

# PHILIPPINE JOURNAL OF PATHOLOGY

The Official Journal of the Philippine Society  
of Pathologists, Inc.

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Welcome to the December 2022 issue of the Philippine Journal of Pathology. Congratulations to the editorial staff for publishing the second issue of the year.

I would like to reiterate our call to the junior and regular members of the society to come up with scholarly research and fascinating case reports and series. Let us continue to sustain this journal by consistently submitting articles for peer-review and eventual publication in our very own platform.

May I take this opportunity to wish you and your loved ones a very happy and festive Christmas season. May this season bring hope, joy and happiness to all of us.

Let us be grateful for the blessings we have received this year and pray for a brighter and a prosperous 2023!

Happy Holidays!

**Alan T. Koa, MD, FPSP**

*President, Philippine Society of Pathologists, Inc.*

## Five Hundred Twenty Five Thousand Six Hundred Minutes



What is in store for PJP for 2023? I am aiming to meet with the board soon not only to take stock of what has been gained over the last five years, but also, to plan for the journal's future. Coming up with clear and actionable strategies to increase copy flow and submissions, boost readership and visibility,

and further enhance operational efficiency and sustainability would be critical, and we would need more brilliant minds to think these through.

Based on my experience, medical journals get indexed when their contents are relevant and contribute to public knowledge, they follow international standards of ethical and scientific publication, and their issues are consistent and sustained. Given PJP's relative stability as a publication, thanks in large part to the support by the Philippine Society of Pathologists, a modest goal I am setting for this year for the journal is to finally be eligible for indexing in the WHO Western Pacific Region Index Medicus, the WHO index of medical/scientific publications for the region. This will first require endorsement by the National Journal Selection Committee of the Philippines under the Department of Science and Technology Philippine Council for Health Research and Development.

As I was recently assigned by the Society as Chair of the Committee on Academic and Research Pathology, I am also hoping to finally get down to actively helping build capacity of PSP's members in generating research outputs. This Committee's work will hopefully bridge the gap between implementing research and publishing findings. Our bottom line is producing local data that will improve not just our profession and practice as pathologists, but also contribute to better patient management.

I guess it is normal to feel sentimental when one year ends and another one begins. For me, this sensation happens as soon as December—not September—hits, beginning as a mild flutter of seasonal excitement. As weeks pass, that anticipation quickly and inevitably degenerates into worry due to parallel deadlines gaining momentum. The feeling culminates to that last minute of the last hour of the last day of the last month of the year, like the final grains of sand sifting through an hourglass. At the final minute countdown, right at the cusp of midnight, I hover somewhere in the middle of anxiety and excitement, between frustration and fulfillment, maybe even suspended between things as polar as happiness and melancholy.

Amid the fireworks lighting up the night sky, the cheers and laughter of family, friends, and neighbors, the savory and social media-friendly feast in most dinner tables, COVID-19 on its third year, there still remains, thankfully, that singular moment to reflect on the year that was, and the promise and potential of the upcoming one. In that brief moment of calm and peace, everyone gets to get back to Day One, filled with hopes of making another trip around the sun. I remember sheepishly the other side of the closing year's highlights, where some things may not have gone the way I would have wanted them to, or the embarrassing mistakes I have made, the previous year's resolutions and plans that did not push through. In that safe minute, I am able to forgive myself and give my Self another chance. Five hundred twenty five thousand six hundred of it.

With the coming 2023, I am expressing my gratitude for the continued support to the journal, not only by the Philippine Society of Pathologists, but also its members who, despite their busy schedules, find time to write and publish, review as peers, cite relevant articles in their research, or read what the journal has to offer.

**Amado O. Tandoc III, MD, FPSP**

*Editor-in-Chief*

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## Institutionalization of the Philippine Health Laboratory System (PHLS)

Hyacinth Joy Balderama, Valerie Anne Tesoro, Terence John Antonio, Richard Albert Ramones

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### INTRODUCTION

In 1966, Section 3 of Republic Act (R.A.) 4688, entitled “An Act Regulating the Operation and Maintenance of Clinical Laboratories and Requiring the Registration of the Same with the Department of Health, Providing Penalty for the Violation Thereof, and for Other Purposes,” provided for the establishment of the Bureau of Research and Laboratories (BRL) under the Department of Health (DOH). The BRL served as the central laboratory that governed the operation of regional public health laboratories. The BRL’s function was delegated to different offices in 2000 by Executive Order 102 s. 1999 entitled “Redirecting the Functions and Operations of the Department of Health,” which was premised in part with Section 78 of the General Provisions of R.A. 8522 (“General Appropriations Act of 1998”) authorizing the President to direct changes in organization and key positions of any department, bureau, or agency.

Towards this direction, the function of the BRL was distributed to different agencies, and the Philippines designated six (6) national reference laboratories that catered to communicable and non-communicable diseases. Five (5) subnational reference laboratories for emerging and re-emerging infectious diseases were established in response to the 2009 Influenza AH1N1 pandemic. The DOH issued Administrative Order No 2012-0021 to establish a national and regional network of laboratories.

### NATIONAL LABORATORY CAPACITY IN RESPONSE TO COVID-19

In the early part of the COVID-19 pandemic, the Philippines reactivated its five (5) existing subnational laboratories capable of molecular testing for emerging and re-emerging infectious diseases to augment the testing capacity of the National Reference Laboratory. However, as the demand for testing increased, the Department had to undertake steps to increase the number of laboratories that performed COVID-19 testing both for government and private health facilities.<sup>1</sup>

Compared with the performance of other countries in terms of testing capacity at the start of the pandemic, the Philippines tested a total of 1.44 persons per 1,000 people.<sup>2</sup> This is relatively lower compared to its neighboring countries: 31.82 per 1,000 people for Singapore, 12.98 per 1,000 people for South Korea, 7.94 per 1,000 people for Malaysia, 4.1 per 1,000 people for Thailand, and 2.68 per 1,000 people for Vietnam.<sup>3</sup> The low cumulative testing figures in the Philippines may be attributed to the limited resources and accredited laboratories especially at the outset of the pandemic, low capacity of testing centers, lack



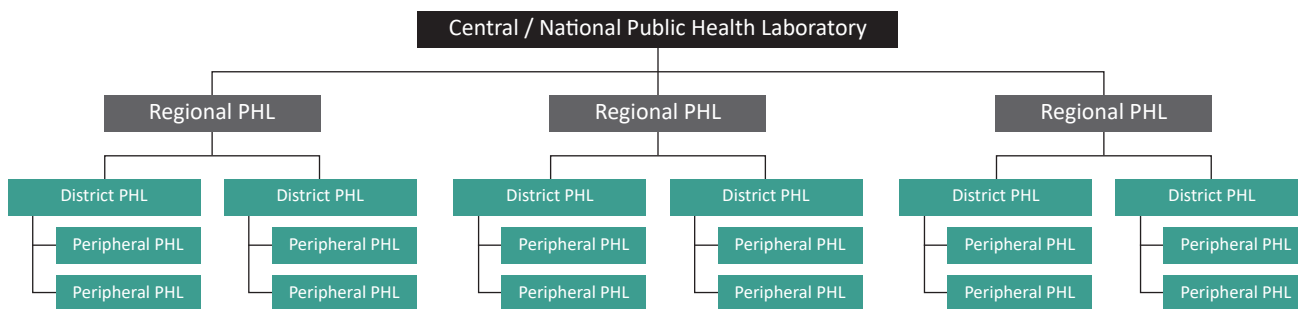


Figure 1. Generic organizational structure of Public Health Laboratories in SEA. 2018.<sup>9</sup>

of trained personnel, inadequate supplies, and distribution of resources across the country, and fragmentation of the laboratory system.<sup>1</sup>

The efficiency and effectiveness of the response of other Southeast Asian countries to the COVID-19 pandemic are associated with their public health preparedness and response plans for communicable disease management.<sup>1</sup> The different approaches among these countries stem from their key interventions (Table 1).

### INTERNATIONAL BENCHMARKING AND MODELS OF LABORATORY SYSTEMS

Based on the World Health Organization Report on Establishment of Public Health Laboratories in South-east Asia, while different countries have different organizational structure for their laboratory network, the generic arrangement of Public Health Laboratories comprises 3-5 levels<sup>1</sup> (Figure 1). In most cases a separate entity / system focuses on public health related services / activities particularly on surveillance, diagnosis, treatment, and control.

#### Taiwan

At the National Level, the Taiwan Center for Disease Control (CDC) serves as the overall governing body for the national laboratory system. Twelve (12) National Reference Laboratories are directly lodged under the country's CDC (Figure 2).

#### Thailand

In Thailand, the Bureau of Laboratory Quality Standards under the Department of Medical Sciences focuses on standards development and facilitates the EQAS provisions (Figure 3). This bureau also functions as the National Reference Laboratories. The Bureau of Laboratory Quality Standards has regional counterparts known as Regional Medical Sciences Centers. They are tasked to manage the budget of the network and coordinate with regional, general and community hospitals.

#### Vietnam

Vietnam's laboratory system consists of approximately 900 laboratories that deal with human infectious diseases including public health diagnostics and reference laboratories, and clinical laboratories at different levels of

Table 1. Approaches of selected Southeast Asian countries in response to COVID-19

Country	Approach/Laboratory Strategy
<b>Vietnam</b>	Vietnam enhanced their surveillance and mass testing approach through target testing of higher risk groups and random testing of households and inpatients. Its maximum testing capacity remains 51,000 tests with 152 laboratories COVID-19 RT-PCR. <sup>4</sup>
<b>Thailand</b>	Thailand is the first country to complete an assessment on how their health system has responded to COVID-19 using a WHO Intra-action Review (IAR) tool. One of their effective systems is integrating epidemiological, laboratory, clinical and logistic data in a new digital information system. Thailand has greatly improved detection through expanding their COVID-19 surveillance as well as their strong capacity to trace and quarantine contacts. <sup>5</sup>
<b>Singapore</b>	Singapore has implemented a whole-of-government, whole-of-society approach to contain the spread of the virus. Singapore's healthcare system provides access to quality healthcare through high government investment in infrastructure, subsidies, financing that has resulted in high coverage of essential health services to support the COVID-19 response. At the start of the COVID-19 pandemic, Singapore performs an average of 8,000 tests per day. <sup>6</sup>
<b>Malaysia</b>	Malaysia has a robust health system with universal health coverage which helped them prepare and respond to the COVID-19 pandemic in a whole-of-government and whole-of-society approach. With a population of 32 million, they have ramped up its testing capacity from 1000 to over 38,000 tests per day. <sup>7</sup>
<b>Australia</b>	Australia has an expert network of public and private laboratories with the capability to detect SARS-CoV-2 and to securely capture and report results. The Australian Public Health Laboratory Network (PHLN) and Communicable Diseases Network Australia (CDNA) were fast to respond and create guidelines as early as Q1 of 2020 on the testing of SARS-CoV-2 hence a more rapid roll out of testing by the laboratory network. <sup>8</sup>

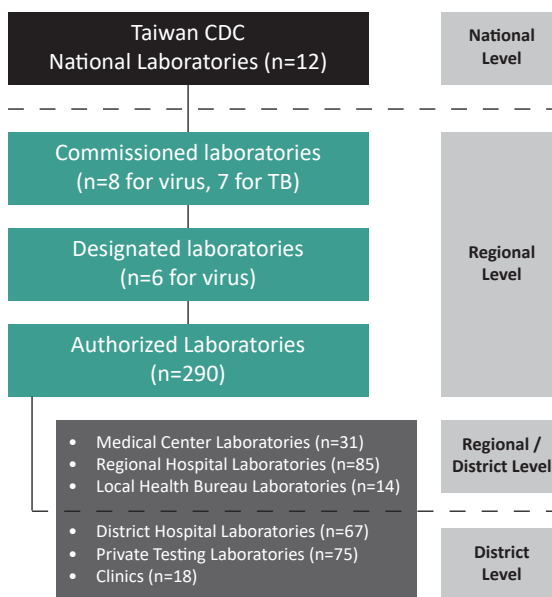
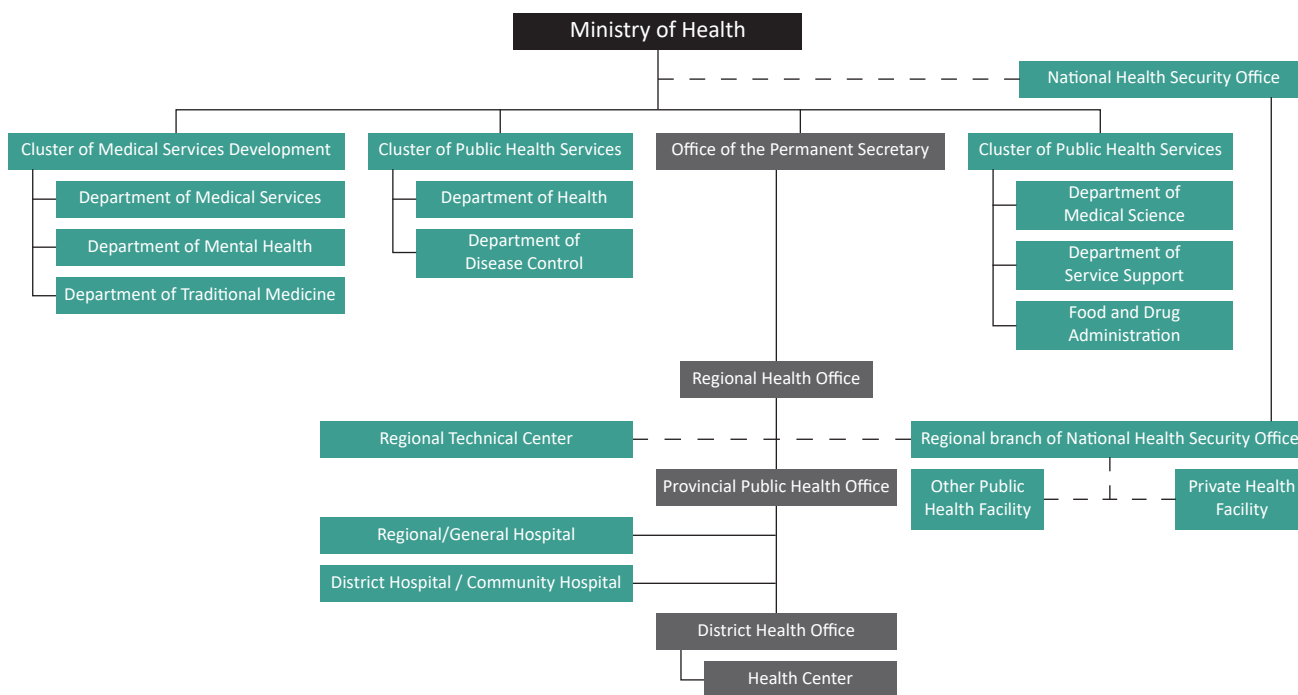
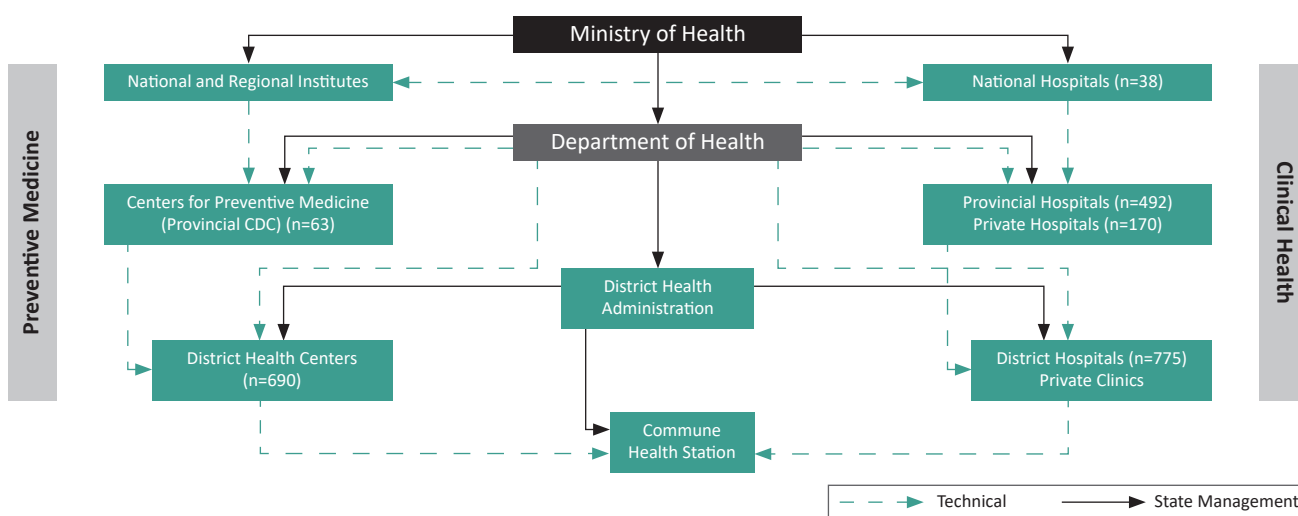


Figure 2. Structure of the National Laboratory System in Taiwan. Adapted from Taiwan's Public Health Laboratory System: success and surveillance, Ji-Rong Yang, Hwa-Jen Teng, Ming-Tsan Liu, et al. 2017.<sup>10</sup>





**Figure 3.** Organizational structure of the Ministry of Public Health, Thailand. Adapted from The Kingdom of Thailand Health System Review. Vol. 5 No. 5, 2015.<sup>11</sup>



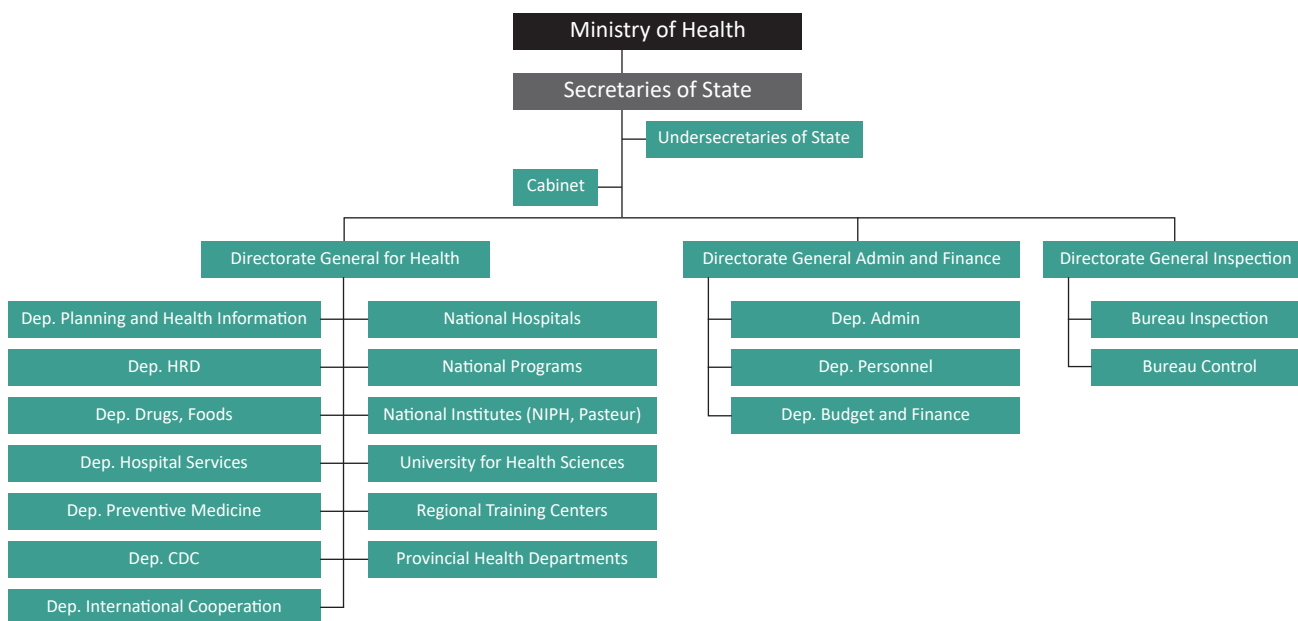
**Figure 4.** Organizational structure of the health system in Vietnam. Adapted from patient complaints in healthcare services in Vietnam’s health system (Bui Thi Thu Ha, Tolib Morzoev, Rosemary Morgan, 2015).<sup>12</sup>

the healthcare system (Figure 4). Of the 73 public health diagnostic and reference laboratories, 4 are national or regional public health laboratories, two non-governmental research laboratories, and two pediatric laboratories. Forty (40) clinical laboratories are at central or regional levels.

**Cambodia**

Cambodia’s laboratory system consists of 94 public laboratories (of which eight are national laboratories) at different levels of the health care system (Figure 5). Aside from laboratories supporting disease-specific programmes (such as HIV/AIDS, tuberculosis, and malaria), Cambodia’s National Institute for Public Health supports surveillance

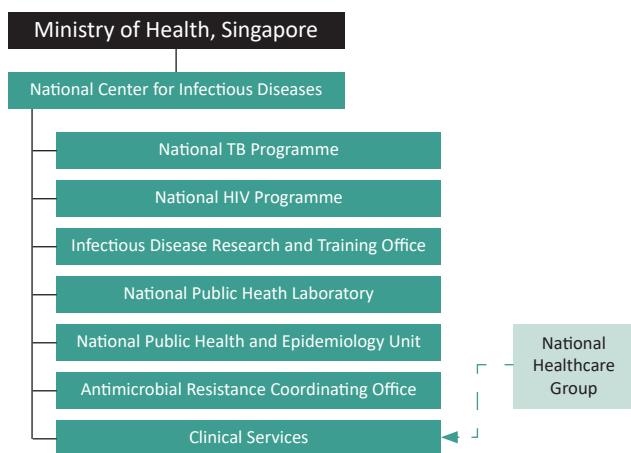
and outbreak response for emerging infectious diseases. It is also the representative body of the ministry of health providing public health and laboratory services, training, and research to improve the health of Cambodians. Under the NIPH is the National Public Health Laboratory (NPHL) known as the National Reference Laboratory, which is operated by highly qualified and experienced technical staff and working on promotion and strengthening of quality laboratory services for public health. Laboratory results particularly for disease diagnosis are ensured through up-to-date technologies and use of Laboratory Quality Management System (LQMS).



**Figure 5.** Organizational structure of the Ministry of Health of Cambodia. Adapted from ReBuild Consortium: country situational analysis, Cambodia.<sup>13</sup>

**Singapore**

The Singapore’s Ministry of Health has established the National Center for Infectious Diseases in 2009 under the Communicable Disease Division (Figure 6). Under this division is the National Public Health Laboratory (NPHL) which is currently involved in the laboratory surveillance and preparedness for detection of highly infectious pathogens, such as MERS-CoV, H7N9, and Ebola Virus. The NPHL facilities include a biosafety-level 3 (BSL-3) containment facility which shall be dedicated for surveillance of infections of public health importance as well as the detection and identification of novel pathogens. The mission of NPHL is to use the best possible science for the laboratory surveillance of infectious diseases and investigation of outbreaks. NPHL also works closely with its partners in government agencies, medical laboratories, and research institutions to enable a coordinated and broad-based response to infectious threats.



**Figure 6.** Organizational structure of the National Center for Infectious Diseases, Ministry of Health Singapore.<sup>14</sup>

The marked similarity among these models includes the following:

1. There is a centrally governing body that focuses on the standards, policies, and operations of the laboratory network under the Ministry or Department of Health.<sup>1</sup>
2. National Reference Laboratories in these countries are stand-alone organizations and not lodged under a hospital.<sup>1</sup>
3. There is a delineation between clinical laboratory services and public health services.<sup>1</sup>
4. Quality Management System, Quality Assurance Program, and Network Development are critical components of a responsive laboratory diagnostic services both for medical and public health services.<sup>1</sup>

**CREATION OF THE OFFICE FOR HEALTH LABORATORIES**

The Office for Health Laboratories (OHL) was created by virtue of Department Order 2021-0421 upon realizing the gaps in our healthcare system during the COVID-19 pandemic. The OHL, under the cluster of the Health Policy and Infrastructure Development Team, shall serve as an interim structure focused on building the foundation of the PHLS, pending the enactment of the Philippine Center for Disease Control and Prevention. At the national level, the OHL shall provide administrative supervision to all Public Health Laboratories and shall stir the implementation of the PHLS with the following core mandates:

- Develop and provide the overall strategic direction, policies, plans, and programs, including infrastructure and equipment investments, in the development of the PHLS;
- Establish and implement systems and programs for (1) national laboratory referral system, (2) nationally coordinated training for HRH Development for clinical and public health laboratories, (3) unified national evidence-based laboratory-related research agenda, (4) national laboratory information system, (5) laboratory

- surveillance, (6) chemical safety and security, and (7) administrative supervision and support;
- Serve as the technical authority in developing the laboratory biosafety and biosecurity standards, policies, plans, and measures to prevent/reduce biorisk and bioterrorism;
- Evaluate the performance of public health and clinical laboratories by ensuring compliance with the laboratory quality management system and quality assurance programs;
- Provide technical and administrative standards and guidelines for the NRLs, SNLs, Public Health Laboratories, and Clinical Laboratories; and
- Participate in inter-agency and international networks for laboratory response to uphold national security and prevent international threats.

### INSTITUTIONALIZATION OF THE INTEGRATED PHILIPPINE HEALTH LABORATORY SYSTEM

The Philippine Health Laboratory System aims to harmonize the entire laboratory system in the country, specifically, to ensure quality, efficient sustainable and accessible laboratory services; and to ensure a functional, efficient, and harmonious network of health laboratories and referral system across different laboratory facilities paving the way to a more responsive medical and public health diagnosis and surveillance.

In order to address these challenges, the Philippine Health Laboratory System (PHLS) was pursued with the vision of Accessible Health Laboratory Services by 2025, and World Class Services by 2035 (Figure 7). Furthermore, to ensure that the development of the Philippine Health Laboratory System is fully realized, the Office for Health Laboratories under the Health Facilities and Infrastructure Development Team shall be created to serve as the interim structure focused on building the foundation for the PHLS, pending the enactment of the mandate for the Philippine Center for Disease Control and Prevention (PH CDC).

Through the Philippine Health Laboratory System, at least one (1) public health laboratory shall be established in each region. The Philippine Health Laboratory System likewise calls for the institutionalization of Subnational Reference Laboratories (SNL) and Regional Public Health Laboratories (RPHL) to ensure the detection of both communicable and non-communicable diseases and health events as guided by the DOH Resource Stratified Framework for Laboratory Facilities.

### THE PHILIPPINE HEALTH LABORATORY SYSTEM STRATEGIC MAP

With a vision of accessible health laboratory services by 2025 and world class services by 2035, the Philippine Health Laboratory System shall ensure the provision of quality, affordable, and accessible laboratory services, and information for the appropriate management of patients and prevention and control of diseases through strengthening key laboratory facilities in the network.

The overall implementation of the PHLS shall have an impact on the daily lives of the Filipinos by providing a responsive clinical and public health diagnostics and surveillance for public health action. The realization of this impact in laboratory diagnostics shall greatly contribute to the overall goals of Universal Health Care.

The following shall be the strategic focus of the PHLS:

#### 1. Functional and efficient network of Clinical and Public Health Laboratories

The following are the strategies to ensure a functional and efficient network of clinical and public health laboratories for appropriate public health and clinical diagnostic response:

- Resource management through long-term efforts to ensure fundings for the infrastructure development, Human Resource development, and provision of supply and equipment through

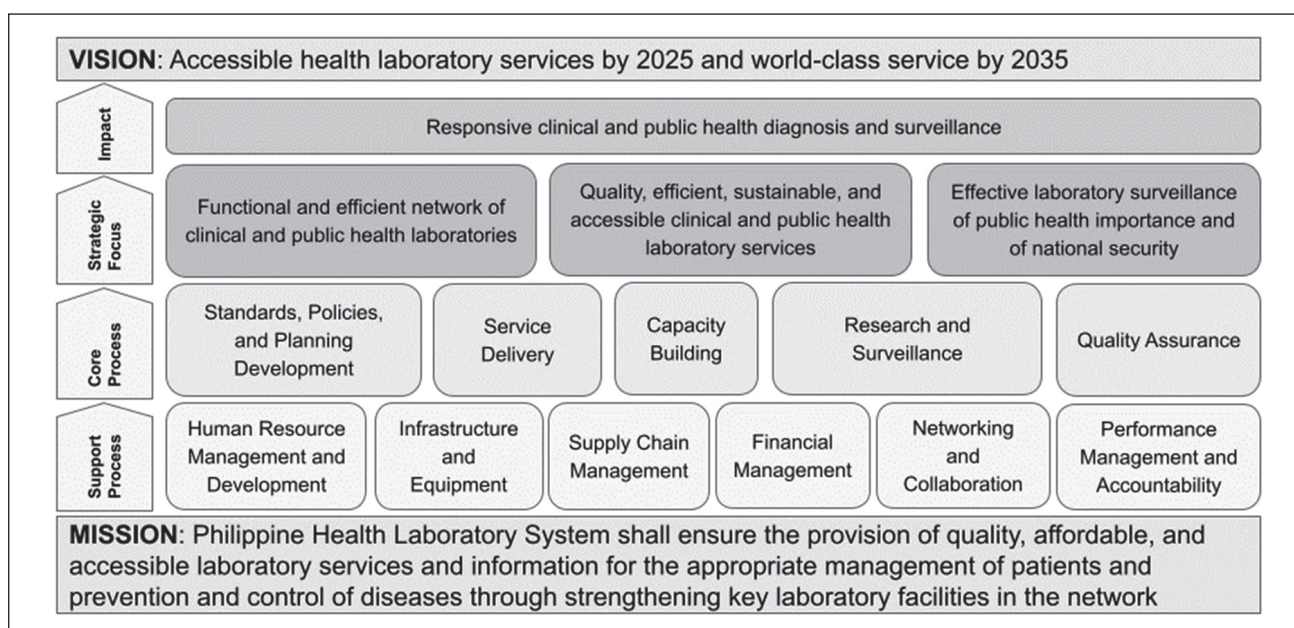


Figure 7. Overall strategic map for the Philippine Health Laboratory System.

the development of a sustainable supply chain management system;

- Partnership with other DOH offices, other stakeholders, different levels of laboratories with clear delineations, roles, and contributions to establish a harmonized network for all DOH programs in terms of laboratory services;
- Provide accessible and quality laboratory services for appropriate management of patients and public health programs;
- Standardize the clinical and public health laboratory policies, operations, and standards aligned with statutory and regulatory requirements, epidemiological criteria, developmental standards, and WHO recommendations.

## 2. Quality, efficient, sustainable, and accessible Clinical and Public Health Laboratory Services

To strengthen this strategy, the following implementing mechanisms shall be enhanced:

- Laboratory Referral System
- Public Health Laboratory Network

## 3. Effective laboratory surveillance of public health importance and of national security

To focus on providing effective diagnostic surveillance, the implementing mechanisms shall be strengthened:

- Laboratory surveillance and management
- Laboratory biological and chemical safety and security
- Integrated laboratory information management system

### PHLS Core Processes

The following are the principal processes for the Public Health Laboratory System based on its strategic map.

#### 1. Standards, policies, and planning development

The Office for Health Laboratories under the DOH Health Policy and Infrastructure Development Team shall lead the institutionalization and implementation of the Philippine Health Laboratory System. The OHL shall develop policies, standards, programs, and plans, and provide the overall strategic direction for the implementation of the PHLS

#### 2. Service delivery

Service capabilities of Public Health Laboratories at various levels from the reference laboratory down to the peripheral laboratories shall be determined by the OHL.

#### 3. Capacity building

A competency framework for the PHLS shall be set by the OHL to guide development of appropriate and effective trainings, workshops and other learning and development interventions.

#### 4. Research and surveillance

The PHLS shall be an important national resource for generation of policy-guiding information through research and surveillance activities.

#### 5. Quality assurance

OHL shall ensure that facilities under the PHLS operate within a functional Quality Management System. It shall lead to the implementation of the National External Quality Assurance Scheme (NEQAS) by the reference laboratories.

### PHLS support functions

To achieve the overall goal set by the PHLS, the following relevant support functions need to be developed and strengthened:

#### 1. Human resource management and development

Capacity building processes needed by the PHLS across all laboratories in the PHLS for continuous professional education shall be supported, to contribute to the improvement and strengthening of the laboratory workforce. This shall include, provision of learning and development interventions, fundings for hiring technical and managerial staff, and strengthening of laboratory interface to support human resources.

#### 2. Infrastructure and equipment

Technical standards for physical infrastructure and equipment appropriate for each laboratory level in the PHLS shall be developed to guide investments.

#### 3. Supply chain management

National policies, guidelines, and standards on laboratory supply chain management guided by the existing statutory and regulatory protocols, policies, and guidelines, shall be established to support the logistics needs of the PHLS.

#### 4. Financial management

The OHL shall work towards obtaining necessary budget to finance the implementation and sustain the operations of the PHLS, to include a dedicated line-item budget in the annual budget appropriations for the Department of Health, retention, and use of income, as well as tapping other potential local and international funding sources.

#### 5. Networking and collaboration

- 5.1 Partnership
  - 5.1.1. Other DOH Bureaus
  - 5.1.2. Other Government Agencies
  - 5.1.3. Non-Government Organization
  - 5.1.4. Private sector
- 5.2 Laboratory Advisory Groups

#### 6. Performance management and accountability

Monitoring and evaluation of the operations and performance of all laboratories in the PHLS through indicators set by the OHL and/or NRL.

### COMPONENT LABORATORIES OF THE PUBLIC HEALTH LABORATORY SYSTEM

Public health laboratories are responsible for providing timely and reliable diagnostic results primarily for disease prevention, control, surveillance, and outbreak emergency response. They perform core public health and

environmental activities, including reference tests for diseases of public health importance.

### National Reference Laboratories (NRLs)

By virtue of DO 2020-0820 “Institutionalizing and Strengthening the National Reference Laboratories in the Philippines,” the National Reference Laboratories (NRLs) serve as the highest level of laboratory in the country performing highly complex procedures, including end confirmatory testing. The NRLs are the responsible entity facilitating National External Quality Assurance Scheme (NEQAS) to ensure compliance of quality standards for regulation and licensing of all laboratories in the Philippines.

The NRLs are housed in six (6) selected host hospitals that cater to disease-specific testing, namely: 1) *Research Institute for Tropical Medicine (RITM)*, 2) *San Lazaro Hospital - STD AIDS Cooperative Central Laboratory (SLH-SACCL)*, 3) *East Avenue Medical Center (EAMC)*, 4) *National Kidney and Transplant Institute (NKTI)*, 5) *Lung Center of the Philippines (LCP)*, and 6) *Philippine Heart Center (PHC)*. These NRLs shall closely collaborate with the OHL as the highest level of laboratories under the PHLS that is mandated to develop standards and policies pertaining to laboratory operations, personnel competence, quality assurance programs, and product evaluation to ensure quality laboratory results across public laboratories and clinical laboratories. They will also play a key role in diagnostic surveillance, research, monitoring and evaluation, and provision of technical assistance to SNLs, RPHLs, Clinical Laboratories, and other Health Laboratories. The NRLs shall serve as the technical authority in evaluating the quality management system performance of SNLs and RPHLs, including the highly technical services offered by Clinical and other Health Laboratories. These NRLs are currently hosted in selected hospitals and shall be operating as a separate department from its host hospital clinical laboratory, with transfer of its administrative and technical supervision to the OHL. The NRLs shall have their own physical infrastructure, equipment, and personnel to deliver the following services.

### Subnational Reference Laboratories (SNLs)

The direction of the PHLS is to establish SNLs across the country with catchment areas according to population density, accessibility, and geographical location, and disease prevalence to streamline services offered by the NRLs into one facility. The SNLs shall be the arm of the NRLs and OHL in rolling out programs, policies, and standards, training, and testing on their catchment area. They shall be technically supervised by the NRLs in conducting selected confirmatory testing by providing accessible laboratory services and decongesting the services of the NRLs down to the regions. In addition, the SNLs shall participate in the conduct of laboratory surveillance and research within their catchment.

### Regional Public Health Laboratories

The RPHLs shall also be established in each region, catering to smaller catchment areas with a less complex service capability than the SNLs and NRLs. The RPHLs shall be established in each region, with administrative governance from the Centers for Health Development. The RPHLs

shall be technically supervised by the NRLs in conducting routine analysis and priority diagnostic testing to support the different DOH programs. The RPHLs shall facilitate roll-out of training programs provided by the SNLs to the clinical laboratories within their catchment area.

### Clinical and other health laboratories

The Philippine Health Laboratory System shall also serve as the overall backbone of the National Framework for Health Laboratories, involving not only public health laboratories but also all clinical and other health laboratories in the country. The Clinical and other Health Laboratories shall comply with regulatory standards to enable an effective and efficient laboratory quality management system as guided by this framework. Delineating its role from Public Health Laboratories, the Clinical Laboratories are mainly focused on individual-based testing and management of patients. In addition, these facilities are involved in the (a) pre-analytical, (b) analytical, and (c) post-analytical procedures and where tests are done on specimens from the human body to obtain information about the health status of a patient for the prevention, diagnosis, and treatment of diseases. These tests include, but are not limited to, the following disciplines: anatomic pathology, clinical chemistry, clinical microscopy, endocrinology, hematology, immunology and serology, microbiology, toxicology, as well as molecular and nuclear diagnostics. All licensed Clinical Laboratories by the DOH shall follow the existing minimum service capabilities for primary, secondary, and tertiary clinical laboratories, as stated in the Administrative Order No. 2021-0037 entitled “New Rules and Regulations Governing the Regulations of Clinical Laboratories in the Philippines.”

In addition, other Health Laboratories are laboratories that do not meet the minimum standards of a Primary Clinical Laboratory but are likewise involved in individual-based testing and research, such as but not limited to the following:

1. Analytical Toxicology Laboratories
2. Drug Testing Laboratories
3. Water Testing Laboratories
4. Bureau of Quarantine Laboratories
5. State Universities and Colleges (SUCs), and Other Academic Laboratories
6. Department of Agriculture Laboratories
7. Department of Science and Technology Laboratories
8. Military Clinical Laboratories

## CONCLUSIONS AND RECOMMENDATIONS

With the challenges faced by the country in the emergence of infectious diseases and other public health threats, the institutionalization of the Philippine Health Laboratory System is an avenue to deliver an effective public health laboratory network across the country by addressing its geographic challenges, to boost laboratory diagnostic capacities and laboratory referral system when and where they are needed, and to serve gateway to mobilize technical expertise, knowledge exchanges and resources in support of future pandemic response. Through the creation of the Philippine Centers for Disease Prevention and Control, these public health laboratories shall serve as the country's laboratory diagnostic arm in providing surveillance

testing, research, and capacity building in support of the Department of Health's disease prevention and control programs to achieve Universal Health Care.

### STATEMENT OF AUTHORSHIP

The authors certified fulfillment of ICMJE authorship criteria.

### AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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## Spit or Swab? The Diagnostic Accuracy of Saliva-based Testing as a SARS-CoV-2 Surveillance Tool

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### ABSTRACT

**Background.** Nasopharyngeal swab/oropharyngeal swab (NPS/OPS) qRT-PCR is the gold standard for detecting SARS-CoV-2. However, it has its own limitations including cost and invasiveness. As an alternative, individual qRT-PCR testing of saliva samples was validated and shown to be comparable in sensitivity and specificity with NP-OP qRT-PCR. To further maximize its utility, the researchers wish to explore antigen and pooled testing methods.

**Objective.** The study aimed to evaluate the diagnostic accuracy of detecting SARS-CoV-2 infection using saliva-based pooled qRT-PCR and rapid antigen test compared with individual saliva qRT-PCR.

**Methodology.** In this retrospective cross-sectional study, saliva specimen from individuals aged 18 years old and above from the outpatient specimen collection station at the Philippine Children's Medical Center were tested individually using qRT-PCR (Mag-bind RNA Extraction Kit/MACURA, Allsheng Extraction Machine, Sansure PCR kit, and MA-600 Sansure Biotech). Non-probability convenience sampling was utilized. Based on the individual results, pools of five (5) individual specimens, which includes one (1) positive sample were tested with qRT-PCR for sensitivity. DNK-2150-1S Dynamiker SARS-CoV-2 Ag Rapid Test (Dynamiker Biotechnology Co., Ltd., Tianjin, China) was also used to test individual saliva specimens.

**Results.** Out of 196 individual saliva specimens, 73 were detected to have SARS-COV-2 by qRT-PCR, while the remaining 123 were negative. Compared with the individual saliva qRT-PCR, rapid antigen tests done showed sensitivity of 46.58% (95% CI 35.13%, 58.02%), specificity of 86.18% (95% CI 80.08%, 92.28%), positive and negative predictive value of 66.67% (95% CI 53.71%, 79.60%) and 73.10% (95% CI 65.89%, 80.32%) respectively. Based on the results of individual saliva-based qRT-PCR, 62 pools were tested and showed sensitivity of 98.39% (95% CI 91.34%, 99.96%).

**Conclusion and Recommendation.** Pooled saliva-based testing for SARS-CoV-2 is comparable with individual saliva-based rapid antigen testing. The use of rapid antigen testing is less sensitive and less specific compared with qRT-PCR consistent with prior reports. Additional studies are recommended to determine optimal conditions for testing.

*Key words: qRT-PCR, rapid antigen test, pooling, SARS-CoV-2, COVID-19*

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### INTRODUCTION

In October 2020, the Philippines entered the top 20 countries with the highest number of COVID-19 cases in the world.<sup>1</sup> Due to the economic harm that this pandemic caused the country, Filipinos are obliged to enter the “new normal” despite the threat of COVID-19 infection. As more Filipinos return to work, demand for a less invasive, more efficient, timely and affordable means of testing for the SARS-CoV-2 virus is at its pinnacle. At present, qRT-PCR of nasopharyngeal (NP) and/or oropharyngeal (OP) specimens is the accepted standard for diagnosis of SARS-CoV-2. This method of testing albeit specific and sensitive incurs high costs limiting the method's availability to the public. The intensive need for trained swabbers and the use of costly materials such as NP and OP swabs, viral transport media, and personal protective equipment increased the cost of these tests. Hence, only a



few sectors of the population can afford standard qRT-PCR SARS-CoV-2 testing.

On August 17, 2020, the Philippine Society of Pathologists Inc. (PSP) published a statement that pooled testing strategies of NP/OP samples can be used to enable mass testing at a lower cost.<sup>2</sup> These strategies allowed asymptomatic individuals in a low positivity rate population to be tested, to be diagnosed faster, and to be reintegrated back to work at reduced costs. Despite pooling strategies, several areas must be improved to adapt to the pandemic. One would be on the issue of safety, as NP and OP swabbing are invasive procedures which may be harmful to patients when done by poorly-trained personnel. Though most patients experience mild discomfort, it is, in general, not a pleasant experience which causes fear and hesitancy in getting the test. Another area that may be improved is the use of disposable materials such as swabs, viral transport media and personal protective equipment (PPE) which not only incur higher costs but also burden the environment with pathologic wastes.

A possible answer to these issues may lie in the utilization of saliva. Saliva qRT-PCR has been reported to have diagnostic sensitivity that ranges from 69.2% to 100.0% in detecting COVID-19.<sup>3</sup> These numbers are better than the sensitivities found in qRT-PCR of NP and OP specimens which range from 63.0% to 73.0% and 32.0% to 61.0% respectively.<sup>3</sup> However there are few studies done on saliva and its potential in the field of COVID-19 diagnosis.

On April 6, 2021, the Department of Health (DOH) issued Department Memorandum No. 2021-0161 which established the guidelines for saliva-based RT-PCR and allowed COVID-19 laboratories certified by the Research Institute for Tropical Medicine (RITM) to perform saliva-based RT-PCR.<sup>4</sup> In compliance with the DOH memorandum, a validation study comparing NP/OP and saliva samples for qRT-PCR was done prior to the commencement of this study. The validation study yielded a sensitivity of 96.77% (CI: 96.58%-96.97%) and specificity of 100% (CI: 100%). The result of the study was submitted to the RITM and the PCMC's COVID laboratory was authorized to perform saliva-based qRT-PCR testing on September 15, 2021(Appendix 3 and 4).

The study aims to answer two questions: (1) How does saliva-based SARS-CoV-2 rapid antigen test and pooled saliva qRT-PCR compare to individual saliva qRT-PCR? (2) What is the diagnostic utility of saliva-based SARS-CoV-2 rapid antigen test and pooled saliva qRT-PCR?

### Significance of the study

Utilization of saliva instead of NP and OP specimens will support patient safety and decrease patient anxiety towards an invasive procedure. More so, validation of pooled saliva qRT-PCR and SARS-CoV-2 rapid antigen testing will decrease the cost of testing by eliminating the need for swabs, viral transport media, and minimize use of PPE as well as hasten the rate of testing by simplifying the method of collection. In addition, pooled saliva-based qRT-PCR when proven to have acceptable sensitivity will allow more individuals to be tested due to the ease of specimen collection and lower cost of testing.

As we ease into a “new normal” transitioning from a pandemic to an endemic phase, there may still be outbreaks that can lead to the rise of new variants if not controlled. However, we predict that many COVID-19 testing laboratories will either shut down or transition to other targets for testing due to the low demand for SARS-CoV-2 testing. Likewise, the teams of trained swabbers consisting mainly of nurses and medical technologists will be demobilized and assigned elsewhere for more productive work. Thus, there may be a scenario where there is an urgent need to test a specific community experiencing an outbreak but the laboratory resources may not be available anymore.

Once saliva pooled testing is found to be a safe, effective and accurate method, it will serve to bridge the gap between the transition phases. Swabbers need not be called back which will disrupt their newly assigned duties and the few remaining laboratories can cope with the volume of testing required. Those who are not symptomatic nor close contacts will just be asked to self-collect saliva samples which will be pooled-tested.

This study focuses on the pooled testing sensitivity since biomarker tests have less than perfect sensitivity, and this is further reduced by the dilution of the specimens to create pools. This is also the most important parameter when pooled testing is applied, since a positive pool will undergo individual testing. Because of this, specificity, positive predictive value and negative predictive value will not be tested.

This study also uses the individual saliva-based testing as the index test as it has been shown that saliva is comparable with NP/OP specimens in sensitivity, using a less invasive method of specimen collection.

### Review of related literature

The gold standard for COVID-19 is still RT-PCR of nasopharyngeal swab. However, this mode of testing has some disadvantages. It is time consuming, entails a certain degree of patient discomfort, requires a health care professional to do the invasive, aerosol-generating procedure, as well as swab collection kits. The above factors may hinder this method of specimen collection from being the regularly done, affordable and convenient test that is needed for COVID-19 diagnosis and monitoring.<sup>5</sup>

Mina et al., explained that due to the long duration of RNA-Positive tail, most infected people are identified as positive after the infectious period has passed and are subjected to unwarranted quarantine measures. Rapid lateral-flow antigens tests and other antigen tests have analytic limits of detection 100 to 1000 times higher than that of RT-PCR. This coincides well with the exponential growth phase of the SARS-CoV2 virus and its most infectious period. Antigen testing serves many purposes: identifying infectious individuals and reducing isolation periods for previously infected persons who had positive Rt-PCR tests thereby allowing more economic activity and downgrading of quarantine regimens without the threat of an outbreak. The key is to perform more frequent testing with the lower sensitivity assays that are cheap, fast and easy to perform in community wide surveillance regimens for SARS-CoV-2.<sup>6</sup>



Saliva is an attractive sample for detection of SARS-CoV-2 due to the documented process of oral shedding of the virus and the rapid and convenient nature of saliva collection which minimizes the need for direct contact between the patient and health care provider with notable savings of PPE. Many reports have shown that saliva contains higher viral loads than in NP swabs which is highest during the first week of infection and consequently the most infectious period of the disease. These reports also suggest that the viral load found in saliva is a good reflection of the transmission potential of a COVID-19 patient. NP qRT-PCR on the other hand remains positive long after the infectious period of the disease.<sup>7</sup>

The median viral load in posterior oropharyngeal saliva and other respiratory specimens at presentation is 5.2 copies per milliliter.<sup>8</sup> The viral load in saliva peaks during the first week of symptoms and subsequently declines in the next few days.

### Use of saliva as qRT-PCR sample

The study of Wyllie et al., demonstrated that saliva samples had comparable sensitivity to nasopharyngeal samples.<sup>9</sup> Their study population consisted of 70 SARS-CoV-2 confirmed cases who were diagnosed 1 to 5 days prior via NP qRT-PCR. They collected secondary NP samples and saliva samples for molecular testing which revealed a positivity rate of 71% and 81% respectively.<sup>9</sup>

The potential of saliva for SARS-CoV-2 testing was cemented by Watkins et. al 2020, when their study on pooled saliva-based qRT-PCR showed promising diagnostic utility. The potential of pooling saliva samples by 5, 10 and 20 prior to qRT-PCR was evaluated and showed sensitivities of 92.52%, 88.89% and 85.19%, respectively.<sup>10</sup> When the prevalence rate exceeds 3%, pools of 5 showed greatest resource savings.

SARS-CoV-2 rapid antigen test is a faster and simpler diagnostic method which enables result reporting in less than 30 minutes.<sup>3</sup> Though it is acknowledged that antigen tests exhibit lesser sensitivity than RT-PCR, the cases that were not detected by the antigen test but were detected by RT-PCR had higher CT values and in about half of the discordant samples that were cultured, none resulted in cytopathic effect indicating absence of viable virus.<sup>3</sup> Subsequent RT-PCR testing of culture supernatants were also negative. Thus, even if antigen tests are not as sensitive as RT-PCR, they still pick up most of the cases that are actually infectious.

The study of Nagura-Ikeda et al., demonstrated the presence of SARS-CoV-2 viral RNA via qRT-PCR in saliva samples of more than half of the asymptomatic individuals included in their study which detected ORF-gene and E-gene in 53.3% and 60.0% of cases respectively.<sup>11</sup> CT values for E-gene were also noted to be significantly lower in positive cases ( $25.4 \pm 1.8$ ) than in negative cases ( $30.8 \pm 2.7$ ). The rapid antigen test (Espline SARS-CoV-2; Fuji Rebio Inc., Tokyo, Japan), however, detected only 11% of the submitted saliva specimens.

Barat et al., evaluated the sensitivity of pooled saliva testing compared to individual nasopharyngeal testing via three

RT-PCR platforms. They noted an average signal loss of 2 to 3 cycle thresholds on pooled saliva specimens with an average sensitivity of 92.6% on all three platforms.<sup>12</sup> The study also noted that the sensitivity of pooled saliva testing increased when evaluating patients with moderate to high viral loads (cycle threshold  $\pm 34$ ).<sup>12</sup>

Rainey et al., adapted the pooled saliva protocol of Ranoa et al., and while adapting the protocol they noted that some individual samples contained an unidentified PCR inhibitor which blocked SARS-CoV-2 gene amplification as well as the amplification of the control RNA.<sup>13</sup> The same individual samples can also block amplification when they are added to pools leading to RT-PCR test failure.<sup>13</sup>

### Use of saliva as rapid antigen test sample

DNK-2105-1SDynamikerSARS-CoV-2(DynamikerBiotechnology (Tianjin) Co., Ltd.) is a lateral flow assay that utilizes SARS-CoV-2 Nucleocapsid protein (N protein) as the target analyte.<sup>14,15</sup> Based on manufacturer's data, it facilitates rapid and dependable testing with a test to result turnaround time of 10 minutes and a 92.98% and 99.07% sensitivity and specificity, respectively.<sup>15</sup> This high specificity is further supported by a multi-center study of Diao et. al wherein they found that all immunochromatographic assays that tested positive and negative for the N protein after three days of fever were in concordance with RT-PCR results.<sup>16</sup>

De Marinis et al., evaluated the sensitivity of four saliva rapid antigen testing platforms (Flowflex, PCL, Pabio and Joinstar) compared to nasopharyngeal and saliva RT-PCR. They noted that for RT-PCR sample cycle threshold values lower than 25, the sensitivity of saliva rapid antigen tests ranged from 43% to 64% while for cycle thresholds higher than 29, the sensitivity ranged from 9% to 19%.<sup>17</sup> The sensitivity of saliva rapid antigen tests also increased when the patient's symptom duration was 5 days or less (average 39.7%) and 10 days or less (average 47.0%).<sup>17</sup> However, for patients with symptom duration longer than 10 days the positive detection rate is 11%.<sup>17</sup> The lower rate of positive case detection of saliva rapid antigen testing was attributed to several factors such as subjects being recruited in the latter part of their COVID illness, some positive PCR tests may not reflect the presence of viable virus and freeze and thaw effect on saliva samples used in the rapid antigen testing.<sup>17</sup>

## OBJECTIVES

This study aimed to evaluate the diagnostic accuracy of saliva as the specimen of choice for detecting SARS-CoV-2 infection and specifically answered the following research objectives: (1) Evaluate the sensitivity of pooled saliva-based qRT-PCR compared with individual saliva qRT-PCR; (2) Evaluate the sensitivity, specificity, positive predictive value and negative predictive value of saliva-based rapid antigen test compared with individual saliva qRT-PCR.

## METHODOLOGY

### Design

This is a retrospective cross-sectional diagnostic accuracy study that determines the diagnostic utility of saliva-based testing using pooled qRT-PCR and Rapid Antigen Test compared with individual saliva qRT-PCR (Figure 1).

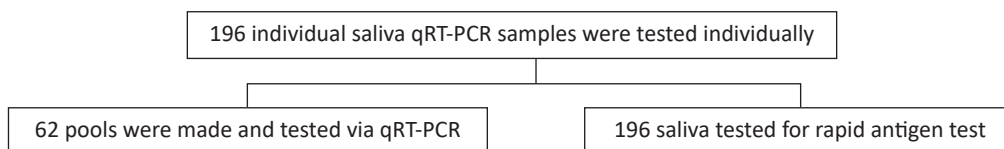


Figure 1. Study workflow diagram.

### Subjects and sample size

Included in this study were 196 saliva specimens taken from individuals aged 18 years old and above from the outpatient specimen collection station at Philippine Children's Medical Center (PCMC). Based on the results of the individual qRT-PCR saliva specimens, non-probability (convenience) sampling was done to determine the pools. Exclusion criteria includes indeterminate saliva qRT-PCR result and contaminated specimen.

The computed sample size for both pooled testing and rapid antigen testing is 73 based on a paper by Hajian-Tilaki which estimates the sample size in diagnostic studies and using the following assumptions: 95% sensitivity, marginal error of 10%, and 25% prevalence based on the Philippine SARS-CoV-2 positivity rate.<sup>18,19</sup>

### Collection and storage of saliva samples

Collection of saliva specimens from an individual is based on the PCMC's standard procedure for saliva-based SARS-CoV-2 qRT-PCR testing. The study participants included in this study were instructed not to eat, drink, brush their teeth, nor gargle for 30 minutes prior to collection. Morning saliva was preferred but not required. Sterile, screw-capped, wide mouth containers were given to the participants, and they were requested to self-collect 5.0 ml of saliva.

The collected saliva specimens were stored and transported to the laboratory at room temperature within 24 hours. Once received in the laboratory, individual saliva-based SARS-CoV-2 qRT-PCR and Rapid Antigen Testing were done immediately. The remaining saliva specimens were stored at 2-8° Celsius while waiting for the individual saliva-based SARS-CoV-2 qRT-PCR results. Based on the results of the individual saliva-based SARS-CoV-2 qRT-PCR, pools were done and tested using qRT-PCR. The remaining samples were kept for 14 days from the time of collection, then discarded using the hospital's biosafety waste disposal policy.

### Saliva processing for individual qRT-PCR

The individual saliva-based qRT-PCR was performed using 200  $\mu$ l aliquot of saliva. The samples underwent nucleic acid extraction using Mag-Bind RNA Extraction Kit/MACCURA and ALLSHENG Extraction Machine. SANSURE PCR KIT and MA-6000/Sansure Biotech were used for qRT-PCR amplification. The samples were subjected to magnetic bead extraction and placed in SANSURE master mix and template prior to qRT-PCR amplification. The rest of the procedures were performed according to the manufacturer's instructions.

### Interpretation of results for individual saliva-based qRT-PCR

All test controls were validated prior to interpretation of patient results. Tests without valid controls were interpreted

as invalid. The Cycle threshold (Ct) cutoff value was set at 38 as per SANSURE kit specification. All results with Ct values  $\leq 40$  and typical S-shape amplification curves as assessed were interpreted as SARS-CoV-2 positive. Results with Ct values  $\geq 40$  were interpreted as SARS-CoV-2 negative.

### Rapid antigen testing of saliva specimens

After an adequate aliquot was obtained for individual saliva-based qRT-PCR test, the saliva specimen was tested within an hour of collection using DNK-2105-1S Dynamiker SARS-CoV-2 Ag Rapid Test (Saliva) using the following instructions:

1. 1.0 ml of saliva was mixed with the rapid antigen kit buffer and placed into the extraction tube.
2. The extraction tube was recapped and mixed completely.
3. 3 drops of the solution were added to the test card.

### Interpretation of results for rapid antigen testing of saliva specimens

The result for the saliva antigen test was interpreted 10 minutes after dropping the saliva and buffer mix to the test card. The presence of two lines: one control and one test, even if faint, were considered as positive results. The presence of a control line only was read as negative. If the control line is not present, the result is invalid. Rapid antigen test kits were identified with the subject's accession number and photographed for data recording purposes and were kept in a secured data file.

### Saliva specimen processing for pooled qRT-PCR

A master list for all individuals selected and the individual saliva-based qRT-PCR results was prepared. From the list, 62 individuals with detected SARS-CoV-2 using saliva-based qRT-PCR and all the saliva samples of individuals which were negative were selected for pooled qRT-PCR.

To create a 1ml pool, one (1) 200  $\mu$ l aliquot of SARS-CoV-2 qRT-PCR-detected saliva specimen was mixed with four (4) aliquots of 200 $\mu$ l SARS-CoV-2 qRT-PCR-negative saliva samples. The 1ml pool was mixed using a vortex mixer for 30 seconds. From the mixed pooled sample, 200  $\mu$ l was obtained to undergo qRT-PCR using the same procedure and interpretation as the individual saliva-based SARS-CoV-2 qRT-PCR test.

### Data processing and analysis

Data was collated and analyzed using Microsoft Excel. The mean age, percentage of male and female participants and presence of symptoms were tabulated to illustrate the characteristics of all the participants.

### Pooled saliva-based RT-PCR

The sensitivity of the pooled saliva-based RT-PCR was computed through the following formula:

$$\text{Sensitivity} = \frac{\text{number of individual samples that were detected/positive using the pooling method}}{\text{total number of individual samples that tested positive using individual RT-PCR}}$$

Comparison of the Ct values of the ORF gene and N gene between the individual positive saliva specimen and the pooled specimen were done using paired T test, however, interpretation is limited due to use of non-probability sampling (Figure 2).

**Saliva-based rapid antigen test**

Diagnostic test evaluation of the saliva-based rapid antigen test was done using a 2 x 2 table. The formula for the calculation of sensitivity, specificity, PPV and NPV were as follows:

$$\begin{aligned} \text{Sensitivity} &= \text{True Positive (TP)} / (\text{TP} + \text{False Negative (FN)}) \\ \text{Specificity} &= \text{True Negative (TN)} / (\text{TN} + \text{False Positive (FP)}) \\ \text{Positive Predictive Value (PPV)} &= \text{TP} / (\text{TP} + \text{FP}) \\ \text{Negative Predictive Value (NPV)} &= \text{TN} / (\text{TN} + \text{FN}) \end{aligned}$$

Box plot was used to illustrate the Ct values of the specimen compared with the rapid antigen test result. Mann-Whitney U test was used to analyze if there is a statistically significant difference between the Ct values of those who tested positive and those who tested negative for the rapid antigen test. Interpretation is limited, however, due to use of non-probability sampling.

**ETHICAL CONSIDERATIONS**

The research was developed in compliance with the Data Privacy Act (2012) and National Ethical Guidelines for Health and Health-Related Research. Informed consents were requested from the participants to allow the use of stored saliva samples for rapid antigen and pooled testing prior to the commencement of the study.

To ensure the protection of the study participants, each data was treated with utmost confidentiality. No personal identifiable information was included and each data set was coded with a control number. Only the investigators were allowed to retrieve and have access to the data.

This study was funded by the Philippine Center for Entrepreneurship Foundation. The individual and pooled saliva-based SARS-CoV-2 qRT-PCR tests and rapid antigen tests were free of charge to the subjects. Official results

for the individual saliva-based SARS-CoV-2 qRT-PCR tests were given to the subjects. The results of the pooled saliva qRT-PCR and saliva rapid antigen testing were not disclosed to the subjects.

Approval was obtained from the Institutional Review Board prior to the commencement of the study (PCMC-IREC 2021-004).

**RESULTS**

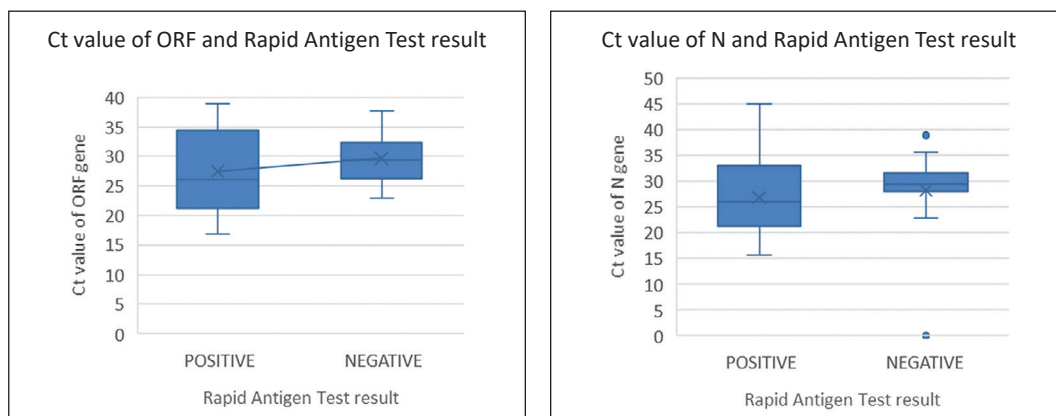
One hundred ninety-six (196) freshly collected saliva were obtained for the study. The characteristics of the study participants are summarized in Table 1. Of the saliva specimens tested, seventy-three (73) were detected to have SARS-CoV-2, while the remaining one hundred twenty-three (123) were not detected to have SARS-CoV-2 by qRT-PCR.

Table 1 describes the characteristic of participants. Among the participants who were detected to have SARS-CoV-2 by saliva qRT-PCR, the mean age is 38 years old. 37% is composed of female participants and 63% are male. 70% reported to have symptoms such as fever, cough, colds and sore throat. Among participants who were not detected to have SARS-CoV-2, 33% are females, 67% are males while only 3% presented with symptoms.

The diagnostic accuracy of the saliva rapid antigen test compared with saliva qRT-PCR as the standard is illustrated in Table 2. Overall, the saliva rapid antigen test has a sensitivity of 46.58% (95% CI 35.13%, 58.02%), specificity of 86.18% (95% CI 80.08%, 92.28%), positive predictive

**Table 1.** Characteristics of participants based on SARS-CoV-2 detection by saliva qRT-PCR

	SARS-CoV-2 Detected (n=73)	SARS-CoV-2 Not Detected (n=123)
Mean Age (years)	38	35
% Female participants	37% (27)	33% (41)
% Male participants	63% (46)	67% (83)
% Symptomatic participants	70% (53)	3% (4)



**Figure 2.** Distribution of the Ct values of the ORF gene and N gene compared with Rapid Antigen Test result.

**Table 2.** Diagnostic accuracy of saliva rapid antigen test compared with saliva qRT-PCR

Rapid Antigen test	Individual Saliva-based qRT-PCR		Total
	Positive	Negative	
Positive	34	17	51
Negative	39	106	145
Total	73	123	196
Sensitivity: 46.58% (95% CI 35.13%, 58.02%)			
Specificity: 86.18% (95% CI 80.08%, 92.28%)			
Positive Predictive Value: 66.68% (95% CI 53.71%, 79.60%)			
Negative Predictive Value: 73.10% (95% CI 65.89%, 80.32%)			

value of 66.67% (95% CI 53.71%, 79.60%), and negative predictive value of 73.10% (95% CI 65.89%, 80.32%).

The mean Ct values of the ORF gene were lower in those who tested positive (27.45) than those who tested negative (29.64) in the rapid antigen test. This is also true for the Ct values of the N gene, in which rapid antigen positive saliva has a lower mean Ct value (26.75) compared with rapid antigen negative saliva specimens (28.23). However, analysis using Mann-Whitney U test showed no statistically significant difference between the rapid antigen test results and the Ct values of the ORF gene ( $p=0.09296$ ) or the N gene ( $p=0.09692$ ). Interpretation of this statistical test may be limited due to use of non-probability sampling.

Among the 73 saliva specimens detected to have SARS-CoV-2, sixty-two (62) were included in the 1:5 dilution for the pooling part of the study since the other eleven (11) saliva specimens were fully consumed. Of the 62 pooled samples, sixty-one (61) were detected to have SARS-CoV-2 by saliva qRT-PCR. Based on this, the overall sensitivity of the 1:5 dilution of pools of saliva specimen is 98.39% (95% CI 91.34%, 99.96%). The ORF Ct value of the individual saliva specimen in the pool not detected is 35.37. From the day of collection up to the pooled saliva qRT-PCR, the number of days of interval ranges from 0 to 2 days, with the specimen stored at 2-8°C. Table 3 details the Ct values of the original individual specimen compared with the pooled saliva samples and the number of days of interval between the specimen collection and pooling.

Using the paired t-test of two sample for means, there was no significant difference ( $p>0.05$ ) noted between the Ct values of the ORF and N gene of the individual saliva specimen compared with the 1:5 dilution of pooled saliva specimen (Table 4). Interpretation of the result is limited by the use of non-probability sampling

## DISCUSSION

At present the use of nasopharyngeal and oropharyngeal (NP/OP) swab specimens for qRT-PCR is the standard for testing. Unfortunately, the use of NP/OP may hinder mass testing since collection will cause patient discomfort and pain and delays due to the involved collection procedure. Furthermore, the use of biosafety barriers and need for additional personnel is costly. These limitations in NP/OP sample collection prompted researchers to explore alternative samples for SARS-CoV-2 testing such as saliva. In the study of Oguri et al., antigen titers and Ct values of saliva samples remain unchanged after 72-hour storage in ambient temperature while Nasopharyngeal and oropharyngeal (NP/OP) swabs specimen displayed

decreased antigen titers when stored at the same temperature and duration. However, they noted that the Ct values in NP/OP swabs remain unchanged.<sup>20</sup>

In a meta-analysis of 51 studies done by Buban et al., it was shown that pooled researches of saliva samples have sensitivity of 84% and specificity of 96%.<sup>21</sup> These data were consistent with the meta-analysis done by Butler-Laporte et al., which showed that saliva NAAT has pooled sensitivity of 83.3% and specificity of 99.2%.<sup>22</sup>

On March 31, 2021, the DOH issued a memorandum on the interim guidelines for the conduct of saliva-based RT-PCR for the detection of SARS-CoV-2. In this memorandum, the use of saliva specimens is allowed for nucleic acid amplification (NAAT) based test, however, strict regulatory requirements, in-house verification and implementation arrangements were developed to ensure that laboratories that will offer saliva-based RT-PCR will perform the procedure correctly and accurately.<sup>4</sup> In relation to this, the Health Technology Assessment Council (HTAC) of the DOH also recommends the use of saliva specimen for RT-PCR as an alternative, provided that the healthcare workers assigned shall provide instructions and directly observe patients on the proper collection of saliva specimens.<sup>23</sup> The recommendation also suggests that the cost of using saliva specimens for public institutions should be significantly less than the government price cap for RT-PCR using NPS/OPS. The same DOH memorandum however does not allow the use of saliva specimens for antigen or antibody tests.<sup>4</sup>

### Saliva rapid antigen tests

As of January 17, 2022, nine (9) saliva-based antigen test kits including the kit used in this study, Dynamiker SARS-CoV-2 Ag Rapid Test is on Stage 2 (Pre-evaluation Stage) of the evaluation being done by the RITM.<sup>24</sup> The findings of this study showed a higher sensitivity for the Dynamiker SARS-CoV-2 Antigen Test compared with the kit used by Nagura et al., in their 2020 study which yielded only 11% sensitivity.<sup>11</sup> The authors attributed the low sensitivity to probable incompatibility of the saliva specimen to the test kits and the freeze-thaw and centrifugation process in their methodology.<sup>11</sup> The sensitivity of the rapid antigen test in this study is closer to the findings by Seitz et al., using COVID-19 Antigen Test Cassette (hypersensitive colloidal gold, Xiamen Zhongsheng Langjie Biotechnology Co., Ltd).<sup>25</sup> Seitz et al., documented an overall sensitivity of 44.4% and concluded that saliva antigen test is not a reliable substitute to RT-PCR.<sup>25</sup>

The results of this study also showed a specificity of 86.16% and positive predictive value of 66.68% for the saliva antigen test. The United States Food and Drug Administration (USFDA) published a report on the Potential for False Positive Results with Antigen Tests for Rapid Detection of SARS-CoV-2, and recommended following the manufacturer's instructions on the appropriate time to read the test result and minimizing the risk for cross-contamination.<sup>26</sup> A study by Patriquin et al., showed that direct sample testing (without the kit buffer) resulted in false positive signals in rapid antigen test kits, and the likely explanation is nonspecific interactions between the SARS-CoV-2 specific conjugated and capture antibodies as pH-induced conformational changes under

**Table 3.** Ct values of the individual saliva specimen compared with the 1:5 saliva pools and the number of days of interval between the specimen collection and pooling

Pool Accession Number	SARS-CoV-2 gene	Ct Values of individual SARS-CoV-2 detected specimen	Ct Values of Pools (1:5 dilution)	Result	Number of days from collection to pooling	Pool Accession Number	SARS-CoV-2 gene	Ct Values of individual SARS-CoV-2 detected specimen	Ct Values of Pools (1:5 dilution)	Result	Number of days from collection to pooling
1102CS-P5A	ORF	30.63	29.43	DETECTED	0	0111RS-P5AF	ORF	23.36	26.44	DETECTED	1
	N	29.10	29.06				N	21.29	23.73		
1102CS-P5B	ORF	31.90	28.17	DETECTED	0	0111RS-P5AG	ORF	38.88	37.17	DETECTED	1
	N	30.59	27.49				N	34.08	36.16		
1105RS-P5C	ORF	22.72	26.72	DETECTED	0	0111RS-P5AH	ORF	22.81	27.88	DETECTED	1
	N	22.45	29.44				N	21.44	27.17		
1105RS-P5D	ORF	23.97	24.54	DETECTED	0	0111RS-P5AI	ORF	17.51	18.69	DETECTED	1
	N	23.08	27.32				N	15.74	17.31		
1105RS-P5E	ORF	29.42	31.26	DETECTED	0	0111RS-P5AJ	ORF	27.22	26.24	DETECTED	1
	N	29.19	35.22				N	26.84	24.55		
0108RS-P5F	ORF	22.90	23.02	DETECTED	0	0111RS-P5AK	ORF	27.59	33.02	DETECTED	1
	N	24.86	25.24				N	26.15	31.91		
0108RS-P5G	ORF	26.26	27.00	DETECTED	0	0111RS-P5AL	ORF	29.40	33.43	DETECTED	1
	N	29.34	29.77				N	28.05	32.62		
0109RS-P5H	ORF	24.02	26.81	DETECTED	1	0111RS-P5AM	ORF	33.01	34.73	DETECTED	1
	N	27.59	25.33				N	31.34	34.06		
0109RS-P5I	ORF	27.38	29.11	DETECTED	1	0111RS-P5AN	ORF	31.13	32.95	DETECTED	1
	N	30.77	28.13				N	28.21	31.25		
0109RS-P5J	ORF	32.27	35.89	DETECTED	0	0111RS-P5AO	ORF	34.62	24.34	DETECTED	1
	N	35.31	34.11				N	31.53	23.95		
0109RS-P5K	ORF	26.90	33.59	DETECTED	0	0111RS-P5AP	ORF	—	39.17	DETECTED	1
	N	30.04	31.74				N	33.80	36.48		
0109RS-P5L	ORF	25.23	29.04	DETECTED	0	0111RS-P5AQ	ORF	34.10	37.83	DETECTED	1
	N	28.75	28.28				N	31.77	36.37		
0109RS-P5M	ORF	24.31	28.01	DETECTED	0	0111RS-P5AR	ORF	24.30	29.09	DETECTED	1
	N	28.53	27.25				N	22.88	27.02		
0109RS-P5N	ORF	16.84	18.54	DETECTED	0	0111RS-P5AS	ORF	37.14	31.63	DETECTED	1
	N	20.16	16.97				N	34.17	31.18		
0109RS-P5O	ORF	27.13	—	DETECTED	0	0111RS-P5AT	ORF	20.82	22.33	DETECTED	1
	N	30.36	33.82				N	18.13	20.72		
0109RS-P5P	ORF	29.33	29.62	DETECTED	0	0111RS-P5AU	ORF	31.98	30.10	DETECTED	1
	N	31.45	29.25				N	28.27	28.62		
0109RS-P5Q	ORF	24.31	25.63	DETECTED	0	0111RS-P5AV	ORF	27.23	26.60	DETECTED	1
	N	26.77	24.50				N	24.38	25.16		
0109RS-P5R	ORF	31.01	30.79	DETECTED	0	0111RS-P5AW	ORF	34.40	39.48	DETECTED	1
	N	33.73	29.93				N	33.98	35.73		
0109RS-P5S	ORF	28.83	33.43	DETECTED	0	0111RS-P5AX	ORF	21.08	26.04	DETECTED	1
	N	33.55	31.77				N	20.32	21.02		
0109RS-P5T	ORF	21.52	23.29	DETECTED	0	0111RS-P5AY	ORF	36.76	22.01	DETECTED	1
	N	23.27	21.37				N	35.58	20.84		
0109RS-P5U	ORF	35.68	38.05	DETECTED	0	0111RS-P5AZ	ORF	34.06	40.47	DETECTED	2
	N	38.87	37.06				N	33.27	36.55		
0109RS-P5V	ORF	24.46	31.27	DETECTED	0	0112RS-P5BA	ORF	30.92	29.31	DETECTED	1
	N	28.67	29.74				N	31.67	27.24		
0109RS-P5W	ORF	25.71	40.97	DETECTED	0	0112RS-P5BB	ORF	30.41	30.55	DETECTED	1
	N	28.23	36.72				N	33.24	28.94		
0109RS-P5X	ORF	35.37	—	NOT DETECTED	0	0112RS-P5BC	ORF	34.03	26.08	DETECTED	1
	N	—	—				N	31.42	24.34		
0111RS-P5Y	ORF	21.40	24.57	DETECTED	1	0112RS-P5BD	ORF	19.81	24.62	DETECTED	1
	N	19.06	22.54				N	21.18	19.74		
0111RS-P5Z	ORF	20.80	30.52	DETECTED	1	0112RS-P5BE	ORF	21.01	25.01	DETECTED	1
	N	19.28	24.37				N	24.46	23.41		
0111RS-P5AA	ORF	37.36	—	DETECTED	1	0112RS-P5BF	ORF	33.25	—	DETECTED	1
	N	34.01	39.32				N	39.21	32.87		
0111RS-P5AB	ORF	36.92	34.16	DETECTED	1	0112RS-P5BG	ORF	26.03	26.81	DETECTED	1
	N	33.55	32.07				N	24.40	22.30		
0111RS-P5AC	ORF	35.99	33.44	DETECTED	1	0112RS-P5BH	ORF	35.05	25.45	DETECTED	1
	N	33.16	32.69				N	29.40	21.76		
0111RS-P5AD	ORF	23.35	26.70	DETECTED	1	0112RS-P5BI	ORF	27.82	32.53	DETECTED	1
	N	22.79	26.06				N	30.88	27.83		
0111RS-P5AE	ORF	24.54	28.51	DETECTED	1	0112RS-P5BJ	ORF	26.29	25.18	DETECTED	1
	N	24.19	27.48				N	27.66	23.60		

**Table 4.** Paired t-test of two sample for means of Ct values of ORF and N gene between individual and pooled saliva specimen (interpretation is limited due to use of non-probability sampling)

SARS-CoV-2 gene	Individual Sample		Pooled Sample		T statistic	df	P (Tst) two-tail
	Mean	Variance	Mean	Variance			
ORF gene	27.7496	43.1424	27.5364	78.3749	0.1523	61	0.8794
N gene	27.8297	40.4573	27.8785	40.8710	-0.0930	61	0.9262

conditions promoting artifact formation.<sup>22</sup> The authors who performed the rapid antigen test strictly followed the manufacturer's instruction on the addition of the buffer, appropriate time interval from application to test kit to reading of the results, and minimized cross-contamination by using clean single-use pipettes.

### Pooled saliva qRT-PCR

In order to provide high-volume screening using saliva, this study looked into the utilization of pooling strategies in saliva samples. Pooling using NP/OP samples was previously demonstrated to be cost-effective and efficient at PCMC with pools of five (5) having the highest sensitivity.<sup>12,27</sup> In this study, pooling of saliva was done using five (5) saliva samples comprising of one (1) aliquot of SARS-CoV-2 detected saliva mixed with four (4) aliquots of SARS-CoV-2 negative saliva. This yielded a sensitivity of 98.39%, and there were no statistical differences observed in the ORF and N-gene Ct-values of the individual and pooled saliva test results.<sup>27</sup>

A study by Barat et al., which included 449 individuals showed that the positive and negative agreement of saliva samples compared to NP swabs were 81.1% and 99.8% respectively. Pooling of the saliva samples using pools of five (5) showed sensitivities of 94%, 90%, and 94% using easyMAG/ABI 7500, Hologic Panther Fusion, and Roche Cobas 6800, respectively. An average decline in pooled Ct-value of 2 to 3 was noted in comparison with individual saliva testing.<sup>12</sup>

In a study done by Sahajpal et al., which used twenty (20) positive and negative pools consisting of five (5) samples per pool, testing done demonstrated 95% positive agreement and 100% negative agreement. The N and ORF1ab gene Ct values of pooled samples were found to be significant compared to individual saliva testing Ct values.<sup>28</sup>

A study by Esteves et al., which used 279 saliva samples for qRT-PCR yielded sensitivity, specificity, positive predictive value, and negative predictive value of 96.6%, 96.8%, 96.6% and 96.8% respectively. Furthermore, pooling strategies were done using 10-sample pools and 20-sample pools producing a sensitivity of 96.9% and 87.5% respectively.<sup>29</sup>

As shown by prior studies, the use of saliva for qRT-PCR showed high sensitivity and specificity when contrasted with NP/OP qRT-PCR. Moreover, pooled saliva testing showed no significant decline in testing sensitivity and congruence of Ct-values between individual and pooled saliva samples was observed in this study.

Despite the promising results of saliva pooling strategies, it is important to take note that in this study one positive sample was not detected using pooled testing. Prior studies also reflected that few samples were not detected using

pooling strategies and some noticed a significant difference in the Ct-value of pooled and individual saliva samples. The small error in detecting SARS-CoV-2-detected saliva samples and incongruence of Ct-values may be due to the limitation of using saliva as a specimen and/or using pooling as a diagnostic strategy.

Saliva as a specimen may differ in viscosity, hence under pipetting may be an issue due to the presence of bubbles and variation in viscosity which may lead to a false-negative result.<sup>7</sup> Moreover, an inherent limitation of pooled testing is the minute risk of not catching weak positive individuals due to sample dilution and the advance technical factors needed to produce consistent pools.<sup>12</sup>

Overall, the results show that pooled saliva testing may be a useful and economical strategy to initiate mass testing in asymptomatic and non-close contact populations such as in work settings and social gatherings. This strategy will eliminate the need for NP/OP swabs, viral transport media, and minimize usage of personal protective equipment thereby lowering the overall cost for the testing laboratory and tested individuals.

## CONCLUSION AND RECOMMENDATIONS

The importance of accurate testing for SARS-CoV-2 during the ongoing pandemic cannot be understated. Reliable diagnostic options should be widely available and commonly known to both clinicians and patients alike. In any setting, but especially the local one, the decision to use a particular testing method needs to take into account the test's sensitivity, potential risks to healthcare providers or patients, estimated costs, simplicity and convenience, and the general population. Scientific literature has demonstrated the effectiveness of using saliva to detect viral load and that it compares favorably with nasopharyngeal swab specimens. The ease with which it can be collected and subsequently tested helps to provide a solution as to how more testing and contact tracing can be done. As testing laboratories become certified to provide this option, it becomes imperative to find ways to improve the process to be more efficient and cost-effective. The data from this study clearly demonstrates that pooled saliva-based testing for SARS-CoV-2 is a reliable and accurate tool to help augment testing. However, it must be emphasized that this study is brand and method-specific and needs to be replicated by other laboratories using their own kits and methods.

For rapid antigen testing using saliva specimens the data is not as clear, though the results from this study are in line with previously published reports. Further studies are needed to determine if there are optimal conditions including particular test kits, reagents, specimen volume, and time to test that can support this applicable method for screening and diagnosis.

Pooled saliva qRT-PCR testing may be an invaluable tool in a pandemic scenario where lower test costs and accurate, faster results are necessary to embark on more widespread testing in outbreak scenarios. Pooled testing is not designed to test symptomatic individuals or their close contacts; rather it is meant to rapidly screen large numbers of people in a locality where community transmission is suspected to prevent further spread of the virus. This method has been and still is being successfully deployed in several countries like China, South Korea and some European countries. But it takes time, effort, personnel and resources to effectively swab and screen large populations that are not at high risk of infection. Using saliva as a sample for pooled testing changes the dynamics with decreased demand for all these factors thus effecting a faster response when results are released earlier.

With the increasingly relaxed restrictions necessary for promoting increased economic activity comes the risk of further outbreaks as people start to congregate in large numbers and indoors. We believe saliva pooled testing will become an important tool to suppress these outbreaks should they occur. We are by no means totally safe when a large segment of the population is still unvaccinated, necessitating control measures such as saliva pooled testing.

Even in a post-pandemic scenario, saliva pooled testing can be useful in routine surveillance for possible outbreaks. In a statement of the PSP on Strategic Testing to Manage Local Outbreaks, it states that limited testing and contact tracing will not address the rapid spread of the virus and thus, more widespread testing in a particular locality under lockdown should be done to effect control of the virus.<sup>30</sup> It proposed pooled testing as a means to mass test at a faster rate, achieve economy of scale with test savings within a certain range of positivity and still achieve regular turnaround time as individual testing. The use of saliva samples for pooled testing will further decrease costs as well as time and personnel resources if used in these situations.

Likewise, it can be a model for mass testing in future pandemics of respiratory pathogens with a significant presence in saliva. There will be no need to mobilize large numbers of swabbers who will have to be trained to perform the procedure safely and efficiently thus ensuring a faster response and may be crucial in determining the course of the pandemic in a particular country. In an archipelagic nation such as the Philippines, immediate border closures followed by mass testing in suspect populations should be sufficient in delaying if not stopping the entry and/or community transmission of a virus and allowing time for the health system to gear up and be prepared for the pandemic.

In conclusion, we have demonstrated the viability of saliva samples for pooled SARS-CoV-2 qRT-PCR testing as a means of fast, efficient and accurate determination of infection status for use in asymptomatic, low positivity rate populations. It will be very useful in both the current pandemic situation as well as in transitioning to an endemic situation. Further, it will also serve as a model for future pandemic response with respiratory pathogens with significant presence in saliva.

## STATEMENT OF AUTHORSHIP

The authors certified fulfillment of ICMJE authorship criteria.

## AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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# Non-parametric Clinical Laboratory Reference Interval Estimation in Volunteer Blood Donors: An Example for Prothrombin Time and Partial Thromboplastin Time

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## ABSTRACT

**Introduction.** To date, there are no reference intervals for prothrombin time (PT) and activated partial thromboplastin time (APTT) based on “normal” Filipino adults. The common practice in most laboratories is to adopt manufacturer provided values or foreign literature even if the importance of establishing or at least verifying laboratory reference intervals has been stressed by Clinical Laboratory Standards Institute (CLSI).

**Objectives.** Here we aim to describe our experience in using a simple non-parametric method to generate reference intervals for PT and APTT, from healthy Filipino volunteer blood donors.

**Methodology.** We used a *de novo*, *a priori* non-parametric estimation method following the CLSI guidelines on establishing reference intervals.

**Results.** The non-parametric lower reference limit for PT is 12.55 seconds, with 90% confidence interval of 12.3 to 12.75 seconds. While the non-parametric upper reference limit for PT is 16.15 seconds, with 90% confidence interval of 15.55 to 16.55 seconds. The non-parametric lower reference limit for activated partial thromboplastin time is 26.12 seconds, with 90% confidence interval of 22.95 to 27.1 seconds, and the non-parametric upper reference limit for activated partial thromboplastin time is 37.44 seconds, with 90% confidence interval of 36.75 to 38.65 seconds. The PT and APTT reference intervals were different from foreign sources and manufacturer provided values in terms of interval width and values of the reference limits by 2 to 4 seconds.

**Conclusion and Recommendations.** Estimation of coagulation reference intervals from volunteer health blood donors is doable, simple, and practical. Collaborative multi-center efforts may be done to expand the pool of reference individuals that are included and increase the representativeness of the reference intervals generated. This simple method can also be used to generate reference intervals for other clinical laboratory assays and may also be extended to at least verify reference intervals in special populations like pregnant women, the elderly, and the pediatric population.

*Key words:* coagulation, non-parametric reference intervals, Filipino, Prothrombin time (PT), Partial Thromboplastin Time (PTT)

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## INTRODUCTION

Physiologic hemostasis, or the prevention/cessation of bleeding, is a tightly regulated process of plasma coagulation, fibrinolysis, and anticoagulation protein systems. Physiologic hemostasis and thrombosis are initiated by factor VIIa and tissue factor, and the latter is also amplified by factor XII activation on injured tissue and platelet thrombus.<sup>1</sup>

The activated partial thromboplastin time (APTT) and prothrombin time (PT) are two assays routinely used to assess coagulation protein abnormalities. Both are extremely useful for assessing the integrity of the blood coagulation system and for recognizing potential bleeding problems in a patient.<sup>1</sup>

The APTT is induced by surface (contact) activation of the system, while the PT is induced by the addition of excess



tissue factor. Contact activation occurs when artificial, negatively charged particles in the reagent autoactivates Factor XII, which in turn, initiates the proteolytic coagulation cascade. With the PT test, the addition of physiologically excessive Tissue Factor (TF) allows factor VIIa to overcome the inhibitory effect of Tissue Factor Pathway Inhibitor (TFPI), favoring the direct activation of Factor X to Factor Xa.<sup>1</sup>

An abnormal APTT is associated with Factor VIII, IX, and XI defects, if the patient is bleeding and with Factor XII, prekallikrein (PK), high molecular weight kininogen, and lupus anticoagulants if there is no bleeding. An abnormal PT is most often due to Factor VII defects. When both the APTT and PT are abnormal, the culprits are usually anticoagulants, disseminated intravascular coagulation (DIC), liver disease, vitamin K deficiency, and massive transfusion.<sup>1</sup>

The major purpose of performing analyte determinations in the clinical laboratory is to aid in the diagnosis and management of disease and in health assessment.<sup>1</sup> And the interpretation of PT and APTT results, just like all other laboratory examinations, involves comparison with reliable reference intervals. Needless to say, reference intervals are essential information used by health professionals in their day-to-day clinical decision making.<sup>2,3</sup>

The reference interval is the interval between and including two numbers, an upper and lower reference limit, which are estimated to enclose a specific percentage (usually 95%) of the values for a population from which the reference subjects are drawn. For most analytes, the lower and upper reference limits are estimated as the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentiles of the distribution of test results for the reference population, respectively.<sup>4</sup>

As defined by Ceriotti, "It is an interval that, when applied to the population serviced by the laboratory, correctly includes most of the subjects with characteristics similar to the reference group and excludes the others."<sup>5</sup>

There are 3 possible means by which to obtain the Reference intervals (RI) of a given analyte for a given population:<sup>3</sup>

1. determine the RI *de novo* from measurements made in reference individuals;
2. transfer a pre-existing RI when a method/instrument is changed; or
3. validate a previously established or transferred RI.

*De novo* determination of RIs is the most frequently used procedure and is the recommended approach in medical and veterinary laboratories, as indicated in the original IFCC recommendations. In this method, reference individuals are selected according to a predefined criteria followed by determination of RIs from the reference values obtained. This approach is most often performed in a single laboratory, but a multicentric procedure also is possible if methods and populations are comparable. In some cases, an a posteriori approach is used in which pre-existing data is exploited to establish reference values.<sup>3</sup> Establishing, as opposed to verifying, reference intervals is clearly more difficult because of the daunting numbers of reference individuals required. But the ability to pool

data from several laboratories using the same method and the availability of new statistical techniques may ease the burden considerably.<sup>6</sup>

It is important to use normal ranges specific to the population being considered because the published normal values may not be entirely applicable. There may be important differences in the values and ignoring this fact may lead to over or under treatment of patients. Examples of this include differences in the serum creatinine (race specific) as well as effect of region on some specific proteins in asians.<sup>7,8</sup> And even if for most examinations, there are few data documenting such differences, it is dangerous to assume that just because there is no documentation, there is no difference.<sup>8</sup>

Unfortunately, reference ranges for PT and APTT have not been established in the Filipino population. Literature search using the UP-Manila Research Database, which includes articles indexed in the Philippine Index Medicus, as well as unpublished theses and dissertations, returned no relevant result. In the Philippine General Hospital (PGH), reference ranges are provided for by the manufacturer of the analyzers and validation of these values is not routinely performed.

Because performing full blown *a priori* reference interval is both expensive and time consuming, it is common practice for laboratories to adopt reference ranges from the manufacturer, foreign laboratories, or from foreign publications, sometimes even without verifying their applicability.<sup>6</sup> But this practice has serious consequences, as described in a study by Brewster et. Al., wherein the manufacturer-defined reference ranges for creatinine was found to be narrower than the one established by the group, resulting in individuals unnecessarily being deprived of cholesterol-lowering medications.<sup>9</sup>

It is therefore still imperative, at the very least, to verify, using as little as 20 reference individuals, the adequacy of reference intervals on a regular basis. And for tests where accuracy is extremely important, laboratories should participate in peer-group quality assessment surveys.<sup>6</sup>

In this paper, we describe our experience in generating a *de novo*, *a priori* non-parametric reference intervals for prothrombin time and activated partial thromboplastin time, using healthy volunteer blood bank donors as the reference population.

## METHODOLOGY

This is a descriptive, cross-sectional study (WHO Classification)<sup>10</sup> done in accordance with the CLSI EP28 A3c recommendations.<sup>4</sup> For a relatively short accrual period of 26 days, from July to August 2011, 122 physically fit, adult Filipinos, with ages ranging from 18 to 55 years old, who came to the PGH Blood bank to donate blood, and who were found to be asymptomatic and physically normal after being interviewed and examined by a medical technologist and a physician, were considered for inclusion in the cohort of reference individuals. We used relevant items in the standardized in-house donor screening criteria as the exclusion criteria for the reference individuals. These

criteria are essentially similar to the CLSI recommended list. No donor identifying information were collected, all samples were de-identified, and all volunteer blood donors signed the informed consent form.<sup>4</sup>

During the conduct of standard procedures in screening volunteer blood donors, licensed medical technologists aliquot about 10 ml of blood during the blood-letting procedure. Five (5) ml aliquot of blood was transferred in a blue-top tube, containing the additive Sodium citrate, and was sent for coagulation examination. The other 5 ml aliquot was collected in a Red-top tube and was sent to the blood bank for routine donor blood testing.

The blood samples were analysed using ACL Elite Pro (Instrumentation Laboratories) following the manufacturer’s manual of procedures, and in accordance with the standard operating procedures of the Blood Bank and Department of Laboratories.

Reference intervals, including the 90% confidence intervals for the upper and lower limits were calculated using the non-parametric method outlined in the CLSI document.<sup>4,11</sup>

Briefly, the nonparametric method as described in section 9.4.1 of CLSI EP28-A3c consists of the following steps:

1. the observations are ranked from smallest to largest (smallest is  $r = 1$ , and largest is  $r = n$ );
2. the non-parametric 95% lower reference limit,  $r_1$ , shall correspond to the value of the observation that is ranked  $r_1 = 0.025*(n + 1)$ ;
3. the non-parametric 95% upper reference limit,  $r_2$ , shall correspond to the value of the observation that is ranked  $r_2 = 0.975*(n + 1)$ ;
4. the rank values of  $r_1$  and  $r_2$  are rounded up to the nearest integer of the calculated values;
5. the non-parametric confidence intervals of the upper and lower non-parametric reference limits are then obtained from Table 8 of CLSI EP28-A3c (which, in turn, was adapted with permission from Solberg HE. Approved recommendations [1987] on the theory of reference values. Part 5. Statistical treatment of collected reference values. Determination of reference limits. Journal of Clinical Chemistry and Clinical Biochemistry. Vol. 25. Berlin, Germany: Walter de Gruyter GmbH & Co. KG; 1987, pp. 645-656. Table 1);
6. the lower and upper 90% confidence interval limits for the lower reference interval limit, correspond to the values of the lower and upper rank numbers indicated in Table 8, at the row corresponding to the sample size of the data obtained;
7. the lower and upper 90% confidence interval limits for the upper reference interval limit, correspond to the values of the ranks:  $(n + 1) - \text{upper rank number}$  and  $(n + 1) - \text{lower rank number}$ , at the row corresponding to the sample size of the data obtained;

Note: The reader is referred to CLSI guidance document EP28-A3c for a more detailed discussion of the methodology and for guided examples on how the method is done using actual data.

## RESULTS AND DISCUSSION

We were able to include blood aliquots from 122 reference individuals. Among these, 109 were from males and 13 were from females - for a male to female ratio of 8 to 1. This disproportionate predominance of males is explained by the fact that there are more males donating blood compared to females in the hospital blood donation unit and is expected of the data. The average age of the reference individuals is 28 years old. The youngest is 18 years old and the oldest is 43 years old. There are no data on the weight and height of the reference individuals.

Using the method outlined by the CLSI EP28-A3c guidance document, the non-parametric lower reference limit for PT was 12.55 seconds, with 90% confidence interval of 12.3 to 12.75 seconds. While the non-parametric upper reference limit for PT was 16.15 seconds, with 90% confidence interval of 15.55 to 16.55 seconds. The non-parametric lower reference limit for activated partial thromboplastin time was 26.12 seconds, with 90% confidence interval of 22.95 to 27.1 seconds, and the non-parametric upper reference limit for activated partial thromboplastin time was 37.44 seconds, with 90% confidence interval of 36.75 to 38.65 seconds (Table 1).

If we incorporate the 90% upper and lower confidence intervals for the reference limits, the reference interval for PT can be as narrow as 12.75 to 15.55 sec or as wide as 12.3 to 16.55 sec, and the reference interval for APTT can be as narrow as 27.1 to 36.75 or as wide as 22.95 to 38.65 seconds.

Computing for the reference interval for INR, which is the quantity commonly reported for PT exams, is complicated as it involves prior estimation of the median normal PT and is not included in this paper. The median normal PT value from this study, however, can be used in the establishment of a reference interval for the INR.

The width of the PGH reference interval for PT is almost the same as that of the manufacturer, data from the Massachusetts General Hospital (MGH), the Merck Manual, and Henry’s Clinical Diagnosis and Management by Laboratory Methods. But the reference limits are different. The lower reference limit is longer by up to 2 seconds and the upper reference limit is longer by up to 3 seconds (Table 2).

The PGH lower reference limit for APTT is up to 4 seconds longer than other sources while the upper reference limit is

**Table 1. Coagulation non-parametric reference intervals with 90% confidence intervals (n = 122; unit = seconds)**

Test	Lower reference limit			Median Reference value	Upper reference limit		
	Estimate	90% LCI	90% UCI		Estimate	90% LCI	90% UCI
PT	12.55	12.30	12.75	14.03	16.15	15.55	16.55
APTT	26.12	22.95	27.10	30.48	37.44	36.75	38.65

**Table 2.** Reference Interval for Prothrombin Time (seconds) from different sources

Source	PGH	Estimated value			
		Manufacturer	Henry's	MGH	Merck Manual
Sample size	122.0	35.0	–	–	–
Upper Reference Limit	16.1	15.1	13	13.7	13
Median Normal Value	14.0	–	–	12.3	–
Lower Reference Limit	12.6	11.8	10	11.0	11

**Table 3.** Reference Interval for Activated Partial Thromboplastin Time (seconds) from different sources

Source	PGH	Estimated value			
		Manufacturer	Henry's	MGH	Merck Manual
Sample size	122.0	37.0	–	–	–
Upper Reference Limit	37.4	38.4	35	33	35
Median Normal Value	30.5	–	–	–	–
Lower Reference Limit	26.1	25.4	25	21	25

also up to 3 seconds longer compared to other limits. The width of the reference interval is narrower (12 seconds) than the manufacturer, but at most only a bit wider than that of MGH, Henry's and Merck (Table 3).

The differences in the reference limits can be attributed to differences in the reference populations or the characteristics of the analytic process itself. The PT and APTT are known to vary among laboratories especially since different preparations of reagents are used. In particular, variability of “normal” APTT is attributed to significant different coagulation factor activities.<sup>13</sup> Nevertheless, institution-specific locally generated reference interval still provide information used for clinical decision making.

By using blood aliquots from healthy volunteers who donated blood in the hospital's blood donation unit, we were able to establish our own reference intervals for PT and PTT. This method, based on CLSI EP28-A3c guidance, is doable, simple, and practical.

Because of the differences in reference interval characteristics found in this study for normal adult population, we highlight the need to establish reference intervals using reference individuals from the population the laboratory primarily caters to. Corollary to this is the need to establish or at least validate reference intervals for pregnant, the elderly, and the pediatric group. Adopting foreign or manufacturer provided reference intervals as is, may not be adequate and applicable to these populations.

**CONCLUSION**

Here we were able to describe an estimation method for coagulation reference intervals, based on the CLSI guidance, that is doable, simple, and practical, by considering healthy volunteers who donate blood in the hospital's blood donation unit. The PT and APTT reference intervals we generated were slightly different from foreign sources and manufacturer-provided values in terms of interval width and values of the reference limits by 2 to 4 seconds. Collaborative multi-center efforts may be done to expand the pool of reference individuals that are included and

increase the representativeness of the reference intervals generated. This simple method can also be used to generate reference intervals for other clinical laboratory assays and may also be extended to at least verify reference intervals in special populations like pregnant women, the elderly, and the pediatric population.

**STATEMENT OF AUTHORSHIP**

The authors certified fulfillment of ICMJE authorship criteria.

**AUTHOR DISCLOSURE**

The authors declared no conflict of interest.

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## Prevalence of CKIT and PDGFRA Mutation in Gastrointestinal Stromal Tumors among Filipinos

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### ABSTRACT

**Background.** Gastrointestinal stromal tumors (GIST) are defined as specific, typically kit (CD117)-positive and CKIT or platelet-derived growth factor receptor alpha (PDGFRA) mutation-driven mesenchymal tumors that can occur anywhere in the GI tract. GIST diagnosis relies heavily on immunohistomorphology. However, with the advent of molecular testing, the classification, diagnosis, and targeted therapy for gastrointestinal mesenchymal tumors have been improved. In the Philippines, molecular testing is not yet readily available as in other countries. The local molecular profile of gastrointestinal stromal tumors is a point of investigation as treatment may be more tailored to the patients' needs.

**Objective.** This study aims to determine the prevalence of CKIT and PDGFRA mutations among formalin-fixed and paraffin embedded gastrointestinal stromal tumors and other gastrointestinal mesenchymal tumors in St. Luke's Medical Center – Quezon City.

**Methodology.** A retrospective cross-sectional study of formalin fixed and paraffin embedded tumor samples diagnosed as Gastrointestinal Stromal Tumor from January 1, 2009 to December 31, 2017 will be analyzed for KIT and PDGFRA mutations.

**Results.** The epidemiology of GIST remains constant in that mean age group is the 5th to 6th decade, with equal gender distribution, and stomach followed by small bowel are the most common sites. Mutational analysis of the GISTs predominantly showed KIT Exon 11 (47.83%) followed by CKIT Exon 9 (13.04%) and PDGFRA Exon 18 (10.87%). For KIT Exon 11, deletion is the most common mutation followed by point mutations. No mutation is detected in 47.83% of GISTs.

**Conclusion.** Mutational analysis for CKIT-PDGFRA is warranted among GIST patients, as it may significantly influence the treatment protocol of patients.

*Key words:* Gastrointestinal Stromal Tumors, GIST, Sequencing, CD117, CKIT, PDGFRA

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### INTRODUCTION

Gastrointestinal stromal tumors (GIST) can be defined as specific, typically kit (CD117)-positive and KIT or platelet-derived growth factor receptor alpha (PDGFRA) mutation-driven mesenchymal tumors that can occur anywhere in the gastrointestinal (GI) tract.<sup>1,2</sup> It is a relatively rare soft tissue sarcoma which commonly arises in the stomach (60%), followed by jejunum and ileum (30%), duodenum (5%), colorectal (<5%), and rarely in the esophagus or appendix.<sup>3</sup> GISTs may also occur as primary tumors outside of the GI tract, in the retroperitoneum or abdomen (e.g., omentum, mesentery), and such tumors have been referred to as extra-gastrointestinal stromal tumors.<sup>1</sup> GISTs arise mostly in middle-aged or older individuals, and some arise as congenital tumors in children, with no sex predilection. These may be asymptomatic or manifest with GI bleeding and abdominal pain. Other clinical symptoms include nausea, vomiting, weight loss and the presence of abdominal mass.<sup>4-6</sup> The vast majority of GISTs are



sporadic with no known associated risk factors, however, approximately 5% are associated with a tumor syndrome, including neurofibromatosis type 1 (NF1), Carney's triad (pulmonary chordoma, paraganglioma, GIST), and familial GIST syndrome.<sup>7,8</sup>

GISTs are either derived from or differentiate toward interstitial cells of Cajal, which act as the pacemaker cells of the gut and serve as intermediaries between the GI autonomic nervous system and smooth muscle cells to regulate GI motility and coordinate peristalsis.<sup>3,9</sup> GISTs were originally considered to be of smooth muscle origin, due to their histology. Due to its spindle cell characteristic, in the past, these tumors were classified as other gastrointestinal muscle tumors (GMT) such as leiomyomas, leiomyosarcomas, leiomyoblastomas and spindle cell neoplasms.<sup>10</sup> Hence, their true frequency is unknown. Epidemiologic data provided by the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program may be difficult to interpret since the early definition "malignant GIST" was taken from the criteria published in 1990, before the molecular classification of GIST.<sup>6</sup> Current epidemiologic studies done showed an annual incidence of 14.5 per million in Swedish,<sup>2</sup> 11 per million in Icelandic<sup>11</sup> and annual incidence of at least 4,000 to 6,000 new cases per year in the United States.<sup>3,6</sup> In Taiwan, the reported incidence is 1.13 per 100,000 in 1998, with an increase to 1.97 per 100,000 in 2008.<sup>11</sup> Shanghai epidemiologic studies showed average crude incidence of GISTs of 2.11 per 100,000 between 2004 and 2008.<sup>12</sup> However, in the Philippines, no studies have been done. Since CKIT and PDGFRA mutation testing has not been previously performed in the Philippines, we will compare the prevalence of mutations in these genes among our GIST cases with from the literature.

At present, GIST is diagnosed in our institution using the following immunohistochemical stains: CKIT/CD117, DOG1, CD34, smooth muscle actin, S100 and desmin. Among these stains, CKIT/CD117, a very specific and sensitive marker in the differentiating GIST from other mesenchymal tumors in the GI tract is most widely used.<sup>13,14</sup> Despite the significant therapeutic implications of CKIT/CD117 positivity, the intensity, extent and patterns of KIT staining does not correlate with the type of KIT mutation or its response to available medications.<sup>15</sup>

Molecular advancements in pathology have established that KIT mutations, majority of which are somatic which cause the constitutive activation of the kinase, are found in 70-80% of GISTs.<sup>1,16</sup> The oncogenic activation of KIT is the dominant pathogenetic mechanism in GIST.<sup>16</sup> However, about 5% of GISTs lacking KIT gene mutations harbor activating mutations in PDGFRA.<sup>3</sup> Molecular findings have led to the development of tyrosine kinase inhibitors, the prototype of which is imatinib. These inhibit the c-KIT and PDGFRA by competing with the adenosine triphosphate-binding site required for phosphorylation and activation of the receptor, hence, inhibiting tumor proliferation.<sup>1,3,16,17</sup> Imatinib has been considered as the standard treatment for GIST. Partial response is achieved in 65 to 70%, but 15-20% maintain stable disease.<sup>3</sup>

GIST is classified into three molecular categories based on the mutations of the KIT and PDGFRA gene: GIST with KIT mutations, GIST with PDGFRA mutations, and non-KIT or PDGFRA somatic mutations that are designated as wild type.<sup>18</sup> The wild type variation is considered complex due to the existence of different subgroups with distinct molecular hallmarks, such as deletion mutations of succinate dehydrogenase subunit A (SDHA) and mutations of neurofibromatosis type 1 (NF1), RAS, or BRAF.<sup>19</sup> Advancement in molecular pathology, has identified PDGFRA mutations, in 5 to 10% of GIST's.<sup>1,16</sup> Mutations of PDGFRA on exon 12, 14, and 18 are mostly implicated. However, PDGFRA exons 12 and 14 mutations have a low frequency of <1%, with PDGFRA exon 18 having a relatively higher frequency of 6 to 7%.<sup>16</sup> PDGFRA, although a close homologue of CKIT, are more gastric in location, and is associated with epithelioid morphology and indolent course.<sup>16,20</sup> Ultimately, GIST can be characterized as a cancer with comparatively small genetic variation; hence, the precise treatment of the cancer gene map for GISTs has become seemingly evident and apt.

Recent data show that GIST patients respond differently to tyrosine kinase inhibitors (drugs like imatinib and sunitinib), depending on the specific mutations displayed by their tumors.<sup>3</sup> Most deletions and deletions preceded by substitutions result to active conformation of the normal kinase activation loop.<sup>16</sup> KIT mutations in exon 11 is the most common mutation, and is seen in 70% of cases. These are commonly seen in the gastric and small bowel and has a higher risk of relapse after surgical resection.<sup>21</sup> The second most common KIT mutation is seen in the extracellular domain encoded by exon 9.<sup>20</sup> It has a frequency of 10 to 15%, and may reach up to 18.1%.<sup>1,16,21</sup> Exon 9 mutations are usually seen in the small bowel, and with an aggressive clinical behavior. Less than 1% of GIST harbor mutations in the exon 13 and 17.<sup>1,16</sup>

Mutational analysis of the KIT gene (exons 11, 9, 13, and 17) and PDGFRA gene (exons 12, 14, and 18) may aid in confirming GIST if immunohistochemical stains fail to support the diagnosis.<sup>16</sup> At present, GIST mutational analysis is recommended in the NCCN (National Comprehensive Cancer Network) and ESMO (European Society for Medical Oncology) clinical recommendations.<sup>16,22,23</sup> Such recommendations have provided clinical significance in therapeutic aspects for its predictive value for sensitivity to molecular-targeted therapy (including dosage) and prognostic value.<sup>16</sup> A study done by Heinrich and Corless et al., indicate a stronger response to imatinib in patients with KIT exon 11 mutations than patients with exon 9 mutations. Patients with an exon 11 GIST mutation were much more likely to have a partial response with imatinib therapy than those with exon 9 or no mutations.<sup>1,3</sup> In contrast, patients with KIT exon 9 mutations, resistant to imatinib, showed better response to a tyrosine kinase inhibitor (sunitinib).<sup>16</sup> GISTs with PDGFRA exon 18 mutation (D842V) show primary resistance to imatinib both in vivo and in vitro.<sup>1,16</sup> Another utility of mutation testing involves the identification of newly acquired secondary mutations, not initially detected in the primary tumor, that can confer drug resistance to imatinib.

The spectrum of mutations in gastrointestinal stromal tumor is still unknown among Filipino patients. The diagnosis and treatment of GIST currently relies on immunohistochemical staining of GIST tumor with CD117 antibody. This study aims to characterize the CKIT and PDGFRA mutations among Filipino patients diagnosed with GIST in our institution.

### METHODOLOGY

Following approval by the institutional review board, a retrospective review of all formalin fixed paraffin embedded (FFPE) tumor samples diagnosed with GIST from the period of January 2009 to December 2017 was performed. All samples were from pre-treatment procedures and were from primary tumor sites. No samples were taken from recurrence or metastatic sites. The age, sex, histopathologic diagnosis, and location of the tumors were recorded.

#### Sample collection

DNA was isolated from FFPE samples after deparaffinization and extraction of 3–5 mm thick paraffin sections in xylene and by using the QIAamp DNA FFPE Tissue Kit (Qiagen) per the manufacturer’s instruction. In samples with DNA concentration of less than 5 ng/ml, a second extraction from another tissue block was performed. Those with DNA concentration less than 5 ng/ml after second extraction were excluded. DNA purity was measured using Nanodrop 1000. A A260/280 ratio of between 1.7-2 was deemed acceptable for subsequent reactions. Suboptimal samples were also excluded.

#### Primer identification

Using data from Ensembl (www.ensembl.org), forward and reverse primers were designed to identify mutations found in the different exons or different regions in a single exon CKIT (exon: 9, 11, 13 and 17) and PDGFRA (exon: 12, 14 and 18) genes (Tables 1 and 2).

**Table 1.** NIH consensus classification criteria for defining risk of aggressive clinical course of primary GISTs

Risk Category	Tumor size in largest dimension	Mitotic count (per 50 HPFs)
<b>Very low</b>	<2 cm	<5
<b>Low</b>	2-5 cm	<5
<b>Intermediate</b>	<5 cm	6-10
	5-10 cm	<5
<b>High</b>	>5 cm	>5
	>10 cm	Any mitotic rate
	Any size	>10

### Sanger sequencing

Sequential testing of mutations was done to determine the CKIT and PDGFRA mutations. PCR amplifications were performed using specific primer pairs to amplify exons 9, 11, 13 and 17 of CKIT gene as well as exons 12, 14 and 18 of PDGFRA gene. The samples negative for CKIT exon 9 and 11 mutations underwent another round of PCR amplification using specific primer pairs to amplify the remaining mutations CKIT (exon 13 and 17) and PDGFRA (exon 12,14 and 18).

### Data analysis

The prevalence of CKIT and PDGFRA mutations for GIST was described. The association of the CKIT and PDGFRA mutations with tumor size, mitotic count, location, and risk stratification<sup>24</sup> (Table 1) was determined using Fisher’s exact test. A *p*-value of <0.05 was considered significant.

### RESULTS

For the duration of the study period, a total of 85 FFPE Gastrointestinal lesions suspected of GIST were retrieved and 58 cases were confirmed by immunohistochemical stain. Table 2 summarizes the characteristics of patients diagnosed with GIST. Out of 58 samples, 46 (79.3%) were resection specimens and 12 (20.7%) were biopsy specimens. The mean age at diagnosis was 60.12 years (29-86 years). Gender distribution was equal (1:1). Among 58 cases, 36 (62.1%) cases were found to have mutations (CKIT or PDGFRA or double mutation) while 22 (37.9%) had no mutations. Overall, the most common tumor site was gastric (63.8%). The patients’ age did not differ between the two groups (*p*=0.090). The presence of mutation was not associated with gender (*p*=0.787) and tumor location (*p*=0.177).

Tumor profile was available in 46 cases (Table 3). Based on risk classification, 37% were classified as low risk, 6.5% as intermediate, and 56.5% as high risk. Mitotic count, tumor size, and risk classification were not associated with presence of mutation (*p*=0.371, *p*=0.660, *p*=0.625, respectively). Immunoreactivity to CD117, DOG1 and CD34 are high at 93.5%, 92.31% and 67.7%, respectively. Some GISTs did test positive for SMA (12.05%) and S100 (5.26%).

Mutational analysis of the GIST cases showed predominantly KIT mutation (29/36, 80.6%). There were 5 (13.9%) PDGFRA mutations and two (5.6%) cases with double mutation (CKIT and PDGFRA). The mutational profile of the cases is summarized in Table 4. CKIT11

**Table 2.** Patient characteristics of GIST cases

Variable	Overall (n=58)	No mutation (n=22)	With mutation (n=36)	P-value
<b>Mean age (range) in years</b>	60.12 (29-86)	56.27 (29-78)	62.47 (38-86)	0.090 <sup>a</sup>
<b>Gender (M:F)</b>	29:29	10:12	19:17	0.787 <sup>b</sup>
<b>Tumor location</b>				
Esophagus	1 (1.7%)	1 (4.5%)	0	0.177 <sup>b</sup>
Gastric	37 (63.8%)	15 (68.2%)	22 (61.1%)	
Duodenum	2 (3.4%)	0	2 (5.6%)	
Jejunum	6 (10.3%)	1 (4.5%)	5 (13.9%)	
Ileum	5 (8.6%)	1 (4.5%)	4 (11.1%)	
Colorectal	4 (6.9%)	1 (4.5%)	3 (8.3%)	
Extra-gastrointestinal	3 (5.2%)	3 (13.6%)	0	

<sup>a</sup>Mann-Whitney U Test; <sup>b</sup>Fisher’s Exact Test



**Table 3. Tumor profile and risk stratification of GIST cases**

Variable	Overall (n=46)	No mutation (n=16)	With mutation (n=30)	P-value
<b>Mitosis (per 50 HPFs)</b>				
<5	35 (76.1%)	12 (75%)	23 (76.7%)	0.371
6-10	5 (10.9%)	3 (18.8%)	2 (6.7%)	
>10	6 (13%)	1 (6.3%)	5 (16.7%)	
<b>Tumor size (cm)</b>				
2-5	14 (30.4%)	6 (37.5%)	8 (26.7%)	0.660
5-10	10 (21.7%)	4 (25%)	6 (20%)	
>10	22 (47.8%)	6 (37.5%)	16 (53.3%)	
<b>Risk stratification</b>				
Low	17 (37%)	7 (43.8%)	10 (33.3%)	0.625
Intermediate	3 (6.5%)	0	3 (10%)	
High	26 (56.5%)	9 (56.3%)	17 (56.7%)	

HPF = High Power Field

**Table 4. KIT and PDGFRA mutational profile**

Gene	Exon	Mutation detected	n (%)
<b>CKIT</b>	9	Internal tandem duplication of AY502-503	6 (16.7)
	11	Deletions between and including K550-G565	14 (38.9)
		Point mutations at Y553, W557, V559, V560	5 (13.9)
		Insertion at D579	3 (8.3)
	9 and 11	N/A	1 (2.8)
<b>PDGFRA</b>	18	V824V silent mutation (GTC to GTT)	2 (5.6)
		Point mutations at D842	3 (8.3)
<b>Double mutation</b>	CKIT11 and PDGFRA18	Deletions between and including K550-G565 Point mutations at D842	2 (5.6)

deletion was the most common mutation (38.9%) followed by CKIT9 tandem duplication (16.7%).

Table 5 summarizes the patient characteristics, tumor profile, and risk stratification between patients with CKIT or PDGFRA mutation. Patient age did not differ between the two groups ( $p=0.851$ ) and gender was not associated with the type of mutation ( $p=0.335$ ). The most common tumor site was gastric in both mutations (62.1% in CKIT and 80% in PDGFRA). Most patients were also classified as high risk with 54.2% of the CKIT mutation and 50% of PDGFRA mutation. However, tumor location, mitotic count, tumor size, and risk stratification were not associated with the type of mutation ( $p$ -values: 0.360, 0.342, 1.00, and 0.547, respectively). The most common morphology was spindle cell at 69.0% and 60% for samples with CKIT mutation and PDGFRA mutation, respectively. Of those with CKIT mutations and spindle cell morphology, 6 (16.7%) had CKIT 9 mutation and 14 (38.9%) had CKIT 11 mutation. Three samples which showed epithelioid ( $n=1$ ), and mixed spindle and epithelioid histomorphology ( $n=2$ ) had CKIT 11 mutations.

**DISCUSSION**

Across geographic regions, the epidemiology of GIST remains constant in that mean age group is between the 5<sup>th</sup> to 6<sup>th</sup> decade, with no gender preponderance, and gastric being the most common tumor site.<sup>25</sup> The same observations were demonstrated in the present study.

The advent of molecular pathology has brought about paradigm shift in the classification, diagnosis and targeted therapy for gastrointestinal mesenchymal tumors.<sup>26</sup> Prior to the wide use of immunohistochemical stains, GISTs were thought to be smooth muscle tumors and classified as cellular leiomyomas, leiomyoblastomas, and leiomyosarcomas

**Table 5. Patient characteristics, tumor profile and risk stratification by CKIT and PDGFRA mutation**

Variable	CKIT mutation (n=29)	PDGFRA mutation (n=5)
<b>Mean age (range) in years</b>	63.14 (43-86)	59.00 (38-78)
<b>Gender (M:F)</b>	13:16	4:1
<b>Tumor location</b>		
Gastric	18 (62.1%)	4 (80%)
Duodenum	2 (6.9%)	0
Jejunum	5 (17.2%)	0
Ileum	3 (10.3%)	0
Colorectal	1 (3.4%)	1 (20%)
<b>Mitosis (per 50 HPF)</b>		
	n=24	n=4
<5	19 (79.2%)	3 (75%)
6-10	1 (4.2%)	1 (25%)
>10	4 (16.7%)	0
<b>Tumor size (cm)</b>		
	n=24	n=4
2-5	7 (29.2%)	1 (25%)
5-10	5 (20.8%)	1 (25%)
>10	12 (50%)	2 (50%)
<b>Risk stratification</b>		
	n=24	n=4
Low	9 (37.5%)	1 (25%)
Intermediate	2 (8.3%)	1 (25%)
High	13 (54.2%)	2 (50%)
<b>Histomorphology</b>		
Spindle	20 (69.0%)	3 (60%)
Epithelioid	1 (3.4%)	1 (20%)
Mixed spindle and epithelioid	2 (6.9%)	0 (0%)
Not specified	6 (20.7%)	1 (20%)

of the GI tract.<sup>10</sup> Histomorphology alone has several limitations as GISTs has a wide morphologic spectrum ranging from spindle cell to epithelioid morphology.<sup>27</sup> The broad histologic differential diagnosis of GIST has brought about the importance of immunohistochemical testing. At present, commonly used immunohistochemical analysis to diagnose GIST includes CD34, CD117 and the much newer DOG1.<sup>28,29</sup> About 95% of GISTs are immunoreactive for CD117, however, more recent studies have shown

that 4 to 15% of these tumors may be negative.<sup>14,30,31</sup> Such occurrence is most commonly observed in gastric GISTs having epithelioid morphology and PDGFRA mutation.<sup>32</sup> In 2004, West R et al., demonstrated that the novel marker, DOG1 is ubiquitously expressed in gastrointestinal tumors. It is more sensitive to CD117 for gastric epithelioid GISTs than those with PDGFRA mutations.<sup>31,33</sup> CD117 and DOG1 have an overall sensitivity of 94.7% and 94.4%, respectively.<sup>32</sup> Nevertheless, in a large-scale study conducted by Miettinen et al., 2.6% of GISTs were noted to be negative to both CD117 and DOG1. In the same study, 11/24 DOG1-negative spindle cell neoplasm was noted to be positive for KIT and PDGFRA mutations, supporting the diagnosis of GIST. Further investigations show that other mesenchymal tumors such as retroperitoneal leiomyomas, peritoneal leiomyomatosis and synovial sarcomas may be immunoreactive to DOG1.<sup>32</sup> Immunohistochemical findings in the present study showed a similar result as majority of the GISTs were positive for CD117, DOG1 and CD34. Several cases also showed positivity to SMA and S100. Most clinical trials on GIST are commonly conducted in Western countries as compared to the limited number in Asia, indicating that Asian GIST patients have limited access to investigational drugs after standard therapy.<sup>34</sup>

Targeted therapy for gastrointestinal stromal tumors was developed with the discovery of KIT mutations.<sup>27</sup> Similar to published literature, the majority of the GIST mutations are that of KIT exon 11. In a review by Szucs et al., 69 to 83% of all GISTs show KIT mutations, specifically exon 11.<sup>35</sup> This is in line with the present data where 81% of mutations were KIT mutations and exon 11 was involved 76% of these cases. Among the mutations of this exon, the most studied is that of deletions. Exon 11 deletions are in 23.2 to 27.7% of all GIST cases.<sup>36</sup> A large-scale study done by Wozniak R et al., showed that tumors with exon 11 deletion, especially those affecting codons 557-558, are usually larger and have high mitosis.<sup>36</sup> Hence, tumors are usually classified as high risk for progressive disease. A similar profile was observed in the current study where 39% of CKIT mutations were exon 11 deletions and 54% with CKIT mutation were classified as high risk. The GISTs mostly have tumors >10 cm, with some accompanied by high mitotic rate. In the same review, contrary to KIT Exon 11 deletions, GISTs with point mutations have an indolent course, with smaller tumors and low mitosis. As seen in the present study, the GIST cases with point mutations have small size, 0 to 1 mitotic rate and are classified as low risk. Although global data suggests an equal distribution of GISTs among genders, CKIT Exon 9 has been reported more in males and may be seen in the lower intestinal tract.<sup>36,37</sup> Clinical behavior of this mutation can be contradicting in some studies. Künstlinger et al., concluded that exon 9 mutations per se do not have a prognostic relevance as they are not associated with high risk and metastasizing tumors.<sup>38</sup> Data in the present study also show that KIT Exon 9 mutations, although located in the lower intestinal tract, have low risk for progression. In spite of this, caution must be taken on Exon 9 mutation. A study done by Zhao et al., indicated the importance of Exon 9 mutation as it may be implicated in the mutations having resistance to Gleevec. A more recent publication showed that Exon 9 mutations have better response to another tyrosine kinase inhibitor, Sutent.<sup>39</sup> PDGFRA

D842V GIST mutations, as previously discussed in recent publications is of importance due to its contradicting behavior and therapeutic response.<sup>40</sup> GISTs with PDGFRA D842V usually have an epithelioid morphology, indolent course and remain localized with low risk of recurrence. However, GISTs harboring this mutation are usually resistant to imatinib.<sup>7</sup> Imatinib was the first FDA-approved as the first-line drug for metastatic and recurrent GIST.<sup>41</sup> However, it was observed in several studies that resistance develops in two years.<sup>42</sup> Recent publications have implicated the presence of a secondary mutation, commonly KIT and PDGFRA as the cause of resistance.<sup>43</sup> Our current two cases were noted to have double mutations seen as KIT Exon 11 and PDGFRA 18 on mutational analysis. On investigation, one of the GIST cases is already on recurrence after treatment with Imatinib.

Ultimately, this study supported by other materials highlights the significance of molecular level analysis to efficiently identify mutations associated with GISTs and recommend individualized treatments depending on the specific mutation's sensitivity. Furthermore, treatment resistance may provide a genetic basis for developing new GIST therapeutic drugs.

## CONCLUSION

Although gastrointestinal stromal tumor is the most common mesenchymal tumor of the gastrointestinal tract, it remains rare compared to other tumors. Given its varying histomorphology, mutational analysis has aided its diagnosis. Mutational analysis also has a significant impact in the treatment and prognosis of gastrointestinal stromal tumors. The presence of resistant mutation (PDGFRA D842V) would warrant alternative treatment. In the Philippines, diagnosis is based on immunohistomorphology of the cases only, and is not optimal for long term management of the patient. As seen in the findings of this study, mutational analysis, in correlation with immunohistomorphology can greatly aid the diagnosis and management of GISTs. Among the 62% of CKIT and PDGFRA wild type GIST, additional testing for other genes (Neurofibromatosis type 1 and Succinate dehydrogenase deficiency) would be warranted.

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## STATEMENT OF AUTHORSHIP

The authors certified fulfillment of ICMJE authorship criteria.

## AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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## APPENDICES

Appendix 1. KIT and PDGFRA mutations to be analyzed		
Gene	Exon	Mutation detected
<i>CKIT</i>	9	Internal tandem duplication of AY502-503
<i>CKIT</i>	11	Point mutations at Y553, W557, V559, V560 Deletions between and including K550-G565 P573R (CCA to CGA) à novel L576P (CTT to CCT) Insertion at D579
<i>CKIT</i>	13	K642E (AAA to GAA) V654A (GTG to GCG)
	17	Point mutations at D816, D820 N822K (AAT to AAA/AAG) Y823D (TAT to TGT) silent mutation (ACT to ATT)
<i>PDGFRA</i>	12	silent mutation (ACT to ATT), S566-E571, Y582-W586 V561D (GTC to GAC) E571K (GAA to AAA) insertions following R560 L580P (CTT to CCT)
	14	K646E (AAG to GAG) N659K, Y (AAC to AAG/TAC)
	18	V824V silent mutation (GTC to GTT) Point mutations at D842 Y849C (TAT to TGT) Deletions between and including D842-846

Appendix 2. List of PCR primer sequences used to amplify all the CKIT and PDGFRA exons for the Sanger sequencing analysis						
Gene	Exon	Mutation Detected	Primer ID	Forward Primer (5' to 3')	Primer ID	Reverse Primers (5' to 3')
<i>CKIT</i>	9	Internal tandem duplication of AY502-503	CKIT9F	ATGCTCTGCTTCTGTACTGCC	CKIT9R	CAGAGCCTAAACATCCCCTTA
<i>CKIT</i>	11	Point mutations at Y553, W557, V559, V560 Deletions between and including K550-G565 P573R (CCA to CGA) à novel L576P (CTT to CCT) Insertion at D579	CKIT11F	CCAGAGTGTCTAATGACTG	CKIT11R	ACCCAAAAAGGTGACATGGA
<i>CKIT</i>	13	K642E (AAA to GAA) V654A (GTG to GCG)	CKIT13F	CATCAGTTTGCCAGTTGTGC	CKIT13R	ACACGGCTTTACTCCAATG
	17	Point mutations at D816, D820 N822K (AAT to AAA/AAG) Y823D (TAT to TGT) silent mutation (ACT to ATT)	CKIT17F	TGTATTACAGAGACTTGGC	CKIT17R	GGATTACATTATGAAAGTCACAGG
<i>PDGFRA</i>	12	silent mutation (ACT to ATT), S566-E571, Y582-W586 V561D (GTC to GAC) E571K (GAA to AAA) insertions following R560 L580P (CTT to CCT)	PDGFRA12F	TCCAGTCACTGTGCTGCTTC	PDGFRA12R	GCAAGGGAAAAGGGAGTCTT
	14	K646E (AAG to GAG) N659K, Y (AAC to AAG/TAC)	PDGFRA14F	TGGTAGCTCAGCTGGACTGAT	PDGFRA14R	GGGATGGAGAGTGGAGGATT
	18	V824V silent mutation (GTC to GTT) Point mutations at D842 Y849C (TAT to TGT) Deletions between and including D842-846	PDGFRA18F	CAGCTACAGATGGCTTGATCC	PDGFRA18R	TGAAGGAGGATGAGCCTGAC

# Programmed Death Ligand 1 (PD-L1) Expression and its Association with Driver Mutations among Patients with Non-Small Cell Lung Cancer in a Private Tertiary Care Setting

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## ABSTRACT

**Objective.** The advent of immunotherapy has significantly changed the treatment and management of patients with advanced non-small cell lung cancer (NSCLC). Prior to initiation of immunotherapy, evaluation of programmed death ligand 1 (PD-L1) expression is required. One factor that affects PD-L1 expression in NSCLC is the presence of oncogenic driver mutations; however, little data on its association is available, especially in the Philippine setting. The study aims to determine the prevalence of PD-L1 expression and its association with driver mutations among patients with non-small cell lung cancer in a private tertiary care hospital in the Philippines.

**Methodology.** The study was undertaken for a period of two years from July 2017-July 2019 at St. Luke's Medical Center and included 446 NSCLC samples. PD-L1 was evaluated by immunohistochemistry using 22C3 anti-PD-L1 antibody clone, EnVision FLEX visualization system on Autostainer Link 48. Patient demographics and data on driver mutation testing were recorded. Statistical analysis was performed using logistic regression.

**Results.** PD-L1 expression was observed in 273 (61.20%) of 446 cases, 119 (61.20%) of which demonstrated high PD-L1 expression while 154 (34.50%) had low PD-L1 expression. There was no significant association between PD-L1 expression and EGFR mutation, ALK mutation, age, and gender. For histologic type, high PD-L1 expression was significantly associated with adenocarcinoma and non-small cell carcinoma, NOS.

**Conclusion.** The overall prevalence of PD-L1 expression in non-small cell lung carcinoma is 61.20% based on the cases included. Although we did not find an association between PD-L1 expression and EGFR and ALK mutation, our study observed that ALK-mutated NSCLCs have 4.7 odds of having high PD-L1 expression, however, a higher sample size is warranted to truly determine significant association. The outcome of this study may provide help in the stratification of patients and predict those who will benefit from immunotherapy.

*Key words: non-small cell lung cancer, programmed death ligand 1, PD-L1, driver mutation*

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## INTRODUCTION

Lung cancer is one of the leading causes of cancer-related mortality worldwide. It is the most common cause of cancer death in the US and UK based on recent data from the American Lung Association (2019) and Cancer Research UK (2016), respectively.<sup>1,2</sup> In the Philippines, data from the Department of Health – Philippine Cancer Control Program showed lung to be the most common site of cancer among Filipino men and the third most common among Filipino women.<sup>3</sup> Moreover, the World Health Organization – Cancer Country Profile (Philippines) in 2020 recorded the highest cancer-related mortality rate for lung (17.9%) followed by the liver (11%) and colorectum (10.2%).<sup>4</sup> Lung cancer is generally categorized into two main types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC accounting for 80% to 85% of cases.<sup>5</sup> Subtypes of NSCLCs include adenocarcinoma (40%), squamous cell carcinoma (25%-30%), and large cell carcinoma (10%-15%).

Immunotherapy has provided good clinical outcomes and is now considered as treatment option in patients with advanced NSCLC.<sup>6</sup> The tumor cells may express an



inhibitory cell surface molecule, called the programmed cell death ligand 1 (PD-L1) which combines with the programmed cell death 1 (PD-1) receptor expressed by T-cells, resulting in the inhibition of T-cell proliferation and activation.<sup>7</sup> This pathway has been used by cancer cells to escape immune surveillance. Hence, by blocking the interaction of PD-L1 with PD-1, immune function is restored and the cancer cells are recognized by the T-cells as foreign. This provides the basis of immunotherapy. The expression of PD-L1 on NSCLC cells, currently evaluated by immunohistochemistry (IHC), predicts the responsiveness of the tumor cells to anti-PD-1/PDL1 drugs.<sup>8</sup> Several of these drugs (nivolumab, pembrolizumab, and atezolizumab) have already been approved by the FDA for the treatment of advanced non-small cell lung cancer.<sup>9</sup>

Somatic mutation in the cancer cell genome is classified into two: driver mutations and passenger mutations. Driver mutations confer growth advantage on cancer cells and are positively selected during cancer evolution whereas passenger mutations do not confer growth advantage and thus do not contribute to cancer development.<sup>10</sup> Oncogenic driver mutations that are recommended for testing by the National Cancer Comprehensive Cancer Network (NCCN) for NSCLC include epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), c-ros oncogene 1 (ROS1), and BRAF as these are the genotypes with approved targeted therapies that have been shown to improve patient's survival.<sup>11,12</sup> Other mutations that are implicated in the pathogenesis of NSCLC include KRAS, MET, LB1, PIK3CA, and RET.<sup>13</sup> EGFR is dysregulated in a number of NSCLCs either by protein overexpression, gene amplification, or mutation.<sup>14</sup> It is mutated in 10% of cases from North America and Western Europe and to as high as 30-50% of patients in East Asia. In a recent study by Nee-Estuye-Evangelista et al., the frequency of EGFR mutation among Filipinos is found to be at 49.4%.<sup>15</sup> EGFR mutation may be detected in both solid tissue biopsies and liquid biopsies.<sup>16</sup> Like those with EGFR mutation, patients with the ALK rearrangement (EML4-ALK fusion) are also commonly found in younger population who are non-smokers.<sup>17</sup> Detection of the EML4-ALK translocation may be done by immunohistochemistry (IHC), fluorescence in-situ hybridization (FISH), and next generation sequencing (NGS) panels.<sup>17</sup> This translocation is found in 3%-7% of NSCLC patients.<sup>18</sup> ROS1, a receptor tyrosine kinase, is seen in 1-2% of NSCLCs that carry a translocation between ROS1 and other genes, the most common being CD74.<sup>19</sup> Like patients with ALK-mutated tumors, patients with ROS1 mutation are also typically found in younger patients who are never smokers.<sup>20</sup> These mutations may be identified by FISH or NGS panels. Lastly, BRAF mutations, most commonly BRAF V600E, have been observed in 1-3% of NSCLCs and found in patients who are smokers.<sup>21</sup> They are typically detected using polymerase chain reaction (PCR) sequencing or NGS panels.

PD-L1 expression on tumor cells has been shown to be predictor of outcomes of immunotherapy and its association with driver gene mutation status has been a focus of several studies in the recent years.<sup>22</sup> A study by Yang et al., concluded that the use of PD-L1 inhibitors is a promising option in the management of advanced NSCLCs with KRAS driver mutation and not with EGFR and

ALK.<sup>23</sup> This is also consistent with the results of the meta-analytic study by Lan et al., where they found that PD-L1 expression in NSCLCs is lower in EGFR-mutated tumors and higher in KRAS-mutated tumors.<sup>24</sup> The association between PD-L1 expression and driver gene mutation in clinical tumor specimens is not well characterized. This study aims to determine the prevalence rate of PD-L1 expression and to determine its association with driver gene mutations among patients in the Philippines diagnosed with non-small cell lung cancer.

## METHODOLOGY

A retrospective cross-sectional study was conducted at the Section of Histopathology and Section of Cellular Immunology and Immunogenetics, Institute of Pathology, at St. Luke's Medical Center. The study was undertaken for a period of two (2) years from July 2017 to July 2019.

### Sample size

All specimens with a histopathologic diagnosis of non-small cell lung carcinoma submitted for PD-L1 testing with or without driver mutation analysis at St. Luke's Medical Center.

### Inclusion criteria

All specimens with a histopathologic diagnosis of non-small cell lung carcinoma submitted for PD-L1 testing at St. Luke's Medical Center from July 2017 to July 2019 with or without driver gene mutation analysis were included.

### Exclusion criteria

Specimens submitted for PD-L1 testing with less than 100 tumor cells were labeled suboptimal and excluded from the study. Specimens submitted with driver mutation testing but with indeterminate/equivocal results were also excluded.

### PD-L1 testing

Immunohistochemistry was performed on formalin-fixed paraffin embedded (FFPE) tissues using 22C3 anti-PD-L1 antibody clone, EnVision FLEX visualization system (Agilent, USA) on Autostainer Link 48, an FDA-approved method. Tumor cells showing membranous staining for PD-L1 were evaluated as positive cells. The immunostaining results were based on tumor proportion score (TPS): (1) No Expression (Negative) – no staining or detected in <1% of tumor cells, (2) Low PD-L1 Expression – staining in ≥1%-49% of tumor cells, and (3) High PD-L1 Expression – staining in ≥ 50% of tumor cells. Two molecular pathologists conducted the evaluation.

### Driver mutation testing

#### EGFR mutation

Detection of the most common EGFR mutations was performed using the Cobas Z 480 Analyzer (Roche Diagnostics, USA). DNA extraction from formalin-fixed paraffin embedded tissues was done followed by PCR amplification and detection of target DNA using complementary primer pairs and oligonucleotide probes labeled fluorescent dyes using Real time PCR analysis. The amplification and detection reagents were provided in the Cobas Roche EGFR Mutation Test v1 kit (Roche

Diagnostics, USA). The most common EGFR mutations detected by the Cobas Z 480 Analyzer includes:

- Exon 18: G719X (G719A, G719C, and G719S)
- Exon 19: deletions and complex mutations
- Exon 20: S768I, T790M, and insertions
- Exon 21: L858R and L861Q

#### ALK rearrangement

Detection of ALK rearrangement was performed either by immunohistochemistry (IHC) or fluorescent in-situ hybridization (FISH). By IHC assay, the anti-ALK (clone D5F3) rabbit monoclonal primary antibody was used, with the OptiView™-diaminobenzidine IHC detection kit and OptiView™ amplification kit, on a fully automated IHC-staining platform. By FISH assay, a total of 50 cells were analyzed using US-FDA approved ALK 2p23dual color break apart Vysis probes from Abbott Molecular. This test was validated on formalin-fixed paraffin-embedded tissues.

#### ROS1 rearrangement

Detection of ROS1 gene rearrangement was performed using fluorescent in-situ hybridization assay. A total of 100 cells were analyzed using laboratory validated ROS1 6q22.1 (genomic location: 117,288,300-117,425,855) dual colorbreak apart Vysis probes from Abbott Molecular. This test was validated on formalin-fixed paraffin-embedded tissues.

#### BRAF mutation

Detection of the most common BRAF exon 15 (codon 600) was performed on Cobas z 480 analyzer using the Cobas BRAF V600 Mutation Test. This is a rtPCR based assay for targeted, qualitative, non-discriminatory detection of the most common mutations in BRAF, including V600E, and some non-V600E mutations (V600D, V600E2 and V600K), which represents approximately 90% of all BRAF mutations. DNA extraction from formalin-fixed paraffin-embedded tissues was followed by rtPCR amplification and detection of target DNA by primers and probes. Mutant and negative quality controls were included in each run.

#### KRAS mutation

Detection of the most common KRAS exons 2 and 3 (codons 12, 13 and 61) was performed on Cobas z 480 analyzer using the Cobas KRAS mutation test. This is a rtPCR based assay for targeted, qualitative, non-discriminatory detection of the most common mutations in KRAS. DNA extraction from formalin-fixed paraffin-embedded tissues was followed by rtPCR amplification and detection of target DNA by primers and probes. Mutant and negative quality controls were included in each run.

#### Statistical analysis

Data was encoded and processed using Microsoft Excel and SPSS. The association between PD-L1 expression and driver gene mutations were analyzed using logistic regression. A *p*-value of <0.05 is considered statistically significant.

#### Ethical clearance

Ethical clearance was obtained from the Institutional Ethics Review Board.

## RESULTS

### Demographic data

A total of 446 subjects were included in the study. The mean age ( $\pm$  standard deviation)  $65.8 \pm 11.3$  years old with a minimum age of 21 years old and a maximum age of 92 years old. Three hundred fifteen (70.6%) subjects were aged 60 years old and above and 131 (29.4%) were less than 60 years of age. There were 263 (59.0%) males and 183 (41.0%) females. Most of the specimens (68.2%) were obtained through biopsy. The most common histopathologic diagnosis was adenocarcinoma (69.7%) followed by non-small cell carcinoma, NOS (19.1%) (Table 1).

### PD-L1 expression in non-small cell lung carcinoma

PD-L1 expression was determined among subjects with non-small cell lung carcinoma. There were 173 (38.8%) of 446 subjects with no PD-L1 expression and 273 (61.20%) subjects with PD-L1 expression. Among the 273 subjects, 154 (34.5%) had low PD-L1 expression and 119 (26.7%) had high PD-L1 expression.

### PD-L1 expression and driver mutations

EGFR mutation testing was only performed on 356 out of 446 subjects. One hundred forty-nine (41.9%) was EGFR-mutated and 207 (58.1%) was EGFR-negative (Figure 1). Among those with EGFR mutation, 140 (39.4%) had single mutation and only 9 (2.5%) had dual mutations. Of the 149 EGFR mutations, 44.30% had mutation of Exon 19 followed by mutation of Exon 21 at 39.6%. The most common dual mutation was the combination of Exon 19 and Exon 20 at 3.36%. There were 283 subjects who underwent ALK mutation determination. Among these 283 subjects, 16 (5.7%) were ALK-mutated. Only 68 of 446 subjects had ROS1 mutation testing and 67 (98.5%) were negative for ROS1 mutation. Of the 26 of 446 subjects who underwent BRAF testing, 25 (96.2%) were negative for BRAF mutation. Two subjects who had KRAS testing were both negative for KRAS mutation.

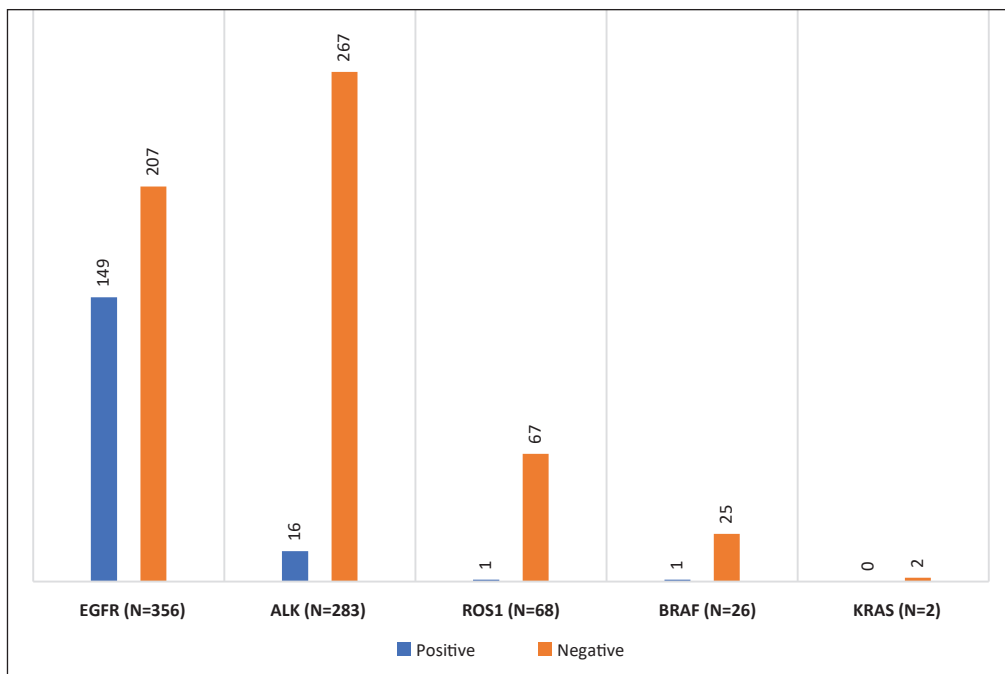
**Table 1.** Demographic data of patients with non-small cell lung carcinoma (n=446)

Age (years)	Frequency (%)
Mean	65.8 $\pm$ 11.3
Median	67.0
Minimum	21
Maximum	92
Age Group	Frequency (%)
<60 years	131 (29.4)
$\geq$ 60 years	315 (70.6)
Sex	Frequency (%)
Female	183 (41.0)
Male	263 (59.0)
Specimen Type	Frequency (%)
Biopsy	304 (68.2)
Fluid cytology	51 (11.4)
Lobectomy/Resection	29 (6.5)
Not available	62 (13.9)
Histopathologic diagnosis	Frequency (%)
Adenocarcinoma	311 (69.7)
Adeno squamous carcinoma	7 (1.6)
Non-small cell carcinoma, NOS	85 (19.1)
Pleomorphic carcinoma	2 (0.4)
Squamous cell carcinoma	41 (9.2)



**Table 2. Association of PD-L1 expression with EGFR and ALK mutation**

Driver mutation	High PD-L1 expression				Low PD-L1 expression			
	N (%)	OR	CI	p value	N (%)	OR	CI	p value
EGFR-mutated	36 (24.2)	0.687	0.402 – 1.174	0.17	53 (35.6)	0.848	0.518 – 1.387	0.51
EGFR negative	62 (30.0)				74 (35.7)			
ALK-mutated	8 (50.0)	4.7	0.973 – 22.873	0.05	6 (37.5)	2.8	0.560 – 14.458	0.21
ALK negative	78 (29.2)				97 (36.3)			



**Figure 1.** Frequency of driver mutations in non-small cell lung carcinoma.

**EGFR**

Low PD-L1 expression (35.7%) is more frequent among EGFR-negative NSCLCs while majority of EGFR-mutated NSCLCs did not express PD-L1 (40.3%) (Figure 2). In this study, however, no significant association is found between EGFR mutation and high PD-L1 expression (OR=0.687, 95% CI: 0.402-1.174, *p*=0.17) and low PD-L1 expression (OR=0.848, 95% CI: 0.518-1.387, *p*=0.51) (Table 2).

**ALK**

Low PD-L1 expression is more frequent among ALK-negative NSCLCs while high PD-L1 expression is more frequent among ALK-mutated NSCLCs (Figure 2). ALK-mutated NSCLCs are 4.7x more likely to have high PD-L1 expression (OR=4.7, 95% CI: 0.973-22.873, *p*=0.05) and 2.8x more likely to have low PD-L1 expression (OR=2.8, 95% CI: 0.560-14.458, *p*=0.21) but the differences are not significant (Table 2).

**ROS1, BRAF, KRAS**

Most of the subjects with ROS1 negative NSCLCs have high PD-L1 expression while those with BRAF-negative NSCLCs have low PD-L1 expression (Figure 2). Only two subjects underwent KRAS mutation testing and both showed positive PD-L1 expression. The relationship between ROS1, BRAF, and KRAS mutation with PD-L1 expression cannot be determined due to the limited number of participants who underwent testing for these driver mutations.

**PD-L1 expression and clinicopathologic factors**

**Age**

The majority of the subjects belong to the elderly age group (≥60 years old). Most of the subjects in both groups did not express PD-L1. There is no significant association between age and high PD-L1 expression (OR=1.05, 95% CI: 0.63-1.76, *p*=0.84) and low PD-L1 expression (OR=0.91, 95% CI: 0.56-1.48, *p*=0.70) (Table 3).

**Gender**

The study population comprised of males (59%) more than females (41%). Most of the male subjects did not express PD-L1 (40.7%). Females have 1.6 odds of having low PD-L1 expression but the difference is not significant (OR=1.6, 95% CI: 1.00-2.49, *p*=0.05) (Table 3).

**Histologic Type**

Adenocarcinoma was the predominant histologic type. Subjects diagnosed with adenocarcinoma are 2.9 times more likely to have high PD-L1 expression than subjects diagnosed with squamous cell carcinoma. In addition, subjects diagnosed with non-small cell carcinoma, NOS are 4 times more likely to have high PD-L1 expression than subjects diagnosed with squamous cell carcinoma. The high PD-L1 expression in adenocarcinoma (OR=2.91, 95% CI: 1.04-8.09, *p*=0.04) and non-small cell carcinoma, NOS (OR=4.4, 95% CI: 1.44-13.40, *p*=0.01) histologic group are both statistically significant (Table 3).

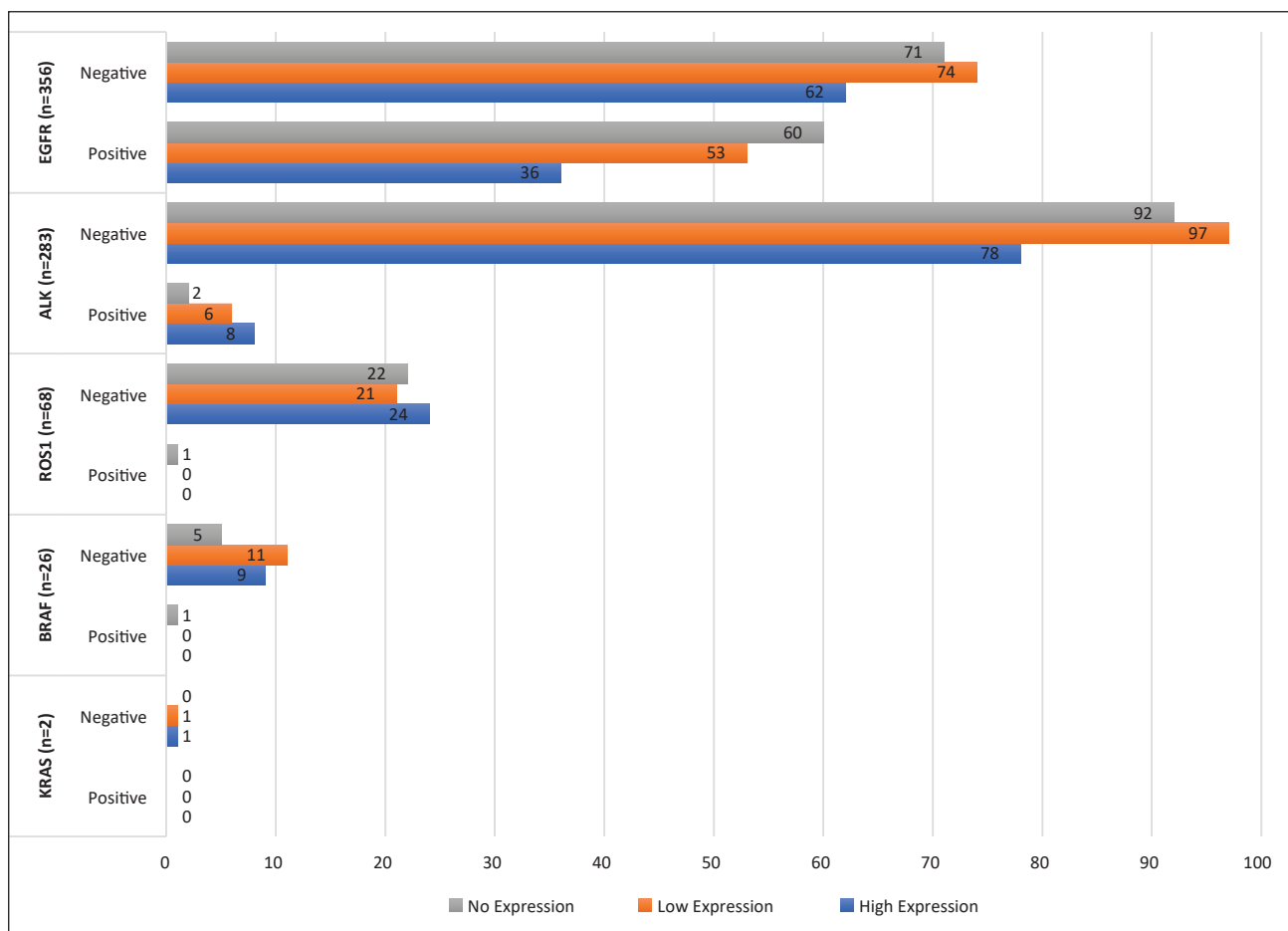


Figure 2. PD-L1 expression and driver mutations.

**Table 3. Association of PD-L1 expression with age, gender and histologic type**

Characteristic	High PD-L1 expression				Low PD-L1 expression			
	N (%)	OR	CI	p value	N (%)	OR	CI	p value
<b>Age group</b>								
<60 years	37 (28.2)	1.05	0.63 – 1.76	0.84	43 (32.8)	0.91	0.56 – 1.48	0.70
≥60 years	82 (26.0)				111 (35.2)			
<b>Sex</b>								
Female	43 (23.5)	0.89	0.54 – 1.46	0.64	74 (40.4)	1.60	1.00 – 2.49	0.05
Male	76 (28.9)				80 (30.4)			
<b>Histopathologic diagnosis</b>								
Adenocarcinoma	84 (27.0)	2.91	1.04 – 8.09	<b>0.04</b>	103 (33.1)	0.91	0.56 – 1.48	0.70
Adenosquamous carcinoma	2 (28.6)	4.09	0.46 – 36.59	0.21	3 (42.9)	1.04	0.50 – 2.16	0.91
Non-small cell carcinoma NOS	27 (31.8)	4.40	1.44 – 13.40	<b>0.01</b>	32 (30.6)	2.36	0.35 – 15.98	0.38
Squamous cell carcinoma	5 (12.2)				15 (36.6)			

**DISCUSSION**

Targeted immunotherapy has been used successfully in the United States since 2014 for the treatment of various advanced cancers such as NSCLCs. In the Philippines, recent approval by the Philippine Food and Drug Administration was given for pembrolizumab, a landmark drug that may be used as first line treatment in advanced NSCLC making immunotherapy a reality for the Filipinos.<sup>25</sup> The drug works by blocking the interaction of PD-L1, present in the cancer cells, with the PD-1 receptor, present in T-cells. Currently, the 2019 National Comprehensive Cancer Network (NCCN) immunotherapy guidelines in the treatment and management of advanced NSCLC require the evaluation

of PD-L1 expression by immunohistochemistry prior to initiation of immunotherapy.<sup>11</sup> This set of guidelines is also followed by many international and local societies in Oncology including the American Society of Clinical Oncology (ASCO), European Society of Medical Oncology (ESMO), and the Philippine Society of Medical Oncology (PSMO). The NCCN strongly recommends single-agent pembrolizumab as first line treatment for patients with high PD-L1 expression (≥50%) because use of single agent pembrolizumab as first line treatment improves overall survival by twofold in patients with high PD-L1 expression. For patients with low PD-L1 expression (≥1%-49%), the treatment varies for non-squamous NSCLC and squamous NSCLC. For patients with low PD-

L1 expression and non-squamous NSCLC, single-agent pembrolizumab is not recommended as first line treatment. Instead, the preferred approach is combination therapy with pembrolizumab/permetrexed with either cisplatin or carboplatin. This combination therapy has resulted in reduced risk of death by 51% vs chemotherapy alone based on the KEYNOTE-189 trial.<sup>26</sup> For patients with low PD-L1 expression and squamous NSCLC, NCCN recommends treatment with pembrolizumab/carboplatin in addition to either paclitaxel or nab-paclitaxel.

Several studies have shown PD-L1 expression to be associated with the presence or absence of driver gene mutations.<sup>12,24,27-37</sup> In patients with driver mutations, the NCCN guidelines recommend targeted therapy as first line treatment since they typically do not respond well to single-agent immunotherapy. Targeted therapy includes (1) tyrosine kinase inhibitors such as osimertinib, erlotinib, gefitinib, afatinib, and dacomitinib for EGFR-mutated NSCLC; crizotinib, ceritinib, and brigatinib for ALK-mutated NSCLC; crizotinib and ceritinib for ROS1-mutated NSCLC; and (2) kinase inhibitors such as dabrafenib + trametinib for BRAF-mutated NSCLC. Since it has been reported that driver mutations are more common in patients of East Asian origin,<sup>14</sup> including those of Filipino descent,<sup>15</sup> the evaluation of PD-L1 expression becomes an important parameter to assess in the treatment planning of patients with NSCLC.

In our study, we retrospectively assessed PD-L1 expression by immunohistochemistry in NSCLC (n=446) for a period of two years. Overall, 61.20% of subjects had PD-L1 expression while 38.80% had no PD-L1 expression. Among those with positive PD-L1 expression, low PD-L1 expression ( $\geq 1\%$ -49%) was detected in 34.50% while high PD-L1 expression ( $\geq 50\%$ ) was detected in 26.70%. This data is comparable with the studies done by Aggarwal et al. (n=3,880/5,879, 66%), Holmes et al. (n=194/264, 73.4%), and Chang et al. (n=334/500, 66.8%), wherein the prevalence of PD-L1 expression in NSCLC was seen in more than 60% of the study population.<sup>38-40</sup>

Among the 446 subjects, only 356 had EGFR mutation testing. Of the 356, 41.9% (n=149) had EGFR mutation and 58.1% (n=207) had negative EGFR mutation. This high EGFR mutation rate is comparable with several studies done in Asian populations which showed EGFR mutation rate ranging from 30% to as high as 76%.<sup>15,41,42</sup> Correlating PD-L1 expression with EGFR mutation, our study found no significant association. This is comparable with previous studies conducted by Cooper et al., Schmidt et al., and Tang et al.<sup>43-45</sup> Other studies, however, demonstrated high PD-L1 expression in EGFR-mutated NSCLCs,<sup>12,27,28</sup> while some showed that it is higher in EGFR-wildtype NSCLCs.<sup>29-31</sup> Moreover, there are also researches that showed lower PD-L1 expression in EGFR-mutated NSCLCs.<sup>24,32</sup> The variation in the results of these studies may probably be explained by differences in study population, antibody clones used for PD-L1 testing, and variable cut offs for PD-L1 expression.

Two hundred eighty-three (283) of the 446 subjects underwent ALK mutation testing. Of the 283, 5.7% (n=16) were positive for ALK rearrangement while 94.3% (n=267)

were negative. In our study, ALK-mutated NSCLCs are 4.7 times more likely to have high PD-L1 expression but this is not statistically significant (OR=4.7, 95% CI: 0.973-22.873,  $p=0.05$ ). Despite a  $p$ -value of 0.05, the confidence interval included the value 1.0 and is very wide which means that we were not able to find a significant difference due to the small sample size (n=283). Hence, we recommend higher sample size for ALK in future studies to truly determine if high PD-L1 expression is correlated with the presence of ALK mutation in NSCLCs. The results of several studies regarding PD-L1 expression and ALK mutation are also conflicting. Some studies have found no association between PD-L1 expression and ALK mutation<sup>24,32,40,46,47</sup> while other studies demonstrated significantly higher PD-L1 expression in ALK-mutated NSCLCs.<sup>32,34</sup> The proposed mechanism behind high PD-L1 expression in ALK-mutated NSCLCs is believed to be due to the upregulation of the MEK/ERK and PI3K/AKT pathway signaling in the tumor cells.<sup>35-37</sup>

The association of PD-L1 expression among clinico-pathologic variables (age, gender, and histologic type) was also determined. In this study, no association is found between low and high PD-L1 expression and age group. This finding is consistent with several studies.<sup>32,41,47-49</sup> For gender, many studies have found PD-L1 expression to be positively associated with male gender,<sup>32,44,46,47,49,50</sup> while some studies did not observe any significant association.<sup>40,48,51</sup> The positive association with male gender could be due to the higher incidence of cigarette smoking among males which is explained by the proinflammatory effects of smoking and that smoking-induced carcinomas also have high mutational tumor burden in which they express neoantigens that trigger anti-tumor immune responses.<sup>32,52</sup> In our study, however, no significant association is observed between PD-L1 expression and gender. For the histologic type, a study by Skov et al., showed significant difference in PD-L1 expression between adenocarcinoma and squamous cell carcinoma with adenocarcinoma having an odds ratio of 1.8.<sup>53</sup> This is also supported by the study of Mu et al., where PD-L1 expression in adenocarcinoma was significantly higher than in squamous cell carcinoma.<sup>54</sup> Their findings are comparable with the result of our study where we observed high PD-L1 expression in adenocarcinoma ( $p=0.04$ ) and non-small cell carcinoma, NOS ( $p=0.01$ ). The clinical significance of this is that patients with advanced lung cancer with a histopathologic diagnosis of adenocarcinoma are more likely to receive and benefit from immunotherapy compared to other histologic subtypes. Other studies, however, demonstrated higher PD-L1 expression in squamous cell carcinoma than adenocarcinoma<sup>24,32,46,50,55</sup> while some studies showed no significant association.<sup>48,56,57</sup> The variation in the results of the studies may again be explained by differences in study population and methodology. The significant association between PD-L1 expression and NSCC, NOS could be because these tumors are more likely adenocarcinoma when immunophenotyping is performed.<sup>58</sup> In this study, however, the cases that were classified as NSCC, NOS, included those wherein the immunohistochemistry results were not conclusive (i.e., TTF-1 and p40 negative cases), as well as those wherein immunohistochemical staining was not performed. Moreover, a formal slide review to confirm the histologic subtype of the tumors was not done.

The histologic subtype was based solely on the final diagnosis in the accompanying histopathology reports.

There are several limitations of the study that should be acknowledged. First, the sample size for the analysis of correlation between PD-L1 expression and driver mutations (EGFR [n=356] and ALK [n=283]) was small; hence, a higher sample size is warranted. Also, we were not able to check for the association of PD-L1 expression with other driver mutations due to the small number of patients who underwent testing for these mutations (ROS1, n=68; BRAF, n=26; and KRAS, n=2). Second, most of the samples from this study are from small biopsies (n=305), from fine needle aspiration and core needle biopsies, which may affect PD-L1 expression due to smaller number of tumor cells available for analysis compared to lobectomy/resection specimens. The tissue samples obtained from small biopsies may not be representative of the whole tumor and may show divergent results due to possible heterogenous expression of PD-L1.<sup>59</sup> It is widely recognized that PD-L1 expression in tumors is heterogenous, which may impact the interpretation on small biopsies versus resections. In a study by Hwang et al., the discordance rates between biopsy and resection specimens were as high as 33.3% (for biopsies greater than or equal to 8 mm<sup>2</sup> area) and 71.4% (for biopsies less than 8 mm<sup>2</sup> area).<sup>60</sup> Lastly, some cases from other hospital institutions that were sent for PD-L1 testing have incomplete data causing them to be excluded from this study and further limiting the sample size.

## CONCLUSION

Our study provided baseline data in the Philippines regarding expression of PD-L1 in NSCLC and its association with driver mutations. The prevalence of PD-L1 in NSCLC was 61.20%, with 34.5% of subjects having low expression ( $\geq 1$ -49%) and 26.71% of subjects having high expression ( $\geq 50$ %). No significant association was observed between PD-L1 expression and EGFR and ALK mutations. For EGFR mutation, determining if there is a significant difference in PD-L1 expression among the specific EGFR exon mutations may also be worth looking into. For ALK mutation, although our study observed ALK-mutated NSCLCs to have 4.7 odds of having high PD-L1 expression, a higher sample size is warranted to truly determine if there is significant association (95% CI: 0.973-22.873, *p*-value=0.05). High PD-L1 expression was significantly associated with adenocarcinoma and non-small cell carcinoma, NOS histology. No significant association was observed between PD-L1 expression and age and gender. The outcome of this study may provide help in the stratification of patients and predict those who will benefit from immunotherapy. Overall, since the evaluation of PD-L1 expression is required prior to initiation of immunotherapy, there is a need to explore factors that may affect its expression. Some of these factors, including the presence of oncogenic driver mutations, age, gender, and histologic type, were already explored in this study; however, a higher sample size is still recommended. Correlation with other clinicopathologic parameters such as smoking status, presence of tumor infiltrating lymphocytes, tumor differentiation, tumor size, lymph node metastasis, and TNM stage, is also recommended for future studies as these factors have been found to affect PD-L1 expression as well.

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The authors certified fulfillment of ICMJE authorship criteria.

## AUTHOR DISCLOSURE

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## Submandibular Secretory Carcinoma in a 10-year-old Filipino

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### ABSTRACT

Secretory carcinoma is a recently described salivary gland neoplasm reported in the fourth edition of the World Health Organization classification of head and neck tumors.<sup>1</sup> We report a case of a primary secretory carcinoma arising from the submandibular gland that was completely excised in a 10-year-old. The histomorphologic features and the immunophenotype studies are compatible with secretory carcinoma. Unless proven otherwise by immunohistochemical stains and cytogenetics, secretory carcinoma should be included as a differential in cases of lesion of the major and minor salivary glands that has the primary differential diagnosis of acinic cell carcinoma. This case report aims to contribute to the limited literature about this disease entity and would be one of the few reported cases of the disease in a school-age child.

*Key words: secretory carcinoma, submandibular gland mass, ETV6-NTRK3 fusion, school-age child*

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### INTRODUCTION

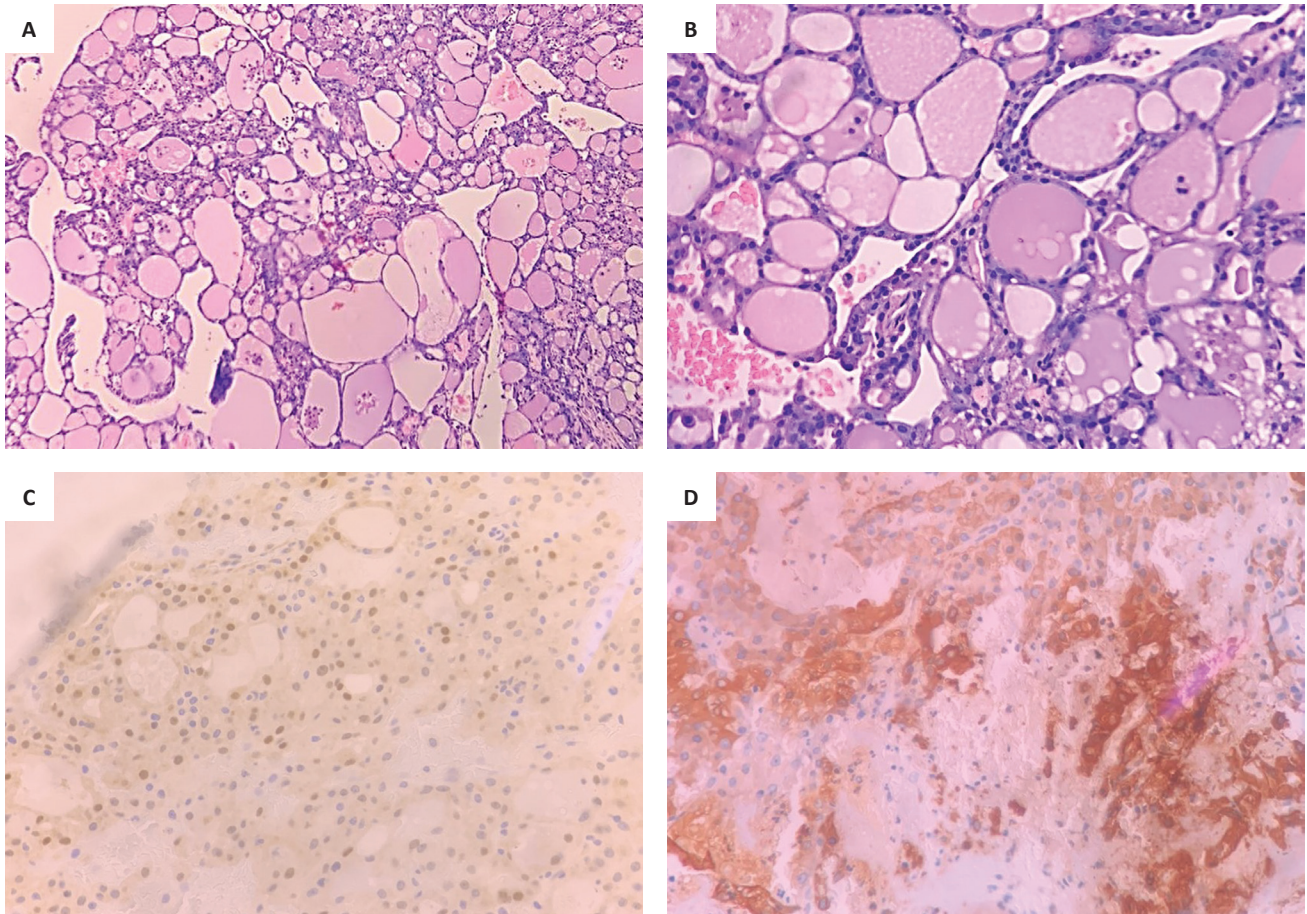
Secretory carcinoma (SC) (formerly known as Mammary analogue secretory carcinoma) is a salivary gland carcinoma that is frequently misdiagnosed as acinic cell carcinoma but is now known to be a distinct entity that is identical to SC of the breast, showing morphologic, immunohistochemical, and genetic similarities between the two entities. This entity was reported by Skalova et al., taking advantage of the fact that mammary SC is associated with the t(12;15) (p13;q25) translocation leading to the *ETV6-NTRK3* gene fusion.<sup>2,3</sup> It was seen to occur predominantly (76%) in the parotid, minor salivary glands of the oral cavity and submandibular gland. It has also been described in the nasal cavity, skin, and thyroid gland. It is more common in adults, with reported cases ranging from 5 to 87 years old, and it is slightly predominant in males (M/F ratio: 1.4:1).<sup>4</sup> Presented here is a 10-year-old male with a rapidly growing left submandibular mass having histopathologic features compatible with SC. The diagnosis of SC is sparsely reported among children and has not been documented in a pediatric patient in any Philippine database, hence this case report serves as an addition to the growing body of literature and adds to the rare entities diagnosed in childhood.

### CASE

This is a case of a 10-year-old male who developed an enlarged left submandibular mass. Consulting in another institution, the parents were advised that their child must undergo excision biopsy. The patient was subsequently diagnosed to have acinic cell carcinoma (ACC). This case was brought to our pediatric center as a slide review. Based on the histomorphologic features, we formulated a differential diagnosis which include lesions with thyroid lineage that encompass ectopic thyroid (ET) and metastatic well-differentiated thyroid carcinoma, ACC, adenoid cystic carcinoma (AdCC) and SC. The microscopic findings on the hematoxylin and eosin-stained slides of representative sections of the lesion presented diagnostic overlaps among the differentials. Immunohistochemistry studies show a





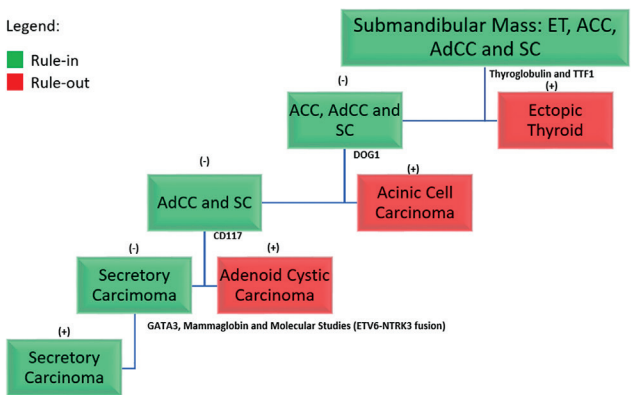


**Figure 1.** (A) Low power view showing varisized lobules composed of tumor cells with eosinophilic, vacuolated cytoplasm (H&E, 100x). (B) High power view showing a vesicular nuclei and conspicuous nucleoli. The lobules contain eosinophilic intraluminal material (H&E, 400x). (C) Focal nuclear immunoreactivity to GATA3 (400x). (D) Strong, diffuse membranous to cytoplasmic immunoreactivity to Mammaglobin (400x).

positive immunoexpression to GATA3 and Mammaglobin (Figure 1), but negative staining to thyroglobulin, TTF1, DOG1 and CD117 antibodies (Figure 2) (Table 1). Molecular cytogenetic testing for *ETV6-NTRK3* is suggested and a final pathologic report of SC was made thereafter.

**DISCUSSION**

It is reported that SC accounts for <0.3% of all salivary gland tumors and SC makes up 4.5% of malignant salivary gland disease processes. In the most recent update, there have been a total of 248 SC cases reported in the literature, and about 24% of these cases arose in minor salivary glands between 2010 and 2017.<sup>13,14</sup> Two patients with oral cavity-originated SC were reported and was subjected to a pooled analysis of previously reported SC cases. It is highly likely that many cases of SC are previously diagnosed as ACC owing to their similar histological findings. The treatment strategy for minor salivary gland-originated SC is similar to ACC; however, SC is an indolent salivary gland malignancy, although 25 % of cases are reported to have lymph node metastasis but distant metastases are rare. Thus, clinical stage and high-grade transformation are the main adverse prognostic factors in which establishing an accurate histopathologic diagnosis, confirming the *ETV6-NTRK3* fusion gene by genetic analysis is important both for diagnostic and prognostic purposes.<sup>15-17</sup> Currently, the guidelines for surgical treatment, chemoradiation, and follow up of these cases have not been standardized due to the small number of cases.<sup>18</sup>



\*ET: Ectopic Thyroid, ACC: Acinic Cell Carcinoma, AdCC: Adenoid Cystic Carcinoma and SC: Secretory Carcinoma.

**Figure 2.** Approach to arriving at our diagnosis.

**Table 1.** Immunohistochemical stains used in this case that we consider coming to the diagnosis of secretory carcinoma<sup>5-12</sup>

Immunohistochemical stain	Staining pattern	Positive staining
<b>GATA3</b>	Nuclear	<ul style="list-style-type: none"> <li>• SC (Diffuse staining)</li> <li>• Other salivary tumors</li> <li>• Metastatic urothelial and breast carcinoma</li> <li>• Metastatic lobular carcinoma of the breast</li> <li>• Squamous cell carcinoma of the skin</li> <li>• Mesothelioma</li> <li>• Peripheral T-cell Lymphoma</li> </ul>
<b>Mammaglobin</b>	Cytoplasmic	<ul style="list-style-type: none"> <li>• Confirmatory for those tumors that exhibit classic SC morphology</li> <li>• Highly specific for breast carcinoma (Circulating or Metastatic Breast Carcinoma)</li> </ul>
<b>Thyroglobulin</b>	Luminal and Cytoplasmic	<ul style="list-style-type: none"> <li>• Specific marker of thyroid differentiation</li> </ul>
<b>TTF1</b>	Nuclear	<ul style="list-style-type: none"> <li>• Marker of thyroid tumors, both primary and metastatic carcinoma (except anaplastic thyroid carcinoma wherein there is complete loss of immunorexpression)</li> </ul>
<b>DOG1</b>	Membranous and Cytoplasmic	<ul style="list-style-type: none"> <li>• ACC (97 to 100% of cases)</li> <li>• AdCC (30 to 71% of cases)</li> <li>• SC (0 to 33% of cases) exhibits focal, weak immunorexpression</li> </ul>
<b>CD117</b>	Cytoplasmic	<ul style="list-style-type: none"> <li>• AdCC exhibits strong staining</li> <li>• SC of the breast</li> <li>• Basal-like breast carcinomas</li> </ul>

SC is a recently described salivary gland carcinoma frequently misdiagnosed as ACC but is now known to be a different entity analogous to the SC of the breast that is also associated with the t(12;15)(p13;q25) translocation leading to the *ETV6-NTRK3* gene fusion. Morphologically, the cases representing this disease entity are characterized by the presence of microcystic and glandular spaces with abundant eosinophilic secretion that is positive for PAS, mucicarmine, MUC1, MUC4, and mammaglobin. The cells have an apocrine appearance with vacuolated, eosinophilic cytoplasm and uniform vesicular nuclei with small nucleoli. Like mammary SC of the breast, the cells are positive for cytokeratin, S-100 protein, BRST-2, and mammaglobin, but are negative for androgen receptor, p63, and DOG-1. They are low-grade carcinomas that recur in approximately 30% of cases but only rarely metastasize or result in death. Rare examples with high-grade transformation (dedifferentiation) have been described and behave more aggressively.<sup>19</sup> In the case presented, it microscopically shows varisized lobules composed of tumor cells with eosinophilic, vacuolated cytoplasm, vesicular nuclei, and conspicuous nucleoli. The lobules contain eosinophilic intraluminal material (Figure 1.A) (Figure 1.B). Immunohistochemically, SC will have 100% diffuse, nuclear immunoreactivity to GATA3, and like its counterpart in the breast, SC express mammaglobin at levels that can be easily detected by routine immunohistochemistry that would establish mammaglobin as a diagnostic tool for confirming SC.<sup>20,21</sup> It must be taken to account that considerable overlaps also exist among the immunohistochemical profiles of the differential diagnoses, and that this should be a caveat in the interpretation of individual immunohistochemical stains. In typical cases, morphology in combination with these markers are sufficient to diagnose SC. Further investigations via molecular study for SC is characterized by *ETV6* gene rearrangement, with its most common translocation partner being the *NTRK3* gene.<sup>22</sup>

## CONCLUSION

We encountered a case of SC arising from the left submandibular gland from a 10-year-old, male child. Patient underwent complete excision of the mass and is currently asymptomatic. The diagnosis is based on the

clinical presentation and morphologic features supported by the immunoreactivity of the tumor cells to GATA3 and Mammaglobin (Figure 1) and negative immunostaining for Thyroglobulin, TTF1, DOG1, and CD117. To the best of our knowledge, this is one of the limited cases, especially given the patient's age, to report this approach to a diagnosis (Figure 2) of SC.

## ETHICAL CONSIDERATION

Patient consent was obtained before submission of the manuscript.

## STATEMENT OF AUTHORSHIP

The authors certified fulfillment of ICMJE authorship criteria.

## AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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## Rapid Respiratory Panel Testing for SARS-CoV-2: Experience in a Private Tertiary Hospital

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### ABSTRACT

SARS-CoV-2 has infected more than 643 million individuals worldwide and accounts for close to 64,950 deaths in the Philippines. Due to COVID-19's clinical overlap with other diseases and non-specific radiologic findings, its diagnosis rests primarily on laboratory methods, including reverse transcription polymerase chain reaction (RT-PCR) and multiplexed molecular platforms for rapid syndromic testing. Compared to RT-PCR which has a turnaround time of 24 to 72 hours, multiplexed molecular platforms can provide alternative diagnoses to COVID-19 in an average of one hour, providing meaningful data that can impact clinical and resource management when handling acute surge of patients with respiratory symptoms.

*Key words: COVID-19, SARS-CoV-2, diagnostics, film array, RT-PCR*

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### INTRODUCTION

COVID-19 is a highly infectious disease that broke out in Wuhan, China in December 2019. Caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), it eventually spread to become a public health emergency of international concern on January 30, 2020 and a pandemic on March 11, 2020. As of December 12, 2022, there have been 643,875,406 confirmed cases around the world, including 6,630,082 deaths.<sup>1</sup> In the Philippines, there has been a total of 4,050,045 confirmed cases with 64,902 deaths.<sup>2</sup> Aside from the clinical effects of the disease, COVID-19 has also led to economic damage and changes in the socio-political climate.

The clinical spectrum of COVID-19 ranges from asymptomatic to mild disease to respiratory failure necessitating mechanical ventilation to multiorgan dysfunction. When symptomatic, the primary clinical presentation of COVID-19 is fever and dry cough.<sup>3</sup> Other common symptoms include sore throat, nasal congestion, malaise, loss of taste and/ or smell, and diarrhea.<sup>3</sup>

Since the disease often manifests as pneumonia, radiologic imaging has a pivotal role in the diagnosis and management of infected patients. Chest x-ray, chest computed tomography (CT) and lung ultrasound may show multifocal alveolar opacities; patchy, multifocal, bilateral ground glass areas with consolidation; and pleural effusion.<sup>3</sup> However, the patterns seen on imaging are often non-specific. Coupled with the significant overlap in clinical presentation with a host of other diseases, diagnosis rests primarily on laboratory methods.

The most sensitive, specific, and widely used test is the reverse transcription polymerase chain reaction (RT-PCR), which involves the amplification of genetic material isolated from upper and/ or lower respiratory tract samples. Current laboratory methods for RT-PCR for COVID-19 have a turnaround time of 24 hours to 3 days, due to



tests being run in batches and to give time allowance for repeat testing in cases of initially indeterminate results. This long turnaround time (TAT) has led to complaints from patients in our institution, especially those from the emergency room awaiting admission, as results are required for triaging and determination of which ward to admit the patient. Clinicians in our institution also raised the question of false negative results in patients presenting with respiratory symptoms and a negative RT-PCR result.

In contrast, multiplexed molecular platforms for rapid syndromic testing, such as the BioFire® Respiratory Panel 2.1 plus (RP2.1plus) (Cepheid, USA), has an average TAT of one hour. These platforms are real time, nested, multiplexed nucleic acid tests that, in addition to detecting SARS-CoV-2, can also simultaneously identify other respiratory viral and bacterial nucleic acids in nasopharyngeal swab samples (Table 1).<sup>4</sup> All necessary reagents for isolation, amplification, and detection of nucleic acids from the aforementioned respiratory pathogens are contained within a closed system disposable pouch. In RP2.1plus, the sample is prepared by bead beating and chemical lysis.<sup>4</sup> Extraction and purification of nucleic acids occurs via magnetic bead technology.<sup>4</sup> Endpoint melting curve data are then used to detect target-specific amplicons which are analyzed to generate a result.<sup>4</sup>

RP2.1plus received emergency-use authorization (EUA) from the US Food and Drug Administration last May 4, 2020 for use on clinical samples and is considered a confirmatory test for SARS-CoV-2 infection by the Philippine Department of Health as part of the national laboratory response. The test has a reported clinical sensitivity of 98% and specificity of 100% for SARS-CoV-2, with a limit of detection at 1.6x10<sup>2</sup> copies/mL.<sup>4</sup> It has a 98% percent positive agreement and a 100% percent negative agreement when compared to other SARS-CoV-2 EUA assays.<sup>5</sup>

In our institution, both RT-PCR and RP2.1plus results are made available to the attending physician through the electronic records and to the patient via an electronic portal.

**METHODOLOGY**

We retrieved the results and demographic data of patients tested for SARS-CoV-2 using RP2.1plus in our institution’s electronic records. The study covers data gathered over three months of testing, which covers an initial period of RP2.1plus being offered in our institution (November 2020) and a subsequent surge in COVID-19 cases in the country (September and October 2021). Our report was deemed exempt from ethical clearance by our institutional review board as it does not include identifiable personal information or patient photographs.

**RESULTS AND DISCUSSION**

Our institution received 2,325 clinical samples for SARS-CoV-2 testing using the RP2.1plus panel during the study period. The majority were samples from adult patients [mean age: 45 years (Table 2)] being admitted through the emergency department.

**Table 1.** Respiratory viral and bacterial nucleic acids that can be detected by the BioFire® Respiratory Panel 2.1plus

Viruses	Bacteria
Adenovirus	<i>Bordetella parapertussis</i>
Coronavirus 229E	<i>Bordetella pertussis</i>
Coronavirus HKU1	<i>Chlamydia pneumoniae</i>
Coronavirus NL63	<i>Mycoplasma pneumoniae</i>
Coronavirus OC43	
Middle East Respiratory Syndrome Coronavirus (MERS-CoV)	
Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)	
Human Metapneumovirus	
Human Rhinovirus / Enterovirus	
Influenza A	
Influenza B	
Parainfluenza Virus 1	
Parainfluenza Virus 2	
Parainfluenza Virus 3	
Parainfluenza Virus 4	
Respiratory Syncytial Virus	

**Table 2.** Demographic data of patients whose samples were tested using RP2.1plus

	Number of samples (n)	Percentage of total samples (%)
<b>Sex</b>		
Male	1,015	43.7
Female	1,310	56.3
<b>Age</b>		
Pediatric (0-18 years)	197	8.5
Adult (> 18 years)	2,128	91.5
<b>Total samples</b>	<b>2,325</b>	<b>100</b>

SARS-CoV-2 was detected in 19.23% of the samples tested while 1.85% had co-infection with other viruses, the most common being Human Rhinovirus / Enterovirus (Table 3). Almost eight percent were positive for an infectious agent other than SARS-CoV-2 while 71.1% of the samples were negative for all viral and bacterial nucleic acids included in the panel. The most common infectious agent in the SARS-CoV-2-negative samples was the Human Rhinovirus / Enterovirus. Note that a negative SARS-CoV-2 result was seen in majority of cases, which then facilitated patient admission to non-COVID wards in our institution.

The human rhinovirus / enterovirus is the most common infectious agent worldwide, afflicting both children and adults.<sup>6</sup> This could then account for the high incidence of this strain in our clinical samples. With an average incubation period of two days, symptom duration of seven to ten days, and a clinical presentation that includes nasal congestion, cough, malaise, and pneumonia, it has considerable clinical overlap with SARS-CoV-2. The prevalence of this causative agent could account for cases who present with a clinical picture suspicious for COVID-19 but who subsequently test negative for SARS-CoV-2 on RT-PCR. For cases that ultimately tested negative for all viruses and bacteria included in the panel, possible explanations include infection with pathogens not detected by RP2.1plus and lower respiratory tract infection which may not be detected with a nasopharyngeal swab.<sup>4</sup>

Viral co-infection in patients with COVID-19 has been previously documented, seen in 4.3% to as many as 47% of SARS-CoV-2 infected patients.<sup>7,8</sup> The most common

**Table 3.** Summary of test results obtained using RP2.1*plus*

	Number of samples (n)	Percentage of total samples (%)
<b>Positive for SARS-CoV-2 only</b>	447	19.23
<b>Positive for SARS-CoV-2 with co-infection</b>	43	1.85
SARS-CoV-2 + Adenovirus	8	—
SARS-CoV-2 + Coronavirus HKU1	1	—
SARS-CoV-2 + Human Rhinovirus / Enterovirus	16	—
SARS-CoV-2 + Parainfluenza Virus 2	2	—
SARS-CoV-2 + Parainfluenza Virus 3	1	—
SARS-CoV-2 + Parainfluenza Virus 4	1	—
SARS-CoV-2 + Respiratory Syncytial Virus	8	—
SARS-CoV-2 + Influenza B	1	—
SARS-CoV-2 + Bordetella pertussis	1	—
SARS-CoV-2 + Multiple other viral strains	4	—
<b>Positive for other viral strain</b>	179	7.70
Adenovirus	16	—
Coronavirus 229E	1	—
Coronavirus HKU1	4	—
Coronavirus NL63	6	—
Human Metapneumovirus	1	—
Human Rhinovirus / Enterovirus	83	—
Parainfluenza Virus 1	2	—
Parainfluenza Virus 2	2	—
Parainfluenza Virus 3	3	—
Parainfluenza Virus 4	3	—
Respiratory Syncytial Virus	37	—
Influenza A	3	—
Influenza B	4	—
Positive for multiple other viral strains	14	—
<b>Positive for bacteria</b>	1	0.04
Bordetella parapertussis	1	—
<b>Negative for all viruses and bacteria included in the panel</b>	1655	71.1
<b>Total samples</b>	<b>2325</b>	<b>100</b>

causative agent in SARS-CoV-2 co-infection varies according to study population. For example, the most common co-infective agent in a cross-sectional study in Indonesia was influenza A virus, followed by influenza B virus.<sup>7</sup> In contrast, the most common co-infective agent in our sample population as well as in a study conducted by Le Glass et al., is human rhinovirus / enterovirus.<sup>8</sup> Of note, in silico analyses of RP2.1*plus* from the manufacturer did not show any loss of sensitivity in detecting SARS-CoV-2, despite the presence of co-infection.

The human rhinovirus / enterovirus causes a predominantly mild and self-limited infection, just like COVID-19.<sup>3,6</sup> However, in patients co-infected with SARS-CoV-2 and rhinovirus, reported cough and dyspnea is significantly more common compared to rhinovirus monoinfection.<sup>8</sup> On the other hand, the likelihood of being transferred to an intensive care unit is not statistically significant between SARS-CoV-2 and rhinovirus co-infection and rhinovirus monoinfection.<sup>8</sup> The impact of co-infection with other causative agents of upper respiratory infections on SARS-CoV-2 replication and transmission is unknown, but a study has shown that human rhinovirus can block SARS-CoV-2 replication by triggering an interferon response.<sup>9</sup>

One limitation of our study is that at the time the study was conducted, circulating SARS-CoV-2 variants of concern only included alpha (B.1.1.7), beta (B.1.351), gamma (P.1), and delta (B.1.617.2). Whether or not the aforementioned frequencies will be similar as SARS-CoV-2 continues to

evolve can be a subject of further study. Additional studies can also expand the dataset to include additional months (or years) of testing; it should be noted that this was only intended to furnish initial data on the utility of rapid syndromic molecular testing in the Philippine setting.

There is no considerable difference between the management of patients with mild SARS-CoV-2 infection and infection with other respiratory pathogens, which consists mainly of symptomatic therapy. However, the ability to definitively rule out SARS-CoV-2 and to simultaneously identify an alternative diagnosis for the patient has potential impact on clinical and resource management when handling such cases.

## CONCLUSION

With a shorter turnaround time and the ability to detect alternative diagnoses and SARS-CoV-2 co-infection, rapid syndromic molecular testing provides meaningful data that can impact clinical and resource management when handling patients with respiratory symptoms. In the emergency room setting, this can facilitate the triaging of patients being admitted to designated hospital wards.

## STATEMENT OF AUTHORSHIP

The authors certified fulfillment of ICMJE authorship criteria.

## AUTHOR DISCLOSURE

The authors declared no conflict of interest.

## FUNDING SOURCE

None.

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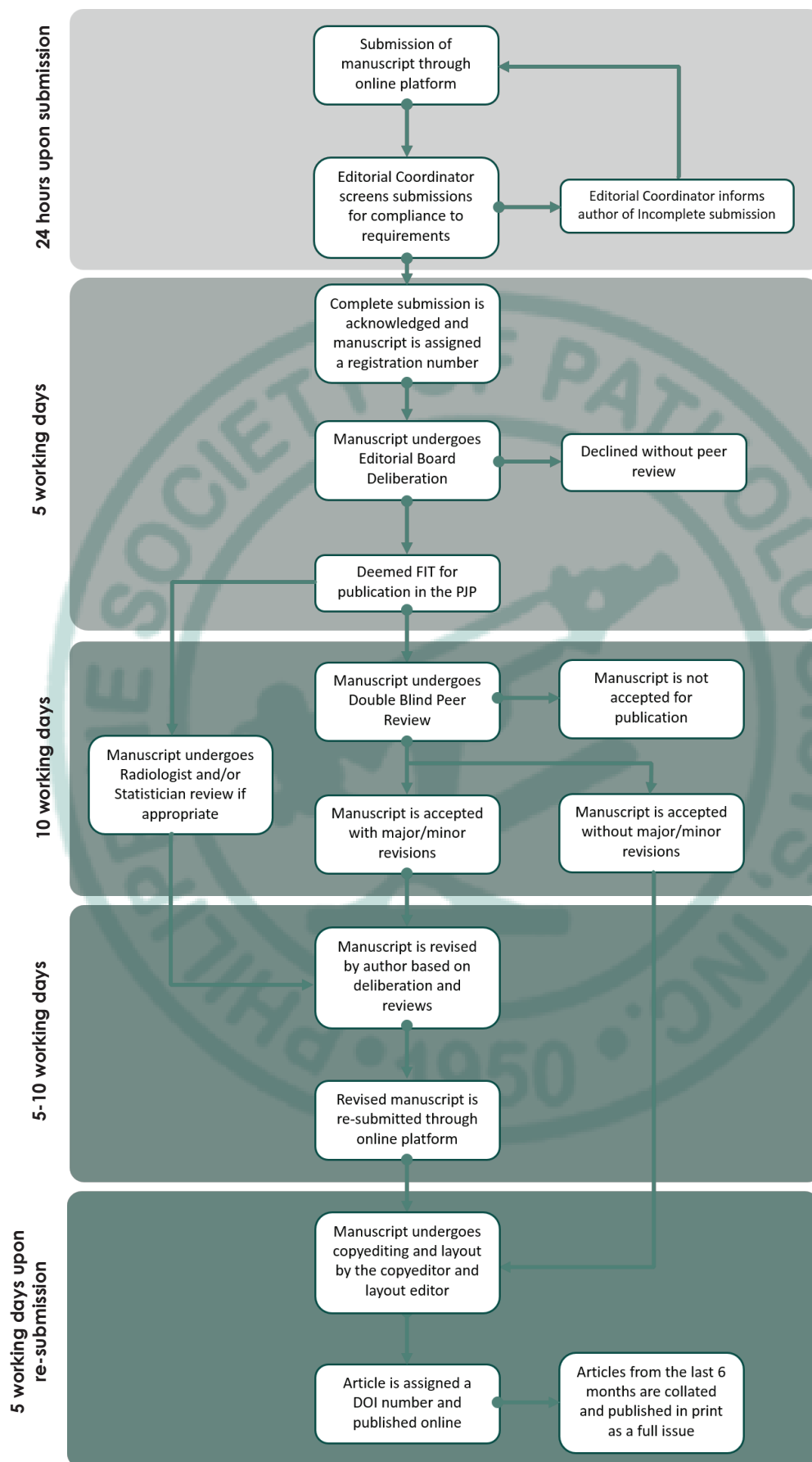


Figure 1. Editorial Process Flow.



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# Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals

**Updated May 2022**

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enforce it. In all cases, authors should use these recommendations along with individual journals' instructions to authors. Authors should also consult guidelines for the reporting of specific study types (e.g., the CONSORT guidelines for the reporting of randomized trials); see [www.equator-network.org](http://www.equator-network.org).

Journals that follow these recommendations are encouraged to incorporate them into their instructions to authors and to make explicit in those instructions that they follow ICMJE recommendations. Journals that wish to be identified on the ICMJE website as following these recommendations should notify the ICMJE secretariat at [www.icmje.org/journals-following-the-icmje-recommendations/journal-listing-request-form/](http://www.icmje.org/journals-following-the-icmje-recommendations/journal-listing-request-form/). Journals that in the past have requested such identification but who no longer follow ICMJE recommendations should use the same means to request removal from this list.

The ICMJE encourages wide dissemination of these recommendations and reproduction of this document in its entirety for educational, not-for-profit purposes without regard for copyright, but all uses of the recommendations and document should direct readers to [www.icmje.org](http://www.icmje.org) for the official, most recent version, as the ICMJE updates the recommendations periodically when new issues arise.

### C. History of the Recommendations

The ICMJE has produced multiple editions of this document, previously known as the Uniform Requirements for Manuscripts Submitted to Biomedical Journals (URMs). The URM was first published in 1978 as a way of standardizing manuscript format and preparation across journals. Over the years, issues in publishing that went well beyond manuscript preparation arose, resulting in the development of separate statements, updates to the document, and its renaming as "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals" to reflect its broader scope. Previous versions of the document may be found in the "Archives" section of [www.icmje.org](http://www.icmje.org).

## II. ROLES AND RESPONSIBILITIES OF AUTHORS, CONTRIBUTORS, REVIEWERS, EDITORS, PUBLISHERS, AND OWNERS

### A. Defining the Role of Authors and Contributors

#### 1. Why Authorship Matters

Authorship confers credit and has important academic, social, and financial implications. Authorship also implies responsibility and accountability for published work. The following recommendations are intended to ensure that contributors who have made substantive intellectual contributions to a paper are given credit as authors, but also that contributors credited as authors understand their role in taking responsibility and being accountable for what is published.

Because authorship does not communicate what contributions qualified an individual to be an author, some journals now request and publish information

about the contributions of each person named as having participated in a submitted study, at least for original research. Editors are strongly encouraged to develop and implement a contributorship policy. Such policies remove much of the ambiguity surrounding contributions, but leave unresolved the question of the quantity and quality of contribution that qualify an individual for authorship. The ICMJE has thus developed criteria for authorship that can be used by all journals, including those that distinguish authors from other contributors.

#### 2. Who Is an Author?

The ICMJE recommends that authorship be based on the following 4 criteria:

1. Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
2. Drafting the work or revising it critically for important intellectual content; AND
3. Final approval of the version to be published; AND
4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

In addition to being accountable for the parts of the work he or she has done, an author should be able to identify which co-authors are responsible for specific other parts of the work. In addition, authors should have confidence in the integrity of the contributions of their co-authors.

All those designated as authors should meet all four criteria for authorship, and all who meet the four criteria should be identified as authors. Those who do not meet all four criteria should be acknowledged—see Section II. A.3 below. These authorship criteria are intended to reserve the status of authorship for those who deserve credit and can take responsibility for the work. The criteria are not intended for use as a means to disqualify colleagues from authorship who otherwise meet authorship criteria by denying them the opportunity to meet criterion #s 2 or 3. Therefore, all individuals who meet the first criterion should have the opportunity to participate in the review, drafting, and final approval of the manuscript.

The individuals who conduct the work are responsible for identifying who meets these criteria and ideally should do so when planning the work, making modifications as appropriate as the work progresses. We encourage collaboration and co-authorship with colleagues in the locations where the research is conducted. It is the collective responsibility of the authors, not the journal to which the work is submitted, to determine that all people named as authors meet all four criteria; it is not the role of journal editors to determine who qualifies or does not qualify for authorship or to arbitrate authorship conflicts. If agreement cannot be reached about who qualifies for authorship, the institution(s) where the work was performed, not the journal editor, should be asked to investigate. The criteria used to determine the order in which authors are listed on the byline may vary, and are to be decided collectively by the author group and not by

editors. If authors request removal or addition of an author after manuscript submission or publication, journal editors should seek an explanation and signed statement of agreement for the requested change from all listed authors and from the author to be removed or added.

The corresponding author is the one individual who takes primary responsibility for communication with the journal during the manuscript submission, peer-review, and publication process. The corresponding author typically ensures that all the journal's administrative requirements, such as providing details of authorship, ethics committee approval, clinical trial registration documentation, and disclosures of relationships and activities, are properly completed and reported, although these duties may be delegated to one or more co-authors. The corresponding author should be available throughout the submission and peer-review process to respond to editorial queries in a timely way, and should be available after publication to respond to critiques of the work and cooperate with any requests from the journal for data or additional information should questions about the paper arise after publication. Although the corresponding author has primary responsibility for correspondence with the journal, the ICMJE recommends that editors send copies of all correspondence to all listed authors.

When a large multi-author group has conducted the work, the group ideally should decide who will be an author before the work is started and confirm who is an author before submitting the manuscript for publication. All members of the group named as authors should meet all four criteria for authorship, including approval of the final manuscript, and they should be able to take public responsibility for the work and should have full confidence in the accuracy and integrity of the work of other group authors. They will also be expected as individuals to complete disclosure forms.

Some large multi-author groups designate authorship by a group name, with or without the names of individuals. When submitting a manuscript authored by a group, the corresponding author should specify the group name if one exists, and clearly identify the group members who can take credit and responsibility for the work as authors. The byline of the article identifies who is directly responsible for the manuscript, and MEDLINE lists as authors whichever names appear on the byline. If the byline includes a group name, MEDLINE will list the names of individual group members who are authors or who are collaborators, sometimes called non-author contributors, if there is a note associated with the byline clearly stating that the individual names are elsewhere in the paper and whether those names are authors or collaborators.

### **3. Non-Author Contributors**

Contributors who meet fewer than all 4 of the above criteria for authorship should not be listed as authors, but they should be acknowledged. Examples of activities that alone (without other contributions) do not qualify a contributor for authorship are acquisition of funding;

general supervision of a research group or general administrative support; and writing assistance, technical editing, language editing, and proofreading. Those whose contributions do not justify authorship may be acknowledged individually or together as a group under a single heading (e.g., "Clinical Investigators" or "Participating Investigators"), and their contributions should be specified (e.g., "served as scientific advisors," "critically reviewed the study proposal," "collected data," "provided and cared for study patients," "participated in writing or technical editing of the manuscript").

Because acknowledgment may imply endorsement by acknowledged individuals of a study's data and conclusions, editors are advised to require that the corresponding author obtain written permission to be acknowledged from all acknowledged individuals.

## **B. Disclosure of Financial and Non-Financial Relationships and Activities, and Conflicts of Interest**

Public trust in the scientific process and the credibility of published articles depend in part on how transparently an author's relationships and activities, directly or topically related to a work, are handled during the planning, implementation, writing, peer review, editing, and publication of scientific work.

The potential for conflict of interest and bias exists when professional judgment concerning a primary interest (such as patients' welfare or the validity of research) may be influenced by a secondary interest (such as financial gain). Perceptions of conflict of interest are as important as actual conflicts of interest.

Individuals may disagree on whether an author's relationships or activities represent conflicts. Although the presence of a relationship or activity does not always indicate a problematic influence on a paper's content, perceptions of conflict may erode trust in science as much as actual conflicts of interest. Ultimately, readers must be able to make their own judgments regarding whether an author's relationships and activities are pertinent to a paper's content. These judgments require transparent disclosures. An author's complete disclosure demonstrates a commitment to transparency and helps to maintain trust in the scientific process.

Financial relationships (such as employment, consultancies, stock ownership or options, honoraria, patents, and paid expert testimony) are the most easily identifiable, the ones most often judged to represent potential conflicts of interest and thus the most likely to undermine the credibility of the journal, the authors, and science itself. Other interests may also represent or be perceived as conflicts, such as personal relationships or rivalries, academic competition, and intellectual beliefs.

Authors should avoid entering into agreements with study sponsors, both for-profit and nonprofit, that interfere with authors' access to all of the study's data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose. Policies that dictate where authors may publish their work violate this

principle of academic freedom. Authors may be required to provide the journal with the agreements in confidence.

Purposeful failure to report those relationships or activities specified on the journal's disclosure form is a form of misconduct, as is discussed in Section III.B.

### **1. Participants**

All participants in the peer-review and publication process—not only authors but also peer reviewers, editors, and editorial board members of journals—must consider and disclose their relationships and activities when fulfilling their roles in the process of article review and publication.

#### **a. Authors**

When authors submit a manuscript of any type or format they are responsible for disclosing all relationships and activities that might bias or be seen to bias their work. The ICMJE has developed a Disclosure Form to facilitate and standardize authors' disclosures. ICMJE member journals require that authors use this form, and ICMJE encourages other journals to adopt it.

#### **b. Peer Reviewers**

Reviewers should be asked at the time they are asked to critique a manuscript if they have relationships or activities that could complicate their review. Reviewers must disclose to editors any relationships or activities that could bias their opinions of the manuscript, and should recuse themselves from reviewing specific manuscripts if the potential for bias exists. Reviewers must not use knowledge of the work they're reviewing before its publication to further their own interests.

#### **c. Editors and Journal Staff**

Editors who make final decisions about manuscripts should recuse themselves from editorial decisions if they have relationships or activities that pose potential conflicts related to articles under consideration. Other editorial staff members who participate in editorial decisions must provide editors with a current description of their relationships and activities (as they might relate to editorial judgments) and recuse themselves from any decisions in which an interest that poses a potential conflict exists. Editorial staff must not use information gained through working with manuscripts for private gain. Editors should regularly publish their own disclosure statements and those of their journal staff. Guest editors should follow these same procedures.

Journals should take extra precautions and have a stated policy for evaluation of manuscripts submitted by individuals involved in editorial decisions. Further guidance is available from COPE ([https://publicationethics.org/files/A\\_Short\\_Guide\\_to\\_Ethical\\_Editing.pdf](https://publicationethics.org/files/A_Short_Guide_to_Ethical_Editing.pdf)) and WAME (<http://wame.org/conflict-of-interest-in-peer-reviewed-medical-journals>).

### **2. Reporting Relationships and Activities**

Articles should be published with statements or supporting documents, such as the ICMJE Disclosure Form, declaring:

- Authors' relationships and activities; and

- Sources of support for the work, including sponsor names along with explanations of the role of those sources if any in study design; collection, analysis, and interpretation of data; writing of the report; any restrictions regarding the submission of the report for publication; or a statement declaring that the supporting source had no such involvement or restrictions regarding publication; and
- Whether the authors had access to the study data, with an explanation of the nature and extent of access, including whether access is ongoing.

To support the above statements, editors may request that authors of a study sponsored by a funder with a proprietary or financial interest in the outcome sign a statement, such as "I had full access to all of the data in this study and I take complete responsibility for the integrity of the data and the accuracy of the data analysis."

### **C. Responsibilities in the Submission and Peer-Review Process**

#### **1. Authors**

Authors should abide by all principles of authorship and declaration of relationships and activities detailed in Sections II.A and II.B of this document.

#### **a. Predatory or Pseudo-Journals**

A growing number of entities are advertising themselves as "scholarly medical journals" yet do not function as such. These journals ("predatory" or "pseudo-journals") accept and publish almost all submissions and charge article processing (or publication) fees, often informing authors about this after a paper's acceptance for publication. They often claim to perform peer review but do not and may purposefully use names similar to well-established journals. They may state that they are members of ICMJE but are not (see [www.icmje.org](http://www.icmje.org) for current members of the ICMJE) and that they follow the recommendations of organizations such as the ICMJE, COPE, and WAME. Researchers must be aware of the existence of such entities and avoid submitting research to them for publication. Authors have a responsibility to evaluate the integrity, history, practices, and reputation of the journals to which they submit manuscripts. Guidance from various organizations is available to help identify the characteristics of reputable peer-reviewed journals ([www.wame.org/identifying-predatory-or-pseudo-journals](http://www.wame.org/identifying-predatory-or-pseudo-journals) and [www.wame.org/principles-of-transparency-and-best-practice-in-scholarly-publishing](http://www.wame.org/principles-of-transparency-and-best-practice-in-scholarly-publishing)).

Seeking the assistance of scientific mentors, senior colleagues, and others with many years of scholarly publishing experience may also be helpful.

Authors should avoid citing articles in predatory or pseudo-journals.

#### **2. Journals**

##### **a. Confidentiality**

Manuscripts submitted to journals are privileged communications that are authors' private, confidential property, and authors may be harmed by premature disclosure of any or all of a manuscript's details.



Editors therefore must not share information about manuscripts, including whether they have been received and are under review, their content and status in the review process, criticism by reviewers, and their ultimate fate, to anyone other than the authors and reviewers. Requests from third parties to use manuscripts and reviews for legal proceedings should be politely refused, and editors should do their best not to provide such confidential material should it be subpoenaed.

Editors must also make clear that reviewers should keep manuscripts, associated material, and the information they contain strictly confidential. Reviewers and editorial staff members must not publicly discuss the authors' work, and reviewers must not appropriate authors' ideas before the manuscript is published. Reviewers must not retain the manuscript for their personal use and should destroy paper copies of manuscripts and delete electronic copies after submitting their reviews.

When a manuscript is rejected, it is best practice for journals to delete copies of it from their editorial systems unless retention is required by local regulations. Journals that retain copies of rejected manuscripts should disclose this practice in their Information for Authors.

When a manuscript is published, journals should keep copies of the original submission, reviews, revisions, and correspondence for at least three years and possibly in perpetuity, depending on local regulations, to help answer future questions about the work should they arise.

Editors should not publish or publicize peer reviewers' comments without permission of the reviewer and author. If journal policy is to blind authors to reviewer identity and comments are not signed, that identity must not be revealed to the author or anyone else without the reviewers' expressed written permission.

Confidentiality may have to be breached if dishonesty or fraud is alleged, but editors should notify authors or reviewers if they intend to do so and confidentiality must otherwise be honored.

#### **b. Timeliness**

Editors should do all they can to ensure timely processing of manuscripts with the resources available to them. If editors intend to publish a manuscript, they should attempt to do so in a timely manner and any planned delays should be negotiated with the authors. If a journal has no intention of proceeding with a manuscript, editors should endeavor to reject the manuscript as soon as possible to allow authors to submit to a different journal.

#### **c. Peer Review**

Peer review is the critical assessment of manuscripts submitted to journals by experts who are usually not part of the editorial staff. Because unbiased, independent, critical assessment is an intrinsic part of all scholarly work, including scientific research, peer review is an important extension of the scientific process.

The actual value of peer review is widely debated, but the process facilitates a fair hearing for a manuscript

among members of the scientific community. More practically, it helps editors decide which manuscripts are suitable for their journals. Peer review often helps authors and editors improve the quality of reporting.

It is the responsibility of the journal to ensure that systems are in place for selection of appropriate reviewers. It is the responsibility of the editor to ensure that reviewers have access to all materials that may be relevant to the evaluation of the manuscript, including supplementary material for e-only publication, and to ensure that reviewer comments are properly assessed and interpreted in the context of their declared relationships and activities.

A peer-reviewed journal is under no obligation to send submitted manuscripts for review, and under no obligation to follow reviewer recommendations, favorable or negative. The editor of a journal is ultimately responsible for the selection of all its content, and editorial decisions may be informed by issues unrelated to the quality of a manuscript, such as suitability for the journal. An editor can reject any article at any time before publication, including after acceptance if concerns arise about the integrity of the work.

Journals may differ in the number and kinds of manuscripts they send for review, the number and types of reviewers they seek for each manuscript, whether the review process is open or blinded, and other aspects of the review process. For this reason and as a service to authors, journals should publish a clear, transparent description of their peer-review process for all types of manuscripts.

Journals should notify reviewers of the ultimate decision to accept or reject a paper, and should acknowledge the contribution of peer reviewers to their journal. Editors are encouraged to share reviewers' comments with co-reviewers of the same paper, so reviewers can learn from each other in the review process.

As part of peer review, editors are encouraged to review research protocols, plans for statistical analysis if separate from the protocol, and/or contracts associated with project-specific studies. Editors should encourage authors to make such documents publicly available at the time of or after publication, before accepting such studies for publication. Some journals may require public posting of these documents as a condition of acceptance for publication.

Journal requirements for independent data analysis and for public data availability are in flux at the time of this revision, reflecting evolving views of the importance of data availability for pre- and post-publication peer review. Some journal editors currently request a statistical analysis of trial data by an independent biostatistician before accepting studies for publication. Others ask authors to say whether the study data are available to third parties to view and/or use/reanalyze, while still others encourage or require authors to share their data with others for review or reanalysis. Each journal should establish and publish their specific requirements for data analysis and post in a place that potential authors can easily access.

Some people believe that true scientific peer review begins only on the date a paper is published. In that spirit, medical journals should have a mechanism for readers to submit comments, questions, or criticisms about published articles, and authors have a responsibility to respond appropriately and cooperate with any requests from the journal for data or additional information should questions about the paper arise after publication (see Section III).

ICMJE believes investigators have a duty to maintain the primary data and analytic procedures underpinning the published results for at least 10 years. The ICMJE encourages the preservation of these data in a data repository to ensure their longer-term availability.

#### **d. Integrity**

Editorial decisions should be based on the relevance of a manuscript to the journal and on the manuscript's originality, quality, and contribution to evidence about important questions. Those decisions should not be influenced by commercial interests, personal relationships or agendas, or findings that are negative or that credibly challenge accepted wisdom. In addition, authors should submit for publication or otherwise make publicly available, and editors should not exclude from consideration for publication, studies with findings that are not statistically significant or that have inconclusive findings. Such studies may provide evidence that, combined with that from other studies through meta-analysis, might still help answer important questions, and a public record of such negative or inconclusive findings may prevent unwarranted replication of effort or otherwise be valuable for other researchers considering similar work.

Journals should clearly state their appeals process and should have a system for responding to appeals and complaints.

#### **e. Diversity and Inclusion**

To improve academic culture, editors should seek to engage a broad and diverse array of authors, reviewers, editorial staff, editorial board members, and readers.

#### **f. Journal Metrics**

The journal impact factor is widely misused as a proxy for research and journal quality and as a measure of the importance of specific research projects or the merits of individual researchers, including their suitability for hiring, promotion, tenure, prizes, or research funding. ICMJE recommends that journals reduce the emphasis on impact factor as a single measure, but rather provide a range of article and journal metrics relevant to their readers and authors.

### **3. Peer Reviewers**

Manuscripts submitted to journals are privileged communications that are authors' private, confidential property, and authors may be harmed by premature disclosure of any or all of a manuscript's details.

Reviewers therefore should keep manuscripts and the information they contain strictly confidential. Reviewers must not publicly discuss authors' work and must not appropriate authors' ideas before the manu-

script is published. Reviewers must not retain the manuscript for their personal use and should destroy copies of manuscripts after submitting their reviews.

Reviewers who seek assistance from a trainee or colleague in the performance of a review should acknowledge these individuals' contributions in the written comments submitted to the editor. These individuals must maintain the confidentiality of the manuscript as outlined above.

Reviewers are expected to respond promptly to requests to review and to submit reviews within the time agreed. Reviewers' comments should be constructive, honest, and polite.

Reviewers should declare their relationships and activities that might bias their evaluation of a manuscript and recuse themselves from the peer-review process if a conflict exists.

## **D. Journal Owners and Editorial Freedom**

### **1. Journal Owners**

Owners and editors of medical journals share a common purpose, but they have different responsibilities, and sometimes those differences lead to conflicts.

It is the responsibility of medical journal owners to appoint and dismiss editors. Owners should provide editors at the time of their appointment with a contract that clearly states their rights and duties, authority, the general terms of their appointment, and mechanisms for resolving conflict. The editor's performance may be assessed using mutually agreed-upon measures, including but not necessarily limited to readership, manuscript submissions and handling times, and various journal metrics.

Owners should only dismiss editors for substantial reasons, such as scientific misconduct, disagreement with the long-term editorial direction of the journal, inadequate performance by agreed-upon performance metrics, or inappropriate behavior that is incompatible with a position of trust.

Appointments and dismissals should be based on evaluations by a panel of independent experts, rather than by a small number of executives of the owning organization. This is especially necessary in the case of dismissals because of the high value society places on freedom of speech within science and because it is often the responsibility of editors to challenge the status quo in ways that may conflict with the interests of the journal's owners.

A medical journal should explicitly state its governance and relationship to a journal owner (e.g., a sponsoring society).

### **2. Editorial Freedom**

The ICMJE adopts the World Association of Medical Editors' definition of editorial freedom (<http://wame.org/editorial-independence>), which holds that editors-in-chief have full authority over the entire editorial content of their journal and the timing of publication of that content. Journal owners should not interfere in the evaluation, selection, scheduling, or editing of individual articles either directly or by creating an environment that

strongly influences decisions. Editors should base editorial decisions on the validity of the work and its importance to the journal's readers, not on the commercial implications for the journal, and editors should be free to express critical but responsible views about all aspects of medicine without fear of retribution, even if these views conflict with the commercial goals of the publisher.

Editors-in-chief should also have the final say in decisions about which advertisements or sponsored content, including supplements, the journal will and will not carry, and they should have final say in use of the journal brand and in overall policy regarding commercial use of journal content.

Journals are encouraged to establish an independent and diverse editorial advisory board to help the editor establish and maintain editorial policy. To support editorial decisions and potentially controversial expressions of opinion, owners should ensure that appropriate insurance is obtained in the event of legal action against the editors, and should ensure that legal advice is available when necessary. If legal problems arise, the editor should inform their legal adviser and their owner and/or publisher as soon as possible. Editors should defend the confidentiality of authors and peer reviewers (names and reviewer comments) in accordance with ICMJE policy (see Section II.C.2.a). Editors should take all reasonable steps to check the facts in journal commentary, including that in news sections and social media postings, and should ensure that staff working for the journal adhere to best journalistic practices including contemporaneous note-taking and seeking a response from all parties when possible before publication. Such practices in support of truth and public interest may be particularly relevant in defense against legal allegations of libel.

To secure editorial freedom in practice, the editor should have direct access to the highest level of ownership, not to a delegated manager or administrative officer.

Editors and editors' organizations are obliged to support the concept of editorial freedom and to draw major transgressions of such freedom to the attention of the international medical, academic, and lay communities.

#### **E. Protection of Research Participants**

All investigators should ensure that the planning, conduct, and reporting of human research are in accordance with the Helsinki Declaration as revised in 2013 ([www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/](http://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/)). All authors should seek approval to conduct research from an independent local, regional, or national review body (e.g., ethics committee, institutional review board). If doubt exists whether the research was conducted in accordance with the Helsinki Declaration, the authors must explain the rationale for their approach and demonstrate that the local, regional, or national review body explicitly approved the doubtful aspects of the study. Approval by a responsible review body does not preclude editors from forming their own judgment whether the conduct of the research was appropriate.

Patients have a right to privacy that should not be violated without informed consent. Identifying information, including names, initials, or hospital numbers, should not be published in written descriptions, photographs, or pedigrees unless the information is essential for scientific purposes and the patient (or parent or guardian) gives written informed consent for publication. Informed consent for this purpose requires that an identifiable patient be shown the manuscript to be published. Authors should disclose to these patients whether any potential identifiable material might be available via the Internet as well as in print after publication. Patient consent should be written and archived with the journal, the authors, or both, as dictated by local regulations or laws. Applicable laws vary from locale to locale, and journals should establish their own policies with legal guidance. Since a journal that archives the consent will be aware of patient identity, some journals may decide that patient confidentiality is better guarded by having the author archive the consent and instead providing the journal with a written statement that attests that they have received and archived written patient consent.

Nonessential identifying details should be omitted. Informed consent should be obtained if there is any doubt that anonymity can be maintained. For example, masking the eye region in photographs of patients is inadequate protection of anonymity. If identifying characteristics are deidentified, authors should provide assurance, and editors should so note, that such changes do not distort scientific meaning.

The requirement for informed consent should be included in the journal's instructions for authors. When informed consent has been obtained, it should be indicated in the published article.

When reporting experiments on animals, authors should indicate whether institutional and national standards for the care and use of laboratory animals were followed.

### **III. PUBLISHING AND EDITORIAL ISSUES RELATED TO PUBLICATION IN MEDICAL JOURNALS**

#### **A. Corrections, Retractions, Republications, and Version Control**

Honest errors are a part of science and publishing and require publication of a correction when they are detected. Corrections are needed for errors of fact. Matters of debate are best handled as letters to the editor, as print or electronic correspondence, or as posts in a journal-sponsored online forum. Updates of previous publications (e.g., an updated systematic review or clinical guideline) are considered a new publication rather than a version of a previously published article.

If a correction is needed, journals should follow these minimum standards:

- The journal should publish a correction notice as soon as possible detailing changes from and citing the original publication; the correction should be on an electronic or numbered print page that is

included in an electronic or a print Table of Contents to ensure proper indexing.

- The journal should also post a new article version with details of the changes from the original version and the date(s) on which the changes were made.
- The journal should archive all prior versions of the article. This archive can be either directly accessible to readers or can be made available to the reader on request.
- Previous electronic versions should prominently note that there are more recent versions of the article.
- The citation should be to the most recent version.

Pervasive errors can result from a coding problem or a miscalculation and may result in extensive inaccuracies throughout an article. If such errors do not change the direction or significance of the results, interpretations, and conclusions of the article, a correction should be published that follows the minimum standards noted above.

Errors serious enough to invalidate a paper's results and conclusions may require retraction. However, retraction with republication (also referred to as "replacement") can be considered in cases where honest error (e.g., a misclassification or miscalculation) leads to a major change in the direction or significance of the results, interpretations, and conclusions. If the error is judged to be unintentional, the underlying science appears valid, and the changed version of the paper survives further review and editorial scrutiny, then retraction with republication of the changed paper, with an explanation, allows full correction of the scientific literature. In such cases, it is helpful to show the extent of the changes in supplementary material or in an appendix, for complete transparency.

## **B. Scientific Misconduct, Expressions of Concern, and Retraction**

Scientific misconduct in research and non-research publications includes but is not necessarily limited to data fabrication; data falsification, including deceptive manipulation of images; purposeful failure to disclose relationships and activities; and plagiarism. Some people consider failure to publish the results of clinical trials and other human studies a form of scientific misconduct. While each of these practices is problematic, they are not equivalent. Each situation requires individual assessment by relevant stakeholders. When scientific misconduct is alleged, or concerns are otherwise raised about the conduct or integrity of work described in submitted or published papers, the editor should initiate appropriate procedures detailed by such committees as the Committee on Publication Ethics (COPE) (<http://publicationethics.org/resources/flowcharts>), consider informing the institutions and funders, and may choose to publish an expression of concern pending the outcomes of those procedures. If the procedures involve an investigation at the authors' institution, the editor should seek to discover the outcome of that investigation; notify readers of the outcome if appropriate; and if the investigation proves scientific misconduct, publish a retraction of the article. There may be circumstances in which no misconduct is proven, but an exchange of letters to the editor could be published to highlight matters of debate to readers.

Expressions of concern and retractions should not simply be a letter to the editor. Rather, they should be prominently labelled, appear on an electronic or numbered print page that is included in an electronic or a print Table of Contents to ensure proper indexing, and include in their heading the title of the original article. Online, the retraction and original article should be linked in both directions and the retracted article should be clearly labelled as retracted in all its forms (abstract, full text, PDF). Ideally, the authors of the retraction should be the same as those of the article, but if they are unwilling or unable the editor may under certain circumstances accept retractions by other responsible persons, or the editor may be the sole author of the retraction or expression of concern. The text of the retraction should explain why the article is being retracted and include a complete citation reference to that article.

Retracted articles should remain in the public domain and be clearly labelled as retracted.

The validity of previous work by the author of a fraudulent paper cannot be assumed. Editors may ask the author's institution to assure them of the validity of other work published in their journals, or they may retract it. If this is not done, editors may choose to publish an announcement expressing concern that the validity of previously published work is uncertain.

The integrity of research may also be compromised by inappropriate methodology that could lead to retraction.

See COPE flowcharts for further guidance on retractions and expressions of concern. See Section IV.A.1.g.i for guidance about avoiding referencing retracted articles.

## **C. Copyright**

Journals should make clear the type of copyright under which work will be published, and if the journal retains copyright, should detail the journal's position on the transfer of copyright for all types of content, including audio, video, protocols, and data sets. Medical journals may ask authors to transfer copyright to the journal. Some journals require transfer of a publication license. Some journals do not require transfer of copyright and rely on such vehicles as Creative Commons licenses. The copyright status of articles in a given journal can vary: Some content cannot be copyrighted (e.g., articles written by employees of some governments in the course of their work). Editors may waive copyright on other content, and some content may be protected under other agreements.

## **D. Overlapping Publications**

### **1. Duplicate Submission**

Authors should not submit the same manuscript, in the same or different languages, simultaneously to more than one journal. The rationale for this standard is the potential for disagreement when two (or more) journals claim the right to publish a manuscript that has been submitted simultaneously to more than one journal, and the possibility that two or more journals will unknowingly and unnecessarily undertake the work of peer review, edit the same manuscript, and publish the same article.

## 2. Duplicate and Prior Publication

Duplicate publication is publication of a paper that overlaps substantially with one already published, without clear, visible reference to the previous publication. Prior publication may include release of information in the public domain.

Readers of medical journals deserve to be able to trust that what they are reading is original unless there is a clear statement that the author and editor are intentionally republishing an article (which might be considered for historic or landmark papers, for example). The bases of this position are international copyright laws, ethical conduct, and cost-effective use of resources. Duplicate publication of original research is particularly problematic because it can result in inadvertent double-counting of data or inappropriate weighting of the results of a single study, which distorts the available evidence.

When authors submit a manuscript reporting work that has already been reported in large part in a published article or is contained in or closely related to another paper that has been submitted or accepted for publication elsewhere, the letter of submission should clearly say so and the authors should provide copies of the related material to help the editor decide how to handle the submission. See also Section IV.B.

This recommendation does not prevent a journal from considering a complete report that follows publication of a preliminary report, such as a letter to the editor, a preprint, or an abstract or poster displayed at a scientific meeting. The ICMJE does not consider results or data contained in assessment reports published by health technology assessment agencies, medical regulators, medical device regulators, or other regulatory agencies to be duplicate publication. It also does not prevent journals from considering a paper that has been presented at a scientific meeting but was not published in full, or that is being considered for publication in proceedings or similar format. Press reports of scheduled meetings are not usually regarded as breaches of this rule, but they may be if additional data tables or figures enrich such reports. Authors should also consider how dissemination of their findings outside of scientific presentations at meetings may diminish the priority journal editors assign to their work.

Authors who choose to post their work on a preprint server should choose one that clearly identifies preprints as not peer-reviewed work and includes disclosures of authors' relationships and activities. It is the author's responsibility to inform a journal if the work has been previously posted on a preprint server. In addition, it is the author's (and not the journal editors') responsibility to ensure that preprints are amended to point readers to subsequent versions, including the final published article. See Section III.D.3.

In the event of a public health emergency (as defined by public health officials), information with immediate implications for public health should be disseminated without concern that this will preclude subsequent consideration for publication in a journal. We encourage editors to give priority to authors who have made crucial data publicly available without delay.

Sharing with public media, government agencies, or manufacturers the scientific information described in a paper or a letter to the editor that has been accepted but not yet published violates the policies of many journals. Such reporting may be warranted when the paper or letter describes major therapeutic advances; reportable diseases; or public health hazards, such as serious adverse effects of drugs, vaccines, other biological products, medical devices. This reporting, whether in print or online, should not jeopardize publication, but should be discussed with and agreed upon by the editor in advance when possible.

The ICMJE will not consider as prior publication the posting of trial results in any registry that meets the criteria noted in Section III.L if results are limited to a brief (500 word) structured abstract or tables (to include participants enrolled, key outcomes, and adverse events). The ICMJE encourages authors to include a statement with the registration that indicates that the results have not yet been published in a peer-reviewed journal, and to update the results registry with the full journal citation when the results are published.

Editors of different journals may together decide to simultaneously or jointly publish an article if they believe that doing so would be in the best interest of public health. However, the National Library of Medicine (NLM) indexes all such simultaneously published joint publications separately, so editors should include a statement making the simultaneous publication clear to readers.

Authors who attempt duplicate publication without such notification should expect at least prompt rejection of the submitted manuscript. If the editor was not aware of the violations and the article has already been published, then the article might warrant retraction with or without the author's explanation or approval.

See COPE flowcharts for further guidance on handling duplicate publication.

## 3. Preprints

Posting of work as a preprint may influence a journal's interest in or priority for peer review and publication of that work. Journals should clearly describe their policies related to the posting and citing of preprints in their Information for Authors. Authors should become familiar with the policies of journals they wish to submit their work to prior to posting work on a preprint server.

### a. Choosing a Preprint Archive

There has been an increase in preprint archives in biomedicine. There are both benefits and harms in dissemination of scientific findings prior to peer review. To maximize potential benefits and minimize potential harms, authors who wish to make preprints of non-peer-reviewed work publicly available should choose preprint archives that have the following characteristics:

- Clearly identify preprints as work that is not peer reviewed;
- Require authors to document disclosures of interest;
- Require authors to indicate funding source(s);

- Have a clear process for preprint archive users to notify archive administrators about concerns related to posted preprints—a public commenting feature is desirable for this purpose;
- Maintain metadata for preprints that are withdrawn from posting and post withdrawal notices indicating the timing and reason for withdrawal of a preprint; and
- Have a mechanism for authors to indicate when the preprint article has been subsequently published in a peer-reviewed journal.

#### **b. Submitting Manuscripts That Are in Preprint Archives to a Peer-Reviewed Journal**

Authors should inform a journal if the work submitted to the journal has been posted on a preprint server and provide a link to the preprint, whether the posting occurs prior to submission or during the peer-review process. It is also helpful to indicate in the text of the manuscript, perhaps in the introduction, that a preprint is available and how reviewers can access that preprint. In addition, it is the authors' (and not the journal editors') responsibility to ensure that preprints are amended to point readers to subsequent versions of the work, including the published article. Authors should not post in the preprint archive the published article nor interim versions that are produced during the peer-review process that incorporate revisions based on journal feedback.

#### **c. Referencing Preprints in Submitted Manuscripts**

When preprints are cited in submitted manuscripts or published articles, the citation should clearly indicate that the reference is a preprint. When a preprint article has been subsequently published in a peer-reviewed journal, authors should cite the subsequent published article rather than the preprint article whenever appropriate. Journals should include the word "preprint" following the citation information in the reference list and consider indicating that the cited material is a preprint in the text. The citation should include the link to the preprint and DOI if the preprint archive issues DOIs. Authors should be cautious about referencing preprints that were posted and never subsequently published in a peer-reviewed journal, but the time interval of concern will vary depending on the topic and specific reasons for citation.

#### **4. Acceptable Secondary Publication**

Secondary publication of material published in other journals or online may be justifiable and beneficial, especially when intended to disseminate important information to the widest possible audience (e.g., guidelines produced by government agencies and professional organizations in the same or a different language). Secondary publication for various other reasons may also be justifiable provided the following conditions are met:

1. The authors have received approval from the editors of both journals (the editor concerned with secondary publication must have access to the primary version).

2. The priority of the primary publication is respected by a publication interval negotiated by both editors with the authors.
3. The paper for secondary publication is intended for a different group of readers; an abbreviated version could be sufficient.
4. The secondary version faithfully reflects the authors, data, and interpretations of the primary version.
5. The secondary version informs readers, peers, and documenting agencies that the paper has been published in whole or in part elsewhere—for example, with a note that might read, "This article is based on a study first reported in the [journal title, with full reference]"—and the secondary version cites the primary reference.
6. The title of the secondary publication should indicate that it is a secondary publication (complete or abridged republication or translation) of a primary publication. Of note, the NLM does not consider translations to be "republications" and does not cite or index them when the original article was published in a journal that is indexed in MEDLINE.

When the same journal simultaneously publishes an article in multiple languages, the MEDLINE citation will note the multiple languages (e.g., Angelo M. Journal networking in nursing: a challenge to be shared. *Rev Esc Enferm USP*. 2011 Dec 45[6]:1281-2,1279-80,1283-4. Article in English, Portuguese, and Spanish. No abstract available. PMID: 22241182).

#### **5. Manuscripts Based on the Same Database**

If editors receive manuscripts from separate research groups or from the same group analyzing the same data set (e.g., from a public database, or systematic reviews or meta-analyses of the same evidence), the manuscripts should be considered independently because they may differ in their analytic methods, conclusions, or both. If the data interpretation and conclusions are similar, it may be reasonable although not mandatory for editors to give preference to the manuscript submitted first. Editors might consider publishing more than one manuscript that overlap in this way because different analytical approaches may be complementary and equally valid, but manuscripts based upon the same data set should add substantially to each other to warrant consideration for publication as separate papers, with appropriate citation of previous publications from the same data set to allow for transparency.

Secondary analyses of clinical trial data should cite any primary publication, clearly state that it contains secondary analyses/results, and use the same identifying trial registration number as the primary trial and unique, persistent data set identifier.

Sometimes for large trials it is planned from the beginning to produce numerous separate publications regarding separate research questions but using the same original participant sample. In this case authors may use the original single trial registration number, if all the outcome parameters were defined in the original registration. If the authors registered several substudies as separate entries in, for example, ClinicalTrials.gov,

then the unique trial identifier should be given for the study in question. The main issue is transparency, so no matter what model is used it should be obvious for the reader.

### **E. Correspondence**

Medical journals should provide readers with a mechanism for submitting comments, questions, or criticisms about published articles, usually but not necessarily always through a correspondence section or online forum. The authors of articles discussed in correspondence or an online forum have a responsibility to respond to substantial criticisms of their work using those same mechanisms and should be asked by editors to respond. Authors of correspondence should be asked to declare any competing relationships or activities.

Correspondence may be edited for length, grammatical correctness, and journal style. Alternatively, editors may choose to make available to readers unedited correspondence, for example, via an online commenting system. Such commenting is not indexed in MEDLINE unless it is subsequently published on a numbered electronic or print page. However the journal handles correspondence, it should make known its practice. In all instances, editors must make an effort to screen discourteous, inaccurate, or libellous comments.

Responsible debate, critique, and disagreement are important features of science, and journal editors should encourage such discourse ideally within their own journals about the material they have published. Editors, however, have the prerogative to reject correspondence that is irrelevant, uninteresting, or lacking cogency, but they also have a responsibility to allow a range of opinions to be expressed and to promote debate.

In the interests of fairness and to keep correspondence within manageable proportions, journals may want to set time limits for responding to published material and for debate on a given topic.

### **F. Fees**

Journals should be transparent about their types of revenue streams. Any fees or charges that are required for manuscript processing and/or publishing materials in the journal shall be clearly stated in a place that is easy for potential authors to find prior to submitting their manuscripts for review or explained to authors before they begin preparing their manuscript for submission ([http://publicationethics.org/files/u7140/Principles\\_of\\_Transparency\\_and\\_Best\\_Practice\\_in\\_Scholarly\\_Publishing.pdf](http://publicationethics.org/files/u7140/Principles_of_Transparency_and_Best_Practice_in_Scholarly_Publishing.pdf)).

### **G. Supplements, Theme Issues, and Special Series**

Supplements are collections of papers that deal with related issues or topics, are published as a separate issue of the journal or as part of a regular issue, and may be funded by sources other than the journal's publisher. Because funding sources can bias the content of supplements through the choice of topics and viewpoints, journals should adopt the following principles, which also apply to theme issues or special series that have external funding and/or guest editors:

1. The journal editor must be given and must take full responsibility for the policies, practices, and content of supplements, including complete control of the decision to select authors, peer reviewers, and content for the supplement. Editing by the funding organization should not be permitted.
2. The journal editor has the right to appoint one or more external editors of the supplement and must take responsibility for the work of those editors.
3. The journal editor must retain the authority to send supplement manuscripts for external peer review and to reject manuscripts submitted for the supplement with or without external review. These conditions should be made known to authors and any external editors of the supplement before beginning editorial work on it.
4. The source of the idea for the supplement, sources of funding for the supplement's research and publication, and products of the funding source related to content considered in the supplement should be clearly stated in the introductory material.
5. Advertising in supplements should follow the same policies as those of the primary journal.
6. Journal editors must enable readers to distinguish readily between ordinary editorial pages and supplement pages.
7. Journal and supplement editors must not accept personal favors or direct remuneration from sponsors of supplements.
8. Secondary publication in supplements (republishing of papers published elsewhere) should be clearly identified by the citation of the original paper and by the title.
9. The same principles of authorship and disclosure of relationships and activities discussed elsewhere in this document should be applied to supplements.

### **H. Sponsorship or Partnership**

Various entities may seek interactions with journals or editors in the form of sponsorships, partnerships, meetings, or other types of activities. To preserve editorial independence, these interactions should be governed by the same principles outlined above for Supplements, Theme Issues, and Special Series (Section III.G).

### **I. Electronic Publishing**

Most medical journals are now published in electronic as well as print versions, and some are published only in electronic form. Principles of print and electronic publishing are identical, and the recommendations of this document apply equally to both. However, electronic publishing provides opportunities for versioning and raises issues about link stability and content preservation that are addressed here.

Recommendations for corrections and versioning are detailed in Section III.A.

Electronic publishing allows linking to sites and resources beyond journals over which journal editors have no editorial control. For this reason, and because links to external sites could be perceived as implying

endorsement of those sites, journals should be cautious about external linking. When a journal does link to an external site, it should state that it does not endorse or take responsibility or liability for any content, advertising, products, or other materials on the linked sites, and does not take responsibility for the sites' availability.

Permanent preservation of journal articles on a journal's website, or in an independent archive or a credible repository, is essential for the historical record. Removing an article from a journal's website in its entirety is almost never justified as copies of the article may have been downloaded even if its online posting was brief. Such archives should be freely accessible or accessible to archive members. Deposition in multiple archives is encouraged. However, if necessary for legal reasons (e.g., libel action), the URL for the removed article must contain a detailed reason for the removal, and the article must be retained in the journal's internal archive.

Permanent preservation of a journal's total content is the responsibility of the journal publisher, who in the event of journal termination should be certain the journal files are transferred to a responsible third party who can make the content available.

Journal websites should post the date that nonarticle web pages, such as those listing journal staff, editorial board members, and instructions for authors, were last updated.

## J. Advertising

Most medical journals carry advertising, which generates income for their publishers, but journals should not be dominated by advertisements, and advertising must not be allowed to influence editorial decisions.

Journals should have formal, explicit, written policies for advertising in both print and electronic versions. Best practice prohibits selling advertisements intended to be juxtaposed with editorial content on the same product. Advertisements should be clearly identifiable as advertisements. Editors should have full and final authority for approving print and online advertisements and for enforcing advertising policy.

Journals should not carry advertisements for products proven to be seriously harmful to health. Editors should ensure that existing regulatory or industry standards for advertisements specific to their country are enforced, or develop their own standards. The interests of organizations or agencies should not control classified and other nondisplay advertising, except where required by law. Editors should consider all criticisms of advertisements for publication.

## K. Journals and the Media

Journals' interactions with media should balance competing priorities. The general public has a legitimate interest in all journal content and is entitled to important information within a reasonable amount of time, and editors have a responsibility to facilitate that. However, media reports of scientific research before it has been peer-reviewed and fully vetted may lead to dissemination of inaccurate or premature conclusions, and doctors

in practice need to have research reports available in full detail before they can advise patients about the reports' conclusions.

An embargo system has been established in some countries and by some journals to assist this balance, and to prevent publication of stories in the general media before publication of the original research in the journal. For the media, the embargo creates a "level playing field," which most reporters and writers appreciate since it minimizes the pressure on them to publish stories before competitors when they have not had time to prepare carefully. Consistency in the timing of public release of biomedical information is also important in minimizing economic chaos, since some articles contain information that has potential to influence financial markets. The ICMJE acknowledges criticisms of embargo systems as being self-serving of journals' interests and an impediment to rapid dissemination of scientific information, but believes the benefits of the systems outweigh their harms.

The following principles apply equally to print and electronic publishing and may be useful to editors as they seek to establish policies on interactions with the media:

- Editors can foster the orderly transmission of medical information from researchers, through peer-reviewed journals, to the public. This can be accomplished by an agreement with authors that they will not publicize their work while their manuscript is under consideration or awaiting publication and an agreement with the media that they will not release stories before publication of the original research in the journal, in return for which the journal will cooperate with them in preparing accurate stories by issuing, for example, a press release.
- Editors need to keep in mind that an embargo system works on the honor system—no formal enforcement or policing mechanism exists. The decision of a significant number of media outlets or biomedical journals not to respect the embargo system would lead to its rapid dissolution.
- Notwithstanding authors' belief in their work, very little medical research has such clear and urgently important clinical implications for the public's health that the news must be released before full publication in a journal. When such exceptional circumstances occur, the appropriate authorities responsible for public health should decide whether to disseminate information to physicians and the media in advance and should be responsible for this decision. If the author and the appropriate authorities wish to have a manuscript considered by a particular journal, the editor should be consulted before any public release. If editors acknowledge the need for immediate release, they should waive their policies limiting republication publicity.
- Policies designed to limit republication publicity should not apply to accounts in the media of presentations at scientific meetings or to the abstracts from



these meetings (see Duplicate Publication). Researchers who present their work at a scientific meeting should feel free to discuss their presentations with reporters but should be discouraged from offering more detail about their study than was presented in the talk, or should consider how giving such detail might diminish the priority journal editors assign to their work (see Duplicate Publication).

- When an article is close to being published, editors or journal staff should help the media prepare accurate reports by providing news releases, answering questions, supplying advance copies of the article, or referring reporters to appropriate experts. This assistance should be contingent on the media's cooperation in timing the release of a story to coincide with publication of the article.

## L. Clinical Trials

### 1. Registration

The ICMJE's clinical trial registration policy is detailed in a series of editorials (see News and Editorials [[www.icmje.org/news-and-editorials/](http://www.icmje.org/news-and-editorials/)] and FAQs [[www.icmje.org/about-icmje/faqs/](http://www.icmje.org/about-icmje/faqs/)]).

Briefly, the ICMJE requires, and recommends that all medical journal editors require, registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication. Editors requesting inclusion of their journal on the ICMJE website list of publications that follow ICMJE guidance ([www.icmje.org/journals.html](http://www.icmje.org/journals.html)) should recognize that the listing implies enforcement by the journal of ICMJE's trial registration policy.

ICMJE uses the date trial registration materials were first submitted to a registry as the date of registration. When there is a substantial delay between the submission of registration materials and their posting at the trial registry, editors may inquire about the circumstances that led to the delay.

The ICMJE defines a clinical trial as any research project that prospectively assigns people or a group of people to an intervention, with or without concurrent comparison or control groups, to study the relationship between a health-related intervention and a health outcome. Health-related interventions are those used to modify a biomedical or health-related outcome; examples include drugs, surgical procedures, devices, behavioral treatments, educational programs, dietary interventions, quality improvement interventions, and process-of-care changes. Health outcomes are any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events. The ICMJE does not define the timing of first participant enrollment, but best practice dictates registration by the time of first participant consent.

The ICMJE accepts publicly accessible registration in any registry that is a primary register of the WHO International Clinical Trials Registry Platform (ICTRP) ([www.who.int/clinical-trials-registry-platform/network/who-data-set](http://www.who.int/clinical-trials-registry-platform/network/who-data-set)) that includes the minimum acceptable 24-item trial registration data set or in ClinicalTrials.gov, which is a

data provider to the WHO ICTRP. The ICMJE endorses these registries because they meet several criteria. They are accessible to the public at no charge, open to all prospective registrants, managed by a not-for-profit organization, have a mechanism to ensure the validity of the registration data, and are electronically searchable. An acceptable registry must include the minimum 24-item trial registration data set (<http://prsinfo.clinicaltrials.gov/trainTrainer/WHO-ICMJE-ClinTrialsgov-Cross-Ref.pdf> or [www.who.int/clinical-trials-registry-platform](http://www.who.int/clinical-trials-registry-platform)) at the time of registration and before enrollment of the first participant.

The ICMJE considers inadequate trial registrations missing any of the 24 data fields, those that have fields that contain uninformative information, or registrations that are not made publicly accessible such as phase I trials submitted to the EU-CTR and trials of devices for which the information is placed in a "lock box." In order to comply with ICMJE policy, investigators registering trials of devices at ClinicalTrials.gov must "opt out" of the lock box by electing public posting prior to device approval. Approval to conduct a study from an independent local, regional, or national review body (e.g., ethics committee, institutional review board) does not fulfill the ICMJE requirement for prospective clinical trial registration. Although not a required item, the ICMJE encourages authors to include a statement that indicates that the results have not yet been published in a peer-reviewed journal, and to update the registration with the full journal citation when the results are published.

The purpose of clinical trial registration is to prevent selective publication and selective reporting of research outcomes, to prevent unnecessary duplication of research effort, to help patients and the public know what trials are planned or ongoing into which they might want to enroll, and to help give ethics review boards considering approval of new studies a view of similar work and data relevant to the research they are considering. Retrospective registration, for example at the time of manuscript submission, meets none of these purposes. Those purposes apply also to research with alternative designs, for example observational studies. For that reason, the ICMJE encourages registration of research with non-trial designs, but because the exposure or intervention in non-trial research is not dictated by the researchers, the ICMJE does not require it.

Secondary data analyses of primary (parent) clinical trials should not be registered as separate clinical trials, but instead should reference the trial registration number of the primary trial.

The ICMJE expects authors to ensure that they have met the requirements of their funding and regulatory agencies regarding aggregate clinical trial results reporting in clinical trial registries. It is the authors', and not the journal editors', responsibility to explain any discrepancies between results reported in registries and journal publications. The ICMJE will not consider as prior publication the posting of trial results in any registry that meets the above criteria if results are limited to a brief (500 word) structured abstract or tables (to include trial

participants enrolled, baseline characteristics, primary and secondary outcomes, and adverse events).

The ICMJE recommends that journals publish the trial registration number at the end of the abstract. The ICMJE also recommends that, whenever a registration number is available, authors list this number the first time they use a trial acronym to refer either to the trial they are reporting or to other trials that they mention in the manuscript.

Editors may consider whether the circumstances involved in a failure to appropriately register a clinical trial were likely to have been intended to or resulted in biased reporting. Because of the importance of prospective trial registration, if an exception to this policy is made, trials must be registered and the authors should indicate in the publication when registration was completed and why it was delayed. Editors should publish a statement indicating why an exception was allowed. The ICMJE emphasizes that such exceptions should be rare, and that authors failing to prospectively register a trial risk its inadmissibility to our journals.

## **2. Data Sharing**

The ICMJE's data sharing statement policy is detailed in an editorial (see Updates and Editorials [[www.icmje.org/update.html](http://www.icmje.org/update.html)]).

1. As of 1 July 2018 manuscripts submitted to ICMJE journals that report the results of clinical trials must contain a data sharing statement as described below.
2. Clinical trials that begin enrolling participants on or after 1 January 2019 must include a data sharing plan in the trial's registration. The ICMJE's policy regarding trial registration is explained at [www.icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html](http://www.icmje.org/recommendations/browse/publishing-and-editorial-issues/clinical-trial-registration.html). If the data sharing plan changes after registration this should be reflected in the statement submitted and published with the manuscript, and updated in the registry record.

Data sharing statements must indicate the following: whether individual deidentified participant data (including data dictionaries) will be shared ("undecided" is not an acceptable answer); what data in particular will be shared; whether additional, related documents will be available (e.g., study protocol, statistical analysis plan, etc.); when the data will become available and for how long; by what access criteria data will be shared (including with whom, for what types of analyses, and by what mechanism). Illustrative examples of data sharing statements that would meet these requirements are provided in Table 1.

Authors of secondary analyses using shared data must attest that their use was in accordance with the terms (if any) agreed to upon their receipt. They must also reference the source of the data using its unique, persistent identifier to provide appropriate credit to those who generated it and allow searching for the studies it has supported. Authors of secondary analyses must explain completely how theirs differ from previous analyses. In addition, those who generate and then share clinical trial data sets deserve substantial credit for their

efforts. Those using data collected by others should seek collaboration with those who collected the data. As collaboration will not always be possible, practical, or desired, the efforts of those who generated the data must be recognized.

## **IV. MANUSCRIPT PREPARATION AND SUBMISSION**

### **A. Preparing a Manuscript for Submission to a Medical Journal**

#### **1. General Principles**

The text of articles reporting original research is usually divided into Introduction, Methods, Results, and Discussion sections. This so-called "IMRAD" structure is not an arbitrary publication format but a reflection of the process of scientific discovery. Articles often need sub-headings within these sections to further organize their content. Other types of articles, such as meta-analyses, may require different formats, while case reports, narrative reviews, and editorials may have less structured or unstructured formats.

Electronic formats have created opportunities for adding details or sections, layering information, cross-linking, or extracting portions of articles in electronic versions. Supplementary electronic-only material should be submitted and sent for peer review simultaneously with the primary manuscript.

#### **2. Reporting Guidelines**

Reporting guidelines have been developed for different study designs; examples include CONSORT ([www.consort-statement.org](http://www.consort-statement.org)) for randomized trials, STROBE for observational studies (<http://stroke-statement.org/>), PRISMA for systematic reviews and meta-analyses (<http://prisma-statement.org/>), and STARD for studies of diagnostic accuracy (<http://www.equator-network.org/reporting-guidelines/stard/>). Journals are encouraged to ask authors to follow these guidelines because they help authors describe the study in enough detail for it to be evaluated by editors, reviewers, readers, and other researchers evaluating the medical literature. Authors of review manuscripts are encouraged to describe the methods used for locating, selecting, extracting, and synthesizing data; this is mandatory for systematic reviews. Good sources for reporting guidelines are the EQUATOR Network ([www.equator-network.org/home/](http://www.equator-network.org/home/)) and the NLM's Research Reporting Guidelines and Initiatives ([www.nlm.nih.gov/services/research\\_report\\_guide.html](http://www.nlm.nih.gov/services/research_report_guide.html)).

#### **3. Manuscript Sections**

The following are general requirements for reporting within sections of all study designs and manuscript formats.

##### **a. Title Page**

General information about an article and its authors is presented on a manuscript title page and usually includes the article title, author information, any disclaimers, sources of support, word count, and sometimes the number of tables and figures.

**Table 1.** Examples of Data Sharing Statements That Fulfill These ICMJE Requirements\*

	<b>Example 1</b>	<b>Example 2</b>	<b>Example 3</b>	<b>Example 4</b>
Will individual participant data be available (including data dictionaries)?	Yes	Yes	Yes	No
What data in particular will be shared?	All of the individual participant data collected during the trial, after deidentification.	Individual participant data that underlie the results reported in this article, after deidentification (text, tables, figures, and appendices).	Individual participant data that underlie the results reported in this article, after deidentification (text, tables, figures, and appendices).	Not available
What other documents will be available?	Study Protocol, Statistical Analysis Plan, Informed Consent Form, Clinical Study Report, Analytic Code	Study Protocol, Statistical Analysis Plan, Analytic Code	Study Protocol	Not available
When will data be available (start and end dates)?	Immediately following publication. No end date.	Beginning 3 months and ending 5 years following article publication.	Beginning 9 months and ending 36 months following article publication.	Not applicable
With whom?	Anyone who wishes to access the data.	Researchers who provide a methodologically sound proposal.	Investigators whose proposed use of the data has been approved by an independent review committee (learned intermediary) identified for this purpose.	Not applicable
For what types of analyses?	Any purpose.	To achieve aims in the approved proposal.	For individual participant data meta-analysis.	Not applicable
By what mechanism will data be made available?	Data are available indefinitely at ( <i>Link to be included</i> ).	Proposals should be directed to xxx@yyy. To gain access, data requestors will need to sign a data access agreement. Data are available for 5 years at a third-party website ( <i>Link to be included</i> ).	Proposals may be submitted up to 36 months following article publication. After 36 months the data will be available in our University's data warehouse but without investigator support other than deposited metadata. Information regarding submitting proposals and accessing data may be found at ( <i>Link to be provided</i> ).	Not applicable

\*These examples are meant to illustrate a range of, but not all, data sharing options.

**Article title.** The title provides a distilled description of the complete article and should include information that, along with the abstract, will make electronic retrieval of the article sensitive and specific. Reporting guidelines recommend and some journals require that information about the study design be a part of the title (particularly important for randomized trials and systematic reviews and meta-analyses). Some journals require a short title, usually no more than 40 characters (including letters and spaces) on the title page or as a separate entry in an electronic submission system. Electronic submission systems may restrict the number of characters in the title.

**Author information.** Each author's highest academic degrees should be listed, although some journals do not publish these. The name of the department(s) and institution(s) or organizations where the work should be attributed should be specified. Most electronic submission systems require that authors provide full contact information, including land mail and e-mail addresses, but the title page should list the corresponding authors' telephone and fax numbers and e-mail address. ICMJE encourages the listing of authors' Open Researcher and Contributor Identification (ORCID).

**Disclaimers.** An example of a disclaimer is an author's statement that the views expressed in the submitted article are his or her own and not an official position of the institution or funder.

**Source(s) of support.** These include grants, equipment, drugs, and/or other support that facilitated conduct of the work described in the article or the writing of the article itself. Inappropriate attribution of funding sources and affiliations are misleading and should be avoided.

**Word count.** A word count for the paper's text, excluding its abstract, acknowledgments, tables, figure legends, and references, allows editors and reviewers to assess whether the information contained in the paper warrants the paper's length, and whether the submitted manuscript fits within the journal's formats and word limits. A separate word count for the abstract is useful for the same reason.

**Number of figures and tables.** Some submission systems require specification of the number of figures and tables before uploading the relevant files. These numbers allow editorial staff and reviewers to confirm that all figures and tables were actually included with the manuscript and, because tables and figures occupy space, to assess if the information provided by the figures and

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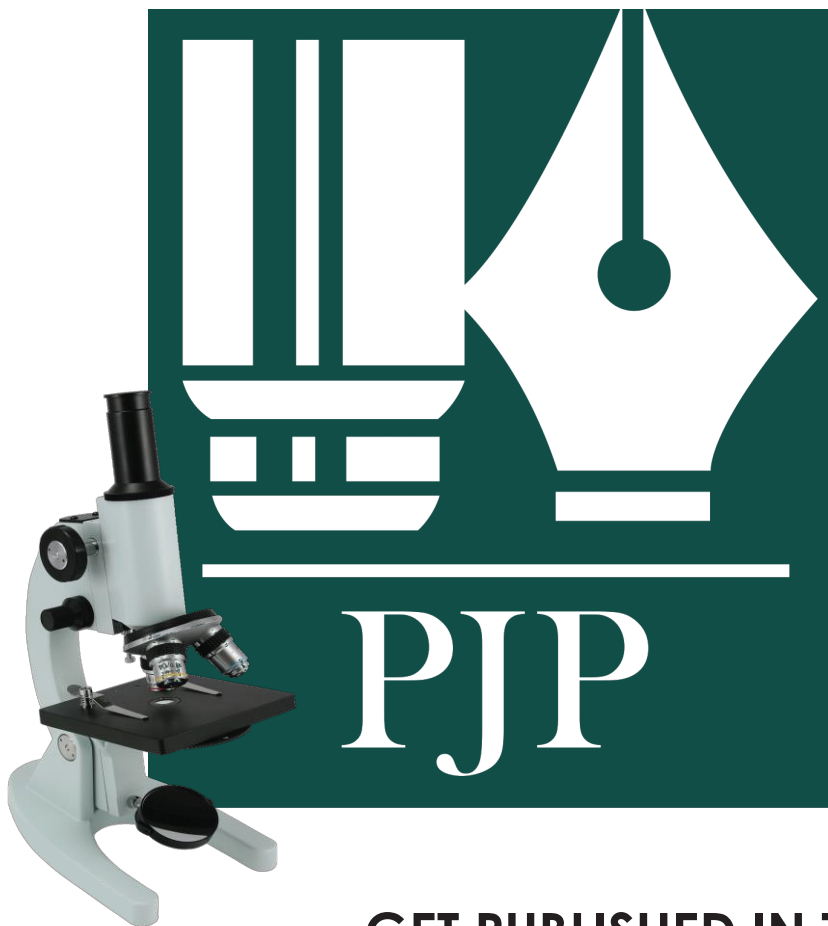
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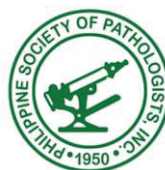
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