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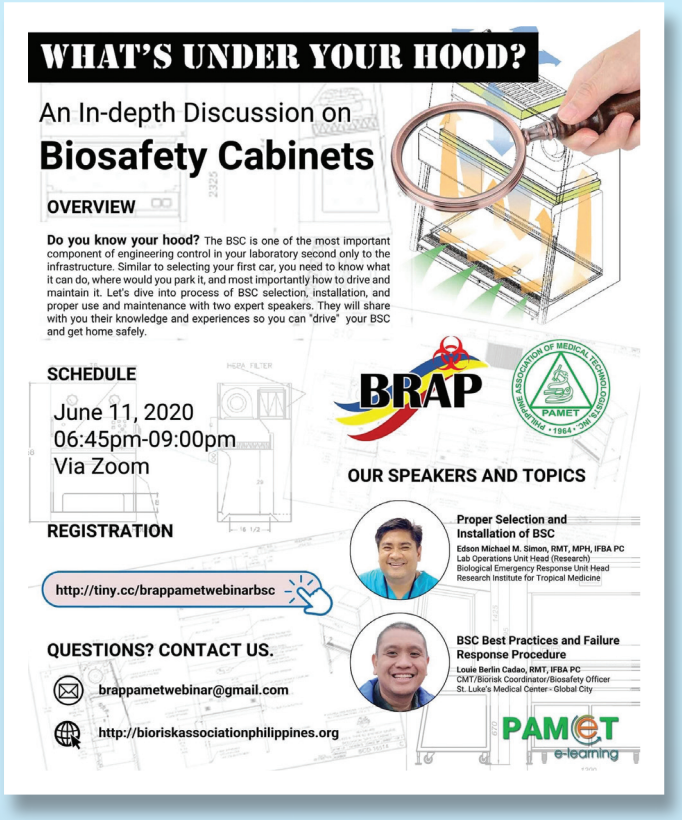

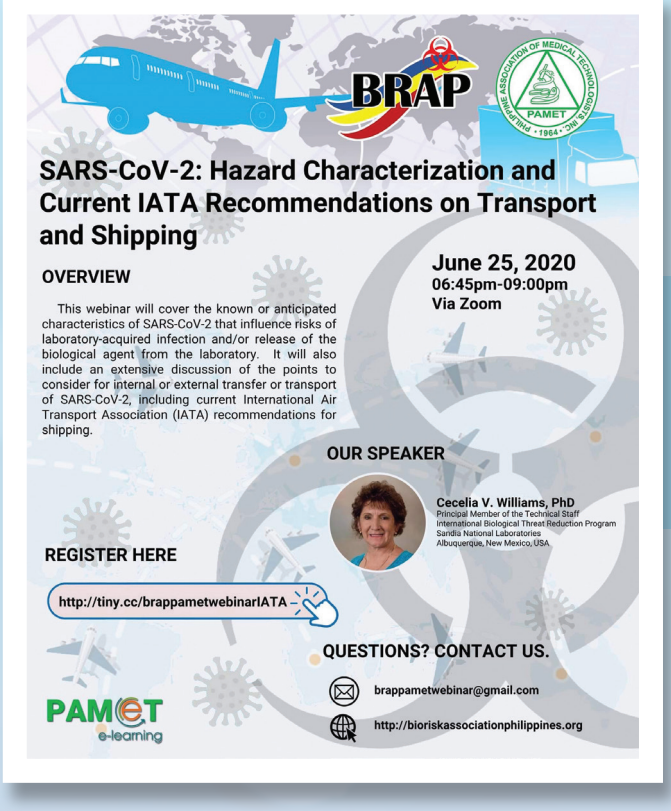

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The Philippine Journal of Pathology (PJP) is an open-access, peer-reviewed, English language, medical science journal published by the Philippine Society of Pathologists, Inc. Committee on Publications. It shall serve as the official platform for publication of high quality original articles, case reports or series, feature articles, and editorials covering topics on clinical and anatomic pathology, laboratory medicine and medical technology, diagnostics, laboratory biosafety and biosecurity, as well as laboratory quality assurance. The journal's primary target audience are laboratorians, diagnosticians, laboratory managers, pathologists, medical technologists, and all other medical and scientific disciplines interfacing with the laboratory. The PJP follows the **ICMJE Recommendations for the Conduct, Reporting, Editing and Publication of Scholarly Work in Medical Journals, EQUATOR Network Guidelines,** and **COPE Guidelines.** The PJP does not charge any article processing or submission fees from authors. It does not charge any subscription fees or download fees to access content.

Warm Greetings!

Welcome to the December 2020 issue of the Philippine Journal of Pathology. Congratulations to the editorial team of the PJP and the PSP Board of Governors for their untiring efforts in coming up with this issue in spite of the COVID-19 pandemic that we are continuously fighting.

The government directives of lock-downs and community quarantines resulted to limitation in our mobility and face-to-face encounter. Added to these is our fear of contracting the deadly COVID-19. These were the challenges that our editorial staff, the Board of Governors, and the PSP members took to work harder and continue to come up with this succeeding issue of the PJP. Their implacable support, commitment, and dedication made all this possible.

We are happy that you are joining us as readers and hope that you will also support us by submitting your scientific study and research paper for publication in PJP.

Let us hope for many more issues. More power to the Philippine Society of Pathologists, Inc. and the Philippine Journal of Pathology.

Wishing you all a Merry Christmas and a healthy and safe 2021!!!

Roberto D. Padua Jr., MD, FPSP, MHA
President, Philippine Society of Pathologists, Inc.

Annus Horribilis, Annus Mirabilis

We all started 2020 with high hopes and expectations, brimming with dreams and full of plans for what was supposedly a lucky year. But 2020 is an *annus horribilis*, a year that this generation will not forget, as we all came face to face with an outbreak of global proportions. That a submicroscopic assembly of protein and nucleic acid can wreak so much havoc to our supposedly modernized lives, bringing economies and health systems down on their knees, reiterates how fragile and ill-prepared the world is.

I welcome all of you to another issue of the Philippine Journal of Pathology, our second for 2020. We feature this time two articles related to the COVID-19 pandemic: the first, a review of the challenges on contamination for SARS-CoV-2 molecular laboratories which should be recognized and addressed to ensure quality laboratory results; and the second, the pragmatic study on pooled testing of the Philippine Society of Pathologists, which now forms a solid basis for the recent policy issued by the Department of Health. Rounding up this issue are 2 interesting case reports on a primary rhabdomyosarcoma in the brain as well as a metaplastic carcinoma post breast augmentation with silicone, and a meta-analysis on the utility of mean platelet volume for diagnosis of acute myocardial infarction.

I am very pleased also to share the editorial byline for this issue with Dr. Raymundo Lo, one of PJP's esteemed editorial board members, a respected columnist tackling issues on laboratory medicine, a member of the DOH COVID Laboratory Experts Panel (CLEP) representing the Philippine Society of Pathologists, and the principal investigator of the pooled testing study, as he traces the journey from germination to fruition of the study amid the changing landscape of clinical pathology in the country. It is my hope that the Society and its members will be inspired by this concrete example of research being translated to policy and practice. Ultimately, this is what research is meant to be for.

In 1905, Albert Einstein published four studies in the scientific journal Annals of Physics. These articles are now known collectively as the *Annus mirabilis* papers – considered as major contributions to the foundation of modern physics, revolutionizing the concepts of time, space, mass, and energy, and which included his most famous formula, $E=mc^2$. Remarkably, all were published in a single "miracle year." While these articles were groundbreaking and continues to break ground up to the present, *our* collective efforts as pathologists in this side of the globe to battle the COVID-19 pandemic with laboratory science, is historic.

2020 is a year that has brought on the worst, and yet brought out the best in us: an *annus horribilis*—what I have described in the last issue as the Year of the Filipino Pathologist—that is nothing short of *mirabilis*.

Amado O. Tandoc III, MD, FPSP
Editor-in-Chief

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A New Era in Infectious Disease Testing

Scarcely a year ago, we heard the rumblings of a looming pandemic. What followed was a journey of self-discovery for the Philippine Society of Pathologists and its members. Early on, we watched helplessly while the pandemic raged on in our country, handicapped by the lack of laboratory resources to test the increasing number of cases. Only the Research Institute for Tropical Medicine (RITM) was equipped and enabled to test for SARS-CoV-2 at that point in time. Most molecular testing was done in private hospitals where the emphasis was on oncology.

The Department of Health launched a program to set up molecular laboratories across the country and pathologists joined in the scramble to capacitate their own laboratories to fight the pandemic. Meanwhile, testing was still very limited and patients were dying without being diagnosed as results were delayed up to two weeks.

The PSP set up a Viber group as a venue for its members to discuss the ongoing events and assist each other with information. This was crucial as the lockdown prevented in person meetings. Upon inquiring with former PSP president Dr. Bernadette Espiritu, the PSP had issued a statement on rapid antibody testing but had not gotten to the point of addressing the society's position on the best response to the pandemic. I suggested we craft a position paper on what we can offer in terms of laboratory testing. What followed was a series of Zoom meetings where we discussed and debated the contents of the PSP statement.

In late May 2020, the PSP released its position paper and almost immediately, the DOH, through USec. Ma. Rosario Vergeire, invited us to present our position paper to the newly formed COVID Lab Experts Panel (CLEP), which at that time did not have a single pathologist in its membership, which was composed mostly of molecular scientists from the academe.

Our position paper was well received and we were encouraged to come up with a proof-of-concept paper on pooled testing which we had advocated as a means of testing on a wider scale than can be done with individual RT-PCR testing. As a result of that meeting, where we pointed out the obvious lack of pathologist representation in the CLEP, Dr. Socorro Yanez and myself were invited to join, a good sign that pathologists are now being taken seriously in the pandemic response.

Again, we had a series of night-long meetings where we crafted the research proposal, which was presented to the CLEP for their approval. It was endorsed to the DOH and subsequently, we were given the go-ahead signal.

During this period, we had the good fortune to be contacted by the Philippine Chamber for Entrepreneurship Foundation Inc., locally better known as GoNegosyo through its RT-PCR Chief Implementor, Dr. Janette Loreto-Garin (Congresswoman of Iloilo). A partnership was born out of the mutual desire to help the ailing Philippine economy suffering its worst contraction since the Marcos years. GoNegosyo graciously agreed to fund the research and we proceeded with alacrity to pursue the research. We are eternally grateful to Mr. Jose Concepcion III, founder of GoNegosyo, Josephine Romero, Project Leader for Project Ark / GoNegosyo COVID-19 Response Initiative, Dr. Criselda Abesamis, PCE Project Consultant and Coordinator for Pooled PCR Testing (a fellow pathologist) and Dr. Garin for forging a lasting relationship with our society.

As a multi-institutional study, it was necessary to clear the Institutional Review-Ethics Committee of each of these institutions, RITM, Philippine Children's Medical Center (PCMC) and University of Perpetual Help-DALTA Medical Center. It took more than a month to secure clearance but in late July we had done Experiment 1 at the RITM, which laid the basis for Experiment 2, done in early August.

When the initial write up of the research was done, the authors met again, this time in person, to finalize the concept paper and provide the necessary framework for pooled testing implementation, including guidelines, quality assurance schemes and training modules for both specimen collection as well as pooled testing and interpretation of results.

By September, we had finished the research and it was presented to the combined panels of CLEP, Technical Advisory Group (TAG) and Health Technology Assessment Council (HTAC), where it was well received.

More hurdles laid ahead. We had to present the pooled testing concept to the Inter- Agency Task Force (IATF) for COVID Response, initially as a concept and later as a finished research complete with the aforementioned accompanying documents. The response was encouraging.

Still, approval was not yet forthcoming. We then initiated pilot project implementation of pooled testing in several LGUs which further resulted in more refinements and process improvements in the pooled testing concept.

Once more, in October, we presented our pilot project results to the CLEP-TAG-HTAC joint meeting. There was consensus that pooled testing will be useful and it was again endorsed to the DOH for final decision. As the HTAC was the last arbiter before approval, it fell on its members to endorse the program to the DOH.

Finally, in late December, our pooled testing program was finally approved with the issuance of the Interim Guidelines on the Conduct of COVID-19 Pooled Testing (dated November 23, 2020 but released December 29, 2020).

It was a long journey but we have proven that we, as pathologists, the real clinical laboratory experts, can contribute to the efforts to mitigate the effects of the pandemic and in the process, make ourselves heard and recognized on the national scene.

Our contribution marks a significant milestone in our Society's history. It is not only a game changer in itself but a first worldwide. Though pooled testing has been done earlier in different countries, no other country has made an effort to systematize and conduct pooled testing on a comprehensive basis such as ours. This complete package of pooled testing will stand as a testament to the hard work, dedication and persistence of members of the PSP who believed they can make a difference.

Our pooled testing process will not only make a difference in our current situation, but I believe it is a lasting contribution to the Philippines, if not the whole world, that will make itself useful in response to future pandemics, and we all know in our hearts and minds, the COVID-19 pandemic will not be the last.

To my fellow pathologists, I salute and thank you for your courage, determination and fortitude in undertaking this journey and in the process, prove to ourselves we have the capacity to lead and to effect change, eventually in recognition of the PSP as leader in the medical field.

The COVID-19 pandemic has indeed changed the entire landscape of clinical laboratory testing. We have gone from being way behind in molecular testing to being quickly capacitated in response to this existential threat to mankind. It has been both a bane and a boon to pathologists. A crisis, as the Chinese proverb says, is both a danger and an opportunity. We grabbed the crisis by the horns ad successfully wrestled it by coming up with our innovation in pooled testing.

It is therefore with great pleasure that we publish this portfolio of work in the Philippine Journal of Pathology as a tribute to the Philippine Society of Pathologists Inc. and the Philippine Chamber for Entrepreneurship Foundation Inc.

May this serve as an inspiration for our Filipino pathologists to engage and be more active in world class research that can be published in our very own Philippine Journal of Pathology.

Mabuhay ang Philippine Society of Pathologists Inc.!

Raymundo W. Lo, MD, FPSP
Principal Investigator

Postscript:

Special thanks to the following who did the legwork with us during the sample collection for Experiment 2, risking their safety by coming face-to-face with our research subjects: Drs. Farrah Fontilla-Santiago, MD, FPSP, Melani Hernandez-Sionzon, MD, FPSP, Socorro Yanez, MD, FPSP, Bernadette Espiritu, MD, FPSP, Agnes Barrientos, MD, FPSP, Mr. Eidelbert Santiago, RN and the PCMC COVID Testing Laboratory Analysts and swabbers.

Also special mention of Amado Tandoc III, MD, FPSP, our indefatiguable Chief of the Laboratory Research Division, RITM who handled Experiment 1 and crafted the Verification Procedure, and Mark Ang, MD, FPSP, who did the statistical analysis for this research.

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OPEN ACCESS – REVIEW ARTICLE

Cross-contamination in Molecular Diagnostic Laboratories in Low- and Middle-income Countries: A Challenge to COVID-19 Testing

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ABSTRACT

At the start of the pandemic, the Philippines had to send swab samples to the Victorian Infectious Diseases Reference Laboratory in Melbourne, Australia for COVID-19 confirmation. With the increasing number of suspected cases needing confirmatory diagnostic testing, there was a demand to rapidly expand the capacity for widescale testing. Remarkably, within 200 days from announcement of the first confirmed COVID-19 case in the Philippines in January 30, 2020, the country has been able to expand its testing capacity from one national reference laboratory, the Research Institute for Tropical Medicine (RITM), to more than 100 licensed reverse transcription-polymerase chain reaction (RT-PCR) and cartridge-based PCR laboratories across the country. Due to the shortage of a trained clinical laboratory workforce, diagnostic centers are forced to hire additional personnel who have limited experience and technical knowledge and skills of molecular assays, especially in processing specimens, interpreting the results, identifying errors, and troubleshooting, in order to meet the demand of increased testing. Thus, the vulnerability to diagnostic errors, including cross-contamination, is increased and with the tendency for generating false-positive results that can compromise the health of the patient and disrupt the efficacy of public health policies and public health response, surveillance programs, and restrictive measures for containing the outbreak. Hence, this review article aims to present the different sources of contamination in the laboratory setting where RT-PCR assays are conducted, as well as provide efficient, effective and feasible solutions to address these issues, most especially in low- and middle-income countries (LMICs) like the Philippines.

Key words: SARS-CoV-2; LMICs; RT-PCR, cross-contaminations, quality control, COVID-19

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INTRODUCTION

The emergence of the coronavirus disease 2019 (COVID-19) has caused a global public health emergency. The rapid escalation in the number of infections resulted in widescale shortages of personal protective equipment (PPE), diagnostic test kits, and essential equipment for patient treatment such as respirators. In resource-limited countries like the Philippines and Indonesia, the overwhelming influx of severe COVID-19 cases has restricted testing to those who have severe symptoms and needing hospitalization.[1,2] Consequently, the inability to rapidly expand the capacity for widescale testing has hindered response efforts. Moreover, the limited efforts for rapid contact tracing even in the absence of diagnostic testing could have also contributed to the rapid transmission of the virus.

Testing of samples for COVID-19 diagnosis is an integral part in resolving the current pandemic. The efforts of the Department of Health (DOH), Research Institute for Tropical Medicine (RITM), the University of the Philippines (UP), Philippine National Red Cross (PNRC) and the World Health Organization (WHO) with respect to building laboratory networks, providing training and proficiency testing, licensing labs, establishing networks for distributed testing, logistics, and addressing the challenges of identifying, validating, and approving test kits for use in the Philippines have been impressive.

Prior to the pandemic, the expertise and capabilities to test for COVID-19 and similar entities were mostly coming from the academe and research centers. In addition, lockdown restrictions hindered molecular biologists from volunteering their services. When specimen collection was heightened, backlogs in testing were experienced as molecular diagnostic laboratories were being constructed across the country.

At the start of the pandemic, the Philippines had to send swab samples to the Victorian Infectious Diseases Reference Laboratory in Melbourne, Australia for COVID-19 confirmation.[3] Remarkably, within two hundred days from announcement of the first confirmed COVID-19 case in the Philippines in January 30, 2020, the country has been able to expand its testing capacity from one national reference laboratory (RITM) to 23 licensed testing laboratories.[4] As of September 4, 2020, RITM has helped accredit a total of 117 reverse transcription – polymerase chain reaction (RT-PCR) and cartridge-based PCR laboratories across the country.[5]

Indeed, molecular diagnostic laboratories play a pivotal role in the diagnosis and management of human diseases, including COVID-19. Considering that RT-PCR remains the gold standard for verifying COVID-19 cases, the diagnostic accuracy for this technique is of utmost importance.[6] Thus, this article aims to present the different sources of contamination in the laboratory setting where RT-PCR assays are conducted, as well as provide efficient, effective and feasible solutions to address these issues, most especially in low- and middle-income countries (LMICs) like the Philippines.

SPECIMEN COLLECTION

When collecting specimens, it is important to properly identify the sample, collect adequate amount or volume as well as practice standard protocols in the transport and proper storage of biological materials to be tested.[6]

LMICs, however, are confronted with limitations in the availability of PPEs and scarcity of manpower while the number of suspected cases needing confirmatory testing is exponentially growing. With limited resources and excessive workload, compliance with the recommended protocols might be challenging but cannot be discounted since breaching them can be an immediate source of cross-contamination that can jeopardize the accuracy and quality of RT-PCR testing as well as a source of laboratory acquired infections.

For healthcare providers collecting specimens or within 6 feet of patients suspected to be infected with SARS-CoV-2, proper infection control must be observed. Prior to specimen collection, all PPEs must be sanitized and worn following the proper sequence. When wearing gloves, it is important to cover part of the forearm while assuring that they remain under the sleeves to minimize skin exposure. Using a second pair of gloves may also be done to cover part of the sleeves. PPEs, including the gown, FFP2 (N95), goggles or face shield, and gloves must be worn all the time.[7] Male health workers are also advised to shave in order to obtain an adequate mask's adherence to the face.[8]

When collecting the sample, the patient must be seated in a comfortable position with the head resting against a plexiglass divider. After collection, the nasopharyngeal or oropharyngeal swabs are placed in sterile test tubes. The tubes are then properly labelled with the patient's personal data and transported to the laboratory in special containers designated for biohazard materials.[8-10] Proper labelling, handling, and storage of collected sample is important not only to avoid a false positive result, but a false negative as well.[7]

It is crucial to change gloves and to clean the work area between each collection to prevent cross-contamination. If this is not feasible or is impractical given the limited resources and manpower on top of a demanding workload, an option is to disinfect the gloved hands with 70% alcohol in a squeeze or spray bottle and then dry with fresh paper towel after each patient. Surfaces of the collection booth, which are made of plastic or metal or sealed with a non-porous cover, should also be disinfected especially when patients made physical contact on the area. WHO has provided guidelines on the use of disinfectants such as sodium hypochlorite or bleach (0.1% for general surface disinfection and 1% for disinfection of sample spills), 62-71% ethanol, 0.5% hydrogen peroxide, quaternary ammonium compounds, and phenolic compounds (used according to manufacturer's recommendations). Although less effective, 0.05-0.2% benzalkonium chloride or 0.02% chlorhexidine digluconate can also be used. Apart from using the correct disinfectant, contact time, dilution and shelf-life should also be considered.[11] Alcohol can also be sprayed, but must be wiped only after at least 20 seconds of contact with the surface. Bleach solutions should be prepared fresh each use.[11] The sample collection boxes or coolers, reusable cold packs, pouches, and racks must also be regularly disinfected. But it must be noted that after disinfection, the technician must wipe the surfaces with paper towel wet with sterile water followed by 70% alcohol dampened paper towel to prevent residue build-up and PCR inhibition.

When sampling is done, removal of PPEs should follow correct sequence while avoiding contact with external surfaces. The suit, shoes, used gloves, and used mask must be placed in a special waste container. The hands are also cleansed with soap and water or sanitized with alcoholic solution.[8]

ANALYTICAL ISSUES

Confirmatory laboratory tests through nucleic acid amplification assay is performed for suspected cases. The use of RT-PCR remains the gold standard for testing wherein unique sequences of the SARS-CoV-2 genome are detected.[12] However, RT-PCR is labor intensive and is an inherently complex assay requiring experience in all aspects of testing, and thus limiting the capacity for quick turnaround time from sample collection to the availability of results. This bottleneck may lead to long wait periods and an exponential demand for testing.[6] With the increasing number of suspected cases needing confirmatory diagnostic testing, laboratory personnel are forced to work under severe pressure in high-throughput settings with an insurmountable workload and with limited

access to personal protective equipment (PPE).[13] Due to the shortage of a trained clinical laboratory workforce, especially in resource-limited countries, diagnostic centers are forced to hire additional laboratory personnel who have limited experience and technical knowledge and skills of molecular assays, especially in processing specimens, interpreting the results, identifying errors, and troubleshooting, in order to meet the demand of increased testing.[14] Thus, the vulnerability of laboratory medical services to diagnostic errors, including cross-contamination, is increased and with the tendency for generating false-positive results that can compromise the health of the patient and disrupt the efficacy of public health policies and public health response, surveillance programs, and restrictive measures for containing the outbreak.[15] In worst cases, a false-positive result may entail unnecessary treatment and may undermine available workforce, especially if the patient is working as a public servant and is forced to self-isolate. Meanwhile, a false-negative result can foster rapid human-to-human transmission of the virus due to the failure in the application of restrictive and containment measures as well as in the identification of other suspected cases, especially those exposed to the patient who is infected with SARS-CoV-2.[6]

Thus, the World Health Organization had released guidelines on biosafety in laboratories handling COVID-19 specimens.[16] Likewise, the Department of Health in the Philippines had released guidelines on how to operate local COVID-19 testing laboratories.[17] This sets the standard to make sure that tests are reliable, and to promote the safety of those operating the laboratory. Aside from this, some studies suggest that there is a high rate of false negative test results from using the RT-PCR diagnostic kits.[18] With this, it is important to avoid unnecessary errors particularly in processing samples.

In the laboratory, RT-PCR is a multi-procedural process which makes it susceptible to cross-contamination. After sample collection, RNA is extracted from the specimen to prepare for RT-PCR. The RNA of SARS-CoV-2 can be easily transferred from a contaminated gloved hand to the working surface, or to the laboratory environment.[19] Although WHO guidelines suggest good microbiological practice and procedure, it is not clear on how often laboratory personnel should change gloves. The guidelines are also more focused on protecting the laboratory personnel. To avoid cross-contamination, it is important to promote changing of gloves as frequently as possible, especially if soiled with solutions containing template RNA. Not only in the use of gloves, but the entire set of the PPE should be changed when moving to a different area of the laboratory. Aside from this, materials such as pens, small equipment, tubes, pipette tips, and other consumables should never be brought from RT-PCR to the pre-PCR area. Laboratory guidelines require unidirectional workflow, as such laboratorians and even the cleaning personnel should be reminded to treat each area as a different room to prevent conveying the amplicons to amplification product-free areas. Moreover, the Philippines' DOH guidelines require the separation of pre-PCR room into two areas: (1) specimen handling or sample preparation room and (2) reagent preparation room. The reagent preparation room is a 'template' free environment which also excludes positive internal reaction controls. Provision of different storage areas and freezers for specimens and reagents is highly encouraged.

Another possible source of cross-contamination is the pipetting of patient samples into the PCR plate or strip. Possibly, samples can also be misidentified as positive due to sample misplacement. Thus, proper pipetting and double-checking sample placement while still following aseptic techniques (use of PPE, use of sterile materials, disinfecting work area) when running RT-PCR analysis should always be followed. Cleaning of the work area, pipettors, freezer handles, and other materials using the appropriate decontaminating agent is also a must before and after PCR work. Racks should be immersed in disinfectant for 10 minutes and then dried with clean paper towel. It is also prudent for clinical molecular laboratories to invest on autoclavable pipettors to lessen cross-contaminations.

Increased frequency of disinfection with the use of disinfectants as provided by the WHO guidelines can also be practiced, either every 30 minutes or after processing of COVID-19 samples.[11] However, for consumables that have been in contact with infectious samples, disposables are recommended.

After RT-PCR analysis, post-PCR is an important step to interpret results for diagnosis. No amplification must be observed in the negative controls provided by the test kit, as well as in the elution buffer (or whichever is appropriate depending on the test kit used) to guarantee that there is no contamination in the process. In case there is possible contamination, the quality of water should be checked, and in some cases contamination of the instrument can also be considered.[20] To avoid these problems, fresh (unopened) water must be used in each run, and the reagents should be prepared in aliquots in sterile containers once the kit is opened. Proper aseptic technique must be observed until the samples are placed in the machine. When placing samples and controls in the multi-well plate, it is recommended for the controls not to be placed next to each other to make sure that no cross-contamination happens while samples are transferred to their designated wells. It is also highly recommended to assign around 3 or more water controls randomly in the multi-well plate to monitor aseptic pipetting.

It is also worth noting that cross-contamination in the laboratory may not be the only cause of unreliable results. In the early stage of the COVID-19 pandemic, a delay in testing in Europe was caused by a contamination in the test kits.[21] This problem was also experienced by the Philippines when locally made test kits were found to have contaminated reagents.[22] Nevertheless, observing proper techniques in COVID-19 testing laboratories should always be strictly followed to avoid unreliable results which are counterproductive in any situation.

Technicians assigned in a COVID-19 testing laboratory can also become infected with the virus and unintentionally contaminate the sample they process and the laboratory environment. Hence, technicians, especially those assigned in the PCR room, are advised to wear goggles or

face shield and disposable surgical cap and mask, which they must be dispose of in proper bins located in the same room before leaving the area. Disposable lab gowns are highly recommended but may not be practical in low resource areas. Hence, it is advised that laboratory gowns must not be brought home by the technicians but have to be washed and sterilized by their hospital linen and laundry services.

CONCLUSION

The COVID-19 pandemic has caused a global public health emergency. Although efforts have been made to prevent the spread of the disease, there is still an increasing number of cases each day. Reliable diagnosis through RT-PCR testing plays an important role in the management of the disease. Thus, it is essential to avoid any cross-contamination when handling biological specimens from patients. Proper laboratory practices should always be observed, with the focus on changing gloves as often as possible and changing PPE when moving from one work area to another. Guidelines from the World Health Organization on how to operate laboratories should be strictly followed, as well as those provided by a country's health ministry. Unidirectional workflow in the laboratory while following aseptic technique in each step is crucial in maintaining the reliability of a molecular diagnostic laboratory.

STATEMENT OF AUTHORSHIP

The authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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An Evaluation of Pooling Strategies for RT-qPCR Testing for SARS-CoV-2 Infection: A Pragmatic Multi-site Parallel Operational Study

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ABSTRACT

Background and Objectives. Sample pooling of COVID-19 PCR tests has been recently proposed as a low-cost alternative to individual tests. This multi-site, laboratory-based, proof-of-concept study explores the feasibility of pooled SARS-CoV-2 RT-qPCR testing, by demonstrating the effect of pooling on sensitivity, specificity, accuracy, number of tests saved, and turnaround time.

Methodology. The research was conducted in two experiments. In Experiment 1, archival nasopharyngeal (NPS) and oropharyngeal (OPS) swab samples were diluted to simulate 5, 10, and 20 sized pools, and tested for SARS-CoV-2 RNA using RT-qPCR. In Experiment 2, actual nasopharyngeal and oropharyngeal swab samples were collected from asymptomatic low-risk volunteers. Aliquots of the samples were pooled following the 5, 10-5, and 20-10-5 multi-staged Dorfman pooling methods and tested. The sensitivity, specificity, accuracy, test savings, and turnaround time for each pooling method were documented.

Results and Conclusions. The study provided evidence that pooling of NP and OP samples for SARS-CoV-2 RNA detection using RT-qPCR is feasible and can be implemented in the Philippines. A 2-stage Dorfman 5 pooling strategy appears to be the best method, because it has the highest over-all accuracy, while still achieving acceptable test savings, and turnaround time. Pooling of nasopharyngeal and oropharyngeal swab samples prior to RT-qPCR testing may be considered by select molecular diagnostic laboratories to further increase testing capacity and at the same time reduce the cost of testing.

Key words: pooled testing, specimen pooling, RT-qPCR, COVID-19, SARS-CoV-2

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INTRODUCTION

The capacity of the Philippine healthcare system to perform NAATs for detecting SARS-CoV-2 nucleic acids was extremely limited at the beginning of the pandemic.[1] Testing capacity steadily increased with the certification and licensing of new COVID-19 diagnostic molecular laboratories, now reaching to about 100. As of August 2020, the testing capacity of the whole country is about 27,800 tests per day.[2] The WHO has suggested around 10 – 30 tests per confirmed case as a general benchmark of adequate testing.[3] In August 2020, the Philippines did 8.2 tests per positive case[4] which placed the country in the "moderate" category. According to OurWorldInData.org, as of September 30, 2020, the Philippines did 12.8 tests per new confirmed case, with a daily new case positive rate of nearly 3,000 for the week of September 26 to October 3, 2020.[3] Based on the above recommendations, the Philippines should be doing 30,000 to 90,000 tests daily. The cost of testing, however, remains a challenge.

The Philippine government gradually transitioned from enhanced community quarantine to more relaxed quarantine regimes.[5] Potential challenges that came with

this reopening of social activities include (1) possible surges in the number of new infections, (2) the need for certain groups of workers to be tested on a regular basis, and (3) the need for patient testing to prioritize access to invasive and surgical procedures.

At present, the gold standard for the diagnosis of COVID-19, is by nucleic acid amplification testing (NAAT), which includes quantitative reverse transcriptase polymerase chain reaction (RT-qPCR). In the case of COVID-19, NAATs amplify a tiny amount of viral genetic material using a nasopharyngeal or oropharyngeal swab sample. The demand for this type of SARS-CoV-2 testing has drastically increased in many health care systems, and resulted in shortages of reagents and materials to conduct the test, or exceeded the capacity limits of the testing laboratories.

Reverse transcriptase polymerase chain reaction (RT-PCR) is a laboratory technique that combines reverse transcription of RNA into DNA and amplification of specific DNA targets. The chain reaction relies on small DNA sequence primers that are designed to specifically recognize complementary sequences on the RNA viral genome and the reverse transcriptase to generate a short complementary DNA copy (cDNA) of the viral RNA (called amplicons). In real-time or quantitative PCR (RT-qPCR), the amplification of DNA is monitored in real time as the PCR reaction progresses. Detection of the viral copies is achieved using a fluorescent dye or DNA probe labeled with a fluorescent molecule and a quencher molecule, as in the case of TaqMan assays. An automated system then repeats the amplification process for about 40 cycles until the viral cDNA can be detected.

The Philippine Society of Pathologists Inc. (PSP), in its position paper of May 29, 2020, recommended the implementation of pooled RT-PCR COVID testing to expand testing capacity, reduce turnaround time and conserve reagents and human resources.[6]

Incorporating specimen pooling strategies into RT-qPCR testing may be a viable solution to facilitate the calibrated exit of the country from community quarantine to a more relaxed economic and social activity. Pooling methods entail getting aliquots from several samples and combining them together in a single tube for RT-PCR testing, reducing the number of actual tests performed.

However, several questions about sample pooling remain. For instance, the largest pooling size, n, such that the sensitivity of detecting low copy numbers of nucleic acids would still be acceptable (sensitivity: 90%) has not yet been established in the local setting. The largest pooling size, n, is the maximum pooling size that can be allowed in a clinical laboratory such that the RT-qPCR would still have a sensitivity of 90 %, a performance characteristic the PSP considers as the minimum acceptable sensitivity for clinical testing.

The effect of pooling on the analytic sensitivity also needs to be studied by looking at how much the Ct-values will change between the original specimen and the pooled (diluted) specimen. Pool sizes with the least change in Ct-values can be considered acceptable.

Prevalence rates are critical in establishing pooling strategies. Unfortunately, with the limited testing done mainly on symptomatic cases, there are no reliable local prevalence data in the general asymptomatic and low risk population. For the purposes of this study, however, an arbitrary but reasonable prevalence rate of 5% is used as a basis to inform the design of pooling methods. Pooling sizes of n=5, n=10, and n=20 are the pool sizes likely to be optimal for disease prevalence ranging from 0.3% to 20% [7,8] and are the pooling sizes that were included in this study.

Several groups, including local scientists, have recommended optimal pooling strategies and sizes by maximizing the expected savings on the number of tests needed using computer simulations.[7–24]

Some of these included feasibility and proof-of-concept studies using actual samples. A summary of the studies and papers are presented in Table 1. A review of their findings is discussed in the succeeding paragraphs.

Well-described and popular pooling methods include the Dorfman, which includes the two-stage hierarchical (D2) and three-stage hierarchical (D3) variations, the repeated sub-pooling method of Sterrett, the halving method by

Table 1. Summary of pooling studies reviewed

Authors	Type of Pooling Study	Study site
(Abdalhamid et al., 2020)	Computer Simulations and testing with actual specimens	Nebraska, USA
	Computer simulations	Nebraska, USA
(Bilder & Tebbs, 2012)	Computer simulations	Manila, Philippines
	Computer simulations	Germany
(Caoili et al., 2020)	Actual specimens	Japan
	Actual specimens	California, USA
(Deckert et al., 2020)	Mathematical theoretical discussion	MA, USA
	Actual specimens	Germany
(Hirotsu et al., 2020)	Mathematical theoretical discussions	Utah, USA
	Mathematical theoretical discussions	Australia
(Hogan et al., 2020)	Computer Simulations	Jerusalem, Israel
	Computer simulations and testing with actual specimens (used automated machines)	Israel
(Litvak et al., 1994)	Mathematical theoretical discussion	USA
	Actual specimens	Spain
(Lohse et al., 2020)	Actual specimens	USA
	Dilution study using actual specimens	Israel

Litvak et al., and the array or matrix based methods by Phatarford and Sudbury.

In the Dorfman method, the individual samples are 1st pooled in n samples and tested. If the master pool is negative, all n samples are classified as negative. If the master pool tests positive, individual testing of each sample is done, and the samples are classified accordingly.[25]

In the repeated sub-pooling method by Sterrett, the strategy starts like the Dorfman methods, but samples included in pools that test positive are tested randomly, rather than exhaustively, until the 1st positive individual sample is found. Once the first positive is found, the rest of the samples that have not yet tested are re-pooled and retested. If the pool tests negative, all other samples in this pool are classified as negative and testing is done on the other pools. The process is repeated until all positives are identified. Sterrett was able to show that the testing efficiency can be increased from 0.8 to 0.86, for example, in a test population with prevalence of 0.01.[20]

The "halving method" proposed by the group of Litvak, splits the samples into two or more sub pools. Further splitting or individual testing can be done on each sub pool that tested positive. All samples in pools that test negative are classified as negative. In this method, each successive split creates two new equally sized sub pools. In real applications, however, the method only involves 3 to 4 levels of sub-pooling. Litvak and his team compared their method with 4 different pooling strategies, including variations of the Dorfman, and further variations of their halving technique which adjusts the number of times positive and negative pools are tested before assigning a classification. In their mathematical discussion, they found that all pooling strategies they tested resulted in cost savings with differences in false negatives and false positives and that the final choice should take into consideration the consequences of these errors in the actual setting.[16]

Square or rectangular matrix or array based pooling methods, with popular designs by Phatarford and Sudbury, are usually used with high throughput screening platforms. In these methods, n x n or n x m matrix-like grids of specimens are created. Each n rows and m columns are tested as a pool. Each pool can be tested twice to increase sensitivity. Samples that lie at the intersection of positive rows and positive columns are tested individually to decode the positives from the negatives. Specimens lying outside of these intersecting rows and columns are declared negative. Phatarford and his colleagues demonstrated mathematically that placing the samples in a square array and pooling rows and columns has substantial advantages, particularly in the reduction of false negatives.[17] They compared the simple Dorfman, to Halving strategy by Fincuan, and the repeated pooling by Sterret.[18,25] The number of tests saved, measured by the number of tests/person as well as the probability of false negative samples were calculated and compared between the Dorfman and the array methods. They were able to mathematically demonstrate the superiority of the array methods compared to the simple Dorfman procedures.[18,25]

SUMMARY OF LITERATURE ON POOLING METHODS ON SIMULATED OR ACTUAL SAMPLES

The group of Hirotsu in Japan validated the feasibility of pooling samples using serial dilution analysis and spike-in experiment using synthetic DNA and nucleic acids extracted from SARS-CoV-2 positive and negative patients. They also studied a total of 1,000 individuals, 667 of which are 'healthy' (195 healthcare workers and 472 hospitalized patients with other disorders than COVID-19 infection) individuals and 333 are infection-suspected patients with cough and fever. Their serial dilution analysis showed a limit of detection of around 10-100 copies. Their spike-in experiment demonstrated that RT-qPCR can detect positive signal in pooling samples of SARS-CoV-2 negative and positive patient at the 5-, 10-, 20-fold dilution. They performed screening using their pooling strategy during the months up to April 2020, and they were able to identify 12 COVID-19 patients in 333 suspected patients (3.6%) and zero in 667 'healthy', using only a total of 538 tests instead of the 1000 which would have been required if done without pooling.[12]

Hogan and colleagues used a simple 2-stage Dorfman pooling strategy using 9 or 10 samples per pool to test and screen for SARS-CoV-2. They were able to screen 292 pools, corresponding to 2740 NP samples and 148 bronchoalveolar lavage samples. They reported only 1 false-positive reading and an expected slight loss in sensitivity.[13]

Perchetti et al., noted a 2 Ct value loss in analytical sensitivity with 1:4 pooling of samples using CDC-based RT-PCR laboratory developed assay.[14]

Mulu et al., demonstrated differences in Ct values in experimental pools of 2 to 10.[15] The group of Lohse evaluated Simple Dorfman pooling methods of varying pool sizes on actual SARS-CoV-2 samples. They were able to show that the difference between the Ct values of pooled and non-pooled specimens ranged up to 5 points. Based on their data, they were able to analyze 1161 samples using only 267 tests to detect 23 positives resulting in large cost savings. Their data suggests that pooling of up to 30 samples per pool can be used but they caution against the possibility of decreased sensitivity in patients 14-21 days after symptomatic infection.[24]

Noriega and his group discussed the applicability of a pooled-sample testing protocol to screen large populations more rapidly and with limited resources using a Bayesian inference analysis. Hierarchical testing stages were implemented, and their sensitivities were benchmarked against early COVID-19 testing data. They calculated the optimal pool size, increases in throughput and case detection abilities as a function of disease prevalence. They concluded that even for moderate losses in test sensitivity due to pooling, substantial increases in testing throughput and detection efficiency can be expected. [17]

Shani Narkiss and colleagues discussed two possible optimized pooling strategies for diagnostic SARSCoV-2 testing on a large scale. The first uses a simple information-theoretic heuristic to derive a highly efficient re-pooling

protocol where an estimate of the target frequency determines the initial pool size and any subsequent pools found positive are re-pooled at half-size and tested again. This was found to reduce the number of tests required dramatically, when the prevalence is less than 5%. The second method is simpler and uses an optimized one-time pooling followed by individual tests on positive pools. They were able to show that this approach is just as efficient for prevalence ranging from 5% to less than 20%. Compared to naive individual testing and alternative matrix methods, they show that their methods can be practical. [8]

Shental and colleagues developed P-BEST - a method for Pooling-Based Efficient SARS-CoV-2 Testing, using a non-adaptive group-testing approach, which significantly reduces the number of tests required to identify all positive subjects within a large set of samples. This method tests samples by pooling into groups. Each sample, however, is part of multiple pools and uses a combinatorial pooling strategy based on compressed sensing method. They evaluated this P-BEST strategy using leftover samples in a proof-of-concept study. They pooled 384 patient samples into 48 pools. Five sets of 384 samples, containing 1-5 positive carriers were tested using the method and all positive carriers in each set were correctly identified.[19]

Torres and colleagues conducted a proof-of-concept study and a mini trial where they evaluated the efficacy of a pooling strategy in Covid-19 testing. They used a total of 20 mini-pools containing either 5 (n=10) or 10 (n=10) nasopharyngeal exudates collected in universal transport medium, each of which included a unique positive NP specimen.[21]

OBJECTIVES

The general objective of this 2-part study is to determine the effect of nasopharyngeal and oropharyngeal swab sample pooling on the test sensitivity, number of tests saved and turnaround time of RT-qPCR testing for SARS-CoV-2 RNA.

Specifically, this study aims to determine which of the pooling sizes (n=5, n=10, and n=20) would retain an acceptable test sensitivity (90%) in identifying samples with low copies of viral RNAs (archival samples with Ct values ranging from 30-38). Pooling strategies are being used routinely in nucleic acid amplification for transfusion-transmissible infections in blood banking.[26,27,28]

This study also specifically aims to compare variations of the Dorfman pooling strategy (2-stage Dorfman 5, 3-stage Dorfman 10-5, and 4-stage Dorfman 20-10-5) to no-pooling and determine test accuracy, test savings and turnaround time.

METHODOLOGY

This study conducted laboratory-based parallel multi-site operational pragmatic experiments using a combination of archival and actual patient samples in 2 phases. Experiment 1 was designed to answer specific objective 1 and was conducted at the Research Institute for Tropical Medicine (RITM). Experiment 2, which was designed to answer specific objective 2, was conducted at the Philippine Children's Medical Center (PCMC) and University of Perpetual Help Dalta Medical Center (UPHDMC). The study protocols, including the informed consent forms, were reviewed and approved by the institutional review boards of RITM (RITM IRB 2020-022), PCMC (PCMC IR-EC 2020-046) and UPHDMC (UPHS-IERB 2020-003).

Interpretation of pooled sample results (Experiment 1)

For Experiment 1, nasopharyngeal and oropharyngeal specimens that have been previously collected and tested in RITM, with Ct values ranging between 30 to 38 were identified and retrieved by convenience sampling. These specimens have been properly stored at 2 ºC to 8 ºC for no more than 72 hours. Specimens that were not tested within 72 hours were stored at -80ºC.

In Experiment 1, we performed an experiment similar to the method used by Abdalhamid et al (2020).[9] Previously characterized positive nasopharyngeal + oropharyngeal (NP+OP) swab specimens with high Ct-values (Ct-value > 30<38) were identified. The Ct-values obtained from previous testing were considered as an indirect measure of the specimen's actual starting nucleic acid copies, with Ct values higher than 30 taken generally to mean low nucleic acid copies. The samples were selected based on the results of their initial real-time RT-PCR runs as well as the quality and remaining volume of the original samples. Undiluted samples were tested along with the diluted samples to ensure that same testing conditions were met for both undiluted and diluted samples.

Fifty (50) uL aliquots from each sample were diluted (as described below) to simulate the different pool sizes at the worst possible pooling scenarios - where only 1 specimen is positive out of the pool:

1. For pool size n = 5, 50 uL of specimen were added to 200 uL of diluent/buffer
2. For pool size n= 10, 50 uL of specimen were added to 450 uL of diluent/buffer
3. For pool size n = 20, 50 uL of specimen were added to 950 uL of diluent/buffer

The diluted samples underwent nucleic acid extraction and RT-qPCR using Qiagen Viral RNA Kit and Maccura SARS-CoV-2 Fluorescent PCR Kit, respectively. ABI 7500 Fast Real-Time PCR was used for real-time PCR amplification of the PCR reaction mix. All procedures were performed strictly according to the manufacturer's instructions for use and followed strict biosafety guidelines and good clinical laboratory practices. Results were recorded and encoded in electronic data collection forms.

For Experiment 2, volunteer employees from a local supermarket chain were interviewed and invited to participate in the study. Supermarket employees were selected for the pooling population as proposed by the Philippine Society of Pathologists Inc.'s position paper entitled "Diagnostic Testing Strategy to Manage COVID-19 Pandemic."[6] In the position paper, an expanded targeted testing for asymptomatic population was listed, including testing employees who are at risk due to higher exposures and contact.

They were selected from a list of potential participants that were provided by the study sponsors. All of them were asymptomatic and were classified under "sub-groups D (patients and healthcare workers with no symptoms but relevant history of travel and/or contact)" as defined in the DOH Department Memorandum 2020-0258: Updated Interim Guidelines on Expanded Testing for COVID-19.[29] Three store locations were pre-selected by the study sponsor: Branch 1: 120 workers, Branch 2: 250 workers, and Branch 3: 80 workers.

Employees who had symptoms of fever, cough, colds, or shortness of breath at the time of interview, those with previous RT-PCR testing, pregnant women, less than 18 years of age and those who were unable to give informed consent were excluded.

The study team conducted an ocular inspection of the swabbing sites a day prior to the actual swabbing of the study participants. Collection sites in open spaces and with good air exchange were identified. Three (3) swabbing booths were provided by PCMC. These swabbing booths are made of an acrylic barrier which minimized contact and provided aerosol protection. The swabbing team consisted of three (3) specimen collectors and 3 supervising consultants. The participants' waiting area were designated in front of the swabbing booths and placed at least 2 meters away. They were large enough to ensure adequate social distancing, at 1 meter apart. Tissue and alcohol dispensers were made available to the study participants. Complete PPEs (closed suit with hood, goggles, 3M N95 mask, shoe cover, double gloves) were worn by the specimen collectors, as prescribed by the CDC (Centers for Disease Control). The swabbing team brought with them yellow trash bags for disposal of PPEs and infectious waste.

A general orientation was conducted, providing information on the swabbing procedure and the pooled testing research. After one-on-one interview and counselling, to make sure that all questions have been satisfactorily answered, the participants who agreed to be part of the study signed an informed consent and fill up a Case Investigation form (CIF). CIFs and Informed consent forms were stored in a locked steel cabinet at the administrative office of the PCMC COVID-19 Testing laboratory. The files are accessible only to the principal investigator and co-investigators.

Swabbing was performed according to standard guidelines and procedures. After swabbing, the VTMs were transported back to the COVID-19 Testing Laboratory of PCMC and UPHDMC following biosafety standards and then stored in the reagent refrigerator until testing.

The samples were accessioned according to standard procedures in each laboratory. They were accessioned according to the date, institution/company in successive numbers. The specimens were then pooled in groups of 5 thus, XXXXXXX -P5A, such that,

- "Accession number" represents the individual specimen accession number
- "Pool Accession Number" (ex.: XXXXXXX P 5 A)
- The first alphanumeric characters represent the desired individual accession code
- "P" stands for pool
- "5" represents the number of unique individuals in the pool
- "A" represents the sequence in which A is for the first 5 samples pooled, B for the next 5 samples, and so on.

Based on volume used for experiment 1, the specimens were divided into at least thirteen (13) 50 uL aliquots (3 each for no pooling, Dorfman 5, and Dorfman 10, and 4 for Dorfman 20+10). A standard accessioning procedure for the aliquots was used. The specimens were then stored in -80°C freezers until ready for processing. Freeze-thaw cycles were minimized.

For the no pooling method, 50 uL aliquots of all the specimens were tested individually using standard laboratory procedures as per manufacturer's specifications using Sansure Novel Coronavirus (2019-nCoV) Nucleic Acid Diagnostic kit. Extraction was done using Natch CS automated extractor and RT-qPCR was performed on MA6000 PCR machine (China).

For purposes of pooled testing, a negative pool is one that shows no target gene amplification. Any target gene amplification (ORF1 and N genes) regardless of Ct value, degree of amplification or curve properties (sigmoid or non-sigmoid) will be considered positive. Individual samples will undergo the same interpretation as per manufacturer's specifications.

For the 2-stage Dorfman 5, sample aliquots were pooled in groups of 5, and each resulting pooled aliquot was tested. All specimens in the pools that tested negative were considered negative. All specimens in the pools that tested positive were then tested individually and classified according to the result of this testing.

For the 3-stage Dorfman 10-5, aliquots were first pooled in groups of 10 and tested. All specimens in the pools that tested negative were considered negative. The specimens in the pools that tested positive were then re-pooled into groups of 5 and each sub-pool of 5 was then tested again. All specimens in the sub pools that tested negative were considered negative. All specimens in the pools that tested positive were then tested individually and classified according to the result of this testing.

For the 4-stage Dorfman 20-10-5, aliquots were first pooled in groups of 20 and tested. All specimens in the pools that tested negative were considered negative. The specimens in the pools that tested positive were then re-pooled into groups of 10 and each sub pool was then tested. All specimens in the sub pools that tested negative were considered negative. The specimens in the pools that tested positive were then re-pooled into groups of 5 and each sub-pool was again tested. All specimens in the sub pools that tested negative were considered negative. All specimens in the sub-pools that tested positive were then tested individually and classified according to the result of this testing.

The individual and pooled samples underwent nucleic acid extraction and SARS-CoV-2 NAAT by RT-qPCR strictly according to the manufacturer's instructions for

use and followed strict biosafety guidelines and good clinical laboratory practices. Results were recorded and encoded in electronic data collection forms.

Interpretation of pooled sample results (Experiment 2)

A pool that shows no gene target amplification whatsoever in any form except for the internal control, is interpreted as negative for all individual samples in that pool. These negative individual samples are reported as negative.

A pool that shows any target gene amplification, regardless of late or low amplification, or unusual or non-sigmoid amplification, is interpreted as a positive pool and deconvolution is done by testing all samples within that pool individually. Results of the individual runs are interpreted according to the manufacturer's instructions.

This procedure was devised based on Experiment 1 that showed loss of sensitivity in pooled samples by increase in Ct values as much as 4.87 in pools of 20. We wanted to be able to detect possible pools with positive samples that have low viral loads and high Ct values, thus reducing the loss of sensitivity to the minimum possible.

All the residual sample aliquots that were used in the study were disposed of in biological waste bags and autoclaved prior to disposal in the hospital bio-waste facility.

The official results of the RT-qPCR testing were based on the initial individual runs and were released according to standard operating procedures of PCMC and UPHDMC and following the guidelines of the DOH.

Experiment data collected were audited, managed and analyzed by a data management and analysis unit. All valid data were encoded into a password-protected Microsoft Excel file. Access to the data was restricted to key study personnel and were subjected to the approval of the Project Leader. Manual backups were performed, and copies were made in password-protected external hard drives with AES-256 encryption and kept in a lock-and-key cabinet at the administrative office of the PCMC and UPHDMC COVID-19 Testing laboratories.

RESULTS

Experiment 1

In Experiment 1, the reference panel consisted of a total of 36 fresh and frozen samples, previously tested positive using A*STAR Fortitude Kit 2.0 COVID-19 Real-Time PCR Test. Fourteen of these were frozen (-80°C) samples collected in July 2020 while 22 were fresh samples prospectively collected during the duration of phase 1 and were stored at 4°C prior to pooling. Among the fresh samples, 18 have been characterized as weak positive (Ct value greater than 30 but less than 38) and 4 were moderate to strong positives. Among the frozen samples, 7 were weak positives and 7 were moderate to strong positives.

Over-all test sensitivity was observed to fall with increasing dilution (simulating dilution by pooling). It decreased to 83% (95% CI 67% - 94%) with pool size of 5, to 72% (95% CI: 55% - 86%) with pool size of 10 and to 67% (95% CI: 49% - 81%) with pool size of 20. Test sensitivity was observed to be maintained at high levels, as high as 100%, in moderate to strong positive samples, even at pool size of 10. This was seen in both fresh and frozen samples. Test sensitivity in weak positive samples decreased with increasing dilutions, from as high as 86%, to as low as 43%. This trend was seen in both the fresh and frozen samples, with the decline more readily seen in frozen samples compared to fresh ones, 86% - 57% - 43% compared to 72% - 61% - 61%. Confidence intervals were calculated using the Clopper-Pearson exact method (Table 2).

Table 2. Effect of pooling as simulated by dilution on test sensitivity

Specimen	pool size = 5				
	N	Pos	Sensitivity	95% CI	
Fresh	22	17	77%	55%	92%
Mod to strong positive	4	4	100%	40%	100%
Weak positive	18	13	72%	47%	90%
Frozen	14	13	93%	66%	100%
Mod to strong positive	7	7	100%	59%	100%
Weak positive	7	6	86%	42%	100%
Total	**36**	**30**	**83%**	**67%**	**94%**

Specimen	pool size = 10				
	N	Pos	Sensitivity	95% CI	
Fresh	22	15	68%	45%	86%
Mod to strong positive	4	4	100%	40%	100%
Weak positive	18	11	61%	36%	83%
Frozen	14	11	79%	49%	95%
Mod to strong positive	7	7	100%	59%	100%
Weak positive	7	4	57%	18%	90%
Total	**36**	**26**	**72%**	**55%**	**86%**

Specimen	pool size = 20				
	N	Pos	Sensitivity	95% CI	
Fresh	22	15	68%	45%	86%
Mod to strong positive	4	4	100%	40%	100%
Weak positive	18	11	61%	36%	83%
Frozen	14	9	64%	35%	87%
Mod to strong positive	7	6	86%	42%	100%
Weak positive	7	3	43%	10%	82%
Total	**36**	**24**	**67%**	**49%**	**81%**

The Ct-value was observed to increase with increasing dilution. The mean increase in Ct value was 2.56 (95% CI: 2.24 – 2.88) with pool size of 5, 3.82 (95% CI: 3.47 – 4.16) with pool size of 10, and 4.87 (95% CI: 4.35 – 5.39) with pool size of 20. This increase in Ct value was consistently observed in both fresh and frozen samples and in both weak positive and moderate to strong positive samples. Confidence intervals were calculated using the standard normal distribution (Table 3).

Over-all, pool size of 5, which results in a 1:5 dilution at its worst case, was observed to have the least drop in test sensitivity, and the smallest mean change in Ct value, even for samples containing low viral RNA (weak positive samples).

Experiment 2

In Experiment 2, a total of 440 asymptomatic volunteer employees of a local supermarket chain were recruited.

SARS-CoV-2 RNA was detected in 12 out of the 440 collected samples, giving an estimated prevalence of

Table 3. Effect of pooling as simulated by dilution on Ct values

Specimen	no.	Dilution = 1:5 mean Ct change	Lower 95% CI	Upper 95% CI
Fresh	22	**2.74**	2.28	3.20
Mod to strong positive	4	**2.05**	1.68	2.42
Weak positive	18	**2.89**	2.36	3.43
Frozen	14	**2.28**	1.91	2.65
Mod to strong positive	7	**2.31**	2.01	2.61
Weak positive	7	**2.24**	1.53	2.94
Total	**36**	**2.56**	**2.24**	**2.88**

Specimen	no.	Dilution = 1:10 mean Ct change	Lower 95% CI	Upper 95% CI
Fresh	22	**4.12**	3.65	4.59
Mod to strong positive	4	**3.90**	2.74	5.05
Weak positive	18	**4.17**	3.64	4.70
Frozen	14	**3.34**	2.94	3.75
Mod to strong positive	7	**3.54**	2.94	4.15
Weak positive	7	**3.14**	2.61	3.68
Total	**36**	**3.82**	**3.47**	**4.16**

Specimen	no.	Dilution = 1:20 mean Ct change	Lower 95% CI	Upper 95% CI
Fresh	22	**4.87**	4.37	5.38
Mod to strong positive	4	**5.11**	3.45	6.77
Weak positive	18	**4.82**	4.30	5.34
Frozen	14	**4.87**	3.75	5.98
Mod to strong positive	7	**5.32**	3.14	7.51
Weak positive	7	**4.41**	3.82	5.01
Total	**36**	**4.87**	**4.35**	**5.39**

Table 4. Ct values of the 12 positive cases

Positive cases	Ct-values FAM ORF1ab gene	ROX N gene
Case 1	34.64	31.71
Case 2	–	34.90
Case 3	39.30	34.74
Case 4	32.33	28.19
Case 5	38.95	34.26
Case 6	34.67	31.29
Case 7	41.19	35.58
Case 8	35.48	31.01
Case 9	37.10	33.19
Case 10	31.63	26.72
Case 11	31.86	27.26
Case 12	33.79	28.49

3% (95% CI: 2% - 5%), for the sampled population of asymptomatic employees. Official results were released and sent to DOH which contacted the individuals for case management including quarantine and contact tracing following standard protocols (Table 4).

Test sensitivity was observed to decrease with increasing pool size. In Dorfman 5-1, only 10 of the 12 positive samples were recovered, resulting in a decrease in sensitivity to 83% (95% CI: 52% - 98%). In Dorfman 10-5-1, only 7 of the 12 positive samples were recovered, for a sensitivity of 58% (95% CI: 28% - 85%). And the largest decrease in test sensitivity was seen in Dorfman 20-10-5-1, where only 6 of the 12 positive samples were recovered, for a sensitivity of 50% (95% CI: 21% - 79%). Test specificity was excellent, estimated at 100% (95% CI:99% - 100%) across the different Dorfman pooling methods (Table 5).

Overall accuracy was observed to be consistently high. In Dorfman 5-1, 438 out of 440 samples were correctly classified for an accuracy of 100% (95% CI: 98% - 100%). Accuracy was the same in Dorfman10-5-1, where 435 out of 440 samples were correctly classified for an accuracy of 99% (95% CI: 97%-99%). The overall accuracy was the same with Dorfman 20-10-5-1, where 434 out of 440 samples were correctly classified, for an accuracy of 99% (95% CI: 97% - 99%) (Table 5).

Test savings were high and ranged from 69% to 83% across the different Dorfman pooling methods. Dorfman 20-10-5-1 resulted in the highest test savings, consuming only 76 tests to generate results for 440 samples, resulting in test savings of 83% (95% CI:79% - 86%). Dorfman 10-5-1 came second, requiring 93 tests for a test saving of 79% (95% CI: 75% - 83%). Dorfman 5-1 showed the least test savings of 69% (95% CI: 64% - 73%), needing 138 tests for the 440 samples) (Table 6).

Delays in turn-around times were seen. For positive samples, the turnaround time was from 2 to 4 batch runs. Dorfman 5-1 had the fastest turnaround time for positive samples with TAT of 2 batch runs, followed by Dorfman 10-5-1 with 3 batch runs, and Dorfman 20-10-5-1 with 4 batch runs. For negative samples, the average turnaround time was from 1.09 to 1.44 batch runs. Dorfman 20-10-5-1 had the longest average turnaround time for negative samples with TAT of 1.44 batch runs. This means that on the average, a proportion of the negative samples will require more than one batch run to be released. Dorfman 10-5-1 had an average TAT of 1.21 batch runs for negative samples, while Dorfman 5-1 had the shortest TAT at 1.09 batch runs. This means that on the average, most of the negative samples tested using Dorfman 5-1 would still be released on the same batch run.

DISCUSSION

Based on results of Experiment 1, which showed reduction of sensitivity to 77% in pools of 5, 54% in pools of 10 and 46% in pools of 20, it was decided to employ measures to mitigate the loss of sensitivity as in the methodology above, where any pool with any form or magnitude of target gene amplification is considered "positive" and its individual samples were tested individually to determine which sample if any, is positive by the manufacturer's specifications.

Notwithstanding this measure, we still missed some positive cases when using aliquots of 50 ul per individual sample for pooling. It is recommended that we use 200 ul as an individual sample contribution to the pool based on a study done by the Korean Society for Laboratory Medicine.[30]

Analysis of the Ct values of positive cases

The Ct values of both ORF1ab and N gene targets of the 12 positive cases are analyzed to determine concordance with the findings of Experiment 1. Compared to the individual run Ct values, there was an increase in Ct values in all

Table 5. Effect of pooling on test performance

	Sensitivity														
	Dorfman 5-1					Dorfman 10-5-1					Dorfman 20-10-5-1				
	x	n	%	95% CI		x	n	%	95% CI		x	n	%	95% CI	
Site A	6	8	75%	35%	97%	3	8	38%	9%	76%	2	8	25%	3%	65%
Site B	4	4	100%	40%	100%	4	4	100%	40%	100%	4	4	100%	40%	100%
Total	**10**	**12**	**83%**	**52%**	**98%**	**7**	**12**	**58%**	**28%**	**85%**	**6**	**12**	**50%**	**21%**	**79%**
	Specificity														
	Dorfman 5-1					Dorfman 10-5-1					Dorfman 20-10-5-1				
	x	n	%	95% CI		x	n	%	95% CI		x	n	%	95% CI	
Site A	212	212	100%	98%	100%	212	212	100%	98%	100%	212	212	100%	98%	100%
Site B	216	216	100%	98%	100%	216	216	100%	98%	100%	216	216	100%	98%	100%
Total	**428**	**428**	**100%**	**99%**	**100%**	**428**	**428**	**100%**	**99%**	**100%**	**428**	**428**	**100%**	**99%**	**100%**
	Accuracy														
	Dorfman 5-1					Dorfman 10-5-1					Dorfman 20-10-5-1				
	x	n	%	95% CI		x	n	%	95% CI		x	n	%	95% CI	
Site A	218	220	99%	97%	100%	215	220	98%	95%	99%	214	220	97%	94%	99%
Site B	220	220	100%	98%	100%	220	220	100%	98%	100%	220	220	100%	98%	100%
Total	**438**	**440**	**100%**	**98%**	**100%**	**435**	**440**	**99%**	**97%**	**100%**	**434**	**440**	**99%**	**97%**	**99%**

Table 6. Test savings and turnaround time

	Prev	Number of tests used		Test Savings				TAT (positives)	TAT (negatives)
	%	Baseline	Pooling	n	%	95% CI		batch runs*	batch runs*
Dorfman 5-1	**3**	**440**	**138**	**302**	**69%**	**64%**	**73%**	**2**	**1.09**
Site A	4	220	74	146	66%	60%	73%	2	1.11
Site B	2	220	64	156	71%	64%	77%	2	1.07
Dorfman 10-5-1	**3**	**440**	**93**	**347**	**79%**	**75%**	**83%**	**3**	**1.21**
Site A	4	220	43	177	80%	75%	85%	3	1.18
Site B	2	220	50	170	77%	71%	83%	3	1.24
Dorfman 20-10-5-1	**3**	**440**	**76**	**364**	**83%**	**79%**	**86%**	**4**	**1.44**
Site A	4	220	29	191	87%	82%	81%	4	1.29
Site B	2	220	47	173	79%	73%	84%	4	1.59

Notes: Controls not yet included in calculation of test savings;
*TAT is measured in number of batch runs required to release a positive (or negative result).
This excludes downtimes and waiting time for the next run.

Table 7. Summary of results for Experiment 2

	Prev	Tests	Sensitivity	Specificity	Accuracy	Tests	Savings	TAT (positives)	TAT (negatives)
	%	n					%	batch runs*	batch runs*
Dorfman 5-1	**3**	**440**	**83%**	**100%**	**100%**	**138**	**69%**	**2**	**1.09**
Site A	4	220	75%	100%	99%	74	66%	2	1.11
Site B	2	220	100%	100%	100%	64	71%	2	1.07
Dorfman 10-5-1	**3**	**440**	**58%**	**100%**	**99%**	**93**	**79%**	**3**	**1.21**
Site A	4	220	38%	100%	98%	43	80%	3	1.18
Site B	2	220	100%	100%	100%	50	77%	3	1.24
Dorfman 20-10-5-1	**3**	**440**	**50%**	**100%**	**99%**	**76**	**83%**	**4**	**1.44**
Site A	4	220	25%	100%	97%	29	87%	4	1.29
Site B	2	220	100%	100%	100%	47	79%	4	1.59

Notes: Controls not yet included in calculation of test savings;
*TAT is measured in number of batch runs required to release a positive (or negative result).
This excludes downtimes and waiting time for the next run.

pool sizes. Three cases had ORF1ab Ct values close to 40 (37.1, 38.95 and 39.3) which did not allow for a definite value when subtracted from the Ct cut-off of 40 and were excluded from the analysis of ORF1ab Ct values. Two cases did not show ORF1ab amplification and were also not included in the calculations. (Table 4).

In the pools of 5, the Ct values of ORF1ab and N gene targets were higher by an average of 0.75 and 2.67 respectively, for an average increase of 2.23. In pools of 10, the Ct values rose by 4.46 and 3.22 respectively with an average of 3.63. The corresponding changes were 4.20 and 3.54 in pools of 20 with an average of 3.67 (Table 8).

These findings are consistent with the Experiment 1 changes in Ct values as well as in previously cited studies which indicate some loss of sensitivity when samples are pooled, especially with Ct values over 35. However, due to the small sample size of positive cases, determination of loss of sensitivity will be best seen in Experiment 1.

The yield was best with pools of 5. Pools of 10 and 20 did not pick up many of the individually positive samples in the original baseline run, especially those with high Ct values. These results are consistent with the observations in Experiment 1, thus, our recommendation is to use pools of 5 in routine testing, based on sensitivity, as

Table 8. Change in Ct value across dilutions

Positive cases	Pools of 5		Pools of 10		Pools of 20	
	FAM ORF1ab gene	ROX N gene	FAM ORF1ab gene	ROX N gene	FAM ORF1ab gene	ROX N gene
Case 1	–	4.39	5.02	4.02	–	5.39
Case 2	–	3.10	–	–	–	0.99
Case 3	–	3.26	–	–	–	1.15
Case 4	–	8.00	–	6.72	–	7.70
Case 6	–	5.32	–	5.16	–	–
Case 7	–	1.03	–	0.87	–	–
Case 9	–	0.19	–	4.33	–	4.08
Case 10	1.46	0.77	2.02	1.98	5.73	2.78
Case 11	0.30	-0.17	7.39	0.85	2.66	1.62
Case 12	0.48	0.81	3.40	1.84	–	4.58
Average Ct-value change	**0.75**	**2.67**	**4.46**	**3.22**	**4.20**	**3.54**
Combined Average	**2.23**		**3.63**		**3.67**	

well as comparable savings in reagents and better turnaround time.

Recent evidence, however, shows that Ct values 35 and over are associated with low viral loads or even viral remnants in persons who are in the process of recovery in which case they are no longer infectious. [31] We need to balance our expectations of pooled testing with its expected slight loss of sensitivity with this in mind. This consideration further supports the expanded use of pooled testing in order to curb transmission in the community since we will detect those who have high viral loads (Ct values 25 or less) without loss of sensitivity at these levels.

Mina et al., argues thus, "The tests we need are fundamentally different from the clinical tests currently being used, and they must be evaluated differently. Clinical tests are designed for use with symptomatic people, do not need to be low-cost, and require high analytic sensitivity to return a definitive clinical diagnosis given a single opportunity to test. In contrast, tests used in effective surveillance regimens intended to reduce the population prevalence of a respiratory virus need to return results quickly to limit asymptomatic spread and should be sufficiently inexpensive and easy to execute to allow frequent testing — multiple times per week. Transmission of SARS-CoV-2 appears to occur days after exposure, when the viral load peaks. This timing increases the importance of high test frequency, because the test must be used at the beginning of an infection to stop onward spread, and reduces the importance of achieving the very low molecular limits of detection of the standard tests."[31]

While Mina et al., claims that the traditional RT-PCR test fails due to its exquisite sensitivity, the use of pooled testing with its slight loss of sensitivity, test savings and applicability to the asymptomatic population lends it well to its being used for frequent testing in order to catch infectious individual in a timely manner.[31]

To quote Mina et al., once again, "A regimen of regular testing works as a sort of Covid-19 filter, by identifying, isolating, and thus filtering out currently infected persons, including those who are asymptomatic. Measuring the sensitivity of a testing regimen or filter requires us to consider a test in context: how often it's used, to whom it's applied, when in the course of an infection it works, and whether its results are returned in time to prevent spread."[31]

Analysis of Turnaround Times

Compared to individual runs, a slight delay in turnaround time is seen in pooled testing, which necessitates repeat individual testing of samples in positive pools. This is to be expected and is more pronounced in the 3 stage pooling schemes. However, with proper time management (scheduling of runs within the day to accommodate deconvolution), the delay **can be** minimized. In return, more subjects can be tested with pooled methods. More positive individuals thus can be identified and isolated and their contacts traced.

Laboratories that will engage in pooled testing should also allot more human resources for pooled testing since some laboratories are running at full capacity and cannot accommodate the additional testing required. More work shifts can be added if not already operating on a 24-hour basis.

The pre-analytical phase can be a source of delays and an increase in turnaround time will ensue if the specimen collection, handling and transport are not properly organized and collection staff are not trained in proper methods for such. We have devised a training module for specimen collection, handling and transport to manage the potential problems that have been identified during the pre-analytical phase of the study.

Analysis of Test Savings

In all pool sizes studied, test savings were substantial and showed progressive increase with bigger pool sizes. Again, this is expected since the positivity rate is low (3%). Even at the lowest savings in pools of 5, 69% savings is seen. However, we must caution that savings is a function of positivity rate and savings will decrease as positivity/prevalence rates increase. The table below illustrates the potential savings at different prevalence rates. To mitigate the reduction of savings, samples from a cluster or sub-group should be pooled together. For example, subjects from the same household or work force in a particular location should be pooled together rather than mixed with other groups.

Table 9. Average savings per batches of 100 specimens, calculated using 10,000 simulations

Prevalence	2 stage Dorfman Pool size			3 stage Dorfman Pool sizes		
	5	10	20	10 & 5	20 & 10	25 & 5
1%	75	80	77	83	84	87
2%	71	72	62	77	73	78
3%	66	64	49	71	64	71
4%	61	56	39	65	56	65
5%	57	50	31	59	48	59
6%	53	44	24	54	42	54
7%	49	38	19	49	36	49
8%	46	34	14	44	31	44
9%	42	29	10	40	26	40
10%	39	25	7	36	21	37
11%	36	21	5	32	17	33
12%	33	18	3	28	14	29
13%	30	15	1	25	10	26
14%	27	12	0	21	8	24
15%	24	9	-1	18	5	21
16%	22	7	-2	15	3	18
17%	20	6	-3	13	1	15
18%	17	4	-3	10	-1	13
19%	15	2	-4	7	-3	11
20%	13	1	-4	5	-4	9

*10,000 batches of 100 specimens each were simulated using the R Programming Language for Statistical Analysis

The amount of savings possible with pooled testing is its main strength. Expanded testing will not be possible without reducing costs of testing and pooled testing actually facilitates more testing. However, to properly manage expectations, preliminary evaluation of the current positivity/prevalence rate of the target populations need to be done. If there is no available data for this, an initial smaller pilot project to determine the positivity rate in the different populations to be tested with the pooled method and the pooled testing adjusted accordingly (Table 9).

Study limitations

Experiment 1 focused on PCR positive samples with Ct values between 30 to 38 with only a few specimens representing medium and high viral loads. Per the protocol, pooling was only simulated by dilution with transport media and not actual pooling with PCR negative samples to demonstrate loss in sensitivity.

In Experiment 2, only 12 positive cases were captured which, although a good thing as it ensured that the pooling methods will perform optimally, it resulted in wide interval estimates for the test sensitivity. In general, estimation of test sensitivity is best done with at least 50 known positives. The test sensitivity estimated in Experiment 1 may be a more reliable basis for policy making. In addition, the estimate of the overall accuracy in Experiment 2 would be a more reliable metric as can be seen in the narrow 95% confidence interval widths of the estimates.

Calculation of the test savings were based solely on the number of tests that were consumed during the testing. A more comprehensive test saving calculation would include tests consumed due to repeats due to any reason, and for running quality controls. We are of the opinion, however, that in the long run, the raw number of tests saved will be an important driver for the overall test and cost savings.

The turnaround time evaluation in this study is based on actual testing hours per run, such that samples in the positive pools were included in the next run. This also does not take into full consideration that larger pool sizes will allow for more samples to be tested in any given batch, which will increase the total number of samples tested per day. The delay due to the increase in turnaround time, thus, can be compensated by the total increase in testing capacity provided by pooling samples.

Sources of potential bias and generalizability

Experiment 1 of this study involved evaluating the effect of pooling on test sensitivity on low amplification samples. Although this may, at first thought, be unrealistic and not representative of actual samples that will be encountered in day to day laboratory operations, using low amplification samples is a good strategy to "stress test" the pooling method and evaluate its performance in extreme situations, where diagnostic errors are more likely to happen and where decision dilemmas frequently occur.

Generalizability

The results of this study are generalizable to other laboratories in as much as the same protocol will be used on the same target tested population. It is recommended to verify that the performance of RT-PCR kits a laboratory uses are at least as good as the performance characteristics of the kits used in this study. Considering the wide range of copy numbers that the different brands of kits can detect (limit of detection [LOD]), it is recommended to use a larger volume per aliquot (200uL or more) to detect samples with low viral loads by providing more template for amplification. It is also recommended that any laboratory intending to implement pooling should carefully evaluate the expected test prevalence of Covid-19 in their tested population. This can be done by reviewing the test positivity rates of the laboratory for the past 2-4 weeks. Any shift in the demographic profile of the tested population that the laboratory services should trigger a review of the test positive prevalence. It is also recommended that laboratories intending to perform pooled testing should conduct initial validation studies with their own RT-qPCR test kits and equipment (Annex A and B). The potential applicability of saliva specimens for pooled RT-PCR testing will further expand the ability to test more individuals with the ease in sample collection it offers.[32-42]

Implications for practice

This pragmatic proof-of concept operational study which demonstrated that Dorfman 5-1 pooling of naso-pharyngeal and oropharyngeal swab samples is a feasible strategy that will result in at least reasonable test savings, with small effects on overall test accuracy and turnaround time.

Intended use and clinical role of pooling

Overall, taking the effects of Dorfman pooling on test sensitivity, specificity, accuracy, test savings, and turn-

around time together, as were observed in this study, it would appear that Dorfman 5-1, with pool size of 5, is the most reasonable pooling method that can be implemented in the laboratory, as long as the test prevalence of COVID-19 is below 10% (Table 9).

CONCLUSION

Pooling is a feasible strategy to further increase testing capacity and decrease cost while keeping accuracy at within acceptable levels. A 2-stage Dorfman 5 pooling strategy appears to be the best method, because it has the highest overall accuracy, while still achieving acceptable test savings, and turnaround time. Pooling of nasopharyngeal and oropharyngeal swab samples prior to RT-qPCR testing may be considered by select molecular diagnostic laboratories to further increase testing capacity and at the same time reduce the cost of testing as a feasible means of adopting more relaxed quarantine schemes.

STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

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ANNEX A. POOLED PCR TESTING WORK INSTRUCTIONS AND GENERAL GUIDELINES

INTRODUCTION

It has been proven that sample pooling can reduce sensitivity of RT-PCR assays for SARS-CoV-2 by several magnitudes i.e., Ct values may increase from 2.56 up to 4.87 higher. This may lead to false negatives and impact virus containment measures. Thus, it is important to make revisions to the interpretation of pooled samples to negate the impact of the loss of sensitivity.

The approach to specimen pooling shall be through pooling of standard volume aliquots of transport media with each containing a single patient sample.

Specimens obtained from different sources or different sample types should not be pooled together, i.e., only nasopharyngeal samples shall be pooled with nasopharyngeal samples, and so on.

The volume of the samples initially collected from an individual must be sufficient for both the pooled testing and individual follow up testing, if needed. This will prevent the need for a second sample collection.

1. SPECIMEN RECEPTION

1.1. The laboratory receptionist shall:
- 1.1.1. Receive the specimen in the receiving room/reception area and check the appropriateness of the transport conditions and the packaging of the specimens.
  - 1.1.1.1. Packaging specimens into groups of five (5) samples must be strictly adhered.
  - 1.1.1.2. If specimens are not received in groups of five (5) samples, these events must be documented and reported to collection teams and/or the source of specimens
- 1.1.2. Cross-checks the details of the patient on the line list/master list, laboratory request form, Case Investigation Form (CIF) and PhilHealth Form CF2 (whichever applies), making sure that the patient name and a second identifier matches the accompanying document.
- 1.1.3. Encode in an electronic line list which will then be transmitted to the staff in charge of specimen handling and inactivation.
- 1.1.4. Retain and file the original copies in the receiving room and store them appropriately based on existing protocols.

1.2. The medical technologist/analyst shall:
- 1.2.1. Receive the triple-packaged samples from the reception area/laboratory receptionist together with the master list/line list, thru a pass box and place it inside a biological safety cabinet.
- 1.2.2. Disinfect the outer container/box with 70% ethanol and wipe with tissue paper
- 1.2.3. Disinfect the inner/second container with 70% ethanol and wipe with tissue paper
- 1.2.4. Remove the samples from the transport box and Individually inspect the samples
- 1.2.5. Individually inspect the samples and asses the specimen integrity via a set criterion for acceptance and rejection, together with a second analyst/laboratory aide
- 1.2.6. Verify the completeness of data in the individual labels on the specimens based on the submitted line list/master list
- 1.2.7. Asses the specimen integrity via a set criterion for acceptance and rejection, together with a second analyst/laboratory aide, taking note of the following acceptance criteria:
  - 1.2.7.1. Swab/s are present in the collection tube
  - 1.2.7.2. Test requisition with patient name and a second identifier
  - 1.2.7.3. Tube label with patient name and a second identifier
  - 1.2.7.4. Collection tube has no leaks and that the cap is intact
  - 1.2.7.5. The specimen is within stability criteria
- 1.2.8. Records any rejected specimens and submits this document to the receptionist and laboratory manager. Rejected specimens shall then be excluded from the pool batch.

2. SPECIMEN ACCESSIONING

2.1. Together with a second analyst in the specimen preparation room, the analyst shall:

2.1.1. Gather specimens into groups of five (5) unique individuals per pool by convenience sampling, or as they are received from the reception area.

2.1.2. Assign a set of unique pooling accession numbers (See attached template) alongside the unique individual accession numbers. An example would be:

Accession Number	Pool Accession Number
A0801PE001	A0801PE-P5A
A0801PE002	
A0801PE003	
A0801PE004	
A0801PE005	

"Accession number" represents the individual unique specimen accession number
"Pool Accession Number" (ex.: XXXXXXX **P 5 A**)

- The first alphanumeric characters represent the desired accession code
- **"P"** stands for pool
- **"5"** represents the number of unique individuals in the pool
- **"A"** represents the sequence in which A is for the first 5 samples pooled, B for the next 5 samples, and so on.

The medical technologist who performed the accessioning relays to another medical technologist in the reagent preparation room the total number of specimens for running, taking into account the number of controls, and that each pool of 5 samples are accounted for a single run, and thus the reagents needed should correspond to only one test. He/she then waits for the cue from the medical technologist in the specimen preparation room when to start the reagent preparation, making sure there are no delays and that only freshly prepared reagents are used.

3. POOLING PROCEDURE

3.1. The recommended dilution factor should be carefully applied considering the characteristics of the target population, and this protocol recommends pools of 5.

3.2. The medical technologist analyst shall:

3.2.1. Identify the samples together with another analyst (buddy)

3.2.2. Arrange the specimens into 5 samples per row and a cryovial labeled with the corresponding pool (i.e., P5A, P5B and so on)

3.2.3. Fill out a printed PCR map template with the accession number corresponding to each pool

3.2.4. With a calibrated pipette with filtered pipette tip, transfer 200uL from each of the 5 individual samples into a 2.0mL cryovial tube, making sure that the sample is properly mixed. When aliquoting and mixing with a pipette, collect the same amount from individual samples and mix in a new container. All pipette tips shall be used only once per sample or at each step. If the sample volume to be collected is 200uL or more, the final volume shall all always be 10% more than the sample for nucleic acid extraction to make a mixed sample.

3.2.5. Transfer an aliquot from the pool using the volume recommended for existing laboratory protocols for extraction.

3.2.6. Pass or communicate the PCR map to the PCR room.

4. POOL EXTRACTION AND PCR

4.1. The medical technologist shall:

4.1.1. Perform sample inactivation according to existing laboratory protocols.

4.1.2. Follow the recommendations of the manufacturer of the nucleic acid extraction reagent, equipment, and PCR reagent for the mixed sample. If the equipment used has a high extraction efficiency, and a large sample volume is used, the possibility of nucleic acid detection in mixed samples is high.

4.1.3. Check the information of the amount of sample used before extraction and the amount and concentration of nucleic acid eluted after extraction.

4.1.4. Store the specimen samples according to existing protocols.

4.1.5. Perform PCR amplification according to existing laboratory protocols.

4.1.6. Set aside and store the pools that test positive, to be individually tested in the next immediate run.

5. QUALITY CONTROL IN DIAGNOSTIC PCR LABORATORIES

5.1. The following are the general guidelines for Quality Control in Diagnostic PCR laboratories for infectious diseases:
- 5.1.1. Maintain separate areas and dedicated equipment (eg. pipettes, microcentrifuges) and supplies (eg. microcentrifuge tubes, pipette tips, gowns and gloves) for assay reagent setup and handling of extracted nucleic acids.
- 5.1.2. Workflow must always be from the clean area to the dirty area.
- 5.1.3. Wear clean disposable gowns and new, previously unworn, powder-free gloves during assay reagent setup and handling of extracted nucleic acids. Change gloves whenever contamination is suspected.
- 5.1.4. Store primer/probes and enzyme master mix at appropriate temperatures (see package inserts). Do not use reagents beyond their expiry dates.
- 5.1.5. Keep reagent tubes and reactions capped as much as possible.
- 5.1.6. Clean and decontaminate surfaces.
- 5.1.7. Do not bring extracted nucleic acid or PCR products into the assay setup area.
- 5.1.8. Use aerosol barrier (filtered) pipette tips only.
- 5.1.9. Use PCR plate strip caps only. Do **not** use PCR plate sealing film.
- 5.1.10. Assay controls should be run concurrently with all test samples. If using a commercial kit, check if the following are already included in the kit:
  - 5.1.10.1. PTC – positive template control with an expected Ct value range
  - 5.1.10.2. NTC – negative template control added during RT-qPCR reaction set-up
  - 5.1.10.3. RP – all clinical samples should be tested for human RNAse P (RNP) gene to assess specimen quality
- 5.1.11. Keep running logs of PTC performance. After each RT-qPCR run of clinical samples, the control Ct values should be recorded.

6. RESULTS ANALYSIS

To ensure the absence of non-specific PCR inhibition of a sample, an internal positive amplification control or internal control is included in each specimen. A sample can be interpreted as negative only if the analysis of the internal positive control indicates that the amplification occurred in the reaction tube but no signal from the target reporter dye has been detected.

6.1. The pathologist shall:
- 6.1.1. Follow the usual validation of negative and positive control samples.
- 6.1.2. Interpret results according to the following:
  - 6.1.2.1. If the pool tests “negative”, report individual samples of that pool as negative or “not detected”.
  - 6.1.2.2. Interpret pools as “positive” if at least one gene target shows any form of amplification (late and low amplification, unusual or non-sigmoid curve).
- 6.1.3. Retest each constituent specimens individually from the pooled samples tagged as “positive” and refer to the individual accession templates described above.
- 6.1.4. Interpret individual runs according to kit manufacturer’s specifications.
- 6.1.5. Report results of individually-ran samples according to existing laboratory protocols.

Reporting of Pooled SARS-CoV-2 Testing Results		
Result	**Interpretations**	**Actions**
Not Detected	Negative	Report each individual in pool as negative
Detected	Positive	Do not report pooled result. Perform diagnostic testing of individual specimens and report each as "positive" or "negative"

7. DECONVOLUTION AND INDIVIDUAL EXTRACTION AND PCR

When pooled samples test positive, samples in these pools should be identified and tested individually.

7.1. The medical technologist shall:

7.2. Obtain another sample from the original specimen and re-test them individually according to the laboratory and manufacturer’s protocol.

7.3. Release individual results and indicate in the report that individual sample testing was done.

8. RESULTS RELEASE

8.1. The staff of the COVID-19 testing laboratory shall release the test results as per the laboratory’s existing protocols on releasing of RT-PCR results.

8.2. Due to the reduction in analytical sensitivity, a pooling strategy should apply risk mitigation procedures such as indicating in the test result/report that the testing procedure involved specimen pooling.

ANNEX B. STANDARD METHOD FOR VERIFICATION OF POOLED TESTING INTERIM GUIDANCE FOR LABORATORIES IN THE COVID-19 LABORATORY NETWORK

As of 26 October 2020

INTRODUCTION

Pooled testing shall be applicable to the performance of SARS-CoV-2 molecular diagnostic tests for the in vitro qualitative detection of RNA from SARS-CoV-2 in respiratory samples for specific target populations only and in line with the Department of Health's latest issuance on testing. Symptomatic patients, as well as people who have high risk exposure to SARS-CoV-2 confirmed cases, shall not be covered by pooled testing.

Pooled testing shall be undertaken only by DOH-selected licensed laboratories of the COVID-19 Laboratory Network only upon review and approval of pooling procedure and method verification data. Only FDA-authorized PCR kits shall be utilized for pooled testing. Pooled testing shall only be performed by qualified proficient laboratory technical staff. The strategy shall only be applied to appropriate target populations with expected low prevalence and low risk. It must be noted that the method may change as more information becomes available.

RATIONALE

In keeping with international standards of good laboratory practice, any new method introduced by the laboratory shall be subject to verification prior to regular performance.

SCOPE

The following contains guidance for the standard method for verification of pooled testing as a testing strategy for SARS-CoV-2 PCR as applicable to respiratory specimens.

Objective

1. To determine the performance, usefulness, practicality, and applicability of pooled testing in a laboratory's particular set-up using its specific PCR reagents, supplies, and laboratory equipment.
2. To verify the method of pooled testing through determination of the percentage agreement between 5-pooled samples and individual samples tested through rRT-PCR prior to adoption of the procedure in the laboratory.

METHOD

1. **Preparation of verification panels**
    a. Positive pools
        i. Using either archived/stored or prospectively collected samples, prepare 20 "positive" pools of five consisting of 80 unique PCR negative samples and 20 PCR positive samples, for a total of 100 samples.
        ii. The 20 PCR positive samples shall consist of:
            1. 25% (n=5) within 2-3 Ct values of the cut-off for the laboratory's PCR assay, to represent low or weak positives
            2. 75% (n=15) with various Ct values representing high and medium positive samples
        iii. Each "positive" 5-sample pool shall consist of 1 PCR positive sample + 4 randomly selected PCR negative samples

    b. Negative pools
        i. Using either archived/stored or prospectively collected samples, prepare 20 "negative" pools of five consisting of 100 unique PCR negative samples.
        ii. If there is sufficient volume, the same negatives used in the preparation of the "positive" pools may be used.

    Note: All samples included in the positive and negative pools should have been tested individually using the laboratory's PCR assay, following manufacturer's instructions, with recording of Ct values.

2. **PCR Testing of positive and negative pools**
    a. The 20 positive and 20 negative pools shall be tested using the laboratory's PCR assay, following the manufacturer's instructions, with recording of Ct values for each gene target.
    b. Ensure that the technical staff conducting the tests on the positive and negative pools are blinded to the results of the individual samples included in each pool.

3. **Analysis of data**
   a. **Data tables**
      i. Data shall be summarized following the table below, showing the Ct values, interpretation of individual results with corresponding pooled test results.

Individual Samples			Pooled Test Result		
Test Result	**Ct Value per gene target**	**Result Interpretation**	**Pool Number**	**Ct Value per gene target**	**Result Interpretation**
Sample Lab ID-001			Pool Number-001		
Sample Lab ID-002					
Sample Lab ID-003					
Sample Lab ID-004					
Sample Lab ID-005					
…			….		
Sample Lab ID-100			Pool Number- 020		

   b. **Percent Agreement (Aggregate)**
      i. Calculate the percent agreement of the pooled samples with respect to the expected results (i.e., if a positive patient sample was included in the 5-sample pools, the expected result was positive).

Samples Tested Individually	Pooled Test Final Result (5-sample Pool)	
Test Result	**Positive**	**Negative**
Positive		
Negative		

Positive Percent Agreement = _____

Formula:

$$\frac{\text{No. of positive results in } \textbf{agreement} \text{ (Pooled test result with individual result)}}{\text{Total number of results (20)}} \times 100$$

   c. **Percent Agreement (Disaggregated to Ct value range)**
      i. Using the table below:

Samples Tested in a 5-sample Pool	Individual Samples with Ct within 3 values within PCR kit cutoff (weak positives)	
Pooled Test Result	**Positive**	**Negative**
Positive		
Negative		
	Individual Samples with Ct values >30 to 37*	
Positive		
Negative		
	Individual Samples with Ct values >20 to 30	
Positive		
Negative		
	Individual Samples with Ct values <20	
Positive		
Negative		
*Note: 37 if kit cut off value is 40, otherwise, indicate 3 values within the PCR kits cutoff value		

Record Keeping

The laboratory shall maintain information on the performance of the pooled testing procedure and all method verification data. These records shall be made available for review and inspection upon request.

Submission of Verification Documents

The accomplished method verification report shall be submitted to the Research Institute for Tropical Medicine for review.

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METHOD VERIFICATION REPORT FOR POOLED TESTING (TEMPLATE)

Date of verification:			
Name of Laboratory:			
Complete Address:			
Laboratory head (Pathologist):			
Chief medical technologist:			
Contact information:	E-mail address:	Landline No.	Mobile No.
Part 1: PCR Assay Intended to be used for Pooled Testing Verification			
Nucleic Acid Extraction kit	• *Brand/manufacturer:* • *Kit description:* • *Attach product brochure and Manufacturer's Instructions for Use* • *Attach Laboratory's SOP*		
Automated Extraction machine	• *Brand/manufacturer:* • *Attach product brochure* • *Attach Laboratory's SOP for operating the machine*		
PCR detection kit	• *Brand/manufacturer:* • *Kit description:* • *Gene targets:* • *Performance data available (FIND, WHO, National Regulatory Agency, RITM)* • *Attach product brochure and Manufacturer's Instruction for Use* • *Attach Laboratory's SOP*		
PCR machine	• *Brand/manufacturer* • *Attach product brochure* • *Attach Laboratory's SOP for operating the machine*		
Part 2: Verification Panel Composition			
Positive pools • *Number/quantity?* • *Archived/stored or prospectively collected?* • *If archived/stored, dates of collection, storage condition (2-8°C, -20°C, -40°C, -80°C)* • *Ct values*			
Negative pools • *Number/quantity?* • *Archived/stored or prospectively collected?* • *If archived/stored, dates of collection, storage condition (2-8°C, -20°C, -40°C, -80°C)* • *Ct values*			
Part 3: Pooling Procedure (Detailed specific steps for pooling undertaken)			

Part 4. Results

Summary table (per gene target)

Individual Samples			Pooled Test Result		
Test Result	**Ct Value per gene target**	**Result Interpretation**	**Pool Number**	**Ct Value per gene target**	**Result Interpretation**
Sample Lab ID-001			Pool Number-001		
Sample Lab ID-002					
Sample Lab ID-003					
Sample Lab ID-004					
Sample Lab ID-005					

Percentage Agreement (Aggregate)

Samples Tested Individually	Pooled Test Final Result (5-sample Pool)	
Test Result	**Positive**	**Negative**
Positive		
Negative		

Notes:

Percentage Agreement (Disaggregated to Ct value range)

Samples Tested in a 5-sample Pool	Individual Samples with Ct within 3 values within PCR kit cutoff (weak positives)	
Pooled Test Result	**Positive**	**Negative**
Positive		
Negative		
	Individual Samples with Ct values >30 to 37	
Positive		
Negative		
	Individual Samples with Ct values >20 to 30	
Positive		
Negative		
	Individual Samples with Ct values <20	
Positive		
Negative		

Notes:

Report prepared by:

Technical Staff
Signature over printed name

Approved by:

Head of Laboratory
Signature over printed name

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ANNEX C. PHILIPPINE SOCIETY OF PATHOLOGISTS RECOMMENDATION FOR POOLED TESTING

The Philippine Society of Pathologists Inc. is an organization of physicians specializing in Pathology and Laboratory Medicine with a membership of over 1,000. The society and its members, with specialization and sub-specialization in various areas of the diagnostic field, including Molecular Pathology and Immunopathology, stands in solidarity with the rest of the nation in its fight against SARS-CoV-2.

The statements issued are based on the preliminary findings of the research study **"An Evaluation of Pooling Strategies for RT-qPCR testing for SARS-CoV-2 Infection: A pragmatic parallel multi-site operational study by the PSP Inc."** This was conducted in Research Institute of Tropical Medicine, Philippine Children's Medical Center, and University of Perpetual Help DALTA Medical Center as principal investigator sites. The research was supported and funded by Philippine Center for Entrepreneurship Inc.

Based on our preliminary research findings, sample pooling can be used as a strategy to enhance COVID-19 testing to increase the number of tests conducted in the country. It will conserve much needed resources, improve turnaround time, and make the test affordable. More importantly as a surveillance testing strategy, it will identify positive asymptomatic persons who are potential spreaders and transmitters of the disease;

To achieve a significant and expanded number of target populations to be tested, the Philippine Society of Pathologists Inc. (PSP Inc.) recommends the following:

1. There must be a comprehensive and cost-effective strategy in place to implement pooled testing;

2. Pooled testing shall not be done on the following:
   A. Symptomatic individuals
   B. Recovered (although asymptomatic) patients or retesting of previously positive individuals
   C. Close contacts (household and family members) of positive individuals

3. Sample pooling is an expanded and targeted testing strategy for screening ASYMPTOMATIC PERSONS. We recommend pooled testing in the following targeted populations:

   A. Low prevalence communities (10% or less) for epidemiologic surveillance and aggressive contact tracing;
   B. Targeted community testing in areas that are under lockdown to identify additional infected individuals and to guide in decisions for lifting the lockdown;
   C. Surveillance of health care workers and all workers in the health care facility
   D. Workplace testing to include factory workers, market vendors, call center agents, transportation workers, and others;
   E. Border testing at airports and seaports for inbound foreign travelers and returning residents;
   F. Overseas deployment of OFWs;
   G. Returning OFWs;
   H. Frontline government workers (police, military, quarantine, immigration officers to name a few);
   I. Locally Stranded Individuals (LSI)
   J. Any other vulnerable populations to be determined in the future

4. The initial recommendation is to use a pool sample of 5, until an accurate prevalence of cases with the presence of the SARS –CoV-2 virus is identified in the population.

5. Quality and accuracy of the sample pooling strategy must be enhanced and maintained thru:
   A. Defined standards and procedures of practice
   B. Training
   C. Quality assurance
   D. Monitoring

6. There is a need to have a technical validation process for all COVID-19 testing laboratories which will participate in the pooled testing;

7. Sample pooling is a strategy to ensure a wider population to be tested in a cheaper, faster, and more efficient manner. More importantly, there is the need to implement contact tracing for those exposed to persons who tested positive for the presence of the SARS-CoV-2 virus in order to break the cycle of transmission at the earliest time possible;

8. Pooled testing will accelerate the analytical process but there is a need to address the pre-analytical and post-analytical phases when dealing with thousands of specimens to be handled at the same time. The pre-analytical and post-analytical processes should ensure an organized, systematic, and streamlined set pf procedures for mass specimen collection, documentation, and reporting, as well as include the use of appropriate Information Technology systems.

9. This is a dynamic situation and PSP will be issuing updated guidelines when circumstances dictate.

In summary, the PSP Inc. stands ready to be in the forefront of diagnostic testing during this pandemic. We are ready to assist in the implementation of these recommendations in an effort to reduce transmission of SARS-CoV-2 in the country.

OPEN ACCESS – ORIGINAL ARTICLE

Diagnostic Performance of Mean Platelet Volume in the Diagnosis of Acute Myocardial Infarction: A Meta-Analysis

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ABSTRACT

Objective. The aim of this systematic review and meta-analysis is to determine summary estimates of the diagnostic accuracy of mean platelet volume for the diagnosis of myocardial infarction among adult patients with angina and/or its equivalents in terms of sensitivity, specificity, diagnostic odds ratio, and likelihood ratios.

Methodology. The primary search was done through search in electronic databases. Cross-sectional, cohort, and case-control articles studying the diagnostic performance of mean platelet volume in the diagnosis of acute myocardial infarction in adult patients were included in the study. Eligible studies were appraised using well-defined criteria.

Results. The overall mean MPV value of those with MI (9.702 fl; 95% CI 9.07 – 10.33) was higher than in those of the non-MI control group (8.85 fl; 95% CI 8.23 – 9.46). Interpretation of the calculated t-value of 2.0827 showed that there was a significant difference in the mean MPV values of those with MI and those of the non-MI controls. The summary sensitivity (Se) and specificity (Sp) for MPV were 0.66 (95% CI; 0.59 - 0.73) and 0.60 (95% CI; 0.43 – 0.75), respectively. The pooled diagnostic odds ratio (DOR) was 2.92 (95% CI; 1.90 – 4.50). The positive likelihood ratio of MPV in the diagnosis of myocardial infarction was 1.65 (95% CI; 1.20 – 22.27), and the negative likelihood ratio was 0.56 (95% CI; 0.50 – 0.64).

Conclusion. The intended role for MPV in the diagnostic pathway of myocardial infarction would perhaps be best as a triage tool. MPV values can discriminate between those who have MI and those without. For a patient with angina presenting with elevated MPV values, it is 1.65 times more likely that he has MI. It is implied that the decision to treat a patient with angina or its equivalents as a case of MI could be supported by an elevated MPV value.

Key words: mean platelet volume, MPV, myocardial infarction, angina, chest pain

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INTRODUCTION

Rationale

The World Health Organization identifies Cardiovascular Diseases (CVD) as the top cause of death worldwide with an estimated 17.9 million deaths annually. Four out of five cardiovascular deaths are due to myocardial infarction and stroke.[1] Acute Coronary Syndrome (ACS) refers to a spectrum of conditions which are consistent with acute myocardial ischemia and/or infarction that are most likely due to an abrupt reduction in coronary blood flow.[2] These conditions include: Unstable Angina (UA), Non-ST Segment Elevation Myocardial Infarction (NSTEMI), and ST-Segment Elevation Myocardial Infarction (STEMI).

The diagnosis of myocardial infarction relies on a constellation of anginal symptoms combined with findings on electrocardiograms (ECGs) and biomarkers of myocardial necrosis. Among these biomarkers, cardiac troponins are the most specific and most sensitive.[2] However, the diagnostic efficiency of these cardiac troponin measurements within 2 to 4 hours of symptom onset is limited.[3] Furthermore, the challenge remains upon Filipino physicians to make rapid and accurate diagnoses in institutions that may not have access to these life-saving diagnostic modalities.

As an integral component in the pathogenesis of myocardial infarction, thrombus formation is brought about by plaque disruption and subsequent exposure of substances that promote platelet activation, adhesion, aggregation, and thrombin generation. Platelets therefore play an important role in its pathologic process.

Previous studies have shown that platelet size may be used as a marker for platelet function, such that larger platelets are more active and have a greater tendency for thrombosis.[4] Mean platelet volume (MPV) is the most accurate measure of the size of platelets and is routinely measured by most automated hematological analyzers together with the complete blood count. Therefore, it is widely available in most healthcare institutions, and the results may be efficiently reported within an hour of blood collection.

Mean platelet volume is an indicator of platelet activation and is a machine-calculated measurement of the average size of platelets.[5] The methods of analysis of platelet parameters utilize either electrical impedance or optical principles, and recent studies imply that this analysis is not routinely subjected to specific standardization and calibration guidelines.[6] Although its measurement provides clinically useful data, MPV remains to be a diagnostic tool that is yet to be included in routine clinical decision making.

Several studies have shown associations between mean platelet volume and cardiovascular risks and outcomes, such as risk of acute coronary syndrome and myocardial infarction, re-stenosis and mortality rates after percutaneous coronary intervention (PCI), and even recurrence of myocardial infarction. Furthermore, some studies demonstrate the diagnostic utility of MPV as an early and independent predictor of acute coronary syndrome in patients presenting with chest pain.[7] However, conflicting data exists such that some studies refute these findings, while most differ on the cut-off point at which to define an MPV value as "elevated." To date, no general consensus exists on the clinical validity of mean platelet volume (MPV) in the diagnosis of myocardial infarction.

RESEARCH QUESTION

Among adult patients presenting with angina and/or its equivalents, what is the diagnostic performance of an elevated mean platelet volume value in the diagnosis of acute myocardial infarction?

OBJECTIVES

General Objective

To determine summary estimates of the diagnostic accuracy of mean platelet volume for the diagnosis of myocardial infarction among adult patients with angina and/or its equivalents.

Specific Objectives

1. To determine the difference of the mean platelet volume values between those with myocardial infarction and those in the non-MI controls.
2. To determine the sensitivity, specificity, likelihood ratios, and diagnostic odds ratio of mean platelet volume in the diagnosis of myocardial infarction among adult patients presenting with angina and/or its equivalents compared to without MI.

METHODOLOGY

Literature search, quality assessment of the included studies and data extraction for diagnostic accuracy were conducted by two reviewers (K.A. and T.V.). Discrepancies were discussed and referred to a third reviewer. This meta-analysis followed a predetermined protocol described in the following paragraph. Standard systematic review techniques, as outlined by the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA), were used for this study.

Approval from the hospital Institutional Review Board was secured.

ELIGIBILITY CRITERIA

Types of Studies. Cross-sectional, cohort, and case-control articles studying the diagnostic performance of mean platelet volume in the diagnosis of acute myocardial infarction in adult patients were included in the study. In the search strategy, studies were included if: (1) CBC was taken upon presentation to the ER or upon admission (within 24 hours of symptom onset); (2) myocardial infarction was diagnosed with serum markers, ECG, or according to accepted guidelines by the Cardiology societies (American Heart Association (AHA), American College of Cardiology (ACC), European Society of Cardiology (ESC); and, (3) if outcomes were measured as significant difference AND/OR sensitivity and specificity. Only publications with English language were selected. No publication status restrictions were imposed. Case reports and/or case series articles were excluded. In addition, studies were also excluded if: study populations comprised of patients with significant comorbidities with expected increase in platelet counts and MPV; articles with inaccessible full text; the study population is not the population of interest (e.g. children) and articles without a control group; however, no restrictions with regards to the type of control were placed (whether the control group are healthy individuals or with non-cardiac chest pain).

Types of participants. Studies with the following participants were included: (1) Adult participants at least 18 years old with angina and its equivalents (dyspnea, shoulder or arm pain, vomiting, diaphoresis), OR (2) Patients with known coronary artery disease; AND the diagnosis of acute coronary syndrome was made using serum markers, ECG, or according to accepted guidelines by the Cardiology societies (American Heart Association (AHA), American College of Cardiology (ACC), European Society of Cardiology (ESC) upon admission or at the emergency department (ED) level.

Language. English

Characteristics of Diagnostic Tests. Studies evaluating the index test (mean platelet volume) in patients with myocardial infarction (MI) compared with those healthy controls or non-MI were examined. The mean platelet

volume should have been measured by automated hematology analyzers; and measurement should have been taken upon presentation to the ER or upon admission (within 24 hours of symptom onset). Only MPV values measured in femtoliter using automated analyzers or cell counters were included in this study, regardless of the machine manufacturer or defined reference ranges imposed by each company. For this study, the reference test was the clinical diagnosis of myocardial infarction (MI) with the aid of serum markers (CK-MB and/or Troponin I) and ECG findings which were according to accepted guidelines by the different Cardiology societies (American Heart Association (AHA), American College of Cardiology (ACC), European Society of Cardiology (ESC). Studies that further grouped the acute coronary syndrome (ACS) spectrum into unstable angina (UA), non-ST elevation segment myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) were also included. Studies that did not specify the type of ACS were excluded. Studies with healthy control groups and control groups with co-morbidities (coronary artery disease) or with initial presentation of angina were allowed.

Time Frame. Studies published and indexed between 1990 until 2020 were included.

Types of Outcome Measures

Primary Outcomes. Diagnostic performance of MPV in the diagnosis of MI that was expressed as summary Sensitivity, Specificity, Positive Likelihood Ratio, Negative Likelihood Ratio, and Diagnostic Odds Ratio; Significant difference in the mean MPV values between those diagnosed with MI and those of the control group.

SEARCH METHODS FOR IDENTIFICATION OF STUDIES

The primary search was done through search in electronic databases like MEDLINE via PUBMED (https://www.ncbi.nlm.nih.gov/pubmed/), Cochrane Review-CENTRAL [Cochrane Central Register of Controlled Trials (http://cochranelibrary-wiley.com/cochranelibrary/search?searchRow.searchOptions.searchProducts=clinicalTrialsDoi)], HERDIN [Health Research and Development Information Network (http://www.herdin.ph)], Google Scholar (http://scholar.google.com), Philippine Journal of Pathology (https://philippinejournalofpathology.org/index.php/PJP), and Philippine College of Physicians Philippine Journal of Internal Medicine (https://www.pcp.org.ph/index.php/pjim/pjim. The reference list of original reports was also searched. Three study authors, one Bangladeshi (Islam 2017), one British (Mathur 2001) and the other a Chinese (Liang 2017), were contacted through e-mail to acquire full text publications of their studies but to no avail.

MeSH terms used for key and text word searching were as follows: "mean platelet volume" OR "MPV OR "mean platelet concentration" AND "myocardial infarction" OR "acute coronary syndrome" OR "chest pain" OR "angina" OR "dyspnea" OR "anginal equivalent". Bibliography for relevant citations were manually searched and experts in the field were contacted to ensure completeness of search strategy. Titles and abstracts of potential articles identified in the primary search were evaluated and a list of potential eligible studies were identified. Studies which fulfilled the selection criteria were included in the meta-analysis. No publication status restrictions were applied.

DATA COLLECTION AND ANALYSIS

Study Selection

The titles and abstracts of each individual study were screened initially to exclude irrelevant reports. Eligibility assessment was performed independently in an unblended standardized manner by two reviewers. Disagreement between the reviewers was resolved by consensus after a thorough discussion among the reviewers. The reviewers started with a large number of identified records that passed the preliminary criteria. The researchers then sequentially excluded records according to the eligibility criteria. Studies which passed the eligibility criteria were then reviewed in its full text publication. The flow diagram summarizing the flow of studies through the selection process is shown in Figure 1.

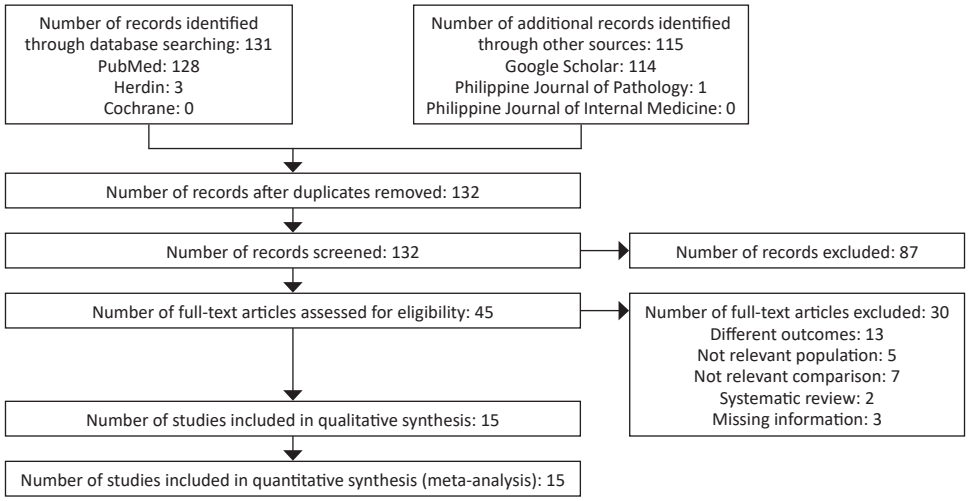

Figure 1. Flow diagram of the Study Selection Process.

Data Extraction and Management

Full manuscripts of eligible studies were independently reviewed by the author and another reviewer. Relevant data were extracted into a data collection electronic form (Appendix 1). The following data were extracted:

a. **Study characteristics:** author, year of publication, country, study design
b. **Population characteristics:** with exclusion criteria, characteristics of control group, study population size and characteristics
c. **Index test characteristics:** timing of sampling, analyzer used
d. **Reference characteristics:** guidelines used in the diagnosis of MI
e. **Outcomes:** mean MPV values in MI, mean MPV values in control, sensitivity, specificity, threshold

The information obtained were summarized and presented in evidence tables (Appendices 2 and 3). Disagreements between the data extractors were resolved after thorough discussion and review of the eligibility criteria before getting into a consensus.

Assessment of Risk of Bias/Quality Appraisal

Quality assessment of the studies using QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies) tool was conducted. Risk of bias assessments was done by one reviewer, with another reviewer providing verification to all of the assessed studies. The QUADAS-2 tool was completed by following stepwise guidelines to judge risk of bias and concerns about applicability for each study (Appendix 4).

STATISTICAL METHODS

Statistical Outcomes

Simple descriptive statistics were used to characterize all included studies. Mean MPV values of those with MI were estimated against those of the control groups across all the included articles.

Standard Mean Difference. Across all included articles, the mean MPV values in those with MI were compared with those of the control. Meta-analysis of the standard mean differences was done using the "metan" command. The results were represented graphically in a Forest Plot. In order to investigate whether or not there was a significant difference in the mean values of MPV in those with MI and in those of the control group, a standardized two-sample t-test with unequal variances was also performed across all included studies.

Diagnostic Test Accuracy Studies. A total of six diagnostic accuracy studies were deemed eligible for inclusion into the meta-analysis. Sensitivity and specificity values of the index test were determined separately for each study. The "MI" and "non-MI" categories were used to allocate patients into positive and negative, respectively. The diagnoses of "unstable angina" and "coronary artery disease" were allocated as negative. Aided by the RevMan calculator, aggregate data (true positives, false negatives, false positives, and true negatives) were extracted from the individual articles. The RevMan calculator was also used to calculate for the Positive Predictive Value (PPV) and Negative Predictive Value (NPV).

Calculation of metrics. Pooled estimates of sensitivity (Se), specificity (Sp), and their 95% confidence intervals were calculated as main outcome measures and were analyzed using the "metandi" command to facilitate the fitting of hierarchical logistic regression models.[8,9,10] The Rutter and Gatsonis HSROC model was used to calculate summary measures of diagnostic accuracy (sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, and diagnostic odds ratio). These measures were pooled using the random effects model. The Rutter and Gatsonis HSROC parametrization model functions of sensitivity and specificity to define a summary ROC curve; whereas the random-effects model assumes that the studies were drawn from populations that differ from each other in ways that could impact on the study effect.[11,12] These models were used to analyze functions of sensitivity and specificity to define the following:

1. the summary ROC curve,
2. the summary operating point (summary sensitivity and summary specificity),
3. a 95% confidence region around the summary operating point, and
4. a 95% prediction region.

Analytical Sensitivity. Analytical sensitivity of the index test (MPV) was evaluated by analyzing the results of the index test (MPV) against the reference standard defined by each study, based on guidelines by the Cardiology societies American Heart Association (AHA), American College of Cardiology (ACC), and/or European Society of Cardiology (ESC).

Variability in Positivity Threshold. As a characteristic source of heterogeneity in meta-analysis of diagnostic test accuracy, presence of the threshold effect was assessed by analyzing the variability of each study's cut-off points to define a positive result. To demonstrate how sensitivity and specificity trade-off with each other as the thresholds vary, summaries of the fitted models were graphically presented as the summary receiver operating characteristic (SROC) curve. Graphical presentations of the summary point, prediction region, and confidence region were also plotted. The "metandiplot" command was used to simplify the plotting of these graphical summaries. Presence of a threshold effect was also evaluated using a Spearman correlation coefficient.

Heterogeneity. In diagnostic test accuracy reviews, heterogeneity has been presumed to exist such that tests for heterogeneity in sensitivity and specificity have not been routinely used since they do not account for heterogeneity explained by phenomena such as positivity threshold effects.[11] No equivalent to the I^2 statistic is currently available for DTA meta-analysis.[13] Instead, computation of variance under the bivariate model was done. For completion of studies, heterogeneity was evaluated by measurement of I^2 values across all included studies.

Statistical Analysis. Data were analyzed using STATA SE13 (StataCorp. 2019. *Stata Statistical Software: Release 16*. College Station, TX: StataCorp LLC).

RESULTS

Description of Studies

Fifteen articles met the inclusion criteria. Eight articles were diagnostic accuracy studies which gave outcome measures of sensitivity and specificity, while the other seven articles were cross-sectional studies which showed outcome measures of mean MPV values in femtoliters across comparison groups. [3,4,7,14-25]

The characteristics of the studies are presented in Table 1. All of the diagnostic test accuracy studies gave outcome measures of sensitivity and specificity specifically for those with MI, except for two studies (Huang 2019; Kaminska 2018) which only gave outcome measures for ACS without further differentiation into the different subgroups. A total of 9748 adult patients were qualified for inclusion. Two-thousand five hundred forty-six patients (2546) were diagnosed with Myocardial Infarction while 7202 patients were allocated to the non-MI group. The studies were conducted from 2001 to 2019. Most of the studies (12 articles) were conducted in Asia, while two were conducted in Europe and one in North America. All of the included studies limited inclusion to adults with chest pain and/or a diagnosis of an acute coronary syndrome or coronary disease. Majority (60%) of the studies employed exclusion criteria, while 40% of the studies did not specify any exclusions.

Quality Appraisal

The risk of bias and applicability concern of each study are presented in Table 2. Two review authors independently assessed risk of bias using the QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies) tool. Risk of bias was assessed in four domains: (1) patient selection, (2) index test, (3) reference standard, and (4) flow and timing. In each domain, the risk of bias was graded as Low, High, or Unclear.

Most of the studies showed low risk of bias. There were applicability concerns regarding patient selection. In 14 out of 15 studies, there was perfect agreement of the QUADAS-2 assessments performed by the 2 review authors. Disagreements were often due to different assessments of the reviewers with regards to patient selection.

Table 1. Characteristics of Included Studies

Study No.	Study 1	Study 2	Study 3	Study 4	Study 5	Study 6	Study 7
Author, Year	Assiri 2012	Cemin 2011	Chu 2011	Dehghani 2014	Huang 2019	Kaminska 2018	Khode 2012
Study Design	Cross-sectional cohort	DTA cohort	DTA cohort	DTA cohort	DTA cohort	DTA case-control	DTA case-control
Country	Saudi Arabia	USA	Taiwan	Iran	Taiwan	Poland	India
Study Population	212 with diagnosis of ACS	1971 with chest pain	282 with chest pain	1046 with chest pain	104 with chest pain	93 with diagnosis of ACS	128 with diagnosis of CAD
w/ Exclusion Criteria	Yes	No	Yes	No	Yes	Yes	Yes
Control Group	With chest pain	With chest pain	With chest pain	Healthy	With chest pain	Healthy	Healthy
Analyzer used	Sysmex	Beckman Coulter	Sysmex	Sysmex	Sysmex	Sysmex	Sysmex
Timing of Test	On admission	At ER/ED	At ER/ED	On admission	At ER/ED	After diagnosis of ACS	On admission
Outcomes							
Mean MPV in MI	8.99	7.925	11	9.7	10.7	10.8	9.65
Mean MPV in Control	8.38	7.875	9.8	9.3	10.0	9.45	9.21
Sensitivity	None	77%	78.60%	72%	None	None	56.40%
Specificity	None	45%	77.6%	40%	None	None	45.9%
Cut-off Threshold	None	7.5 fl	10.65 fl	9.15 fl	None	None	9.25 fl

Table 1. Characteristics of Included Studies *(continued)*

Study No.	Study 8	Study 9	Study 10	Study 11	Study 12	Study 13	Study 14	Study 15
Author, Year	Kilicli-Camur 2015	Lippi 2009	Mirzaie 2012	Ozlu 2013	Senaran 2001	Wang 2016	Yaghoubi 2016	Yilmaz 2008
Study Design	DTA cohort	Cross-sectional cohort	Cross-sectional cohort	Cross-sectional case-control	Cross-sectional case-control	DTA cohort	Cross-sectional case-control	Cross-sectional case-control
Country	Turkey	Italy	Iran	Turkey	Turkey	China	Iran	Turkey
Study Population	200 who underwent angiography	2304 with chest pain	851 with chest pain	79 with diagnosis of ACS	57 with diagnosis of ACS	1574 with chest pain	631 with diagnosis of ACS	216 with diagnosis of ACS
w/ Exclusion Criteria	Yes	No	No	No	No	Yes	Yes	Yes
Control Group	With chest pain	With chest pain	With chest pain	Healthy	Healthy	With chest pain	With chest pain	With stable CAD
Analyzer used	Sysmex	Siemens	Sysmex	Coulter	Coulter	Sysmex	Sysmex	Sysmex
Timing of Test	First 24 hours	At ER/ED	At ER/ED	First 24 hours	On admission	On admission	First 24 hours	First 24 hours
Outcomes								
Mean MPV in MI	11.75	8.175	9.92	8.78	8.2	9.4	10.14	10.4
Mean MPV in Control	10.89	7.7	9.57	7.78	6.6	7.9	9.34	8.9
Sensitivity	46%	None	None	None	None	69.69%	None	None
Specificity	87%	None	None	None	None	50%	None	None
Cut-off Threshold	12 fl	None	None	None	None	9.0 fl	None	None

Table 2. Quality Assessment of Included Studies using QUADAS-2 Tool

Study	Risk of bias				Applicability concerns		
	Patient Selection	Index Test	Reference Standard	Flow and Timing	Patient Selection	Index Test	Reference Standard
Assiri 2012	☺	☺	☺	☺	☺	☺	☺
Cemin 2011	☹	☺	☺	☺	☺	☺	☺
Chu 2011	☺	☺	☺	☺	☺	☺	☺
Dehghani 2014	☹	☺	☺	☺	☺	☺	☺
Huang 2019	☺	☺	☺	☺	☺	☺	☺
Kaminska 2018	☹	☹	☺	?	☺	☺	☺
Khode 2012	☹	☹	☺	☺	☺	☺	☺
Kilicli-Camur 2015	☺	☺	☺	☺	☺	☺	☺
Lippi 2009	☹	☺	☺	☺	☺	☺	☺
Mirzaie 2012	☹	☺	☺	☺	☺	☺	☺
Ozlu 2013	☹	☹	☺	☺	☺	☺	☺
Senaran 2001	☹	☹	☺	☺	☺	☺	☺
Wang 2016	☺	☺	☺	☺	☺	☺	☺
Yaghoubi 2013	☹	☹	☺	☺	☺	☺	☺
Yilmaz 2008	☹	☹	☺	☺	☺	☺	☺

☺ Low Risk ☹ High Risk ? Unclear Risk

Outcomes

Pooled Standard Mean Difference. MPV values were higher in patients with myocardial infarction (9.702 fl; 95% CI 9.07 – 10.33) than in those of the non-MI control group (8.85 fl; 95% CI 8.23 – 9.46). Meta-analysis across all fifteen included studies showed a pooled standard mean difference of 1.131 (95% CI; 0.81 – 1.45) using the Hedges method with random effects model. This value of 1.131 was within the reported normal analytical variation according to a study by Buoro, et al. [26] This could imply that although there is heterogeneity in the included studies, the variability in MPV values among patients with MI was still within the expected normal range (no significant outliers or deviations). Forest plots for the mean values of MPV in the diagnosis of myocardial infarction are seen in Figure 2. The pooled estimate favors a positive association (increased values) of MPV. Overall heterogeneity for the included studies was measured using I^2 with a value of 96.3%.

Significant Difference Between Means. Results of the two-sample t-test with unequal variances and Hedges random effects model showed a t-value of 2.0827 with degrees of freedom at 27.9, and a t-distribution table value of 2.048. Since the calculated t-value was greater than the t-distribution table value (2.0827 > 2.048), we can reject the null hypothesis and accept the alternative hypothesis that there is a significant difference in the mean MPV values of those with MI and those of the non-MI controls.

Summary Sensitivity and Specificity. Subgroup analysis of the six diagnostic test accuracy studies showed that the summary sensitivity (Se) and specificity (Sp) for MPV were 0.66 (95% CI; 0.59 - 0.73) and 0.60 (95% CI; 0.43 – 0.75), respectively (Table 3). Forest plots for Se and Sp in the diagnosis of myocardial infarction are presented in Figure 3. Heterogeneity was measured by Cochran's Q statistic and quantified using the I^2. The calculated I^2 was 80% for the pooled Se, and 97% for the pooled Sp.

Table 3. Summary points of diagnostic performance

Summary Point	Value	95% Confidence Interval
Sensitivity	0.66	0.59 - 0.73
Specificity	0.60	0.43 - 0.75
DOR	2.92	1.90 - 4.50
LR+	1.65	1.20 - 2.27
LR–	0.56	0.50 - 0.64
Mean Threshold	9.59 fl	7.97 - 11.21

Summary Positive LR, Negative LR, DOR. The positive likelihood ratio of MPV in the diagnosis of myocardial infarction was 1.65 (95% CI; 1.20 – 2.27), and the negative likelihood ratio was 0.56 (95% CI; 0.50 – 0.64). The pooled diagnostic odds ratio (DOR) was 2.92 (95% CI; 1.90 – 4.50).

Summary Positive and Negative Predictive Values. The positive predictive value (PPV) of MPV in the diagnosis of myocardial infarction was 0.3180, and the negative predictive value (NPV) was 0.8189.

Mean Cut-off Value and Threshold Effect. Different positivity thresholds were used across all six diagnostic accuracy studies in this meta-analysis. The mean cut-off value for positivity was 9.59 ± 1.55 fl (95% CI; 7.97 – 11.21 fl). A threshold effect was already presumed in this study; however, the investigator opted to measure the Spearman correlation coefficient which was 0.314 (p value of 0.544).

Summary ROC Curve. Because of the presence of heterogeneity, the diagnostic indices were calculated using the HSROC model and the random effects model. The summary ROC curve is shown in the figure below (Figure 4). Also shown in Figure 4 are the ff: the summary operating point (summary sensitivity and summary specificity), the 95% confidence region around the summary operating point, and the 95% prediction region. The confidence region is a measure of within-study uncertainty caused by sampling variability, while the prediction region is a measure of between-study variability.[8,13] Estimates of variance under the bivariate model showed (logitSe) of 0.1239 and (logitSp) of 0.7084.

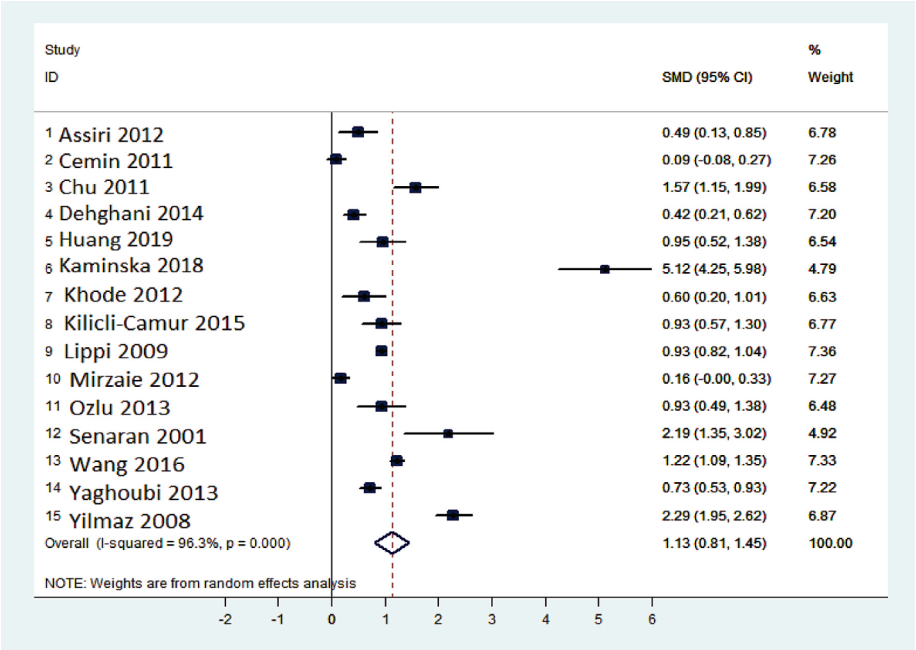

Figure 2. Forest plot of standard mean difference of MPV in those with MI and non-MI controls.

Study	TP	FP	FN	TN	Sensitivity (95% CI)	Specificity (95% CI)
Cemin 2011	102	1011	31	827	0.77 [0.69, 0.84]	0.45 [0.43, 0.47]
Chu 2011	22	57	6	197	0.79 [0.59, 0.92]	0.78 [0.72, 0.83]
Dehghani 2014	138	512	54	342	0.72 [0.65, 0.78]	0.40 [0.37, 0.43]
Khode 2012	22	48	17	41	0.56 [0.40, 0.72]	0.46 [0.35, 0.57]
Kilicli-Camur 2015	32	17	38	113	0.46 [0.34, 0.58]	0.87 [0.80, 0.92]
Wang 2016	613	347	267	347	0.70 [0.67, 0.73]	0.50 [0.46, 0.54]

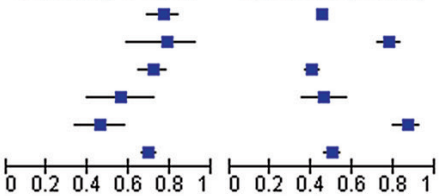

Figure 3. Forest Plot of Diagnostic Accuracy Studies.

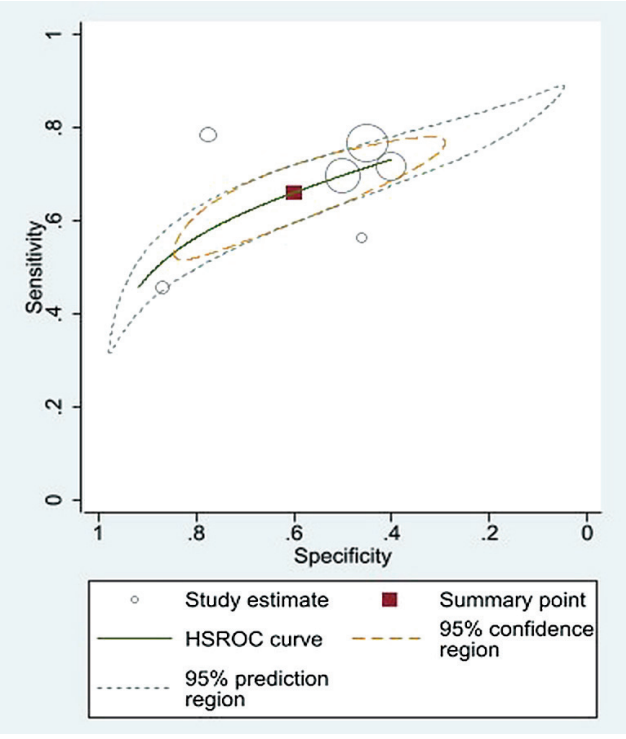

Figure 4. Summary ROC Curve of MPV in the Diagnosis of Myocardial Infarction.

DISCUSSION

Summary of Main Results

This meta-analysis compiled cross-sectional studies and diagnostic accuracy studies on the diagnostic performance of mean platelet volume in the diagnosis of myocardial infarction among adult patients with angina and/or its equivalents. A total of fifteen articles met the inclusion criteria and were deemed eligible for subsequent meta-analysis, and among which, six studies were diagnostic accuracy studies. The overall mean MPV value of those with MI (9.702 fl; 95% CI 9.07 – 10.33) was higher than in those of the non-MI control group (8.85 fl; 95% CI 8.23 – 9.46). Interpretation of the calculated t-value of 2.0827 showed that there was a significant difference in the mean MPV values of those with MI and those of the non-MI controls. The summary sensitivity (Se) and specificity (Sp) for MPV were 0.66 (95% CI; 0.59 - 0.73) and 0.60 (95% CI; 0.43 – 0.75), respectively. The pooled diagnostic odds ratio (DOR) was 2.92 (95% CI; 1.90 – 4.50). The positive likelihood ratio of MPV in the diagnosis of myocardial infarction was 1.65 (95% CI; 1.20 – 22.27), and the negative likelihood ratio was 0.56 (95% CI; 0.50 – 0.64). The positive predictive value (PPV) of MPV in the diagnosis of myocardial infarction was 0.3180, and the negative predictive value (NPV) was 0.8189. Across all fifteen included studies, a high degree of heterogeneity

was measured at I^2 of 96.3%. Across the six diagnostic accuracy studies, estimates of variance under the bivariate model showed logitSe of 0.1239 and logitSp of 0.7084. Under the HSROC model, the 95% prediction region was larger than the 95% confidence region, signifying high heterogeneity.[13]

Summary of Statistical Findings

It was established that there was a significant difference between the mean MPV values in those with MI compared to those of the non-MI controls. This study also showed that overall, mean MPV values of those with MI were higher than in those without. However, in diagnostic accuracy studies, further statistical data must be studied in order to view this information in terms of values that are of actual clinical value.

A high degree of heterogeneity was expected in meta-analyses of diagnostic test accuracy. The most common cause of which was the varied cut-off points or thresholds used by each study.[11,13] Because different positivity thresholds were used across all the diagnostic accuracy studies in this meta-analysis, a SROC curve was used to summarize diagnostic performance. For this meta-analysis, the generated SROC curve was located slightly above the line of symmetry, which implies that MPV had a low discriminatory power in identifying the presence or absence of MI. This data coincided well with the pooled diagnostic odds ratio (DOR) of 2.92, which indicates that MPV indeed has discriminatory power, but at a low level.

The pooled sensitivity and specificity were at 0.66 and 0.60, respectively. This suggests that MPV demonstrates the ability to detect MI, as the ability to correctly identify those without the disease.

Clinically, likelihood ratios are much more useful than sensitivity and specificity because they provide a summary of how many times more (or less) likely patients with the disease are to have that particular result than patients without the disease.[27] In this meta-analysis, the calculated positive likelihood ratio was 1.65. Since LR+s greater than 1 mean that a positive test is more likely to occur in people with the disease than in people without the disease, this means that for a patient with elevated MPV values, he is 1.65 times more likely to have MI.[27] On the other hand, LR-s less than 1 mean that a negative test is less likely to occur in people with the disease compared to people without the disease.[27] In this meta-analysis, the negative likelihood ratio was 0.56. Thus, this means that a normal MPV value is 0.56 times less likely to occur in individuals with MI than in those without the disease. However, it must be emphasized that since the LR+ of 1.65 is quite low (below 10), an elevated MPV value cannot effectively "rule-in" MI. Moreover, since the LR- of 0.56 is not very low (below 0.1), a normal MPV value also cannot effectively "rule-out" MI.

In addition, the PPV of 0.3180 tells us that among those with an elevated MPV value, the proportion of patients that can correctly be identified as having MI was 31.80%. Moreover, the NPV of 0.8189 shows that among those with a normal MPV value, the proportion of patients that can correctly be identified without the disease was 81.89%. This data suggests that a normal MPV can correctly identify patients without MI 81.89% of the time.

In addition, identification of the appropriate threshold or cut-off of a test was not possible with the SROC curves.[13] For this study, the computed mean threshold value was 9.59 fl, which is below the usual upper limit of normal employed by many hospital laboratories. At this point, it should be noted that several studies have determined varying normal reference ranges for healthy individuals, with a minimum value of 7.0 fl to a maximum value of 11.7 fl.[5,6,28,29] The study by Korniluk et al., signified the need to establish individual reference values for MPV by laboratory, and in order to do so, each laboratory should enroll adequate number of individuals with respect to gender, age, and ethnicity.[29,30]

Quality of evidence

The assessment for quality of evidence is summarized in the table of Summary of Findings (Table 4). The outcome on the significant difference in mean MPV values was deemed of moderate quality. [31]

Strengths and weaknesses of included studies

This analysis contained several studies with similar results and conclusions: that MPV was significantly higher in those with MI; and that elevated MPV values are associated with MI. The studies also occurred in similar population groups. However, it is important to note that six out of fifteen articles were case-control studies. This may affect the estimation of accuracy since there was an inherent bias in first choosing cases of the disease before looking for a correlation with MPV. Upon quality assessment using the QUADAS2 tool, several factors contributed to weakness of the included studies. Six out of 15 articles did not employ exclusion criteria. Under the index test domain, six case-control studies already had knowledge of the results of the reference standard before interpreting the index test. Lastly, under concern for applicability, one article used a study population of patients who underwent angiography.

Strengths and weaknesses of the review process

Limitations of search strategy. There were no limitations imposed on the search strategy. No filters, as well as no language restrictions were employed.

Quality assessment and data extraction. There were a few studies that were identified with missing information. The authors were contacted by the reviewers. Most did not respond, and the few who did could not give the information that was inquired because either the data was already inaccessible, or that the data was not part of the planned dataset of the study.

Limitations in the review analyses. There were few systematic reviews, but this was the first meta-analysis that attempted to summarize diagnostic accuracy measures of MPV values in the diagnosis of myocardial infarction. This analysis was limited by substantial heterogeneity especially with the threshold value. The threshold effect could not be explored due to statistical limitations.

Table 4. Summary of Findings

Among adult patients presenting with angina and/or its equivalents, what is the diagnostic performance of an elevated mean platelet volume value in the diagnosis of acute myocardial infarction?				
Population	Adult patients with angina and/or its equivalents			
Setting	Tertiary hospitals (majority in Asia with some in Europe and North America)			
Index Test	Mean platelet volume measure by automated hematology analyzers			
Reference Standard	Diagnostic criteria and guidelines set by Cardiology societies			
Studies	Fifteen studies consisting of eight diagnostic accuracy articles and seven cross-sectional studies.			
Outcomes	**Summary**	**No. of participants (studies)**	**Implications**	**Quality of Evidence (Grade)**
Significant difference in mean MPV (in 15 studies)	Mean in those with MI 9.702 fl (95% CI; 9.07 – 10.33) Mean in non-MI control group 8.85 fl (95% CI 8.23 – 9.46) SMD 1.131 (95% CI; 0.81 – 1.45) t-value 2.0827 with degrees of freedom at 27.9	9748 (15)	MPV values are higher in those with MI than those without. A positive association is seen between increasing MPV values and those with MI. There is a significant difference in the mean MPV values of those with MI compared with those without.	+ + + - Moderate
Diagnostic performance of MPV (in 6 studies)	Sensitivity 0.66 (95% CI; 0.59 - 0.73) Specificity 0.60 (95% CI; 0.43 – 0.75) DOR 2.92 (95% CI; 1.90 – 4.50) LR+ 1.65 (95% CI; 1.20 – 22.27) LR- 0.56 (95% CI; 0.50 – 0.64) PPV 0.3180 NPV 0.8189	5017 (6)	In patients with MI, 66% of them can be identified with elevated MPV values. In those without MI, 60% can be correctly identified by normal MPV. MPV values can discriminate between those who have MI and those without, but at low levels. For a patient with elevated MPV values, he is 1.65 times more likely to have MI. On the other hand, a normal MPV value is 0.56 times less likely to occur in individuals with MI than in those without the disease. The PPV of 0.3180 tells us that among those with an elevated MPV value, the proportion of patients correctly diagnosed with MI was 31.80%. The NPV of 0.8189 shows that among those with a normal MPV value, the proportion of patients correctly identified without the disease was 81.89%.	+ + - - Low

Caution: The results on this table should not be interpreted in isolation from the results of the individual included studies contributing to each summary test accuracy measure. These are reported in the main body of the text of the review.

Overall completeness and applicability of evidence

As a diagnostic test for prediction of MI, perhaps the most appropriate function of mean platelet volume is for triaging adult patients with angina and/or its equivalents who are suspected of having myocardial infarction. This can be supported by the findings of this study, which has determined that a significant difference exists in the mean MPV values between those with MI and those without. Additionally, it has been established that those with MI have greater MPV values than those without.

The threshold at which we define an MPV value as "elevated" or "normal" ideally should have been identified with this study. However, a high degree of heterogeneity posed a challenge to this objective such that current statistical methods for obtaining diagnostic performance summaries do not allow for this identification. The reviewers thus opted to get the mean threshold value at which the articles defined an elevated value, which was 9.59 fl.

The main evidence obtained by this meta-analysis was a summary of diagnostic accuracy estimates across similar studies. Although the sensitivity and specificity were moderately high, what were perhaps more valuable for this study were the predictive values, likelihood ratios and diagnostic odds ratio, which tell us that MPV indeed has discriminatory value but at a low level.

Limitations of the Study

This study employed only articles that differentiated the subgroups of ACS. Those that did not identify subgroups were not included in this study. Further, investigation of heterogeneity ideally should have been done. A funnel plot could have been generated to examine for the presence of asymmetry, which would suggest possible publication bias which usually occurs where studies with negative results are less likely to be published than studies with positive results.[32] However, a funnel plot could not be generated with the program used for the meta-analysis. The statistical command package "metandi" in the program STATA which was used in this study currently has no capability to generate a funnel plot. Meta-regression analysis and a funnel plot analysis ideally should have been done to investigate the threshold effect and identify any outlier studies that may have affected the outcome of this study.

Potential biases in the review process

Few articles in this study had a case-control design and therefore induced bias in the domains of patient selection and interpretation of the index test. Additionally, articles that did not differentiate the ACS subgroups were not included in this study, and it is of interest to note the outcome if these studies provided the needed information.

CONCLUSION

Implications for practice

The intended role for MPV in the diagnostic pathway of myocardial infarction would perhaps be best as a triage tool. Since MPV results are more readily available, it has the potential to guide early diagnostic decisions especially in patients who are suspected of having MI. With a DOR of 2.92, MPV values can discriminate between those who have MI and those without. To support this, it was also concluded that there is a significant difference in the mean MPV values of those with MI compared with those without; and that MPV values are higher in those with MI. Therefore, it can be implied that in a patient with a probable diagnosis of MI, a high MPV value (greater than 9.59 fl) can discriminate cases of actual disease and warrant further testing according to the reference standard. Additionally, it can also be implied that a diagnosis of MI is less likely in a patient with normal MPV values.

For a patient with angina presenting with elevated MPV values, it is 1.65 times more likely that he has MI. For patients with the disease, a substantial 66% of these cases can be correctly flagged by an elevated MPV value. Additionally, the NPV of 0.8189 shows that among those with a normal MPV value, the proportion of patients correctly identified without MI was 81.89%. Thus, it is implied that the decision to treat a patient with angina or its equivalents as a case of MI could be supported by an elevated MPV value. Moreover, a normal MPV value can correctly identify patients without MI 81.89% of the time. With that said, it should be taken into consideration that MPV's low discriminatory power and its levels of sensitivity and specificity cannot warrant its use as a screening tool to decide who gets treated for the disease.

Implications for research

The main drawback of meta-analyses of diagnostic test accuracy studies is that summarized data obtained from analysis are indirect forms of evidence and cannot be used to make generalized conclusions. Being the first meta-analysis of its kind, this study has shown that marked heterogeneity can be expected especially with studies that have varied thresholds. The reviewers therefore recommended that for further studies, the following measures may be implemented. A subgroup analysis of studies comparing those with ACS and those without may be done. Meta-regression analysis and funnel plot graphs may be done to identify outlier studies. Investigation into the threshold effect would be most beneficial. Perhaps in future studies, more advanced statistical methods may already be available which can correctly identify the threshold cut-off for positivity. In addition, further efforts to identify unpublished studies may be done in order to ensure a greater range of included studies. Broad guidelines in the implementation of this study are tabulated in Appendix 5.

Abbreviations used in this protocol study. *MPV* (mean platelet volume); *MI* (myocardial infarction); *ACS* (acute coronary syndrome); *AUC* (area under the curve); *ROC* (receiver operating characteristics); *SROC* (summary of ROC); *HSROC* (hierarchical SROC); *CBC* (complete blood count); *NSTEMI* (non-ST segment elevation MI); *STEMI* (ST segment elevation MI); *UA* (unstable angina); *ECG* (electrocardiogram); *DTA* (diagnostic test accuracy)

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CONFLICT OF INTEREST

No conflict of interest was declared.

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STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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APPENDICES

Appendix 1. PRISMA Checklist

Section/Topic	#	Checklist Item	Reported on Page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome-level assessment (see Item 12).	
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group and (b) effect estimates and confidence intervals, ideally with a forest plot.	
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., health care providers, users, and policy makers).	
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review level (e.g., incomplete retrieval of identified research, reporting bias).	
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	

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Appendix 2. Table for full-text review

Specification	Details
Authors	
Year	
Title	
Journal	
Volume	
Issue / Number	
Pages	
Population	
Inclusion criteria	
Exclusion criteria	
Index test	
Comparator	
Outcomes	
Methodology	
Study design	
Time frame	
Decision (Include/Exclude)	
Basis for decision	

Appendix 3. Data Extraction Table

Study No.	Study 1	Study 2	Study 3	Study 4
Author, Year				
Study Design				
Country				
Study Population				
w/ Exclusion Criteria				
Control Group				
Analyzer used				
Timing of Test				
Outcomes				
Mean MPV in MI				
Mean MPV in Control				
Sensitivity				
Specificity				
Cut-off Threshold				

Appendix 4. QUADAS-2 Tool

Domain	Patient Selection	Index Test	Reference Standard	Flow and Timing
Description	Described the method of patient selection Describe included patients	Describe the index test and how it was conducted and interpreted	Describe the reference standard and how it was conducted and interpreted	Describe any patients who did not receive the index tests or reference standard or who were excluded from the 2 x 2 table. Describe the interval and any interventions between index tests and the reference standard
Signalling questions (Yes, No, Unclear)	Was a consecutive or random sample of patients enrolled? Was a case-control design avoided? Did the study avoid inappropriate exclusions?	Were the index test results interpreted without knowledge of the results of the reference standard? If a threshold was used, was it prespecified?	Is the reference standard likely to correctly classify the target condition? Were the reference standard results interpreted without knowledge of the results of the index test?	Was there an appropriate interval between index tests and reference standard? Did all patients receive a reference standard? Did all patients receive the same reference standard? Were all patients included in the analysis?
Risk of bias (Yes, No, Unclear)	Could the selection of patients have introduced bias?	Could the conduct or interpretation of the index test have introduced bias?	Could the reference standard, its conduct, or its interpretation have introduced bias?	Could the patient flow have introduced bias?
Concerns about applicability	Are there concerns that the included patients and setting do not match the review question?	Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Are there concerns that the target condition as defined by the reference standard does not match the review question?	

OPEN ACCESS – CASE REPORT

Metaplastic Carcinoma with Mesenchymal Differentiation in Augmented Breast using Liquid Silicone Injection: A Case Report

Lara Mae Lorenzo, Sarah Jane Datay-Lim, Jose Carnate Jr.

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ABSTRACT

The relationship between the use of liquid silicone for breast augmentation and carcinogenesis remains undetermined due to limited data reported, especially regarding its risks for acquiring cancer. We documented a case of an 81-year-old woman who presented with bilateral enlarging breast masses with a known history of breast augmentation using liquid silicone. On microscopic examination, the malignancy showed both mesenchymal and epithelial components in a background of stromal changes related to liquid silicone. Based from morphology and immunohistochemistry studies (p63, CK, HMW-CK, and CK5/6, CD34, and BcL-2), this case was signed out as metaplastic carcinoma with mesenchymal differentiation. This rare case of metaplastic carcinoma with mesenchymal differentiation coexisting with liquid silicone, provides evidence supporting the link between cancer development and siliconomas.

Key words: metaplastic breast carcinoma, liquid silicone, breast augmentation, invasive breast carcinoma

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INTRODUCTION

The practice of using liquid silicone for breast augmentation was popular between 1944 to the early 1990s[1,2] but was eventually banned by the USA Food and Drug Administration. This was due to findings of breast complications such as inflammatory changes and fibrosis in patients who had liquid silicone breast augmentation.[1]

To this date, liquid silicone injection for breast augmentation is still performed illegally in the Philippines by unlicensed and unskilled practitioners. A five-year retrospective study was able to report the benign complications of foreign body injection to the breast.[3] Yet, no literature was published and reported locally (HERDIN Plus and Philippine E-Journals) regarding the coexistence of silicone mastopathy and a malignant breast neoplasm, specifically with a metaplastic carcinoma.

This paper reports the finding of breast malignancy in a patient with breast augmentation. Microscopically, the tumor was adjacent to stromal changes associated with liquid silicone.

CASE

This is a case of an 81-year old woman who presented with bilateral enlarging breast masses, 5 years prior to consultation. Patient had a 30-year history of using liquid silicone injection as augmentation. On physical examination, the palpable masses were both firm, movable and irregular. The left breast mass was noted to be more tender, larger and heavier in size. Patient underwent excision of the masses and the specimens were sent for histopathologic evaluation. No radiological examination was done prior to excision biopsy.

On gross examination, two pink-tan ovoid doughy tissues were received and with the following measurements: "Right breast mass" – 240 grams and 11.2 x 8.5 x 3.2 cm;

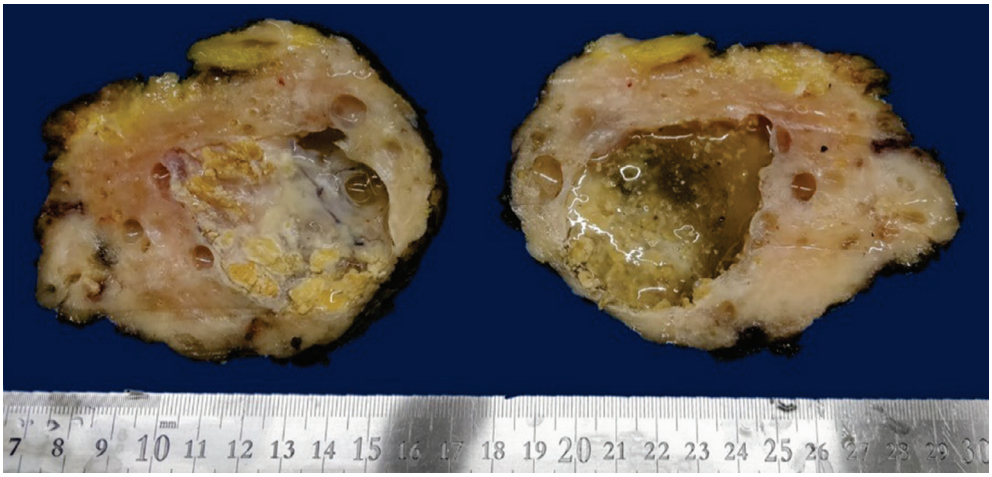


Figure 1. Gross appearance of the excision biopsy specimen of the right breast mass. Cut section of the mass show multiple cystic spaces filled with clear viscous material, largest cyst having multiple yellow-tan gritty tissue fragments.

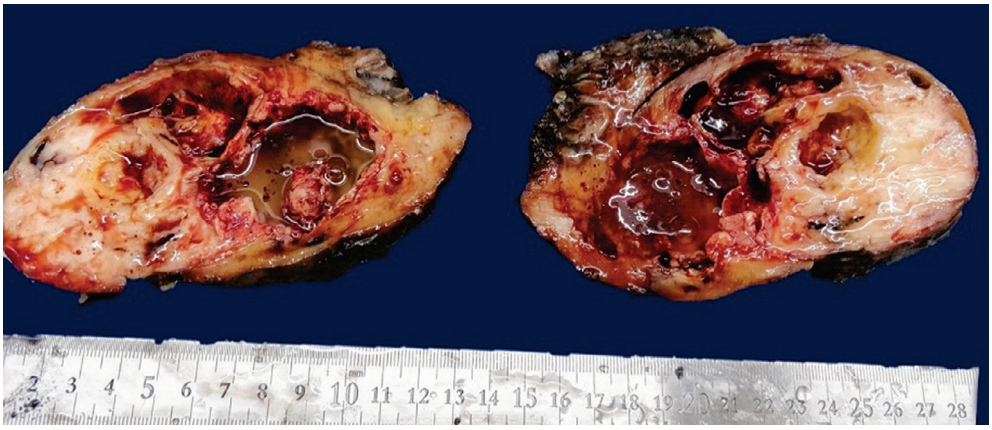


Figure 2. Gross appearance of the excision biopsy specimen of the left breast mass. Cut sections show a cream to red-tan variegated and gritty cut surface, with multiple cystic cavities filled with clear viscous material and red-brown clot-like material. The inner lining of the largest cavity was nodular.

"Left breast mass" –580 grams and 13.4 x 9.5 x 7.1 cm. Cut sections of the right breast mass showed multiple cystic cavities, measuring up to 6.0 cm in widest dimensions, filled with clear viscous material (Figure 1). Cut sections of the left breast mass showed multiple cystic cavities which measure up to 4.5 cm in widest dimensions, filled with clear viscous material and red-brown clot-like material (Figure 2). The inner lining of the largest cavity is nodular with a tan-white gritty cut surface. The rest of the left breast mass had a cream to red-tan variegated and gritty cut surface.

Histopathologic examination of the right breast showed foreign-body changes with numerous cystic spaces and vacuoles, consistent with silicone mastopathy (Figure 3).

However, microscopic examination of the left breast mass showed a malignancy composed of tumor cells surrounded by stroma with varying degrees of ossification and chondromyxoid changes (Figures 4 to 5). Tumor cells were noted to have enlarged, markedly pleomorphic and hyperchromatic nuclei, prominent nucleoli, coarse

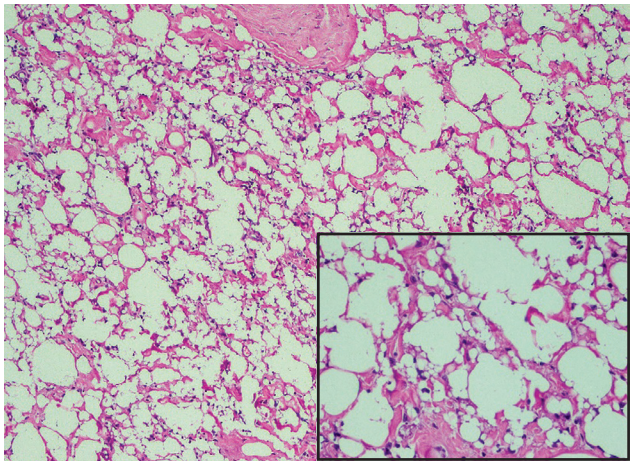


Figure 3. Extensive involvement of silicone to the breast tissue (right). The background is composed of chronic inflammation and reactive fibrosis. Silicone has been leached from the fixation processed and is visualized as empty vacuoles or refractile colorless material on closer magnification (Hematoxylin-Eosin, 100x, Inset 400x).

chromatin pattern and ample eosinophilic cytoplasm (Figure 6). Adjacent to the carcinoma are findings of foreign-body changes with numerous cystic spaces and vacuoles, consistent also with silicone mastopathy (Figure 4).

Immunohistochemistry studies were done and showed that the tumor cells stained weakly and focally positive for p63, CK, HMW-CK, and CK5/6, and stained negative for CD34 and BcL-2 (Figure 7). For the hormone receptor assay, ER, PR and HER-2/neu were negative (Figure 7). Given the morphologic and immunohistochemical profile of the mass, this case was signed out as metaplastic carcinoma with mesenchymal differentiation (syn. Matrix-producing metaplastic carcinoma).

DISCUSSION

Metaplastic breast carcinomas with mesenchymal elements subtype, formerly called as matrix-producing metaplastic carcinoma, are invasive carcinoma with direct transition to osseous and/or cartilaginous matrix, with or without an intervening spindle cell component.[4,5,6] Considered as extremely rare and aggressive[4,7,8], the mean age of patients with this malignancy is 56 years.[9] Clinically and mammographically, presentation is similar to invasive ductal carcinoma.[8] Microscopically, they are mainly composed of two kinds of populations: the mesenchymal and epithelial. The mesenchymal elements would often include chondroid, osseous, rhabdomyoid and even neuroglial differentiations. The epithelial areas would be present in the forms of glandular differentiation, in tubules or in solid clusters, and/or foci of squamous differentiation.[6]

Differential diagnoses for a metaplastic carcinoma with a mesenchymal differentiation would include the following: high-grade phyllodes tumor, primary breast sarcoma, extraskeletal osteosarcoma, and myoepithelial carcinoma. Just like in our case, identification of overt epithelial component would be difficult especially if most tumor cells have already undergone metaplastic changes. Hence, immunohistochemical studies are helpful, wherein the epithelial component would stain positive for cytokeratin (CK), while the mesenchymal / matrix-producing component will stain negative for CK but positive for S100.[8]

For this carcinoma (or even any malignancy), diagnostic dilemmas arise with a concomitant foreign body injectable material or even the use of implants. On mammography, prior use of liquid silicone, silicone gel-filled and saline-filled implants, can hinder the accurate delineation between a silicone granuloma and breast carcinoma. The opaque densities (similar to fat) would obscure the malignancy, ultimately hindering the early diagnosis of a coexisting neoplasm.[10,11] Augmented patients would have a higher false-negative mammography rate compared to nonaugmented patients. Despite the augmented group of patients showing a slightly greater risk of invasive tumors, higher frequency of palpable mass and a higher incidence rate of metastasis to the axillary lymph nodes, both groups showed no statistically difference in terms of disease stage, tumor size, recurrence rates, and survival

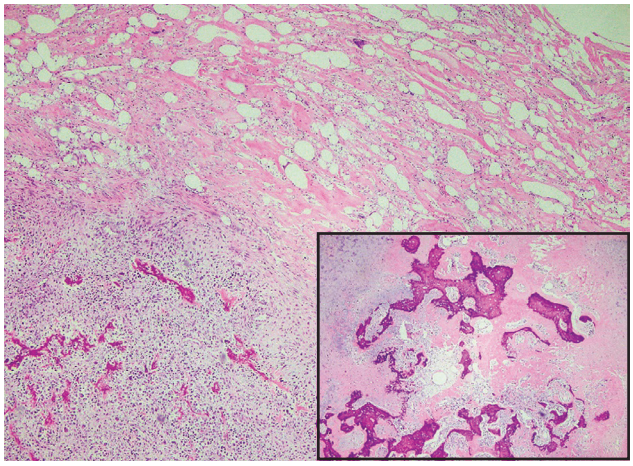


Figure 4. Metaplastic carcinoma with mesenchymal differentiation with adjacent silicone mastopathy of the left breast. On closer magnification, it is composed of an overt carcinoma with direct transition to an osseous stromal matrix (Hematoxylin-eosin, 40x, Inset 100x).

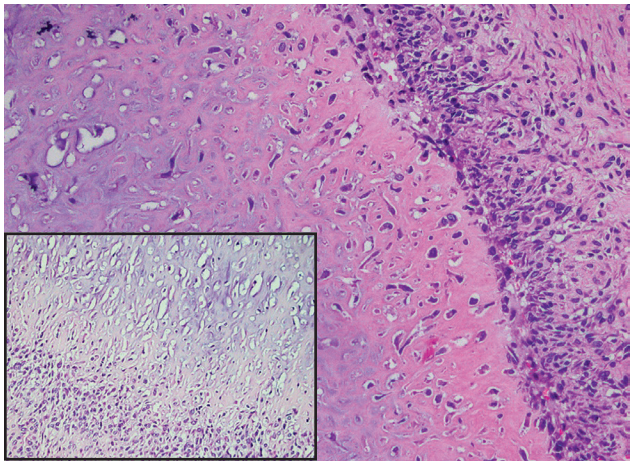


Figure 5. Matrix-producing metaplastic carcinoma with poorly differentiated carcinomatous cells at the periphery of a chondromyxoid stroma, left breast (Hematoxylin-eosin, 200x, Inset 200x).

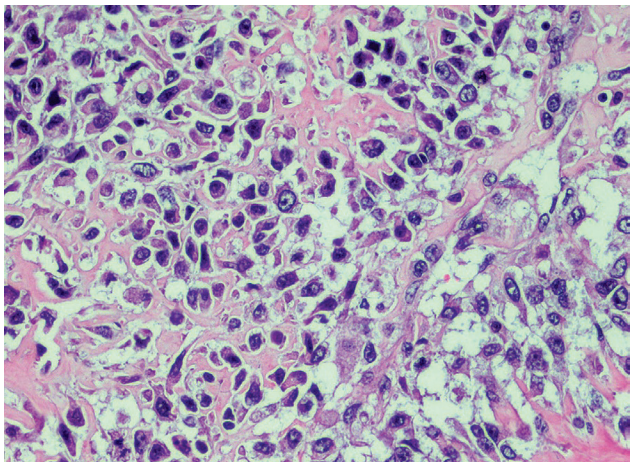


Figure 6. Tumor cells exhibiting enlarged, markedly pleomorphic and hyperchromatic nuclei, prominent nucleoli, coarse and vesicular chromatin patterns and ample eosinophilic cytoplasm (Hematoxylin-eosin, 100x).

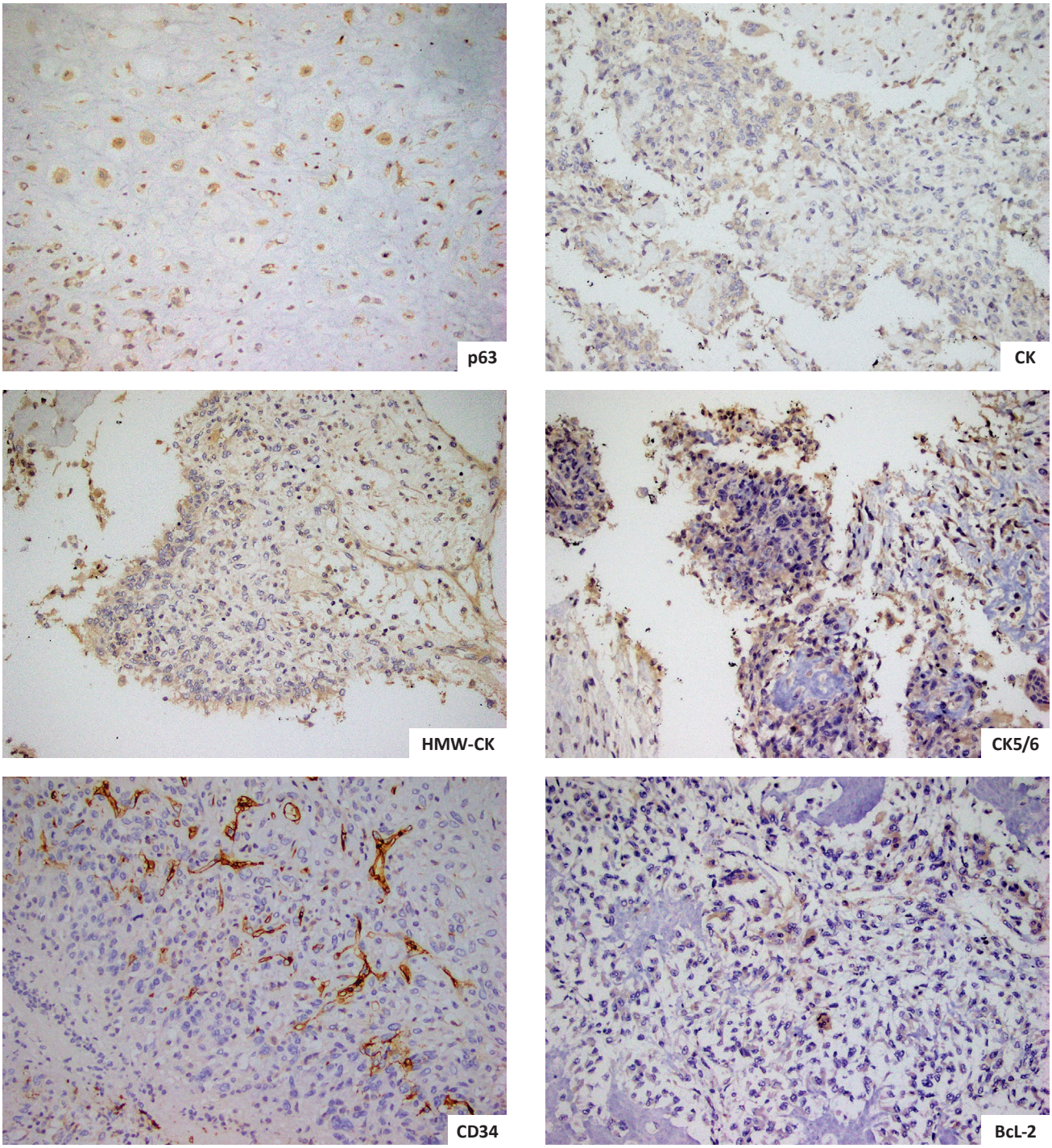
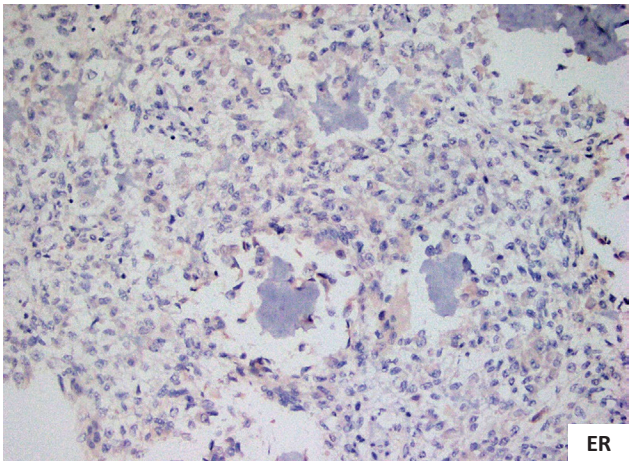
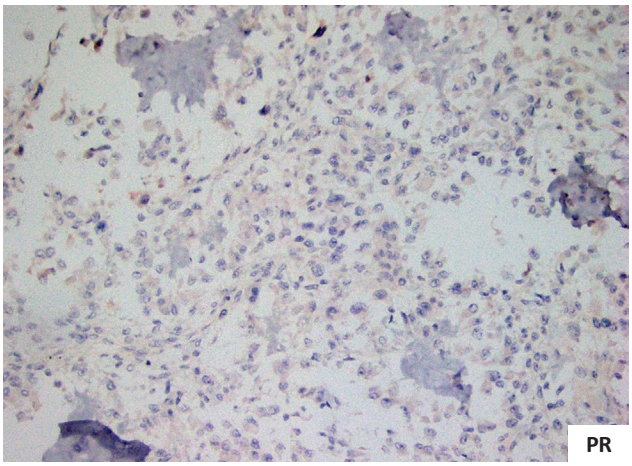


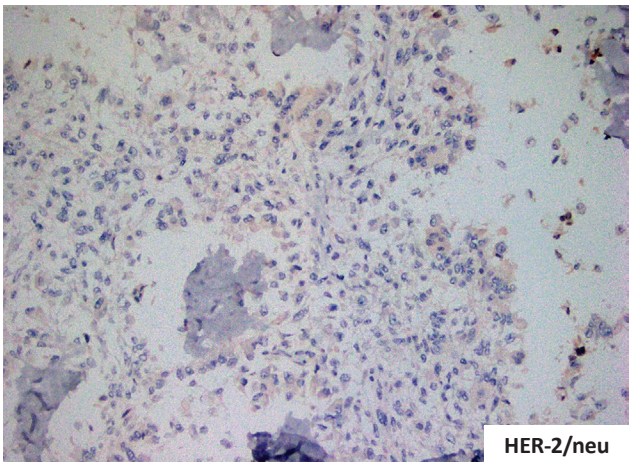
Figure 7. Immunohistochemistry showing: focal and weak nuclear positivity staining for p63; focal and weak cytoplasmic positivity staining for CK, HMW-CK and CK5/6; negative staining for CD34 and BcL-2, and a triple negative receptor (ER, PR, HER-2/neu) assay *(see next page)* (Horse radish peroxidase method, 100x).

rate. One explanation would be that the presence of implants would help facilitate the palpation of the tumor, thus are easier to clinically examine compared to the nonaugmented.[10]

On the histopathologic context, liquid silicone appears as empty vacuoles or spaces, which has been washed out during tissue preparation. Foreign body giant cells, vascular olibiterans, stromal fibrosis, and chronic inflammation would also be appreciated.[1] For its causal relationship with carcinogenesis, there is still no definite consensus regarding it.[2,12] According to a study done by Stivala et al., major cohort studies were done prior which demonstrated a 0.2% to 2.7% breast cancer frequency rate following augmentation.[13] Although many cases of breast cancer with prior augmentation have been reported,[3,10,13] data specific to liquid silicone is still limited.[2,11,14] Most studies were attributed from using silicone gel implants.[10] However, Morgenstern et al were able to discuss their findings of 12 patients with free silicone and breast cancer.


ER


PR


HER-2/neu

Their study postulated that free silicone may provide a more attractive medium for tumor cells to proliferate. Other pathologic findings were the lack of desmoplastic reaction of the tumor when admixed with silicone and tumors showing usual desmoplastic reaction showed little fibrosis in areas away from silicone. A study done by Tanaka et al reported that the malignant cells of the tumors diagnosed as invasive micropapillary carcinoma had close contact with the liquid silicone, which they considered as an evidence as a link between carcinogenesis and use of silicone.[12] And to support the higher incidence of metastasis to the axillary lymph nodes, the pathologic findings of the same study suggested that free silicone causes the following: an abnormal opening of the lymphatic channels, facilitating the spread of tumor cells; the inhibition of tumor-induced desmoplasia; or from an altered immune mechanism associated with silicone granuloma.[2] Despite these observations and evidences reported, data was still considered limited by the authors due to lack of thorough investigation between breast cancer and liquid silicone.[3,12,14]

Metaplastic breast carcinomas with mesenchymal elements subtype are hormone receptor negative (ER, PR, and HER-2), and radiotherapy and chemotherapy are of limited effectiveness. No standard treatment has been established yet. Recent clinical trials have shown targeted gene therapy plays a role following genetic profiling.[15] But for silicone mastopathy with coexistence of this carcinoma (and even this carcinoma alone), surgery is still considered the treatment of choice.[2,14]

CONCLUSION

Metaplastic carcinoma with mesenchymal elements is considered by most publications as rare. To the authors present knowledge, there have been no local reported cases of having this malignancy after breast augmentation with liquid silicone. Although there is no established relationship between liquid silicone and carcinoma, our findings suggest a link between the two.

ETHICAL CONSIDERATION

All attempts were done to acquire a consent from the patient, however, was lost to follow-up. This case report was made in accordance to the principles based on the Declaration of Helsinki.

STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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None.

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OPEN ACCESS – CASE REPORT

Metachronous Primary Cerebral Rhabdomyosarcoma After Treatment of Oral Primitive Neuroectodermal Tumor/ Ewing's Sarcoma in a School-Aged Girl: A Case Report

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ABSTRACT

Multiple primary malignancies are defined as two or three malignant neoplasms arising in different organ systems. Several cases of multiple primary malignancies are emerging in recent years due to the advancement in medical therapy and diagnostics. Multiple primary malignancies are not uncommon occurring at 0.7-16% of cancer patients, however reported cases of multiple primary sarcomas are sparse. Presented in this report is a pediatric patient diagnosed with primary metachronous cerebral rhabdomyosarcoma after being treated for primitive neuroectodermal tumor/Ewing's sarcoma of the oral cavity. Despite limited cases addressing multiple primary sarcomas, this entity must not be overlooked as it is associated with a meager outcome compared to an index case of sarcoma alone.

Key words: multiple primary malignancies, multiple primary sarcomas, metachronous malignancies, PNET/ Ewing's sarcoma, rhabdomyosarcoma, Immunohistochemistry, fluorescence in situ hybridization, reverse transcription-polymerase chain reaction

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INTRODUCTION

Multiple primary malignancies (MPM) are an emerging clinical entity.[1] These are further divided into synchronous and metachronous malignancies, temporally separated by six months.[2-5] Presently, there are adequate number of scholarly articles on MPM, however reported cases of multiple sarcomas are rare. This is especially uncommon in the pediatric population, and based on the authors literature search, there are no other reported case of multiple primary sarcomas in the head and neck region and central nervous system occurring in the pediatric population.

CASE REPORT

This is a case of an 11-year-old girl diagnosed with primitive neuroectodermal tumor (PNET)/Ewing's sarcoma stage IV of the left oral cavity mass on September 2017. She has no family history of malignancy nor known environmental exposure to carcinogens. On December 2018, the patient was in remission after undergoing 7 cycles of PNET/ Ewing's sarcoma chemotherapy protocol and 30 cycles of radiation therapy (RT). Monitoring cranial and chest computerized tomography (CT) scan done on January 25, 2019 showed, normal study of the brain, polysinusitis, soft tissue fullness on the left pterygoid muscles and left para-pharyngeal space. The chest CT scan shows unremarkable findings. After these radiologic studies, she was advised close follow-up and repeat scan after 3 months.

On September 24, 2019, the patient had 4 to 5 episodes of tonic-clonic seizures, several bouts of projectile vomiting, weakness of all extremities, decreased verbal output and involuntary movement of both upper extremities. Pertinent physical examination showed a Glasgow Coma Scale (GCS) of 11 (E4M6V1), upper extremity myoclonus,

and the manual muscle test (MMT) of all extremities are 4/5.

Cranial CT-scan with contrast was done which shows a rimed, heterogeneously enhancing mass measuring 6.6 x 6.7 x 8.0 cm in the region of the left fronto-temporo-parietal lobe extending inferiorly to the left cranial fossa and is contiguous with the soft tissue lesion in the left pterygoid muscle. There are hyperdensities seen within the mass which may represent tumor bleed (Figure 1).

The patient underwent left temporal craniotomy with excision of the tumor. Histopathologic and cytogenetic studies of the cranial mass and slide review of the mandibular tumor were done which revealed rhabdomyosarcoma with spindle cell features of the brain occurring two years after the diagnosis of PNET/Ewing's sarcoma.

Three months after the diagnosis and initiation of treatment for rhabdomyosarcoma, the patient was admitted due to loss of consciousness. Plain and contrast cranial CT scan done showed an ill-defined, non-enhancing mass in the left tempo-parietal lobes measuring 7.0 x 6.5 x 8.0 cm. There is noted soft tissue mass with calcifications and lytic destruction of the adjacent bone structures. Also noted are hydrocephalus, polysinusitis, right subfalcine herniation and brain edema of the left hemisphere (Figure 2). The consideration is tumor recurrence. The patient eventually succumbs to her illness and died.

PATHOLOGIC EXAMINATION AND CYTOGENETIC STUDIES

Oral cavity mass

Slide review of hematoxylin and eosin (H&E) stained smears show fibroconnective tissues containing salivary glands interspersed by malignant round-cell neoplasm with marked crushing artifact. The preserved, small round cells contain finely granular nuclei, prominent nucleoli and indistinct to clear cytoplasm. Immunohistochemical stains done showed strong, diffuse, membranous staining for cluster of differentiation (CD) 99 and negative for desmin and myogenin, supporting the diagnosis of a PNET/Ewing family of tumors (Figure 3). Cytogenetic studies for EWSR1 gene alteration to support PNET/Ewing and FLI-1 to rule out rhabdomyosarcoma were further recommended.

The case was subsequently referred to a pediatric tertiary hospital. Immunohistochemistry performed at the said institution showed that the neoplastic cells are positive for CD99 and has diffuse nuclear positivity for myoblast determination protein 1 (MyoD1). Immunohistochemical staining for desmin, myogenin, CD34, S100 and SRY-Box transcription factor (SOX) 10 are all negative. Fluorescent in situ hybridization (FISH) targeting FOXO1 gene is negative. Reverse transcription polymerase chain reaction (RT-PCR) is positive for fusion transcript consistent

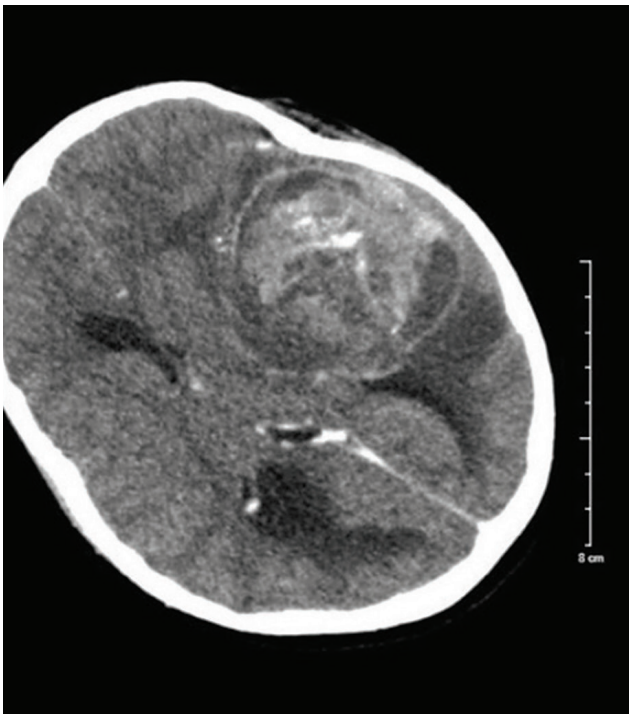

Figure 1. Cranial CT scan with contrast showed a 6.6 x 6.7 x 8.0 cm (AP T CC) mass, exhibiting rim and heterogeneous internal enhancement, in the region of the left fronto-temporo-parietal lobe. Hyperdensities are seen within the mass.

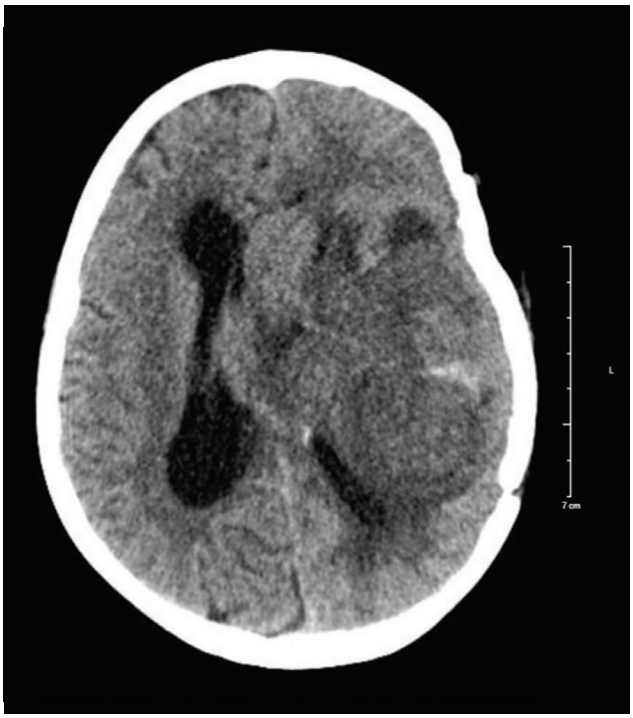

Figure 2. Plain and contrast CT scan showed a post craniotomy defect in the left fronto-parietal bones where in excision of the previous mass was noted. There is shift in the midline structures to the right with a finding of ill-defined non enhancing mass in the left fronto-parietal lobes measuring 7.0 x 6.5 x 8.0 cm replacing the malacic focus in the left parietal lobe. This lesion compresses the left lateral and third ventricles. There is effacement of the brain parenchyma in the left cerebral hemisphere and minimal perilesional edema above the lesion.

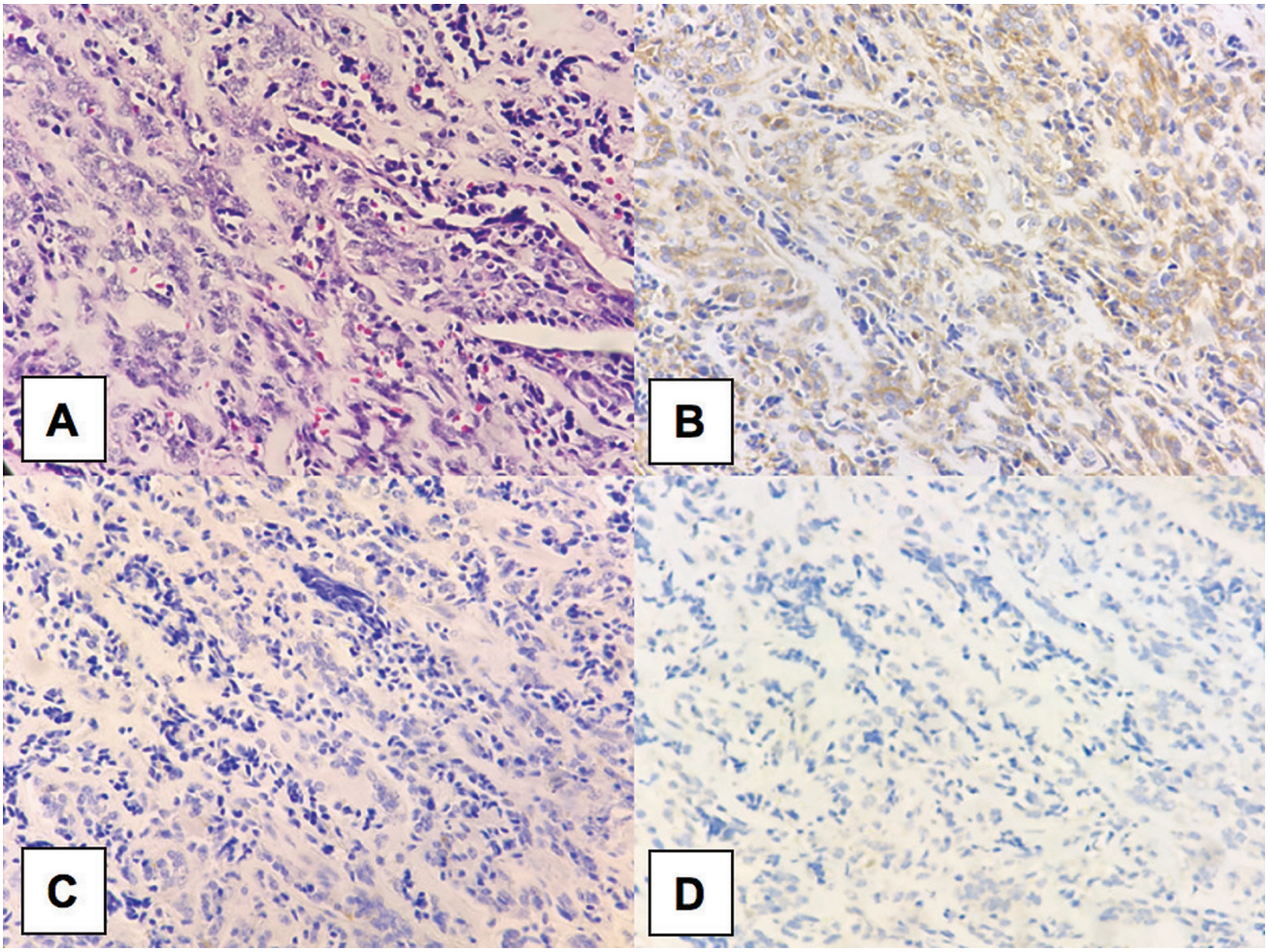

Figure 3. **(A)** H&E shows small round blue neoplasm with marked crushing artifacts. The well preserved small, round cells have fine granular nuclei, some prominent nucleoli and indistinct to clear cytoplasm. **(B)** CD99 shows strong, diffuse, membranous immunohistochemical staining. **(C and D)** Immunohistochemical stains for desmin and myogenin are negative.

with Ewing's sarcoma/PNET family of tumors. These immunohistologic and cytogenetic findings are diagnosed as malignant round cell neoplasm with evidence of fusion transcript consistent with Ewing's sarcoma/PNET family of tumors by RT-PCR.

Cerebral mass

Two specimens are submitted for pathologic evaluation. One specimen is labeled as "Brain tumor 1" consisting of several cream, irregularly shaped, soft tissue fragments measuring 6.5 x 6.5 x 2.0 cm from which twenty-two representative sections are taken. The other specimen is labeled as "Brain tumor 2", consists of three cream, irregularly shaped, soft tissue fragments measuring 7.5 x 6.7 x 4.0 cm, 2.3 x 2.0 x 0.5 cm, and 1.5 x 1.3 x 1.0 cm from which 24 representative sections are taken.

Microscopic examination shows sheets of medium to large spindle cells interspersed with patchy areas of necrosis. The cells contain increased nuclear to cytoplasmic ratio. The spindle cells have round to oval, vesicular nuclei with clumped chromatin patterns and slightly eosinophilic, scant cytoplasm. Admixed within the malignant cells are few round, spindle, and stellate cells containing ample eosinophilic cytoplasm with peripherally located nucleus and prominent nucleoli. There are 8-10 mitosis seen per high power field. Immunohistologic staining of the malignant cells show strong, nuclear reactivity for myogenin, strong, diffuse cytoplasmic reactivity for vimentin and rare cytoplasmic positivity for CD99 and BCL2 (Figure 4). The immunohistochemical stains are negative for CD34, CK and EMA. These findings are suggestive of rhabdomyosarcoma with spindle cell features.

The case was also referred to the previous referral institution, immunohistochemical stains done showed heterogeneous pattern of expression with desmin, myogenin and MyoD1. The tumor cells are negative for S100 and SOX10 with rare p53 expression. In line with the clinical history of PNET/Ewing sarcoma, reverse transcription polymerase chain reaction (RT-PCR) and fluorescence in situ hybridization (FISH) assay were performed in the brain lesion. These molecular tests turned out negative for EWSR1 gene alteration, suggesting that the two lesions are likely unrelated. This case was signed as morphologic and immunohistochemical findings consistent with rhabdomyosarcoma.

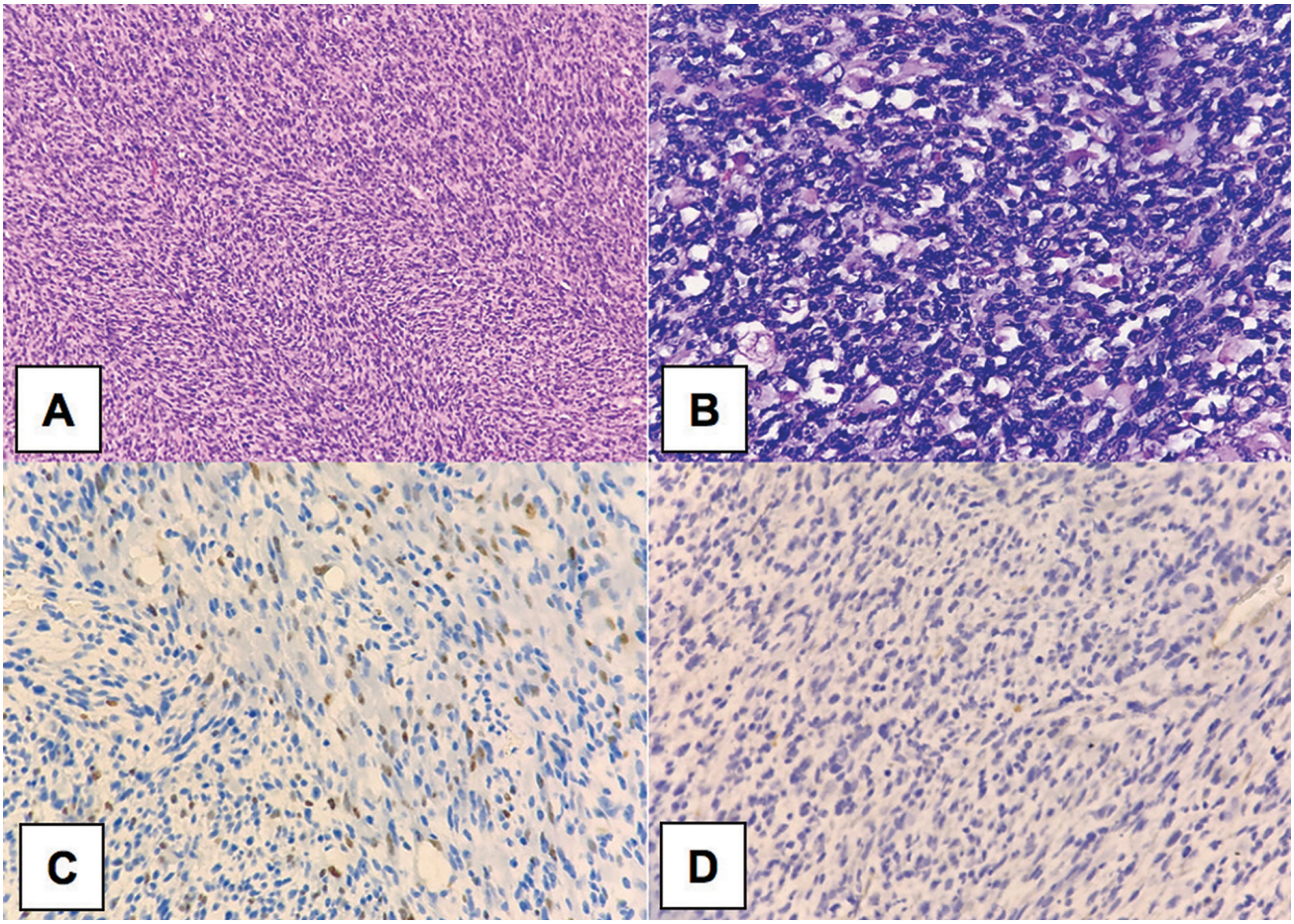

Figure 4. **(A and B)** Scanner and High Power Objective show sheets of medium to large spindle cells. These spindle cells have increased nuclear to cytoplasmic ration, slightly eosinophilic, scant cytoplasm, vesicular nuclei and clumped chromatin patterns. **(C)** Immunohistochemical staining for myogenin shows strong, and patchy nuclear reactivity. **(D)** Immunohistochemical staining for CD99 shows rare, cytoplasmic staining.

DISCUSSION

Multiple primary malignancies (MPM) are defined as two or more tumors arising from different organ systems.[1] The entity was first published by Billroth in 1889, who noticed gastric carcinoma in a patient previously diagnosed with spinoepithelioma of the ear.[2,6] Presently, the diagnosis of MPM follow the criteria established by Warren and Gates in 1932; 1. Both the index and succeeding tumors are histologically different and confirmed as malignant, 2. The tumors occur in different organ system and separated by a normal mucosa, 3. The probability of metastasis from the index tumor is excluded.[1,3] Furthermore, these malignancies are divided into two: synchronous – malignancies that are separated by less than six months and metachronous – if the succeeding malignancies occur more than six months from the diagnosis of the index tumor.[1,2,4]

The incidence of MPM ranges from 0.7% to 16%. Moreover, synchronous and metachronous primary malignancy account for 25-36% and 67-75% respectively.[1,2,5] Most of the index tumor are located in the head and neck, breast, gastrointestinal tract, gynecologic organs and genito-urinary tract. On the other hand, secondary primary malignancies are more common in the gastrointestinal tract, lungs, gynecologic organs, breast and genito-urinary tract.[1,4]

In spite of the abundant literatures addressing MPM, only few tackled multiple primary sarcomas. In a multicenter review involving 7351 patients previously diagnosed with soft tissue sarcomas in three tertiary orthopedic hospitals in Canada and United Kingdom, only six (0.08%) had second primary sarcoma.[7] The following factors are associated with new primary soft tissue sarcomas following previous resection of primary soft tissue sarcomas: 1. Genetic syndromes such as Li-Fraumeni syndrome, Hereditary Retinoblastoma, and Familial Adenomatous Polyposis, 2. History of radiation and chemotherapy, 3. Chronic inflammation, 4. Lymphedema, and 5. Exposure to chemical carcinogens.[8] The 5-year overall survival rate of multiple primary soft tissue sarcomas is 50%, a steep decline from the 83.3% 5-year survival rate of the index cases of soft tissue sarcomas.[7]

There are few reported sarcomas involving both PNET/Ewing's sarcoma and rhabdomyosarcoma. Among these are five cases occurring simultaneously in the female genital tract. All of these cases are in the uterus with an average age of 39 years old and one reported pediatric case. Three of the cases are Fédération Internationale de

Gynécologie et d'Obstétrique (FIGO) stage III with lymph node involvement, and two cases are localized in the uterus. It was further stated that the rhabdomyosarcoma component of the two localized neoplasms are of embryonal subtype, however the three other neoplasms were not histologically subclassified. Despite these neoplasms being immunohistomorphologically classified as PNET of the uterus, gene rearrangement for EWSR1 gene are not detected in all of the cases.[9-13] De Alva et al., reported a case of a 13-year-old girl with a left arm mass who underwent fine needle aspiration biopsy. Cytologic evaluation showed strands of small cells with scant cytoplasm and hyperchromatic nuclei. Molecular study of this neoplasm showed simultaneous expression of the EWSR-FLI1 gene and PAX2-FKR gene, both are specific for Ewing's family of tumors (EFT) and alveolar rhabdomyosarcoma.[14] Another series done in Canada, reported five cases of round cell neoplasms with immunohistomorphologic features compatible with rhabdomyosarcoma and simultaneous fusion of the EWSR1/FLI1 gene.[15]

In contrast to the above mentioned cases, our case of an 11-year-old girl is unique since the diagnosis of rhabdomyosarcoma in the brain occurred two years after the diagnosis of PNET/Ewing's sarcoma in the oral cavity. Additionally, cytogenetic evaluation of both masses showed that EWSR1/FLI1 gene fusion seen in the oral cavity mass is not present in the brain mass, hence the conclusion that these tumors are likely unrelated. To the authors knowledge, there are no other case report on metachronous PNET/Ewing's sarcoma and rhabdomyosarcoma in the head and neck region and in the pediatric population.

The PNET/Ewing's sarcoma are grouped under EFT. These small, round cell neoplasms are neuroectodermal in origin.[16] Microscopically, the cells are composed of small, round or oval cells with cytoplasm containing glycogen.[17] Immunohistochemistry shows strong, and diffuse immunoreactivity for CD99 and Vimentin.[16-17] An estimate of 85% of Ewing sarcomas has a somatic translocation involving t(11;22)(q24;q12) that fuses EWSR1 to FLI1.[18] This translocation generates the EWSR1-FLI1 oncoprotein.[18] Molecular diagnostic techniques such as RT-PCR and FISH allow an accurate detection of the EWSR1-FLI1 oncoprotein. The detection of this oncoprotein facilitates the differentiation from rhabdomyosarcoma, malignant lymphoma, desmoplastic small round cell tumor and other round cell neoplasms.[16,17]

A newly recognized group of malignancies known as "Ewing-like" neoplasms are emerging as diagnostic pitfalls.[16-18] These group of neoplasm are morphologically similar to PNET/Ewing's sarcoma however lacks the molecular evidence supporting their inclusion in EFT.[16-18] Majority of the "Ewing-like" neoplasms have nuclear immunoreactivity for WT1 and 84% of these are positive for CD99 in a patchy staining pattern.[17] The most common reported translocation in this group of tumors are the CIC-DUX4 fusion with few reported cases of BCOR-CCNB3 and CIC-FOXO4 fusion.[17,18] It is important to recognize these tumors since the 5-year survival rate of "Ewing-like" neoplasms are 43%, as compared to the 77% 5-year survival of Ewing's sarcoma.[17]

Rhabdomyosarcoma is a soft tissue neoplasm in which cells have propensity for myogenic differentiation.[19] The World Health Organization (WHO) recognized four histologic variants of rhabdomyosarcoma. These variants are embryonal rhabdomyosarcoma, alveolar rhabdomyosarcoma, pleomorphic rhabdomyosarcoma and spindle cell/sclerosing rhabdomyosarcoma.[18] The histologic examination of rhabdomyosarcoma varies per subtypes, but mostly these cells are heterogeneously shaped.[19] Some of these tumors contain undifferentiated cells, round to ovoid cells, "tadpole-like" cells, and spindle cells with eosinophilic cytoplasm and peripherally located nuclei known as rhabdomyoblasts.[17-19] The rhabdomyoblasts may contain eosinophilic cytoplasm with thick and thin filaments appearing as striations.[18]

The most common subtype, Embryonal rhabdomyosarcoma shows tumor cells that are small and spindle in shape.[17-18] A diagnostic feature of this subtype is the presence of highly cellular areas surrounding blood vessels alternating with variably cellular regions in a background of abundant myxoid material.[17] Alveolar rhabdomyosarcoma contains small, round, or oval cells that are arranged in nests of connective tissue septa.[17] Pleomorphic rhabdomyosarcoma are composed of large, atypical, and multinucleated cells that are difficult to differentiate from other soft tissue sarcomas.[17] Diagnostic clues of pleomorphic rhabdomyosarcoma are the presence of rhabdomyoblasts and myognenin or MyoD1 immunoreactivity of malignant cells.[17-18] Spindle cell rhabdomyosarcoma are predominantly composed of spindle cells with ovoid or elongated nuclei, vesicular chromatin, inconspicuous nucleoli and scant, pale eosinophilic cytoplasm.[18] Presence of necrosis and high mitotic counts are common in this subtype.[18] Few rhabdomyoblasts may be seen scattered within the spindle cells.[18]

Special examinations used to confirm the diagnosis of rhabdomyosarcoma are electron microscopy, immunohistochemical staining, and molecular studies. Electron microscopy allows the identification of sarcomere structures; however, these may not be present in poorly differentiated cells.[17] Highly sensitive and specific immunohistochemical stains like myogenin and MyoD1 are commonly used to diagnose rhabdomyosarcoma.[16] A specific indicator of muscle differentiation such as Desmin may be used, but this can stain both smooth and striated muscles.[17] Cytogenetic testing for the diagnosis and prognosis of alveolar rhabdomyosarcoma to detect PAX3-FOXO1 and PAX7-FOXO1 genes using FISH and RT-PCR may be used.[16-19] In recent literature, approximately 75-78% of alveolar rhabdomyosarcomas are associated with the translocation t(2;13) or t(1;13), which result in the gene fusion PAX3-FOXO1 and PAX7-FOXO1.[17-18]

In a review article by Schiffman and Wright, subsequent malignant neoplasms after Ewing's sarcoma are subclassified into solid tumors and hematologic malignancies. The most common solid tumor is osteosarcoma (50-60%) and the most common hematologic malignancy is acute myeloid leukemia or myelodysplastic syndrome (60%). The article reviewed three censuses on the most common risk of childhood cancers that develop second malignancies; in both the British Census and the Surveillance, Epidemiology

and End Result Program (SEER), Ewing's sarcoma is second, and in the multicenter cohort study done in France and United Kingdom, Ewing's sarcoma is ranked first. Based on the cumulative review, the onset of secondary malignancies due to Ewing's sarcoma 10 years after the diagnosis is at 5-6%. In the report the main etiologies are the use of high dose (>60Gy) radiation therapy, DNA damage to hematopoietic stem cells due to activation of G-CSF by chemotherapy and the possibility of genetic abnormalities are being investigated. It was further stated that there is no predisposing genetic syndrome associated with Ewing's sarcoma, however translocation that encodes the EWS/FLI-1 protein can alter the RB and p53 pathway.[20]

CONCLUSION

Although there is no clear pathophysiology yet for MPM, the most common conjectures are genetic predisposition, iatrogenic and environmental exposure to carcinogens. Upon excluding metastasis, a low threshold for diagnosis of new primary malignancy should be suspected in previous cancer patients. An early diagnosis is essential for the management of multiple sarcomas since it has a remarkable decline in prognosis. Catching these malignancies at an early stage could be achieved by a harmonious coordination among the primary physician, hematologist and oncologist, radiologist and pathologist.

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ETHICAL CONSIDERATION

Patient consent was obtained before submission of the manuscript.

STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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None.

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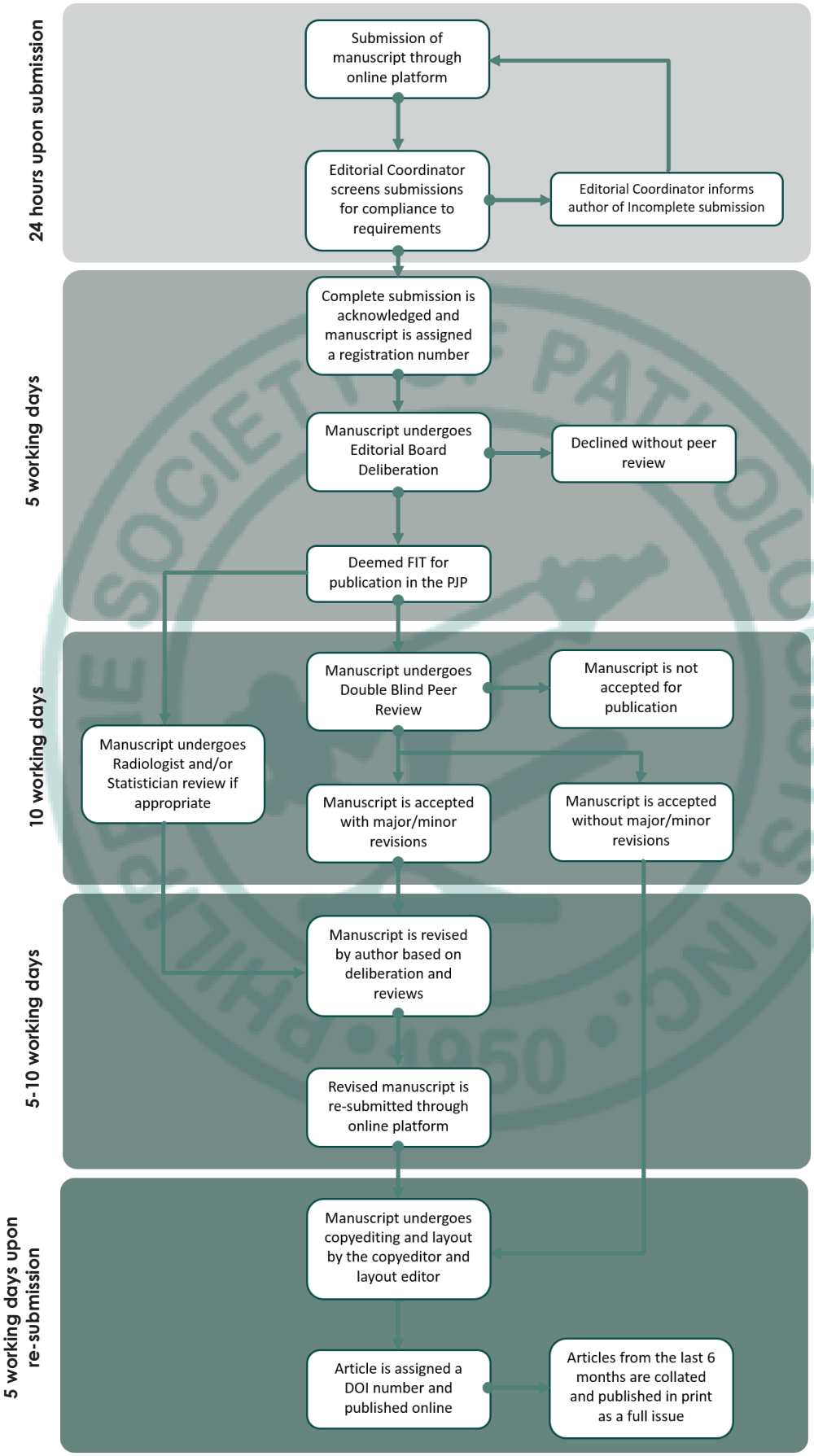

Figure 1. Editorial Process Flow.

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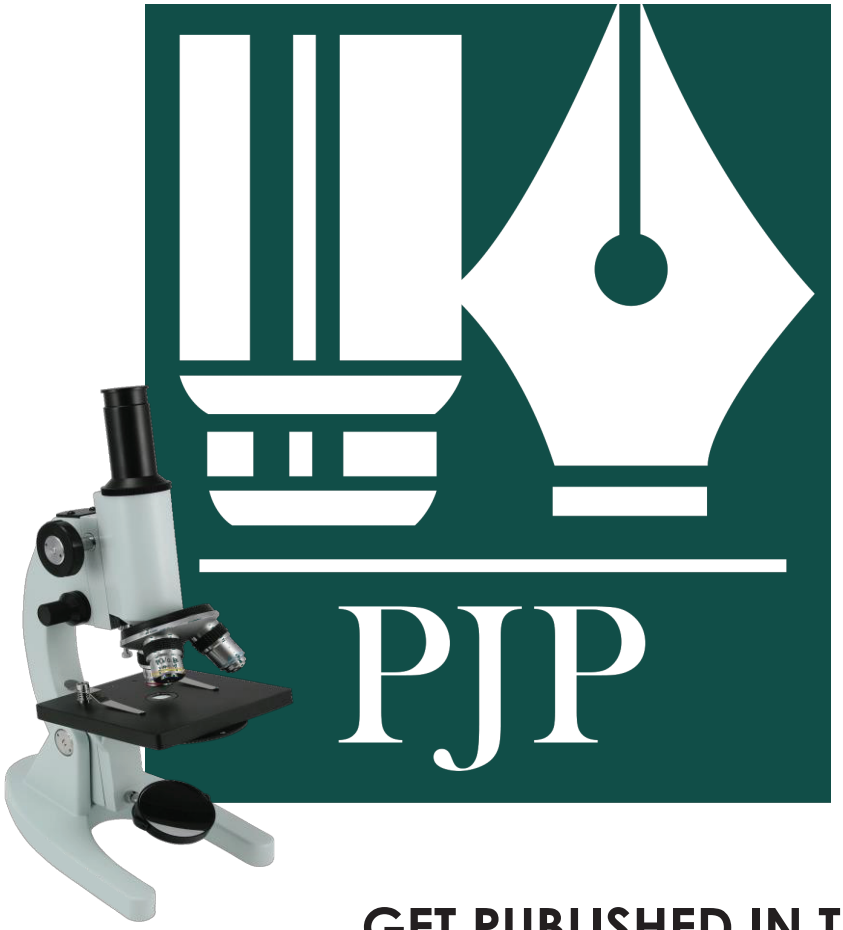

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