner that can carry out the Loop-Mediated Isothermal Amplification (LAMP) assay; implementing qPCR at the point of care is operationally difficult. LAMP may be a substitute molecular assay to help the development of yaw eradication efforts, since real-time PCR is only available in a few national number and reference laboratories worldwide. The cost-effectiveness evaluation of the LAMP assay was not performed; however, such an evaluation should consider the cost of the assay itself, the cost per assay, and how well each assay performs in comparison to the others to determine the cost per case detected. According to our results, the *T. pallidum* and *H. ducreyi* Loop-

Mediated Isothermal Amplification (TPHD-LAMP) test, however, may be a more affordable option than qPCR, particularly at the point of care.[14]

In this study, *T. pallidum* DNA was found in tissues from genitourinary ulcers, enabling the laboratory detection of primary syphilis. Genetic studies of the 23S rRNA gene target allowed for laboratory detection of the high incidence of *T. pallidum* strains exhibiting macrolide resistance in Shanghai.[8] The genome *T. pallidum* showed in Figure 1.

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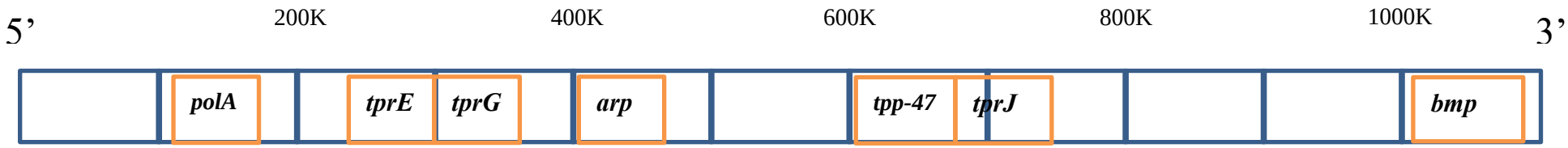

Figure 1. Genome *T. pallidum.*

Table 1. Molecular method for *T. pallidum* in The Previous Study.

Species	Specimen	Detection	Method	Primer	Amplicon (bp)	References
Treponema pallidum	Skin biopsy	*tp47* gene	Polymerase Chain Reaction	sense KO3A (5’-GAAGTTT GTCCCAGTTGCGGTT-3’) anti-sense KO4 (5’-CAGAGCCATCA GCCCTTTTA-3’)	260	Buffet M. et al.[9]
Treponema pallidum subsp. endemicum	Genital/anal ulceration or skin lesions	*polA*, *tp47*, and 16S rDNA loci	Real Time PCR	Not available	Not available	Noda AA, et al.[10]
Treponema pallidum	Genital ulcer swab	*polA* gene	Taqman real-time PCR assay	forward primer 5’-GGTAGAAGGGAGGGCTAGTA-3’, reverse primer 5’-CTAAGATCTCTATTTTCTATAGGTATG-3’and the Taqman probe 5’-ACACAGCACTCGTCTTCAACTCC-3’	116	Koek AG et al.[11]
Treponema pallidum subspecies pertenue	Swabs yaws-like ulcers	*PolA and 16S ribosomal RNA*	TPHD-LAMP assay	Not available	Not available	Becherer L, et al.[14]
Treponema pallidum	Genital ulcers	*arp and tpr genes*	PCR	Not available	Not available	Martin IE, et al.[8]
Treponema pallidum	blood	*Not available*	Taqman-locked nucleic acid (LNA) qPCR	TP forward primer 5’-AGGTCATTATCGTGGTGTTAC-3’ TP reverse primer 5’-CAATCCATCCGTTTCACAATC-3’ TP Probe ROX-CACACCATTCGCACACG-Eclipse	103	Zhou L, et al.[15]
Treponema pallidum	Genital ulcer and whole blood	*DNA polymerase I gene*	PCR-RFLP	*polA*-forward (5’-TGCGCGTGTGCGAATGGTGTGGTC-3’) reverse primer *polA*-reverse (5’-CACAGTGCTCAAAAACGCCTGCACG-3’)	377	Sutton MY, et al.[16]
Treponema pallidum	Blood and CSF	*arp, tpr subfamily II genes, tprC gene,tprD gene locus, tprE, tprG, and tprJ*	RFLP	Sense: 5’-CAGGTTTTGCCGTTAAGC-3’ antisense: 5’-AATCAAGGGAGAATACCGTC-3’	735 - 1575	Marra CM, et al.[17]

Treponema pallidum* ssp. *pallidum	TPA isolate Street Strain 14	*Complate genome*	Oligonucleotide array	Not available	Not available	Matějková P, et al.[18]
Treponema pallidum	Swab of suspected early syphilis lesion	*47kDA lipoproptein*	PCR	Reverse 5'-AGG GGA AGG TGC TGACCATAG-3' & Forward 5'-GGGAGTGAAATCCGCAGAGAG-3' and hydrolysis probe 5'-AGCCTAAGC TTGTCAGCGATCAAG C-3'	Not available	Shields M, et al.[19]
Treponema pallidum	Genital swabs, whole blood, sera, and cerebrospinal fluid	*Treponemal repeat genes, arp and tpr.*	PCR	Not available	Not available	Martin IE, et al.[20]
Treponema pallidum	Swab supernatant, whole blood, and blood serum	*polA and tmpC*	Nested PCR	Not available	Not available	Flasarová M, et al.[21]
Treponema pallidum subsp. pallidum	Genital, anal, oral or rectal lesion swabs and tissue sample.	*Whole genome*	Nested PCR and DNA sequencing	Not available	Not available	Grillová L, et al.[22]
Treponema pallidum* ssp. *pallidum	Treponemal cells	*Whole genome*	DNA sequencing	Not available	Not available	Pětrošová H, et al.[23]
Treponema pallidum* sp. *pallidum	Serum from whole blood	*polA and antibody*	Simple PCR and IgG/IgM ELISA	Forward primer 5'-AGACGGCTGCACATCTTCTCCA-3'; and reverse primer 5'-AGCAGACGTTACATCGAGCGGA-3'	209	Casal CAD, et al.[24]
Treponema pallidum subsp. pallidum	Isolate Propagation	*tprK*	DNA sequencing	Forward primer (5'-AGTAATGGTTTTCGGCATCG-3') and reverse primer (5'-CCATACATCCCTACCAAATCA-3')	175	LaFond RE, et al.[25]
Treponema pallidum	Blood and Tissues	*flaA*	Real-Time PCR	Forward primer 5'-GCGGTTGCACAGTGGGAG-3' and reverse primer 5'-CAGCATGGGCGACAGGAT-3'	61	Salazar JC, et al.[26]
Treponema pallidum subsp. pallidum	Bacterial pellets and swab samples	*tprK*	PCR amplification	Forward primer 5'-ATATTGAAGGCTATGCGGAGCTG-3' and reverse primer 5'-TACCCCACACTCGTAATACCC-3'	182 - 1380	LaFond RE. et al.[27]
Treponema pallidum	Genital ulcers	*polA*	PCR	Forward (F1) primer (5'- TGCGCGTGTGCGAATGGTGTGGTC-3') (nucleotides 1759 to 1783, corresponding to amino acids CACANGVV) (in *polA* 1390 to 1413) and reverse (R1) primer (5'-CACAGTGCTCAAAAACGCCTGCACG-3') (nucleotides 2111 to 2135, corresponding to amino acids RAGVFEHCA); than for forward (F2) primer (5'-CGTCTGGTCGATGTGCAAATGAGTG-3') (nucleotides 1539 to 1563, corresponding to amino acids TSG RCANEC) and reverse (R2) primer (5'-TGCACATGTACACTGAGTTGACTCGG-3')	377 and 395	Liu H, et al.[28]

Treponema pallidum	Genital ulcers	*arp and tpr*	PCR	Forward primer ARP1A 5′-CAAGTCAGGACGGACTGTCCCTTGC-3′) and reverse primer ARP2A (5′-GGTATCACCTGGGGATGCGCACG-3′), than forward primer B1 (5′-ACTGGCTCTGCCACACTTGA-3′) and reverse primer A2 (5′-CTACCAGGAGAGGGTGACGC-3′)	Not available	Pillay A, et al.[29]
Treponema pallidum	Swabs and blood	*Tpp47*	PCR	Tps (5'-TTCGATGCAGTTTCTCGCGCCAACC-3'); Tpe (5'-CTACTGGGCCACTACCTTCGCACG-3'); KO5 (5'-CCCGTTCGCAATCAAAGTCAGCCT-3'); and KO3B (5'-GACGCGAGCTACACCAATCTGATG-3')	1103	Grange PA, et al.[30]
Treponema pallidum	(ano)genital ulcer and skin scraping	*polA*	Real-Time PCR	Forward primer 5'-GGTAGAAGGGAGGGCTAGTA-3', and reverse primer 5'-CTAAGATCTCTATTTTCTATAGGTATGG-3'. Then for TaqMan probe 5'-FAM-ACACAGCACTCGTCTTCAACTCC-BHQ1-3'	Not available	Heymans R, et al.[31]
Treponema pallidum	Whole blood	*polA*	PCR	forward primer 5'-TGCGCGTGTGCGAATGGTGTGGTC-3' and reverse primer 5'-CACAGTGCTCAAAAACGCCTGCACG-3'	377	Marfin AA, et al.[32]

Molecular method for *T. pallidum* in the previous study are showed in Table 1. Quantitative Reverse Transcription PCR (qRT-PCR) has the benefit of being quicker and more sensitive when compared to conventional techniques like reverse transcription-polymerase chain reaction (RT-PCR), Enzyme-linked Immunosorbent Assay (ELISA), Indirect Fluorescent Antibody (IFA), and culture virus. Such assays were consequently created recently, and have swiftly emerged as one of the most crucial techniques for pathogen detection. It is extremely difficult to build a multiplex qRT-PCR assay because each target pathogen requires the use of different optimized primer pairs, probes, and ion concentrations. In fact, the planning and improvement of the reaction system require more attention. qRT-PCR allows the measurement of numerous fluorophores in a single tube and the execution of multiplex assays, allowing for the simultaneous testing of multiple target sequences in a simple reaction. Its superior analytical capacity is evident when a number of potential infections need to be detected quickly. It is a good alternative to conventional approaches because it can run tests concurrently rather than sequentially. Multiplex PCR has been frequently used to simultaneously detect several diseases.[15]

For RFLP analysis of the *tpr* gene, MseI was used to digest the unpurified PCR amplicons from the second stage of nested PCR amplification. Restriction fragments were detected by ethidium bromide staining after separation by electrophoresis on a agarose gel (2%) at 100 V for 60 min.[16]

The Centers for Disease Control and Prevention (CDC) technique has been utilized in several earlier studies to examine the prevalence of *Treponema pallidum* subtype prevalence in communities. For instance, during a syphilis outbreak in Phoenix, Arizona, Sutton, and colleagues identified CDC and *T. pallidum* subtypes using DNA taken from vaginal ulcer swabs and blood samples.[16]

The improved typing technique described here has biological, clinical, and epidemiological value. This marks a considerable improvement in the concept of the molecular epidemiology of syphilis and provides a chance to discover more about the pathophysiology of diseases of the central nervous system.[17]

The protein family (tpr*)* consists of 12 *T. pallidum* replicate proteins, which are only present in this bacterium and share sequence similarities with Treponema denticola's main sheath proteins. Sequence alterations were found to affect 8 of the 12 *tpr* genes (66%), a larger percentage than the rate for the entire genome (13.1%). The *tpr* genes contained locations exhibiting either interstrain, intrastrain, or both variability. The *tpr* genes contained a total of 53 SNPs and 38 intrastrain variable nucleotide locations, with at least one allele matching the Nichols genome sequence (V1–V7 regions of *tprK* were excluded from the analysis). Given the high degree of DNA similarity among *tpr* genes, it is anticipated that differences might be overestimated because of the limits of the hybridization approach for repetitive sequences.[18]

Improved specimen collection may increase the sensitivity of the PCR assays. Serum was extracted from carefully chosen secondary lesions using the edge of a large-bore needle or scalpel. This procedure may also increase the sensitivity of the PCR.[19]

Based on the examination of the repeat genes *arp* and *tpr*, a molecular substyping strategy was devised for *T. pallidum* was devised before. Therefore, direct amplification from clinical samples is necessary to characterize these genes. According to this approach, strains can be categorized based on the number of sixty-base pair tandem repeats that are present in

their *arp* genes; up to this point, repeat counts ranging from 3 to 22 have been recorded. The genes *of tpr*, notably *trpE, tprG*, and *tprJ* of *tpr* subfamily two, allow restriction fragment length polymorphism analysis, which permits the classification and letter designation of starins based on a combination of fragment sizes.[20]

Developing effective and affordable typing methods that may be applied in any laboratory with access to sequencing resources, wherever in the world. These techniques would offer data on the frequency of different strain types and variations over time, as well as trends among antibiotic-resistant strains and whether specific strain types are linked to particular patient groups.[22]

Strain Mexico A's high quality entire genome sequence was discovered utilizing the "next generation" sequencing method (Illumina). Despite the fact that this strain's genome had fewer significant rearrangements than those of other treponemal genomes, two genes incorporated sequences from the subspecies pallidum or pertenue. The apparent mosaic nature of these two genes is probably due to the concurrent infection of a single host, Pallidum and Pertenue, which recombinate with one another.[23]

PCR may be a promising option for syphilis diagnosis in the mother and for contributing to the early detection of the disease, especially primary, secondary, and latent syphilis), which, if undiagnosed and untreated, can have harmful effects on the fetus. Studies of this strategy have also demonstrated its value as a method for control of maternal and congenital syphilis epidemiology in Brazil, lowering public spending on treatable and avoidable diseases. This study found a discrepancy in the results of IgM FTA-Abs (fluorescent treponemal antibody absorption) versus IgG ELISA and PCR versus IgG ELISA due to the low sensitivity of polymerase chain reaction and FTA-Abs. It should be noted that the majority of patients had indeterminate syphilis according to clinical diagnosis and that 19 of the women in this group had received treatment for the disease before the study pregnancy, which could be explained by the serological finding of antibodies from an earlier infection or by the presence of a minor number of circulating treponemes.[24,33-36]

The distribution of germs in the different components of the blood under investigation also suggests that samples (serum) are a less trustworthy source than whole blood or plasma for detecting circulating spirochetes. The decreased amounts in serum could be due to fibrin, fibrin breakdown products, spirochetal adhesion to cellular components, or the clot itself.[26]

When ABI 310 equipment is utilized for analysis, the theoretical detection limit of polA PCR is 200 organisms per milliliter, or two organisms per PCR. This cap was established using data from a two hunderd-ml sample that underwent DNA isolation and yielded 100 ml of eluted DNA. The flow PCR procedure ran a PCR with 5 ml of the collected DNA. The sensitivity increased to approximately one organism per PCR, making it comparable to that of Rabbit Infectivity Testing (RIT). However, it is vital to remember that the sampling technique has a cap on the sensitivity of detection. The detection of organisms at concentrations less than one per 5 ml may be hampered by sampling errors.[28,37-40]

STRENGTH AND LIMITATION

This review article describes the development of molecular methods for the detection of *T. pallidum* targeting various genes. In addition, this article also describes the primers used in the molecular method so that it can be used as a reference for the detection of *T. pallidum* to develop a diagnostic system. This article is limited to laboratory research reports and does not include molecular methods used for patient diagnostics.

CONCLUSIONS

Early detection of primary, secondary, and latent syphilis, made possible by quick molecular methods, particularly PCR results, will result in prompt treatment and a reduction in the amount of time the patient's sexual partners are exposed to the disease, as well as the prevention of disease progression. Thus, it is sufficient to check for this disease using a whole blood specimen.

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CONFLICT OF INTEREST

The authors claim to have no conflicts of interest.

ETHICAL CLEARANCE

This study did not require an ethical review because the data were obtained from the existing literature and did not use human subjects.

FUNDING

There is no funding for this study.

CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in writing this manuscript.

AUTHOR CONTRIBUTION

All authors contributed to the preparation of this review article. Author WS in this study was in charge of collecting data, analyzing data, and writing article reviews. Meanwhile, NW is the principal investigator who provides study ideas and validates the data.

REFERENCES

1. Gerbase AC, Rowley JT, Heymann DH, Berkley SF, Piot P. Global prevalence and incidence estimates of selected curable STDs. Sex Transm Infect. 1998 Jun;74 Suppl 1:S12-6
2. Wijesooriya NS, Rochat RW, Kamb ML, Turlapati P, Temmerman M, Broutet N, Newman LM. Global burden of maternal and congenital syphilis in 2008 and 2012: a health systems modelling study. Lancet Glob Health. 2016 Aug;4(8):e525-33
3. Kusumawaty M, Djawad K, Massi MN, Adam AM, Wahab S, Bahar B. Sero-epidemiology and risk factors of syphilis in Makassar, Indonesia. Serbian J Dermatology Venereol. 2019;11(2):43-49
4. Burstain JM, Grimprel E, Lukehart SA, Norgard MV, Radolf JD. Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. ASM. 1991;29(1):62-69
5. Tramont EC. *Treponema pallidum* (syphilis), in Mandell GL, Douglas RG, and Bennett JE, Priciples and practice of infectious diseases, 3rd ed. New York: John Wiley & Sons, Inc.
6. Strouhal M, Šmajs D, Matějková P, Sodergren E, Amin AG, Howell JK, Norris SJ, Weinstock GM. Genome differences between *Treponema pallidum subsp. pallidum* strain Nichols and *T. paraluiscuniculi* strain Cuniculi A. Infect Immun. 2007; 75(12):5859-5866
7. Pillay A, Liu H, Chen CY, Holloway B, Sturm AW, Steiner B, Morse SA. Molecular subtyping of *Treponema pallidum subspecies pallidum*. Sex Transm Dis. 1998; 25(8):408-14

8. Martin IE, Gu W, Yang Y, Tsang RSW. Macrolide resistance and molecular types of *Treponema pallidum* causing primary Syphilis in Shanghai, China. Clin Infect Dis. 2009;49:515-21
9. Buffet M, Grange PA, Gerhardt P, Carlotti A, Calvez V, Bianchi A, Dupin N. Diagnosing *Treponema pallidum* in secondary syphilis by PCR and Immunohistochemistry. J Invest Dermatol. 2007;127:2345-2350
10. Noda AA, Grilloá L, Lienhard R, Blanco O, Rodríguez, Šmajs D. Bejel in Cuba: molecular identification of *Treponema pallidum subsp. endemicum* in patients diagnosed with veneral syphilis. Clin Microbiol Infect. 2018;24:1210.e1-1210.05
11. Koek AG, Bruisten SM, Dierdorp M, van Dam AP, Templeton K. Specific and sensitive diagnosis of syphilis using a real-time PCR for *Treponema pallidum*. Clin Microbiol Infect. 2006;12:1233-1236
12. Šmajs D, Matějková P, Woznicová V, Vališová Z. Diagnosis of syphilis by polymerase chain reaction and molecular typing of *Treponema pallidum*. Rev Med Microbiol. 2006;17(4):93-99
13. Gayet-Ageron A, Sednaoui P, Lautenschlager S, Ferry T, Toutous-Trellu L, Cavassini M, Yassir F, de Tejada BM, Emonet S, Combescure C, Schrenzel J, Perneger T. Use of *Treponema pallidum* PCR in Testing of Ulcers for Diagnosis of Primary Syphilis. Emerg Infect Dis. 2015;21(1):127-129
14. Becherer L, Knauf S, Marks M, Lueert S, Frischmann S, Borst N, von Stetten F, Bieb S, Adu-Sarkodie, Y, Asiedu K, Mitjà, Bakheit M. Multiplex Mediator Displacemen Loop-Mediated Isotermal Amplification for Detection of *Treponema pallidum* and *Haemophilus ducreyi*. Emerg Infect Dis. 2020; 26(2):282-288
15. Zhou L, Gong R, Lu X, Zhang Y, Tang J. Development of a multiplex Real-Time PCR assay for the detection of *Treponema pallidum*, HCV, HIV-1, and HBV. Jpn J Infect Dis. 2015;68:481-487
16. Sutton MY, Liu H, Steiner B, Pillay A, Mickey T, Finelli L, Morse S, Markowitz LE, St. Louis ME. Molecular subtyping of *Treponema pallidum* in an Arizona Country with increasing Syphilis morbidity: Use of specimens from ulcers and blood. J Infect Dis. 2001;183:1601-6
17. Marra CM, Sahi SK, Tantalo LC, Godornes C, Reid T, Behets F, Rompalo A, Klausner JD, Yin Y, Mulcahy F, Golden MR, Centurion-Lara A, Lukehart SA. Enhanced molecular typing of *Treponema pallidum*: Geographical distribution of strain types and association with Neurosyphilis. J Infect Dis. 2010;202(9):1380-1388
18. Matějková P, Strouhal M, Šmajs D, Norris SJ, Palzkill T, Petrosino JF, Sodergren E, Norton JE, Singh J, Richmond TA, Molla MN, Albert TJ, Weinstock GM. Complate genome sequence of *Treponema pallidum ssp. Pallidum* strain SS14 determined with oligonucleotide arrays. BMC Microbiol. 2008;8(76):1-12
19. Shields M, Guy RJ, Jeoffreys NJ, Finlayson RJ, Donovan B. A longitudinal evaluation of *Treponema pallidum* PCR testing in early syphilis. BMC Infect Dis. 2012;12(353):1-6
20. Martin IE, Tsang RSW, Sutherland K, Anderson B, Read R, Roy C, Yanow S, Fonseca K, White W, Kandola K, Kouadjo E, Singh AE. Molecular typing of *Treponema pallidum* strains

in Western Canada: Predominance of 14d subtypes. Sex Transm Dis. 2010;37(9):544-548
21. Flasarová M, Pospíšilová P, Mikalová L, Vališová Z, Dastychová E, Strnadel R, Kuklová I, Woznicová V, Zákoucká H, Šmajs D. Sequencing-based molecular typing of *Treponema pallidum* strains in the Czech Republic: All identified genotypes are related to the sequence of the SS14 strains. Act Derm Venereol. 2012;92:669-674
22. Grillová L, Bawa T, Mikaloá L, Gayet-Ageron A, Neiselt K, Strouhal M, Sednaoui P, Ferry T, Cavassini M, Lautenschlager S, Dutly F, Pla-Díaz M, Krützen M, González-Candelas F, Bagheri HC, Šmajs D, Arora N, Bosshard PP. Molecular characterization of *Treponema pallidum subsp. pallidum* in Switzerland and France with a new multilocus sequence typing scheme. PloS ONE. 2018;13(7):e0200773
23. Pětrošová H, Zobaníková M, Čejková D, Mikalová L, Pospíšilová P, Strouhal, M, Chen L, Qin X, Muzny DM, Weinstock GM, Šmajs D. Whole genome sequence of *Treponema pallidum ssp. Pallidum*, strain Mexico A, suggests recombination between Yasws and Syphilis strains. PloS Negl Trop Dis. 2012;6(9):e1832
24. Casal CAD, da Silva MO, Costa IB, Araújo EC, de Oliveira Corvelo TC. Molecular detection of *Treponema pallidum* sp. Pallidum in blood samples of VDRL-seroreactive woman with lethal pregnancy outcome: a retrosprective observational study in northern Brazil. Rev Soc Bras Med Trop. Aug 2011;44(4):1-6
25. LaFond RE, Centurion-Lara A, Godornes C, van Voorhis WC, Lukehart SA. TprK sequence diversity accumulates during infection of Rabbits with *Treponema pallidum subsp. pallidum* Nichols Strain. Infect Immun. Mar 2006;73(3):1896-1906
26. Salazar JC, Rathi A, Michael NL, Radolf JD, Jagodzinski LL. Assessment of kinetics of *Treponema pallidum* dissemination into blood and tissues in experimental Syphilis by Real-Time Quantitative PCR. Infect Immun. June 2007;75(6):2954-2958
27. LaFond RE, Centurion-Lara A, Godornes C, Rompalo AM, van Voorhis WC, Lukehart SA. Sequence diversity of *Treponema pallidum subsp. pallidum* tprK in human syphilis lesions and rabbit-propagated isolates. J Bacteriol. Nov 2003;185(21):6262-6268
28. Liu H, Rodes B, Chen CY, Steiner B. New tests for syphilis: rational design of a PCR method for detection of *Treponema pallidum* in clinical specimens using unique regions of the DNA polymerase I gene. J Clin Microbiol. May 2001;39(5):1941-1946
29. Pillay A, Liu H, Ebrahim S, Chen CY, Lai W, Fehler G, Ballard RC, Steiner B, Sturm W, Morse SA. Molecular typing of *Treponema pallidum* in South Africa: Cross-sectional studies. J Clin Microbiol. Jan 2002;40(1): 256-258
30. Grange PA, Gressler L, Dion PL, Farhi D, Benhaddou N, Gerhardt P, Morini JP, Deleuze J, Pantoja C, Blanchi A, Lassau F, Avril MF, Janier M, Dupin N. Evaluation of a PCR test for detection of *Treponema pallidum* in swabs and blood. J Clin Microbiol. March 2012;50(3):546-552
31. Heymans R, van der Helm JJ, de Vries HJC, Fennema HSA, Coutinho RA, Bruisten SM. Clinical value of

Treponema pallidum Real-Time PCR for diagnosis of syphilis. J Clin Microbiol. Feb 2010;48(2):497-502
32. Marfin AA, Liu H, Sutton MY, Steiner B, Pillay A, Markowitz LE. Amplification of the DNA polymerase I gene of *Treponema pallidum* from whole blood of persons with syphilis. Diagn Microbiol Infect Dis. 2001;40:163-16
33. Kusumawati D, Martodihardjo S. Syphilis in pregnancy. Berkala Ilmu Kesehatan Kulit dan Kelamin. 2013-12;25(3):199-204
34. Lubis YH, Susilawati S. Analysis of HIV and Syphilis transmission prevention programs for adolescents. Indonesian Journal of Health Administration. June 2023;11(1):120-130
35. Susilawati TN, Satria YAA. Genital tract infection during pregnancy and its association with preterm delivery. Indonesian Journal of Tropical and Infectious Disease. 2021;9(1):46-57
36. Purnamasari I, Hidayati AN, Kusumastuti EH, Effendy I. The histopathological features of syphilis and its mimickers. Folia Media Indonesiana. 2023;59(1):63-69
37. Griffith C. Surface sampling and the detection of contamination. Handbook of Hygiene Control in the Food Industry. 2016:673-696
38. Downey AS, Silva SMD, Olson ND, Filliben JJ, Morrow JB. Impact of processing method on recovery of bacteria from wipes used in biological surface sampling. Appl Environ Microbiol. 2012;78(16):5872-5881
39. Löder MG, Gerdts G. Methodology used for the detection and identification of microplastics-A critical appraisal. Marine Anthropogenic Litter. 2015:201-227
40. Masyeni DAPS, Hadi U, Kuntaman, Yohan B, Margyaningsih NI, Sasmono RT. Expression of four cytokine/chemokine genes in peripheral blood mononuclear cells infected with dengue virus. Indonesian Journal of Tropical and Infectious Disease. 2019;7(4):75-78

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Original Article

Nigella sativa 3% Inhibition Test of Natural Toothpaste Compared *Cetylpyridinum chloride* (CPC) Toothpaste 0.01-0.1% on *Aggregatibacter actinomycetemcomitans*

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ABSTRACT

Periodontitis generally begins with gingivitis progresses to alveolar bone increasing the risk of systemic disease. The primary etiological factor in the etiology of periodontal disease is *Aggregatibacter actinomycetemcomitans* bacteria. *Cetylpyridinium chloride (CPC) has a bactericidal effect by disrupting the function of bacterial membranes in the cytoplasm. CPC can also absorb negative charges from bacteria, increase bacterial cell wall permeability, decrease cell metabolism, and reduce bacterial attachment to teeth. Use of antimicrobial toothpaste, such as Cetylpyridinium chloride (CPC), is one strategy to prevent periodontal disease, but CPC is hazardous in some quantities. As a result, it should be compared to a natural toothpaste, specifically Nigella sativa toothpaste. The objective of this study is to compare the antibacterial activity of natural toothpaste containing Nigella sativa 3% with toothpaste containing CPC 0.01% - 0.1% on Aggregatibacter actinomycetemcomitans. Experimental studies are used in this kind of research. The* colony count method was used to assess the natural toothpastes *Nigella sativa* 3% and toothpaste containing CPC 0.01- 0.1% for their capacity in inhibiting the *Aggregatibacter actinomycetemcomitans.* Natural toothpaste containing *Nigella sativa* 3% was completely inhibiting *Aggregatibacter actinomycetemcomitans,* compared to 0.01-0.1% CPC toothpaste. The significance level for the statistical test results was 0.000 ($p<0.05$). The conclution of this research are the natural toothpaste containing *Nigella sativa* 3% and toothpaste containing CPC 0.06%–0.1% can effectively suppress the growth of the microorganisms *Aggregatibacter actinomycetemcomitans.*

Keywords: : Periodontitis, *Aggregatibacter actinomycetemcomitans, Nigella sativa toothpaste 3%, Cetylpyridinium chloride* toothpaste 0.01-0.1%, and natural toothpastes.

Highlights: This research are expected to provide information about tooth paste contain *Nigella sativa* 3% and *Cetylpyridinium* chloride 0.01-0.1% which can inhibit the growth of *Aggregatibacter actinomycetemcomitans*.

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INTRODUCTION

Periodontitis, an inflammatory and pathological that impacts the connective tissue connection of the teeth, affects the majority of the adult population of the world. The Gram-negative microorganisms typically form a biofilm and cause it to be characterized by an increased host reaction against them, which ultimately results in tooth loss.[1] Pathogenic bacteria in the sulcus gingiva are the main cause of periodontitis, which has a complex etiology. *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are two bacteria that are recognized as being significant periodontal pathogens and responsible for the devastating periodontal disease.[2]

Periodontitis can be prevented and treated at a low cost by maintaining oral hygiene with practices like cleaning your teeth.[3,4] According to Maria et al., periodontal pathogens in dental plaque were reduced when teeth were brushed with antibacterial toothpaste.[5] Periodontal therapy aims to restore lost shape, function, and aesthetics of all supporting structures and tissues (gingiva, periodontal ligament [PDL], cementum and alveolar bone) of the periodontal tissues and prevent periodontitis. The goal of standard periodontal therapy is to reduce the overall bacterial burden and change environmental factors affecting the microbial habitat so as to maintain homeostasis in the periodontal microbiota.[2] Abrasives are found in toothpaste and are used to polish teeth. A surface-active element in toothpaste known as a foaming agent is detergents.[5,6] Moreover, herbal extract is employed because it has beneficial benefits, such as anti-inflammatory, that prevent gingivitis and maintain oral health.[7,8]

The medical herb *Nigella sativa*'s antibacterial and anti-inflammatory properties have been studied.[9–11] Among the evidence-based herbal remedies, *Nigella sativa* is hailed as a "miracle herb".[10,12,13] The perennial plant *Nigella sativa*, commonly known as black cumin, black seed, habbatul barakah, black caraway, kalojeera, kalonji, or kalanji, is native to several Middle Eastern and Mediterranean countries as well as southern Asia. It is a member of the *Ranunculaceae* family.[12–15] The herb *Nigella sativa* contains tannins, thymoquinone, flavonoids, and thymol. The anti-inflammation and anti-oxidant properties of *Nigella sativa*, mint, cloves, aniseed, and olive leaf extracts increase tooth paste performance.[3,7,13] *Nigella sativa* may therefore be utilized as an adjuvant in periodontal therapy.[7,16] According to a preliminary investigation, supragingival plaque bacteria could not grow when used with toothpaste containing 2% SLS and *Nigella sativa* extract (Dentomaxxima). *Nigella sativa* extract also prevented bacterial plaque.[3,7]

Currently available toothpaste contains *Cetylpyridinium chloride* (CPC) as one of its primary active ingredients, an antibacterial agent.[17] The quaternary ammonium compound CPC possesses a range of antimicrobial properties. Water, ethanol, chloroform, benzene, and water are all solvents for CPC.[18] After two weeks of consistent use, the state of the oral cavity will improve. CPC is an anti-bacterial, anti-plaque, and gingivitis treatment.[19] In a prior investigation into the efficiency of CPC-containing mouthwashes through a clinical trial, Rawlinson et al. shown that two different types of CPC-containing mouthwashes with concentrations of 0.05% and 0.1% may clinically suppress plaque growth.[20] CPC also has a bactericidal effect by interfering with the function of the bacterial cytoplasmic membrane and disturbing bacterial metabolism, resulting in reduction of cell development and, eventually, cell death. Plaque index can be decreased by reducing the number of bacteria present in the plaque.[18,19,21]

Cetylpyridinium chloride (CPC) works by infiltrating the bacterial cell

membrane, producing leaking inside the cell, and ultimately killing the bacteria.[18] CPC has the power to absorb bacterial negative charges, improve bacterial cell wall permeability, slow down cell metabolism, lessen bacterial adhesion to tooth surfaces, and prevent bacterial cell growth. The drawback of these artificial substances is that they leave dark stains on the teeth.[22]
According to literacy about CPC as an antibacterial agent, few have undertaken research on the inhibition of growth the *Aggregatibacter actinomycetemcomitans* bacteria. Therefore, the purpose of this study was to learn more about the inhibition of growth the *Aggregatibacter actinomycetemcomitans* by toothpaste containing CPC and *Nigella sativa*, as well as to compare the differences in CPC's inhibition of *Aggregatibacter actinomycetemcomitans* at concentrations of 0.01% - 0.1%.

MATERIALS AND METHODS

This research was performed at the Airlangga University Faculty of Dentistry Research Center in Surabaya, East Java. The research design in this study was a post-test-only laboratory experiment with a control group. The sample used in this investigation was the bacteria *Aggregatibacter actinomycetemcomitans*, which is available at the Faculty of Dental Medicine Airlangga University Research Center. Federer's formula **(n-1) (t-1)≥15**, where n=sample size for each intervention and t=number of interventions, for determining the number of samples was used to determine the number of samples used in the study.[22] The total number of group in this study was 12 made up of 11 treatment groups (*Nigella sativa* toothpaste 3% 1 group, and CPC toothpaste 10 groups), and 1 control groups (control media). Each concentration was repeated 3 times. Therefore, three samples are required at a least for each treatment to be repeated.

The independent variable in this study is toothpaste consisting of CPC 0.01-0.1% and *Nigella sativa* 3%. In this study, 3% extract was used because, in the previous study, formulation stable with the concentration of the active ingredient viscous extract of black cumin seeds (*Nigella sativa*) by 3%. The dependent variable in this study was the growth of the *Aggregatibacter actinomycetemcomitans* bacteria. The controlled variables in this study were the incubation time and temperature of *Aggregatibacter actinomycetemcomitans* bacteria, the sterility of the materials, and the skills of the operators during the study.

Research Procedure

This research is based on the development of previous research by Setiawati et al.,[23] and the procedure is based on research conducted by Toar et al.[22]

1. Tool Sterilization
   To prevent microorganism contamination, all tools and materials used throughout the investigation must be sterile and clean. Metal and glass instruments are sterilized by carefully washing, drying, and wrapping them in aluminum foil. Metal tools were sterilized for 60 minutes at 121°C in the autoclave, and glass tools were sterilized at 110°C for 15 minutes. Plastic tool sterilization is possible with 70% ethanol.

2. Preparation of *Aggregatibacter actinomycetemcomitans* Bacterial Culture Media

3. *Aggregatibacter actinomycetemcomitans* was used to create bacterial culture medium for the study. Brain Heart Infusion – Broth (BHI-B) media was then used to inoculate the bacteria, resulting in the suspension of the *Aggregatibacter actinomycetemcomitans* bacteria.

4. Preparation of *Aggregatibacter actinomycetemcomitans* Bacterial Suspension.

   The following procedure is to make an *Aggregatibacter actinomycetemcomitan* bacterium suspension. A sterile ose needle was used to collect bacterial colonies that had been grown on agar media and placed inside a test tube's BHI-B medium. After then, the test tube was kept at 37°C for 24 hours. *Aggregatibacter actinomycetemcomitan* bacteria must be suspended in a solution that meets the McFarland turbidity requirement of 0.5. Two ingredients make up McFarland's 0.5 standard solution: 1% Barium chloride ($BaCl_2$) and 1% Sulfuric acid (H_2SO_4). The two solutions are combined in the necessary amount, shaken, and then combined. The concentration of $1.5x10^8$ CFU/ml in McFarland's standard solution is equivalent to a bacterial cell suspension. If the BHI-B media appears more turbid than the 0.5 McFarland solution, the solution can be introduced into the BHI-B media a little slowly to bring it up to the 0.5 McFarland turbidity standard.

5. Inhibitory test of the growth of *Aggregatibacter actinomycetemcomitans* bacteria.

6. The next step was to prepare test tubes that were labeled for testing the inhibition of Brain Heart Infusion – Broth (BHI-B) and CPC media at concentrations of 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, and 0.1% on the growth of the bacteria *Aggregatibacter actinomycetemcomitans*. The tube also contained *Nigella sativa* 3%. The tubes were all then filled with 0.1 mL of bacterial suspension, and they were all cultured at 37°C for two consecutive days. Take 0.1 mL of the suspension from each tube, spread it on the Mueller Hinton Agar (MHA) media, and incubate the MHA media for two cycles of 24 hours at 37°C. The next procedure was to calculate the number of bacterial colonies on MHA media and perform data analysis.

7. The Shapiro-Wilk normality test, the homogeneity test utilizing Levene's test, and the One-Way Anova parametric test were employed in this study's data analysis, which comprised more than two sample variables. To evaluate if the data are regularly distributed or not, the Shapiro-Wilk normality test is utilized. If it is, the parametric test can be performed. If (p-Value) > 0.05, then homogeneous data are obtained from two or more data groups, according to the homogeneity test with Levene's test, which tries to determine the homogeneity of the data. Parametric one-way Anova test. This test is run when the data is discovered to be regularly distributed. One-Way Anova seeks to determine whether there is a significant difference between the means of two or more sets of data. Then proceed with the post hoc test with the Games-Howell test if the data is not homogeneous, test it aims to find out which groups are different significant.

RESULTS

After being incubated for two consecutive 24-hour periods at 37°C, *Aggregatibacter actinomycetemcomitans* bacteria underwent a colony count test on MHA media. The results revealed that there were changes in the number of colonies that grew. Table 1 shows that the number of *Aggregatibacter actinomycetemcomitans* colonies decreases with increasing CPC concentration. *Aggregatibacter actinomycetemcomitans* bacteria did not grow in MHA media with CPC

concentrations of 0.06% to 0.1%. The following graph illustrates in Figure 1 the percentage of inhibition on *Aggregatibacter actinomycetemcomitans* bacteria growth.

The results of the inhibition test data were reviewed to see if the data were normally distributed using the Shapiro-Wilk test. If the study's data are normally distributed (p>0.05), the statistical test is conducted using the one-way Anova parametric procedure. The SPSS 27 program and statistical tests were used to examine the study's findings. The following table displays the results of the Shapiro-Wilk normality test.

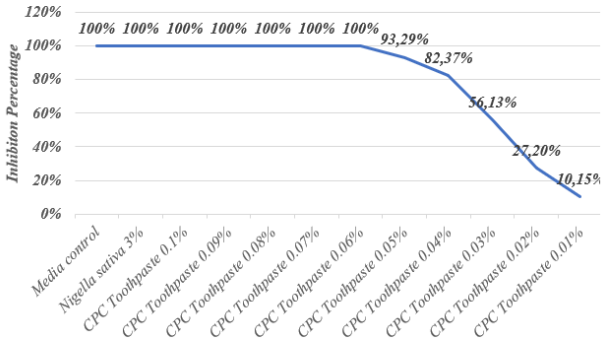

Figure 1. Graph of Percentage of Bacterial Growth Inhibition *Aggregatibacter actinomycetemcomitans*

Table 1. Shapiro-Wilk Normality Test

Sample Group	*Sig. (p)*	*Description*
Media Control	0	Non-normal Distribution
Nigella sativa toothpaste *3%*	0	Non-normal Distribution
CPC toothpaste 0.1%	0	Non-normal Distribution
CPC toothpaste 0.09%	0	Non-normal Distribution
CPC toothpaste 0.08%	0	Non-normal Distribution
CPC toothpaste 0.07%	0	Non-normal Distribution
CPC toothpaste 0.06%	0	Non-normal Distribution
CPC toothpaste 0.05%	0.637	Normal Distribution
CPC toothpaste 0.04%	0.298	Normal Distribution
CPC toothpaste 0.03%	0.688	Normal Distribution
CPC toothpaste 0.02%	0.380	Normal Distribution
CPC toothpaste 0.01%	0.567	Normal Distribution

Note: t>0,05 (Normal Distribution)

The normality test results in Table 1 reveal that the data are normally distributed, and the significance level is more than 0.05 (t>0.05) in several groups, especially in the CPC toothpaste group whose values are 0.05%, 0.04%, 0.03%, 0.02%, and 0.01% respectively. In contrast, the data was not normally distributed and there was no significant change in the 0.06% to 0.1% group because there was no bacterial colony formation. Levene's test is then used to do a homogeneity test on the normally distributed data.

Table 2. Levene's Test Homogeneity Test Results

Levene's Test				
Variable	Levene Statistic	df1	df2	Sig.
Number of Colonies	8.522	12	29	0.000

In Table 2, the data on the total number of colonies in the sample group was not homogenous, according to the results of the Levene homogeneity test, because the p-value was less than 0.05 (p = 0.000). If the data is normally distributed but not homogeneous, data analysis is done using a parametric test technique using the One-Way Anova test with a follow-up test using Games Howell.

Table 3. One-Way ANOVA Test Results

ANOVA			
Variable	df	F	Sig
Number of Colonies	12	654.973	0.000

Note: p < 0.05 (Significantly Different)

Based on the ANOVA test on Table 3 findings in Table 3, a significance value (p) of 0.000 (p>0.05) was found, indicating a significant difference in the growth of the

bacterial colonies of *Aggregatibacter actinomycetemcomitans* in each sample group.

DISCUSSION

Cetylpyridinium chloride (CPC) and *Nigella sativa* toothpaste at concentrations of 0.01%, 0.02%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, and 0.1% were the subjects of this laboratory experiment to test their ability to inhibit the growth of *Aggregatibacter actinomycetemcomitans* bacteria. The colony counting technique counts the number of colonies that have developed on MHA media in order to show the antibacterial activity of CPC and *Nigella sativa*. Based on the percentage of CPC inhibition at each concentration, the Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of *Aggregatibacter actinomycetemcomitans* were determined.

A genuine infection can be contracted via exposure to the exogenous bacterium *Aggregatibacter actinomycetemcomitans.* It is capable of generating virulence factors.[24] *Aggregatibacter actinomycetemcomitans* tissue-destructive virulence factors include released proteins such as cell stress protein and lipopolysaccharide (LPS), which is found on the bacterial cell wall. In vitro and in vivo studies have shown that LPS increases bone resorption.[25] However, it is thought to be a less potent cytokine inducer than the released protein. Because it acts as an osteoclast "growth factor" and encourages bone resorption, the cell stress protein chaperonin 60 is thought to be a powerful bone-degrading agent.[26]

Aggregatibacter actinomycetemcomitans bacterial colonies are growing in CPC at concentrations of 0.01%, 0.02%, 0.03%, 0.04%, and 0.05%, according to the results of the colony count. When compared to the CPC concentration, which is 0.06%, 0.07%, 0.08%, 0.09%, and 0.1%, no colony growth was observed. This demonstrates that the number of *Aggregatibacter actinomycetemcomitans* bacterial colonies grown on MHA media decreased with increasing CPC content. In order to make it simple to determine the values of MIC and MBC, this study also determined the percentage of CPC inhibition. The Minimal Inhibitory Concentration (MIC) for CPC is in the 0.05% concentration range. CPC, a quaternary ammonium molecule has a broad-spectrum antibacterial impact on both Gram-positive and Gram-negative bacteria. Because CPC has a cationic group, it is simpler to bind to the bacterial cell membrane's surface negative charge, which will alter permeability and damage the cell membrane, leading to the leaking of cell components and cell death.[19]

Nigella sativa 3% did not exhibit colony growth *Aggregatibacter actinomycetemcomitans* bacteria, according to the results of the colony. Setiawatie et al. research on which showed that *Prevotella intermedia* and *Porphyromonas gingivalis*, the two bacteria that cause periodontitis, were vulnerable to *Nigella sativa*'s antibacterial effect, confirmed this result.[7] Recently, it was discovered that toothpaste containing *Nigella sativa*, 2% Sodium lauryl sulfate, and non-Sodium lauryl sulfate had no effect on fibroblasts. According to these findings, *Nigella sativa* may be the main component of dental paste used as an adjuvant therapy for periodontitis.[7]

In a study conducted by Kapil et al., the antibacterial activity of 0.2% thymoquinone gel was evaluated *in vitro*.[27] The results showed that *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans*, and *Prevotella intermedia* were all very sensitive to 0.2% thymoquinone gel up to dilution levels of 10-9 and 10-8, respectively. This demonstrated that each of the three biological strangles was advised that the drug's effectiveness be assessed against more periodontal infections due to the intricacy of periodontal diseases in this area.[1,27] Thymoquinone was proposed as

a potential natural product source by Kouidhi et al. to its activity in altering resistance and its selective antibacterial efficacy against oral bacteria.[1]

Thymoquinone and *Nigella sativa* have been addressed as anti-inflammatory and antioxidant mediators with therapeutic benefits in a number of studies.[13,28,29] Thymoquinone causes an antioxidant effect by scavenging a variety of free radicals, and it is as effective at scavenging superoxide anions as superoxide dismutase is at doing so.[30] Thymoquinone has been shown to have substantial anti-inflammatory benefits in clinical studies as well. Pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and *tumor necrosis factor*-α (TNF-α), and prostaglandin E2 (PGE2) are reduced whereas anti-inflammatory cytokine IL-10 is elevated as a result of decreased macrophage production and lower Nitric oxide (NO) levels.[13,29–31] Thymoquinone's potential method of action for its anti-inflammatory and anti-oxidant activity may be related to its ability to suppress eicosanoid synthesis. Through the inhibition of COX and LOX molecular pathways, thymoquinone and *Nigella sativa* extracts have been shown in experiments to significantly limit lipid peroxidation and the production of *eicosanoids,* particularly thromboxane B and leukotrienes B4.[15,29,32] According to the current review, thymoquinone may be crucial in avoiding the beginning and progression of periodontal disease due to its potential anti-inflammatory and anti-oxidant properties.[13,14]

The active component, thymoquinone, accounts for 30% to 48% of the seeds of *Nigella sativa*.[3] *Nigella sativa* may be utilized as a treatment or adjuvant in bacterial illnesses because of its strong anti-bacterial action.[3,12,14] The substances thymoquinone, thymol, and tannins found in black cumin have been shown to be helpful in preventing the growth of the subgingival and supragingival plaque bacteria that are the main cause of periodontitis. Both Gram-positive and Gram-negative bacteria are known to be resistant to the growth of these substances.[33,34] The quinon derivative thymoquinone contains the element thymol. Quinon has strong antimicrobial qualities. The adhesin surface, polypeptide cell walls, and membrane-bound enzymes are among the targets in bacterial cells. Thymoquinone is known to combine with nucleophilic amino acids in proteins to generate irreversible compounds that can inactivate proteins and cause malfunctions. The periodontitis-causing supragingival and subgingival plaque bacteria are effectively stopped by the antibacterial THQ in black cumin extract.[33,35]

Nigella sativa, a traditional treatment, is frequently employed to treat a range of diseases. The *Nigella sativa* extract showed significant antibacterial effectiveness against the germs that cause pulpitis and periodontitis.[4,10,36] According to Setiawatie et al., *black cumin* extract demonstrates free radical scavenging activity at a concentration of 3%.[3] According to the results of the cytotoxic test performed on cell cultures of fibroblasts and osteoblasts, 3% of the *Nigella sativa* extract had viability levels above 90%.[7,16] The active ingredients in *Nigella sativa* have been connected to the plant's positive effects on health. Alkaloids, saponins, and 28–36% protein, as well as 0.4–2.5% essential oil, are all present in the seeds. Although *Nigella sativa* has many pharmacologically active chemicals, thymoquinone, dithymoquinone, thymol, and thymohydroquinone are the most commonly reported active components.[11,34]

The active component thymoquinone may be responsible for *Nigella sativa*'s anti-inflammatory and anti-destructive properties. Thymoquinone has been utilized to illustrate the primary pharmacological characteristics of *Nigella sativa*, such as its anti-inflammatory, antioxidative, anti-bacteriostatic, analgesic, hypoglycemic, and anti-carcinogenic properties.[3,13,34] It has also been demonstrated that thymoquinone inhibits pro-inflammatory cytokines such as

ILs, TNF-α, and MMP8.[1,3,14,29] By stopping the development of biofilms in *Porphyromonas gingivalis* and *Prevotella intermedia*, thymoquinone additionally demonstrated its antibacterial activity.[7,36] These results suggest that *Nigella sativa* has antibacterial, anti-inflammatory, and anti-destructive characteristics, especially when applied to tissues with periodontitis.[3,7,36] The findings of this study can be used to develop an alternative antibacterial agent in the form of toothpaste since natural toothpaste has less negative effects on tooth and mucosal discoloration and because *Nigella sativa* toothpaste can be created in alcohol-free preparations.[3,8,33,37] In the area of periodontal disease, toothpaste containing *Nigella sativa* can stop supragingival plaque bacteria from growing. A reduced periodontal index and a significantly lower quantity of sub-gingival bacteria were observed in Wistar rats administered with *Nigella sativa* extract in drinking water in animal tests as compared to the control group. Other studies looked into the *Nigella sativa* extract's potential to stop periodontal inflammation in its tracks. Because it slows down alveolar bone resorption, *Nigella sativa* taken orally aids in the prevention of periodontal disease. In comparison to the chitosan group, the administration of periodontal chips containing *Nigella sativa* significantly improved the clinical condition of patients with chronic periodontitis.

Nigella sativa extract is ideal for application in herbal medicine in the field of dentistry as toothpaste, mouthwash, root canal irrigation material, pulp capping material, and dental implant coating due to its considerable antioxidant, antibacterial, anti-inflammatory, and cytoprotective qualities. By researching the development of *Nigella sativa* formulations in nanobiotechnology, future dental materials can be created.

STRENGTH AND LIMITATION

Only a few have researched the inhibition of *Aggregatibacter actinomycetemcomitans* bacteria. So, in this study, the researchers wanted to find out more about the inhibition ability of toothpaste containing *Nigella sativa* 3% and CPC 0.01-0.1% against *Aggregatibacter actinomycetemcomitans* bacteria. In addition, future studies are needed to determine the effectiveness of toothpaste containing *Nigella sativa* 3% and *CPC* 0.01-0.1% in inhibiting other bacteria.

CONCLUSIONS

According to the study's findings and analysis, *Aggregatibacter actinomycetemcomitans* bacteria can be inhibited from growing when natural toothpaste containing *Nigella sativa* 3%, which is comparable to CPC 0.06%–0.1%, is used.

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ETHICAL CLEARANCE

The research protocol was approved by Faculty of Medicine Research Ethics Commission Udayana University, ETHICAL CLEARANCE No: 1078/UN 14.2.2.Vll.14/LT/2022.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this research.

AUTHOR CONTRIBUTION

EMS : Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Writing – review & editing. PW: Conceptualization, Formal analysis, Software, Validation, Visualization, Roles/Writing – original draft. RPR : Conceptualization, Formal analysis, Investigation, Methodology. AE: Conceptualization, Formal analysis, Software, Validation, Visualization, Writing. DS : Resources, Supervision, Validation. IJS : Investigation, Project administration, Validation, Writing. LB : Investigation, Project administration, Validation, Writing. RSM : Investigation, Project administration, Validation, Writing.

REFERENCES

1. Kouidhi B, Zmantar T, Jrah H, Souiden Y, Chaieb K, Mahdouani K, et al. Antibacterial and resistance-modifying activities of thymoquinone against oral pathogens. Ann Clin Microbiol Antimicrob. 2011;10(1):29.
2. Gaybullaev E. Clinical and Biochemical Features of the Use of Black Cumin Oil in the Treatment of Chronic Generalized Periodontitis of Moderate Severity. 2020;2.
3. Setiawatie EM, Gani MA, Rahayu RP, Ulfah N, Kurnia S, Augustina EF, et al. Nigella sativa toothpaste promotes anti-inflammatory and anti-destructive effects in a rat model of periodontitis. Archives of Oral Biology. 2022 May;137:105396.
4. Al-Attass SA, Zahran FM, Turkistany SA. Nigella sativa and its active constituent thymoquinone in oral health. Saudi Med J. 2016 Mar;37(3):235–44.
5. Maria Martina N, Regina T, Raiyanti IGA. The Effectiveness of Tooth Brushing and Gargling Using Toothpaste and Mouthwash of Beluntas Leaf Ethanol Extract in Reducing Streptococcus mutansBacteria Number in Tooth Plaque [Internet]. 2008 [cited 2023 Dec 1]. Available from: http://repository.poltekkes-denpasar.ac.id/3805/
6. Sakr O. Evaluation of Simulated Toothbrushing with different Dentifrices on Enamel Resin Infiltrated Teeth Surface Roughness and Gloss. Journal of International Dental and Medical Research. 2020;13(4):1416–21.
7. Ernie Maduratna Setiawatie -, Desi Sandra Sari -, Badai Sapta Wahyudadi -, Eka Fitria -, Shafira Kurnia Supandi -, Lambang Bargowo -, et al. Viability of Nigella sativa Toothpaste with SLS Compared Non-SLS on Fibroblast Cell Culture. Journal of International Dental and Medical Research. 2021;14(2):525–8.
8. Singh K, Singh P, Oberoi G. Comparative studies between herbal toothpaste (dantkanti) and non-herbal tooth paste. IJDR. 2016 Sep 30;4(2):53.
9. Ismail NFA, Rostam MA, Jais MFM, Shafri MAM, Ismail AF, Arzmi MH. Review: exploring the potential of Nigella sativa for tooth mineralization and periodontitis treatment and its additive effect with doxycycline. IIUM Journal of Orofacial and Health Sciences. 2022 Mar 4;3(1):136–46.
10. Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK, Siddique NA, et al. A review on therapeutic potential of Nigella sativa: A miracle herb. Asian Pac J Trop Biomed. 2013 May;3(5):337–52.
11. Forouzanfar F, Bazzaz BSF, Hosseinzadeh H. Black cumin (Nigella sativa) and its constituent (thymoquinone): a review on antimicrobial effects. Iran J Basic Med Sci. 2014 Dec;17(12):929–38.

12. Yimer EM, Tuem KB, Karim A, Ur-Rehman N, Anwar F. Nigella sativa L. (Black Cumin): A Promising Natural Remedy for Wide Range of Illnesses. Evid Based Complement Alternat Med. 2019 May 12;2019:1528635.
13. Mekhemar M, Hassan Y, Dörfer C. Nigella sativa and Thymoquinone: A Natural Blessing for Periodontal Therapy. Antioxidants. 2020 Dec;9(12):1260.
14. Kulyar MF e A, Li R, Mehmood K, Waqas M, Li K, Li J. Potential influence of Nagella sativa (Black cumin) in reinforcing immune system: A hope to decelerate the COVID-19 pandemic. Phytomedicine. 2021 May;85:153277.
15. Farkhondeh T, Samarghandian S, Shahri AMP, Samini F. The Neuroprotective Effects of Thymoquinone: A Review. Dose Response. 2018 Apr 11;16(2):1559325818761455.
16. Mohammed SJ, Amin HHH, Aziz SB, Sha AM, Hassan S, Abdul Aziz JM, et al. Structural Characterization, Antimicrobial Activity, and *In Vitro* Cytotoxicity Effect of Black Seed Oil. Evidence-Based Complementary and Alternative Medicine. 2019 Aug 18;2019:e6515671.
17. Radzki D, Wilhelm-Węglarz M, Pruska K, Kusiak A, Ordyniec-Kwaśnica I. A Fresh Look at Mouthwashes—What Is Inside and What Is It For? Int J Environ Res Public Health. 2022 Mar 25;19(7):3926.
18. K N, V SK, K MSK. A Review on Cetylpyridinium Chloride. International Journal of Research and Review. 2021;8(4):439–45.
19. LeBel G, Vaillancourt K, Morin MP, Grenier D. Antimicrobial Activity, Biocompatibility and Anti-inflammatory Properties of Cetylpyridinium Chloride-based Mouthwash Containing Sodium Fluoride and Xylitol: An In Vitro Study. Oral Health Prev Dent. 2020;18(1):1069–76.
20. Rawlinson A, Pollington S, Walsh TF, Lamb DJ, Marlow I, Haywood J, et al. Efficacy of two alcohol-free cetylpyridinium chloride mouthwashes – a randomized double-blind crossover study. Journal of Clinical Periodontology. 2008;35(3):230–5.
21. Sreenivasan PK, Haraszthy VI, Zambon JJ. Antimicrobial efficacy of 0·05% cetylpyridinium chloride mouthrinses. Letters in Applied Microbiology. 2013 Jan 1;56(1):14–20.
22. Toar AI, Posangi J, Wowor V. Daya Hambat Obat Kumur Cetylpyridinium Chloride Dan Obat Kumur Daun Sirih Terhadap Pertumbuhan Streptococcus Mutans. Jurnal Biomedik:JBM. 2013 [cited 2023 Dec 1];5(1). Available from: https://ejournal.unsrat.ac.id/v3/index.php/biomedik/article/view/2639
23. Setiawatie EM, Valentina R, Meiliana RS. Effectiveness of Cetylpyridinium Chloride in Reducing the Growth of Bacteria that Cause Periodontal Disease. e-GiGi. 2023 Feb 7;11(2):115–20.
24. Raja M, Ummer F, Dhivakar CP. Aggregatibacter Actinomycetemcomitans – A Tooth Killer? J Clin Diagn Res. 2014 Aug;8(8):ZE13–6.
25. Syahiran S, Wan Taib WR, Jaffar N. Aggregatibacter actinomycetemcomitans: The virulence factors and relation to persistence biofilm formation. Biomedicine. 2021 Jan 1;40(4):429–35.
26. Herbert BA, Novince CM, Kirkwood KL. Aggregatibacter actinomycetemcomitans, a potent immunoregulator of the periodontal host defense system and alveolar bone homeostasis. Mol Oral Microbiol. 2016 Jun;31(3):207–27.
27. Kapil H, Suresh DK, Bathla SC, Arora KS. Assessment of clinical efficacy of locally delivered 0.2% Thymoquinone gel in the treatment of periodontitis. The Saudi Dental Journal. 2018 Oct 1;30(4):348–54.
28. Varela-López A, Bullón P, Giampieri F, Quiles JL. Non-Nutrient, Naturally Occurring Phenolic Compounds with Antioxidant Activity for the Prevention and Treatment of Periodontal Diseases. Antioxidants (Basel). 2015 Jun 24;4(3):447–81.

29. Shaterzadeh-Yazdi H, Noorbakhsh MF, Hayati F, Samarghandian S, Farkhondeh T. Immunomodulatory and Anti-inflammatory Effects of Thymoquinone. Cardiovasc Hematol Disord Drug Targets. 2018;18(1):52–60.
30. Kassab R, El-Hennamy R. The role of thymoquinone as a potent antioxidant in ameliorating the neurotoxic effect of sodium arsenate in female rat. Egyptian Journal of Basic and Applied Sciences. 2017 Jul 1;4.
31. Bargi R, Asgharzadeh F, Beheshti F, Hosseini M, Sadeghnia HR, Khazaei M. The effects of thymoquinone on hippocampal cytokine level, brain oxidative stress status and memory deficits induced by lipopolysaccharide in rats. Cytokine. 2017 Aug;96:173–84.
32. Mostofa AGM, Hossain MK, Basak D, Bin Sayeed MS. Thymoquinone as a Potential Adjuvant Therapy for Cancer Treatment: Evidence from Preclinical Studies. Front Pharmacol. 2017 Jun 12;8:295.
33. Masya RN, Ulfah N, Hadinoto MEK, Sugito BH, Septa B, Setiawatie EM. Inhibition Activity of Black Cumin Toothpaste Contain With Detergent Compared with Black Cumin Extract Non Detergent to the Growth of Supragingiva Plaque Bacteria. 2(2).
34. Tavakkoli A, Mahdian V, Razavi BM, Hosseinzadeh H. Review on Clinical Trials of Black Seed (Nigella sativa) and Its Active Constituent, Thymoquinone. J Pharmacopuncture. 2017 Sep;20(3):179–93.
35. Forouzanfar F, Bazzaz BSF, Hosseinzadeh H. Black cumin (Nigella sativa) and its constituent (thymoquinone): a review on antimicrobial effects. Iran J Basic Med Sci. 2014 Dec;17(12):929–38.
36. Tantivitayakul P, Kaypetch R, Muadchiengka T. Thymoquinone inhibits biofilm formation and virulence properties of periodontal bacteria. Archives of Oral Biology. 2020 Jul 1;115:104744.
37. Cvikl B, Lussi A, Gruber R. The in vitro impact of toothpaste extracts on cell viability. Eur J Oral Sci. 2015 Jun;123(3):179–85.

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4. Vidyadaran S, Ramasamy R, Seow HF. Stem cells and cancer stem cells: Therapeutic Applications in Disease and Injury. In: Hayat MA, editor. New York: Springer; 2012.

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5. Canadian Dental Hygienists Association. Dental hygiene: definition and scope. Ottawa: Canadian Dental Hygienists Association; 1995.

E-book

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Journal article with more than 6 authors

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Journal article in press

3. Clancy JL, Patel HR, Hussein SM, Tonge PD, Cloonan N, Corso AJ, et al. Small RNA changes enroute to distinct cellular states of induced pluripotency. Nature communications.2014; 5:5522. Epub 2014/12/11.

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