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Original Article

Portable and Battery-Operated Isothermal Amplification Device Validation for Onsite Analysis of *M. tuberculosis* "DNA Hunter"

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ABSTRACT

Point-of-care (POC) devices play an important role in the protection of public health by providing rapid diagnosis of infectious diseases, patient management, and effective treatment. Fast, easy-to-interpret, environmentally resistant, and cost-effective POC tests that can be used practically in the field are gaining more and more importance every day. There is a need for portable devices that will enable rapid diagnosis kits to be used in the field for early diagnosis and treatment. The aim of this study is to evaluate the DNA hunter device that was developed in terms of providing the required temperature for *M. tuberculosis* (MTB) diagnosis of the loop-mediated isothermal amplification (LAMP) assay and visually evaluating the analysis results. The device in this study; handheld (total weight 430 g, outer dimensions 70 x 175 x 80 mm), the average operating time can reach a maximum temperature of 110 degrees in 2 minutes with a fully charged battery, and the processing time is about 90 minutes without being connected to electricity. It can display the pre-evaluation result on the screen with the full digital color sensor. The device can be adjusted to the desired reaction temperature and time. It also has software where sample registration numbers can be entered. DNA Hunter can be used for all analyses performed by the LAMP method and the results can be evaluated colorimetrically, thus it is well suited for POC testing.

Keywords: handheld device; loop-mediated isothermal amplification (LAMP); *M. tuberculosis* (MTB); Point of care (POC)

Highlights: A portable device has been developed that allows an important public health pathogen such as tuberculosis infection to be screened with the LAMP method without the need for complex laboratory infrastructure. The most important aspect of this device is that it is small enough to fit in the palm and can work independently of electricity for a certain period of time.

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INTRODUCTION

Analyses that can be performed quickly outside of the laboratory are known as Point-of-Care (POC) tests.[1] POC tests are required for disease screening in the diagnosis of infectious diseases, particularly in countries with limited laboratory facilities, a high disease burden, and a low income. The need for POC tests for the rapid screening of infectious diseases is rapidly expanding.[2] The main advantages of POC tests are to reduce procedures, and costs associated with hospitalization and prevent the risk of hospital-acquired infections by determining the infection factor detected during hospitalization, rapid diagnosis in epidemics and pandemics, and the ability to work with fewer samples compared to traditional methods.[3]

POC tests, as well as antimicrobial use control, rapid treatment initiation, and outbreak monitoring and control, all contribute to the investigation of unknown pathogens.[4] With a compound annual growth rate of 11.4%, the global POC market was valued at USD 29.5 billion in 2020 and is expected to reach USD 50.6 billion in 2025. The production of devices for POC analysis has economic and commercial importance, as shown by these statistics.[5] Tuberculosis is one of the oldest known diseases, is an infectious disease caused by the *Mycobacterium tuberculosis* complex (*M. tuberculosis, M. bovis, M. africanum, M. microti*). This disease is characterized by the presence of granulomas in infected tissues involving the respiratory tract or other organs. Although tuberculosis has fluctuated in its incidence over thousands of years of human history, it has remained a permanent threat to public health.[6] Tuberculosis disease treatment takes a long time, it can be transmitted from patients with positive sputum smears via respiration to healthy people and can cause mortality. The fight against tuberculosis requires a continuous and disciplined public health practice. Because of droplet infection, each patient should be diagnosed early and treated effectively to protect public health.[7,8] Nucleic acid amplification methods are widely used to identify *M. tuberculosis* (MTB), which is difficult to see by microscopy and takes a long time to produce in culture.[9,10] In parallel with the developments in molecular techniques such as Polymerase chain reaction (PCR), real-time PCR, and transcription-based amplification (TMA) have been developed for the diagnosis of tuberculosis.[11;13] However, the most important disadvantage of molecular methods is that they mostly require a laboratory environment and cannot be applied as the POC tests.[14;16]

Nucleic acid amplification tests (PCR, Real-Time PCR) can detect trace amounts of genetic material (DNA or RNA) of various pathogens in the early stage of the disease. However, the thermal cycling condition adds complexity to the way PCR devices operate.[15,17] Recently, various isothermal amplification methods have been developed, such as rolling circle amplification (RCA), recombinase polymerase amplification (RPA), and loop-mediated isothermal amplification (LAMP). Among these methods, LAMP is the most popular isothermal nucleic acid test for detecting viruses, bacteria, fungi, and parasites due to its low cost and operation at a single temperature.[16];[19]

Loop-mediated isothermal amplification (LAMP) reaction is performed with four or six primer sets for DNA/RNA amplification.[18] The most important advantage of the LAMP method is; It provides the opportunity to reproduce target nucleic acid sequences under isothermal conditions (60-65°C) in a miniaturized environment with low energy consumption.[20] Proliferation; turbidity can be monitored with dyes that show the amount of fluorescence or free magnesium bound to nucleic acids. Therefore, it does not require any expensive device, allowing the results to be evaluated with a simple optical system or with the naked eye. The LAMP method gives more sensitive results than other amplification

methods because it provides sequence-specific visual detection of the 4/6 region on the target gene.[21] However, LAMP assay requires a heating block system in order to be operated as in other diagnostic techniques. Therefore, new generation smart devices are needed to perform analysis in places without laboratory infrastructure. The portability of these devices, low cost, robustness, ease of use, less need for trained personnel, easy-to- interpret results, and ability to produce accurate and reliable results quickly are important.[22]

The aim of this study was to develop a hand-held portable device (DNA Hunter) for rapid and accurate diagnosis of MTB and enabling analysis without the expert in the field as point-of- care testing. DNA Hunter (total weight 430 g, outer dimensions 70 x 175 x 80 mm) can be adjusted to the desired reaction temperature and time also has software where sample numbers can be entered. The device has 6 aluminum chambers and the average operating time can be realized around 90 minutes with a fully charged battery, without being connected to electricity. Reservoir and the cover section have a heating function reaching a maximum temperature of 110°C in 2 minutes. The color change is measured and evaluated positively and negatively by a full digital color sensor and the result can be displayed and compared with the reference values on the screen.

MATERIALS AND METHODS

Materials

The 93 sputum samples (68-culture positive, 25-culture negative) were used in this study, and the *M. tuberculosis* H37Rv Pasteur Institute standard strain was provided by Atatürk Chest Diseases Hospital within the scope of the TUBITAK project (115R002). ARB staining, Löwenstein-Jensen culture (BD, New Jersey, USA), and Geneexpert (Cepheid, California, USA) analyses were routinely performed by the institutional laboratory where sputum samples were obtained.

The QIAamp DNA mini kit was purchased from (QIAGEN, Hilden, Germany). LAMP amplification reagents (WarmStart Colorimetric LAMP 2X Master Mix) were purchased from New England Biolabs (Massachusetts, USA) and Loopamp MTBC Detection Kit was purchased from Eiken Chemical Co., Ltd. (Tokyo, Japan). LAMP primers used in this study were synthesized Microsynth AG (Balgach, Switzerland) as HPLC grade.

Methods

LAMP primers targeted specifically for the MTB IS6110 gene (GenBank accession number: X17348) were designed using the PrimerExplorer V5 program. The primers were optimized according to the protocol described in the previous study.[23] The LAMP primers consisted of two outer primers (F3 and B3) and two inner primers [FIP (F1c + F2) and BIP (B1c + B2)], and two loop primers [FLP: (forward loop primer) and BLP (backward loop primer)].[21]

For the preparation of samples, the standard strain of *M. tuberculosis* H37Rv was used. H37Rv pure culture produced in Lowenstein Jensen (LJ) broth was homogenized with PBS (pH 6.8) in a sterile glass beaded tube, its density was adjusted according to McFarland 1 and accepted as the main dilution. It was then diluted up to 10^1 in 10-fold serial dilutions starting with the main dilution. ARB negative sputum samples were spiked with main stock and its serial dilution from 1/10 to 1/100000. A nonspiked sputum sample was used for negative control. DNA of all sputum samples was extracted using QIAamp DNA mini kit, according to the manufacturer's instructions. The purity and quality of DNA were controlled using the Implen NanoPhotometer (Implen GmbH, Germany). Then extracted DNA was stored at –20°C until used. For the colorimetric assay, NEB Warmstart colorimetric LAMP 2X master mix kit (Table 1) was used and the process was performed according to the manufacturer's instructions.

Table 1. Colorimetric LAMP Reaction Mixture

Content	Stock Const.	Final Const.	1X (µl)
2x Master mix	2 µL	1	12,5
10x Primer Mix	10 µL	1	2,5
DNA	40 ng/µL		1
Ultrapure water			9
Total		25	

The limit of detection (LOD) of the *M. tuberculosis* LAMP test was determined as 10^2 CFU/mL in the previous study.[23] In this study to test whether the DNA hunter device performed the LAMP reaction correctly, five different concentrations (10^1-10^5 CFU/mL) were studied above and below the LOD limit. A bacteria-free sputum sample was used for negative control. The colorimetric LAMP method works on the principle of changing the color of the pH indicator dye added to the reaction with the hydrogen ions formed during the reaction changing the pH of the environment.[24] LAMP results were evaluated based on color change, with yellow color indicating positive and pink color examined as negative in visual evaluation. However, the pH-sensitive dye in the colorimetric LAMP reaction mix may not be seen as clear yellow or pink due to the amount of nucleic acid in the sample or some substances that come with the sample. The especially orange color formation can cause problems in evaluating the test. After LAMP reaction orange color is formed, which is considered an intermediate result in some studies even though the reaction color change from pink to yellow was not clear. In order to interpret the unclear results, a color scale was created and shown in Figure 1.[25,26]

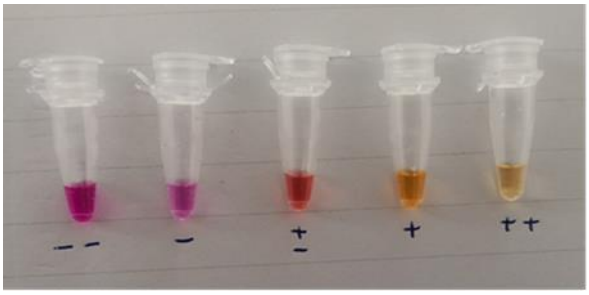

Figure 1. LAMP Reaction Results; Positive: Yellow, Negative: Pink
(--) Strongly Negative, (-) Negative, (±) Positive/ Negative (Unclear), (+) Positive, (++) Strongly Positive.[25,26]

Fabrication of DNA Hunter Device

The LAMP method requires a constant temperature (approximately 65 degrees) during the analysis. In the first design of the device, Peltier was used as the heater. However, the Peltier tends to generate an excessive amount of heat, so instead of using the Peltier as a heater, it was decided to use a resistor. The heater is designed to reach a maximum temperature of 110°C in 2 minutes at room temperature. The mechanical and electronic materials used were chosen to withstand temperatures of 150°C for a short period of time (maximum 2 minutes) and 120°C for an indefinite time. The temperature resistance between the top of the heater and the cover is very high. In order to keep the top cover temperature low a Teflon plate with a thickness of 4 mm was used. It is one of the materials with a very low thermal conductivity coefficient (5.10^{-4} Cal/c m. s. degree).

To prevent the heat from affecting the motherboard and graphics cards, the internal heat dissipation should be designed well. For this reason, the device's internal structure was changed several times and the air outlet was adjusted with the appropriate fan placement. Active cooling time was measured as 30 seconds while the fans were running, and the cooling time on its own (passive) was more than 10 minutes due to thermal insulation. It was observed that the warm-up cooling time was less than 2 minutes between 20 degrees and 60 degrees. DNA Hunter device was designed with 6 wells using Solidworks® software (DSS Solidworks 2016) and shown in Figure 2.

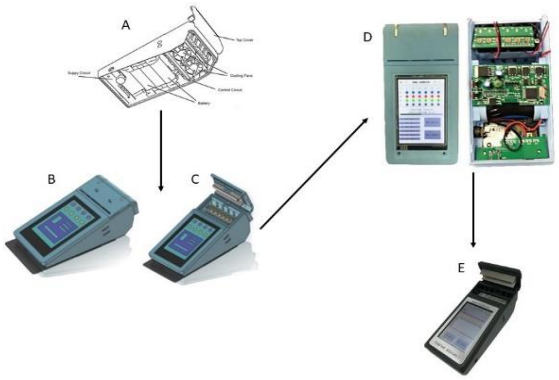

Figure 2. Design of the device (A), off mode (B), and open mode (C), processor card of the device system (D), and the completed device (E)

RESULTS AND DISCUSSION

Battery and Display Features

Since DNA Hunter was designed to be handheld, it was equipped with a Li-Ion battery with a low power consumption feature. With a fully charged battery, the total power drawn was about 10 W on average, and the running time was about 90 minutes. Every measuring cell was equipped with six high-precision full-color digital sensors. As a result of the system's evaluation, the color change was measured during the test and compared to the reference values that were displayed on the screen.

The total weight of the unit is 430 g, and the exterior dimensions are 70 x 175 x 80 mm. The device does not require expertise to use; test protocols can be accessed by simply entering the transaction code, thanks to the 15 different protocol storage processes. It assures also three different temperature set values and warm-up times for each test process. The user is also provided with temporary or permanent correction options.

Test Program Features

Protocols can be quickly accessed by entering transaction codes, owing to the program's memory capacity for 15 different operations. The protocols determine three separate temperature set values and warm-up times for each test process (Figure 3).

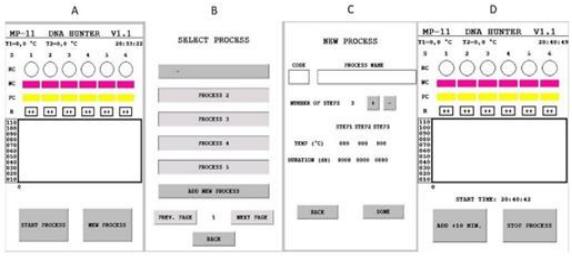

Figure 3. Program features (A) and (B), process selection page (C), and adding new process (D)

The user is given the option of temporary or permanent correction if appropriate. The results of the tests can be transferred to a microSD card and then to the host machine. In order to enter sample information on the device, a touch screen suitable for alphanumeric information entry and appropriate software was arranged. Test recipes and procedures can be entered on the device with the help of the same touch screen. In order to record the date and time of the tests, a battery-protected real-time clock was used.

Monitoring Temperature Changes of DNA Hunter

MTB-LAMP assay requires a constant temperature which is at 65°C for 30 minutes (Figure 4). To check the temperature stability of the device, every 6 wells were tested separately and also 10 repetitions were read for each well. Temperature adjustment is provided in the device with an accuracy of ± 0.1°C between 50-100°C.

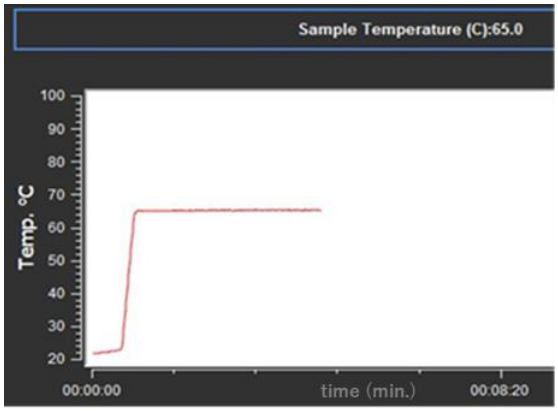

Figure 4. Temperature Curve of "DNA Hunter" Device

After 30 minutes, the device turned off the heating and indicated the end of the operation with an alarm sound before turning itself off at the end of the run.

Visualization of MTB

The colorimetric LAMP method works on the principle of changing the color of the pH indicator dye depending on the hydrogen ions formed during the reaction. In this study, a colorimetric LAMP master mix kit was used according to the optimized protocol.[23] LAMP assay was performed by a thermal cycler (Bio- Rad, Hamburg, Germany) at the same time as carrying out the DNA Hunter device. LAMP reaction operated under the same conditions (65°C for 30 minutes). As shown in Figure 1 the color change from pink to yellow in the tubes was considered positive, and the absence of color change (pink) was considered negative at the end of the result.[24] In our study, (orange), that is, unclear color formation, which creates problems in the evaluation of the results colorimetrically, was not observed.

LOD values on both devices were remarkably similar, which was 10^2 CFU/ml to monitor the performance stability of DNA Hunter 6 PCR wells in the device, were studied with samples contaminated with 5 different concentrations of bacteria, to evaluate whether there was a performance difference between the wells.[23] For this study, 10 readings were repeated for each well. The performance measurement chart is shown in Table 2.

Table 2. Performance Evaluation Results of the Device Wells

	1st Well					2nd Well					3rd Well					4th Well					5th Well					6th Well				
★	▼	■	●	□	△	▼	■	●	□	△	▼	■	●	□	△	▼	■	●	□	△	▼	■	●	□	△	▼	■	●	□	△
1	-	-	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
2	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
3	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	+	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
4	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	+	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
5	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
6	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
7	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
8	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
9	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	-				+	-				+	-				+	-				+	-				+	-				+
10	-	-	-	+	+	-	-	+	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+	-	-	-	+	+
	-				+	-				+	-				+	-				+	-				+	-				+

★ Number of readings, ▼(- -) Strongly Negative,
■(-) Negative, ●(±) Unclear, □ (+) Positive,
△(++) Strongly Positive

Sample Analysis

ARB staining, Löwenstein-Jensen culture, and Genexpert analyses were performed by the institutional laboratory where sputum samples were obtained. These sputum samples were subjected to DNA extraction using the QIAamp DNA mini kit in our laboratory. All isolates were analyzed in parallel with the thermal cycler using the Eiken Loopamp kit and the DNA hunter using the in-house LAMP method.[23] The results were demonstrated in Table 3 and Table 4.

Table 3. Sputum Sample Results

Sample No	*ARB	**LJ Culture	Gene Expert	Eiken Loopamp/ Thermal cycler	Inhouse LAMP/ DNA Hunter
1	++	+	+	+	+
2	+	+	+	+	+
3	++	+	+	+	+
4	-	-	-	-	-
5	++	++	+	+	+
6	+	++	+	+	+
7	++	++	+	+	+
8	++	++	+	+	+
9	-	-	-	-	-
10	-	-	-	-	-
11	++	+	+	-	+
12	++	++	+	-	-
13	++	+	-	-	+
14	+	++	+	-	+
15	++	+	+	+	+
16	++	++	+	+	+
17	-	-	-	+	-
18	++	++	+	+	+
19	++	+	-	-	-
20	++	+	+	+	+
21	++	+	+	+	-
22	+	-	+	+	+
23	-	-	-	-	-
24	++	+	+	+	+
25	+	+	-	+	+
26	+	++	+	+	+
27	+	-	+	+	+
28	-	-	+	+	+
29	++	+	-	+	+
30	++	+	-	+	+
31	-	-	-	-	-
32	-	-	-	-	-
33	++	+	+	+	+
34	++	+	+	+	+
35	+	-	-	-	-
36	+	-	+	+	+
37	++	+	+	+	+
38	++	-	+	+	+
39	-	-	-	+	+
40	+	-	+	+	+
41	-	-	-	-	-
42	+	-	+	+	+
43	+	+	+	+	+
44	+	+	+	+	+
45	++	++	+	+	+
46	++	++	+	+	+
47	-	-	-	-	-
48	-	-	-	-	-
49	-	-	-	-	-
50	-	-	-	+	-
51	++	++	+	+	+
52	+	+	+	+	+
53	++	+	+	+	+
54	++	++	+	+	+
55	-	-	-	-	-
56	+	+	+	+	+
57	+	+	+	+	+
58	+	-	+	+	+
59	++	++	+	+	+
60	-	-	-	-	-
61	++	++	+	+	+
62	++	+	+	+	+
63	++	++	+	+	+
64	-	-	-	-	-
65	++	++	+	+	+
66	++	+	+	+	+
67	-	-	+	+	+
68	+	+	+	+	+
69	++	++	+	+	+
70	+	-	+	+	+
71	+	-	+	+	+
72	++	++	+	+	+
73	-	-	-	-	-
74	-	-	-	-	-
75	+	-	+	+	+
76	++	++	+	+	+
77	+	+	+	+	+
78	++	+	+	+	+
79	+	-	+	+	+
80	-	-	-	-	-
81	-	-	-	-	-
82	+++	+	+	+	+
83	++	++	+	+	+
84	+	++	+	+	+
85	++	+	+	+	+
86	-	-	-	-	-
87	++	++	+	+	+
88	++	+	+	+	+
89	-	-	-	-	-
90	++	+	+	+	+
91	-	-	-	+	-
92	++	++	+	+	+
93	+	+	+	+	+

(+) Positive, (++) Strongly Positive, (-) Negative
*Microscopy results;
100 microscope scanned area:
no ARB (-), 1-9 ARB (+),10-99 ARB (++).27
**Culture media (LJ) results;
50-100 CFU (1+), 100-200 CFU (2+).[28]

Table 4. Analysis Results of Sputum Samples in Different Techniques

Results	ARB Staining	LJ Culture	Gene Expert	Eiken Loopamp	DNA Hunter
Positive	68	56	63	62	64
Negative	25	25	23	19	22
False Positive	-	-	2	6	2
False Negative	-	12	5	6	5
Total	93	93	93	93	93

According to the ARB staining method of the sputum samples obtained, it was found 68/93 positive, and 25/93 negative. Compared with the ARB results; With the LJ culture, 12 samples were obtained as false negatives. The Geneexpert method detected 5 ARB positive samples as negative. The false negative detection rate of the DNA hunter device was the same as the Gene Expert method. Finally, 6 false positives and 6 false negative samples were detected by the Eiken Loopamp LAMP kit. the DNA hunter device with the in-house LAMP method was successful with only 2 false positives and 5 false negatives results.

Although various articles were published about the detection of pathogenic microorganisms by LAMP assay, the use of the LAMP method is not limited to pathogens, its application in the diagnosis of allergens, GMOs, and cancer was reported. The importance of the LAMP method increased especially during the Covid19 pandemic period. The popularity of LAMP depends on its ability to operate at a constant temperature. For this reason, simple heaters have been developed by researchers for usage in the field. Papadakis et al. developed a real-time quantitative colorimetric LAMP (qcLAMP) device. Their device is 3D-manufactured and operates via an in-house developed smartphone application. The size and weight of this device are (11×10×10 cm;370 g.) The device employed a mini digital camera for monitoring in real-time the transition during colorimetric LAMP amplification. The device's clinical evaluation is demonstrated in cancer mutations-analysis and COVID-19 testing.[29] Kaygusuz et al. also developed a device called DiamonD which is used for GMO detection. The device features are 108 g, 6 × 6 × 3 cm. The physical parts of the device were manufactured by using a 3D printer. In this device, Peltier is used as a heating element, different from our study. The LAMP reaction result was evaluated colorimetrically using HNB.[30]

Liang et al. developed a handheld, automatic, and detection system-free thermal digital microfluidic (DMF) device for LAMP assay. Droplet manipulation and real-time temperature control systems were integrated into a handheld device[31] called LampPort that performed detection of Trypanosoma brucei, a blood parasite (Table 5). In addition to that Hu et al., studied Salmonella contamination on eggs with the LAMP method by using Genie II (OptiGene, UK) instrument. This device is commercially available for LAMP analysis and products can be visualized under UV light.[32]

Table 5. Comparison of Isothermal Amplification Devices and LAMP Applications

LAMP applications	Devices	Portable	Monitoring of LAMP results
MTB in sputum	DNA Hunter	Yes	Colorimetric
Cancer mutations-analysis, COVID-19 testing (Papadakis et al., 2022)	qcLAMP	Yes	Colorimetric
GMO in soybean (Kaygusuz et al.,2019)	DaimonDNA	Yes	Colorimetric
Trypanasoma brucei in blood (Liang et.al.,2019)	LampPort	Yes	UV light
Salmonella ser. Enteritidis in egg products (Hu et al., 2018)	Genie III OptiGene	Yes	UV light
Zika Virus detection (Song et al., 2016)	Disposible cassette	Yes	Colorimetric
Fecal bacteria analysis in water (Lee et al.,2019)	LAMP PCR device	Yes	Colorimetric

Song et al. reported a simple, easy-to-use, LAMP assay for the detection of the Zika virus. the system has a disposable cassette that carries out all the unit operations from sample introduction to detection. The device reported in this study is different from the other devices shown in Table 5, it can operate independently of electricity, as in our device.[33] Lee et al., studied LAMP assay on a

portable device for the detection of indicator microorganisms in environmental water samples.[34]

In our device, the ability to work for 90 minutes independently without electricity is provided by a low-power consumption Li-Ion battery. This feature of our device provides important benefits besides being portable for analysis in the field. DNA Hunter was successfully used in our other study on the detection of *Streptococcus* type A (GAS).[35]

STRENGTH AND LIMITATION

The strength of this work was supported as a research project and developed over a 48-month period. There was no time or financial hardship. The LAMP method used in device tests was completed as a project work package and the results were published in other articles. The weakness of the study is that the article writing phase is delayed and takes a long time after the project is completed.

CONCLUSIONS

MTB LAMP assay with the portable device; It can be used in natural disasters and war situations and/or in places with insufficient laboratory infrastructure. DNA Hunter can also be used as a screening test for those who stay in prisons, immigrants, refugees, asylum seekers, come from other countries with a high incidence of tuberculosis, and the homeless. MTB-LAMP is a simple molecular assay that requires less than one hour to perform and can be analyzed by the naked eye.

Following a review of the latest research, WHO suggests that MTB-LAMP can be used as a replacement for microscopy in the diagnosis of pulmonary MTB in adults with signs and symptoms of tuberculosis.

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CONFLICT OF INTEREST

The author declare that she has no conflict of interest.

AUTHOR CONTRIBUTION

As a project coordinator and researcher, I carried out the relevant studies and completed the article writing.

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Case Report

Severe Leptospirosis (Weil's Disease) with Multiple Organ Failure in Urban Setting: A Case Report

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ABSTRACT

Leptospirosis is a rare disease that could cause multiple organ failures and death if left untreated. The correct treatment will determine the recovery of patients. A 28-years old male came to the Emergency Department with profuse diarrhea. No prior medical history; worked as a private employee recently assigned to collect rat traps one week before. Laboratories show severe thrombocytopenia, acute liver failure, and acute renal failure support by imaging with the conclusion of hepatomegaly with normal kidney size. During observation in the emergency room, the patient worsens into septic shock. The patient was treated in intensive care, diagnosed with Weil's disease, and treated given antibiotics with aggressive fluid therapy; dialysis was postponed, and close monitoring of the patient's symptoms and organ function. After five days of care, clinical symptoms and organ function improved, and the patient was discharged well. Diagnosis of Leptospirosis is challenging with a combination of signs and symptoms that are not commonly found. Therefore, primary treatment is antibiotic and supportive care such as renal replacement therapy is not routinely needed as long there are improvements in close monitoring. This objective is to increase awareness and treatment option for further severe leptospirosis cases

Keywords: dialysis; fluid therapy; leptospirosis, multi organ failure; Weil's Disease

Highlights: . Novelty in this case is Weil's Disease could manifest as severe acute kidney injury without prominent icteric whilst hepatomegaly with increase liver function occur will be reversable with appropriate conservative management. It benefits as reference to postpone dialysis with proper conservative management.

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INTRODUCTION

Leptospirosis is a zoonotic infection that affects both humans and animals.[1]According to WHO, in 2019, there were 920 cases reported in Indonesia, with 122 deaths. However, this reported case number is a severe underestimate of leptospirosis occurrence in Indonesia, given that the annual morbidity of leptospirosis in the population was recently estimated at 39.2 per 100,000 people.[2]Clinical symptoms are undistinguishable from other infectious diseases such as hepatitis, dengue, and typhoid. Severe cases, rather known as Weil's Syndrome, are the triad of haemorrhage, jaundice, and acute kidney injury.[3]The primary treatment for leptospirosis is antibiotic such as penicillin and supportive care. Hemodialysis as the early supportive therapy for kidney injury did not associate with the mortality rate in a critically ill patient.[4] The objective of this report is to increase awareness and as the reference consideration to treat severe leptospirosis in further cases.

CASE REPORT

A 28th-year-old man from Kemayoran, Central Jakarta, was admitted to the Emergency Department with profuse diarrhoea, nausea, yellowish-red sclera, malaise, and muscle pain, especially below the knee. The stool is brown-yellow with soft consistency without blood; meanwhile, the urine is dark. Symptoms occur around two days before admission with a fever that has never been felt before. There is no prior medical history or high-risk lifestyles such as needle injection and promiscuity. Patient work as a private employee. One week before admission, he was assigned to collect rat traps around the corner of a warehouse. He did not catch a single rat and managed to clear up the trap. No evidence of rat bite or prior flood was recorded during that time.

Physical examination on admission shows vital signs of low blood pressure (109/75 mmHg), regular pulse, breath and no fever (36.5°C). Conjunctival suffusion, normal breath and heart sound, abdominal pain at the epigastric, and no swelling nor jaundice on the extremities. The patient was suspected of having a Hepatitis A infection.

Laboratories and imaging did the further investigation. Laboratory finding shows anaemia, leucocytosis, thrombocytopenia, hyponatremia, increased bilirubin level, slight hypo albumin, normal blood coagulation test, liver injury and renal failure—serologic tests of anti-HAV, HBsAg, anti-HCV, and anti-HIV show negative results (Table 1). Chest x-ray (Figure 1) was clear, and abdominal ultrasonography (Figure 2) shows non-specific hepatomegaly without other organs abnormality. By the time examination was done, blood pressure had dropped to 85/34 mmHg, pulse rate 102 times per minute, respiratory rate 25 times per minute, with a normal temperature of 38.2°C, fall into the diagnosis of septic shock then given norepinephrine 0,1 mcg per bodyweight per minute. Transfusion of one unit thrombocyte concentrate followed by hydration of NaCl 3% 500 ml with crystalloid 2000 ml over 24 hours, antibiotic, proton-pump inhibitor (PPI) and attapulgite was given as initial therapy. The patient was admitted to the ICU for further monitoring.

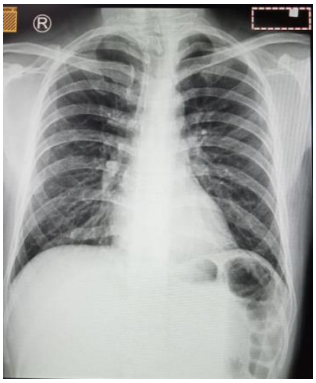

Figure 1. Chest X-Ray, Shows No Abnormality in Lungs and Heart

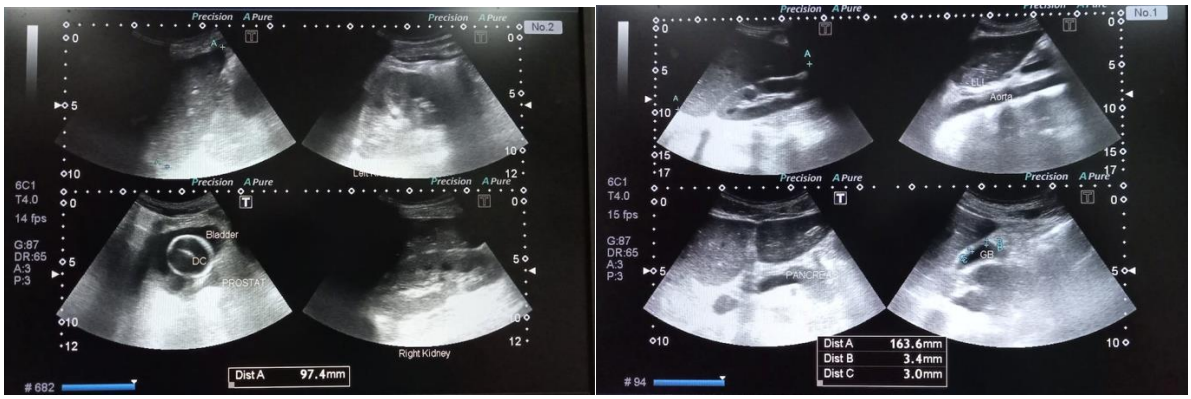

Figure 2. Abdominal Ultrasonography, Shows A Non-Specific Hepatomegaly with Normal Kidney Structure

During intensive care, the patient clinical was improved with normal vital signs, decreasing icteric, and no other symptoms. Follow-up laboratory findings were done with anaemic, improving leucocytes, thrombocytes, and liver and renal function. Norepinephrine support was tapered down, and the patient planned to move to the general wards. Additional test IgM anti-leptospirosis was done and shows a positive result. Treatment of antibiotics, rehydration, and PPI was continued. The patient was hospitalized for another three days. On the last day, the patient clinically improves, and symptoms are all gone but icteric slightly remains. Stool and urine are within normal colours. The patient then discharges with antibiotics and PPI as home medicine.

Table 1. Laboratory Examination

Examination	Result				
	22/8	**23/8**	**24/8**	**25/8**	**26/8**
Hemoglobin (g/dL)	10.3	9.5	9.8	10.1	10.2
Hematocrit (%)	27.8	25.3	27.0	27.8	28.1
Leucocyte (/uL)	20,400	27,680	12,600	6,080	5,950
Thrombocyte (/uL)	36,000	71,000	91,000	119000	163,000
Natrium (mmol/L)	126.0	-	-	-	-
Kalium (mmol/L)	3.67	-	-	-	-
Chloride (mmol/L)	95.5	-	-	-	-
ALT (g/dL)	79.7	-	-	-	-
AST (g/dL)	170.4	-	-	-	-
Albumin (mg/dL)	2.8	-	-	-	-
Creatine (mg/dL)	11.45	8.76	-	-	2.29
Urea (mg/dL)	236.1	283.1	-	-	145.7
eGFR (mL/min/1.73m^2)	6	8	-	-	39
Anti HAV	Non-reactive	-	-	-	-
HBsAg	Non-reactive	-	-	-	-
Anti HCV	Non-reactive	-	-	-	-
Anti HIV	Non-reactive	-	-	-	-
Total Bilirubin (mg/dL)	8.0	-	-	-	5.05
Conjugated Bilirubin (mg/dL)	6.45	-	-	-	3.93
Unconjugated Bilirubin (mg/dL)	1.55	-	-	-	1.12
PT (second)	17.8	-	-	-	-
Control PT	14	-	-	-	-
APTT (second)	31.4	-	-	-	-
Control APTT	31.3	-	-	-	-
IgM Anti-Leptospira	-	-	-	-	Reactive

Abbreviations: g = grams; dL = deciliter; uL = microliter; mm = millimeter; U = unit; L = liter; mEq = milliequivalent, min = minutes

DISCUSSION

In this report, we have described a case of severe leptospirosis or known as Weil's Disease.[1,3] On admission, the patient presented with fever, conjunctiva suffusion, dark urine, and myalgia with leucocytosis, thrombocytopenia, AKI, liver failure, and hyperbilirubinemia. Patients experience septic shock in the ER and are given norepinephrine as support. Treatment given was antibiotics and aggressive hydration. Dialysis was postponed while watchful waiting for the improvement of kidney functions by fluid therapy. Strict monitoring of kidney function and haematology was done. Symptoms and kidney function then recover with the treatment given.

Leptospira is a zoonotic disease that is an emerging global public health problem. Indonesia, with a high incidence of flooding and subsequent presence of stagnant water and poor sanitation conditions in some housing areas, is at high risk for leptospirosis. The transmission from infected animals through their urine (rodents, dogs, livestock, pigs, horses, wildlife) can survive for weeks to months in water and soil. A human can be infected through direct contact with the urine, urine-contaminated water, and wet soil, or ingestion of urine-contaminated food or water.[1,5,6] In the present case, there is no contact with water or soil, but our patient does risk contact with a rat trap which could be contaminated with rodent urine. High-risk infection activities include wading, swimming, boating, and activities that could lead to skin abrasion and water or soil exposure.

Leptospirosis symptoms are usually a flu-like illness of sudden onset, fever, headache, nausea, vomiting, abdominal pain, conjunctival suffusion, and myalgia, typically on the calves and lower back. Severe cases have a classic presentation known as Weil's syndrome consists of the triad of haemorrhage, jaundice, and AKI.[1,3,5] Incidence of severe leptopirosis estimated 5% to 15% of patients.[7]Symptoms that occur in our patients fulfil the severe symptoms. Thrombocytopenia is common in leptospirosis, which suggested a mechanism caused by peripheral platelet consumption due to widespread haemorrhages, immune-mediated platelet destruction caused by antiplatelet antibodies, and inhibited platelet production by bone marrow.[8] It aggravates hemorrhagic manifestation, as does installation access for dialysis if needed. Therefore, transfusion of thrombocyte concentrate was given as a preventative strategy.[9]Septic shock occurs because of severe infection from leptospira which causes vasculitis and systemic inflammatory response syndrome.[10] It could develop into an immunosuppressive state as it evolves until the death of the host.[11] Early administration of the vasoactive drug norepinephrine is beneficial in restoring organ perfusion in septic shock patients.[12]

Treatment of leptospirosis consists of antibiotics and supportive therapy. Antibiotics chosen are penicillin group or cephalosporin such as ceftriaxone that was given to our patient. Leptospira are highly susceptible to a broad range of antibiotics. A Jarisch-Herxheimer reaction may occur as a response to the clearance of spirochetes from the circulation. It is an acute inflammatory response characterized by fever, rigors, and hypotension with a 21% incidence according to Guerrier et al which is not found in this report.[13,14] Supportive therapies are based on clinical manifestation with renal replacement therapy, ventilatory support, and blood products. A study in Brazil shows that leptospirosis patients with complications of acute respiratory distress syndrome and AKI benefit from daily hemodialysis to lower the mortality rate.[15] While the STARRT-AKI (Standard versus Accelerated Initiation of Renal-Replacement Therapy in Acute Kidney Injury) investigation concluded that among critically ill patients with AKI, an accelerated renal-replacement strategy within 12 hours was not associated with a lower risk of death than the standard strategy.[4] This study supports the present case in which dialysis, as renal replacement therapy, was not given to the patient and, as a result of clinical laboratories, does improve with aggressive

fluid therapy alone. The choice made was risky yet convenient and promising as for the patient condition and psychology that he did not need dialysis.

STRENGTHS AND LIMITATIONS

The strength of this study were the detail information given from patient history prior medication to condition and treatment given until discharge. The limitation of this study were treatment decision are based on physician experience and patient profile therefore not always applicable in every cases.

CONCLUSIONS

Overall, diagnosis and treatment of leptospirosis are challenging. Typical infection symptoms of fever, when followed by icteric, hemorrhagic, and AKI, should be asked for further anamnesis of contact with rodent or other leptospirosis risks to ensure the diagnosis. Treatment given for leptospirosis is mainly antibiotics and close monitoring. In contrast, other supportive care, such as renal replacement therapy, is not routinely needed because renal failure will recover itself as the infection diminishes.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest in this report.

AUTHOR CONTRIBUTION

Conceptualization and supervision: SH. Data curation, writing-original draft, review, and editing: BAH.

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Original Article

Germ Tube Induction Test Comparing Total of Six Liquid and Three Solid Media in *Candida albicans*

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ABSTRACT

Invasive candidiasis (IC) has a high mortality rate of 70%, thus diagnosis should be established without delay. Given its fast result, serological test such as β-d-glucan (BDG) test is one alternative diagnosis modalities. However, it lacks specificity. *Candida albicans* germ tube antibody (CAGTA) test is an alternative serological test which has a high sensitivity of 76.2% and specificity of 80.3%. Manufacturing CAGTA serological test requires provision of specific germ tube antigen. In this study, various culture media were tested to find the best media for germ tube induction. This study was an experimental in vitro study. The number and length of the germ tube were recorded in two- and three-hour incubation periods. A total of six samples containing one C. albicans ATCC 90028, four *C. albicans* wild type strains, and one *C. krusei* wild type strain were used. Nine media were tested to induce germ tube formation: human and sheep serum, fetal bovine serum, mueller hinton agar and broth, tryptic soy agar and broth, brain heart infusion agar and broth. At both incubation periods, the medium with the highest number of germ tube was human serum (p=0.001 and p=0). The longest germ tube was found in sheep serum at two-hour incubation period (p=0.005). Mueller hinton broth (MHB) showed comparable results with human and sheep serum (p>0.05). Human serum is a superior inducer of morphogenesis. However, the use of MHB is recommended in this study, since provision of fresh human and sheep serum on a regular basis is impractical.

Keywords: *Candida albicans*; germ tube; human serum; mueller hinton broth; sheep serum

Highlights: Several media could induce not only numbers of germ tube, but also its length. Therefore, they could benefit for easier diagnosis and also higher amounts of germ tube protein.

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INTRODUCTION

Candidiasis is a disease with a high prevalence rate globally. This disease generally affects the skin and mucosal tissue, causing mild conditions such as oral and vulvovaginal candidiasis.[1] At the systemic level, it has high mortality and morbidity, referred to as invasive candidiasis (IC). It is associated with prolonged intensive care unit (ICU) admission and immunocompromised conditions such as acquired immune deficiency syndrome (AIDS).[2] Globally, candidiasis occupies the top three incidences of diseases caused by fungi in the year 2017—the first is oral candidiasis with an incidence rate of 2,000,000, followed by esophageal candidiasis with 1,300,000, and then IC with 750,000 incidences.[1] Invasive candidiasis yields a high mortality rate of 70%.[3]

Invasive candidiasis is caused by *Candida spp*. with the most common etiology being *Candida albicans*.[4] In humans, this fungus is a normal flora of the skin, oropharynx, digestive, and urogenital tract.[5] Infection occurs when there is hyphal growth and biofilm formation in the tissue. These mechanisms also allow resistance of *C. albicans* to traditional antifungal agents.[6]

Timely diagnosis is required in order to reduce mortality. Currently, IC is diagnosed through the findings of hyphae on microscopic examination or through a time- consuming culture.[1] Serological tests provide relatively faster and easier way to diagnose IC. β-d-glucan (BDG) test is one widely used *Candida* serological test. However, it lacks specificity due to cross reaction with other fungi.[7] A serological test detecting antibody against germ tube could be used as an alternative to diagnose IC, namely *C. albicans* germ tube antibody (CAGTA) test.[8] Germ tubes are formed by *C. albicans* in a number of conditions such as starvation, presence of serum or N-acetylglucosamine, physiological temperature, and CO_2.[9] It has high sensitivity of 76.2% and specificity of 80.3% since morphological transition from yeast to germ tube and hyphae is important for pathogenicity of *C. albicans.*[8,10] Combination of BDG and CAGTA serological tests is recommended for IC early diagnosis.[11]

The first step in manufacturing CAGTA serological test is isolation of the germ tube antigen. It is important to seek the best medium for *C. albicans* since this antigen is obtained by inducing its growth in a suitable environment. Various media can be used for induction of germ tube, each with unique compositions and function. Human serum is the most used medium for germ tube test.[12] Its main limitation is the requirement of fresh human serum on a regular basis. For this reason, this study aims to find the best media in inducing germ tube formation of *C. albicans.* While previous studies mainly assessed the sensitivity of each medium for germ tube test, this study also measured the number and length of germ tube formed after certain incubation period.

MATERIALS AND METHODS

Study Design

This experimental in vitro study was conducted in the Parasitology Laboratory, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia from August 2020 to October 2020. Ethical clearance was obtained from the Atma Jaya ethical committee with the number 01/06/KEP-FKUAJ/2020.

Fungi Strains

Candida albicans wild type, *C. albicans* ATCC 90028, and *C. krusei* wild type were used in this study. All *C. albicans* wild type were obtained from patient's sputum in Microbiology Laboratory, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, while *C. albicans* ATCC 90028 and *C. krusei* wild type were obtained from the collection of Department Parasitology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia. Each strain

was identified and confirmed through macroscopic and microscopic examination. Macroscopic identification was made using CHROMAgar (Oxoid, United Kingdom) to analyze the color and characteristics of the fungal colonies. *Candida albicans* was characterized by the formation of green colonies, in contrast with *C. krusei* which appeared as pink colonies.[13] Microscopic identification was made through lactophenol cotton blue (LPCB) staining and germ tube test. Light microscope (Olympus CX21) is used to identify the morphologies. Ovoid and spherical yeast cell shapes are a characterization of *C. albicans*, distinguished from *C. krusei* that commonly appear as a more elongated (long grain rice) shape. A positive germ tube test is also only found in *C. albicans.*[14,15] A total of six isolates containing one *C. albicans* ATCC 90028, four *C. albicans* wild type, and one *C.krusei* wild type were used. *Candida albicans*ATCC 90028 and *C. krusei* were used as the positive and negative control, respectively.

Medium

The media used for induction of germ tube were serum, broth, and agar. Sera used were human serum, sheep serum, and fetal bovine serum (FBS, Biowest, France). The FBS used was not diluted with 100% concentration. Human serum was prepared by centrifugating blood from a healthy donor.[16] The broth and agar media used were mueller hinton agar (MHA, Oxoid, United Kingdom), mueller hinton broth (MHB, Conda, Spain), tryptic soy agar (TSA, Oxoid, United Kingdom), tryptic soy broth (TSB, Merck, Germany), brain heart infusion agar (BHIA, Oxoid, United Kingdom), and brain heart infusion broth (BHIB, Oxoid, United Kingdom). All media were obtained from the Department of Microbiology, Parasitology, and Pharmacology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia.

Broth and agar media were prepared by combining 1 L of aquadest with 38, 21, 45, 30, 53, and 37 g of MHA, MHB, TSA, TSB, BHIA, and BHIB, respectively. These suspensions were heated until completely dissolved, followed by sterilization using an autoclave at 121˚C with a pressure of 15 Psi for 15 minutes. Broth medium was poured into the 1.5 ml test tube, while agar medium was poured into a petri dish. For all media, pH was adjusted at 7.4 which is confirmed by pH meter.

Gem Tube Induction

Candida albicans and *C. krusei* were inoculated in sabouraud dextrose agar (SDA, Oxoid, United Kingdom) for 48 hours at room temperature (25°C). Two hundred µL of 3 McFarland fungi suspension was added into 800 µL of each serum and broth medium. The mixture was incubated for 24 hours at 37°C.[12] The number and length of the germ tube were recorded in two-, three-, and 24-hour incubation periods. Ten µL of the mixture was dripped into an improved Neubauer counting chamber and the germ tube was observed under the microscope (Figure 1a).[17] All processes were performed in duplicate.

Germ tube induction in agar media was conducted by dripping 10 µL of 0.5 McFarland fungi suspension into 1 x 1 cm^2 agar.[18] Cover slip was placed to facilitate easier examination. The agar was then incubated for three hours at 37°C. The number and length of germ tube induction were recorded in two- and three-hour incubation periods using the microscope (Figure 1b). All processes were performed in duplicate.

Germ Tube Calculation

This study measured the number and length of germ tubes formed. The number of germ tube was calculated in five small squares of the counting chamber using standardized formula (Figure 2).[19] Germ tube length measurement was conducted by comparing the length of the germ tube and the counting chamber small squares. The longest germ tube in the five small squares was

recorded. Germ tube length measurement was done only in serum and broth media. There are no difficulties in performing the measurement methods.

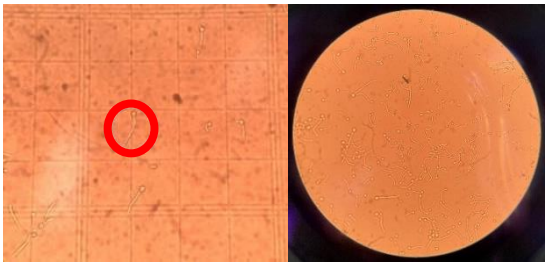
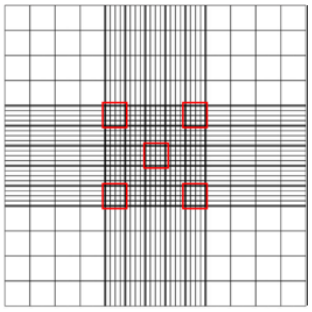


Figure 1. Germ tube appearance under the microscope in different preparations. (a) Improved Neubauer Counting Chamber – Germ tube (red circle); (b) Slide culture



$$Number\ of\ germ\ tube\ /\ \mu L = \frac{n}{V}$$

$$V = improved\ Neubauer\ counting\ chamber\ volume = \frac{1}{50} mm^3$$

$$n = total\ of\ germ\ tube\ in\ 5\ small\ squares$$

Figure 2. Improved Neubauer Counting Chamber

Calculation of the number of germ tube on agar media was carried out by taking three representative images using the high-power field (HPF) microscope. Then the number of germ tubes was grouped into several categories (Table 1).

Table 1. Germ Tubes Counts Categories Using Agar Media Inductions

Categories	Germ Tube Count/HPF
1+	1-10
2+	11-20
3+	21-30
4+	31-40
5+	41-50
6+	>50

HPF – high-power field

Data Analysis

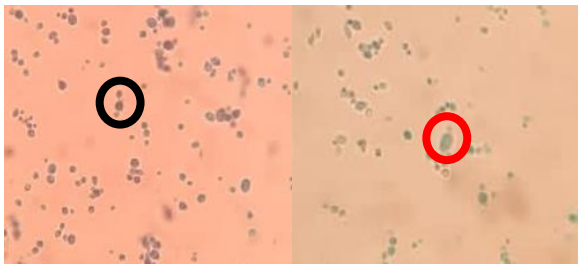
Data analysis was done using Statistical Product and Service Solution (SPSS) version 22. Data on serum and broth medium was analyzed using a One-Way ANOVA statistical test or Kruskal-Wallis test, depending on data normality. It was then followed by a Bonferroni post hoc test if significant results were found. In agar medium, Fisher-Exact or Chi-Square test were used depending on the terms and criteria of the statistical test. A significant value was yielded if $p<0.05$.

RESULTS AND DISCUSSION

Fungi Identification

Macroscopic identification of all samples was done using CHROMagar. It is depicted in Figure 3. Moreover, the morphologies found in microscopic identification using LPCB confirmed the samples' species (Figure 4).

Figure 3. Macroscopic characterizations on CHROMagar. The green colonies are *C. albicans* and the pink colony is *C. krusei*



(a) **(b)**

Figure 4. Microscopic characterizations of *Candida* species grown in SDA. (a) Ovoid appearance of *C. albicans* (black circle) (b) Elongated appearance of *C. krusei* (red circle)

Germ Tube Induction in Serum and Broth

All Candida samples were used in germ tube induction. *Candida albicans* ATCC 90028 and *C. krusei* function serve as a positive and negative control, respectively. In all media, *C. albicans* ATCC 90028 showed positive germ tube results, while *C. krusei* had negative results. *Candida albicans* wild type was used for the evaluation of each media's ability to induce germ tube formation.

Several media could facilitate germ tube formation. Results were variable between media at two- and three-hour incubation period. At two-hour incubation period, it was found that the medium with the highest number of germ tube was human serum. Sheep serum also showed comparable results in germ tube induction. Roughly, the order of media that could facilitate germ tube induction was as follows: human serum, sheep serum, FBS, MHB, TSB, and BHIB; while at three-hour incubation period, the order was as follows: human serum, MHB, sheep serum, FBS, TSB, and BHIB as shown in Table 2.

Table 2. Number of Germ Tubes Formed on Serum and Broth Media (number/µL)

Time	Fungi	Media						p-value
		Human Serum	Sheep Serum	FBS	MHB	TSB	BHIB	
2 hours	CA 1	38125	40625	26875	11250	5625	5000	0.001
	CA 2	23125	20625	10000	22500	11250	3750	
	CA 3	25000	23125	8750	15625	13750	0	
	CA 4	21250	15000	18125	11250	0	625	
3 hours	CA 1	42500	16250	13750	15625	3750	0	0
	CA 2	26250	15000	5000	37500	15625	6875	
	CA 3	32500	20625	7500	25000	16250	0	
	CA 4	31875	13125	18750	15000	3750	0	

*Post hoc (2 hours) Human Serum vs. BHIB p=0.003; Human Serum vs. TSB p=0.024
**Post hoc (2 hours) Sheep Serum vs. BHIB p=0.006
***Post hoc (3 hours) MHB vs. BHIB p=0.004
****Post hoc (3 hours) Human Serum vs. BHIB p=0; Human Serum vs. Sheep Serum p=0.03; Human Serum vs. TSB p=0.001; Human Serum vs. FBS p=0.003

No difference was shown between MHB and human serum in post hoc analysis (p>0.05). The number of germ tubes between broth media were highest in MHB, compared to TSB and BHIB (p=0.015 and p=0.009 at two- and three-hour incubation periods, respectively) as shown in Table 3. At the 24-hour incubation period, the fungi experienced rapid growth into hyphae. Therefore, further analysis was not performed in this incubation period.

Table 3. Number of Germ Tubes Formed on Broth Media (number/µL)

Time	Fungi	Media			p-value
		MHB	TSB	BHIB	
2 hours	CA 1	11250	5625	5000	0.115
	CA 2	22500	11250	3750	
	CA 3	15625	13750	0	
	CA 4	11250	0	625	
3 hours	CA 1	15625	3750	0	0.009
	CA 2	37500	15625	6875	
	CA 3	25000	16250	0	
	CA 4	15000	3750	0	

*Post hoc (2 hours) MHB vs. BHIB p=0.015
**Post hoc (3 hours) MHB vs. BHIB p=0.009

Table 2. Number of Germ Tubes Formed on Serum and Broth Media (number/μL)

Time	Fungi	Media						p-value
		Human Serum	Sheep Serum	FBS	MHB	TSB	BHIB	
2 hours	CA 1	14	28.89	22.22	17.775	24.445	2.22	0.005
	CA 2	26	38	37.78	22.22	26	17.78	
	CA 3	20	33.335	26.665	23.33	31.115	0	
	CA 4	30	55.555	38	33.335	0	10	
3 hours	CA 1	26.665	53.33	26	31.115	14	0	0.155
	CA 2	57.78	50	42	77.775	95.555	48.89	
	CA 3	28.89	36	34	37.78	57.78	0	
	CA 4	77.78	44.665	22	73.335	26.665	0	

*Post hoc (2 hours) BHIB vs. Sheep Serum p=0.003
*Post hoc (2 hours) BHIB vs. FBS p=0.039

In the measurement of length, the longest germ tube was found in sheep serum at two-hour incubation period, followed by FBS, MHB, TSB, human serum, and BHIB. No difference was found at the three-hour incubation period as shown in Table 4. No broth media was found superior to the other in terms of the germ tubes length as shown in Table 5.

Table 5. Length of the Germ Tube Formed on Broth Media (μm)

Time	Fungi	Media			p-value
		MHB	TSB	BHIB	
2 hours	CA 1	17.775	24.445	2.22	0.998
	CA 2	22.22	26	17.78	
	CA 3	23.33	31.115	0	
	CA 4	33.335	0	10	
3 hours	CA 1	31.115	14	0	0.132
	CA 2	77.775	95.555	48.89	
	CA 3	37.78	57.78	0	
	CA 4	73.335	26.665	0	

Germ Tube Induction in Agar

The number of germ tubes formed was highest in MHA. However, results were not significant both in two- and three-hour incubation periods as shown in Table 6.

Table 6. Length of the Germ Tube Formed on Agar Media (μm)

Time	Fungi	Media			p-value
		MHA	TSA	BHIA	
2 hours	CA 1	+3	+1	0	0.408
	CA 2	+6	+1	+2	
	CA 3	+4	+1	+1	
	CA 4	+1	0	0	
3 hours	CA 1	+1	0	0	1
	CA 2	+6	+1	+1	
	CA 3	+2	+1	+1	
	CA 4	0	0	0	

Hyphal morphogenesis is one of the most investigated virulence attributes of *C. albicans*. The ability of *C. albicans* to undergo reversible morphological transition could be triggered by variety of environmental condition.[20–22] Serum, especially human serum, contains several important components that promote germ tube formation; therefore, it accounts as strong inducer for yeast-to-hyphae formation. Burch et al. (2018) stated that human serum fraction revealed signs of bacterial peptidoglycan (PGN)-like molecules which highly active for hyphae induction.[23] Glucose could also act as morphogen, which in the certain amount could stimulate morphogenesis of *C. albicans*.[24,25]

Human serum, sheep serum, and FBS have approximately 1 mM to 10 mM of glucose.[26–28] This amount of glucose is optimal for germ tube formation according to previous study.[24] Moreover, combined with exposure of 37°C and neutral pH environment, serum could inhibit NRG1 transcription, a potent inhibitor for hyphal formation (Su et al. 2018).[29] This exposures to 37°C and neutral to alkaline pH, could induce hyphal growth through the Cek1 mitogen-activated protein kinase pathway (MAPK pathway) and the Rim101-pH sensing pathway, respectively.[21] Studies by Hilmioglu et al. (2007) found that human serum was superior with the highest number of positive germ tube.[30] In concordance to previous data, present study found that human and sheep serum were capable of inducing the highest number and longest germ tube, respectively, compared to other

media. This study also found that extended incubation period on serum led to an increasing number of hyphae. Long incubation period causes deprivation of nutrient and energy which leads to more effective hyphal growth.[31,32]

Utilization of human serum for germ tube induction has few drawbacks despite its superiority to other media. Serum has to be fresh otherwise stored serum could decrease germ tube production.[12] Some serum could have biological inhibitor present in it. Wich et al. (2021), found that human serum antibodies have the capability to inhibit adherence of *C. albicans* to epithelial cells.[33] Moreover, Ding et al. (2014) stated that germ tube formation in RPMI 1640 medium was delayed in the initial stage of the culture (within 90 min) in the presence of serum, although the number of hyphae was gradually increased to normal after extension of incubation period (from 2h to 3h).[31] Presence of these biological inhibitor could cause inconsistent result in different batches of serum. Lastly, serum preparation poses possible risk of biohazard.[12] In this study, several standardized media were studied to find comparable alternative of serum. Application of commercially available media also facilitates further culture and production of germ tube antigen for serological test.

Broth medium also have essential substances for the growth and morphogenesis of *C. albicans*. MHB, TSB, and BHIB were tested in their ability to promote germ tube formation. As stated above, elevated temperature and neutral pH were the one main inducer for morphogenesis.[29] Moreover, all media contains nutritive compounds that are necessary for fungal growth. Amino acids are one potent inducer present in the medium. It promotes yeast-to-hyphal transition through the cAMP-PKA pathway.[21] In this study, MHB, TSB, and BHIB contain amino acids which further facilitate hyphal formation.[21,22] Furthermore, in MHB, *C. albicans* showed the highest number of germ tube formation. One possible explanation is that it contained starch components with protective colloid roles against toxic compounds in the medium.[34] BHIB is the most nutritious medium with combination of brain and beef infusion (a total of 17.5 g/l), protease peptone (10 g/l), and dextrose (2 g/l) which provide carbon, nitrogen, amino acids, and other nutrients.[34] However, *C. albicans* showed lowest number of germ tube in BHIB. An exact explanation of this phenomenon is unknown. It seems that high nutrient provision is not an inducer of morphogenesis. According to Mba et al. (2020), *C. albicans* exhibits metabolic flexibility and filamentous growth in the condition of nutrient starvation.[34] It is difficult to identify which media facilitate better morphogenesis based on previous studies, since results were mostly conflicting. Hilmioglu et al. (2007) showed that BHIB surpassed TSB as morphogenesis inducer, while Yakasiri et al. (2020) concluded that TSB exceeds MHB and BHIB.[30,35] Different results could be attributed to different strain, media quality, and research conditions.

Interestingly, Atalay et al. (2017) found that MHA was the best media for germ tube induction compared to human serum.[18] Distinct factor affects the yeast-to-hyphal transition in agar media. Villa et al. (2020) stated that *C. albicans* hyphal growth in agar is influenced by the embedded surroundings or conditions. This is achievable through the upregulation of CZF1 transcription factor when the fungi are inoculated within the agar matrix.[9] In present study, higher number of germ tube was found in MHA compared to other agar media although the result was not significant.

STRENGTH AND LIMITATION

To our knowledge, this is the first study that compares several media by measuring the number and length of the germ tubes. However, this study has several limitations. Lack of fungal strain prevents generalization of the result to other strain of *C. albicans*.

Wider variety of culture medium could be used, since RPMI 1640 and YEPD broth also showed potential in previous studies.[12,31]

CONCLUSIONS

This study showed that certain media in a specific environmental condition could facilitate hyphal growth that initially appears as germ tube formation. Human serum is a strong inducer of morphogenesis. Incubation of *C. albicans* in standardized medium such as MHB and TSB, coupled with 37°C environmental temperature and neutral pH, is also adequate to facilitate such phenomenon. Those media are preferred to human serum because it is readily available, routinely used in daily microbiology laboratories, and it provides stable result between batches.

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ETCHICAL CLEARANCE

Ethical clearance was obtained from the Atma Jaya ethical committee with the number 01/06/KEP-FKUAJ/2020.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTION

Designed the study, collected and analyzed the data, and also prepared the manuscript: RR and EAS. A scientific adviser in the field of mycology: SS and SVK. All authors read and approved the final manuscript.

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Original Article

Epidemiological and Clinical Features of Critical and Non-Critical Elderly COVID-19 Patients in Udayana University Academic Hospital: A Retrospective Study

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ABSTRACT

Elderly COVID-19 patients have been associated with worse outcomes and have been presented with the highest mortality rate. However, studies on the clinical features and the differences between critical and non-critical elderly COVID-19 patients in Indonesia and even other countries are still lacking and rare. In this retrospective study, the epidemiological and clinical features of critical and non-critical elderly COVID-19 patients admitted to Udayana University Academic Hospital between April 2020 and March 2021 were analyzed and then compared. Of the 280 medical records analyzed, 60.7% were male and the median age was 65.0 years old. Based on the medical records, 18.2% of elderly patients met our criteria of critical patients. The most common symptoms presented in both category upon admission included fever and coughing. The most common comorbidity found in critical patients was heart disease and hypertension in non-critical patients. Laboratory results differences included leukocytes, neutrophils, lymphocytes, Neutrophil-to-Lymphocyte Ratio, platelets, SGOT, SGPT, and urea. Only 9.9% of critical patients and 6.1% of non-critical patients were given antiviral therapy. In contrast, 68.6% of critical patients and 76% of non-critical patients were given antibiotics. The mortality rate in critical patients was 70.6% and 0.4% in non-critical patients. Based on the results, a multimodal approach in the treatment of elderly COVID-19 patients is very essential. The higher mortality rate in elderly patients should be able to be reduced by giving early and timely antiviral therapy with the addition of effective choice of drugs.

Keywords: Covid-19; epidemiology; elderly patients; geriatric; SARS-CoV-2

Highlights: The novelty of this study is that it is the first study focusing on the clinical profile of elderly COVID-19 patients in Bali. The benefit of this study through its description and comparison is for clinicians to be able to provide a more structured and comprehensive approach towards elderly COVID-19 patients.

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INTRODUCTION

Novel coronavirus disease 2019 (Covid-19) has been a worldwide phenomenon since it was initially found in China in December 2019. It was first declared as a worldwide pandemic by the World Health Organization (WHO) in March 2020.[1] Indonesia reported its first cases in early March 2020 and by late May 2020 the number of cases had undergone significant increase, reaching a total of 21430 cases with 1326 deaths. A study on the epidemiology of COVID-19 in Indonesia, which observed a total of 8211 cases between March 2nd and April 24th 2020, showed that 16% of the patients were from the age group of above 60 years old. Patients from this age group were also presented with the highest mortality rate of 43.6%.[2] Elderly patients have been associated with a higher susceptibility to COVID-19 and worse outcomes due to several reasons such as immunosenescence and comorbidities which usually worsen with aging.

Deterioration of pathophysiological functions in the body systems of elderly patients might also contribute to higher mortality rates.[3] However, studies on the clinical features of elderly Covid -19 patients and the differences between critical and non-critical elderly COVID-19 patients in Indonesia and even other countries are still lacking and rare. Through this descriptive study, we aimed to summarize the clinical features and to provide a comparison between critical and non-critical elderly COVID-19 patients who were admitted to Udayana University Academic Hospital in hope that this could act as a reference for physicians to have a better understanding and to provide better treatment for elderly COVID-19 patients in the future.

MATERIALS AND METHODS

Study Design and Setting

This study was designed as an observational cross-sectional study conducted at Udayana University Academic Hospital which serves as a health facility in Jimbaran, Bali and was the province's main referral site for COVID-19 patients.

Data Collection

Secondary data of all elderly COVID-19 patients admitted to Udayana University Academic Hospital between April 2020 and March 2021 were collected through medical records using the method of total sampling. The inclusion criteria for our study were all elderly COVID-19 patients who were aged 60 to 90 years old. Although there are different ways to classify elderly age range, several studies such as the study by Alterovitz and Mendelsohn[4] in Journal of Aging Studies had used 60 years old as the baseline age for elderly people according to the US census bureau age divisions as well as to previous researches on physical and cognitive aspects of aging. In addition, three other studies which discussed about clinical characteristics of elderly COVID-19 patients in Jakarta, Hunan, and Hainan had also used 60 years old as the baseline age for their population of elderly patients.[5–7]

The patients were then divided into two categories, including critical and non-critical elderly COVID-19 patients. Critical patients were patients who met any of the following criteria, including patients with Acute Respiratory Distress Syndrome (ARDS), shock, and/or sepsis; patients who were admitted to the ICU; and patients who died in a short duration upon admission, whereas non-critical patients consisted of the rest. Patients were excluded if they were not aged 60 to 90 years old and/or were not diagnosed with Covid-19. The collected data only consisted of epidemiological features, clinical manifestations, comorbidities, laboratory results, treatment, and patients' outcome, whereas any data regarding patients' identity were not disclosed due to patients' right to privacy. Patients who decided to opt out of the study were also given the opportunity to contact the authors

and all data were only accessible to the authors. This study had been approved by the Ethical Commission of Udayana University (No. 1010/UN14.2.2.VII.14/LT/2020).

Data Analysis

All statistical analyses were performed using IBM SPSS Statistics 20. All data, except sex and prognosis, were tested using Kolmogorov-Smirnov Z test and continued by either Independent-Samples T test if the asymptotic significance 2-tailed was > 0.05 or Mann-Whitney U test if the asymptotic significance 2-tailed was < 0.05. Sex and prognosis variables were tested using Chi-square test. Continuous variables are described in median (interquartile range, IQR) and categorical variables are presented as n (%).

RESULTS AND DISCUSSION

A total of 280 elderly COVID-19 patients were included in this study and all patients were admitted to Udayana University Academic Hospital, Bali. The median age of all elderly patients were 65.0 years old. This finding correlates with a study in China where COVID-19 had the epidemiological characteristics which commonly affect age group ranging from 30 to 79 years old.[8] Other specific studies on elderly COVID-19 patients also showed similar results where a study in Jakarta by Azwar *et al.*[5] found that the majority of its patients were aged 60 to 69 years old whereas another study in Hunan by Guo *et al.*[6] found that the median age of all its elderly patients was 67 years old.

More than half of the patients in this study were constituted by male patients in both critical and non-critical criterion. The data samples in several other studies from different countries were also predominantly constituted by male patients. A study in a tertiary hospital in North India by Soni *et al.*[9] reported that 57.8% of its patients were males and an even higher percentage were reported from a study in Al Ain Hospital of United Arab Emirates by Ismail *et al.*[10] where 84.6% of its critical patients were also males. Aside from the prevalence of COVID-19 cases among males, it was also found that there was a difference in terms of fatality rate where males showed higher results.[11] There are several mechanisms which may contribute to the possible correlation between male sex and a higher incidence of COVID-19 cases among them. The SARS-CoV-2 is known to infect humans by binding to angiotensin-converting enzyme 2 (ACE2) receptor. After binding to the ACE2 receptor, the virus is then able to enter human tissue through cell surface fusion mediated by transmembrane protease serine 2 (TMPRSS2).[12]

In molecular perspective, ACE2 gene is found to be located on the X chromosome which means there should be alleles that regulates resistance towards Covid-19.[11] In addition, ACE2 was also found to be a constitutive product of Leydig cells which is a factor affecting testosterone secretion in males. To further support this incidence, it was also found that androgen receptor activity had been considered as a factor in the transcription of TMPRSS2 gene, thus implying that the expression of TMPRSS2 modulated by testosterone might contribute to male predominance in COVID-19 cases.[13] Despite several supporting factors which may explain male predominance in COVID-19 cases, there are also several studies which had higher cases among females rather than males.[6,14]

Table 1. Demographics and Clinical Characteristics of Elderly COVID-19 Patients

	All Patients (*n* = 280)	**Critical (*n* = 51)**	**Non-critical (*n* = 229)**	***p* value**
Age, years	65.0 (62.0-72.0)	68.0 (63.0-74.0)	65.0 (62.0-71.0)	0.061
Sex				0.059
Male	170 (60.7)	37 (72.5)	133 (58.1)	
Female	110 (39.3)	14 (27.5)	96 (41.9)	
Symptoms				
Fever	276 (98.6)	51 (100)	225 (98.3)	0.344
Cough	252 (90)	46 (90.2)	206 (90)	0.959
Cold	46 (16.4)	2 (3.9)	44 (19.2)	0.000
Sore Throat	116 (41.4)	17 (33.3)	99 (43.2)	0.187
Cephalgia	78 (27.9)	8 (15.7)	70 (30.6)	0.015
Myalgia	90 (32.1)	10 (19.6)	80 (34.9)	0.020
Diarrhea	15 (5.4)	5 (9.8)	10 (4.4)	0.223
Comorbidities				
Hypertension	132 (47.1)	17 (33.3)	115 (50.2)	0.026
Heart Disease	68 (24.3)	23 (45.1)	45 (19.7)	0.000
Diabetes	72 (25.7)	13 (25.5)	59 (25.8)	0.968
Kidney Disease	12 (4.3)	2 (3.9)	10 (4.4)	0.888
Malignancy	5 (1.8)	1 (2)	4 (1.7)	0.917
HIV	0 (0)	0 (0)	0 (0)	
Autoimmune	0 (0)	0 (0)	0 (0)	

Data are presented in median (IQR) or *n* (%)

The study of critical patients by Ismail *et al.*[10], showed that the most common symptoms presented in its patients were coughing followed by fever. Another study from China by Liu *et al.*[7] which compared the clinical features manifested in elderly patients with young patients also showed that the most common symptoms in both age groups were fever and cough. These clinical features are in accordance with our findings where both non-critical and critical patients were most commonly presented with symptoms of fever followed by coughing. While ACE2 acts as an entry point for Covid-19, it also has a crucial anti-inflammatory role by converting angiotensin II, which is a perpetrator of inflammation, to angiotensin 1-7 in the renin-angiotensin signaling system. The expression of ACE2 declines with aging and in patients with cardiovascular diseases. When SARS-CoV-2 binds to ACE2 receptors in patients, it further reduces the ACE2 cell surface expression. Hence, elderly COVID-19 patients with cardiovascular comorbidities are suspected to have a significantly low level of ACE2 which contributes to the predisposition of a more severe outcome.[15]

A study from Central Sulawesi by Faustine[16] which focused on the severity profile of COVID-19 patients with hypertension concluded that there was no significant correlation between high blood pressure and the severity and mortality of COVID-19 patients. On the other hand, it was stated that cardiovascular comorbidities other than hypertension were associated with the severity of Covid-19.[16] Another study stated that the presence of heart lesion in COVID-19 patients was associated with poor prognosis where these patients were five to ten times more at risk. The cardiac manifestations found were predominated by acute myocardial damage.[17] These findings may provide an explanation to the difference found in terms of the most common comorbidity suffered by the elderly patients in our study where hypertension was more prevalent in non-critical elderly patients whereas heart disease was more prevalent in critical elderly patients. All data on demographics and clinical characteristics are presented in Table 1.

Table 2. Laboratory Results of Elderly COVID-19 Patients

	All Patients (*n* = 280)	**Critical (*n* = 51)**	**Non-critical (*n* = 229)**	***p* value**
Hemoglobin, d/dL	13.2 (12.1-14.1)	13.7 (11.9-14.3)	13.2 (12.1-14.0)	0.967
Hematocrit, %	39.0 (36.1-41.5)	39.1 (35.5-42.4)	38.9 (36.2-41.5)	0.718
Leukocyte count, $x10^3$µL	6.77 (5.1-8.8)	8.1 (6.1-12.4)	6.3 (4.9-8.3)	0.000
Neutrophil count, $x10^3$µL	4.5 (3.2-6.6)	6.9 (4.4-10.5)	4.2 (3.1-5.9)	0.000
Lymphocyte count, $x10^3$µL	1.2 (0.8-1.7)	0.7 (0.5-1.2)	1.35 (0.9-1.9)	0.000
Neutrophil-to-Lymphocyte Ratio	3.4 (2.0-5.7)	10.0 (4.7-16.1)	3.1 (1.9-4.6)	0.000
Platelet count, $x10^3$µL	210.5 (165.0-272.0)	187.0 (163.0-234.0)	219.0 (165.5-276.0)	0.037
Liver function				
SGOT, U/L	33.0 (26.0-50.0)	52.0 (34.3-70.0)	31.0 (25.0-45.0)	0.000
SGPT, U/L	29.0 (21.5-47.0)	44.5 (28.0-67.5)	28.0 (20.0-40.5)	0.000
Kidney function				
Blood urea nitrogen, mg/dL	15.0 (11.0-21.0)	20.0 (15.0-30.5)	14.0 (11.0-19.0)	0.000
Creatinine, mg/dL	0.8 (0.6-1.1)	0.9 (0.7-1.2)	0.8 (0.6-1.0)	0.122
Random blood sugar, mg/dL	114.5 (97.0-152.0)	121.0 (107.0-152.0)	114.0 (96.0-152.0)	0.959

Data are presented in median (IQR) or *n* (%)

Similar to our study, the study by Faustine *et al.*[16] found that most patients' laboratory test showed an increase in neutrophils level but a decrease in lymphocytes level. According to several other studies, majority of the COVID-19 cases also displayed low lymphocytes level, especially in critical patients. The SARS-CoV-2 virus is known to induce the manifestation of cytokine storm during infection which causes an excessive inflammatory reaction. The persistent stimulation in this phenomenon may lead to a reduction in lymphocytes.[18] The higher value of neutrophil and lower value of lymphocyte in critical patients when compared to the lower value of neutrophil and higher value of lymphocyte resulted in a disparity of NLR value where the median NLR in critical patients was significantly higher. The value of NLR was found to be constantly higher in severe COVID-19 patients in several other studies. A study which focused on the predictive values of NLR found that NLR has good specificity and sensitivity, thus makingit a good predictive value on the severity and mortality of COVID-19 patients.[19]

The median platelet level of critical patients was found to be approximately 32 x 10^3/µL lower than non-critical patients in this study. Mild thrombocytopenia had been reported as one of the laboratory findings in 58-95% of severe COVID-19 cases. Viral infections are able to cause thrombocytopenia through various causes. The development of thrombocytopenia in response to viral infections is generally mediated via enhanced platelet clearance. Viruses are also known to interact with megakaryocytes and reduce platelet synthesis.[20]

The correlation between elevated levels of SGOT and SGPT in liver function test and COVID-19 is still a subject of debate and needs further investigation.[21] Direct cytopathic effect may not be the main mechanism for SARS-CoV-2 to induce liver damage since ACE2 receptors are found to be more abundant in cholangiocytes than in hepatocytes.[22] However, other factors such as Covid-19-induced cytokine storm, sepsis, or drug-induced liver injury should be considered as possible mechanisms of Covid-19-related liver injury. In addition, COVID-19 may also worsen underlying chronic liver disease which contributes to a higher mortality outcome.[23] The disparities of kidney function test in median urea between critical and non-critical patients may be due

to the role of COVID-19 in causing kidney damage. The infection of SARS-CoV-2 may contribute to the impairment of kidney through multiple mechanisms. The viral load of SARS-CoV-2 was found to be able to directly induce cytotoxicity of renal resident cell. Symptoms manifested in COVID-19 patients such as fever, vomiting, diarrhea, and shock could also cause kidney hypoperfusion. In addition, the cytokine storm induced by SARS-CoV-2 should also be considered.[24] All data on laboratory results are presented in Table 2.

Table 3. Treatment and Outcomes of Elderly COVID-19 Patients

	All Patients (*n* = 280)	**Critical (*n* = 51)**	**Non-critical (*n* = 229)**	***p* value**
Treatment				
Antiviral Therapy	19 (6.8)	5 (9.8)	14 (6.1)	0.353
Antibiotics	209 (74.6)	35 (68.6)	174 (76)	0.035
Vitamin C	262 (93.6)	43 (84.3)	219 (95.6)	0.038
Anticoagulants	48 (17.1)	14 (27.5)	34 (14.8)	0.066
Prognosis				0.000
Discharge	243 (86.8)	15 (29.4)	228 (99.6)	
Death	37 (13.2)	36 (70.6)	1 (0.4)	

Data are presented in median (IQR) or *n* (%)

A significantly higher overall mortality rate in elderly COVID-19 patients was found in this study and the study conducted by Azwar, et al.[5] in Jakarta when compared to the study by Guo et al.[6] in Hunan, China. The mortality rate in our study and the one in Jakarta was 13.2% and 23% respectively in contrast to the only 2.9% in Hunan.[5,6] There are several factors that we supposed may have contributed to higher mortality rate in Indonesian studies when compared to China. These factors included the lower usage of antiviral therapy and choice of antibiotics administered to patients. Only 6.8% from all the elderly patients in our study were given antiviral therapy with the choice of either Aluvia (Lopinavir-Ritonavir), Favipiravir, or Remdesivir. The administration of antibiotics was much higher with the choice of either Azithromycin, Levofloxacin, or combination of both. In comparison, 93.3% of the elderly patients in Hunan were given antiviral therapy and the most common antibiotic administered was moxifloxacin.[6]

A study by Wu *et al.*[25] found that patients experiencing mild symptoms received earlier initiation of antiviral therapy, thus indicating that early and timely administration of antiviral therapy may contribute to the slowing of COVID-19 progression into a more severe state and may improve the prognosis of patients under care. In a randomized controlled trial, which assessed the clinical efficacy and safety of moxifloxacin compared to levofloxacin plus metronidazole in treating community-acquire pneumonia (CAP), it was found that moxifloxacin monotherapy was more effective in treating CAP with a clinical cure rate of 76.7% compared to 51.5% in the levofloxacin plus metronidazole group. The administration of moxifloxacin also showed lower incidence of adverse events with a more convenient dosing regimen.[26] All data on the treatments and outcomes of patients are presented in Table 3.

STRENGTHS AND LIMITATIONS

The strengths of this study were being the first few studies to focus on the clinical profile of elderly COVID-19 patients and to further compare the characteristics of critical and non-critical patients in Indonesia and even Southeast Asia. The limitations of this study were not including the data of the second wave of COVID-19 in Indonesia, which was predicted to have higher

mortalities, and not further classifying the elderly age group into youngest-old (65-74 years), middle-old (75-84 years), and oldest-old (≥ 85 years) for a more in-depth comparison.

CONCLUSIONS

In conclusion, elderly patients are more susceptible to develop a severe outcome with COVID-19. There is a diverse number of possible factors which affect bodily functions in elderly patients and contribute to the progression of the disease. Hence, a multimodal approach in the treatment of elderly COVID-19 patients is very essential. The higher mortality rate in elderly patients should be able to be reduced by giving early and timely antiviral therapy with the addition of effective choice of drugs.

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ETHICAL CLEARANCE

The research protocol was approved by the Ethical Commission of Udayana University (No. 1010/UN14.2.2.VII.14/LT/2020).

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CONFLICT OF INTEREST

The authors declared that there was no conflict of interests that might bias or fabricate the information and work stated within the paper.

AUTHOR CONTRIBUTION

Study design and data collection: CAWP. Clinical advice and data collection: IKAS. Data analysis and report writing: DJ and RCS. Report writing and manuscript review: IKHA, GVP, IGWP, J, and PKW. Manuscript review and revision: DAFPS and IGNASD.

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Original Article

Moringa oleifera Leaf Ethanol Extract Inhibits *Toxoplasma gondii* Tachyzoites Replication

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ABSTRACT

The various infection routes of *Toxoplasma gondii* that are close to daily life strongly support the incidence of toxoplasmosis. The emergence of drug-resistant *Toxoplasma gondii* strains raises future concerns. Moringa leaf ethanol extract has been shown to have several anti-pathogen activities, which could have an anti-*Toxoplasma* effect. This research was conducted to analyze the anti-*Toxoplasma* effect of moringa leaf ethanol extract against tachyzoites replication in *Toxoplasma gondii* and the correlation between extract doses with the number of tachyzoites. Mice were divided into five groups. The negative control group (Group I) received CMC-Na solution. The positive control group (Group II) received spiramycin 100 mg/kg BW. The treatment groups received moringa leaf ethanol extract 250 mg/kg BW (group III), 500 mg/kg BW (group IV), and 1000 mg/kg BW (group V), respectively. Mice were injected with 1 x 10^5 tachyzoites/0.1 mL/mice intraperitoneally on the first day. Moringa leaf ethanol extract and spiramycin were given orally once daily for three days. The number of tachyzoites in the intraperitoneal fluid was calculated on the fifth day. The results have shown that there were significantly lower differences ($P < 0.05$) in group IV ($P = 0.021$) and group V ($P = 0.022$) compared to group I. There was also a significant negative correlation between the extract doses and the number of tachyzoites ($P = 0.000$; $r = -0.781$). *Moringa oleifera* leaf ethanol extract has an anti-*Toxoplasma* effect by inhibiting the tachyzoite replication at 500 mg/kg BW and 1000 mg/kg BW.

Keywords: *Moringa oleifera*; tachyzoites; *Toxoplasma gondii*

Highlights: This research provides the first study that proved the effectiveness of *Moringa oleifera* leaf ethanol extract in inhibiting *Toxoplasma gondii* tachyzoites replication.

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INTRODUCTION

Toxoplasma gondii is an obligate apicomplexan intracellular protozoan that causes toxoplasmosis in warm-blooded animals, including humans. It has been reported that approximately 30-50% of the world's human population is infected by this parasite.[1] The prevalence of toxoplasmosis seropositivity in humans has also reached 32,6% among 9 of 47 primary health centers in Makassar, Indonesia.[2] The high incidence of toxoplasmosis makes this disease a global health problem that needs attention because it can cause severe clinical manifestations in immunocompromised patients and permanent fetal disability.[3]

The various infection routes of *T. gondii* that are close to daily life could strongly support the incidence of toxoplasmosis. This zoonotic infection can occur in several ways: accidentally ingesting cat faeces that contain oocysts; eating undercooked meat that contains tissue cysts; transplacental transmission from an infected mother to a fetus; and other possibilities, such as receiving a blood transfusion or an organ transplant from an infected donor.[4]

Recent studies in clinical cases of toxoplasmosis have shown that drug resistance in *T. gondii* is ongoing. The emergence of drug-resistant *T. gondii* strains raises future concerns, not only in terms of treatment failure but also of increasing clinical severity in immunocompromised patients.[5]

Using natural ingredients such as plants or fruits as herbal medicine can be an alternative. This alternative is also considered less toxic than synthetic drugs and is better in terms of economy, practicality, and accessibility. Research on natural resources in Indonesia should always be carried out due to the vast and abundant biodiversity in Indonesia, which has excellent potential to bring benefits to the health sector.

Moringa (Indonesian: kelor) or *Moringa oleifera* is a plant often found in Indonesia. Parts of the plants that can be utilized are roots, stems, fruits, flowers, seeds, and leaves. *In vitro* study of *M. oleifera* seeds has been shown to inhibit the replication of tachyzoites.[6] The leaves are part of the plant that is often consumed by Indonesian people and have been shown to have anti-inflammatory, antifungal, and antibacterial effects.[7-9] It also acts as a larvicidal.[10]

A phytochemical analysis of *M. oleifera* leaf ethanol extract revealed alkaloids, phenolics, flavonoids, tannins, saponins, and terpenes as their bioactive compounds.[8,11-13] It has rutin as its major flavonoid, gallic acid as its major phenolic acid, and lutein as its major carotenoid. Several alkaloid compounds were also detected, such as pyrazoline alkaloids, piperidine alkaloids, and quinoline alkaloids.[14,15]

Quinoline alkaloids are one of the typical deoxyribonucleic acid (DNA) intercalating alkaloids that have cytotoxic and antiparasitic effects from their intercalating actions between the nucleotide pairs of the parasite.[16] *M. oleifera* leaf ethanol extract could have an anti-*Toxoplasma* effect through its DNA-damaging compounds. Therefore, this research was conducted to prove the potential of *M. oleifera* leaf ethanol extract as the new anti-*Toxoplasma* drug against tachyzoites replication in *T. gondii*.

MATERIALS AND METHODS

Experimental Materials and Tools

M. oleifera leaves were purchased from and identified by the Technical Implementation Unit of the Herbal Laboratory, Materia Medica Batu, East Java, Indonesia (reference number 074/656/102.20-A/2022). The RH strains of *T. gondii* tachyzoites were obtained from the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia. *Deutschland-Denken-Yoken* (DDY) mice (male, 20-30 grams, 2-3 months old) were purchased from a certified local experimental animal breeder. Other materials used in this research were spiramycin (500 mg,

Spirasin®, SANBE, Bandung, Indonesia) and sodium chloride 0.9% (Otsu®, PT Otsuka Indonesia, East Java, Indonesia).

Tools used in this research were Neubauer counting chamber (0.1 mm depth, Assistent®, Germany), cover glass (22 x 22 mm, OneLab®, Indonesia), light microscope (Nikon®, Nikon Corporation, Japan), disposable syringe 3 cc (Terumo®, Terumo Company, Tokyo, Japan), sterilized falcon tubes (NEST®, NEST Biotech, China), micropipette (DLAB®, DLAB Scientific Co., Ltd., Beijing, China), and hand tally counter (MARAS®, Togoshi Seiki, Taiwan).

Plant Extraction

M. oleifera leaves were washed, dried at 40ºC temperature using an oven, and ground into powder. The powder was macerated with 96% ethanol for 24 hours while being stirred occasionally. The first maceration results were filtered, and the residue was re-macerated with the same stage until the second maceration results were obtained. Both maceration results were mixed and evaporated using a rotary evaporator at 40ºC until they became a dense mass.[17]

Phytochemical Analysis

Table 1. Chemical Reaction Tests for Some Bioactive Compounds from *M. Oleifera* Leaf Ethanol Extract[7,17]

Constituent	Method
Alkaloids	Mayer test
Flavonoids	Ammonium test
Phenolics	Ferric chloride test
Steroids	Lieberman-Burchad test
Saponins	Froth test
Tannins	Ferric chloride test

The phytochemical analysis was performed to determine secondary metabolites (alkaloids, flavonoids, phenols, steroids, saponins, and tannins) present in the *M. oleifera* leaf ethanol extract using the color and precipitate reaction methods (Table 1).

Thin-Layer Chromatography Analysis

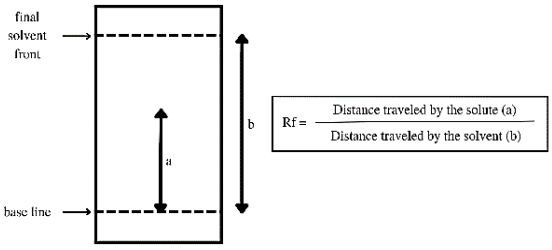

Figure 1. Retention factor (Rf) Values Calculation Formula in Thin-Layer Chromatography Analysis.

The thin-layer chromatography (TLC) analysis was performed to isolate the specific compounds present in the *M. oleifera* leaf ethanol extract. The extract was applied on silica gel 60 F_{254} plates as the stationary phase. The mobile phases were (chloroform: methanol: water) (50:65:10) for alkaloids and steroids, (n-butanol: acetic acid: water) (4:1:5) for flavonoids, phenols, and tannins. After leaving the developed plates to dry, they were observed under Ultra-Violet (UV) light at both 254 nm and 366 nm, then sprayed with iodine reagents to detect the bands.[18] The movement of the separated compounds was expressed by retention factor (Rf) values, which were calculated by the formula (Figure 1).

Animals

All the mice have been declared healthy by the veterinarian. The mice were acclimated for one week under laboratory conditions in wire-covered cages with paddy husk as bedding at a temperature of 24±4ºC, relative humidity of 44-56%, and 12 hours of light and dark cycle. Four mice per cage were given free access to distilled water and standard mouse food.

Parasite Culture

The tachyzoite culture was performed *in vivo* on male DDY mice. They were maintained by routine intraperitoneal passage every 72 hours. The number of tachyzoites was determined by counting them in a counting chamber, then diluted to sodium chloride 0.9% solution before being inoculated into the experimental mice.[19] The toxoplasmosis induction used in this research was 1 x 10^5 tachyzoites/0.1 mL/mice.

Toxoplasmosis Drug Reference

This research used spiramycin as the toxoplasmosis drug reference.[20] It was administered orally at a dose of 100 mg/kg BW. The tablets were crushed into powder and dissolved with sodium carboxymethyl cellulose (CMC-Na) solution until they became a homogenous suspension.

Experimental Design and Protocol

Mice were divided into five *T. gondii*-infected groups (n = four mice for each group). The infected group were divided as follows: negative control group (Group I) received CMC-Na solution 0.5 mL/mice orally as a placebo, positive control group (Group II) received spiramycin 100 mg/kg W, group III received *M. oleifera* leaf ethanol extract 250 mg/kg BW, group IV received *M. oleifera* leaf ethanol extract 500 mg/kg BW, and group V received *M. oleifera* leaf ethanol extract 1000 mg/kg BW.

Mice in the infected group were injected with 1 x 10^5 tachyzoites/0.1 mL/mice intraperitoneally on the first day. *M. oleifera* leaf ethanol extract and spiramycin were diluted into 0.5 mL of CMC-Na solution and given orally once daily for three days from the second to the fourth day. On the fifth day, all mice were sacrificed with cervical dislocation, and the intraperitoneal fluid was collected to count the tachyzoites.

Intraperitoneal Fluid Collection

The outer skin of the peritoneum was cut using scissors and tissue forceps, then gently pulled back to expose the inner skin lining the peritoneal cavity. The peritoneal cavity was washed with 3 mL of normal saline. The abdomen was shaken slowly to dislodge the tachyzoites into the saline solution. Aspiration of the intraperitoneal fluid was carried out using a syringe.[21]

Count of Parasites

The number of parasites was carried out by blind-direct examination using a counting chamber at 400x magnification of a light microscope.[19] Blind-direct examination means the counter does not know from which group the sample was taken. The mean of tachyzoites was expressed in a multiplication factor of 10^4.

Statistical Analysis

The research results were analyzed using Statistical Product and Service Solutions software (IBM Corp., Armonk, NY) version 25. The significant differences were statistically determined using the Kruskal-Wallis test, followed by the Mann-Whitney test. Values at $P < 0.05$ are considered significant. The Pearson correlation coefficient (r) was used to determine the correlation between extract doses and the number of tachyzoites.

RESULTS AND DISCUSSION

Phytochemical Analysis

Table 2. Phytochemical Analysis Results of *M. oleifera* Leaf Ethanol Extract

Constituent	Result	Interpretation
Alkaloids	Development of cream-yellow precipitate	Positive
Flavonoids	Development of red or pink color	Positive
Phenols	Development of dark green color	Positive
Steroids	Development of blue color	Positive
Tannins	Development of brownish-green color	Positive
Saponins	No formation of stable foam	Negative

The phytochemical analysis of *M. oleifera* leaf ethanol extract revealed the absence of saponins and the presence of alkaloids, flavonoids, phenols, steroids, and tannins (Table 2). These results were not aligned with the previous research, which revealed that the *M. oleifera* leaf ethanol extract contained saponins as its secondary metabolite compound.[7,10,12] The absence of saponins in this research could have occurred due to several factors that affected the extraction process. Factors influencing the maceration process results are temperature, solvent types and concentration, duration, and other factors.[22]

The extraction of *M. oleifera* leaves using methanol as a solvent with 72 hours of maceration showed positive saponin results on the phytochemical screening.[7] Positive saponin results were also obtained in the extraction using ethanol as the solvent with a maceration time of 72 hours.[12] Another study using ethanol as a solvent with 48 hours of maceration time showed negative screening results for saponins, which were the same as the results of the phytochemical analysis in this research using the same type of solvent but with 24 hours of maceration time.[11]

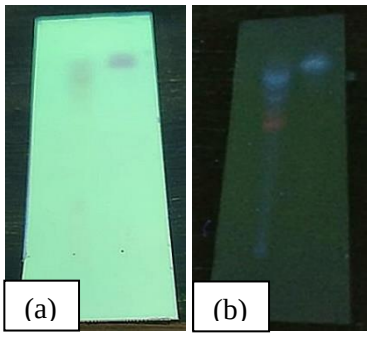

Figure 2. TLC results of alkaloids under UV light (a) 254 nm (b) 366 nm

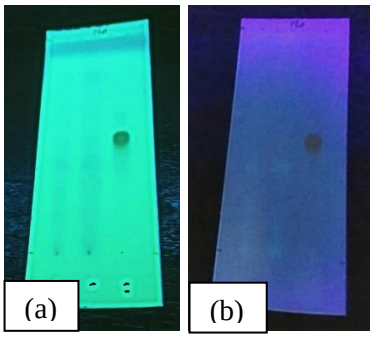

Figure 3. TLC results of flavonoids under UV light (a) 254 nm and (b) 366 nm

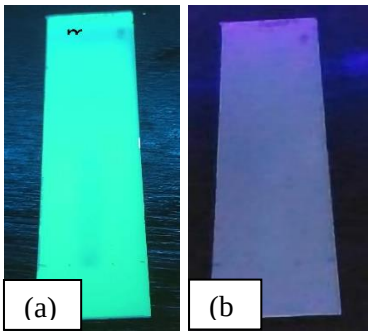

Figure 4. TLC results of phenols and tannins under UV light (a) 254 nm and (b) 366 nm

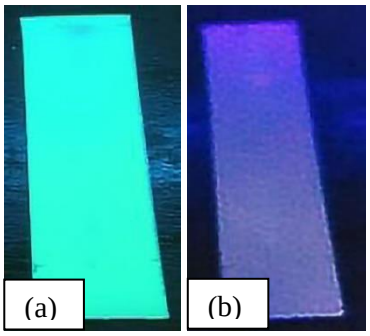

Figure 5. TLC results of steroids under UV light (a) 254 nm and (b) 366 nm

Table 3. Rf values and colors of peaks of each compound of *M. oleifera* leaf ethanol extract

Constituent	Rf values	Colors of peaks
Alkaloids	0.63	Red
	0.73	Blue
	0.81	Blue
Flavonoids	0.35	Yellow
	0.43	Yellow
	0.48	Yellow
	0.80	Yellow
	0.88	Yellow
Phenols	0.78	Blackish green
Steroids	0.75	Yellowish blue
	0.88	Yellowish blue
	0.93	Yellowish blue
Tannins	0.78	Blackish green

The positive bioactive compound results were also confirmed with TLC analysis as shown in Figure 2–5. Alkaloids were detected with Rf values of 0.63, 0.73 and 0.81. Flavonoids were detected with Rf values of 0.35, 0.43, 0.48, 0.80, and 0.88. Phenols were detected with Rf values of 0.78. Steroids were detected with Rf values of 0.75, 0.88, and 0.93. Tannins were detected with Rf values of 0.78 (Table 3).

Number of Tachyzoites

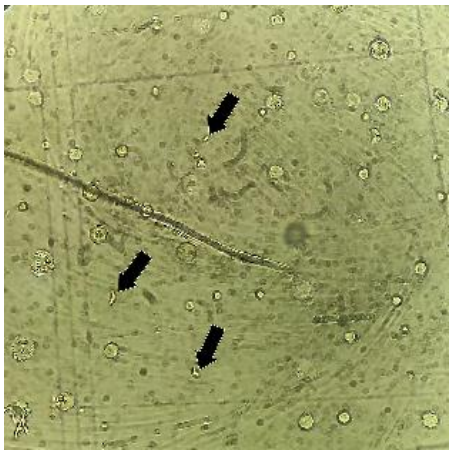

Figure 6. *T. gondii*-infected group. Tachyzoites (black arrows) on intraperitoneal fluid from the infected group under a light microscope with 400x magnification.

Identification of tachyzoites was carried out based on its distinctive morphology, which is a crescent-like shape, sharp at the anterior and blunt at the posterior, with a length of about 4-8 µm and a width of about 2-3 µm. The color of tachyzoites in the intraperitoneal fluid preparation was clear and transparent, accompanied by movement, indicating that the protozoa are still alive and have motility (Figure 6).

Table 4. Tachyzoites count results in the intraperitoneal fluid of the *T. gondii*-infected group (n = four mice per group)

Group	Infection	Mean±SD (x 10^4)
I	+	8.91±3.45
II	+	2.88±2.14*
III	+	6.41±1.25
IV	+	3.03±1.08*
V	+	2.28±0.12*

Group I: Negative Control Group; Group II: Positive Control Group; Group III: Treatment Group 1; Group IV: Treatment Group 2; Group V: Treatment Group 3; +: Infected; SD: Standard Deviation; *: $p < 0.05$ compared to Group I

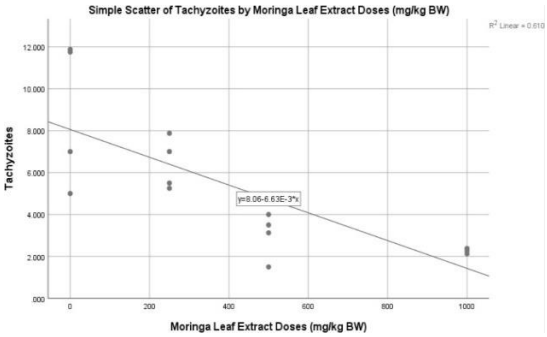

Figure 7. Simple scatter plot of tachyzoites count results (y-axis) by the *M. oleifera* leaf ethanol extract doses (x-axis). The number of tachyzoites tends to increase as the extract doses decrease, with $P = 0.000$ and $r = -0.781$

The highest tachyzoites were in group I with a total of 8.91±3.45 x 10^4. Group II was significantly lower than group I, with a total of 2.88±2.14 x 10^4. There were two extract treatment groups with a significantly lower number of tachyzoites compared to group I: group IV with a total of 3.03±1.08 x 10^4 and group V with a total of 2.28±0.12 x 10^4. The number of tachyzoites in group III, with a total of 6.41±1.25 x 10^4, was not significantly different compared to group I as shown in Table 4.

The toxoplasmosis intraperitoneally induction used in this research was 1 x 10^5 tachyzoites/0.1 mL/mice. Tachyzoites can invade almost all the host nucleated cells

and replicate rapidly.[3] The cell will eventually suffer damage and rupture due to this state, and the released tachyzoites will continue to look for other cells, perpetuating the cycle. The severity of the clinical symptoms depends on the degree of tachyzoite replication, which means that an individual's immune system is crucial in defining the clinical manifestations.[23]

Administration of spiramycin at 100 mg/kg BW orally for three days effectively inhibited tachyzoite replication, as proved by the significantly lower difference in the mean number of tachyzoites present in the peritoneal fluid between group II and group I (Table 4). Spiramycin is an antibiotic and an antiparasitic macrolide agent that is considered the drug of choice against *T. gondii* in pregnancy. The mechanism of action of the drug was to inhibit the synthesis of proteins and the growth of protozoan cells.[24] Its effectiveness as a toxoplasmosis drug has also been proven through previous research, which could reduce the number of *T. gondii* cysts in brain tissues.[20]

The doses of *M. oleifera* leaf ethanol extract used in this research were 250 mg/kg BW, 500 mg/kg BW, and 1000 mg/kg BW. The tachyzoite count differences were significantly lower in groups IV and V compared to group I. There was no significant difference in the mean number of tachyzoites between group III and group I (Table 4).

The results revealed that *M. oleifera* leaf ethanol extract at doses of 500 mg/kg BW and 1000 mg/kg BW could inhibit tachyzoite replication, but the dose of 250 mg/kg BW could not. These results occurred because the concentration of chemical properties in *M. oleifera* leaf ethanol extract increased with increasing doses (Figure 7). Our results are also align with other research on *Plasmodium yoelii*, in which the higher the dose of *M. oleifera* leaf ethanol extract, the greater the inhibitory activity against the parasites.[25]

Quinoline alkaloids (*3-methylquinoline*) are typical DNA intercalating compounds found in the *M. oleifera* leaf ethanol extract.[15] Their antiparasitic effect occurred through their hydrophobic, aromatic, and planar properties, which allow them to intercalate between the nucleotide pairs of the parasite. These cause mutations, such as deletions or frame-shift mutations, which will disrupt the replication of the parasite.[16] If the mutation occurs in an essential protein-coding gene, it causes the death of the parasite.[13] This theory also aligns with previous research, which proved that the moringa seeds extract promotes apoptosis-like death in *T. gondii* tachyzoites *in vitro*.[6]

A simple scatter plot showed a negative correlation between the extract doses and the number of tachyzoites (Figure 7). The decrease in tachyzoite count results, along with increasing doses of *M. oleifera* leaf ethanol extract, occurred because the concentration of alkaloids in the extract is directly proportional to the dose. The higher concentration of alkaloids causes more intercalated DNA in the parasites, resulting in more disruption in the replication of the tachyzoites.

This research proved the effectiveness of *M. oleifera* leaf ethanol extract in inhibiting tachyzoites replication. Future research needs to conduct more specific studies on the effect of the extract as an anti-*Toxoplasma*, whether isolating the specific antiparasitic bioactive compound and examining the histopathological variables on *T. gondii* target organs or other variables of its pathway of antioxidant properties.

STRENGTH AND LIMITATION

The blind-direct examination method in this research provided minimal occurrence of bias due to the subjective perspective of the researcher. This research also verified and explained the antiparasitic mechanism of *M. oleifera* leaf ethanol extract through the combination of phytochemical screening, TLC analysis, and the count of parasites.

Although we adopt the blind-direct examination for the count of parasites method, it does not provide full objective

results in this research. More objective parameters, such as histopathological or hematological variables, in order to examine the impact of the *T. gondii* tachyzoites replication, should be carried out in future study to support the results of this research.

CONCLUSIONS

The *M. oleifera* leaf ethanol extract has an anti-*Toxoplasma* effect by inhibiting the tachyzoite replication at 500 mg/kg BW and 1000 mg/kg BW.

ACKNOWLEDGEMENT

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ETHICAL CLEARANCE

The research protocol was approved by the Health Research Ethics Committee (HREC) of the Medical Faculty of Widya Mandala Catholic University, Surabaya, Indonesia (reference number 0326/WM12/KEPK/DSN/T/2022).

This research was carried out following the ethical principles outlined in the Council for International Organizations of Medical Sciences (CIOMS) and World Health Organization (WHO) International Ethical Guidelines for Health-Related Research Involving Humans.

FUNDING

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

AUTHOR CONTRIBUTION

Experimental design: LW, GLW, CPT. Extract preparation: GLW, CPT. Materials preparation: LW, CPT. Research implementation: LW, CPT, HP. Research supervision: LW, HP. Data analysis: LW, GLW. Manuscript writing: LW, CPT. Manuscript editing: LW, GLW, CPT, HP.

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Review Article

Impact of Hypertension and Cardiovascular Diseases to Immune Response in COVID-19 Vaccination: A Systematic Review

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ABSTRACT

To determine impact of hypertension and cardiovascular diseases towards effectivity and safety of COVID-19 vaccination. Systematic review based on PRISMA statement was done. Searching was conducted in PubMed, ScienceDirect, Scopus, and ProQuest and resulting in 6 studies involving 4,053 participants which deemed on good quality according to Joanna Briggs Institute tools for critical appraisal. After thorough analysis, we found that two out of four studies assessing mRNA-based vaccine found out that hypertension lower antibody response significantly. Two out of two studies assessing inactivated virus vaccine shown that hypertensive patients tend to have lower antibody titers compared to control. One of studies mentioned above found that antibody titer was not different between populations with cardiovascular diseases and control. Hypertension lessened response to COVID-19 vaccination regardless of vaccine type used. However, lack of studies on cardiovascular disease suggested that more studies should be conducted, along with hypertension, in-order to make meta-analysis possible to provide better evidence.

Keywords: antibody; cardiovascular disease; COVID-19; efficacy; hypertension

Highlights: The discovery of the phenomenon of hypertensive patients having lower antibody titers when vaccinated against COVID-19

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a global pandemic resurging from Wuhan, China. It involve mild to severe respiratory symptoms which could be left fatal at various cases. In order to end its pandemic status, World Health Organization (WHO) has mandated vaccine to be developed and applied. COVID-19 vaccine was first introduced in late 2020 and early 2021, with implementation vaccine begun ever since. In general, COVID-19 vaccine consists of either mRNA or inactivated virus as its base. Recent meta-analysis shown that mRNA-based and inactivated virus COVID-19 vaccines provided efficacy of 94.6% (95% CI 93.6–95. 4) and 80.2% (95% CI 98.0–98.4) respectively.[1] It was also proven safe in pregnancy. A meta-analysis studying mRNA vaccines shown that efficacy rate was 89.5% (95% CI 69.0–96.4) along with low risk of stillbirth and no addition to risk of miscarriage, earlier gestation at birth, pulmonary embolism, placental abruption, and maternal death.[2]

Emergence of newer variants, which known as variants of concern also did not damper its effectivity, with another study shown that fully vaccinated patients shown efficacy of 88.0%, 73.0%, 63.0%, 77.8%, and 55.9% to alpha, beta, gamma, delta, and omicron variants respectively. Boosted patients were more immune to delta and omicron variants with effectivity of 95.5% and 80.8% respectively.[3] Systematic review by Mohammed shown that COVID-19 vaccines deemed to suppress infection rate among population and severity, hospitalization rate, and mortality among COVID-19 patients.[4] A study by Gram found that COVID-19 vaccines successfully reduced hospitalization rates for 14–30 days by 98.1%, 98.1%, and 95.5% for alpha, delta, and omicron variant respectively.[5] Even though several reports have shown that COVID-19 vaccine effectiveness wanes as weeks pass, COVID-19 has been proven to protect population from severity and mortality because of COVID-19 and to improve health and well-being.[5,6]

Response to COVID-19 vaccination was not the same for every recipient, there were several factors playing part. A study in Japan showed that age which older than 60 years, hypertension, high HbA1c (>6.5%), and sedentary lifestyle were significant for inhibiting immune response in COVID-19 vaccination.[7] Other studies mention age, sex, nutritional status, obesity, gut microbiota, polymorphisms, and immune system as determinants.[8] There were several limitations for populations with high blood pressure and cardiovascular disease to take COVID-19 vaccines, even though the limitations have been leniently loosened.[9,10] However, impact of hypertension and cardiovascular diseases to immune response to COVID-19 vaccination is not fully known. Therefore, we conducted a systematic review to determine its relationship to provide better knowledge on COVID-19 vaccination.

MATERIALS AND METHODS

Materials

We conducted systematic review based on The Preferred Reporting Items of Systematic Review and Meta-Analysis (PRISMA) Statement.[11] Searching was conducted on PubMed, Scopus, ProQuest, and ScienceDirect published in 2022 using specific keywords and medical subheading (MeSH).

Methods

Searching was conducted on PubMed, Scopus, ProQuest, and ScienceDirect using specific keywords and medical subheading (MeSH) terms (Table 1). We applied following inclusion criteria: (1) clinical studies; (2) studying population of people with hypertension and/or cardiovascular disease; (3) studying all sort of COVID-19 vaccine as intervention; (4) studying effectivity as outcome. In addition, we applied following exclusion criteria: (1) co-

existence of other comorbidities; (2) language other than English. Selected studies were appraised using The Joanna Briggs critical appraisal tools.12 Studies were extracted for characteristics and result. Qualitative analysis was conducted to determine the relationship between variables.

Table 1. Keywords Being Used for Searching.

Database	Keywords	Filters
PubMed	("COVID-19 Vaccines"[Mesh]) AND (("Cardiovascular Diseases"[Mesh]) OR "Hypertension"[Mesh])	
Scopus	("COVID-19 vaccine") AND (("hypertension") OR ("cardiovascular disease"))	
ProQuest	("COVID-19 vaccine") AND (("hypertension") OR ("cardiovascular disease"))	"Scholarly Journals", "COVID-19 Vaccines"
ScienceDirect	("COVID-19 vaccine") AND (("hypertension") OR ("cardiovascular disease"))	"Research Articles"

RESULTS AND DISCUSSION

We found total six studies after application of searching strategies and criteria (Figure 1).[13–18] There were three studies across Asia, two across Europe, and one American study involving total 4,053 subjects. There were two studies studying CoronaVac, which is an inactivated virus, and four studies studying BNT162b2 vaccine which is based on mRNA. All studies were eligible to be included in this study after appraisal using Joanna Briggs Institute critical appraisal tools (Table 2). Studies characteristics could be seen in Table 3. All four studies studying mRNA vaccines shown that hypertensive patients tend to have lower antibodies level compared to control, but only two deemed significant.[13–15,17] On the other hand, hypertensive patients which underwent inactivated virus COVID-19 vaccination shown significantly lower antibody level compared to control based on both two studies.[16,18] One of the studies stating that cardiovascular diseases yet to contribute on antibody level. [16] All results could be seen on Table 4.

Table 2. Critical Appraisal Results of Selected Studies.[12]

Studies	Aspect											Overall
	1	2	3	4	5	6	7	8	9	10	11	
Watanabe et al, 2022	Y	Y	Y	Y	Y	Y	Y	Y	Y	NA	Y	Include
Ebinger et al, 2022	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Include
Delgado et al, 2022	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Include
Soegiarto et al, 2022	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Include
Parthymou et al, 2022	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Include
Rifai et al, 2022	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Include

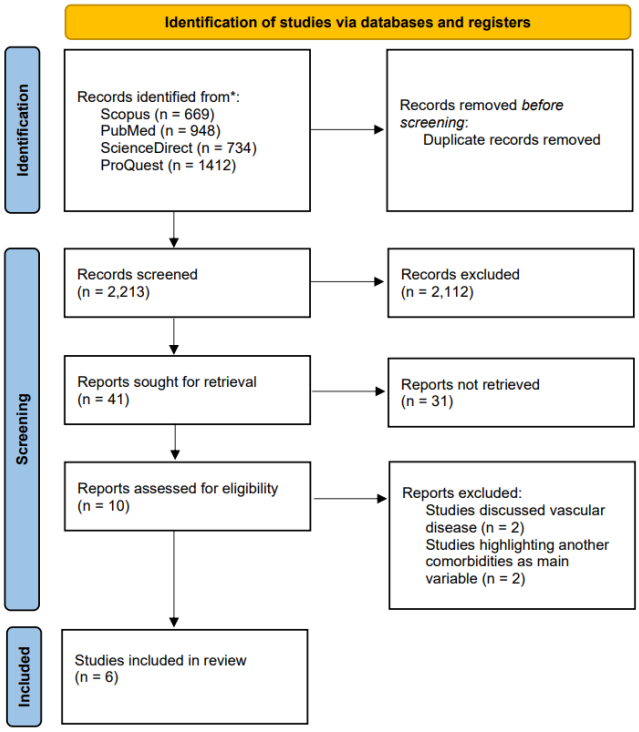

Figure 1. Schematic Workflow of Studies' Finding.[11]

Table 3. Characteristics of Selected Studies

Author	Year	Location	Sample Size	Ab	Vaccine	Dose	Measurement (Weeks after Dose 2)	Age (Years)	Male (%)	BMI (kg/m^2)	Hyper-tension (%)	Dia-betes (%)	Smokers (%)
Watanabe et al[13]	2021	Japan	68	IgS	BNT162b2	2	1-4	29.0 (17.0)	39.5	22.4 (5.5)	15.3	2.4	31.7
Ebinger et al[14]	2022	USA	843	IgS	BNT162b2	2	1, 2, 8, 16, 24, 32, 40	45.0 (13.0)	30.0	-	15.2	-	-
Delgado et al[15]	2022	Spain	2174	IgS	BNT162b2	2	12	45.9	19.9	24.1	8.1	-	22.2
Soegiarto et al[16]	2022	Indonesia	101	IgG	CoronaVac	2	4, 12, 20	47.7 (18.9)	59.5	-	23.7	17.8	10.9
Parthymo u et al[17]	2022	Greece	712	IgS	BNT162b2	2	3, 12	50.8 (11.4)	37.6	26.7 (4.9)	16.2	7.0	34.4
Rifai et al[18]	2022	Indonesia	155	IgG	CoronaVac	2	8, 24	39.0 (9.2)	48.3	27.9 (7.3)	18.7	-	-

Table 4. Results of Selected Studies

Author	Vaccine	Results
Watanabe et al[13]	mRNA	Hypertensive patients presented lower antibody response compared to normotensive (650 ± 1192 vs 1911 ± 1364, p = 0.001). Hypertensive patiens shown significant beta coefficient on univariate and multivariate analysis with -1033.16 (p = 0.005) and -973.27 (p = 0.036) respectively.
Ebinger et al[14]	mRNA	Hypertensive patients shown significant beta coefficient on multivariate analysis with -0.17 and SE of 0.08 (p = 0.041).
Delgado et al[15]	mRNA	Hypertensive patients shown insignificant fold changes with -1.02 (p = 0.8584).
Soegiarto et al[16]	Inactivated	Hypertensive patients shown significant beta coefficient on multivariate analysis with -11.208 (p = 0.038). Patients with history of cardiovascular diseases shown non-significant beta coefficient on multivariate analysis with -10.040 (p = 0.969)
Parthymou et al[17]	mRNA	Hypertensive patients shown insignificant beta coefficient on multivariate analysis with -0.0454 (p = 0.3276).
Rifai et al[18]	Inactivated	Patients with high systolic blood pressure and high diastolic blood pressure shown significant correlation with lower antibody response with R coefficient of -0.172 (p = 0.016) and -0.139 (p = 0.043) respectively second months after vaccination, and R coefficient of -0.284 (p = 0.046) and -0.475 (p = 0.006) respectively six months after vaccination.

Hypertension accounted for lower antibody response in COVID-19 vaccination which was stated in all adjuvant vaccine studies and in most of mRNA vaccine studies. However, some studies showed that there were reports of non-significant differences between groups. Study by Delgado *et al* involving mRNA vaccines reported there were positively increased anti-S protein antibody level after vaccination in patients with older age, more BMI, and arterial hypertension, but exclusive to infected subjects which explained the non-significant of result.[15] However, another mRNA vaccines study by Parthymou *et al* reported that non-significant difference of immune response between hypertensive and non-hypertensive groups was due to confounding factors and differences in size, age, and self-reporting of the populations.[17]

It is known that vaccine response was based on cascades of immune system responses. It depends on the role of T helper 2 (Th2) and B cells to provide a connection to produce long-lived plasma cells which secrete antibodies with high affinity.[19] However, there is differences between mRNA vaccine and adjuvant vaccine in terms of immune response, whereas mRNA vaccine is stimulating cellular immune response and adjuvant vaccine stimulates humoral immune response. Hypertension played role in impairing both of mechanisms. Hypertensive patients had lower Th2 and interleukin 4 (IL-4) levels significantly, thus immune response was impaired.[20] In addition, hypertensive patients developed proinflammatory T cells as a result of high blood pressure which could produce cytokines relating to Th1 and Th17 such as interferon-gamma and interleukin 17A (IL-17A).[21] Another piece of evidence found that angiotensin II, which was over-activated on hypertension, was accounted for the increase in Th1 production and Th2 suppression.[22] Th1 will inhibit humoral immune response, thus inhibiting antibody production.[23] Many other evidences have stated similar hypertension's role in modulating T cell immune metabolism.[24–26] In addition, another study stated that chronic inflammatory due to hypertension will release cytokines due to endothelial dysfunction which included reactive oxidative species (ROS) and interleukins such as IL-1-beta, IL-6, IL-8, IL-17, IL-23, and TNF-alpha. All of these cytokines were responsible for dysfunction of angiotensin II which worsen blood pressure. These cytokines also could alter immune response in hypertension.[27]

Besides applying a damper effect to the immune response of COVID-19 vaccination, hypertension accounted for more severe COVID-19 outcomes.[28–29] Hypertension was found to be the most common comorbidity observed in COVID-19 infection and alongside cardiovascular disease accounted for 2.36 folds higher chance of mortality compared to control.[30] Not only as comorbid, hypertension also played its role as an adverse event towards COVID-19 vaccination. A meta-analysis showed that 3.20% of patients who underwent COVID-19 vaccination showed an abnormal increase in blood pressure, with 0.6% of patients developed hypertensive urgencies and emergencies.[31] This was further confirmed by other studies which stated similar findings.[31–36] Therefore, hypertension provided difficult challenges for healthcare workers who administered COVID-19 vaccine. Not only being impactful to lessen antibody response, but it also accounted for more severe COVID-19 outcomes and more risk towards adverse events. Therefore, hypertension in populations who were prospective for COVID-19 vaccine administration should be taken cautiously and seriously in order to prevent adverse events or severe outcomes. Vaccine developers should be able to make sure that COVID-19 vaccine provided the expected antibody response when given to hypertensive populations in a safe fashion.

Relation between cardiovascular diseases and antibody response is still yet to be known with unclear mechanisms. However, it is

suspected to accounted towards blood circulation and component. Therefore, more studies should be conducted further to determine relation and mechanism of cardiovascular disease impact towards COVID-19 vaccination.

This was a systematic review which provided information on the impact of hypertension and cardiovascular diseases and hypertension towards COVID-19 vaccine response. However, there were limited studies available. In addition, studies included in this review is limited to wide-scope of hypertension which is yet to be graded or classified. This make reviewer could not determine stage which is more responsible for impairment of immune response after vaccination. Therefore, it was recommended that more high-quality studies which involved graded hypertension should be done to make meta-analysis possible to provide a better understanding and knowledge of this field.

STRENGTH AND LIMITATION

The strength of this study was a comprehensive literature search and a bias study was carried out. The limitation of this study is that the amount of literature found is very small.

CONCLUSIONS

Hypertension was linked with lesser antibody response to COVID-19 vaccination in both mRNA-based and inactivated virus-type vaccines. However, cardiovascular diseases are yet to be linked to COVID-19 vaccination response. Due to the few studies which have been retrieved, more studies should be conducted to make a meta-analysis with higher and stronger evidence to be conducted to provide better knowledge on this field.

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This study did not receive any funding.

CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

AUTHOR CONTRIBUTION

Writer, literature searcher, collecting data from literature: KDF. Conceptor and supervision: GS. Review and supervision: LW and DP.

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Original Article

Electronic Nose (E-Nose) for Quality Detection of Tuna *(Thunnus thynnus)* Contaminated Bacteria

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ABSTRACT

Tuna *(Thunnus thynnus)* is a food that is often consumed raw to support raw food diet activities, so it has the potential to be contaminated with *Salmonella typhi* bacteria. Fish can be contaminated by bacteria due to their high water and protein content. Indonesia is the world's main tuna producer. Salmonella typhi detection in fresh tuna in Indonesia must be negative for Salmonella microbial contamination in order to meet food safety requirements. Microbial testing has drawbacks, such as long delays. An electronic nose was used to detect *Salmonella typhi* bacteria in tuna fish. The sample used consisted of 3 kinds of samples: *Salmonella typhi* bacteria, tuna, and tuna with *Salmonella typhi* contamination. The research was conducted with a shelf life of 48 hours and a sensing period every 6 hours with a sensor array of 8 sensors. The sensor output data is processed using the PCA (Principal Component Analysis) method. Through the PCA method, each variation of bacterial treatment can be classified. The result of the cumulative percentage variance of the two main components (PC) in the classification test between *Salmonella typhi*, tuna, and tuna with *Salmonella typhi* bacteria contamination was 90.5%. The most influential sensors in this study are TGS 825 for PC1 with a loading value of 0.625 and TGS 826 for PC2 with a loading value of -0.753. Therefore, it can be concluded that an electronic nose can classify between pure tuna and tuna contaminated with *Salmonella typhi* bacteria.

Keywords: array gas sensor; electronic nose; principal component analysis; *Salmonella typhi*; Tuna *(Thunnus thynnus)*

Highlights: The most influential sensors in this study are TGS 825 for PC1 with a loading value of 0.625 and TGS 826 for PC2 with a loading value of -0.753. Therefore, it can be concluded that an electronic nose can classify between pure tuna and tuna contaminated with Salmonella typhi bacteria.

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INTRODUCTION

People's habits and behavioral patterns have changed as a result of the times. Knowledge of something, including information about food, enables a person to alter his behavior. Humans require food to survive. Food is therefore crucial for humans. The raw food diet is one change in lifestyle that can be influenced by food knowledge.

A raw food diet is a way of eating that involves only consuming unprocessed, uncooked, or unheated food. Due to the growing propensity of people to eat healthy foods and create a society that values health, this trend has spread more widely in recent years. This is in line with research on the perceptions of the Surabaya population towards organic food.[1] By using a quantitative exploratory research method and a multidimensional scaling technique, it was discovered that respondents' perceptions of the quality and safety of their food were, on average, 3.26, with the highest values occurring between the intervals of 2.6 and 3.4. This suggests that the Surabaya population, or respondent, has perceptions of food quality that are very favorable.

Despite having a higher nutritional value than processed food, raw food has the potential to be contaminated with pathogenic bacteria, according to microbiological hazard identification research by the Foodborne Illness Investigation (FII). The cleanliness and absence of pathogenic microorganisms that could potentially cause disease are indicators of good food quality. This illness is referred to as a foodborne illness.

The majority of the bacteria identified as histamine producers are gram negative (87% of isolates), and the majority of these isolates (80%) are members of the *Enterobacteriaceae* family. *Morganella morganii* was the organism most frequently and actively producing histamine in canned tuna fish. Along with several strains of *Enterobacter cloacae* and *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and other potent histamine-producing bacteria were also discovered during the canning process. Some workers have previously experienced similar outcomes. 73% of the former histamine-producing strains that were isolated and identified were from *Morganella* and *Enterobacter spp*.

Salmonella typhi bacteria is one of the microorganisms that frequently contaminate raw food. The maximum contamination limit for *Salmonella sp* is negative per 25 grams of food. *Salmonella typhi* is a gram-negative bacterium that causes typhoid fever. The disease may occur anywhere in the world, but is most prevalent in developing countries, including in Indonesia. The incidence of typhoid fever in Indonesia is thought to be between 300 and 810 cases per 100,000 people per year, with a range of 600,000 to 1,500,000 cases per year. Effective prevention measures are required because this disease has a 1-5% patient mortality rate.[3]

Salmonella typhi bacteria may be present in tuna (*Thunnus thynnus*), which is one of the foods frequently consumed in a raw state to support raw food diet activities. Additionally, due to the high water and protein content of fish meat, bacteria can easily contaminate fish.[4] Indonesia is the primary producer of tuna in the world, according to the Food Agriculture Organization (FAO).

Salmonella sp must test negative in every gram of fresh tuna for the specific type of *Salmonella* microbial contamination test in order to meet quality and food safety requirements in Indonesia. An organization called BKIPM (Fish Quarantine Agency, Quality Control and Safety of Fishery Products) is in charge of vetting the safety and quality requirements for fishery products.[5] Microbiological tests in accordance with Indonesian National Standard (INS) are used as a detection, isolation, and confirmation mechanism for *Salmonella bacteria* in *tuna*. However, due to the lengthy turnaround time (1-3 days) required for test results and the high absorption capacity of both the domestic and international tuna markets, there are a number

of weaknesses with the microbiological method of detection.[6] The United States was Indonesia's primary export market for fisheries products. The quantity of tuna products exported by Indonesia to the United States from 8,504 tons in 2015 to 10,788 tons in 2016, the number of states increased. As of May 2017, Indonesian tuna exports were registered at 65,875 metric tons, valued at USD 226 million. It was anticipated that the volume of fishery goods exported would grow, possibly leading to more products being returned. The primary cause of the rejection of shrimp commodities was microbial contamination. The most frequently reported microorganisms that caused the illnesses were Salmonella, E. coli, and Vibrio cholerae. The challenge faced by exporters from Indonesia, the occurrence of variations in sample collection techniques and testing microorganisms between Indonesian laboratories and destination nations. Testing technology and laboratory infrastructure (methods and tools) change in order to provide results with varying degrees of precision.[7]

As anticipated, precooking tuna fish significantly reduced the numbers of all bacterial groups under investigation. However, the bacterial burden in tuna fish continued to rise following the precooking stage. For precooking tuna fish, high heat (100–105 °C for 110 min) is typically employed. The time-temperature link is sufficiently strong to significantly lower the bacterial load. After precooking, tuna was allegedly left at room temperature, allowing damaged germs to quickly recover, multiply, or get decontaminated by the environment. *Mesophilic* and *psychrotrophic* bacteria counts rose concurrently during canning, but with a modest advantage for the latter. The temperature of the water where the tuna fish was caught (between 8 and 15 °C) and the length of time it was kept frozen are likely to be to blame for the larger number of *psychrotrophic* organisms. In frozen tuna fish, *Enterobacteriaceae* and coliform counts have always been low and only made up 0.34 percent of the overall bacterial burden. But as the tuna was handled during the canning process, the number of *Enterobacteriaceae* grew until it made up 2.18 percent of the bacterial load.

An instrument called the *Electronic Nose* (*E-Nose*) mimics how the sense of smell functions. As an alternative to olfactory receptors, which are responsible for detecting smells or scents, the *E-Nose* is made up of a variety of gas sensors. The aroma picked up by numerous gas sensors will then take the form of a specific pattern.[8] *E-Nose* has applications in the areas of microbiological detection and food safety.[9,10] *E-Nose* has the benefits of being non-destructive, real-time, quick, and inexpensive.

According to the research's conclusions, E-Nose could differentiate between samples of beef, pig, or a combination of the two based on the fragrance pattern each sample created. *E-Nose* has been extensively used in a variety of fields and industries, including those related to food, drink, chemicals, defense, health, etc.[11] Research on early detection and classification of pathogenic fungi that attack strawberry farming is one application of *E-Nose* in the food industry for monitoring production processes.[12]

Principal Component Analysis (PCA) is one technique for analyzing the data produced by Electronic Nose. By using the PCA method, it is possible to replace some of the original, correlated variables with a new, smaller set of uncorrelated variables. In order to make it simpler to interpret the data, the main goal of this method is to reduce the dimensions of the interconnected and numerous variables. The authors intend to conduct research on the pattern of data generated by the *E-Nose* gas sensor array in an effort to detect the content of *Salmonella typhi* in tuna *(Thunnus thynnus)* using PCA method because the national standard for microbial testing used to detect the content of *Salmonella typhi* in *tuna* (Thunnus thynnus) has a drawback, namely it takes a long time.

By using a gas sensor that can react to specific scents, the *E-Nose* device imitates how a mammal's nose detects smells.[13] As an alternative to olfactory receptors, which are responsible for detecting smells or scents, the *E-Nose* is made up of a variety of gas sensors. The aroma picked up by various gas sensors will then take on a particular pattern. In order to analyze and identify the signal response produced by the *E-Nose* to a specific scent, pattern recognition software will be used. *E-Nose* has a wide range of uses, such as assessing food quality, tracking air pollution, and identifying various gases and toxins.[14]

E-Nose uses the biological nose's operating principle to characterize various gas mixtures. The human smell system is divided into three layers, namely.[15]

1. A layer of approximately one billion olfactory cells
2. Olfactory vesicles have three main functions: to control, regulate, and amplify messages from olfactory cells.
3. The brain's olfactory center, which defines signals and organizes the different types of smells that can be detected.

The *E-Nose* has three main components, sample handling, detection systems, and data processing systems.[16] The *E-Nose* operates on a similar principle to the human nose, which contains a variety of receptors for identifying scents. The sensors on the *E-Nose* serve as a replacement for these receptors, and each remaining receptor reacts differently to the same aroma vapor.

The stages in the *E-Nose* system are signal pre-processing, signal processing, and pattern recognition system processing. The sensor array is initially exposed to the scent that needs to be detected. These sensors perform nearly as well as olfactory cells in humans. An *analogue* to *digital converter* (ADC) will convert the *analogue* data from the sensor into *digital* data, which can then be saved to a computer and used for further analysis. Preprocessing will be done on the ADC data first. Processing is used to get the signal ready so that a pattern recognition machine can process it quickly. Similar to the vesicle layer in the human sense of smell, this stage performs almost the same functions. The pattern recognition system processes the data in the final step. This section seeks to categorize and forecast unidentified samples. This component's function is comparable to that of the brain's olfactory center.[17]

The following list of necessary components is provided by Gardner and Bartlett as a definition of an electronic nose device's fundamental requirements:

1. A sensor array system with an aroma delivery system that transfers volatile aromatic molecules from the source material.[18]
2. The environment in which the sensor is located: normally, the temperature and humidity are fixed, as this would prevent the aroma molecules from being absorbed otherwise.
3. Electrical signals are transformed into chemical signals by electronic transistors.
4. A *digital converter* that transforms electrical (*analogue*) signals into *digital*.
5. A computer microprocessor that reads the digital signal and outputs it after statistical analysis is carried out to classify or identify a sample.

Each of the gas sensors in the *E-Nose* will react to changes in smell or aroma. Each gas sensor will respond to aroma or odor by changing its resistance.[19]Each gas sensor's resistance will fluctuate, changing the voltage as a result. This voltage changes yielded data in the form of *digital* computer data. From this point forwards, a data processing device will be used to process the data. Figure 1 displays the block diagram for the *E-Nose*.

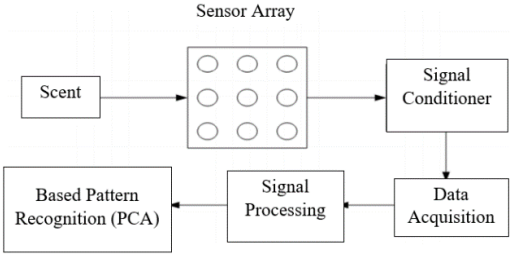


Figure 1. Electronic Nose Block Diagram

A sensor is a piece of technology used to identify symptoms or signals brought on by changes in energy, including electrical, mechanical, chemical, biological, and other types of energy. A transducer is a device that, when powered by an energy in a transmission system, transmits the energy to the following transmission system in the same form or in a different form. This energy transmission may be thermal, optical (radiation), mechanical, chemical, or electrical (heat). In other words, the sensor is a part that can be used to transform a certain quantity into an analogue unit so that an electronic circuit can read it. The sensor is the main part of a transducer, and the transducer is a supporting system that enables the sensor to have the desired output and to be directly readable at the output.

Principle component analysis (PCA) is a mathematical technique that transforms a set of potential correlated variable observations into a set of principal components, which are linearly uncorrelated variable values.[10] Data from *electronic nose* output is processed using PCA, which can classify data based on the type and concentration of bacteria.

In order to obtain a smaller amount of data with the greatest data variation in the new coordinate system, PCA reduces variable data by transforming it linearly. Figure 2 displays thePCA transformation's outcomes.

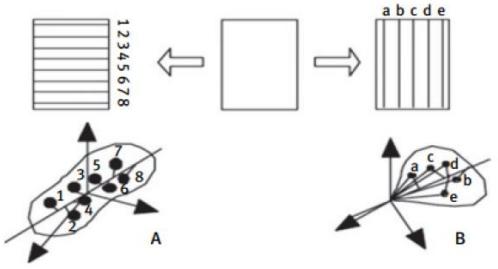


Figure 2. Results of PCA transformation.[20]

The PCA method aims to reduce the dimensions of the observed variables, thus simplifying them. This is accomplished by changing the original independent variable into a new variable that is completely uncorrelated, thereby removing the correlation between the independent variables. These components become new independent variables once several components of the PCA results that are independent of multicollinearity are obtained. One benefit of the PCA method is that correlations can be effectively eliminated without reducing the number of initial variables.

Direct observation of the *E-Nose* sensor output makes it challenging to distinguish between different samples. Since the gas sensors used by the *E-Nose* are non-selective and cross-sensitive, multivariable pattern recognition techniques like PCA are required to represent the data for simple analysis.

MATERIALS AND METHODS

Materials

The materials used by the bacteria in this study were *Salmonella typhi* isolates, tuna fish (*Thunnus thynnus*), cotton, plastic wrap, physiological graphic water, TSA, TSB, 70% alcohol, tissue, distilled water, aluminum foil.

Methods

The first sample, specifically *Salmonella typhi bacteria*, will be cultured and incubated for 48 hours until it forms a biofilm; once a biofilm has formed, the bacterial culture will release a more overpowering odor. Both the second and final samples of tuna contain *Salmonella typhi* bacteria. The electronic nose functions as a "sensing system" made up of three components: a sampling system, a chemical gas sensor array that produces a range of signals when exposed to gases, vapors, or scents, and a system for classifying the resulting pattern. The sensor in the *E-Nose* will generate a voltage that varies depending on the sample time and the sensor's sensitivity in order to detect odors from the sample. At each data retrieval, a voltage will be measured and sent to a computer for analysis.

The process for using the gas sensor array system and its basic operation is as follows: after turning on the power source, the tool

warms up the sensor for a minute before the sensor can be used to detect reactants. Eight sensors are used, and each one will send a response voltage that is converted to digital form. There are 8 TGS sensors in this sensor array. A 10 ml beaker was used to hold the sample. Eight gas sensors will then enter and pick up the aroma from the sample. Excel will be used to plot the output voltage that is produced. In Figure 3, systematic data collection is displayed.

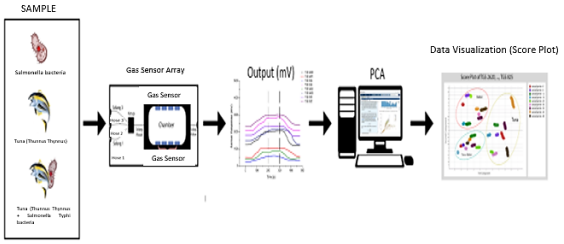


Figure 3. Systematics of data collection

The target clean air will be inhaled by hose 3 during the preheating process as a control, and it will flow through the inlet hose into the chamber with the valve shutting off hoses 2 and 1 to prevent the clean air from mixing with the smell of the sample. Because all sensors are in a steady state during that time, the preheating process takes 60 seconds. The valve closes hose 1 and opens hoses 2 and 3 during the sensing process to allow the target odor to enter the chamber. As the smell of the sample gradually fills the chamber, the sensor responds by outputting a specific voltage.

The sensing process takes 300 seconds to complete. The valve closes hoses 1 and 2 and opens hose 3, draining the desired clean air into the chamber where it will be expelled through the outlet hose during the purging process. The gas inside the chamber is supposed to be cleaned with fresh air during the purging procedure. 120 seconds pass during this process. alternately flowing the target gas into the chamber through a number of processes. The sensing mechanism by the gas sensors kicks in when the target gas is in the chamber, allowing each gas sensor to generate an output in the form of a voltage.

Sensor Response Test

Tuna (Thunnus thynnus), Salmonella typhi biofilm, and a combination of the two were tested for sensor response with each sample being given a time variation of 0, 6, 12, 18, 24, 30, 36, 42, and 48 hour.

Sensor Validity Test

The output data from the sensor is then tested to prove the validity of the data. The data validity test includes sensor precision tests and sensor accuracy tests. Accuracy is the degree to which the results of a measurement closely resemble the actual value of the quantity being measured. In order to assess the correctness of the findings from the analytical tests that have been conducted, it is required to evaluate the percentage recovery (% recovery). At 10% recovery tolerance, or between 90% and 110%, accuracy is regarded as being good.

Data analysis

The results of the sample test using the array of sensors are then processed using a personal computer, and the data is stored as a spreadsheet table in the form of a voltage value obtained from the output of the sensor series. The following are the steps in data processing:

1. Feature extraction is the process of obtaining the most pertinent and instructive values that can represent the general characteristics of the sensor response.
2. Data representation using radar graphs can show differences in the shape of the web between one and the other and serves to display data from 8 sensors in the gas sensor row. This type of radar chart displays a graph with the appearance of a spider's web. in comparison to other samples. The average value of the feature extraction results is used to create the radar graph.
3. Using the Principal Component Analysis (PCA) technique, data on variations in the aroma of tuna *(Thunnus thynnus)* were categorized. By reducing the number of variables, the PCA method is used to

reduce the dimensions of the data. Next, the variance value of each principal component (PC) is obtained. The initial data set used to create PCA is the value obtained from feature extraction. Orange Data Mining and Minitab were the two pieces of software used in this study's PCA analysis. PC data, eigenvectors, eigenvalues, and cumulative proportion of PCA data are obtained using Minitab software.

The score plot graph is used as the final classification outcome and is used to represent the data using the principal component graph of the first and second principal components' values.

RESULTS AND DISCUSSION

Gas Sensor Response Test Results

The goal of the sensor response test is to ascertain the *E-Nose* sensor's response value when testing samples. In comparison to samples or compounds with weak aromas or low concentrations of gaseous compounds, E-Nose sensors respond more strongly (signal amplitude) to samples with stronger aromas and higher concentrations of gaseous compounds.

Preheating Gas Sensor Array

Before the sensor is used to detect and respond to gases that alter the output's resistance value, it is heated. A stable output during data collection is achieved by optimizing the preheating time of each sensor. To get the sensor ready for a steady state condition, preheating treatment is applied. Preheating is done in a clean environment with a room temperature. Figure 4 depicts the preheating process graph.

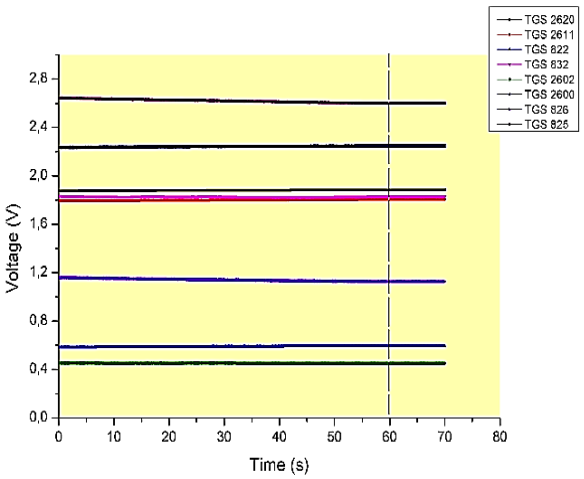


Figure 4. Graph of Preheating Sensor

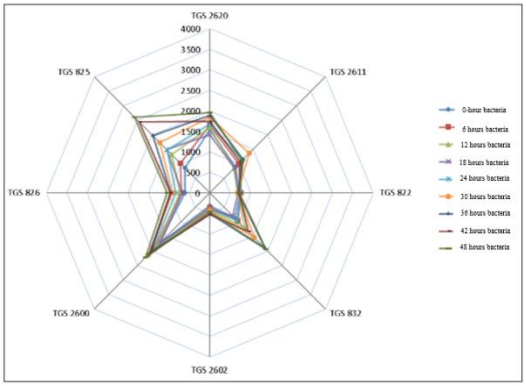
Time sensing is performed after 60 seconds because the sensor is ready to use at that point, as shown in Figure 4 where all sensors produce a stable voltage output at 50–60 seconds.

Sensing the Gas Sensor Array to the Sample

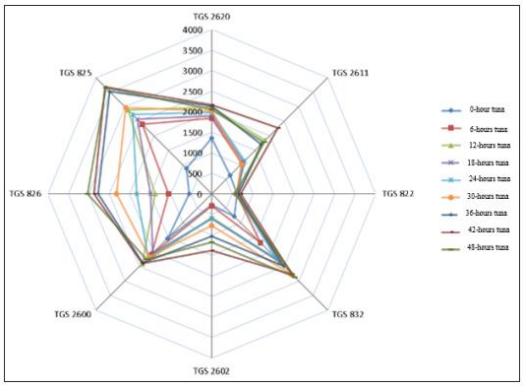
The *E-Nose* device's sample sensing treatment was performed based on the shelf life, which was as follows: 0 hours, 6 hours, 12 hours, 18 hours, 24 hours, 30 hours, 36 hours, 42 hours, and 48 hours. Two replications of each sensing data collection procedure were run on each sample for a total of 5 minutes. The information derived from the *E-Nose* output includes stress on the smell of *tuna (Thunnus thynnus)* with varying shelf life, stress on the smell of *Salmonella typhi bacteria* with varying shelf life, and stress on the smell of *tuna (Thunnus thynnus)* contaminated with *Salmonella bacteria*. variations in the shelf life of typhi.[21] The figure depicts the sensor array's response to varying time variations during the preheating, sensing, and purging processes for each type of sample.

Following observation, it was determined that the sensor output response is stable between 100 and 200 seconds, so the output data between 100 and 200 seconds was used to analyze using PCA after first being visualized as a line plot graph. The line plot graph is useful for displaying the range of data from each sensor. Line plots with time variations are created in this study so that the range of data can be seen during each sensing period.

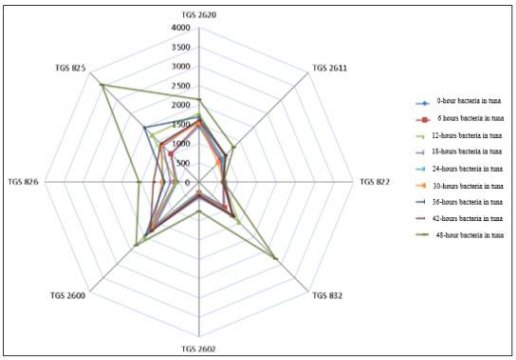
Each sample generates a unique voltage output, which results in a unique graphic pattern. Radar graphs were used to visualize the data based on the sample type and shelf life. The radar graph interprets the sensor array's response for each sensing period. It is evident that the *E-Nose* generates various sensor outputs for various sample types, resulting in various radar graphic patterns. The radar chart pattern of the three samples, however, is not noticeably different at 0 hour. The radar graph in Figure 5 interprets the sensor array response for each type of sample.



(a)



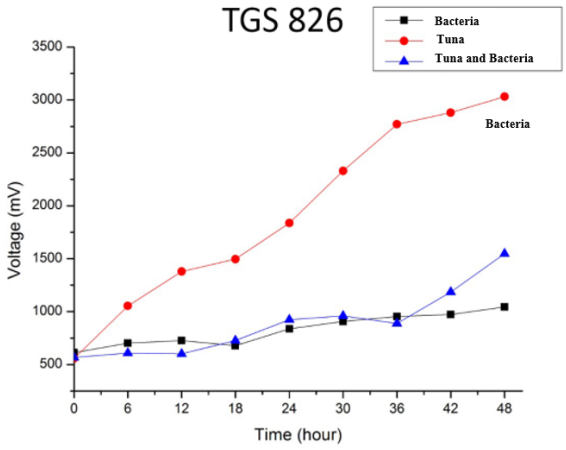
(b)



(c)

Figure 5. Radar Sensor Graph for Each Sample Type (a) *Salmonella typhi* bacteria; (b) tuna (*Thunnus thynnus*), and (c) tuna (*Thunnus thynnus*) contaminated with *Salmonella typhi* bacteria

Each sensing period saw an increase from the TGS 826 sensor in the *tuna* sample (*Thunnus thynnus*). In the meantime, each sensing period saw an increase in the *Salmonella typhi bacteria* sensor TGS 825 sample. It is clear from the radar graph that the sensor output response to the sample yields various values. Each sample has a unique set of odor characteristics, which accounts for the variation in the radar chart pattern. Because the sensor will produce a higher voltage when reacting to the target gas, which has a higher gas concentration as well, an increase in output voltage is obtained for the same sample with variations in shelf life at each shelf-life period. Figure 6 displays the output value for each sensor in the sample with varying shelf lives.



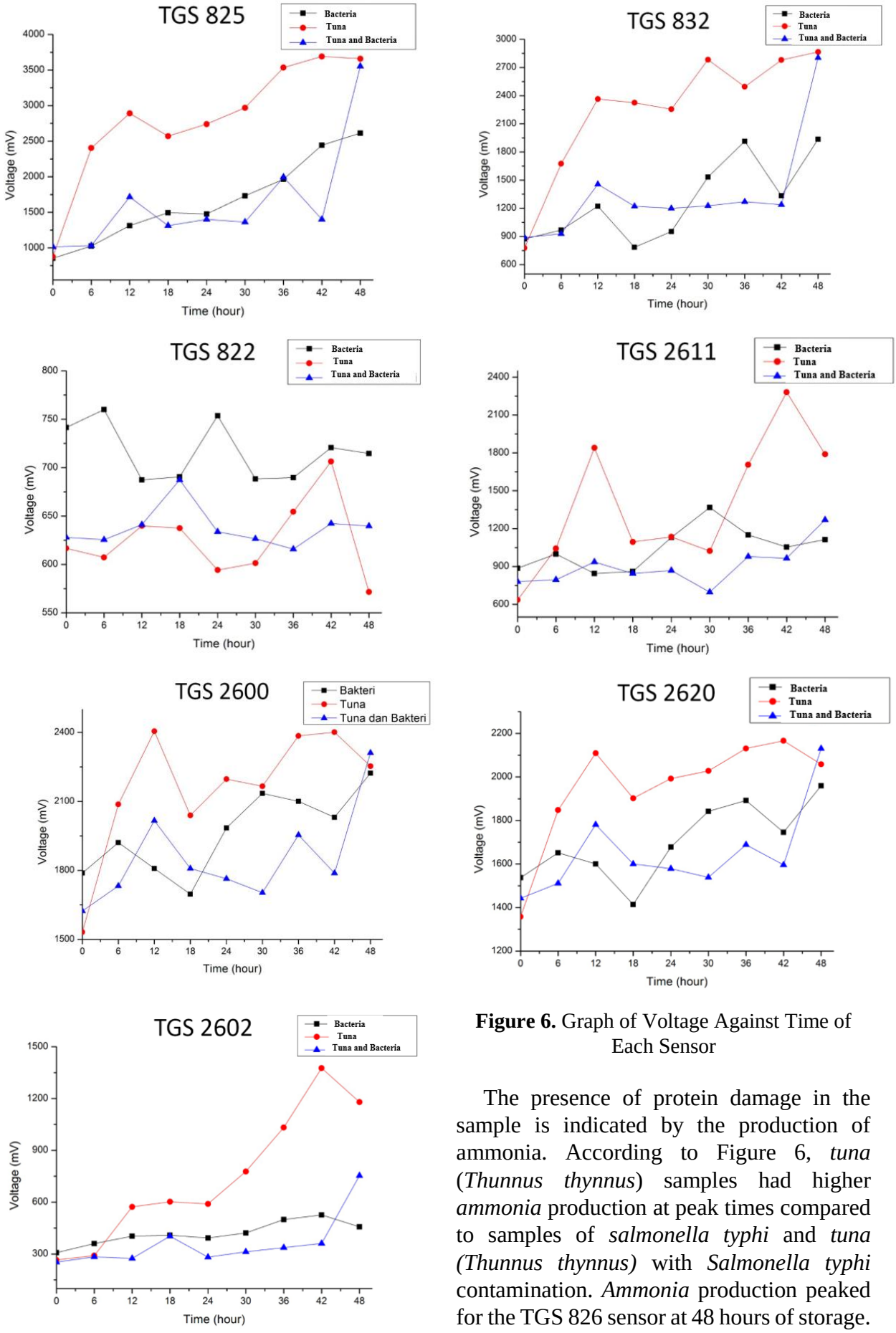


Figure 6. Graph of Voltage Against Time of Each Sensor

The presence of protein damage in the sample is indicated by the production of ammonia. According to Figure 6, *tuna* (*Thunnus thynnus*) samples had higher *ammonia* production at peak times compared to samples of *salmonella typhi* and *tuna (Thunnus thynnus)* with *Salmonella typhi* contamination. *Ammonia* production peaked for the TGS 826 sensor at 48 hours of storage.

When using the TGS 825 sensor to detect H_2S, it was discovered that all samples produced the most H_2S after 48 hours of storage, with *Salmonella typhi bacteria* producing the most H_2S overall. Because ammonia and H_2S are the main gases produced by samples of tuna and *Salmonella typhi bacteria*, TGS 825 and TGS 826 also have sensor production peaks.

Sensor Validation Results

Accuracy is the closeness of conformity between the results of a measurement and the true value of the quantity measured. It is necessary to test the percentage recovery to measure the accuracy of the results from the analysis tests that have been carried out. Accuracy is considered good at 10% recovery tolerance, or within the range of 90%–110%. The results of testing the accuracy of the H_2S gas detected by the TGS 2602 and TGS 825 sensors are shown in Table 1.

Table 1. Sensor Accuracy Test Results

Sensor	Recovery (%)				
	1 ppm	2 ppm	3 ppm	4 ppm	5 ppm
TGS 2602	95.89	99.29	102.59	101.2	98.534
TGS 825	100.00	100.00	99.27	98.984	100.756

From Table 1 it is known that the TGS 2602 and TGS 825 sensors, which function to detect H2S gas, meet the validation parameters, which are categorized as good because the percentage of H2S gas recovery ranges from 90% to 110%. The percent recovery value farthest from 100% is produced by the TGS 2602 sensor when it detects standard H_2S gas with a concentration of 1 ppm and a recovery value of 95.899%, and the value closest to the standard concentration is produced by the TGS 2602 sensor when it detects H2S gas with a concentration of 2 ppm and a recovery value of 99.297 percent.

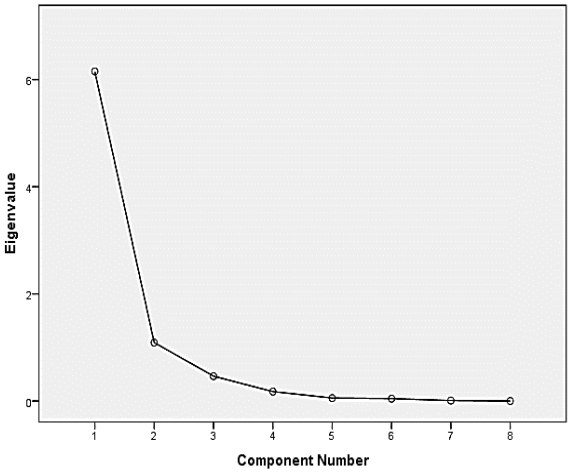


Figure 7. Graph of Eigenvalue Relationship to Principal Component

***Principal Component Analysis* (PCA) Results**

To find the correlation between each variable, the PCA method searches for a covariance matrix. The eigenvalue of each variable is then determined using the covariance matrix. The data information formed at the new coordinates (*principal component*) is described by its eigenvalue. Figure 7 depicts the connection between eigenvalues and principal components.

Score Plot

A graph that displays where data clusters are located is the PCA score plot graph. The similarity of grouped data can be displayed on the score plot graph. Two or more data distributions are present when data are grouped together to form a cluster. The two variables, *Principal Components* 1 and 2, which are not correlated, are substituted for the eight sensor variables that are correlated with one another to create the Score Plot.

The graph shows the score plot of a sample of contaminated tuna (*Thunnus thynnus*), *Salmonella typhi bacteria*, and *tuna fish*. Figure 8 illustrates a time variation of 0-48 hours with *Salmonella typhi bacteria*.

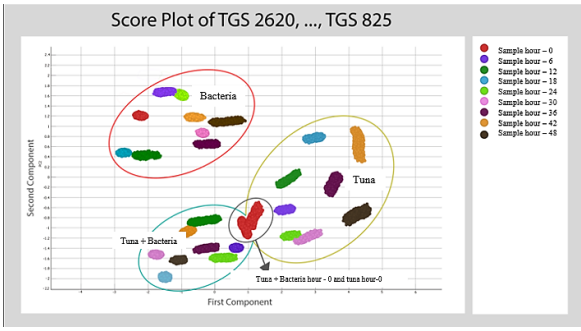


Figure 8. PCA Score Plot Graph

According to the type of sample, Figure 8 depicts clusters forming among the samples. Plotting differs for each type of sample with time variation. However, overlap was seen in samples of *tuna* and *tuna* that had *Salmonella typhi* contamination at the 0th hour of shelf-life variation. This is so because the sample's distinctive odor hasn't yet developed into a distinct or different characteristic. The organoleptic test classified the samples of *tuna* and *tuna* contaminated with *Salmonella typhi bacteria* as fresh. The organoleptic values for pure *tuna* and *tuna fish* with *Salmonella typhi* contamination were 8.4 and 8.3, respectively, making it impossible to tell the two samples apart based on their odor characteristics.

The percentage of variance criterion is used to determine the maximum number of components that can be formed. The *principal component* is a linear combination of variables and a type of variable transformation.[13] The number of main components that have a cumulative percentage of variance of at least 80% will be used in the cluster analysis. Sensor output data can be classified using PCA analysis according to sample type and sample time variation, with a total cumulative variance value of 90.5% and specifics for PC 1 and PC 2 variants of 73.9% and 13.6%, respectively.

Because each sample has different sensor output characteristics, each sensor's significance for the newly formed variable varies, resulting in the cluster on the score plot (*principal component*). The most significant variables are interpreted on the loading plot graph based on the relationship between their principal components.

Salmonella typhi must test negative in every gram of fresh *tuna* for the specific type of *Salmonella microbial* contamination test in order to meet quality and food safety requirements in Indonesia. Microbiological testing for detection has a number of drawbacks, including a lengthy turnaround time (more than 10 days) for test results.[14] Utilizing an *E-Nose* with a gas sensor can solve the issue of lengthy test times.

Each sensing period saw an increase from the TGS 826 sensor in the tuna sample (*Thunnus thynnus*). Given that the TGS 826 sensor measures *ammonia* content and that *ammonia* is one of the odors produced by bacteria that cause rot, it is obvious that the longer *tuna fish* are stored, the rottener tuna fish there will be. Because it contains a lot of free amino acids, which are necessary for microorganism metabolism, ammonia production, biogenic amines, organic acids, ketones, and sulphur components, fish is known as a food that is both high in nutritional value and perishable.[24] Increasing storage time can accelerate bacterial growth. The rate of autolysis and the expansion of spoilage bacteria both decrease with increased handling speed.

An instrument called the *Electronic Nose (E-Nose)* mimics how the sense of smell functions. As an alternative to olfactory receptors, which are responsible for detecting smells or scents, the *E-Nose* is made up of a variety of gas sensors. The aroma picked up by various gas sensors will then take on a particular pattern. Eight semiconductor sensors from the TGS2620, TGS2611, TGS822, TGS832, TGS2602, TGS2600, TGS826 and TGS825 family of E-Nose devices were used in this study.

When SnO_2 (tin dioxide) metal oxide crystals are heated to a high temperature in air, oxygen will be adsorbate on the crystal surface with a negative charge due to the presence of electron donors on the crystal surface. This negative charge is transferred to the adsorbate oxygen, creating a positively charged space layer. As a result, a surface potential will be created that has the ability to prevent the flow of electrons, which causes electrical resistance.

The density of negatively charged oxygen adsorbed on the semiconductor surface of the sensor decreases in the presence of a reducing gas, which lowers the barrier height at the grain boundary. The resistance of the grain sensor in the gas environment decreases as the barrier height is reduced. The resistance value decreases as the gas concentration in free air increases. Additionally, if a lower gas concentration value is detected in free air, a higher resistance value will be detected.[22]

Principal Component Analysis (PCA) is one technique for analyzing the data produced by *E-Nose*. By using the PCA method, it is possible to replace some of the original, correlated variables with a new, smaller set of uncorrelated variables. This method's primary goal is to reduce the dimensions of the variables that are connected and have a sufficient number of variables so that the data will be easier to interpret.[23]

Fish gills and stomachs are where *spoilage bacteria* are most commonly found to accumulate. Because so many organs in a fish's body degrade quickly to rot when it dies, the stomach and gills of fish are parts of the body that are very susceptible to microbial growth[25]. The largest source of microbes in the body is the stomach. The muscles, gills, and guts of fish are likely a source of bacteria because they naturally contain bacteria. With longer storage, there are more bacteria present. an ideal environment for bacterial growth that encourages optimum bacterial growth.

STRENGTH AND LIMITATION

The strength of this study was that the direct observation of the E-Nose sensor output makes it challenging to distinguish between different samples The limitation of this study was Since the gas sensors used by the E-Nose are non-selective and cross-sensitive, multivariable pattern recognition techniques like PCA are required to represent the data for simple analysis

CONCLUSIONS

The classification test between tuna fish, *(Thunnus thynnus)*, and *tuna fish* contaminated with *Salmonella typhi* bacteria yielded results showing a cumulative variance of the two main components (PC) of 90.5%. TGS 825 for PC1 and TGS 826 for PC2 had loading values of 0.625 and -0.753, respectively, making them the most significant sensors in this study. Thus, *E-Nose* can tell the difference between tuna that is pure and tuna that has been tainted with *Salmonella typhi bacteria*.

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CONFLICT OF INTEREST

The authors confirm that they have no conflict of interest.

AUTHOR CONTRIBUTION

Conceptualization, methodology, writing review, validation, editing, funding acquisition, and supervision: SDA. Conceptualization, methodology, validation, original draft preparation: ABM. Writing review and editing, conceptualization, methodology, validation: AR. Writing review and editing, Conceptualization, validation: AKY. Conceptualization, methodology, validation original draft preparation: YS. Conceptualization, methodology, validation: AKA.

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Case Report

A Young Female with Acute Acalculous Cholecystitis Associated with Hepatitis A Viral Infection: A Case Report

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ABSTRACT

Most hepatitis A infections are acute, self-limiting, and asymptomatic. In rare instances, extra hepatic complication, such as acute cholecystitis, may emerge. Acute cholecystitis is inflammation of the gallbladder wall and is classified into calculus and acalculus. About 90–95% of cases are brought on by bile duct stones. Acute acalculous cholecystitis can be brought on by structural and functional abnormalities in the gallbladder brought on by viral hepatitis infection. Here we present a 20 years old female patient with acute acalculous cholecystitis associated with hepatitis A infection. Gallbladder distention, thickening of the gallbladder wall, absence of acoustic shadow or biliary sludge, perivesical liquid buildup, and absence of dilatation of the intra- and extrahepatic bile ducts are among the ultrasonographic criteria for diagnosing acute acalculous cholecystitis. The viral hepatitis serology revealed acute hepatitis A infection with positive anti-HAV IgM. Hepatitis A testing should be considered in patients suspected with acalculous cholecystitis of undefined etiology in markedly deranged liver function test adult patients.

Keywords: acalculous cholecystitis; acute cholecystitis; gallbladder inflammation; hepatitis A infection; viral cholecystitis

Highlights: A rare entity of extrahepatic complication from hepatitis A viral infection in the form of acalculous cholecystitis. Recognized and treated the acalculous cholecystitis could prevent the morbidy and mortality.

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INTRODUCTION

The hepatitis A virus, which causes hepatitis A infection, is spread by the ingestion of contaminated food and drink. Transmission through sharing needles and sexual activity is also possible, however this is unusual.[1–4] Acute cholecystitis is inflammation of the gallbladder wall and is classified into calculus and acalculus.[5] As many as 90–95% of cases are brought on by bile duct stones.[6,7] Acute acalculous cholecystitis can be brought on by structural and functional abnormalities in the gallbladder brought on by viral hepatitis infection, including gallbladder wall thickening, perivascular edema, and poor bile filling.[8,9]

There are extremely few cases of acute acalculous cholecystitis as a result of hepatitis infection. Only few previous studies have ever reported it.[1–3] Even while this condition normally resolves on its own, it can occasionally proceed to gangrene, perforation, and even death.[10] In acute acalculous cholecystitis instances that do not exhibit the traditional signs of acute cholecystitis, delayed identification and treatment can result in serious consequences and high death rates.[11] Here we reported a case of young female who was came with acalculous cholecystitis associated with hepatitis A virus infection which a rare entity that needs to be recognized and treated to prevent morbidity and mortality.

CASE REPORT

This is a 20 years old single female patient, who came to emergency department with 4-day history of epigastric pain, nausea, anorexia, and generalized fatigue. The pain increased over the last 2 days with the radiation to right hypochondriac area along with feeling of rising in body temperature. The patient also complain vomiting five times before she came. She denied any pale stool and dark colored urine. Past medical illnesses were only reported tonsillectomy procedure two years ago. Same complains in her family was denied. Upon physical examination, vital sign was in normal state with maximum temperature was 37°C, her sclera is anicteric, abdominal examination revealed epigastric and right hypochondriac area tenderness.

Blood investigations showed normal complete blood count, normal electrolytes, normal renal function test, normal prothrombin time (PT) and partial thromboplastin time (PTT), normal urinalysis, and negative qualitative pregnancy test. There was significant raise in liver function test with alanine aminotransferase (ALT) 1716 u/L (normal 0–31u/L) and aspartate aminotransferase (AST) 1564 u/L (normal 0– 30 u/L), total bilirubin 3.98 mg/dL (normal 0.1-1 mg/dL) and direct bilirubin 2.07 mgl/dL (normal <0.2 mg/dL). Her WBC is 5100, Hb is 13.8 gm/dL, platelets are 188,000, mild elevation in ESR 25 mm/hour (normal 0-20 mm/hour). Abdominal ultrasound (Figure 1) indicated a collapsed gallbladder and ±12 cc of free fluid in the pelvic cavity. No stones or sludge were seen inside the gallbladder, and neither the intra- nor extrahepatic bile duct was dilated. Abdominal CT (Figure 2) demonstrated diffuse thickening of gallblader wall (8 mm) and pericholecystic fluid without any calculus found.

The serology for viral hepatitis suggested acute hepatitis A infection with positive anti-HAV IgM and was negative for other viral hepatitis causes, where HBsAg (-) and anti-HCV (-).

Thus, the diagnosis was acute acalculous cholecystitis due to viral hepatitis. Patient was treated with supportive therapy of intravenous (I.V.) fluid, antinausea and vomiting, analgetic, hepatoprotector, and low fat diet. The abdominal pain is gradually diminishing but her scleral found to be mild icteric since the second day of hospitalization.

Repeated liver function test in day 4th showed resolution of ALT 815 u/L and AST

463 u/L. Her symptoms of abdominal pain and nausea improved gradually and settled completely by day 5th. Evaluation of liver function test in day 7th showed the following: ALT 355 u/L, AST 110 u/L, total bilirubin 1.93 mg/dL, and direct billirubin 0.97 mg/dL. The patient was then discharged with good general condition and a medical clinic appointment for follow up was given.

During the follow up at the 16th day, she had no complaint and the liver function test showed resolution ALT 31.5 u/L, AST 27.9 u/L, direct billirubin 0.6 mg/dL, and total bilirubin 1.03 mg/dL.

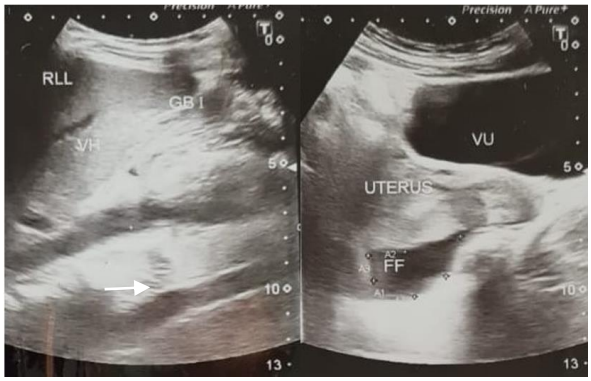

Figure 1. Abdominal ultrasound indicated a collapsed gallbladder and free fluid in the pelvic cavity. (RLL : Right Liver Lobe; VU: Vesica Urinary; GB: Gallbladder; FF: Free Fluid)

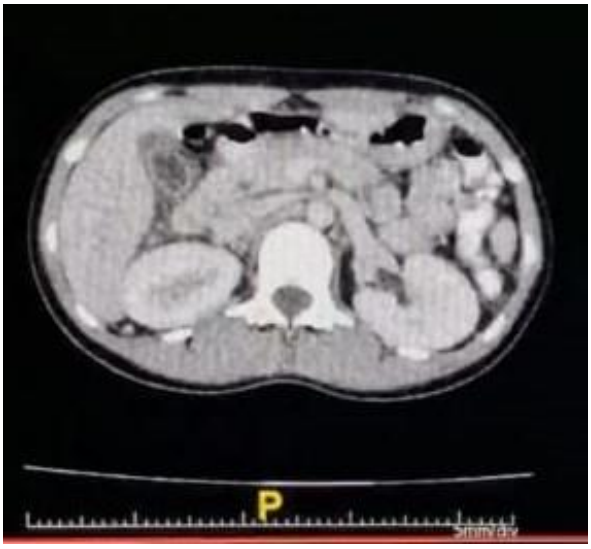

Figure 2. Abdominal CT demonstrated diffuse thickening of gallblader wall (8 mm) and pericholecystic fluid without any calculus found (white arrow).

DISCUSSION

In this report, we have described a case of severe leptospirosis or known as Weil's Disease.[1,3]On admission, the patient presented with fever, conjunctiva suffusion, dark urine, and myalgia with leucocytosis, thrombocytopenia, AKI, liver failure, and hyperbilirubinemia. Patients experience septic shock in the ER and are given norepinephrine as support. Treatment given was antibiotics and aggressive hydration. Dialysis was postponed while watchful waiting for the improvement of kidney functions by fluid therapy. Strict monitoring of kidney function and haematology was done. Symptoms and kidney function then recover with the treatment given.

Most hepatitis A infections are acute and self-limiting. Infection is usually asymptomatic, but in rare instances, fulminant hepatitis, which can be fatal, or extra hepatic symptoms, such as acute acalculous cholecystitis, may emerge.[4,12] Acute cholecystitis is an emergency that often occurs in the emergency department.[3,13] An inflammation of the gallbladder without the presence of stones or sludge is known as acute acalculous cholecystitis.[10,14] Acute acalculous cholecystitis caused by viral hepatitis has a significant morbidity and death rate due to changes in gallbladder conditions such as gangrene, perforation, and empyema, even though the precise pathophysiology of the disorder is yet unknown.[6]

Up to 95% of cases of acute cholecystitis are caused by stones that block the bile duct or gallbladder neck, with the remaining cases being inflammation without the presence of stones or sludge. About 5–15% of cases of acute cholecystitis are acalculous, and 47% of these cases follow surgery, extended immobility, prolonged starvation, such as long-term intravenous feeding, elderly patients, patients receiving care in the intensive care unit, and septic conditions.[7,10,14,15] Hepatitis A infections linked to pancreatitis and cholecystitis happen in 5% of instances as a result of the

virus's direct invasion.[16] Acute acalculous cholecystitis is a very rare condition and has not been widely reported.[6,12,17,18] acute acalculous cholecystitis is an uncommon gallbladder infection without gallstones, yet it progresses rapidly. Infection in acute acalculous cholecystitis cases progresses faster than in acute calculous cholecystitis cases, and 10% of patients also have consequences including gangrene or perforations. While acute acalculous cholecystitis instances are generally encountered in men and older adults in their sixth decade of life, acute calculous cholecystitis cases are typically seen in women in their fourth and fifth decades of life.[11]

Uncertainty surrounds the precise pathophysiology of acute acalculous cholecystitis. The influence of bile chemicals on the epithelium, ischemia in the gallbladder epithelium, formation of bile wall immune complexes leading to bile fluid stasis, and bacterial invasion are some of the claims made as the reason.[9,10,17] Hepatitis A infections can lead to the virus to invade the gallblader and bile duct epithelium because of high viral antigen levels followed by an immune complex-mediated immune response.[9,10,12,17] The existence of hypoalbumin conditions, the localized spread of the inflammatory process, and increased portal venous pressure, which results in edema of the gallbladder wall, sludge development, and reduced volume during fasting, are structural and functional alterations in the gallbladder.[6,10,18] Some of the mechanisms thought to be the pathogenesis of acute acalculous cholecystitis include direct injury to the mucous and muscular layers due to direct invasion of the hepatitis A virus in the gallbladder, impaired production and excretion of bile substances due to damage to hepatocytes, and spread of inflammatory mediators from surrounding organs due to hepatocyte cell necrosis.[8,10,19]

The majority of hepatitis A infections are asymptomatic, however common symptoms often include a mix of gastrointestinal, cholestasis, and flu-like illness.[4] While adult HAV infection rates are thought to be more than 70%, around 70% of HAV infections in children are asymptomatic.[1] Hepatitis A infection is frequently accompanied by diarrhea, stomach discomfort, fever, anorexia, nausea, vomiting, fever, malaise, dark urine, pale stool, and jaundice.[1,17] Hepatitis A infection symptoms are quite similar to those of acute cholecystitis patients in terms of their complaints.

Acute renal failure, autoimmune hemolytic anemia, pleural or pericardial effusion, acute pancreatitis, encephalopathy, ascites, and cholecystitis are only a few of the extremely uncommon extrahepatic problems that might develop.[1,3,18,19] The clearance of the hepatitis infection typically results in an improvement of the symptoms caused by these consequences.[18] Although it is mentioned that the etiology of ascites in hepatitis A infection is still unknown, it is believed to be caused by an increase in portal venous pressure because of damage to the structure and cells of the liver organ.[19] This patient had mild ascites.

It is difficult to separate hepatitis A from other viral hepatitis types solely on the basis of clinical characteristics. Serologic testing, which recognizes the presence of immunoglobulin M (IgM) anti-HAV in the acute phase of infection and immunoglobulin G (IgG) anti-HAV in the convalescent phase of infection, is necessary for a definitive diagnosis of hepatitis A.[2] Although not specific, laboratory testing of cases with acute acalculous cholecystitis as a complication from hepatitis A infection revealed an increase in white blood cells (WBC), C-reactive protein (CRP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and anti-HAV IgM.[1,11]

When acute gallbladder inflammation is unrelated to gallstones or sludge, the diagnosis of acalculous cholecystitis is established.[6] Due to its low cost, ease of accessibility, quick examination time, and absence of ionizing radiation, ultrasonography (US) continues to be the recommended first imaging modality for the assessment of suspected acute cholecystitis. High sensitivity and specificity in identifying gallstones, as well as the ability to elicit "Murphy's sign" using the ultrasonic transducer, are key benefits of US over other imaging modalities.[15] Gallbladder distention, thickening of the gallbladder wall (>3.5 mm), absence of acoustic shadow or biliary sludge, perivesical liquid buildup, and absence of dilatation of the intra- and extrahepatic bile ducts are among the ultrasonographic criteria for diagnosing acute acalculous cholecystitis.[4,12,20] Although the usual gallbladder wall is thin-hairline or undetectable, a modestly thicker wall was not included.[20] The specificity and accuracy of ultrasonography for the identification of acute acalculous cholecystitis are 97.8 and 96.1%, respectively, while the sensitivity is 88.9%.[12]

Computed tomography (CT) scans must be conducted in the event of non-contributory ultrasound imaging or clinical warning signals in order to prevent erroneous diagnoses and identify gangrenous forms necessitating a change in therapeutic approaches.[3] Overextended gallbladder, mural thickness, mural enhancement with intramural gas buildup, pericholecystic fat stranding, pericholecystic fluid, and enhanced hyperenhancement of the neighboring liver are all characteristics of acute acalculous cholecystitis on CT.[14,15,20]

The discovery of VHA antigen during immunohistochemical analysis in the gallbladder wall of a patient exhibiting ANC while VHA confirms the causal relationship between VHA and ANC.[3] Hepatitis A individuals with noticeably abnormal liver function tests should be tested if they are suspected of having acalculous cholecystitis of unknown cause.[4,6,12]

Depending on the clinical appearance, several acute acalculous cholecystitis related with acute viral hepatitis treatments are available.[12] The majority of cases are self-limited, and with therapy for the underlying systemic condition, the gallbladder may spontaneously decompress in about two weeks.[10] Surgery may be indicated by related problems such gallbladder perforation and worsening of abdominal symptoms.[10,12] When the viremia drops within a few days and gallbladder wall thickness recovers to normal under conservative care. These cases don't need to have surgery.[18] Hepatoprotective maintenance therapy is sufficient for the majority of mild and self-limiting cases.[8]

If medical treatment alone often is ineffective in treating acute acalculous cholecystitis, early cholecystectomy or percutaneous cholecystostomy application is advised as a treatment; nonetheless, cholecystectomy may be a difficult procedure for the surgeon.[11] The recommended course of treatment for complex acute cholecystitis is an early cholecystectomy performed within 7 days of the beginning of symptoms.[13]

Acute acalculous cholecystitis is an extremely rare complication of acute viral hepatitis, and the mortality from acute acalculous cholecystitis with viral hepatitis is extremely low in comparison to acute acalculous cholecystitis of other origin that needs urgent surgical intervention.[10] Some of these are self-limiting and heal spontaneously, while a limited number of cases progress to a gangrenous state, gallbladder perforation, and even to death.[14] Mortality rates range from 10%–50% due to the severity of underlying illness.[9]

STRENGTH AND LIMITATION

The strength of this study was reporting rare case which need to be recognized as soon as possible to prevent the morbidity and mortality. The limitation of this study was no

follow-up results were done in the form of ultrasound or abdominal ct scan.

CONCLUSIONS

Acute Acalculus cholecystitis is a rare condition and although it was extremely rare it needs to be considered as a complication in cases with viral hepatitis infection. On the other hand, hepatitis A testing should be considered in patients suspected with acalculous cholecystitis of undefined etiology in markedly deranged liver function test adult patients. Early diagnosis and treatment in acute acalculous cholecystitis cases that do not show classical acute cholecystitits symptoms could prevent the case progressed to emergence of severe complications and high rates of mortality.

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AUTHOR CONTRIBUTION

Data curation, writing–review and editing, validation: BNA. Data curation, writing–review, and editing: NN. Data curation, supervision, validation: DHP.

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