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Editorial

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A simple method for isolation of rest of trypsinized stem cells with magnetic beads

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It is a a common practice that human stem cells like mesenchymal stromal stem cells (MSC) can be isolated from different sources like umbilical cord tissue, placenta, adipose tissue and bone marrow as these are multipotent cells, which can differentiate into different kinds of cells like adipocytes, osteocytes, chondrocytes and so on.[1] During the isolation, the passaging of the cells is needed to get the required minimum number of stem cells for experimental and therapeutic applications. As the stem cells grow very slowly, therefore their needed number may be many times difficult to obtain. The stromal stem cells are adherent to the plastic surface of cell culture flasks during their selection and growth, hence these cells are obtained through the trypsin treatment because such treatment will detach them from the surface. Trypsin treatment is one of the common solutions used in laboratories around the world. After the trypsin treatment, the user collects the solution containing detached cells in a tube and they are centrifuged to get a pellet for further applications. MSC has different markers e.g. CD90, CD105, CD73 and they are negative for CD34, HLA DR, CD45 depending upon the source of isolation. Usually, the user disposes of the cell culture flasks as waste after trypsin treatment. Till now, there is no laboratory in the world, which makes thoughts that there may be still stem cells remaining in the flasks after the trypsin treatment. The user checks the presence of stem cells during trypsinization with the microscope whether the whole population of stem cells has been collected. Magnetic beads are being used around the world to isolate different kinds of cells like CD4, CD8, CD34, and other cells. One of the advantages of magnetic isolation is that they can be used to isolate cells from solutions containing even a low number of targeted cells. We conducted the experiments to see whether all remaining MSC can be obtained from trypsin treated flask with magnetic beads isolation.

Human Umbilical cord tissue was cut into 1 mm sized small pieces and placed in flasks to get MSC in DMEM (Lonza) containing 1% Streptopenicillin antibiotics (Biochrome) and 10% fetal calf serum (Biochrome). On the 7th day, the growth of the stem cells was observed under a microscope. The MSC after reaching 80% confluency were trypsinized with trypsin solution (Biochrome) and they were processed to get passage 1 (P1) in a new flask in PBS (Biochrome) after obtaining the pellet through centrifugation. The P1 stem cells were grown for the next 8 days to harvest with trypsin solution to get passage P2. The 15 ml PBS was added to the trypsinized flask (which should be without any cells) and observed under a microscope to see whether they are still the rest of the cells.

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It was found that there are no cells in the flask under the microscope. This 15 ml PBS was collected in a 15 ml tube. To it, 50 ul of magnetic beads specific for CD90 (Genekam) were added. This was kept at room temperature for 30 minutes. After that, the beads were removed in a magnetic rack (Genekam). The removed magnetic beads were suspended in 5ml of DMEM solution and they were observed under a microscope. After that, the cells were cultured and observed under a microscope for further growth for 7-10 days.

Stem cells grew out of small pieces of umbilical tissue in flasks around 6-9th day as adherent cells on the plastic bottom of flasks. They were trypsinized to obtain successfully a pellet for passage 1. First passage P1 was done successfully. The stem cells show their typical structure under the microscope. Under the microscope after trypsin treatment to get second passage P2, we were unable to see any cell in 15 ml PBS from trypsinized flasks. During the magnetic isolation with CD90 specific Magnetic beads, it was found under the microscope that there are huge numbers of cells being attached to beads. These cells are subcultured and they were grown in MSC cells (Picture 1), where the magnetic beads are attaching to parts of cells. These cells were used for further passage and gave new stem cells, which were adherent to plastic. The results indicate that there are still a huge number of rest cells in the trypsinized flask but most of the users think that they have isolated all cells, which is not true and they are throwing away valuable stem cells. The magnetic beads can be used to isolate the rest of MSC because for many applications, there is a need of the largest number of stem cells for conducting studies like flow cytometry, molecular testing along with pre-clinical and clinical studies. The magnetic beads method is simple and can be performed within a short period. It does not need any extra expensive instruments except a magnetic rack (Picture 2). Such a magnetic rack can be used for a very long time and for different cells to be isolated. There are a large number of remaining stem cells after the trypsin treatment in the flasks and they can be successfully removed with CD90 magnetic breads for further culturing.

DISCLOSURE STATEMENT

The author declare that they have no conflict of interest.

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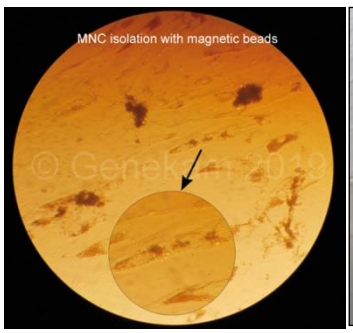

Picture 1

Picture 2

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Scientific Letter

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Absolute leukocytes count and NLR as a diagnostic and prognostic biomarkers for severity of COVID-19 infection

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The current COVID-19 pandemic challenges not only the lack of awareness of the disease, but also the rapid diagnosis, the prediction of severe early disease, the clinical characterization of mortality and severity of patients, and the effective management that affects the global scale, resulting in low capacities of healthcare systems, the resilience of systems and global economics. Therefore, early differential diagnosis and prediction of SARS-COV-2 infection severity are needed. CRP and NLR are diagnostic marker commonly used, most available, effective, and economically used primarily to evaluate the ongoing systemic inflammatory response. By developing the rapid diagnosis of SARS-CoV-2 using RT-PCR, the diagnosis of SARS-CoV-2 was facilitated by WHO network laboratory protocols.[1] RT-PCR results can give false negatives depending on the test, sample, and period of the disease.[2]

Elevated CRP values have been previously reported in patients with COVID-19 during hospitalization with higher ICU patient levels.[3] Recent study concluded that CRP was a predictive and prognostic marker for early severe COVID-19.[4] An innate immune response is the first-line for clearance of viral infections, CRP might be linked to the overproduction of inflammatory cytokines in severe patients and may lead to dysfunction of various organ systems in COVID-19-infected patients.[5]

Most patients with extremely extreme COVID-19 types (ICU) show that deregulation of the immune system results in a rapid decrease in the number of lymphocytes (lymphopenia) and a rise in the number of neutrophils (neutrophilia).[6] Although lymphopenia is an early marker, neutrophilia is a COVID-19 late-onset maker.[3] The proposed lymphopenia mechanisms are either chemokine-mediated redistribution and sequestration of lymphocytes in the lungs or suppression of the bone marrow via CD13 or CD66; the most powerful suggested mechanism is the condition of 'hypercytokinemia' or cytokine storm, demonstrated in COVID-19 patien.[7] Neutrophil Lymphocyte Ratio (NLR) is a commonly used marker for evaluating the prognosis of cancer, inflammatory and infectious diseases in patients and more accurately reflects the balanced relationship between the severity of inflammatory reactions and the immune

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state.[8] Despite a significantly higher NLR value in severe patients on admission, non-ICU patients remain borderline NLR value during hospitalization.[6, 9,10]

Recent studies found that during the entire hospital stay, NLR correlates well with severity (significantly increased at week 2 (threshold) and significantly decreases down by week 3).[10] Several studies have shown that the level of CRP and the value of NLR are independent risk factors for severe COVID-19 and have concluded that high NLR and CRP can serve as an early marker of severe COVID-19.[6, 9]

This observation illustrates the fact that it can be useful to combine the NLR value with the level of CRP as well as lymphopenia and neutrophilia to predict terrible outcomes at admission. In addition to rapid intervention, we concluded early detection and risk stratification of COVID-19 by warning signs within clear and convenient predictive measures (high values of CRP, NLR, as well as lymphopenia and neutrophilia) to guide clinical practice will greatly reduce mortality, improve the rate of cure and shorten the period of hospital stay.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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Original Research

OPEN ACCESS

Conserved B-cell epitope identification of envelope glycoprotein (GP120) HIV-1 to develop multi-strain vaccine candidate through bioinformatics approach

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Abstract: HIV-1 gp120 can be recognized by the immune system because it is located outside the virion. The conserved region is identified in gp120, and it is recognized by an immune cell which then initiates specific immune responses, viral mutation escape, and increase vaccine protection coverage, a benefit derived from the conserved region-based vaccine design. However, previous researchers have little knowledge on this conserved region as a target for vaccine design. This paper explains how the conserved region of gp120 HIV-1 is a major target for vaccine design through a bioinformatics approach. The conserved region from gp120 was explored as a vaccine design target with a bioinformatics tool that consists of B-cell epitope mapping, vaccine properties, molecular docking, and dynamic simulation. The peptide vaccine candidate of B5 with the gp120 HIV-1 conserved region was found to provoke B-cell activation through a direct pathway, produce specific antibody, and increase protection from multi-strain viral infection.

Keywords: AIDS; Bioinformatics; Conserved Region; HIV; Vaccine Design

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) has been identified from US patients since 1981. AIDS is caused by infection with the human immunodeficiency virus type 1 (HIV-1) which is a retrovirus. AIDS is an opportunistic conditionwherein the immune system fails to respond to pathogens such as bacteria, fungi, protozoa, and other viruses.[1] Viral attachment of HIV-1 is regulated by structural and non-structural proteins. A structural protein is located in the envelope and consists of gp120 and gp41. gp120 is formed through protein cleavage with furin and protease in the endoplasm reticulum.[2]

HIV-1 infectivity is higher than HIV-2 due to differences in mutation rates.[3] Epidemic cases of HIV-1 are more common because mutations have produced various strains. HIV-2 has less virulent strains and is endemic in West Africa.[4] The current HIV-1 strains consist of A, B, C, D, E, F, G, H, & J from Asia, Africa,

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Australia, Europe, and America.[5] The gp120 protein from these various strains was the main target of the vaccine design in this study.

Currently, vaccine candidates entering the clinical trial stage consisting of HTVN702, CR106152, CR108068, and HTVN106 do not use conserved regions as vaccine design targets. Previous studies have shown the discovery of conserved regions in gp120. These regions play an important role in the formation of the gp120-host receptor complex, produce specific immune responses, and increase vaccine protection coverage.[6] However, previous researchers know little about this conserved region HIV-1 as a target for vaccine design, leading to less protection from viral mutation.

Based on this, the HIV-1 vaccine with conserved regions in gp120 must be developed through a bioinformatics approach to predict the molecular mechanism of peptides vaccine candidates and provoke an immune response. The vaccine design method used in bioinformatics consists of prediction of B-cell epitopes on HIV-1 gp120, antigenicity, similarity, molecular docking, and dynamic simulation.[7] This study used a conserved region of gp120 from various HIV-1 strains as a vaccine design target through the bioinformatics approach. The peptides of the vaccine candidate are expected to initiate B-cell activation, production of specific antibodies, and increased protection coverage. This study aims to determine the potential for conserved regions in gp120 HIV-1 as a candidate for multi-strain vaccines through a bioinformatics approach.

MATERIAL AND METHOD

a. Conserved Identification of gp120 HIV-1

NCBI database (*www.ncbi.nlm.nih.gov*) was used to collect HIV-1 envelope gp120 protein sequences with the keyword "HIV-1 envelope glycoprotein". Then, a screening sample was carried out to identify the full record sequence. Information such as country of origin, strain, and length (mer) was obtained.[8,9] MEGA X for 64-bit Windows software was used to identify the conserved region position on gp120 HIV-1 through sequence alignment. Construction of the 3D gp120 HIV-1 template structure was done through SWISS-MODEL (*https://swissmodel.expasy.org/*) with the homology modelling, then model validation and structure quality determine were carried out on the Ramachandran.[10,11]

b. B-cell Immune Epitope Prediction

Prediction of B-cell epitopes in the gp120 HIV-1 region was performed using BepiPred 1.0 and Emini Surface Accessibility through the IEDB server (*http://tools.iedb.org/bcell/*). Both methods were used to identify probabilities as B-cell epitopes in the antigen region with positive predictions. These predictions were based on values above the threshold, calculation of the probability scale, and Hidden Markov Model for probability prediction of the epitope-based on the amino acid query sequence.[12]

c. Properties Analysis & Peptide Modeling

The peptide sequences obtained from B-cell epitopes on HIV-1 gp120 were then analyzed for their antigenicity, similarity, toxicity, and physiochemical. VaxiJen v2.0 (*http://www.ddg-pharmfac.net/ vaxijen/VaxiJen/VaxiJen.html*) was used for antigenic peptides prediction.[12] The antigenic peptide was identified with <70% similarity through BLASTp (*https://blast.ncbi.nlm.nih.gov/ Blast.cgi?PAGE=Proteins*).[13] An antigenic peptide with a low similarity score identified the level of toxicity in ToxinPred (*http://crdd.osdd.net/raghava/toxinpred/*) (Gupta et al. 2013). A 3D structure construction of the candidate peptide was carried out on the PEP-FOLD 3.5 server (*https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms*).

d. Molecular Docking and Dynamic

Molecular docking with Cluspro 2.0 server was performed on the peptide binding simulation of B-cell receptor (BCR) to determine binding energy in peptide-BCR molecule complex form. This method was done by superimposing the atoms to the lowest energy to determine possible binding positions.[14,15] A 3D structure visualization was displayed through PyMol software with molecular selection.[16] The result of docking simulation was used to analyze the chemical bond interaction and position on the peptide-BCR complex through Discovery Studio 2017.[17] Molecular dynamic simulations were carried out through CABS-flex 2.0 server (*http://biocomp.chem.uw.edu.pl/CABSflex2/index*) to determine stable RMSF of vaccine candidate peptides in hotspot interaction with BCR activity.[18]

RESULTS AND DISCUSSION

This study used 80 HIV-1 envelope isolates from the continents of Asia, Europe, Australia, America, and Africa with types of strains A, B, C, D, E, F, G, H, & J that were obtained from NCBI. The results of the alignment analysis proved that 50 isolates were showing the conserved region identified on the inner, outer, and bridging sheet of gp120 HIV-1. The inner, bridging sheet and outer regions of gp120 HIV-1 through in vitro studies can be used as vaccine design targets as they have a role in the viral fusion initiation process, interacting directly with CD4+ receptors and CCR5 co-receptors during viral attachment mechanisms, and generating responses such as neutralization of antibodies.[19] The conserved region is a DNA or protein sequence that is retained during each virus generation and may serve as a vaccine design target.[20] The results of this study showing a conserved region in the HIV-1 gp120 structure can act as a vaccine design target and initiate the production of antibodies to bind the specific region recognized by immune cells.

Activation of B-cell through the direct pathway plays a role in initiating the production of IgM isotype antibodies which play a role in the neutralization, opsonization, and efficiency of phagocytosis against viruses.[21] The gp120 HIV-1 can be recognized as epitopes by B-cell. Eight peptides were obtained through BepiPred and Emini Surface Accessibility parameters. This prediction generates threshold values of 0,35 and 1,00 (Figure 1). Sequences on gp120 HIV-1 with values above the threshold included positive predictions, B-cell epitopes that were identified in this study were two peptides from the outer area, five inner peptides, and one bridging sheet peptide (Table 1). Regions from the gp120 HIV-1 consist of outer, inner, and bridging sheets that could be recognized by B-cell and initiate activation through the direct pathway.

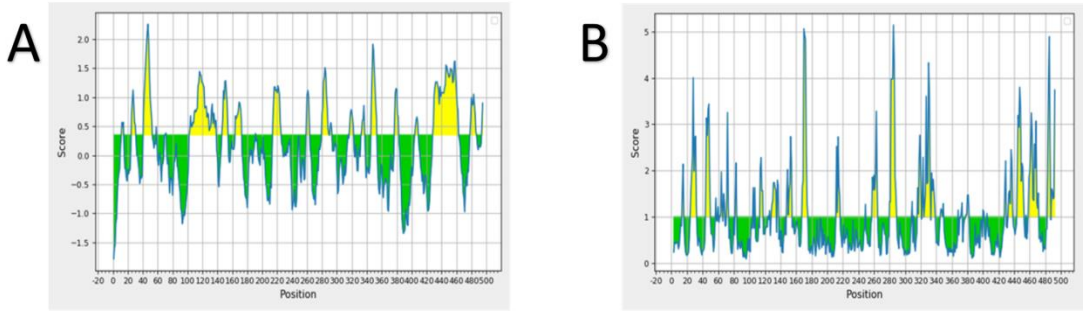

Figure 1. B-cell epitope mapping of gp120 HIV-1, positive predictions are shown in yellow areas while green is negative. Prediction graphs (A) BepiPred & (B) Emini Surfaces Accessibility.

Table 1. B-cell epitope prediction of gp120 HIV-1

Position	Prediction Methods	Peptide Length (mer)	Peptide Number	Epitope	Location
41-52	BepiPred	12	B1	CVPTDPNPHEEF	*Inner*
103-138	BepiPred	36	B2	NVNDTNVNCTTNSTTN GNCTTKGNISEWERVE QGDL	*Inner*
214-225	BepiPred	12	B3	KKFSGTGPCKNV	*Inner*
280-289	BepiPred, Emini Surface Accessibillity	10	B4	TRPNNNTRKS	*Outer*
428-462	BepiPred	35	B5	DGGNRINSTDNSTIGYS NDNNTETFRPGGGNMK DN	*Outer, Inner, Bridging Sheet*
458- 471	Emini Surface Accessibillity	14	B6	NMKDNWRSELYKYK	*Inner*

Peptide vaccine candidates derived from B-cell epitopes of gp120 are identified using vaccine properties prediction which consists of antigenicity, similarity, and toxicity. Antigenic peptides must have an antigenicity threshold value of ≥0.4.[22] Furthermore, the similarity value must be >70% so that it can initiate the antibodies escaping from an autoimmune response, and the toxicity level is below the threshold of 0,1.[8,23] The result of antigenicity and similarity prediction shows that the three peptides from the B-cell epitope consists of B2, B3 & B4. The antigen had values above the threshold of ≥0.4 and had a low <70% which is similar to the sequences from cell receptors surface from a human. The results of toxicity analysis showed that peptides B2, B3, & B4 were non-toxic because their values were lower than the 0.1 thresholds (Table 2). The presence of conserved residues on the peptides from B-cell epitope can trigger specific immune cell responses because they are antigenic, have low similarity, not toxic, and escape autoimmune reactions. The antibodies can bind to the conserved region of gp120 HIV-1. Peptide B5 was identified as having a conserved region consisting of 'DRGG' residue numbers 1, 26, 29, & 30 at the inner and outer locations of gp120 HIV-1.

Table 2. Vaccine properties of B-cell epitope

Peptide Number	Peptide	Vaccine Properties		
		Antigenicity	Similarity	Toxicity
B1	CVPTDPNPHEEF	Non-Antigen (0,37)	-	-
B2	NVNDTNVNCTTNSTTNGNC TTKGNISEWERVEQGDL	Antigen (0,88)	Non-Similar (<70%)	Non-Toxin (-0,83)
B3	KKFSGTGPCKNV	Non-Antigen (0,20)	-	-
B4	TRPNNNTRKS	Antigen (0,56)	Non-Similar (<70%)	Non-Toxin (-0,50)
B5	DGGNRINSTDNSTIGYSND NNTETFRPGGGNMKDN	Antigen (0,41)	Non-Similar (<70%)	Non-Toxin (-1,03)
B6	NMKDNWRSELYKYK	Non-Antigen (0,14)	-	-

This study used a 3D structure of BCR (ID: 5IFH) which was obtained from the RCSB PDB database (https://www.rcsb.org). Docking simulation of B5 and BCR was performed with fast rigid-body method on Cluspro 2.0 to determine the lowest energy value which is from the molecular complex after superimposing action.[14] Then, the docking results of peptide-protein interactions were identified through Discovery Studio to determine the position, distance, and types of chemical bond interactions in molecular complexes.[24] Weak bond interactions from molecular docking are non-covalent types consisting of electrostatic, Van der Waals, hydrophobic, pi, and hydrogen interactions.[25] This type of bonding interaction plays a role in protein response activity when there is a ligand interaction in the specific binding region. Types of bonds and specific amino acid residues can initiate various responses in proteins such as activation, inhibition, stability, etc.[26]

B10 peptide binds to the BCR region initiating electrostatic, hydrogen, and hydrophobic interactions. About four amino acid residues make up the B5 peptide resulting in twelve interactions in the L chain regions: Glu163, Thr164, & Thr166) and H: Lys43, Asp117, Thr113, Val114, Ser115, Glyc42, & Pro41) on BCR. The lowest energy value was -510.1 kcal/mol. Visualization of the docking results was carried out on PyMol software in transparent surfaces and cartoon's structure. The result of molecular dynamic analysis showed that B5 peptide has residual fluctuations when interacting with regions L and H chain on BCR. RMSF values consisted of 1.0-1.5 Å in peptides and 2.0-3.0 Å in forming stable molecular complexes (Figure 2).

Binding between antigens and Lys & Val residues in the H-chain BCR can initiate B lymphocyte activation, proliferation, and maturation into plasma cells.[27] With the RMSF value being ≤4.0 Å, a stable molecular complex can trigger fluctuating residues that contribute to receptor response activity.[28] The results showed that the conserved region consists of Asp30 & Arg26 from peptide B5 that binds to H chain BCR with hydrogen bonds. It initiates biological responses activation with residues stable binding of Lys43 and Val114 through hydrogen bonds interaction. Peptide B5 is predicted to be an effective vaccine candidate because it can be recognized by B cells as an epitope, is antigenic, has low similarity, is not toxic, and forms a stable molecular complex with BCR. Peptide B5 allows being constructed in further tests in a wet lab. However, the immune response triggered by B cell epitope recognition in this study has not yet produced memory because there is no mechanism for B cell activation through helper T cells.

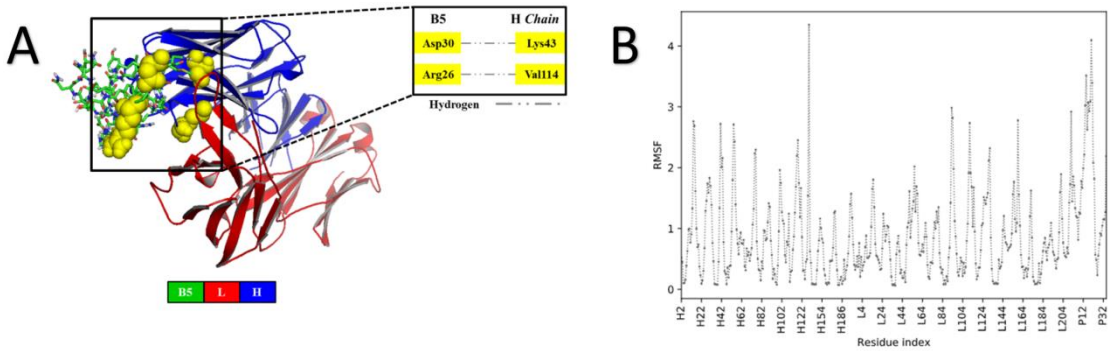

Figure 2. Molecular docking and dynamic simulations representation. (A) 3D structure visualization and interaction hotspots (B) RMSF graph of fluctuative peptide B5 and BCR residues.

CONCLUSION

Peptide B5 can be used as a good candidate for the HIV-1 vaccine because it has a conserved region to initiate B-cell activation through the BCR functional region, production of specific antibodies, and increased protection against multi-strain viral infections. It is further recommended that the results of this research be further tested through in vitro and in vivo approach.

AUTHORS' CONTRIBUTIONS

Viol Dhea Kharisma and Arif Nur Muhammad Ansori prepared the samples, designed the protocols, executed the protocols, and wrote the manuscript. Arif Nur Muhammad Ansori, Gabrielle Ann Villar Posa, and Wahyu Choirur Rizky reviewed the manuscript. Sofy Permana and Arli Aditya Parikesit reviewed and supervised the manuscript. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute in this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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Original Research

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Does gender affect immune response in HIV patients?

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Abstract: Gender differences affect the frequency and course of many diseases. This study aimed to determine the gender distribution in HIV-infected patients and investigate the relationship between gender and immune response. The study included HIV-infected patients who followed up in our hospital in 2018. The patients were divided into HIV RNA negative patients (Group 1) and HIV RNA positive patients (Group 2). Patients with diseases that may affect the immune system and those using drugs that affect the immune system were excluded from the study. The evaluation was made of 549 patients, as 305 patients (45 females 14.75%) in Group 1 and 224 patients (23 females, 9.43%) in Group 2. When the CD4/CD8 ratio of male and female patients was compared in both groups, a lower rate was determined in females (0.71-0.58) than males (0.82-0.93). A negative correlation was determined between HIV RNA and the CD4/CD8 ratio in premenopausal females (p=0.045) and males (≤45 years p=0.0001). Clinical studies of HIV infection have demonstrated better initial viremia control in females with primary infection, faster disease progression, and stronger immune activation than males for the same level of viral replication.

Keywords: HIV; AIDS; Gender; Immune Response.

INTRODUCTION

Approximately 38 million people worldwide are infected with the human immunodeficiency virus (HIV). As most of these individuals do not have access to appropriate treatment, HIV infection eventually leads to serious immune deficiency, resulting in a condition called AIDS (Acquired Immune Deficiency Syndrome), resulting in morbidity and mortality.[1,2,3] However, the CD4: CD8 ratio is usually less than 1 in HIV infection. The primary mechanism leading to immunodeficiency, opportunistic infections, and ultimately death in HIV infection is the continuous loss of CD4 T cells.[4]

Gender differences affect the incidence and course of many diseases. Gonadal sex steroids are particularly focused on gender-specific risk factors.[5] A combination of environmental factors, host genetics, and viral characteristics determines transmission and pathogenesis in HIV infection.[6] The objective of this study was to determine the gender distribution in patients infected with HIV

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In Istanbul, the most populous city in Turkey, and to investigate the relationship between gender and immune response in this patient group.

MATERIAL AND METHOD

Approval for this retrospective study was granted by the Local Ethics Committee(2019-18/16.09.2019). The study included HIV-infected patients who followed up in our hospital in 2018. The patients were divided into HIV RNA negative (Group 1) and HIV RNA positive patients (Group 2). Patients were divided into two groups as under 45 age and 45 and above age according to sex hormones effects. Patient information was obtained from the hospital data system and immunology laboratory. The informed consent form was not obtained from the patients due to the retrospective study. The identity of the patients or the information to disclose their identity was never used, and all patient data was stored in encrypted files on personal computers. Patients were excluded if they were aged <18 years, pregnant, had any disease affecting the immune system, a diagnosed malignancy, chronic renal failure, receiving estrogen-progesterone or androgen-containing hormone therapy, or if they were receiving estrogen-progesterone or androgen-containing were taking drugs that could affect the immune system. Flow cytometric analyses were performed with Navios cytometer (BECLS)-Kaluza Software. Whole blood was stained with anti-human FITC-CD45, PE-CD4, ECD-CD8, PE-CY5.5-CD3 (Beckman Coulter, Brea, California) antibodies. The percent and count of CD4+ and CD8+ cell are expressed CD3+CD4+ and CD3+CD8+ cells in CD45+ leukocyte gating. The HIV RNA level was measured with the PCR method (Real-time PCR, Rotor gene Q, QIAGEN, Germany).

Statistical analyses were performed using NCSS 11 software (Number Cruncher Statistical System, 2017 Statistical Software). Chi-square analysis was applied to determine relationships between categorical variables. The Independent Samples t-test was used to compare two groups of continuous independent variables with normal distribution. The Mann-Whitney U test was used to compare two independent groups of variables that did not fulfill the normal distribution assumption. Spearman correlation analysis was applied to determine the correlational relationships between variables that did not meet the normal distribution assumption. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The evaluation was made of a total of 549 patients, comprising 68 females and 481 males. Group 1 included 305 patients (45 females, 14.75%) HIV RNA negative after appropriate antiretroviral therapy (ART). Group 2 included 224 patients (23 females, 9.43%) who had not yet received treatment or had not completed treatment and were HIV RNA positive. In both groups, there was no significant age difference between male and female patients (Table 1). When the female patients were evaluated alone, WBC, CD4 T cells, and CD4 / CD8 values were lower, and lymphocyte and CD8 T cells values were higher in Group 2, although not statistically significant (Table 2). The CD4 / CD8 ratio was 0.71 ± 0.56 in Group 1 and 0.58 ± 0.47 in Group 2 ($p>0.05$). When the male patients have evaluated alone, WBC and CD4 cells values were significantly lower ($p <0.0001$), and CD8% and CD4 / CD8 values were significantly higher ($p <0.0001$) in Group 2 (Table 3). The CD4 / CD8 ratio was 0.82 ± 0.9 in Group 1 and 0.93 ± 6.64 in Group 2 ($p <0.0001$). The CD4 / CD8 ratio of female and male patients in Group 1 was compared; the CD4 / CD8 ratio in females was 0.71 ± 0.56, which

was lower than that of males (0.82 ± 0.9). The ratio in patients in Group 2 was similar to that in Group 1, with a CD4 / CD8 ratio of 0.58 ± 0.47 in females and lower than in males (0.93 ± 6.64). A significant negative correlation was determined between HIV RNA and the CD4 T cells in premenopausal women ($p = 0.005$, $r = -0.700$) and males (≤45 years $p < 0.0001$, $r = -0.322$, >45 years $p < 0.001$, $r = -0.486$). There was a positive correlation between HIV RNA and the CD8 T cells in males (45 years $p = 0.022$, $r = 0.178$). A significant negative correlation was determined between HIV RNA and the CD4 / CD8 ratio in premenopausal women ($p = 0.045$, $r = -0.542$) and males (≤45 years $p = 0.0001$, $r = -0.486$, >45 years $p = 0.001$, $r = -0.447$) (Table 4). When the RNA count was compared in Group 2, the median RNA value was lower in females than in males (433-655 copy/ml) (Table 5).

The progression of the disease in HIV infection is determined by the number and percentage of CD4 T cells. CD4 is a Tcell helper T cell and regulates the immune response by interacting with other immune cells via inflammatory cytokines such as interleukin-1 (IL-1), IL-6, or tumor necrosis factor α (TNF-a).[7,8] Specific cytotoxic CD8 Tcells recognize virus antigens on the surface of HIV-infected cells and need help in immunological memory and the cytolytic response of CD4 T cells while killing cells by lysis through antigen-specific cytotoxic mechanisms.[9]

In the early stage of infection with HIV, CD8 T cells initiate a severe specific HIV response and increase in number by approximately 20-fold. Specific anti-HIV cytotoxic CD8 + T cells are present even before specific antibodies are detected.[10] In acute infection, most of the cells in the immune response become active and contribute to infection progression. In the current study, CD8+ T cells levels were high in both male and female patients in the group with acute HIV infection (Group 2). CD8 + T cells exhibit antiviral activity with soluble factors that inhibit the HIV replication they secrete and with their HIV-specific cytotoxins.[11]

The absolute number of CD4 T cells is considered the main criterion in determining the risk of opportunistic infection, deciding to initiate ART, and predicting AIDS-related cancers and deaths. However, the CD4 / CD8 ratio is also used as a parameter to predict prognosis.[12,13] In the general population, a CD4 / CD8 ratio of <1.0 is considered the predictor of immunosenescence and is an independent predictor of overall mortality.[14]

Studies have shown that in both HIV-infected and non-HIV-infected populations, females have higher CD4 cell counts than males.[15,16] Zapata et al. showed that the CD4 / CD8 ratio of male patients was lower than that of females, regardless of age and duration of treatment.[17] However, in contrast to that study, the CD4 / CD8 ratios were higher in males in both Group 1 and Group 2 in the current study. In a long-term cohort study by Sacarino et al., multivariate analyses including age, sex, rare CD4, CD4 Tcell count, and ART duration were performed in 407 patients to predict non-AIDS events, including malignancies, cardiovascular and renal diseases. A low CD4 / CD8 ratio was an independent factor for both non-AIDS morbidity and mortality in long-term HIV-positive patients independent of the absolute CD4 T cell count.[18] As the current study did not include long-term follow-up data, no comment on mortality and morbidity can be made. All these studies show that the CD4 T cell number has lost its predictive value, while the CD4 / CD8 ratio remains a predictor of morbidity and mortality associated with AIDS or not, even after prolonged ART.[19]

The incidence, severity, and course of some diseases may be different for men, and women.[20] Autoimmune diseases, in particular, are more common in females.[21] In addition, the incidence, and severity of many microbial infections, including malaria and tuberculosis,[22] influenza,[23] hepatitis[24], and HIV, have been reported to differ between the genders.[25] In the current study, 87.64% (n: 481) of the patients who were followed up for HIV infection and included in the study

within one year were male, and there was a statistically significant male predominance in the current patient population. In HIV infection, clinical research has shown that females have faster disease progression and stronger immune activation than males for the same level of viral replication and better control of the first viremia in women during primary infection.[26] In the current study patient population, 66,17% (n: 45) of the female patients had viremia control, and HIV RNA was negative, whereas this rate was 54.05% (n: 260) for males, similar to the above-mentioned study.

Despite advances in the pathogenesis and treatment of HIV infection, the biological mechanisms leading to gender-specific differences have not been fully elucidated. Gender-specific environmental risk factors, differences in the microbiome by sex,[27] steroid hormones secreted by gonads[28], and the direct effects of X and Y chromosome-related factors[29] may be responsible for this. Of these factors, gonadal sex steroids have mainly been focused on.

The level of immune activation is an essential predictor of the course of HIV disease.[30] The level of immune activation contributes to the progression of HIV disease and has been shown to differ between males and females.[28]

In a multi-center cohort study that included many countries such as the United States of America, Australia, Canada, and Brazil, it was seen that in primary HIV infection, females tend to have lower plasma viral load levels than men.[31] Similarly, in the current study patient population, the viral load in female patients in Group 2 was much lower than in male patients. Another study stated that the risk of developing AIDS was 1.6 times higher in HIV-infected females with the same viral load as males.[32] In the current study, as the HIV RNA increased in premenopausal patients, the CD4 / CD8 ratio decreased, and the absence of this decrease in postmenopausal women supports this view. Differences in immunoreaction in reproductive and postmenopausal periods in women suggest that sex-related changes in HIV infection may be due to gonadal sex hormones in the foreground. Sex hormones can affect the susceptibility of CD4 T cells to infection by altering the density of coreceptors and receptors in the target cells. The effects of progesterone on human cervical cells have also been investigated and shown to increase expression of HIV receptors CD4, CCR5, and CXCR4 in cervical CD4 + T cells.[33]

CONCLUSION

Clinical studies of HIV infection have demonstrated better initial viremia control in females with primary infection, faster disease progression, and stronger immune activation than males for the same level of viral replication. Further studies are needed to clarify the role of gender in HIV pathogenesis. Particular focus on gonadal sex steroids in the pathogenesis may be of guidance for new trends in preventing or treating HIV infection.

AUTHORS' CONTRIBUTIONS

Gülçin Şahingöz Erdal	:	Conceived, designed the review, and wrote the manuscript.
Ramazan Korkusuz	:	Performed the search of articles and gathered the information and valid biomarkers from the articles, and wrote the manuscript.
Pınar Kasapoğlu	:	Performed the search of articles.
Nilgün Işıksaçan	:	Read the literature and applied the exclusion criteria, and interpret the data.
Kadriye Kart Yaşar	:	Substantial contribution to the interpretation of data.

All authors critically revised the manuscript for important intellectual content, approved the final version to be submitted, and agreed to be accountable for the manuscript's content.

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The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

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Table 1. Gender Distribution of the Groups

Gender	Group 1 n (%)	Group 2 n (%)	p-value
Female	45	23	0.060
Male	260	221	
Chi-square test			

Table 2. Comparison of Variables of Female Patients According to Groups

Female	Group 1 n=45 Mean±SD Med. (Min.-Max.)	Group 2 n=23 Mean±SD Med. (Min.-Max.)	p-value
Age (years)	42.31±9.3 41- (26-65)	41.39±13.52 38- (18-61)	0.673
white blood cell 10*3/ul	6542.89±2159.65 6650- (2670-11410)	6070±1472.41 6240- (3710-9120)	0.292
Lymphocytes %	31.82±9.38 32.3- (8.9-51.7)	34.56±8.62 36.8- (20.6-51)	0.247
CD3+	74.34±11.12 76.3- (48.7-92)	74.53±11.55 76.9- (49.8-92.8)	0.945
% CD3+CD4+	26.08±11.63 26.4- (1.53-56.2)	22.57±12.43 20.8- (1.4-42.9)	0.291
CD3+CD4+ Cell/ul	545.62±319.97 514- (11-1432)	508.04±345.53 503- (20-1230)	0.657
%CD3+CD8+	43.44±12.22 44.1- (19.2-72.9)	47.47±13.98 47.3- (25.4-72)	0.224
CD3+CD8+ Cell/ul	873.87±401.49 882- (144-2022)	1029.22±504.27 823- (431-2284)	0.484
CD4/CD8	0.71±0.56 0.51- (0.03-2.32)	0.58±0.47 0.47- (0.02-1.64)	0.235
Mann Whitney U test Independent Samples t-test			

Table 3. Comparison of Variables of Male Patients According to Groups

Male	Group 1 n=260 Mean±SD Med. (Min.-Max.)	Group 2 n=221 Mean±SD Med. (Min.-Max.)	p-value
Age (years)	38.11±11.87 36-(18-70)	37.18±11.28 35-(18-69)	0.437
white blood cell 10*3/ul	7350.04±2081.98 7080-(3620-16980)	6548.42±1959.86 6290-(2270-13470)	<0.0001*
Lymphocytes %	34.48±9.03 34.8-(6.2-67.2)	33.15±10.05 32.4-(6.6-67.1)	0.129
CD3 Cell/ul	75.77±8.57 76-(50.1-96.4)	77.02±10.36 78.4-(32-96.5)	0.133
%CD3+CD4+	28.39±9.83 28.1-(0.9-56.4)	20.88±9.67 19.8-(0.76-46.9)	<0.0001*
CD3+CD4+ Cell/ul	697.62±309.38 672-(11-1703)	452.86±266.93 431-(2-1531)	<0.0001*
%CD3+CD8+	41.64±11.53 41.5-(16.9-83)	51.55±29.69 49.6-(16.6-447)	<0.0001*
CD3+CD8+ Cell/ul	1025.08±528.43 871-(10.56-3826)	1076.3±553.59 961-(129-4051)	0.259
CD4/CD8	0.82±0.9 0.67-(0.01-13)	0.93±6.64 0.4-(0-99)	<0.0001*

Mann Whitney U test
* Independent Samples t-test

Table 4. Relationships between variables with HIV RNA level according to the age limit of 45 years in males and females (Group 1)

		Female		Male	
		≤45 years	>45 years	≤45 years	>45 years
CD4/CD8	**r**	-0.542*	0.083	-0.486*	-0.447*
	p	0.045	0.831	0	0.001
	n	14	9	165	56

**Spearman Correlation analysis*

Table 5.Examination of HIV RNA positive cases by Gender

	Female (n=23) Mean±SD Med. (Min.-Max.)	Male (n=221) Mean±SD Med. (Min.-Max.)	p-value
HIV RNA Copy/ml	45230±1460 433-(27-695017)	200842106±2962484 655-(5-44041392)	0.506

Mann Whitney U test

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Evaluation of favipiravir treatment before intensive care in COVID-19 patients

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Abstract: The purpose of this study is to investigate the healing effect of favipiravir used in pre-intensive care treatment of patients diagnosed with COVID-19 in order to elucidate the pathogenesis and complications of coronavirus. The data regarding the clinical findings of the patients in the hospital information system and the biochemical parameters made standard in the treatment or follow-up of COVID 19 were taken from the system and evaluated retrospectively. In addition, it was examined as a whole with mild, moderate, and severe pulmonary involvement compared to CT findings. Hemogram, coagulation, and biochemistry parameters used in the diagnosis and follow-up of COVID-19 were evaluated. SPSS 22.0 statistics program for Windows was used in statistical analysis to evaluate the data obtained from patient files and hospital information systems. There is no standard treatment protocol within the scope of treatment. Drug studies are currently ongoing. In this study, the first clinical findings, treatment types, and recovery times of patients diagnosed with COVID-19, the healing effect of favipiravir used before intensive care were determined. Between-group 1 (those who started treatment within 0-5 days) and group 2 (those who started treatment within 6-10 days), after five days of favipiravir treatment, when serum parameters were compared, favipiravir treatment was statistically significantly lower in the first group that was started early, WBC, Neutrophil, Creatine, CK, CRP, D-Dimer, PCT, LDH. By collecting the data obtained as a result of the research, early deaths can be prevented worldwide. Our study recommending alternative treatment approaches is vital for the protection of patients' quality of life. In this study, when all biochemical markers were evaluated together, it was evaluated that starting Favipiravir treatment early was beneficial in treating COVID-19 disease.

Keywords: Coronavirus; Favipiravir; Covid-19; Intensive Care; Pandemic.

INTRODUCTION

Antiviral drug development is determined by the viral life cycle through viral replication stages and cellular processes that support viral replication. The effect of antivirals targeting a viral replication step can be enhanced by causing synergies with secondary effects through an antiviral or drug metabolism that hits a different viral target or a cell process. The stages of antiviral drug development begin in silico design. They are tested in single-cell types (organotypic cell lines or primary cells) and animal models, clinical trials, and ultimately regulatory approval/market complex models to determine IC_{50} / CC_{50} = SI. A significant obstacle in developing antivirals is that many compounds that exhibit in vitro

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activity have little effect in animal models. Human organoids/complex in vitro infection models (e.g., Barrier models) can provide a bridge to predict activity in clinical trials. It is well known that even the threat of biological attack will cause mass hysteria accompanying economic cuts. Only visible preparation with visible infrastructure, drug and vaccine stocks, and well-thought-out emergency plans will allow governments to provide the necessary assurances when necessary to prevent negative consequences.[1] While many virus infections are asymptomatic, new or improved antiviral drugs are needed to prevent and/or treat several important conditions caused by viruses that cannot be controlled by alternative measures, including vector control, immunization, and current antiviral therapy. Antiviral compounds that are effective in infections caused by tropical and vector viruses have been a neglected subject of international antiviral research until recently.

Some compounds are currently in clinical trials, and few have regulatory approval or are available on the market. FDA approval is pending for the use of favipiravir to treat filovirus infections. Several animal pilot studies in non-human primates (NHP) have recently demonstrated the efficacy of favipiravir.[2] Toyama Chemical Co., Ltd. Favipiravir (T-705), discovered and synthesized, shows a wide range of activities against influenza virus, arenaviruses, bunyaviruses, West Nile virus, foot and mouth disease virus, yellow fever virus, and various RNA viruses.[3] Favipiravir is converted to its active form, ribofuranosyl-5-triphosphate, by host enzymes and inhibits viral RNA polymerase in host cells. Only a few reports have resisted favipiravir in vitro.[4,5] In research conducted by Janowski A. et al. on the treatment of favipiravir in 2020, Astrovirus VA1 (VA1) replication had a 10-fold reduction.

However, only a 44% reduction in human astrovirus 4 (HAstV4) replication was observed at 1000μM.[6] No significant decrease in cell viability was observed in any favipiravir concentration. The results show that favipiravir inhibits VA1 replication. The *in vitro* EC_{50} values defined for the drug have similar activities for other enteric viruses such as enteroviruses and noroviruses. Favipiravir is also used in the treatment of Thrombocytopenia Syndrome with Severe Fever (SFTS). Studies of potential antiviral drugs for SFTS-specific therapy have been performed on existing or newly discovered agents *in vitro* and *in vivo*. Ribavirin and favipiravir are the most promising candidates. Although animal experiments and retrospective studies have proven the limited efficacy of ribavirin, ribavirin is thought to be effective in patients with a viral load of $< 1\times10^6$ copies/mL.

Favipiravir showed higher efficacy than ribavirin against SFTS in vitro experiments. Higher efficacy was observed in animal models administered even three days after virus inoculation.[7] *In vivo* efficacy of favipiravir has been studied using animal models. Intraperitoneal (i.p.) administration of favipiravir at a dose of 60 or 300 mg/kg/day for five days ensured that mice were fully protected from death upon SFTS infection. On the other hand, 40% of mice treated with ribavirin (i.p.) at a dose of 25 or 100 mg/kg/day lost body weight and died from SFTS infection, reducing the rate of case death. All mice treated with favipiravir survived when treatment was initiated three days after infection or earlier, whereas mice treated 4 and 5 days after infection exhibited 83% and 50% survival, respectively. Generally, favipiravir is administered orally to humans as in the mouse model.[8] In addition, it may be desirable to use intravenous administration as SFTS patients with severe symptoms may have difficulty taking oral medications.[7] Favipiravir also has a protective effect against Nipah virus infections in the hamster model[9] and inhibits some paramyxoviruses *in vitro.*[10] Favipiravir resistance mechanism has been described in the influenza virus.[5]

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MATERIAL AND METHOD

Preliminary permits for the feasibility of the study were obtained from the Public Health Directorate before the start of the study. This study was approved ethically by the decision of Siirt University Non-Interventional Clinical Research Ethics Committee dated 02/06/2020 and number 08.02. In this study, patients who applied to Siirt State Hospital who complained of high fever, difficulty breathing, and weakness were diagnosed with COVID-19 and were administered Favirpiravir before intensive care were examined. The patient groups consisted of those who started treatment up to the first five days after hospitalization and started treatment after five days in the second group. That is, the first group was determined as those who started treatment within the first five days after hospitalization (together with favipiravir hydroxychloroquine + azithromycin + oseltamivir), and the second group (hydroxychloroquine + azithromycin + oseltamivir before the favipiravir) (Table 4 and 5). According to the science committee's guidelines, the Ministry of Health of the Republic of Turkey has been moving. The groups were compared among themselves. Hemogram, coagulation, and biochemistry parameters (Ferritin, Creatine, CK, CRP, D-Dimer, PCT, LDH) were used to diagnose, and follow-up of COVID-19 was evaluated. SPSS 22.0 statistics program for Windows was used in statistical analysis to evaluate the data obtained from patient files and hospital information systems. The biochemical parameters of patients were also evaluated retrospectively. In the biochemistry laboratory, biochemical parameters were studied using Siemens Dimension RxL Max Integrated Chemistry System.

Statistical analyzes using chi-square to show the differences between the time of starting the drug and demographic data. In cases where the expected value was below 5, chi-square Fisher's Exact Test was used. Spearman correlation test was used to determine the relationship between the data. Independent t-Test was used to determine the significance of differences between the time to start medication and biochemical parameters.

RESULTS AND DISCUSSION

When comparing demographic data, chronic diseases, smoking, tomography involvement, and initial symptoms between group 1 (those who started treatment in 0-5 days) and group 2 (those who started treatment in 6-10 days), no statistically significant difference was found. (Table 1). Serum parameters were evaluated between group 1 (those who started treatment within 0-5 days) and group 2 (those who started treatment within 6-10 days) at the time of treatment, CRP and D-Dimer values were in group 1; Although CK, LDH and Ferritin values were higher in the 2nd group, no statistically significant difference was found between the two groups (Table 2). Serum WBC, neutrophils, creatine, CK, CRP, D-Dimer, PCT, and LDH levels of patients whose drug start time is 0-5 days were statistically significantly different (Table 3) compared to the patients whose drug start time is 6-10 days serum levels ($p < 0.05$). Between-group 1 (those who started treatment within 0-5 days) and group 2 (those who started treatment within 6-10 days), after five days of favipiravir treatment (Table 4), when serum parameters were compared, favipiravir treatment was statistically significantly lower in the first group that was started early, WBC, Neutrophil, Creatine, CK, CRP, D-Dimer, PCT, LDH. It is crucial that CRP, D-Dimer markers, which are stated as harmful prognostic factors, especially in covid-19 disease, are lower than the second group and may indicate a better clinical response if treatment is started early. Ferritin value, a prognostic marker, was found lower in the first group but was not statistically significant. When the lymphocyte values, one of the diagnostic criteria and prognostic markers, were examined, it was higher in the first group but not statistically significant (Table 3).

In order to elucidate the pathogenesis and complications of coronavirus, the healing effect of favipiravir used in pre-intensive care treatment of patients diagnosed with COVID-19 was investigated. It has also been compared with its applications during intensive care in the world. Republic of Turkey Ministry of Health Sciences Board has published numerous guidelines during the pandemic. As can be seen in the guides, While favipiravir treatment was recommended to intubated (critical) patients in intensive care at the beginning of the pandemic, it was recommended to start before intensive care in patients with moderate-severe pneumonia / non-responding to the first-line treatment (hydroxychloroquine-azithromycin-oseltamivir) in the following guides. Favipiravir was approved for the new flu treatment on February 15, 2020, in China, but clinical trials are ongoing to treat COVID-19. Favipiravir is a new type of RNA-dependent RNA polymerase (RdRp) inhibitor. In addition to its anti-influenza virus activity, favipiravir can block the replication of flavi-, alpha-, filo-, bunya-, arena-, noro- and other RNA viruses.[11] Favipiravir is converted to an active phosphorylated form in cells and recognized as a substrate by viral RNA polymerase, thereby inhibiting RNA polymerase activity.[12] Therefore, it was thought that favipiravir might have a potential antiviral effect on SARS-CoV-2, an RNA virus.

On February 14, a clinical trial on favipiravir for the treatment of COVID-19, initiated by the National Infectious Diseases Clinical Medical Research Center and Shenzhen Third People's Hospital, had promising results. Initial results of a total of 80 patients (including the experimental group and the control group) showed that favipiravir had a more substantial antiviral effect than lopinavir+ritonavir.[12] There were no significant adverse reactions in the favipiravir treatment group and significantly fewer adverse events than the lopinavir+ritonavir group.[13] Favipiravir has complex, nonlinear, time- and dose-dependent pharmacokinetics influenced by weight.[14,15] Because favipiravir is metabolized and inhibited by aldehyde oxidase, initial oral loading is required to obtain adequate blood levels, and the plasma half-life is 4 hours. In persons with hepatic dysfunction, blood concentration should be monitored and the dose adjusted. Favipiravir or its metabolites have been detected in semen and breast milk. Although favipiravir showed no pharmacokinetic interaction with oseltamivir, overexposure to acetaminophen was observed when coadministered with acetaminophen in healthy volunteers.[14,15] When used together with acetaminophen (paracetamol), the dose of acetaminophen should not exceed 3000 mg/day (less than in liver failure). When used together with theophylline, the possibility of side effects increases as the blood concentration of the drug increases. Therefore, caution should be exercised when using them together.[16,17,18] The most common side effects are diarrhea, increase in serum uric acid level, increase in serum transaminase (ALT, AST, ALP) and total bilirubin levels, and decrease in neutrophil levels. Attention should be paid to the use of the drug.[18]

In a study by Janowski *et al*., the EC_{50} values for ribavirin and favipiravir were quantified against two human astrovirus strains, astrovirus VA1 (VA1) and human astrovirus 4 (HAstV4). VA1 replication was inhibited 10-100-fold by both ribavirin (EC_{50}=154 μM) and favipiravir (EC_{50}=246 μM). In contrast, ribavirin inhibited HAstV4 replication (EC_{50}=268 μM), but favipiravir only reduced replication by 44% at the highest dose.[6] In a study by Tani *et al.,* Favipiravir exhibited higher effectiveness than ribavirin in *vitro* and *in vivo* studies.[7] In a study by Yamada *et al.,* Favipiravir (T-705), a broadspectrum antiviral drug against RNA viruses, is effective against Rabies virus *in vitro* but ineffective *in vivo.*[19] These data suggest that favipiravir is a promising drug for the treatment of infections by a specific virus and a wide range of RNA viruses. Favipiravir is efficacious in multiple types of Influenza viruses, regardless of sensitive or

resistance to existing anti-influenza drugs. A specific note is that favipiravir is active against a wide range of other RNA viruses *in vitro* and *in vivo*. *In vitro* studies indicate no emergence of resistance to favipiravir.[20] Favipiravir with these unique profiles will be a promising therapeutic agent for unremedied infections by RNA viruses in the near future.

CONCLUSION

Evidence to date from clinical studies shows that favipiravir is well tolerated in humans. By collecting the data obtained as a result of the research, early deaths can be prevented worldwide. Our study recommending alternative treatment approaches is vital for the protection of patients' quality of life. In this study, when all biochemical markers are evaluated together, it is evaluated that starting Favipiravir treatment early is beneficial in treating COVID-19 disease.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

FOUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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Table 1: Patients group statistics with demographic data, chronic diseases, smoking, tomography involvement, and initial symptoms.

		Drug Start Time		
		0-5 days (n=28) %	**6-10 days** (n=8) %	P* χ^2
Gender	Male	50	75	p= 0.257
	Female	50	25	χ^2= 1.58
DM	+	21.4	37.5	p= 0.384
	-	78.6	62.5	χ^2= 0.857
HT	+	21.4	12.5	p= 1.00
	-	78.6	87.5	χ^2= 0.317
KBH	-	100	100	-
Cardiovascular	+	17.9	0	p= 0.566
	-	82.1	100	χ^2= 1.66
Pulmonary Disease	+	14.3	12.5	p= 1.00
	-	85.7	87.5	χ^2= 0.017
Cigarette	+	42.9	50	p= 1.00
	-	57.1	50	χ^2= 0.129
Bt	Mild-Middle	75.7	62.5	p= 0.167
	Severe	14.3	37.5	χ^2= 2.141
Latest Status	Discharged	96.4	87.5	p= 0.400
	Ex	3.6	12.5	χ^2= 0.945
Clinical Response	+	85.7	75	p= 0.596
	-	14.3	25	χ^2= 0.514
Nausea Vomiting	+	21.4	0	p= 0.302
	-	78.6	100	χ^2 = 2.06
Body Pain	+	17.9	25	p= 0.639
	-	82.1	75	χ^2= 0.203
Headache	+	25	25	p= 1.00
	-	75	75	χ^2= 0.00
Cough	+	87.5	62.5	p= 0.167
	-	14.3	37.5	χ^2= 2.141
Shortness of breath	+	46.4	37.5	p= 0.709
	-	53.6	62.5	χ^2= 0.201
Fever	+	46.4	37.5	p= 0.691
	-	53.6	62.5	χ^2 =0.643

*Fisher's Exact Test

Table 2. Effect of Drug Start Time on Serum Parameters (Before)

Parameters	Drug Start Time		p-Value
	0-5 days (n=28)	6-10 days (n=8)	
WBC	5.5±0.5	5.77±0.76	p>0.05
Lymphocytes	1.06±0.06	1.05±0.15	p>0.05
HGB	12.62±0.5	13.54±0.52	p>0.05
HTC	41.39±0.95	41.56±1.43	p>0.05
Neutrophils	4.06±0.46	4.05±0.85	p>0.05
PLT	183.96±12.14	160±10.98	p>0.05
Creatine	0.94±0.04	0.97±0.05	p>0.05
AST	44.71±4.9	43.75±5.07	p>0.05
ALT	33.61±4.25	41.63±9.62	p>0.05
CK	130.79±26.91	205.88±73.13	p>0.05
CRP	80.28±11.69	72.15±29.55	p>0.05
BUN	29.6±2.38	27.52±2.87	p>0.05
D-Dimer	1365.46±386.66	886±135.59	p>0.05
PCT	0.14±0.01	0.16±0.03	p>0.05
LDH	330.21±20.99	401±55.74	p>0.05
Ferritin	592.67±89.21	642.75±147.74	p>0.05

Data are given as mean ± standard deviation

Table 3. Effect of Drug Start Time on Serum Parameters (After)

Parameters	Drug Start Time		p-Value
	0-5 days (n=28)	6-10 days (n=8)	
WBC	6.28±0.36	11.46±4.12	p<0.05
Lymphocytes	1.51±0.14	1.23±0.23	p>0.05
HGB	12.73±0.29	12.94±0.59	p>0.05
HTC	40.25±0.8	41.7±1.72	p>0.05
Neutrophils	4.22±0.35	*9.42±4.11	p<0.05
PLT	322.96±23.99	269.25±28.55	p>0.05
Creatine	0.89±0.04	*1.13±0.17	p<0.05
AST	43.14±4.75	48.13±5.95	p>0.05
ALT	53.14±8.23	65.88±18.81	p>0.05
CK	61±9.84	*304.38±161.8	p<0.05
CRP	28.53±7.76	*74.18±39.61	p<0.05
BUN	30.31±3.31	41.16±13.39	p>0.05
D-Dimer	964.29±236.07	*4041.13±2291.99	p<0.05
PCT	0.13±0	*0.28±0.1	p<0.05
LDH	264.21±13.82	*395.38±88.85	p<0.05
Ferritin	601.01±100.83	753.06±204.77	p>0.05

Table 4. Patient Correlation ****

	Age	DM	Ht	Cardiovascular	Pulmonary Disease	Cigarette	Start Time	Latest Situation	Clinical Response
Gender	0.140	0.000	-0.126	0.036	-0.287	0.800**	-0.209	0.027	-0.100
Age		-0.380*	-0.551**	-0.379*	-0.159	0.135	0.029	0.181	-0.011
DM			0.365*	0.139	-0.046	0.129	-0.154	0.140	0.086
Ht				0.412*	0.006	-0.157	0.094	-0.187	-0.157
Cardiovascular					-0.161	-0.036	0.215	-0.253	-0.036
pulmonary disease						-0.198	0.021	-0.253	-0.036
Cigarette							-0.060	-0.027	-0.050
Start Time								0.162	0.120
Latest Situation									0.542**

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
No statistically significant correlation was found between the time to start medication and other parameters

Table 5. The relationship between Start Time with tdsck and tdspct.

	tdsck	tdspct
Start Time	0.335*	0.374*

* Correlation is significant at the 0.05 level (2-tailed)
A weak positive correlation was found between the time of starting the drug and tdsck ($r = 0.335$, $p < 0.05$) and tdspct ($r = 0.374$, $p < 0$)

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Original Research

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Molecular identification of pathogenic bacteria causing foodborne disease in Caulerpa racemosa

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Abstract: *Caulerpa racemosa* is a green algae consumed by people in northern coastal areas. *C. racemosa* has a habitat attached to the shallow seabed. *C. racemosa* usualy consumed fresh without any cooking process so that the contamination of microorganisms can be eaten. Molecular identification using 16S rRNA is needed to determine the type of bacterial contaminants in *C. racemosa.* The isolates of *C. racemosa* were cultured in HIA, BAP, and BHI media. Bacteria from BHI media were isolated by DNA, PCR for 16S rRNA gene, and sequencing. Bacteria isolate *C. racemosa* was found to have the α-hemolytic ability in BAP media. The sequencing analysis showed that the three bacterial colonies of *C. racemosa* isolate had high similarity with *V. parahemolyticus, Caldalkalibacillus mannanilyticus*, and *Exiguobacterium profundum*.

Keywords: Bacteria identification; Caulerpa racemose; Foodborne disease.

INTRODUCTION

Bacterial growth, especially in waters, is often associated with or contaminating marine organisms, one of which is *C. racemosa*.[1] The presence of bacteria can threaten the hygiene of marine biota and even human health who consume polluted marine biota.[2] Bacterial identification is needed to find out what types of bacteria live in symbiosis with *C. racemosa*. Identification of pathogenic bacteria is crucial to provide information regarding the safety of *C. racemosa* when consumed raw. BAP media is an enriched and differential selective medium that can differentiate pathogenic bacteria based on their ability to hemolysis erythrocytes.[3] Microbiological identification of bacteria has the disadvantage of taking a long time from bacterial culture to biochemical tests.[4]

In addition to the culture method, identification of bacteria can be carried out using a molecular-based method with a high level of sensitivity by analysis using the 16S rRNA gene (16S ribosomal Ribonucleic acid / Ribonucleic acid encoding the 16S ribosome, S represents Svedberg, which is the unit of measurement for ribosomes). The gene encoding rRNA is the most conserved gene, so the 16S rRNA gene is used as a universal primer in the use of Polymerase Chain Reaction and determination of nucleotide sequences through sequencing (PCR).[5]

There is still limited research related to identifying pathogenic bacteria that cause foodborne disease in *C. racemosa*, it is necessary to research to provide new insights to the public regarding hygiene in the consumption of fresh

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C. racemosa. Molecular identification of bacteria causing foodborne disease in *C. racemosa* have high sensitivity and specificity regarding the types of bacteria. The 16S rRNA gene was chosen because all bacteria have this gene, so it is possible to find new species that are pathogenic and specifically found in *C. racemosa*

MATERIAL AND METHOD

A sampling of C. racemosa was carried out in the waters of Jepara, Central Java, Indonesia. Isolation to obtain pure bacteria from C. racemosa culture was carried out at the Microbiology Laboratory. In contrast, DNA isolation from C. racemosa culture bacteria and molecular identification was carried out at the Molecular Biology Laboratory, Medical Laboratory Technology, Faculty of Nursing and Health, University of Muhammadiyah Semarang. The 16S rRNA gene sequencing analysis was carried out at PT. Indonesian Genetics Science.

Isolation of bacteria culture from C. racemosa

Three grams of C. racemosa were put into a clean and dry plastic to be mashed. Then one spoon of C. racemosa was put into 5 ml of Physiological NaCl, homogenized. The suspension of C. racemosa was isolated by culturing it on NA and then incubated at 37°C for 24 hours. Then the bacteria were isolated again on BAP at 37°C for 24 hours. Hemolysis formed on BAP was observed against a bright light background. Colonies on BAP with a whole clear zone and clear green zone were inoculated on HIA (Heart Infusion Agar) fertilizing medium for 24 hours at 37°C in the Incubator. Colonies on HIA (Heart Infusion Agar) were cultured on BHI (Brain Heart Infusion) for 48 hours at 37°C in an incubator.

Isolation of DNA from C. racemosa bacterial culture

Bacterial colonies of C. racemosa culture from HIA were instilled in 5 ml of liquid BHI, then incubated at 37°C for 24 hours. The incubation results were centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was discarded, then the pellet was added with 750 µl of lysis buffer, vortexed for a few seconds. 20 µl of proteinase K was added, shaken for 15 minutes using a shaker, then incubated at 55°C for 30 minutes, then centrifuged for 10 minutes at 12000 rpm at 4°C.

The supernatant solution was transferred to a 1.5 ml Eppendorf tube, and 700 µl of phenol CIAA was added, stirred slowly for 30 minutes, then centrifuged at 12000 rpm for 10 minutes at 4°C. The topmost part (aqueous phase) was transferred to an Eppendorf tube, then 96% ethanol was added in a 1:1 ratio, mixed gently until fine threads were seen, then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant solution was discarded, the pellet was washed with ethanol 70%, then centrifuged at 12000 rpm for 10 minutes at 4°C.

The supernatant was discarded, then allowed to dry, then 100 µl of TE was added to dissolve the bacterial DNA to be seen on 1% agarose gel electrophoresis. The results of DNA isolation were pipetted as much as 2 µl, and the absorbance was measured using a UV nanodrop spectrophotometer at a wavelength of 260/280 nm. DNA purity has a ratio limit between 1.8-2.0. Obtained results of absorbance and concentration of DNA purity.[6]

Amplification of C. racemosa cultured bacteria

Nuclease Free Water was added as much as 7.5 µl and added 12.5 µl of taq polymerase enzyme into a PCR microtube. The forward and reverse primers were added 2 µl each, and 1 µl DNA samples were added to the microtube. The PCR temperature is set at the initial stage of denaturation (pre-denaturation) with 95°C for 6 minutes, followed by a denaturation stage at 95°C for 30 seconds, then annealing stage at 55°C for 30 seconds, extension at 72°C for 2 minutes.

The final extension stage was carried out at 72°C for 10 minutes and the cooling down the stage at 4°C for 6 minutes. The results of PCR amplification and markers were read using 2% agarose gel electrophoresis. PCR product approximately 1500 bp.

Data analysis

The purified 16S rRNA gene PCR product then proceeds to the sequencing stage to determine the sequence of nucleotide bases in the PCR product. The PCR product is sent to PT Genetics Science Indonesia for further processing to the sequencing analysis stage. The nucleotide sequence obtained is then used to compare the sequence data available at Genbank through the BLAST (Basic Local Alignment Search Tool) program at (NCBI), https://blast.ncbi.nlm.nih.gov/. The results of the sequencing were aligned and made a phylogenetic tree using Mega X.

RESULTS AND DISCUSSION

The bacterial culture of *C.racemosa* obtained four bacterial colonies. Bacterial colonies were grown on BAP media and produced a partial erythrocyte lysis zone. 4 Colonies of isolates of *C. racemose* had α-hemolysis type on BAP media (Figure 1).

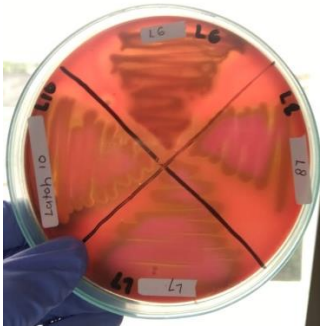

Figure 1. Characteristics of 4 bacterial colonies isolate *C.racemosa* in BAP

Four colonies of isolates of *C. racemosa* (CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK) were pathogenic in humans because they were able to lyse some erythrocytes in BAP media. Each bacterial colony was purified, and Gram stain was performed to determine gram properties, morphology and confirm purity. CR1-AIK and CR6-AIK are Gram-positive bacilli. CR7-AIK is a slight rod-shaped gram-negative.

Each isolated bacterial colony of *C.racemosa* (CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK) that had been purified was cultured on BHI media, and DNA isolation was performed using the phenol-CIAA method. The results of DNA isolation were used as a template for amplification of the 16S rRNA gene.

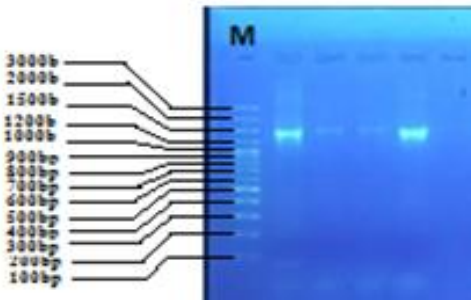

Figure 2. PCR product of about 1500 bp of C.racemosa isolate bacteria (CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK).

Bacterial isolates CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK had PCR products of about 1500 bp. The DNA band length of 1500 bp is specific to the target gene, namely 16S rRNA. In addition, the results of electrophoresis of PCR isolates of bacteria CR1-AIK, CR6-AIK, CR7-AIK, and CR8-AIK formed a single band (Figure 2). The PCR product of the 16S rRNA gene on bacterial isolates CR1-AIK, CR6-AIK, and CR7-AIK was then sequenced.

C.racemosa is processed without a cooking process, so that it is feared that there will be contaminant bacteria that can harm consumers. The habitat of *C. racemosa*, which is at the bottom of the waters, causes all parts of the harvested algae to contain sand, even sea pebbles*. C. racemosa* has abundant epibiotic bacteria and diatoms that adhere to the outer surface and form biofilms.[7] Physical defenses such as mucus production in *C.racemosa* and diverse aquatic environments can form bacterial communities on the algal surface. Research related to bacteria from the surface of *C. racemosa* found no antimicrobial effect. Bacteria that live on the surface of *C. racemosa* form biofilms and produce secondary metabolites similar to algae.[8]

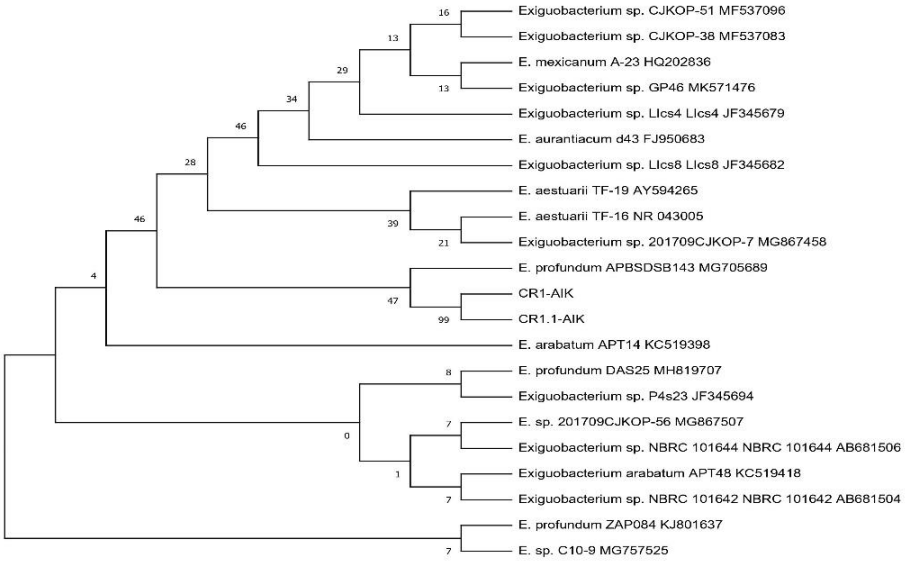

Figure 3. Phylogenetic analysis of the bacterial isolate CR1-AIK has a close similarity to *E. profundum*

Based on tree phylogenetic analysis using Mega X, the bacterial isolate CR1-AIK had similarities with the bacterium *Exiguobacterium profundum* (Figure 3). *E. profundum* has gene sequences with high similarity to the bacterial isolate CR1-AIK found from the Bay of Bengal Visakhapatnam Coast. Bacteria, firmicutes, bacilli, bacillales, Exiguobacterium. One of the bacteria found in *C.racemosa* is *Exiguobacterium profundum*.

Exiguobacterium profundum is a thermophilic bacteria that can produce lactic acid isolated from deep-sea hydrothermal. *E. profundum* is an anaerobic, halotolerant, moderately thermophilic bacterium and a bacterium that does not produce spores. *E. profundum* was isolated from the sea under hydrothermal conditions with an average depth of 2600m. *E. profundum* is a gram-positive bacterium, motile rod-shaped with peritrichous type and lives optimally at 45 °C, pH 7.2% NaCl. (+)-L-Lactate is the primary organic acid detected from carbohydrate fermentation by producing formate, acetate, and ethanol

compounds. Colonies formed circular, have a creamy or orange color. The characteristics of *E. profundum* are chemoorganotrophic and facultatively anaerobic. Biochemical tests showed that *E. profundum* had positive catalase and negative oxidase abilities. The substrates used for growth under anaerobic conditions were aesculin, amygdalin, arbutin, cellobiose, D-fructose, D-galactose, N-acetyl-D-glucosamine, D-glucose, gentiobiose, maltose, D-mannitol, D-mannose, D-ribose, salicin, starch, sucrose, and trehalose.[9] The genus Exiguobacterium is not only found in *C.racemosa* but also red algae. *Exiguobacterium aestuarii* has been isolated from red algae, *Gracillaria corcata*. *E. aestuarii* produces magnesium ammonium phosphate (struvite) and can lyse agar (agarolytic*). E aestuarii* can produce struvite, a bioremediation-based fertilizer on industrial phosphate waste.[10] The discovery of the genus Exiguobacterium in the aquatic environment has the potential as a bioagent to control the explosion of the cyanobacterial population, namely *Microcystis aeruginosa.*[11]

The genus Exiguobacterium is also found in marine organisms such as shrimp and fish. *Exiguobacterium arabatum* isolated from the stomach of shrimp (*Penaeus vannamei*) has potential as a probiotic because it has no resistance. Gene and only one virulence gene.[12] In addition, *Exiguobacterium arabatum* was also found in fish.[13]

Several genera of Exiguobacterium cause disease in humans. Exiguobacterium was found in periodontitis, myeloma, and infectious endocarditis patients, all of which isolates formed orange-yellow colonies on blood agar.[14] Phylum Firmicutes species Exiguobacterium were also found in dishwashing water that had formed biofilms. Several species in this genus are known for their ability to proliferate in extreme environments such as hot, alkaline, and marine environments.[15] The genus Exiguobacterium causes pathogens in humans because it has virulence factors including tlyC, MprR, MCP, Dam, which play an essential role in lethal infections.[16]

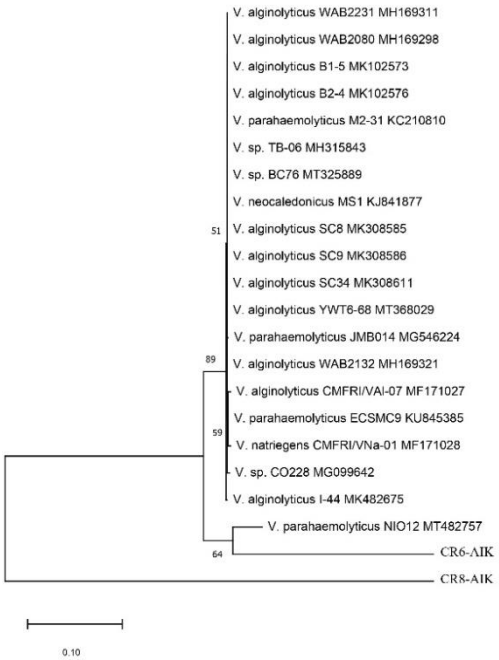

Figure 4. phylogenetic analysis of bacterial isolate CR6-AIK has a close similarity to *V. parahaemolyticus*

The results of the phylogenetic tree analysis using Mega X showed that the bacterial isolate CR6-AIK had a close relationship with *Vibrio parahaemolyticus* found in aquatic areas (figure 4). *Vibrio parahaemolyticus* is a bacteria, proteobacteria, gammaproteobacteria, vibrionales, Vibrionaceae, and the genus Vibrio.

The genus Vibrio is a bacterium that is mainly found in marine environments. Vibrios develop in the bodies of marine animals. Vibrios that live in the bodies of marine animals are mutualism, for example, *Vibrio fischeri*-squid. Vibrio interactions with the host are also parasitic and pathogenic to humans, for example, *Vibrio harveyi*-shrimp. The genus Vibrio is vast and is divided into several species groups that have different ecological environments. Vibrio is not only found in *C. racemosa*, but also many other marine organisms. For example, *V. harveyi* is a species associated with disease-causing various marine animals. *Vibrio coralliilyticus* is involved in bleaching events on corals. *V. splendidus* a group of species involved with a disease in mollusks, *V.halioticoli* is a species that have the potential to have mutualistic relationships with abalones organisms and act as probionts. V cholera is a type of vibrio pathogen in humans.[11,17]

CR6-AIK is closely related to *Vibrio parahaemolyticus*. *Vibrio parahaemolyticus* is naturally found in aquatic areas and is associated with gastroenteritis either from contaminated raw food or undercooked seafood[18,19] and is life-threatening in immunocompromised patients. *V parahaemolyticus* is a halophilic Gram-negative bacterium. Several *V parahaemolyticus* were identified to cause opportunistic diseases in humans, causing subacute gastroenteritis with dehydration, fever, and of various ages, as well as gender.[20] *V parahaemolitycus* causes foodborne disease. *V parahaemolitycus has* different genetic distribution, genetic elements, and pathogenicity depending on the environment.[21] *V parahaemolyticus* isolated from 3 main water areas in China has different genetic diversity and population structures.[20,22] Based on the analysis using WGS, there were differences in the pathogenicity of *V parahaemolitycus* in various aquatic environments resulting from the combination of horizontal genome transfer, pathological distribution elements.[11,13,18] *V. parahaemolyticus* was also found to cause death in shrimp due to acute hepatopancreatic necrosis disease.[23]

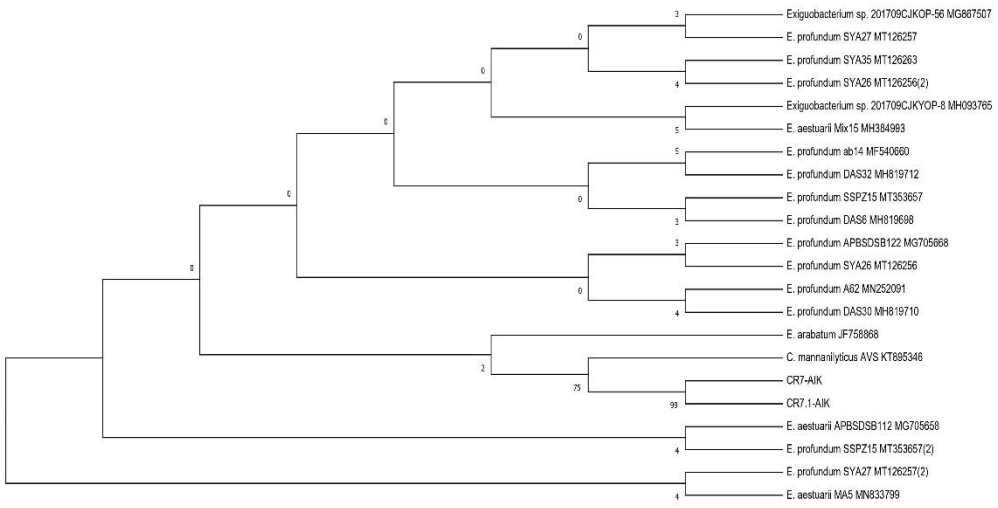

Figure 5. Phylogenetic analysis of the bacterial isolate CR7-AIK has a close similarity to *Caldalkalibacillus mannanilyticus*

Based on phylogenetic analysis using Mega X Isolate, CR7-AIK bacteria have a relationship with *Caldalkalibacillus mannanilyticus* (Figure 5). C*. mannanilyticus* has been found in textile factory wastewaters.[23] *C. mannanilyticus*, bacteria, firmicutes, bacilli, bacillales, bacillacae, caldalkalibacillus. Besides being found in C*. racemose, C. mannanilyticus* were found on the bottom of the water in hypersaline aiding lake conditions. One of the alkaliphilic bacteria that can be used for industrial enzyme applications is *Bacillus mannanilyticus* sp. *Bacillus mannanilyticus* is capable of producing alkaline enzymes such as proteases. *Caldalkalibacillus thermarum* produces extracellular enzymes that are resistant to high pH and high-temperature conditions. Aerobic, endospore-forming, obligate alkaliphilic.[24,25,26]

CONCLUSION

The bacterial isolate CR1-AIK had a high similarity with the bacterium *Exiguobacterium profundum*, commonly found in marine areas. Isolate bacteria CR6-AIK has similarities with bacteria *Vibrio parahaemolyticus*, which can cause foodborne disease. Genus Exiguobacterium and Vibrio can cause infectious diseases in humans. CR7-AIK has a similarity with the bacteria *Caldalkalibacillus mannanilyticus* and is widely used in the enzyme industry. Consumption of fresh *C.racemosa* must go through a clean wash because the algae contain pathogenic bacteria that cause foodborne disease.

AUTHORS' CONTRIBUTIONS

Aprilia Indra Kartika	:	Analysing sequencing data, compiling publication manuscript
Meutia Srikandi Fitria	:	Bacteria culture, NA, BAP, BHI
Vanny Oktaviola	:	DNA extraction, PCR

All authors contributed equally to this work.

FOUNDING INFORMATION

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request. Sequencing data in FASTA format, bacteria culture from NA, BHI, and BAP is very open to be accessed by anyone.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals

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Original Research

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Multi drugs resistance to Diabetes Mellitus patients with tuberculosis in Manado City

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Abstract: Diabetes mellitus (DM) with pulmonary tuberculosis (TB) is an infectious disease if not educated regularly, there will be a high risk of drug resistance and even some anti-tuberculosis drugs. This study aims to identify anti-tuberculosis drug resistance in DM patients with TB in Manado City. The population in the study types 2 DM patients as amount 80 patients. Based on TCM/GenExpert examination from 47 respondents, there were 17 respondents positive multi drugs resistance rifampicin (RR). Sampling taking based on inclusion criteria, i.e., have had type DM for five years, had suffered TB MDR RR based on GenXpert examination as much as 17 respondents followed in the resistance test with Sputum TB culture and MGIT method. The result of the study showed that MDR DM-TB with MGIT method as followed is obtained from 17 samples, six samples (35.30%) resistance INH 0.4 mg and 1 sample (5.88%) MDR canamycin, and still sensitive INH 0.4 mg and camaycin is ten samples (58.82%). This study results could be used to program planning of prevention and controlling efforts TB-DM in this treatment obedience and regimen anti-tuberculosis medicine for MDR-TB patients.

Keywords: Diabetes Mellitus; Tuberculosis; Multi Drugs Resistant

INTRODUCTION

The risk factors that cause TB to become MDR TB are HIV coinfection, malnutrition, and smoking. Type 2 diabetes mellitus is a chronic metabolic disorder caused by impaired function of pancreatic beta cells which causes progressive insulin resistance and chronic inflammation.[1] Wan-Mei conducted the research in China on 2004-2007 got founding that MDR-TB is more highest on DM patients than non DM.[2]

Other studies have reported the association between diabetes and MDR TB, but the results were controversial, and their samples were not enough. In addition, there remains a paucity of information about the relationship between diabetes and various subgroups of primary DR-TB and clinical characteristics of TB-DM cases, especially in China, which had the second-highest burden of TB in 2017.[2]

Compounding the problem, increasing drug resistance also poses a grave threat to TB control it requires more sophisticated laboratory infrastructure. However, the development of Xpert/MTB/RIF, a real-time PCR (rt-PCR) assay that can diagnose TB and detect rifampicin resistance concurrently has revolutionized the diagnosis of drug-resistant tuberculosis (DR TB). Very

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importantly, in the same policy statement which emphasizes that DST for anti-TB medicines other than rifampicin should also be offered.[3] Thus, along with TB diagnosis, drug susceptibility testing (DST) has become an urgent clinical requirement for a timely constitution of proper and effective TB treatment. However, TB diagnostic and laboratory capacity are still deficient in many areas of highly TB-burdened countries. It causes a crucial barrier to detecting human immunodeficiency virus (HIV)-associated and drug-resistant tuberculosis.[4]

MATERIAL AND METHOD

The population in the study are types 2 DM patients as much 80 patients with 46 respondents had comorbidities. Based on TCM/GenExpert examination from 47 respondents, there were 17 respondents positive multi drugs resistance rifampicin (RR). Sampling taking based on inclusion criteria, i.e., have had type DM for five years, had suffered TB MDR RR based on GenXpert examination as much as 17 respondents followed. The resistance test with Sputum TB Culture and MGIT Method. The tools used in this research include TCM cartridges, GeneXpert, and Bactec MGIT 960.

RESULTS AND DISCUSSION

The results of the research carried out can be seen in the following picture

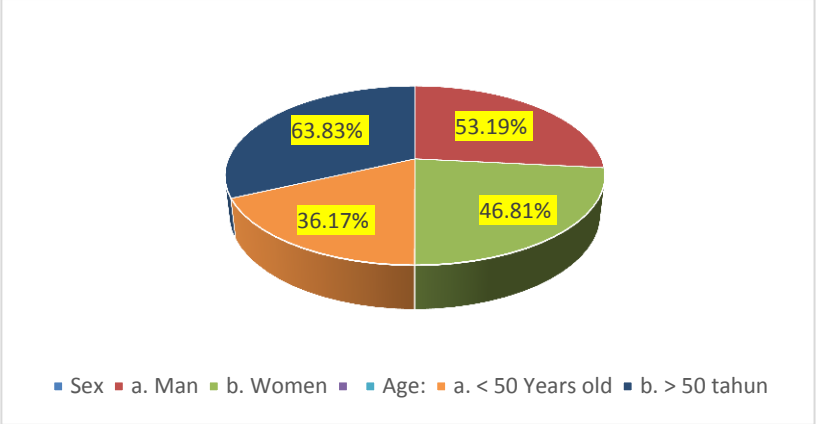

Figure 1. Distribution Sex and Age MDR-TB Patients

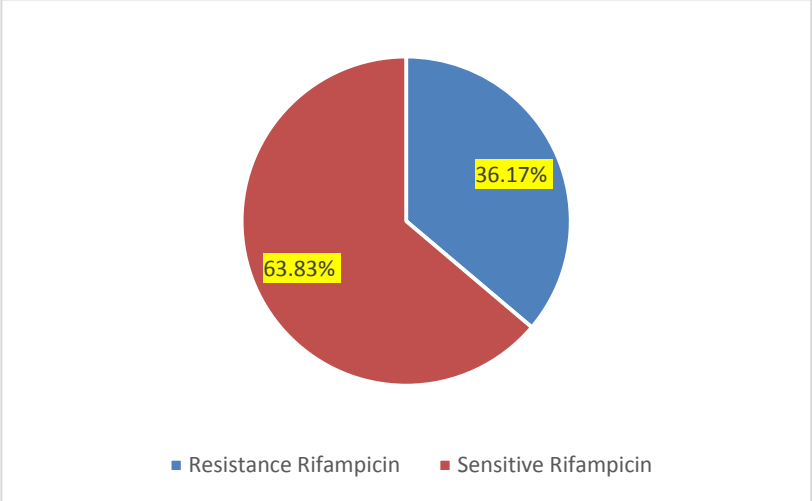

Figure 2. Results of TCM/GenXpert MDR DM-TB Patients

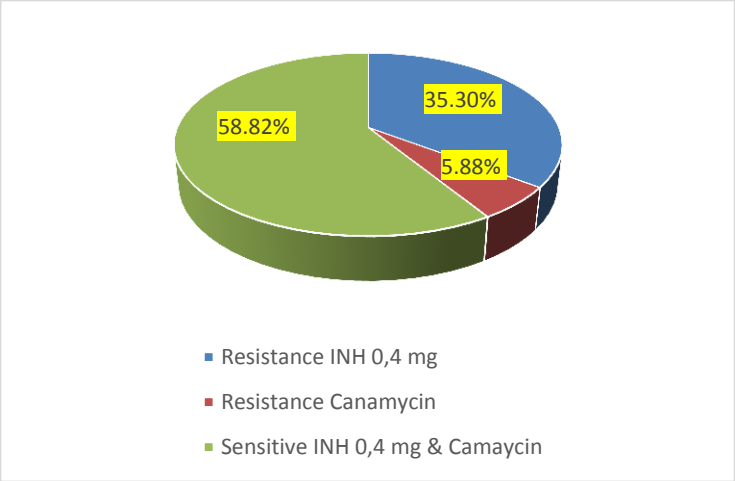

Figure 3. Result of MDR DM-TB with MGIT Method

While data in figure 3 showed that examination results about MDR DM-TB with MGIT method as followed is obtained from 17 samples, six samples (35.30%) resistance INH 0.4 mg and 1 sample (5.88%) MDR canamycin, and still sensitive INH 0.4 mg and camaycin is ten samples (58.82%). TCM as tools which could existence *Mycobacterium tuberculosis* detection as soon. The sensitivity level of tools reaches 98.3%, and sensitivity is 99%.[5,6]

The growing prevalence of TB-DM comorbidity worldwide has provided a new challenge to clinical management and health systems control strategy. It was observed that patients who have DM complicated with TB often experience delayed sputum culture conversion, increased risk of death and recurrence. What is more, the emergence of MDR-TB makes the adverse anti-TB treatment outcomes in TB-DM comorbidity even worse, which may increase the treatment-related economic burden, promote the transmission of MDR-TB, and even accelerate the generation of extensively drug-resistant-TB (XDR-TB).[7,8]

The reasons for a higher MDR rate in patients with TB-DM comorbidity are not thoroughly understood, however, they may differ by different MDR type (primary or secondary).[7] The combination of the impaired immune system in DM and bacterial genetics might be a reasonable explanation for primary MDR. It has been reported that poor glucose control is often associated with dysfunction of phagocytosis, reactive oxygen species (ROS) production, chemotaxis, and T-cell reaction in DM patients. On the other hand, MDR strains are shown to be less virulent due to heterogeneous mutations, and they are less likely to lead to secondary TB cases compared with drug-sensitive strains 41–43. Then the less fit MDR strains are more likely to flourish in immunocompromised DM patients, which leads to the higher primary MDR-TB in those patients. The situation seems to be more complex concerning the mechanisms of secondary MDR-TB in DM. Possible explanations include higher mycobacterial burden, altered pharmacokinetics of anti-TB drugs, and lower treatment adherence, which promote the selection of MDR strains by anti-TB drugs.[8,9,10] The drug-susceptible *M. tuberculosis* isolates were susceptible to all four (rifampicin, isoniazid, ethambutol, and streptomycin) drugs tested (pansusceptible strains).[9,10]

Drug susceptibility testing results were retrieved for 502 isolates from 490 patients. Ten patients had results for two isolates at different time points, and one patient had results for three isolates. Among the 490 patients with a DST result, 55.0 and 63.1% had resistance to pyrazinamide and ethambutol, respectively.[10] Although these drugs are not used in regimen 1 or 2, the high resistance rates might be explained by easy access to antibiotics without prescription in Vietnam. HIV co-infection, positive baseline AFB smear, older age, and previous treatment with second-line drugs are the main risk factors for poor treatment outcomes in

our cohort, which were also observed in Estonia, Latvia, Philippines, Russia, Peru, and Ukraine. Malnutrition was common (57.8%) and a risk factor for poor outcome (OR: 0.81 for every 1 kg/m2 increase of BMI). Low BMI might be a consequence of severe disease and low socioeconomic status, which are well-known risk factors for poor outcomes in TB.[10] On the other hand, studies conducted in Thailand and Fiji indicated that the death rate was similar in both TBDM and TBNDM patient groups.[11] DM prevalence increased, and TB disease incidence declined, DM was predicted to play a significant and growing role in TB epidemiology.[12]

More extended sputum conversion describes more drugs to which *M. tuberculosis* is resistant. The effectiveness of second-line anti TB drugs in MDR-TB patients is best evaluated in the first eight weeks of administration.[13] The TB and DM interactions potentially cause adverse impact by increasing each other's complications, making diagnosis and treatment more difficult, worsening disease course and outcome.[14] Current TB treatment and control levels in the public sector have led to lower levels of MDR TB prevalence than if TB treatment had been provided exclusively by private-sector clinics that do not follow effective TB treatment protocols. If DOTS had never been implemented and instead the private sector had expanded to cover half of the population, MDR TB prevalence would be approximately 33% larger in 2038 – rising from 32 per 100,000 in 2013 to 56 per 100,000 in 2038.[15,16,17] MDR-TB, especially untreated MDR-TB, would rise rapidly under China's current MDR-TB control strategies. Interventions designed to promote effective detection and treatment of MDR-TB are imperative in the fights against MDR-TB epidemics.[18] Expansion of diagnosis and treatment of MDR-TB, even using current sub-optimal second-line regimens, is expected to decrease MDR-TB incidence at the population level significantly. Focusing MDR diagnostic efforts on previously-treated cases is an efficient first-step approach.[19]

CONCLUSION

Only 0.4 mg IN and clindamycin can be used to treat TB in Manado City. This study results could be used to program planning of prevention and controlling efforts TB-DM in this treatment obedience and regimen anti-tuberculosis medicine for MDR-TB patients.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

FOUNDING INFORMATION

The funding for this research was sourced from the Health Polytechnic of the Manado Health Ministry.

DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Original Research

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Digital communication in health promotion in handling tuberculosis sputum in the COVID-19 pandemic era

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Abstract: The Covid-19 pandemic that has caught the attention of not only Indonesia but the rest of the world. The 3M movement, namely using masks, washing hands and keeping a distance to minimize the transmission of tuberculosis, is very useful for the eradication of TB tuberculosis. However, this is a challenge in itself for microscopic officers at the Microscopic Reference Center (MRC) in education and handling of sputum for tuberculosis patients who are currently being treated. The purpose of this study was to test 4 (four) animated communication videos, namely VisKomLAM 1, 2, 3 and 4 in bridging communication between officers in sputum management education for tuberculosis sufferers at each arrival during the Covid-19 pandemic. The research locations took the Tuminting, Wawonasa and Tikala Baru MRC in Manado City and the Telaga MRC in Gorontalo Regency. Analysis with paired t-test showed a significance below 5%, which means VisKomLAM 1, 2, 3 and 4 can bridge education from officers to tuberculosis sufferers in the Covid-19 pandemic era. It is recommended that health workers always strive to improve tuberculosis health in achieving tuberculosis elimination by 2050.

Keywords: COVID-19; Tuberculosis Elimination; Health Promotion; Video Animation.

INTRODUCTION

In the current era of the Covid-19 pandemic, handling tuberculosis (TB) must remain a concern.[1,2,3] The World Health Organization (WHO) reports that Indonesia is in the third position with the highest cases of tuberculosis (TB) in the world. While the first and second positions are currently India and China.[4,5]

Large-scale restrictions on society due to covid-19 are very beneficial in the spread of tuberculosis in a large community, but the attention of the entire world community at this time, which is focused on covid-19 causes the elimination of public concentration, especially if you see cough for more than 2 (two weeks) that is an early sign / symptom of tuberculosis.[6,7] Early diagnosis in people with early symptoms of tuberculosis cannot be made quickly due to the pandemic.[8]

A good initial diagnosis is very much needed in increasing the Case Detection Rate (CDR) by paying attention to the pandemic area.[9,10] It is not only CDR that is disrupted during this pandemic, but also the Success Rate (SR) Tb.[11]

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This is not only health workers who are responsible for this, but also all parties. The obstacle identified was the limitation of community gatherings, which also made it difficult for officers to find TB sufferers quickly and increase the success of their treatment. It takes a wise way to deal with this.

Like it or not,[12,13] Pulmonary Tuberculosis Program (OPTP) officers are required to know how to use digital media facilities to fulfill program achievements,[14] including delivering detailed and correct education through messages to TB sufferers about the correct way to cough; collect purulent sputum; take medication regularly; regularly and return to check themselves at the appointed time play an important role in achieving tuberculosis elimination and increasing SR during the Covid-19 pandemic. Digital media is currently recognized as making it easier to serve health workers. This is not only the duty of the officers, but also the tuberculosis sufferers themselves, cadres, household members, treatment reminders (PMO) and officers in health services.

MATERIAL AND METHOD

2.1. Research design

This research is an action research that uses applied design with the main objective to facilitate social change. Ethics certificate is issued by Health Research Ethics Committee Health Polytechnic of the Ministry of Health, Manado number KEPK.01 / 03/043/2021.

2.3. Population and sample research

Population : TB sufferers; cadre; Direct Contact; and Microscopic Officer at MRC Tuminting; Wawonasa; Tikala Baru Manado City and MRC Telaga Gorontalo District

Sample : each type of population was taken 5 for each MRC

2.4. Materials and research tools

VisKomLAM 1 : During the first sputum inspection when the suspect Tb. The emphasis is on sputum collection, because it is their first time doing ARB examinations, plus the mandatory return after undergoing treatment for the second month. Of course other health promotion is also important.

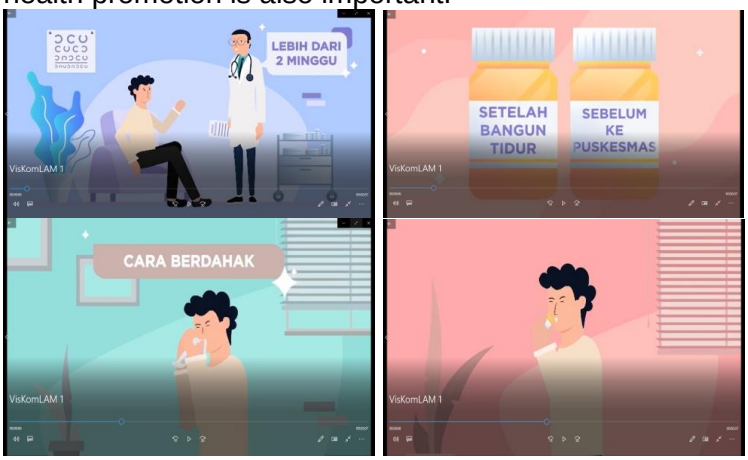

Figure 1. Some of the scripts on VisKomLAM 1

VisKomLAM 2 : At the second sputum examination, after 2 months of treatment. The emphasis is on having to return to the sputum examination after undergoing the fifth month of treatment, because by the fifth month of treatment, the patient feels very healthy.

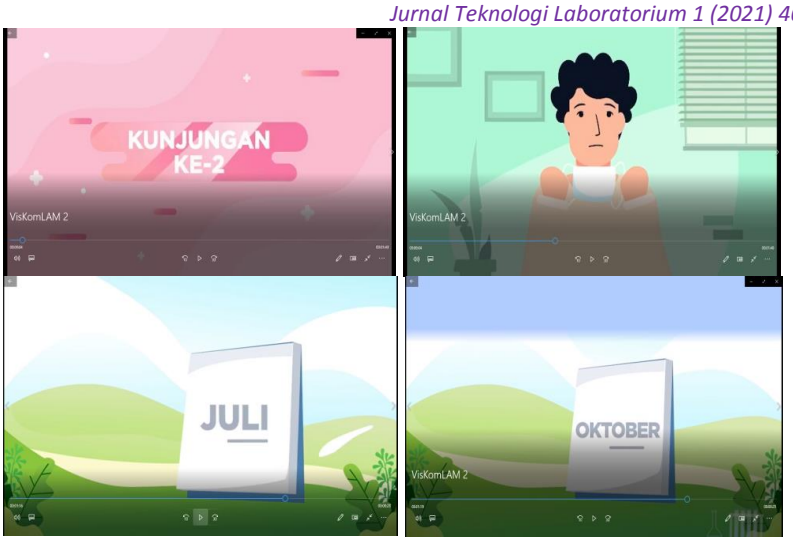

Figure 2. Some of the scripts on VisKomLAM 2

VisKomLAM 3 : At the third sputum examination, after 5 months of treatment. It is mandatory to return after undergoing treatment is complete, because when the treatment is complete, the patient feels that he does not need another examination. In some cases, there are sufferers who require further treatment for two months.

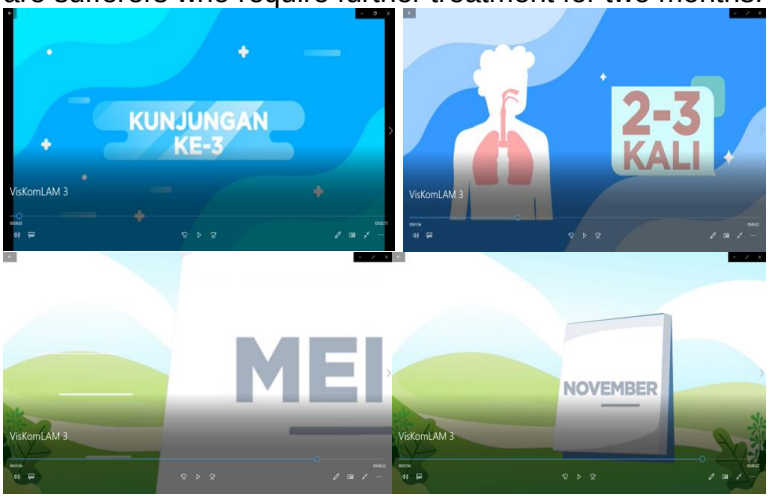

Figure 3. Some of the scripts on VisKomLAM 3

VisKomLAM 4 : At the fourth sputum examination, at the end of 6 months of treatment. It is obligatory to carry out a sputum examination while maintaining a clean and healthy lifestyle. The important thing to overcome the stigma in society is that tuberculosis is a disease that can be cured, "I am an example".

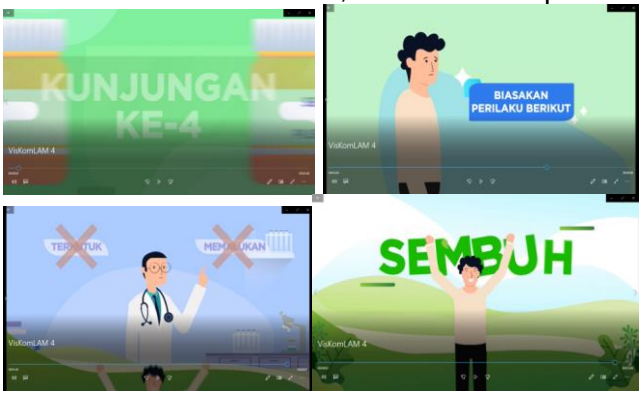

Figure 4. Some of the scripts on VisKomLAM 4

2.5. Collection / research stages

1) Out of 4 (four) ARB, namely 3 in Manado City and 1 in Gorontalo District, each is taken :
   5 respondents suspect
   5 respondents 2 months treatment
   5 respondents 5 months treatment
   5 respondents completed 6 months of treatment
2) Given a pretest
3) Watch the video based on the respondent's visit category
4) Given a post test

2.6. Data analysis

Univariate data analysis used descriptive and multivariate using t paired test

.

RESULTS AND DISCUSSION

Tuberculosis is an almost neglected disease during the Covid-19 pandemic, this can be seen from the decrease in Case Rate Detection (CDR) in 2020.[5] The stigma of being infected with COVID-19. this requires attention in TB research and funding.[15]

When someone with a cough for more than 2-3 weeks comes to the MRC and is referred to a BTA sputum examination, they should receive education on how to collect purulent sputum, how to collect it in a pot.[1,16,17] Online communication using digital communication is a current solution that can be taken as a bridge between officers and sufferers.[18] This can be seen from the increase in respondents' knowledge and understanding after watching VisKomLAM 1 with a significance of 0.000 (table 1).

After undergoing treatment for 2 (two) months, the TB patient came to the MRC to do another sputum BTA examination. Education about taking medicine regularly, periodically changing sleeping equipment, maintaining air circulation and the sun in the room and at home, exercising regularly and having a clean and healthy lifestyle, eating nutritious food and returning to the next examination after five months of treatment.[19] The significance of 0.000 in 2 MRCs, namely Wawonasa Kota Manado and Telaga Kabupaten Gorontalo for MRC Tuminiting 0.020 and Tikala Baru 0.010 in increasing the knowledge and understanding of respondents after watching VisKomLAM 2 (table 1).

At 5 months of treatment, TB sufferers must come even though they do not feel any pain so that cases do not drop out.[20] Microscopic officers still have to educate about symptom recognition, how to cough properly, handle sputum, take medication regularly, periodically change bedding, maintain air and sun circulation in the room and house, exercise regularly and have a clean and healthy lifestyle as well. eat nutritious food through getting to know the nutritious menu and must return at the end of treatment, which is the sixth month. Increased knowledge and understanding of respondents after watching VisKomLAM 3 can be seen the significance level in 3 MRCs in Manado City 0,000; 0.010 in MRC Telaga Gorontalo District in increasing knowledge and understanding after watching VisKomLAM 3 (table 1).

After 6 months of treatment, TB sufferers are required to be examined at the MRC to ensure a negative smear sputum result. Microscopic officers still have to educate about symptom recognition, how to cough properly, handle sputum, take medication regularly, periodically change bedding, maintain air and sun circulation in the room and house, exercise regularly and have a clean and healthy lifestyle and eat. nutritious food through knowing nutritious menus and breaking the stigma of society that TB is a curse disease and cannot be cured and the proof is that I am already cured.[21] In table 1, it can be seen that the significance at 4 MRCs is below 0.05: PRM Wawonasa 0.0180; Tuminting

0.0200; Tikala Baru and MRC Telaga Gorontalo District 0.0010 after watching VisKomLAM 4.

Table 1: Significance of VisKomLAM 1, 2, 3 and 4 in 4 PRM

Significance	Microscopic Referral Health Center (MRC)			
	Wawonasa	Tuminting	Tikala Baru	Telaga
VisKomLAM 1	0.000	0.000	0.000	0.000
VisKomLAM 2	0.000	0.020	0.000	0.010
VisKomLAM 3	0.000	0.000	0.000	0.000
VisKomLAM 4	0.180	0.020	0.010	0.010

Preventing TB patients from becoming MDR[22] can be done using digital communication,[23] as well as increasing CDR and SR TB in the Covid-19 pandemic era which can be bridged with VisKomLAM 1, 2, 3 and 4 digital communications.

CONCLUSION

Digital communication using VisKomLAM 1, 2, 3 and 4 in the era of the Covid-19 pandemic in handling sputum, how to cough properly, taking medication regularly, periodically changing sleeping equipment, maintaining air and sun circulation in the room and house, doing sports regularly and behave in a clean and healthy life as well as eating nutritious food through knowing a nutritious menu is very helpful in bridging communication between microscopic officers and TB sufferers.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

FOUNDING INFORMATION

The funding for this research was sourced from the Health Polytechnic of the Manado Health Ministry.

DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Air-Dried and wet fixation on Fine Needle Aspiration Biopsy (FNAB) specimen

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Abstract: Two fixation methods can affect the quality of cytological staining derived from fine-needle aspiration biopsy, but both fixation methods have drawbacks. Dry fixation can cause cell rupture, while wet fixation requires a longer time and costs more, so dry fixation is more often used. The purpose of this study was to compare the results of the Diff-Quick staining of cytological preparations that were fixed with the dry fixation method and the wet fixation method on FNAB samples. This study was an experimental study with 36 samples of cytology preparations from FNAB, divided into three groups, the control group, dry fixation group, and wet fixation group. Staining dry fixation preparations gave four poor results, five good preparations, and three very good preparations while staining wet fixation preparations gave 0 poor results, eight good preparations, and four very good preparations. This shows that the staining results of wet-fixed preparations tend to be of better quality and more consistent than the staining results of dry-fixed preparations. Even though statistically through the test *Post-Hoc* showed no difference, the quality of Diff-Quick staining of cytological preparations fixed by wet fixation method was better than dry fixation, so the wet fixation method was more recommended for cytological preparations than FNAB.

Keywords: Air-dried fixation; Wet fixation; FNAB; Diff-Quick.

INTRODUCTION

Cytology specimens are easy to obtain in the laboratory, but proper technique in collecting cytological specimens needs to be considered because it can affect the examination results.[1] Cytological specimen collection techniques are divided based on the type of specimens such as rinses in the stomach, bladder, and bronchi, or *scraping* as in the Pap *Smear*, as well as those collected directly such as sputum and urine.[2] In addition, specimens can also be obtained from FNAB (*Fine Needle Aspiration Biopsy),* which is widely used and is a quick and inexpensive diagnostic technique.[3] The FNAB technique can be used to diagnose *breast* cancer*,* namely to determine the presence of cell malignancies.[4] In addition, evaluation of samples from FNAB is vital for the pathologist to confirm sample adequacy, additional tests and establish an initial cytologic interpretation for the tumor.[1,5]

Specimens from FNAB are usually received directly in the form of smears taken by medical personnel who have met the requirements or have received training.[6,7] *The smear* from the FNAB then will be fixed and stained with the appropriate method and technique.[2] Two types of fixation can be carried out on cytological specimens in the form of *smears,* namely wet fixation and dry fixation.[7] Wet fixation using alcohol and stained with Papanicolaou or

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Hematoxylin Eosin. While dry fixation using Romanonowsky staining.[8,9] Wet fixation with Papanicolaou stain is recommended for staining core morphology for specimens from FNAB and effusions, while dry fixation is recommended for viewing details of the cytoplasm.[7]

Fixation on cytological smear preparations can affect the condition and stability of cells, prevent loss of cell content, display reactive sites for staining, and increase cell membrane permeability for dyes.[10] Dry fixation and wet fixation methods are commonly used in the fixation of cytological preparations. The wet fixation method can keep the cell conditions as close as possible to the conditions when they were still in the body.[11,12] Fixation solutions used in the wet method include alcohol, methanol, propanol, isopropanol, and formalin. The dry fixation method can be done by drying the preparation in the open air or heating by hairdryer.[13] The dry fixation method is relatively more manageable, cheaper, and shorter in operation than the wet fixation method and can prevent the occurrence of artifacts. However, dry fixation can result in an increase in cell size, an apparent effect on the size and shape of the cell nucleus.[7,8,14] The speed with which the results of the cytological examination are released is essential. Therefore the fixation is not too long to reduce the waiting time required of the patient to obtain the results.[10,12] Health agencies will usually choose a fixation method according to the needs and the available reagents and tools. The choice of fixation method for cytology preparations is adjusted to the quality of the preparation, time, and cost-efficiency.

The fixation of cytological preparations is one of the factors that can affect the staining of the preparations. Papanicolaou stain is commonly used for cytological samples but has the disadvantage, its takes a long time in the staining process.[15] One of the commonly used Romanowsky stains because it is easy to do, inexpensive, and does not require a long time so that the test results can be issued quickly is the Diff-Quick stain.[16,17] Research that compares the results of Diff-Quick staining on cytological preparations from FNAB with the wet fixation method and the dry method needs to be done to determine which fixation method is better to use. Similar research has never been done before; therefore, this research needs to be done. This research will reference the use of a good and efficient method of fixation of cytological preparations from FNAB samples, both in terms of time and in terms of costs.

MATERIAL AND METHOD

This type of research is an experimental design with *Post-test Only Group Design*. The research was carried out in August 2020 at the Anatomical Pathology Laboratory at one of the General Hospitals in Semarang. The samples used were cytology preparations of patients in the Laboratory of Pathology and anatomy obtained through the FNAB method as 36 preparations. The preparations obtained were divided into three groups based on the fixation method used, namely the dry fixation method, wet fixation method, and control as a comparison. The dry fixation method was carried out by drying the preparation using a *hairdryer* until the preparation was dry. The wet fixation method is done by immersing the preparation in 95% alcohol solution for 1 minute. The control fixation group is the fixation used in the Pathology anatomic Laboratory, where the research was conducted by combining dry fixation and wet fixation. After all, preparations have been fixed, proceed with staining the preparations using the Diff-Quick stain, which is available in a Staining Kit. The staining procedure begins with each preparation dipping in 8 dips of Diff-Quick I (Eosin) solution, then dried. Then it was dipped in 8 diff-Quick II (Methylene blue) solution. The preparation is then rinsed in running water, then allowed to dry. An Anatomical Pathologist Specialist read the dried preparations. The criteria for reading the preparations are following Table 1.

Table 1. Criteria for Assessment of Preparation Quality

Criteria	Category		
	Not good	**Well**	**Very good**
Cell shape	Unclear	Clear	Very clear
Nucleus	A colored core is not clear	The core is colored	The colored core is very clear
Nucleolus (child nucleus)	Not visible	Seen	See clearly
Cytoplasm and intracellular components	Unclear	Clear	Very clear

RESULTS AND DISCUSSION

The results of the microscopic study showed that the staining quality of the preparations fixed by the dry fixation method had less than optimal results. Poor staining results were only found in the preparations fixed by the dry fixation method, while similar results were not found in the preparations using the wet fixation method and controls (table 2). Several factors that can cause this to happen include inadequate drying so that there is still water in the preparation. The presence of water content in the preparation is a phenomenon known as a *drying artifact*.[18] This phenomenon can be observed in the picture of red blood cells that are round or sickle, with cells that look like holes or have a structure like a refractile vacuole in Figure 2. In addition, the presence of water content in the smear preparation will cause the cells to become moist, which will eventually result in the dye cannot bind perfectly to the cell components in the smear preparation. The result is that the preparation is not appropriately stained, such as the results of research by Jhala that dry fixation is a fixation method that is often used in diff-quick staining, but if the drying process is not suitable, it can cause poor staining results and detail pictures.[19]

The results also showed that the microscopic appearance of cells for the preparations fixed by the dry fixation method was less than optimal. In addition, cell details such as the nucleus and nucleolus are less observable. The slow drying process can cause the cell to become hypertonic due to the evaporation of water that occurs by increasing the temperature, which causes the rupture of the cell membrane and nuclear membrane.[10] This is also in line with the study results that the preparations fixed by the dry fixation method had relatively fewer cells that could be observed compared to the preparations fixed by the wet method or in the control preparations. Factors allow this to occur due to cell rupture due to drying using a *hairdryer*.[18]

Preparations fixed with alcohol can cause the blood smear to be insoluble in water, whereas this is not found in preparations fixed by drying. This occurs because of the effect of the alcohol fixation solution, which reduces the electric charge, and the decrease in the dielectric constant, which allows for stronger attractive bonds between protein molecules.[20] This is also one of the factors causing poor results in the preparations fixed by the dry fixation method because the cells can also dissolve with water. After all, the final process of diff-quick staining is rinsing the preparation with running water.

Good and very good staining results were obtained in all fixation methods because the three fixation methods can trigger the denaturation process. The denaturation process occurs due to the presence of solvents such as alcohol and also high temperatures. This condition causes an increase in density and a decrease in cell elasticity, and an increase in reactivity to acid-base dyes such as diff-quick dyes. The reaction is further because it involves the opening of globular proteins that increase the number of *reactive groups* capable of binding to acid-base dyes.[20] According to the research results, the dye can bind well to the cells so that the preparation is stained well or even very well.

The results of the Kruskal-Wallis statistical test (p-*value* > 0.05) showed that the quality of the staining results of the preparations fixed by the dry method and the wet method had no difference. The results of *Post Hoc* analysis between the staining results of wet-fixed and control preparations (p-*value* > 0.05) and between dry fixation and control (p-*value* < 0.05), it can be interpreted that the preparations fixed by the wet method have similar staining results with statistical control when compared with the staining results of the preparations that were fixed by the dry method. However, microscopically, the staining results of the preparations fixed by the dry method did not show the same results as wet fixation. In addition, the results of staining with preparations fixed by the dry method were also inconsistent, as evidenced by the results obtained that were too varied compared to the wet fixation method, which did not show poor results and was consistent with good staining results. The results obtained in this study are in accordance with the research results conducted by Rupinder that wet fixation gave better results than a dry fixation on *cervical smear* samples.[11] The same results were also shown by Randal & van Amerongen[21] and the results of Safnect[22]. Different results were shown by Shiddam[3] on the FNAB and Gupta samples[7] on *cervical smear* samples, dry fixation showed better staining results compared to wet fixation.

CONCLUSION

The research found that the staining quality of the cytological sample preparation from FNAB on diff-quick staining with wet fixation was better than dry fixation. According to the results of this study, wet fixation was preferred for cytological samples from FNAB compared to dry fixation. The results also showed that combining the two fixation methods as the control group in this study showed the best results for the FNAB sample.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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Table 2. Percentage of Preparation Based on Fixation Method

	Category						Total	
	Not good		Well		Very good			
	n	%	n	%	n	%	n	%
Control	0	0	4	33.3	8	66.7	12	100%
Dry Fixation	4	33.3	5	41.7	3	25.0	12	100%
Wet Fixation	0	0	8	66.7	4	33.3	12	100%

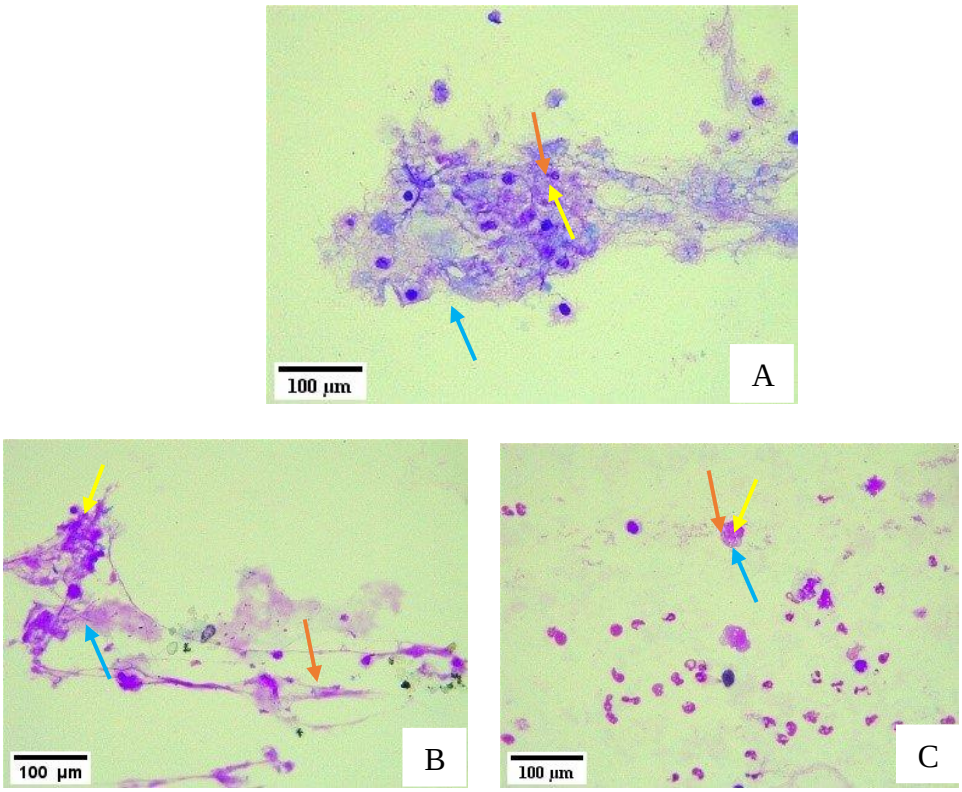

Figure 1. Result for a Well Good Staining Criteria

(A) Dry Fixation
(B) Wet Fixation
(C) Control
Cell form (→); Nucleous (→): Nucleoulous (→)

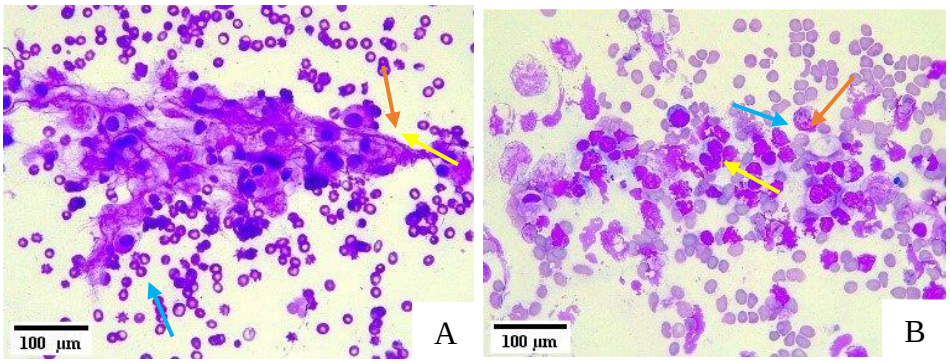

Figure 2. Result for a Very Good Staining Criteria

(A) Control
(B) Wet Fixation
Cell form (→); Nucleous (→): Nucleoulous (→)