



PHILIPPINE JOURNAL OF PATHOLOGY

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

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Warm Greetings!

Welcome to the July 2020 issue of the Philippine Journal of Pathology. Congratulations to the editorial team and the PSP Board of Governors for the job well done in spite of the COVID-19 pandemic that we are currently fighting.

Though we have encountered many challenges from the time we started this scholarly work, the support, commitment, and dedication of our editorial staff, the Board of Governors, and the PSP members inspired us more to work harder and continue to come up with succeeding issues of the PJP.

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

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

A handwritten signature in black ink, appearing to read 'R. Padua Jr.', written over a large, faint watermark of the Philippine Society of Pathologists, Inc. logo.

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

The Year of the Filipino Pathologist



Sitting here in the quarantine facility, waiting for my rTPCR result, after getting exposed to a positive colleague, I now get a sort of breather: a reprieve from the frenzied activities related to the COVID-19 laboratory response over the last six months.

Outside, everything looks the same but feels different at the same time. Limited outside activities, social distancing, mass wearing of masks have become the new normal. Strict curfews, relatively less traffic, closed stores and deserted streets, now describe our once-busy evenings. Work itself has adapted through virtual meeting places and shared online documents. The pandemic has redefined and continues to redefine life as we all know it in this generation.

At this point, our Institute has tested more than 150,000 specimens, the largest number of specimens our agency has tested for a single public health threat. Thousands of results have been generated and transmitted to the Department of Health as part of the laboratory's contribution to the response. But one molecular laboratory cannot do it alone. After several thousands of miles and hours of travel time, our technical team has assessed, trained, provided technical assistance to, and activated over 90 molecular laboratories proficient for SARS-CoV-2 testing, giving rise to a living, breathing laboratory network. We now have a daily capacity greater than 20,000 specimens compared to 300 when we started in February.

Outside, the Philippine Society of Pathologists has become more active in voicing out its collective stand on COVID-19 laboratory issues, issuing a position paper on strategies to improve the accessibility and scope of testing. In the last two months, we noted the increasing representation of pathologists in various tri-media platforms, talking and being consulted about COVID-19 laboratory testing. The Society has also supported a group of pathologist-researchers in incubating, finalizing, and implementing a pragmatic, operations research on pooled testing, findings of which we hope to soon read in our journal.

Virtual technical working groups have been established to discuss antibody-based testing, instrument-based serologic assays, closed molecular systems, and other issues. Enabled by technology, the pandemic has catalyzed more technical, management-related, and quality assurance discussion among heads of laboratories.

With laboratory testing considered as the central basis for contact tracing and treatment strategies, and the increased recognition of the role of the laboratory in public health, 2020 is turning out to be the year of the Filipino pathologist. We can only hope that the momentum is sustained, and pathologists continue to assert their expertise and provide leadership and direction in concerns related to the laboratory.

Like PCR testing which cannot simply be done by a single laboratory entity, understanding our enemy cannot be accomplished by one agency alone. We are all together in fighting this submicroscopic entity and a lot still remains to be fathomed about the pathology it causes. Research should not cease because of the pandemic.

I am issuing this call to all to all Filipino pathologists: I enjoin you to contribute to the global knowledge base on COVID-19. Publish your researches, case reports, interesting images and findings, related to the pandemic in our journal. Engage with your clinical colleagues in their studies on the management of this disease. Share your assay validations of PCR and serologic test kits, operational improvements, biosafety-biosecurity enhancements and best practices in your laboratories. Let us learn from each other and help each other beat this disease.

The PJP is one with the Society in the fight against SARS-CoV2. We are here. At your service.

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

<https://doi.org/10.21141/PJP.2020.01>



PHILIPPINE SOCIETY OF PATHOLOGISTS, INC.

Diagnostic Testing Strategies to Manage COVID-19 Pandemic

Proposed by the Philippine Society of Pathologists, Inc.

The **Philippine Society of Pathologists, Inc. (PSP)** is an organization of physicians specializing in Pathology and Laboratory Medicine. It is one of the eight recognized specialty members of the **Philippine Medical Association**. It has a membership of over 1,000, whose specialization and sub-specializations are in various areas including Molecular Pathology and Immunopathology. It stands in solidarity with the rest of the nation in its fight against SARS-COV-2.

To date, the country has not gained inroads in producing significant number of SARS- COV-2 PCR testing. In over 3 months only 307,813 of cumulative tests, 283,147 tests of cumulative unique individuals have been deployed (*doh.gov.ph/covid19tracker*; May 25, 2020). This only represents less than 1% of over 110 million population who have been tested in the Philippines. A 7.6 % incidence has been established from a total of 21,643 positive cases identified, out the 283,147 unique samples tested. Country incidence rates and prevalence studies have not yet been established.

The RT-PCR which is the gold standard for SARS-COV-2 testing is a laborious procedure requiring high levels of sophisticated equipment, engineering and biosafety standards, controls, and personnel proficiency. The molecular testing technology is expensive and is not readily available in the country, although there is now a widely concerted effort to establish molecular pathology laboratories nationwide.

To flatten the COVID-19 curve, the PSP is proposing the formulation of an **expanded targeted testing strategy**. This will enlarge the population base of persons to be tested in the country in order to identify and survey the asymptomatic carriers who shed the SARS-COV-2 virus and unknowingly infect the community.

The proposed **expanded targeted testing strategy** will not test all of the 110 million Filipinos, but rather **escalate** the testing population base to the **Asymptomatic Population** who are at risk and those communities with high prevalence.

For this purpose, **expanded targeted testing** will be defined as an enhanced, wider based purposive testing, designed to test a greater number of **asymptomatic** populations at risk, based on robust scientific and epidemiologic data.

We propose an expanded targeted testing for screening **asymptomatic populations** to be conducted in the following situations:

- A. High prevalence communities for epidemiologic surveillance and aggressive contact tracing
- B. Health care workers with low risk exposure
- C. Workplace testing
- D. Border testing at airports and seaports for inbound foreign travelers and returning residents
- E. Overseas deployment of OFWs
- F. Returning OFWs
- G. Frontline government workers (police, military, quarantine, immigration officers to name a few)

Predictably, this will entail millions of PCR tests to be conducted. In a resource-poor environment, this is not practical. In addition, countries all over the world are suffering from the unavailability and shortage of diagnostic tests and supplies as cases continue to rise.

The disparity between the required number of reagent kits and the actual number needed to be tested has impeded efforts of DOH to identify and isolate infected individuals. In most cases, testing is restricted mainly towards symptomatic cases, with the vast number of asymptomatic cases going undetected.

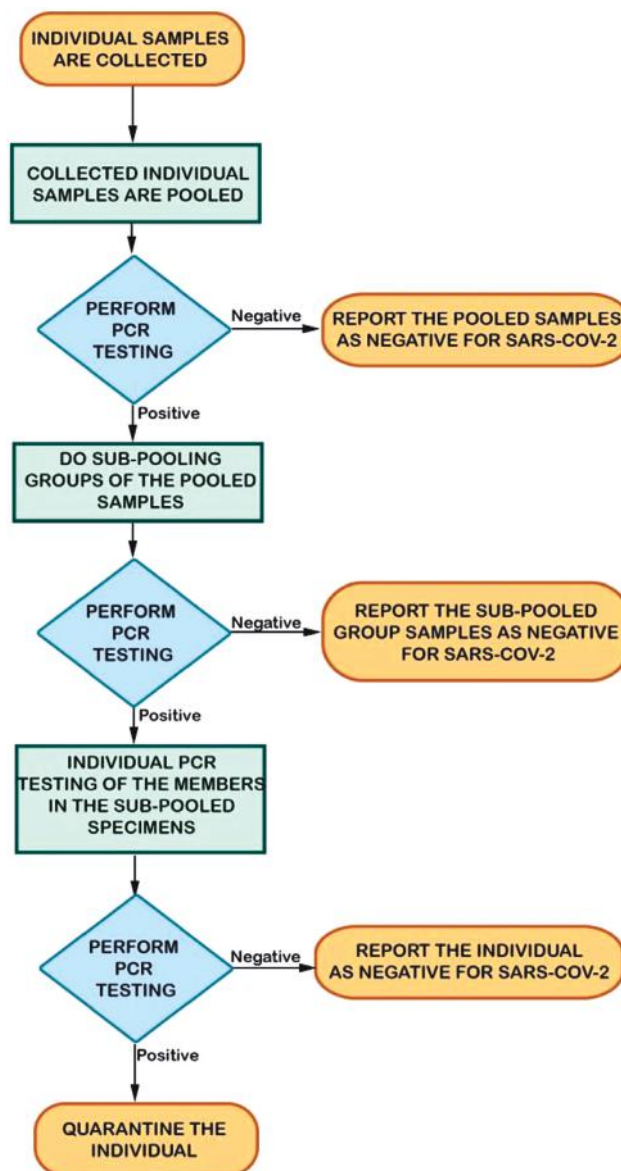
In order to achieve a significant and expanded number of target populations to be tested, there must be a comprehensive and cost-effective strategy in place. The Philippine Society of Pathologists, Inc. proposes the following:

I. THE USE OF MOLECULAR BASED PCR TESTING

A. "Smart Pooled Sample Testing"

This procedure will help meet the high demand of testing SARS-COV-2 in the early identification and isolation of **asymptomatic individuals**. The pooling of samples before nucleic acid testing is a safe and well-established procedure in blood banking. We recommend using this as well, in expanded and targeted screening for SARS-COV-2.

We propose two algorithms in the conduct of pooled testing of **asymptomatic individuals** during expanded targeted testing using:



Algorithm A. Algorithm for PCR pooled testing in asymptomatic individuals.
 *Size of pools and subpools will depend on the prevalence of COVID-19 in the target population.

A1. Pooled nasopharyngeal / oropharyngeal samples for direct RT-PCR testing (Algorithm A)

*Samples from several individuals are collected and uniquely identified from nasopharyngeal or oropharyngeal swabs. The samples are then pooled and tested together in a single tube using standard RT-PCR method. If a **POSITIVE** result is achieved from a pooled sample, this will be tested individually. If a negative pooled sample is achieved, all the samples are issued a **NEGATIVE** result.

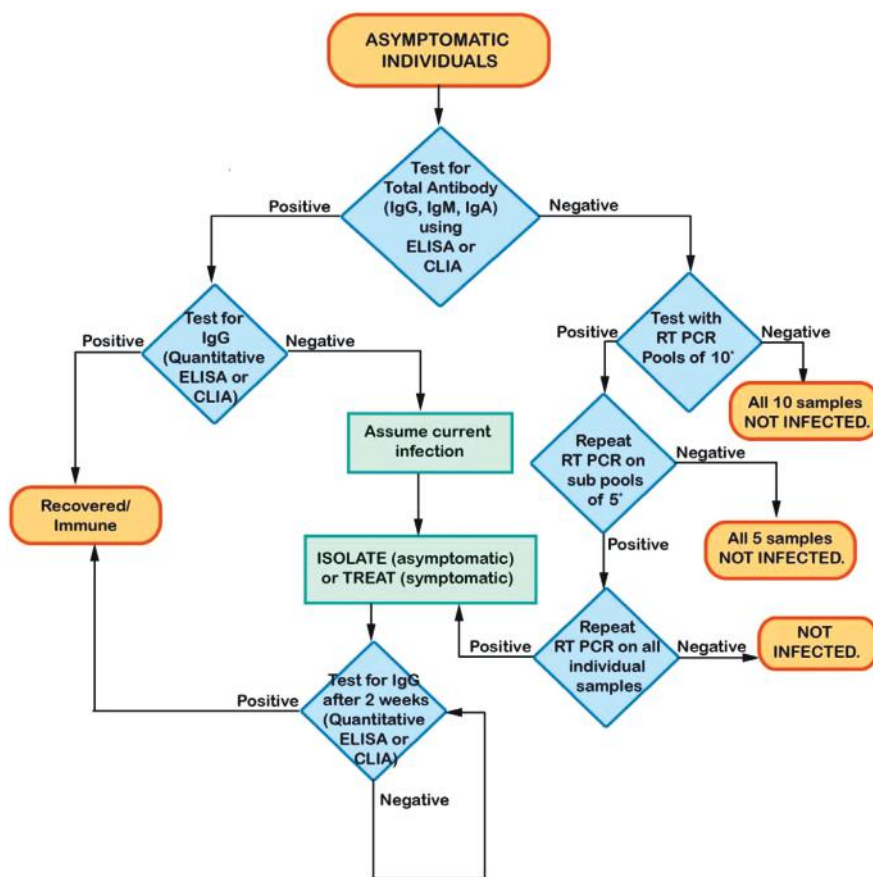
The number within the pooled sample would have to be guided and determined by robust statistical and epidemiologic data. When the infection rate in a given population is low and only a few people are infected, pool testing can significantly expand the testing capacity of the existing laboratory infrastructure.

In other countries (Pakistan, India, Israel, some EU countries), the pooled sample number ranged from 5-63

pooled samples. The higher the prevalence, the lower the recommended sample pool.

In our setting, we recommend a pooled sample range of 5-30 based on the risk, prevalence and suspected incidence. In case one of these 5-30 pooled samples turn out to be positive, same will be subjected to sub-pooling containing lower number of samples for PCR. The pooled sample that will turn out to be positive, will then be individually tested by PCR. This method would need to be scientifically validated by our COVID-19 PCR testing laboratories.

This strategy will considerably reduce the workload and conserve the much-needed PCR resources in the country (approximately 60-80% reduction); provide a more cost-effective method of testing and ramp up the number of populations to be screened for appropriate contact tracing and surveillance.



Algorithm B. Algorithm for identifying COVID-19 cases using combined serological and pooled polymerase chain reaction testing. *The size of the initial pooled samples are best determined by the PCR laboratory based on available epidemiological data and the succeeding sub-pooling of individuals can be based on the size of the initial pooled sample. Sub-pooling may also be skipped if the initial pool size is small (e.g. 5 or less).

A2. Initial antibody testing using instrumented laboratory-based methods like ELISA and ECLIA. (Algorithm B)

The rationale of the algorithm using instrumented antibody testing (Algorithm B) is that laboratory-based antibody tests are already available in the market. The instrumentation for such tests is already in place in many hospital laboratories, thus eliminating the need for capacity building, both in equipment and personnel. This is much faster and cheaper than molecular methods for screening purposes but will not replace it, rather they are meant to conserve and supplement molecular assays.

The laboratory based instrumented assays are superior to rapid antibody tests as they have undergone more scrutiny and validation, both internal and external before their introduction.

We recommend the pooling method for RT-PCR assays to reduce the volume of testing and facilitate clearance of low risk individuals. Negative serological tests will be subjected to pooled samples for PCR.

High risk individuals (symptomatic, frontline health care workers and those with history of exposure) should be tested individually. The pooling method will greatly reduce the limited resources, workload of the RT-PCR laboratories and reduce work-related injuries and illnesses due to fatigue.

We recommend the use of instrumented method of antibody testing for reasons of increased throughput that is required in expanded testing, coupled with higher analytical performance (better accuracy, sensitivity, specificity and lower limits of detection).

The use of Rapid Antibody Test kits is NOT recommended because of poor sensitivity and specificity, leading to higher false negatives and false positive which will ramp up for the need of PCR testing.

B. Ensuring efficient proactive supply chain management of PCR test kits/supplies and streamlining operations in COVID-19 testing

B1. Putting up a dedicated agency within DOH which will efficiently provide the following functions:

- Central management and tracking of inventories of reagents and supplies to ensure adequacy and availability;
- Repository of information on the source, origin, updated prices of reagent and supplies for end users. The PSP can assist DOH in the securing of this information.

C. Streamlining operations in COVID-19 testing.

- C1. Reduce the documentation requirements to be sent to various DOH agencies that do not add value to the testing process and increases the length of the turn-around-time, e.g. reporting for negative data, sub-national laboratories submission of daily work sheets to RITM.*
- C2. Review the reporting protocol for COVID-19 testing to reduce unnecessary documentation.*

D. Simplify licensing requirements for COVID -19 testing laboratories

- D1. To comply with the training requirements for COVID-19 testing, the PSP is willing to assist DOH/RITM by conducting recognized training modules in Molecular Pathology and Biosafety/ Biosecurity procedures for pathologists, medical technologists and other relevant health care workers.*
- D2. Review and harmonize the licensing requirements of Rapid PCR testing that are not appropriate for the technology, like facility site, equipment, training and personnel.*

E. Use of Rapid PCR testing like GeneXpert technology

- E.1 Ensure that the donated GXP cartridges be deployed in hospital settings which require immediate results for management and decision making, e.g. critical care, pre-operative requirements and medical emergencies.*
- E.2. The donated cartridges should be deployed to the hospitals to allow immediate access and service delivery.*

II. THE EFFECTIVE, EFFICIENT UTILIZATION OF ANTIBODY TESTING

A. Antibody testing has a role in COVID-19 management. It is cost-effective strategy for the following situations:

- Workplace and conditions for return to work
- Epidemiologic surveillance for prevalence and population immunity
- Research

B. There are differences in analytical performance of different serologic methods. The prudent use of these should be based on performance characteristics of the different analytical methods in order to reduce incidence of false negatives and false positives.

C. We recommend the use of instrumented assays for Antibody testing using ELISA and ECLIA on the following basis:

- Higher throughput and turn-around-time, desirable in an expanded testing program
- Earlier detection of antibody levels in patients who are exposed
- Excellent analytical specificity and sensitivity
- Lower detection limit which allows to identify population with low antibody titers
- Less interferences and cross-reactivities
- Use of quantitative immunoassays and will allow for detection of neutralizing antibodies

D. Based on the Clinical laboratory Law (RA No. 4688, June 18, 1966) and AO 2007- 0027, laboratory tests must be performed and supervised by a Clinical Pathologist and results are issued by a licensed clinical laboratory. This is to ensure quality of test results and to assure the protection and safety of both personnel and environment.

In summary, we, the **Pathologists** stand ready to be in the forefront of diagnostic testing during this pandemic. We propose expanded targeted testing algorithms combining molecular virus detection and laboratory-based antibody assays in an effort to reduce transmission of SARS-COV-2 in the Filipino population. We will win this battle and together we can heal as one.

For the Philippine Society of Pathologists, Inc:



ROBERTO D. PADUA JR., MD, FPSP
President

May 29, 2020

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31st October 2019

RE: Notification of the journal evaluation result for ACI

Title: Philippine Journal of Pathology
ISSN / E-ISSN : 0118-3265 / 2507-8364
Owner : the Philippine Society of Pathologists, Inc., Philippines

Dear Editor,

The title mentioned above has been evaluated for inclusion in ASEAN Citation Index (ACI) database by the ACI Secretariat. The review of this title is now completed and the ACI Steering Committee has advised **to accept** the title for ACI inclusion in April 2019. Congratulations! The reviewers also suggested that the journal should include more editorial board members from abroad to increase visibility.

To record all the bibliographic data and references of all the journal articles into the System, the following information is required :-

1. General information of the journal (the form is attached) ;
2. Journal Subject Categories for ACI (the list is attached) ;
3. PDF files of all issues of this journal published in 2019.

Please kindly send us the needed information to aci.submitting@gmail.com as soon as possible so that we can proceed with the indexing.

Thank you for your kind co-operation. We look forward to receiving all the requested information.

Your sincerely,

A handwritten signature in black ink, appearing to be 'Narongrit Sombatsompop', written over a horizontal line.

(Prof.Dr. Narongrit Sombatsompop)
Chairman of the ACI Steering Committee

Comparison of Digital Image Analysis and Conventional Microscopy In Evaluating Erythrocyte Morphology in Peripheral Blood Smears*

Erick Martin Yturralde, Karen Bulseco-Damian, Nelson Geraldino

Department of Laboratories, Philippine General Hospital, University of the Philippines-Manila

ABSTRACT

Background and Objectives. The use of conventional microscopy still forms the basis for the morphologic evaluation of erythrocytes despite widespread use of automated tests in the hematology laboratory. This requires a considerable length of time and expertise, and have the potential of becoming a source of errors and delay in reporting. Advances in image processing and machine learning in recent years have shown acceptable performance characteristics and have promising applications in the diagnostic laboratory. Use of these newly-developed technologies can address the stated problems and provide an alternative approach in the microscopic analysis of erythrocytes.

Methodology. This prospective validation study compared digital image analysis using a machine-learning based image recognition algorithm with conventional microscopy performed by a trained microscopist, which served as the reference standard. Random deidentified anticoagulated peripheral blood samples submitted to the hematology laboratory were assessed.

Results. A total of 956 erythrocytes were evaluated after image processing using support vector machine and routine microscopy as classifiers of erythrocytes into three categories: size, central pallor, and shape. The tested software was able to achieve a strong level of agreement compared to conventional microscopy, having kappa values ranging from 0.81 to 0.86. Accuracy for size, central pallor and shape were 89.88%, 93.72% and 87.89%, respectively.

Conclusion. The validated image recognition software is an acceptable diagnostic test in determining erythrocyte morphology in peripheral blood smears. Its integration can potentially minimize hands-on time and improve the diagnostic laboratory workflow.

Registration. Philippine Health Research Registry (PHRR) ID: PHRR191211-002348; University of the Philippines Manila Research Ethics Board (UPMREB): 2019-356-01

Key words: erythrocyte morphology, digital imaging, microscopy

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**Presented in the 25th Pathology Resident's Research Forum held in the Philippine General Hospital, on the 8th of November 2019.*

INTRODUCTION

Background

The complete blood count (CBC) is one of the most commonly requested tests in a diagnostic laboratory, providing the clinician a reflection of the cellular constituents of blood which includes red blood cell (RBC), white blood cell (WBC) and platelet counts, as well as, red cell indices and white cell differential counts. With the advent of laboratory automation, the CBC result can be released by a hematology laboratory in a relatively rapid and reliable manner leading to faster turn-around times and specimen reporting. Depending on the manufacturer, these hematology analyzers (Beckman Coulter Hematology Analyzer, Siemen's ADVIA®, Sysmex Hematology Analyzer, and Abbott CELL-DYN Sapphire®) rely on the principles of electrical impedance and light scatter, with the conventional microscopy in peripheral blood smears still forming the basis in erythrocyte morphologic classification.¹ In high-volume settings, the use of peripheral blood smears remains a laborious and time-consuming process that can take



several minutes from slide preparation to microscopic reading. In smaller laboratories handling relatively smaller number of specimens, the lack of expertise of the microscopist can be an attributable factor to incomplete or inaccurate results.² With the considerable reliance of clinical hematology in diagnostic laboratory procedures for establishing a diagnosis, the need for accurate and timely results are of utmost priority.

As automated image recognition of cellular elements started becoming more accessible, the advantages of its application in the diagnostic field became apparent, from minimizing technologist hands-on time to improving work flow and decreasing turn-around time of specimens. In recent years, the rapid advance of artificial intelligence, specifically machine learning, provided benefits in data processing by focusing on the desired input-output behavior rather than manually coding for the desired response for all possible inputs. The image recognition software used in analyzing urine sediments is a good example of the latter, relying on a sizeable database provided by the equipment manufacturer. Depending on the machine learning algorithm used, the program can be 'trained' into appropriately classifying an input data into a desired output classifier. Applying this in the field of diagnostic hematology laboratory, an input data can be represented as captured microscopic images of red cells and the output classifier being represented by the size, pallor and shape characteristics. By providing the algorithm multiple images of, for example, echinocytes, the software, in theory, will be able to correctly identify other red cells with the same morphology.

Objectives of the Study

With the above presented concepts in mind, the present study aimed to prospectively validate a newly developed image recognition software that utilized machine learning algorithm in the classification of erythrocyte morphology and compared it to conventional microscopy performed by a trained microscopist. The specific objectives were as follows: 1) Using photomicrographs of erythrocytes, the cells were classified according to its morphologic characteristic (size of cell, area of central pallor and shape of cell) by a microscopist, which served as the reference standard for the purposes of the study, 2) the same images were classified by the image recognition software, 3) statistical agreement between the two methods was determined using kappa statistics, 4) sensitivity, specificity and accuracy of the image recognition software were calculated.

Significance of the Study

This study aims to provide a novel method in the identification of erythrocyte morphology in the diagnostic clinical laboratory and if proven as an acceptable alternative, will be beneficial to the following groups:

Pathologists. The results of this study will expand the knowledge of pathologists regarding the potential applications, advantages and inherent limitations of machine learning in the laboratory. This knowledge could help pathologists provide relatively accurate results with minimal subjectivity and improved turn-around times.

Medical Technologists. The results of this study can improve the test process by minimizing hands-on time in handling specimens, allowing the technologist to focus on other important aspects of the laboratory workflow. The study can also provide new knowledge regarding how machine-learning based algorithms can be applied in a diagnostic laboratory.

Clinicians and Patients. The expected improved turn-around time gained from the method can provide clinicians time-sensitive laboratory information and aid them in formulating treatment plans that can ultimately benefit the patient.

Review of Related Literature

Ever since the microscope was introduced in the field of health sciences, the microscopic study of blood elements has brought about several observations in the characteristics of erythrocytes, from the size of the cell, central pallor, and alterations in its shape. Through various observations, several disease states were correlated with the characteristic morphologic alteration of erythrocytes.^{3,4} In the 1930s, Wintrobe introduced the evaluation of red cell indices such as Mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), which to this day are still used in the evaluation of erythrocytes.⁵ In many forms, these elements of the complete blood count have been adopted in the processes employed by automated hematology analyzers. However, the practice of evaluating peripheral blood smears for identifying erythrocyte morphology has endured despite the widespread use of automated analyzers.^{2,6}

Currently, the need to perform peripheral blood smears in the evaluation of erythrocytes after using automated analyzers are set by individual laboratories and employ specific reporting methods and terminologies.^{7,8} Reasons as to why automated analyzers are currently unable to characterize red cell morphology has highlighted their limitations, specifically: 1) the analytical principles employed by automated hematology analyzers such as cell impedance and light scattering are physically unable to identify the specific red cell morphologies, 2) the methods employed intentionally alter the red cells as they pass through a measuring aperture used to calculate the cell volume, 3) the use of specialized procedures (three-dimensional optical measurement of erythrocytes, detection of high oxygen affinity hemoglobin) requires highly specialized equipment and reagents that are not readily available and uneconomical as part of routine screening, and compared to these methods, the manual peripheral blood smears are more feasible as a screening or adjunct method in specimens flagged by automated analyzers.^{4,9}

The persistence of the manual counting and evaluation of the peripheral smears requires a significant amount of hands-on time, making it predisposed to interobserver variability and bias. The additional use of laboratory resources and the possible consequences in the test accuracy can lead to prolonged test reporting.^{6,8}

The rapid development of machine learning algorithm as reflected by the number of published papers in the last decade has provided several glimpses on its application in laboratory medicine.¹⁰⁻¹² Machine learning is different from the so-called “expert systems” that are commercialized for specific laboratory use. The latter follow predefined lines of code that state specific parameters, and by following logic rules, the system checks if the specimen characteristics fall into which set parameter.¹³ This rigid approach is vastly different from machine learning, where the parameters are “learned” by only providing input and output information. Laboratory parameters and data are ripe for application of machine learning systems because of the use of quantitative data that are relatively easy to categorize or coded. With the growing complexity and amount of data produced by the laboratory, machine learning systems can provide immediate feedback not only to the healthcare team, but to the patient as well.¹³⁻¹⁵

Computer software-driven morphological cell analysis relies on mathematical algorithms to identify cellular features. It employs several processing techniques such as edge detection, image enhancement, skeletonization and pattern recognition to produce a geometric figure that can be analyzed.¹⁶ With the use of machine learning, these set of complex data can yield better performance in resolving the task of cellular morphological analysis. Support vector machine, one of the models of machine learning, utilizes supervised learning methods wherein the algorithm categorizes a set of training examples. It is able to resolve binary problems by maximizing margins of errors to make hyperplanes from support vectors and handle discriminatory functions.^{16,17} By providing several examples, a decision boundary and model is formed able to predict new examples into its category. Appropriate tuning of the model is performed by providing additional training examples and regression from erroneous results. The inputs from the dataset are known while the outputs are learned in the form of responses. These methods can increase reliability and accuracy, making its utilization more acceptable in the diagnostic laboratory.¹⁸

METHODOLOGY

Ethical Considerations

The study was accomplished after seeking ethical approval from the University of the Philippines Manila Research Ethics Board (UPMREB). A waiver of consent was requested and approved as there were no risks to the study participants. All peripheral blood specimens and smears analyzed in the study were deidentified to ensure anonymity and confidentiality of the patients during data collection, specimen handling and storage.

Study Design

The study design used a validation of a novel assay platform in the form of machine learning-based algorithm in comparison with the conventional microscopic method used in erythrocyte morphologic assessment. Figure 1 summarizes the study methodology. Both procedures involved the evaluation of peripheral blood smears presenting with various erythrocyte parameters in accordance with the inclusion criteria stated below.

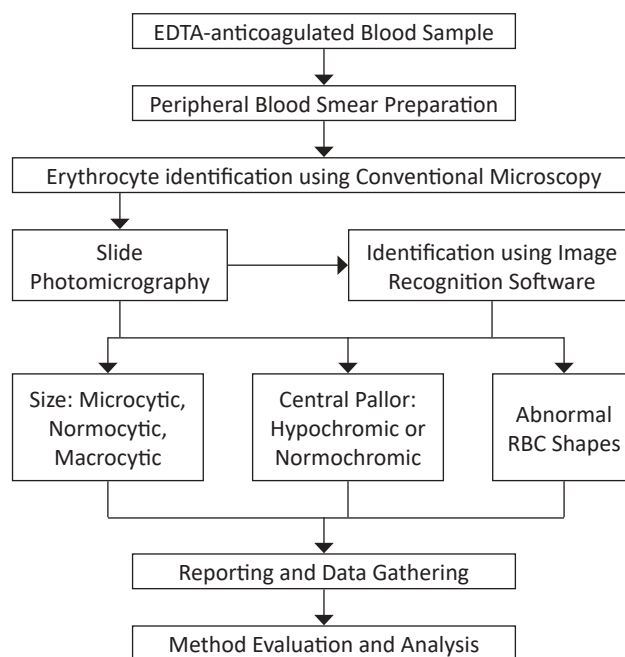


Figure 1. Study workflow diagram.

Sample Size

Study sample sizes were computed based on a test agreement between two raters using kappa statistic to detect a true kappa value of at least 0.80 (almost perfect agreement). The sample sizes are computed based on three (3) categories: for erythrocyte chromicity or central pallor, requiring at least 172 subjects, for erythrocyte size, requiring at least 144 subjects and erythrocyte shape, requiring 135 subjects. These power calculations are based on a significance level of 95%. Overall, this study needed at least 172 subjects. Random samples from the hematology laboratory were collected within two months (October to November 2019).

Inclusion Criteria

- 1) Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood from patients submitted to the section of Hematology for complete blood count with normal red cell counts and red cell indices.
- 2) Submitted EDTA-anticoagulated blood from patients for complete blood count and flagged by the hematology analyzer due to abnormal red cell counts or cell indices, requiring peripheral blood smears.
- 3) Submitted EDTA-anticoagulated blood from patients for complete blood count with physician-requested peripheral blood smear.

Exclusion Criteria

- 1) Blood from patients flagged due to improper specimen collection or presence of blood clots.
- 2) Blood from patients with hyperleukocytosis or other conditions presenting with inclusions and intracellular parasites which might impair red cell visualization.

Data collection

Cases were collected prospectively from EDTA-anticoagulated patient blood samples submitted to the section of Hematology submitted from October to

November 2019. Blood smears were manually prepared and generated by placing a small drop of blood on one end of a clean glass slide and smeared using a spreader slide. The smear is then allowed to air-dry and stained with Wright-Giemsa stain in strict accordance with laboratory protocols to maintain standardization among all smears. No patient data or clinical information were collected in the study.

Each blood smear was evaluated by a single trained microscopist, who is an experienced medical technologist working in the hematology laboratory, and photomicrographs of the identified red cells were captured using a camera (Raspberry Pi camera module) attached to the eyepiece of a microscope (Olympus CX23) under the oil immersion objective (100X magnification). The field of the smear where the erythrocytes will be optimally evaluated will be initially identified by the microscopist and images will be taken. Depending on the number of optimal fields present, five to ten images were taken for each smear. The camera attachment is directly interfaced with a single board computer (Raspberry Pi) with LCD monitor which also contains the image recognition software (Photograph 1). The same identified cells in the photomicrograph will then be evaluated using an image recognition software as developed by Divina & Felices using Python OpenCV. The software utilized several image processing techniques (Photograph 2) and formed a machine learning

model using Support vector machine in classifying erythrocytes as shown by the flow chart below (Figure 2).¹⁹ The microscopist and computer engineer operating the image analyzer were blinded to the results of the other group.

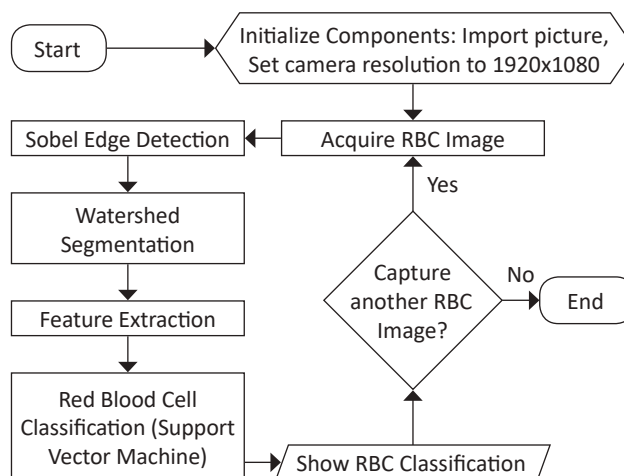
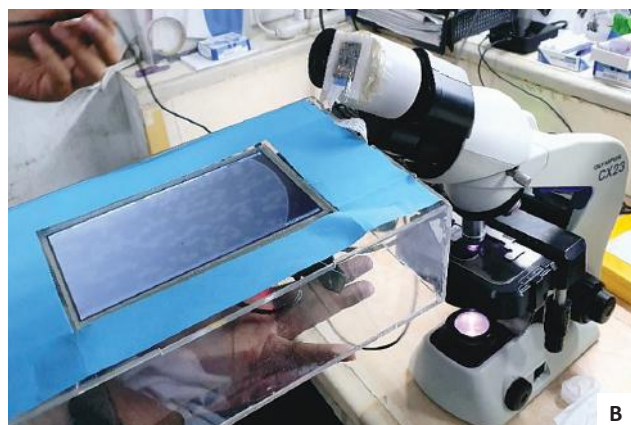
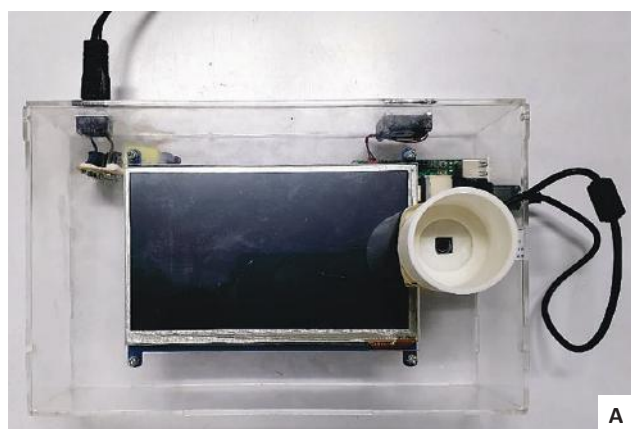


Figure 2. Image recognition software process flow.

RBC morphologies were classified by the microscopist and the image analysis software as to RBC size (normocyte, microcyte, and macrocyte), central pallor (hypochromic and normochromic), and RBC shape (normal, dacrocytes, echinocytes, elliptocytes, spherocytes, stomatocytes, and target cells). All data generated by the software are automatically compiled in a CSV file format (Microsoft® Office Excel®) for future reference.

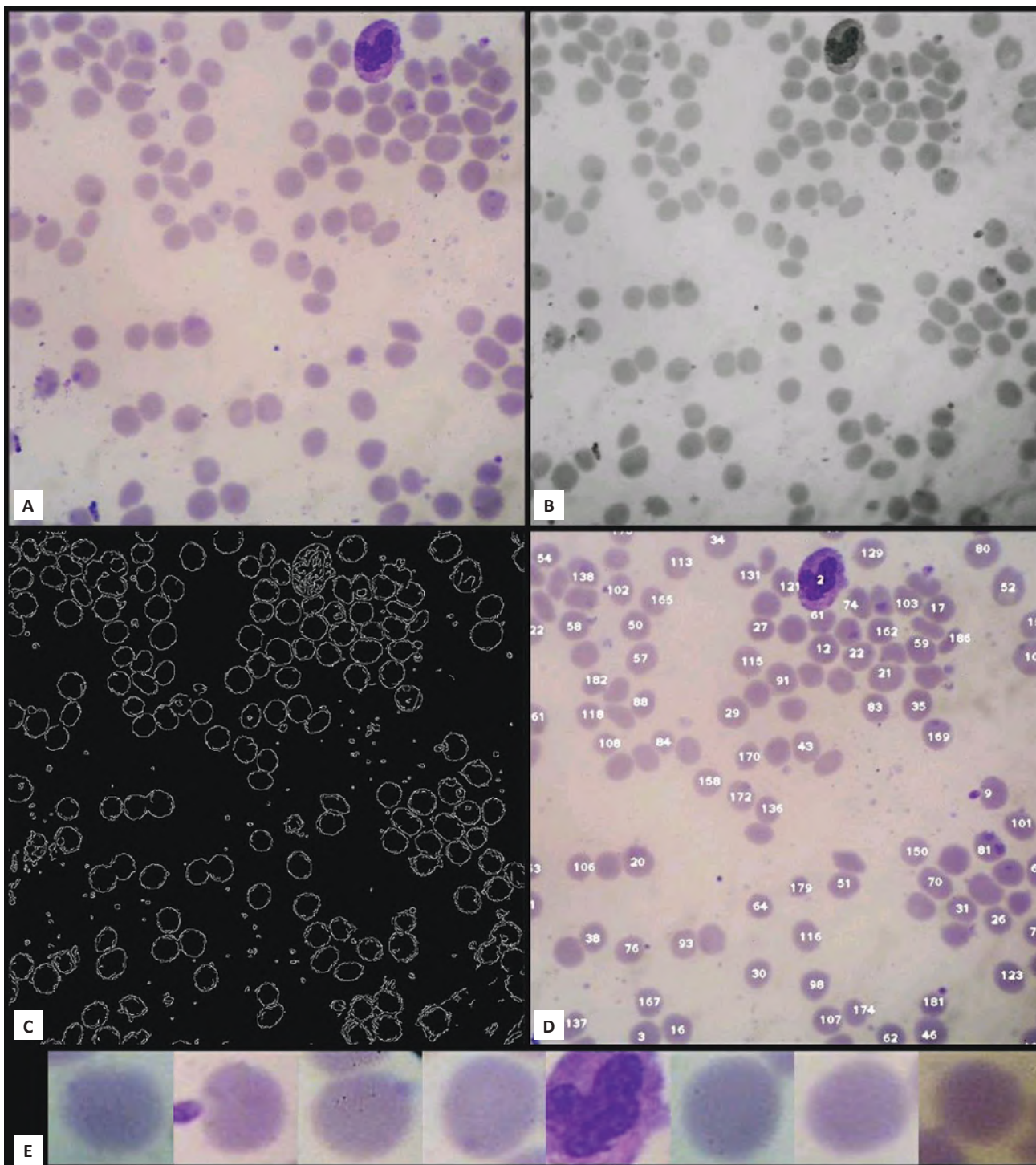
Data analysis

The erythrocyte findings of the microscopist were considered the “true” value for the purposes of the study. Three confusion matrix tables were generated for each RBC classification (central pallor, size and shape) to demonstrate the performance of the image recognition software while the determination of the reliability between the two modalities was analyzed using Cohen’s kappa. This is a widely used method in the evaluation of machine learning-based systems. The kappa statistic guidelines established by McHugh²⁰ was used in the interpretation of the values as seen in Table 1. Accuracy, sensitivity (true positive rate or recall) and specificity (true negative rate) were also computed from the generated confusion matrices. The 95% confidence intervals (CI) for sensitivity and specificity were computed using “exact” Clopper-Pearson confidence intervals.



Photograph 1. Prototype with camera attachment for the microscope (A) and prototype with microscope set-up during photomicrography (B).

Table 1. Interpretation of Cohen's Kappa		
Value of Kappa	Level of Agreement	Percentage of Data that are Reliable
0-0.20	None	0-4%
0.21-0.39	Minimal	4-15%
0.40-0.59	Weak	15-35%
0.60-0.79	Moderate	35-63%
0.80-0.90	Strong	64-81%
Above 0.90	Almost Perfect	82-100%



Photograph 2. Image processing algorithm utilized by the software. Shown here are the original image (A), watershed segmentation (B), Sobel edge detection (C), output after image processing (D) and sample of cropped cells identified by the software (E).

RESULTS

The image recognition software was able to effectively isolate and identify each red blood cell (RBC) according to size, central pallor and shape. For classification, the software identified seven features (area, perimeter, diameter, shape geometric factor, deviation value, central pallor and target flag) and analyzed the image using the machine learning algorithm based on acquired patterns

from the training set. Based on these features and the training datasets, a confidence value will be assigned by the software as to how definite is its classification on a particular erythrocyte. A confidence value of at least 70 is sufficient for the software to assign a specific classification. The images used for the machine learning algorithm training datasets were acquired from a different set of cases in relation to the current validation study.

Table 2. Erythrocyte central pallor confusion matrix

		Conventional Microscopy		Total
		Hypochromic	Normochromic	
Image Recognition Software	Hypochromic	111	7	118
	Normochromic	6	83	89
	Total	117	90	207
Kappa	0.8571	Level of Agreement		Strong
p-value	<0.0001	Expected Agreement		49.30%
% Agreement	92.75%	Accuracy		93.72%

Table 3. Erythrocyte size confusion matrix

		Conventional Microscopy				Total
		Microcytic	Normocytic	Macrocytic	Unknown	
Image Recognition Software	Microcytic	73	10	0	3	86
	Normocytic	10	191	2	0	203
	Macrocytic	0	8	38	1	47
	Unknown	0	0	0	0	0
	Total	83	209	40	4	336
Kappa	0.8141	Level of Agreement			Strong	
p-value	<0.0001	Expected Agreement			45.57%	
% Agreement	89.88%	Accuracy			89.88%	

Table 4. Erythrocyte shape confusion matrix

		Conventional Microscopy								Total
		Normal	Target Cell	Echinocyte	Elliptocyte	Dacrococyte	Spherocyte	Stomatocyte	Unknown	
Image Recognition Software	Normal	70	0	0	0	0	2	0	0	72
	Target Cell	8	63	0	0	0	0	0	0	71
	Echinocyte	2	0	41	0	0	1	0	0	44
	Elliptocyte	0	0	1	29	8	1	0	3	42
	Dacrococyte	3	1	1	5	37	3	0	1	51
	Spherocyte	1	0	0	0	0	61	0	1	63
	Stomatocyte	7	0	0	0	1	0	62	0	70
	Unknown	0	0	0	0	0	0	0	0	0
	Total	91	64	43	34	46	68	62	5	413
Kappa	0.8549	Level of Agreement						Strong		
p-value	<0.0001	Expected Agreement						14.89%		
% Agreement	87.65%	Accuracy						87.89%		

A total of 956 erythrocytes from 24 peripheral blood smears were assessed using manual microscopic evaluation and image recognition software. For central pallor, red cell size, and red cell shape, a total of 207, 336, and 413 cells were tested, respectively. Confusion matrix tables for each red cell morphology classification are listed below (Tables 2, 3 and 4). Interpretation of the kappa values for each classification system showed a “strong” level of agreement between conventional microscopy and the image recognition software. Accuracy for each classification system were 93.72% for central pallor, 89.88% for size, and 87.89% for shape. Cell types identified as “Unknown” are images taken by the software that have low confidence value, insufficient for the machine to provide a definite cell morphology.

Table 5 summarizes the different cell morphologies, from the different classifications systems, that were isolated and identified by the software. Their corresponding sensitivity (true positive rate or recall) and specificity (true negative rate) were computed from the confusion matrices, and 95% confidence intervals (CI) for the sensitivity and specificity were computed using “exact” Clopper-Pearson confidence intervals.

Table 5. Sensitivity and specificity of the image recognition software

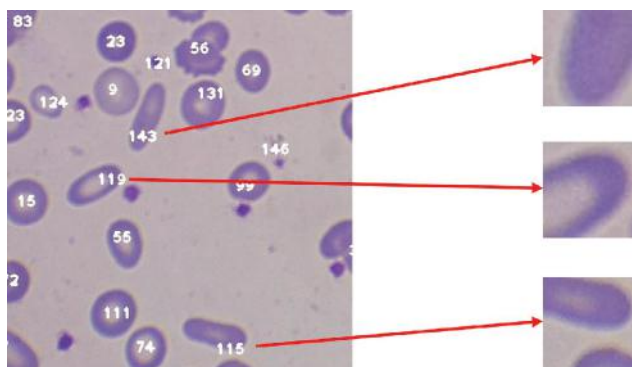
Red Cell Classification	Morphology	Sensitivity [95% CI]*	Specificity [95% CI]
Size	Microcytic	87.95% [78.96-94.07%]	94.86% [91.37-97.24%]
	Normocytic	91.39% [86.73-94.82%]	90.55% [84.08-95.02%]
	Macrocytic	95.00% [83.08-99.39%]	95.95% [94.31-98.60%]
Central Pallor	Hypochromic	94.87% [84.63-96.82%]	92.22% [89.17-98.10%]
	Normochromic	92.22% [89.17-98.10%]	94.87% [84.63-96.82%]
Shape	Normal	76.92% [66.91-85.11%]	99.38% [97.77-99.92%]
	Target Cell	98.44% [91.60-99.96%]	97.71% [95.53-99.01%]
	Dacrococyte	80.43% [66.09-90.64%]	96.19% [93.68-97.90%]
	Elliptocyte	85.29% [68.94-95.05%]	96.57% [94.21-98.16%]
	Spherocyte	89.71% [79.93-95.76%]	99.42% [97.92-99.93%]
	Stomatocyte	100% [94.22-100%]	97.72% [95.96-99.01%]
	Echinocyte	95.35% [84.19-99.43%]	99.19% [97.65-99.83%]

* Confidence Interval

DISCUSSION

In this study, we evaluated a machine-learning based digital image analysis of red blood cells in peripheral blood smears. Comparison of this system with manual microscopy showed a high kappa value representing a “Strong” level of agreement. The performance characteristics of the system based on its sensitivity and specificity demonstrates its acceptability as an alternative method to the routine blood smear evaluation.

Most of the encountered errors were from the evaluation of red cell shapes, specifically dacrocytes and elliptocytes, due to deviation of the cell from the original ovoid shape of erythrocytes, and, stomatocytes and target cells, due to variation in the features of the central pallor. During the image processing phase, segmentation and cropping of individual cells appear to be incomplete leading to classifying an elliptocyte as dacrocyte and vice versa (Photograph 3). This was reflected in the relatively lower sensitivity values (80.43% for dacrocytes and 85.29% for elliptocytes) of these cell types. For stomatocytes and target cells, the software has a propensity to classify them erroneously as normal erythrocytes (sensitivity of 76.92%). Overlapping cells were classified by the software into an unknown category as accurate segmentation is required to properly identify abnormal red cell morphology.



Photograph 3. Incomplete red cell segmentation and cropping after image processing.

The intrinsic processes of a digital image analysis can provide the laboratory several key advantages. Review of digital images can provide timely consultation with experts and inter-institutional referral. These images can be easily stored for future review, education, and training purposes. Machine parameters can also be adjusted to the specific needs of the laboratory in flagging results for further review.

Other published works on erythrocyte classification have focused on classification of erythrocytes into normal or abnormal,²¹ limited number of erythrocyte shapes,²²⁻²⁴ or on correlation with specific disease processes based on the combination of extracted features from RBCs.²⁵ These studies also demonstrated accuracy rates ranging from 80-91% while the validated method performed relatively similar with accuracy rates of 87.89-93.72% depending on the erythrocyte classification. The Cellavision® DM systems Advanced RBC Application (ARBCA) is a commercially available automated digital morphology

analyzer that can classify RBCs into 21 categories based on size, shape, color and inclusions. This platform is considered an acceptable screening method prior to definitive classification by a microscopist.²⁶ The systems in the aforementioned studies used artificial neural networks in classifying erythrocytes. Our study evaluated a machine-learning system that utilizes support vector machine providing optimum performance characteristics. Support vector machine has been shown to achieve higher accuracy rates from generated classification models after undergoing only a few rounds of training datasets.²⁷ Several authors have used this machine-learning method in successfully classifying blasts and other abnormal lymphoid cells in the peripheral blood smear.^{28,29}

With the ultimate goal of integration in any diagnostic laboratory setting, the evaluated system used relatively inexpensive components and widely available laboratory equipment in its application. Implementation of the software using a fully-automated and computerized system will require more specialized machines in smear preparation, staining and slide imaging. Several studies and commercially available instruments are tackling these issues with promising, albeit, proprietary technology.^{26,30}

CONCLUSION

We have demonstrated satisfactory performance of a newly-developed and machine-learning based digital image analysis system that is able to satisfactorily identify and classify erythrocytes in blood smears. The image recognition software provides an acceptable diagnostic method in a clinical laboratory setting.

During the conduct of the study, several observations for future implementation and opportunities for research were encountered. Variations in cell morphology secondary to the number of manual processes involved during smear preparation and staining process can be mitigated by using automated systems in smear preparation and staining. Adding more cell classes or features for detection, such as red cell inclusions, and other cell classes, such as leukocytes and platelets will not only provide a more robust software in peripheral blood analysis and refine the image processing. Studies on the effect of the image recognition software on turnaround time and feasibility in a diagnostic laboratory can be undertaken to determine the definite advantages or disadvantages in its utilization.

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All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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A Fifteen-year Report of Serotype Distribution and Antimicrobial Resistance of *Salmonella* in the Philippines

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ABSTRACT

Background. *Salmonella enterica* ser. Typhi and *Salmonella enterica* ser. Paratyphi are agents of typhoid fever, a severe systemic disease, which remains to be a public health concern in the Philippines. Infection due to non-typhoidal *Salmonella* (NTS), on the other hand, most often results in a self-limiting acute gastroenteritis but may result in invasive disease in some cases. There is scarcity of information on the *Salmonella* serotypes in the Philippines which limits understanding of the distribution, transmission and antimicrobial resistance of these bacteria.

Objective. This study describes the serotype distribution and antimicrobial resistance of *Salmonella* in the Philippines over a 15-year period.

Methodology. *Salmonella* isolates were collected through the Philippine Department of Health-Antimicrobial Resistance Surveillance Program (DOH-ARSP) from January 1, 2004 to December 31, 2018. The isolates were serotyped using Sven Gard method for slide agglutination using antigens from Denka Seiken (Japan), and S and A serotest (Thailand). Antigenic formula obtained were classified according to White-Kauffmann-LeMinor scheme. Antimicrobial susceptibility testing for ampicillin, ceftriaxone, cefotaxime, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole, were performed using both automated and conventional methods (Kirby Bauer disk diffusion and gradient diffusion method). Antimicrobial susceptibility results were interpreted using Clinical and Laboratory Standards Institute (CLSI) 2018 interpretive criteria (M100Ed28E).

Results. A total of 2,387 isolates were collected from human specimens during the 15-year study period. There were 69 serotypes of *Salmonella* identified with the most common being *Salmonella enterica* ser. Typhi: n=1895 (79.39%), *Salmonella enterica* ser. Enteritidis: n=182 (7.62%), *Salmonella enterica* ser. Typhimurium: n=87 (3.64%), *Salmonella enterica* ser. Weltevreden: n=24 (1.00%), *Salmonella enterica* ser. Paratyphi A: n=17 (0.71%), *Salmonella enterica* ser. Stanley: n=17 (0.71%), *Salmonella enterica* ser. Anatum: n=13 (0.54%), *Salmonella enterica* ser. Heidelberg: n=12 (0.50%), *Salmonella enterica* ser. Choleraesuis var. Kunzendorf: n=9 (0.38%). The multidrug resistant *Salmonella* serotypes reported in this study were mostly resistant to ampicillin, cefotaxime, ciprofloxacin combinations.

Conclusion. This present study showed that prevailing *Salmonella* serotypes in the Philippines were similar with *Salmonella* serotypes reported from other Asian countries. Typhoidal isolates were high among 6-17 years old and were mostly from males. The antimicrobial resistance rates for typhoidal *Salmonella* isolates to ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, ciprofloxacin, ceftriaxone and cefotaxime were lower compared with the antimicrobial resistance rates for non-typhoidal *Salmonella* isolates. Multidrug resistance for both *Salmonella* Typhi and NTS were relatively low. Continued and enhanced surveillance is needed to monitor the rising levels of antimicrobial resistance, determine risk factors and exposures associated with *Salmonella* Typhi and NTS infection to guide prevention and control measures.

Key words: *Salmonella* Typhi, NTS, serotype distribution, antimicrobial resistance, multidrug resistant

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INTRODUCTION

Salmonella enterica is the agent of typhoid and paratyphoid fever (enteric fever), as well as of salmonellosis and nontyphoidal infections. Globally, 14.3 million estimated cases of enteric fever (10.9 M typhoid fever and 3.3 paratyphoid fever) and 535,000 cases of nontyphoidal *Salmonella* (NTS) invasive disease were reported in 2017.¹ Agents of enteric fever (*Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi) are transmitted through fecal contamination of food or water by ill or asymptomatic chronic carriers while agents of salmonellosis and nontyphoidal *Salmonella* are transmitted through consumption of contaminated water, food animal products or fresh produce, and contact with animals or their environment.²

Enteric fever due to *Salmonella* Typhi (SAT) and *Salmonella* Paratyphi serotypes A, B and C is a severe systemic disease requiring antibiotic therapy. In the Philippines, there were 11,140 reported cases of typhoid fever from January 1 to July 2018, with 20 reported deaths.³ Locally, cases of uncomplicated typhoid fever are treated with ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.⁴ Based on national AMR surveillance in the country, these antibiotics have remained effective against agents of enteric fever with resistance rates remaining under less than five percent for these antibiotic from 2004-2018 (Figure 1A and 1B).⁵

Infection due to Non-typhoidal *Salmonella* is commonly a self-limiting acute gastroenteritis. Treatment is thus primarily directed to replacement of fluid and electrolytes and antimicrobials are not routinely recommended for uncomplicated NTS gastroenteritis. Invasive infections, however, may occur in less than 5% of patients for which oral therapy may include fluoroquinolone, trimethoprim-sulfamethoxazole or amoxicillin. Figure 1C and 1D shows resistance rates of NTS to these antibiotics based on the national AMR surveillance in the country.

Emergence of multidrug resistance among typhoidal and non-typhoidal *Salmonella* attributed to transferable plasmids, however, have been observed. In a particular strain - SAT H58, antimicrobial resistance (AMR) genes have been associated with an IncHI1 plasmid.⁶ In the 1990s, there was worldwide emergence of multidrug resistant *Salmonella* Typhimurium (phage type 104 or DT104) which were resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline.

Salmonella enterica is highly diverse bacterial pathogen with over 2,600 known serotypes.⁷ Identifying *Salmonella* serovar plays an important role in understanding the epidemiology of the pathogen, is used to establish link between cases as well as to track potential source of infection. The World Health Organization (WHO) reported that *Salmonella* serovars Enteritidis, Typhimurium, Typhi, Heidelberg, Infantis, Virchow, Hadar, Saintpaul, Montevideo and Agona were the most common serotypes isolated from human sources worldwide.⁸

There is scarcity of information on *Salmonella* serotypes in the Philippines, particularly for NTS, which limits

understanding of the distribution and transmission of these bacteria. This report provides valuable information on the prevailing *Salmonella* serotypes in the country from 2004 to 2018 (Figure 2). Antimicrobial resistance among these bacteria will also be described.

METHODOLOGY

Isolates

Salmonella isolates from clinical specimens were collected through the Philippine Department of Health-Antimicrobial Resistance Surveillance Program (DOH-ARSP) from January 1, 2004 to December 31, 2018. The DOH-ARSP is a laboratory based antimicrobial resistance surveillance with 24 sentinel sites representing 16 regions in the country. Case finding for ARSP is based on priority specimens sent routinely to sentinel sites laboratories for clinical purposes. The initial bacterial identification and antimicrobial susceptibility of the isolates were confirmed by automated (Vitek 2) and conventional biochemical tests systems in the Antimicrobial Resistance Surveillance Reference Laboratory (ARSRL). Results are managed and analyzed using WHONET software.

Serotyping

The isolates were serotyped using Sven Gard method for slide agglutination using antigens from Denka Seiken (Japan), and S and A serotest (Thailand). Antigenic formula obtained were classified according White-Kauffmann-LeMinor scheme, as recommended by the World Health Organization Collaborating Centre for Reference and Research on *Salmonella*.⁹

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing for ampicillin, ceftriaxone, cefotaxime, chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole, were performed using both automated (Vitek 2) and conventional methods (Kirby Bauer disk diffusion and gradient diffusion method). Antimicrobial susceptibility results were interpreted using Clinical and Laboratory Standards Institute (CLSI) 2018 interpretive criteria (M100Ed28E).

Data Extraction

The research data were derived from the laboratory-based surveillance software (WHONET). Data gathered included identification (ID) and antifungal susceptibility (AST) and routine demographic information. No clinical information from the patients' chart were included thus, factor such as timing of specimen collection cannot be identified. A data collection tool was used to extract information of the isolates.

Ethical Considerations

During the biobanking (preservation) process, a biobank form is completed indicating the assigned accession number of the isolates. Isolate information in the biobank form includes ID and susceptibility profile, age, specimen type, and birthday of the patient; the name of the patient is not indicated. All of the information required in the data collection tool for this study were extracted exclusively from the biobank form. Since ARSRL department only handles referred isolates from its sentinel sites, no patient researcher interaction

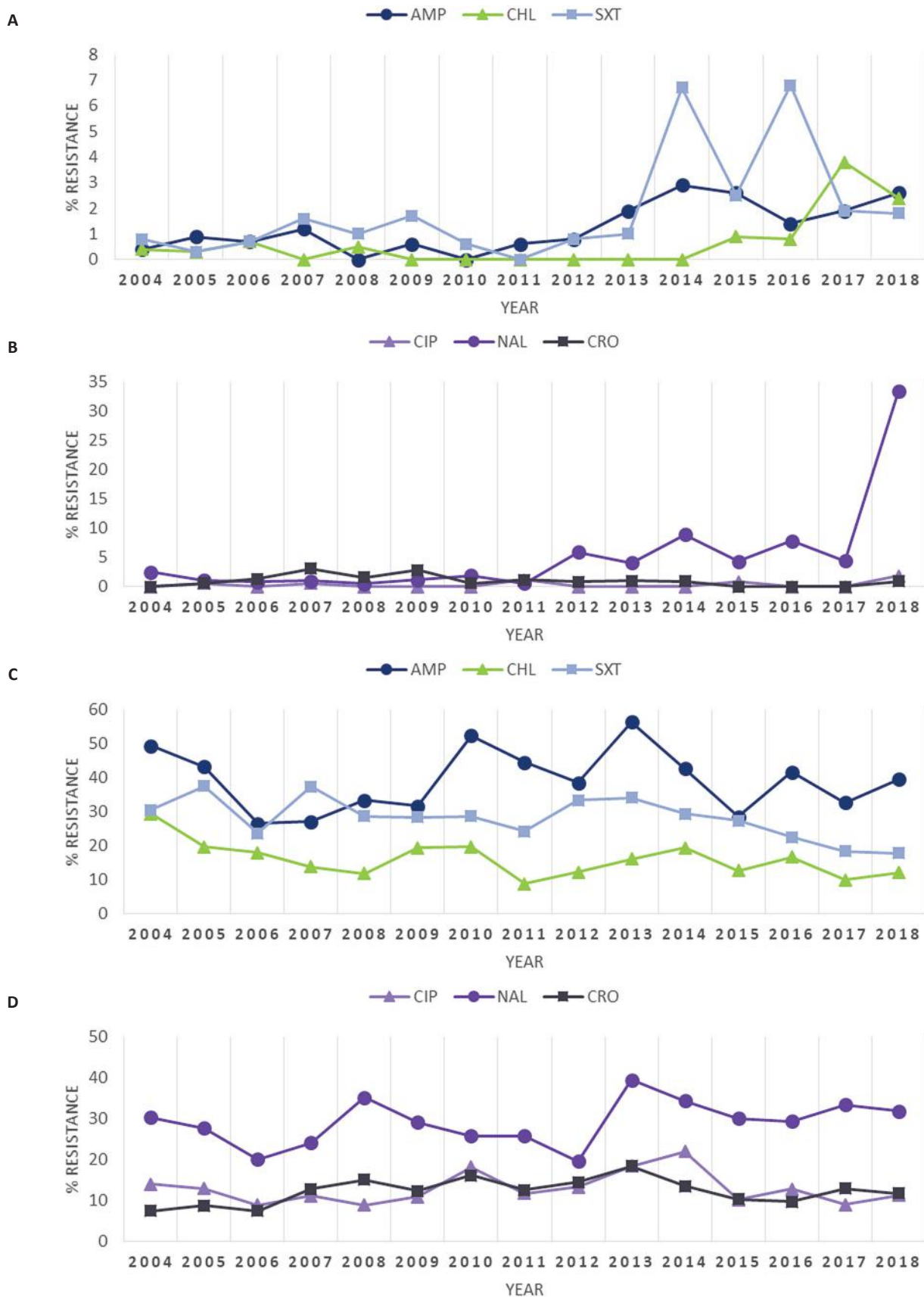


Figure 1. Yearly antimicrobial resistance rates of *Salmonella* Typhi isolates from 2004-2018 to **(A)** ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole; **(B)** ciprofloxacin, nalidixic acid and ceftriaxone. Yearly antimicrobial resistance rates of NTS isolates from 2004-2018; **(C)** ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole; **(D)** ciprofloxacin, nalidixic acid and ceftriaxone.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2004	16	12	9	16	11	10	6	10	11	3	13	12	129
2005	17	13	9	7	11	17	9	15	15	12	25	14	164
2006	20	19	33	25	32	26	21	33	22	15	11	9	266
2007	14	14	12	13	14	7	9	16	13	11	9	7	139
2008	11	5	15	13	14	15	15	19	7	6	8	6	134
2009	8	10	14	15	13	4	11	6	14	17	16	15	143
2010	12	10	10	11	11	9	13	9	9	9	12	7	122
2011	17	12	7	9	14	12	12	16	16	6	7	9	137
2012	8	11	14	13	6	12	11	8	5	6	10	3	107
2013	5	9	2	6	6	15	14	5	4	3	3	7	79
2014	6	8	4	6	6	7	7	6	6	14	10	6	86
2015	8	10	6	6	5	9	10	14	8	16	7	8	107
2016	10	6	3	9	5	11	5	11	16	25	6	9	116
2017	9	16	12	17	9	12	5	9	13	11	6	4	123
2018	6	10	7	8	6	1	6	5	4	8	4	6	71
Average	11	11	10	12	11	11	10	12	11	11	10	8	128.2
	11	11	10	12	11	11	10	12	11	11	10	8	

LEGEND:



Figure 2. Frequency distribution of Typhoidal *Salmonella* per year from 2004 to 2018 (n=1,923).

was established. It was noted that, data obtained for this study were part of an ongoing surveillance in the Philippines and surveillance reports in the country does not require ethical evaluation when used for research purpose, moreover, no informed consent were required from all the patients involved.

Statistical Analysis

No statistical software or tool was used in the study since the nature of the research is purely descriptive. Moreover, no analytical computations were required, and simple quantitative treatment of data were represented (e.g number of species over total number of isolates expressed in %).

RESULTS

A total of 2,387 isolates were collected from human specimens during the 15-year study period. There were 69 serotypes of *Salmonella* identified with the most common being *Salmonella* Typhi: n=1895 (79.39%), *Salmonella* Enteritidis: n=182 (7.62%), *Salmonella* Typhimurium: n=87 (3.64%), *Salmonella* Weltevreden: n=24 (1.00%), *Salmonella* Paratyphi A: n=17 (0.71%), *Salmonella* Stanley: n=17 (0.71%), *Salmonella* Anatum: n=13 (0.54%), *Salmonella* Heidelberg: n=12 (0.50%), *Salmonella* Choleraesuis var. Kunzendorf: n=9 (0.38%) (Table 1).

Salmonella Typhi was the most common typhoidal serotype observed in this study (n=1895, 98.54%). *Salmonella* Paratyphi A (n=17, 0.88%), *Salmonella* Paratyphi B

(n=9, 0.46%) and *Salmonella* Paratyphi C (n=2, 0.10%) accounted for the rest. Most of the serotypes were from blood specimens (SAT=1753, *Salmonella* Paratyphi A=15, *Salmonella* Paratyphi B=6 and *Salmonella* Paratyphi C=1). Typhoidal serotypes was 15.8% more commonly isolated from males (n=1087; compared to females n=785) and most were from age group 6-17 years (n=915) (Figure 3). Many were collected from the Visayas region (Table 2) and 93.03% (1789 out of 1923) were from sterile sites. The most number of isolates were collected in 2006 (n= 266). No trend was observed on the number of salmonella isolates reported per month in the 15-year period.

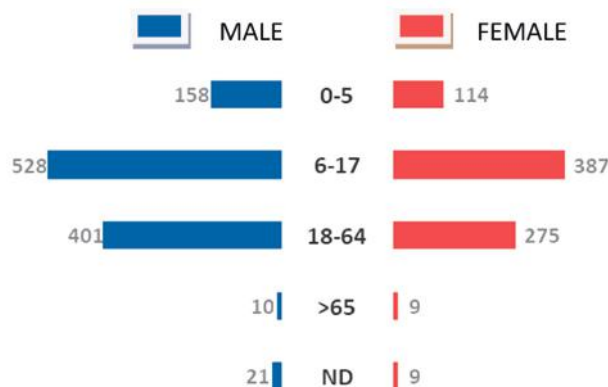


Figure 3. Distribution of Typhoidal *Salmonella* serotypes as to age group and sex.

The yearly antimicrobial resistance rates for typhoidal *Salmonella* serotypes from 2004 to 2018 are shown in Figure 4A. Resistance to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole remained low from

2007-2018. Chloramphenicol resistance was reported only in 2004 (n=127) (0.8%) and 2006 (n=262) (0.4%). Resistance to ciprofloxacin, ceftriaxone and cefotaxime likewise have remained low throughout the study period (Figure 4B). There were statistically significant increases in ceftriaxone resistance in 2008 (0.4%) to 2010 (0.8%) as well as from 2012 (0%) to 2013 (1.3%).

Table 1. Frequency of *Salmonella* isolates, ARSP, 2004 TO 2018

<i>Salmonella enterica</i> Serotype	Total Number of Isolates
Typhoidal <i>Salmonella</i>	1923
<i>Salmonella</i> Typhi	1895
<i>Salmonella</i> Paratyphi A	17
<i>Salmonella</i> Paratyphi B	9
<i>Salmonella</i> Paratyphi C	2
Non-typhoidal <i>Salmonella</i>	464
<i>Salmonella</i> Enteritidis	182
<i>Salmonella</i> Typhimurium	87
<i>Salmonella</i> Weltevreden	24
<i>Salmonella</i> Stanley	16
<i>Salmonella</i> Anatum	13
<i>Salmonella</i> Heidelberg	12
<i>Salmonella</i> Choleraesuis var. Kunzendorf	9
<i>Salmonella</i> Group B	9
<i>Salmonella</i> Virchow	8
<i>Salmonella enterica</i> ss. arizonae	7
<i>Salmonella</i> Kentucky	6
<i>Salmonella</i> Rissen	6
<i>Salmonella</i> Albany	5
<i>Salmonella</i> Choleraesuis	5
<i>Salmonella</i> Group C	5
<i>Salmonella</i> Newport	4
<i>Salmonella</i> Schwarzengrund	4
<i>Salmonella</i> Bardo	3
<i>Salmonella</i> Derby	3
<i>Salmonella</i> Hissar	3
<i>Salmonella</i> Lexington	3
<i>Salmonella</i> Saintpaul	3
<i>Salmonella</i> Agona	2
<i>Salmonella</i> Bonn	2
<i>Salmonella</i> Corvallis	2
<i>Salmonella</i> Essen	2
<i>Salmonella</i> Abardeen	39 (1 isolate each)
<i>Salmonella</i> Ajiobo	
<i>Salmonella</i> Bareilly	
<i>Salmonella</i> Bournemouth	
<i>Salmonella</i> Brazaville	
<i>Salmonella</i> Breda	
<i>Salmonella</i> Chailey	
<i>Salmonella</i> Denver	
<i>Salmonella</i> Eastbourne	
<i>Salmonella</i> Emek	
<i>Salmonella</i> Farcha	
<i>Salmonella</i> Group A	
<i>Salmonella</i> Group E1	
<i>Salmonella</i> Group K	
<i>Salmonella</i> Haifa	
<i>Salmonella</i> Hillingdon	
<i>Salmonella</i> Houtenae	
<i>Salmonella</i> Indica	
<i>Salmonella</i> Irumu	
<i>Salmonella</i> Itami	
<i>Salmonella</i> Javiana	
<i>Salmonella</i> Lome	
<i>Salmonella</i> Lomita	
<i>Salmonella</i> London	
<i>Salmonella</i> Mbandaka	
<i>Salmonella</i> Mgulani	
<i>Salmonella</i> Montevideo	
<i>Salmonella</i> Ndolo	
<i>Salmonella</i> Nnessziona	
<i>Salmonella</i> Ohio	
<i>Salmonella</i> Potsdam	
<i>Salmonella</i> Regent	
<i>Salmonella</i> San Diego	
<i>Salmonella</i> Sangera	
<i>Salmonella</i> Saphra	
<i>Salmonella</i> Seftenberg	
<i>Salmonella</i> Tallahassee	
<i>Salmonella</i> Tumodi	
<i>Salmonella</i> Wanatah	

Salmonella Enteritidis: n=182 (39.22%) were the most common NTS followed by *Salmonella* Typhimurium: n=87 (18.75%) and *Salmonella* Weltevreden: n=24 (5.17%) (Table 1). It was noted that NTS were isolated more from males for all serotypes (Figure 6) except for *Salmonella* Stanley (Male= 7, Female= 10) and *Salmonella* Anatum (Male= 5, Female= 8). The serotypes with the highest number of isolates for 0-5 years old were *Salmonella* Typhimurium (n=45), *Salmonella* Stanley (n=13), *Salmonella* Anatum (n=6), and *Salmonella* Heidelberg (n=10). Highest number of *Salmonella* Weltevreden isolates were seen among 6-17 year olds. On the other hand, *Salmonella* Enteritidis (n=81) and *Salmonella* Choleraesuis var. Kunzendorf (n=5) have the most number of isolates for 18-64 years olds. For more than 65-years population, *Salmonella* Enteritidis (n=21) and *Salmonella* Typhimurium (n=15) were the two serotypes with the most number of isolates. Metro Manila shared the biggest number of isolates for all serotypes except for serotypes *Salmonella enterica* serovars Weltevreden and Heidelberg. Overall, NTS were mostly isolated from blood (n=227, 49%) and stool (n=159, 34%). The most number of NTS isolates were collected (n= 68) in 2018; however, no trend in the reported number of NTS isolates per month was observed for the 15-year period.

Resistance to ampicillin was the highest (63.3%) in 2004 with fluctuations on the resistance rates from 2005 to 2018 (Figure 7). Chloramphenicol and trimethoprim-sulfamethoxazole resistance among NTS was highest in 2008 (33.3%) and remained in the range of 10-20% from 2009-2018. *Salmonella* Typhimurium showed the highest resistance to ampicillin (65.1%) among the NTS. Trimethoprim-sulfamethoxazole resistance rates were lowest for *Salmonella* Stanley (50%) and *Salmonella* Heidelberg (66.7%). Combined ampicillin and ciprofloxacin resistant phenotypes in *Salmonella enterica* serovars Enteritidis (n=8), Typhimurium (n=10) and Choleraesuis var. Kunzendorf (n=3) were also noted.

Table 2. Distribution of *Salmonella* sp. ARSP, 2004 TO 2018

Demographics	Typhoidal (N=1,923)	NTS (N=464)
Island Group		
Metro Manila	321 (16.69%)	202 (43.53%)
Luzon	348 (18.09%)	116 (25%)
Visayas	815 (42.38%)	100 (21.55%)
Mindanao	430 (22.36%)	46 (9.91%)
Specimen Type		
Blood	1775 (92.30%)	227 (48.92%)
CSF	2 (0.10%)	5 (1.07%)
Stool	85 (4.42%)	159 (34.26%)
Urine	18 (0.93%)	17 (3.66%)
Respiratory	4 (0.20%)	3 (0.64%)
Wound	14 (0.72%)	23 (4.95%)
Tissue	5 (0.26%)	0
Fluid	7 (0.36%)	21 (4.52%)
Other	4 (0.20%)	9 (1.93%)

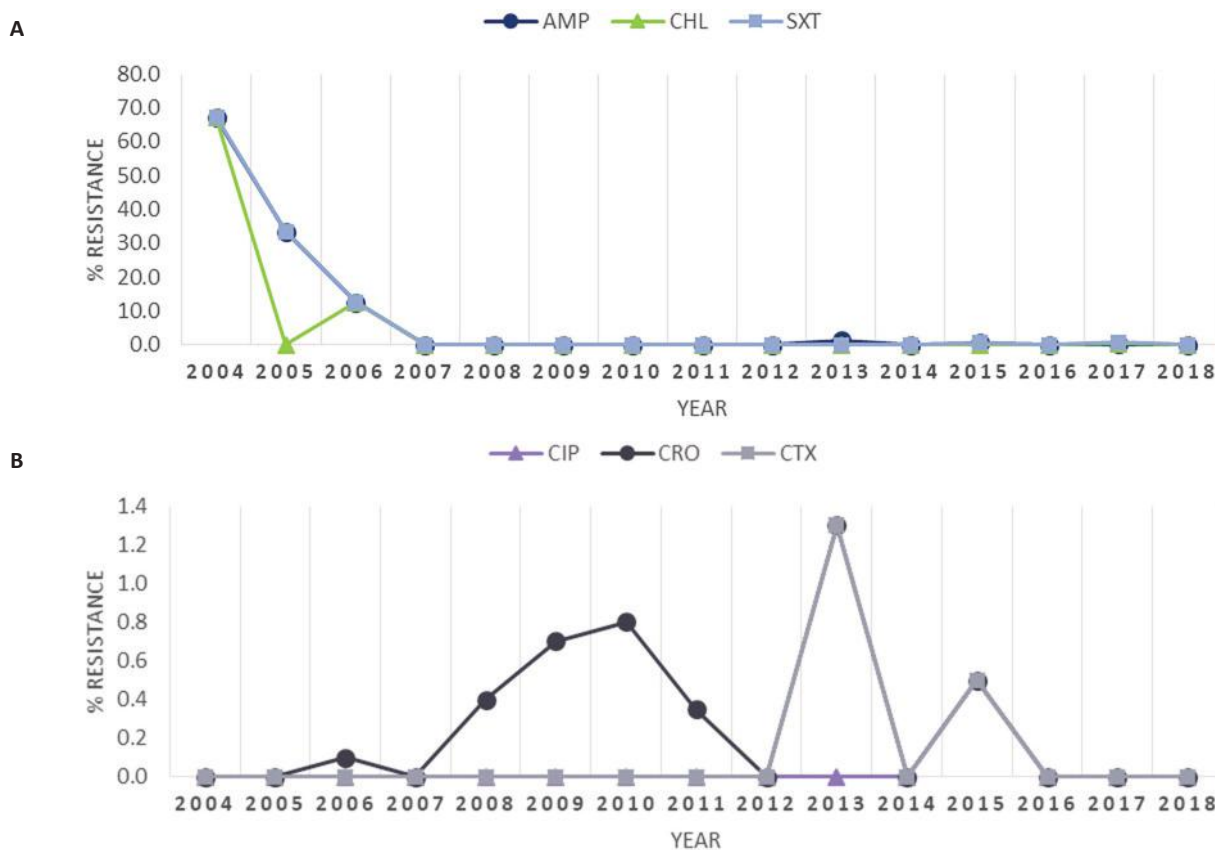


Figure 4. Yearly antimicrobial resistance rates of Typhoidal *Salmonella* serotypes from 2004-2018 to (A) ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole; (B) ciprofloxacin, ceftriaxone and cefotaxime.

Among the typhoidal *Salmonella* isolates, combined cefotaxime-ciprofloxacin resistance phenotype was seen in 17 *Salmonella* Typhi isolates. Multidrug resistance (resistance to ampicillin-ciprofloxacin-chloramphenicol) was seen among *Salmonella* Paratyphi B and *Salmonella* Paratyphi C. No multidrug resistance was noted among *Salmonella* Paratyphi A. On the other hand, combined ampicillin- ciprofloxacin resistance was noted in *Salmonella* enterica serovars Enteritidis (n=8), Typhimurium (n=10) and Choleraesuis var. Kunzendorf (n=3).

DISCUSSION

A more recent report by Epidemiology Bureau Public Health Surveillance Division of the Department of Health in January 1-to February 23, 2019, revealed that there were 2,720 reported cases of typhoid fever collated nationwide.¹⁰ The number of typhoid fever cases decreased by 16%, from 3,524 cases in 2018 to 2,720 in 2019. There were, however, two confirmed deaths (CFR=0.07%) out of the 2,720 cases reported. The report confirms the persistence of *Salmonella* Typhi infections in the country.

There is scarcity, on the other hand, of data on occurrence of NTS infections, including its distribution and antimicrobial resistance, in the country (Figure 5).

Typhoidal *Salmonella*

The present study showed that both *Salmonella* Typhi and *Salmonella* Paratyphi A were more commonly isolated from males. While available national census data shows higher

percentage of males more than females in the country from 2004-2015,¹¹ the present observation may also be attributed to male behavioral factors such as higher risks in food handling, preparation and consumption.¹²

The most number of typhoidal isolates in this study were collected from the Visayas region. This is in contrast with the Department of Health data from January 1 to February 23, 2019 which showed that the Mindanao region has the most number of *Salmonella* Typhi cases. The relative numbers per island group in the present study may not necessarily indicate relative prevalence of *Salmonella* infections but could be a reflection of the diagnostic practices of clinicians as well as technical capabilities, including laboratory resources, of the sentinel sites of the ARSP. Nevertheless, it can be noted that most areas of these two island groups – Visayas and Mindanao – were rural areas.

The Philippine Progress Report on the Millennium Development Goals 2010 stated that in 1990-2008, 18% percent of the rural areas in the country still practice open defecation.¹³ Moreover, the direct exposure of individuals to livestock animals which is prevalent in the country might also increase risk of human *Salmonella* infection. These factors may contribute to the continued presence of *Salmonella* infections in the country.

As antibiotic therapy is the mainstay for the treatment of typhoid fever and the complications associated with it, the emergence of resistance to antibiotics used against

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
2004	16	12	9	16	11	10	6	10	11	3	13	12	129
2005	17	13	9	7	11	17	9	15	15	12	25	14	164
2006	20	19	33	25	32	26	21	33	22	15	11	9	266
2007	14	14	12	13	14	7	9	16	13	11	9	7	139
2008	11	5	15	13	14	15	15	19	7	6	8	6	134
2009	8	10	14	15	13	4	11	6	14	17	16	15	143
2010	12	10	10	11	11	9	13	9	9	9	12	7	122
2011	17	12	7	9	14	12	12	16	16	6	7	9	137
2012	8	11	14	13	6	12	11	8	5	6	10	3	107
2013	5	9	2	6	6	15	14	5	4	3	3	7	79
2014	6	8	4	6	6	7	7	6	6	14	10	6	86
2015	8	10	6	6	5	9	10	14	8	16	7	8	107
2016	10	6	3	9	5	11	5	11	16	25	6	9	116
2017	9	16	12	17	9	12	5	9	13	11	6	4	123
2018	6	10	7	8	6	1	6	5	4	8	4	6	71
Average	11	11	10	12	11	11	10	12	11	11	10	8	128.2
	11	11	10	12	11	11	10	12	11	11	10	8	

LEGEND:



Figure 5. Frequency distribution of NTS per year from 2004 to 2018 (n=464).

it – chloramphenicol, ampicillin, sulphamethoxazole-trimethoprim, quinolones and cephalosporins – is a concern. Although the origin of antibiotic resistance genes among *Salmonella* Typhi is still unclear, it has been recognized that the two factors that mediate antibiotic resistance are foreign genes acquisition via plasmids and chromosome mutation.¹⁴ Moreover, resistance may be through inactivation of the antimicrobial agent, efflux or transport of the antimicrobial, modification of the antimicrobial target site and reduced permeability of the antimicrobial agent.

Antimicrobial resistance in developed countries has been linked to the utilization of antimicrobial drugs in livestock animals and environmental usage.

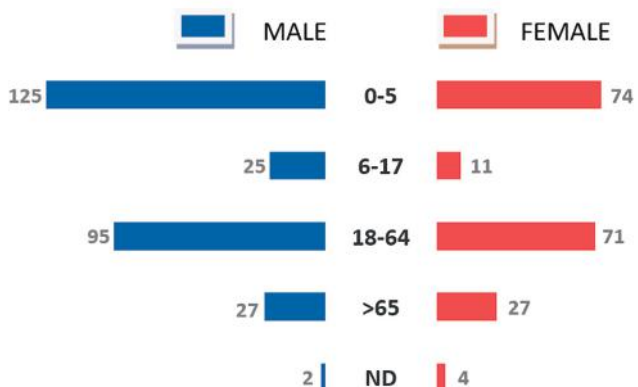


Figure 6. Distribution of NTS serotypes as to age group and sex.

Among developing countries, however, episodes of antimicrobial resistance of both Non-typhoidal and typhoidal *Salmonella* spp. were associated with the use of antimicrobials for medication in humans.¹⁵

Multidrug resistant strains of *Salmonella* resistant to first line antibiotics (ampicillin, chloramphenicol, and cotrimoxazole) have been identified as early as 1989.¹⁶ The widespread use of these drugs facilitated the emergence of resistance to chloramphenicol and subsequently to ampicillin and co-trimoxazole, leading to MDR typhoid. In the present study, however, the percent resistance of *Salmonella* Typhi among ampicillin (0.3%), ceftriaxone (0.1%), cefotaxime (0.4%), ciprofloxacin (0.1%) and sulfamethoxazole-trimethoprim (0.3%) have remained low in the country from 2004-2018.

The multidrug resistant *Salmonella* Typhi reported in this study were mostly resistant to ampicillin, cefotaxime, ciprofloxacin tandems or combinations. On the other hand, no multidrug resistance was noted for *Salmonella* Paratyphi A. No multi-locus sequence typing (MLST) was performed in the present study. It will be interesting to determine the genotypic characteristics of the MDR clone in the country and its relation, if any, with the multidrug-resistant (MDR) *Salmonella* Typhi H58 clone which is the dominant MDR type that circulates in the Indian subcontinent and Southeast Asia¹⁷⁻¹⁹ as well as clones from India, Pakistan and Vietnam which had higher rates of MDR isolates of *Salmonella* Typhi than Indonesia and China.²⁰



Figure 7. Yearly antimicrobial resistance rates of Non-typhoidal *Salmonella* serotypes from 2004-2018 to (A) ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole; (B) ciprofloxacin, ceftriaxone and cefotaxime.

Non-typhoidal *Salmonella*

Three of the most frequently isolated NTS serotypes in this present study – *S. Enteritidis*, *S. Typhimurium*, and *S. Weltevreden* - are included in the 2006 WHO list of commonly isolated *Salmonella* strains. ARSP had been part of the WHO *Salmonella* Surveillance and has contributed data to this surveillance since 1994. It has been reported by WHO that serotype distribution varies with geographical location, age group affected and socio-economic status of the region. It was noted that NTS serotypes reported in the present study were similar to that of Thailand, a developing nation like the Philippine. We report in this study the sporadic occurrence over the 15-year period of the following isolates which were not reflected in the 2006 WHO list of commonly isolated *Salmonella* strains: *Salmonella* Stanley, *Salmonella* Anatum, *Salmonella* Choleraesuis var. Kunzendorf.

Although NTS gastroenteritis are typically self-limiting and non-fatal to immunocompetent individuals, invasive NTS (iNTS) infections can lead to death among susceptible populations suffering from malaria, malnutrition and people living with HIV.²¹ Case fatality rate of NTS related community acquired bacteremia in African regions was reported to be as high as 20.6%.

In this present study, there were 212 invasive NTS isolates with the most common serotypes being *Salmonella enterica* serovars *Enteritidis*, *Dublin*, and *Typhimurium*. Serovars associated with iNTS in the present study is similar with serovars causing iNTS In Vietnam

(*Salmonella* Enteritidis and *Salmonella* Typhimurium). Most of the patients with iNTS were adult males, HIV positive and with history of drug use.²² Future studies on iNTS may consider identifying risks for this type of infection. Further consideration for risk factor evaluation may also include other potential predisposing factors such as previous medication (acid blockers and antimicrobial pre-treatment).²³

NTS infections in this study were noted to be higher in urbanized area such as Metro Manila. While this could be due to the urban in-migration that caused expansion and generation of urban slums, wherein there is resultant increased risk of food and waterborne diseases linked to poor water, sanitation and hygiene infrastructures,²⁴ the high number of NTS may also be accounted for by the diagnostic practices of physicians, capacity of the laboratories, and the relative number of patients in the area.

Reservoir of NTS was previously thought to be exclusively of animal origin. Personal hygiene and regulated food handling were then considered sufficient control measures to prevent NTS infection. Human reservoir of iNTS from Burkina Faso in West Africa, however, has since been confirmed.²⁵ With the emerging reports of human iNTS reservoir, vaccines has been increasingly explored as a control measure for iNTS. Continuous and comprehensive surveillance of iNTS would be an invaluable information towards vaccine development for agents of iNTS.

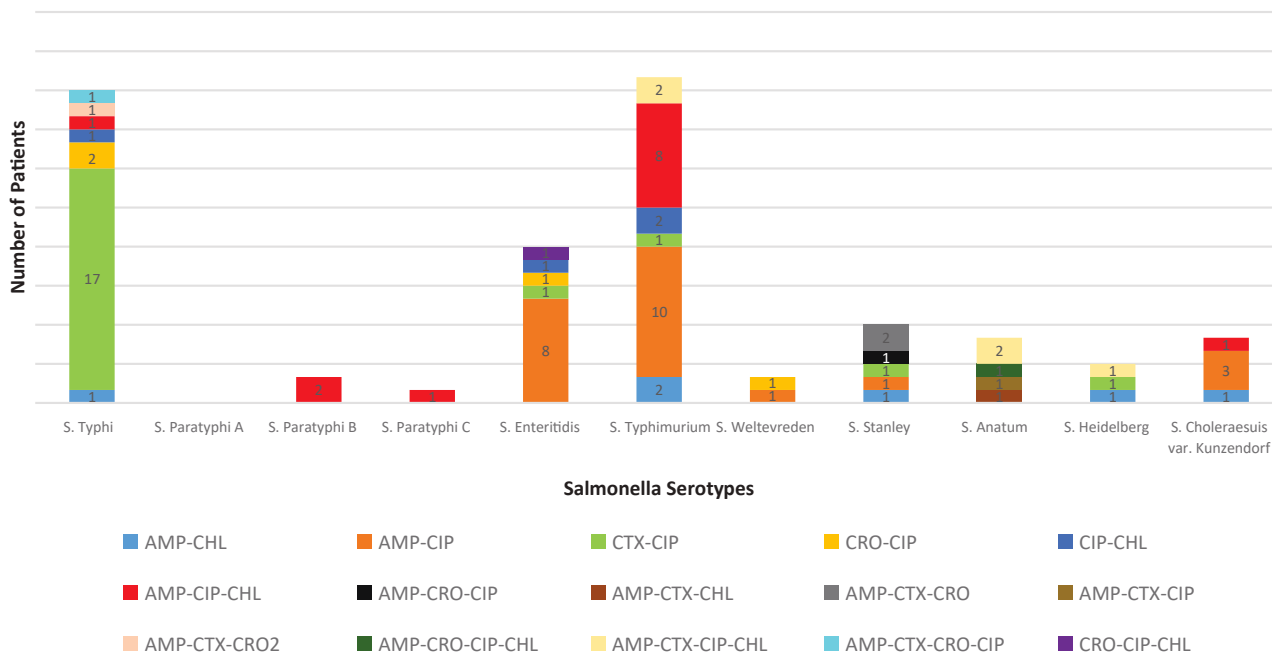


Figure 8. Multidrug resistant *Salmonella* serotypes from 2004-2018.

For NTS in this present study, resistance to antibiotics – chloramphenicol, ampicillin and sulfamethoxazole-trimethoprim among *Salmonella* enterica serovars Typhimurium, Anatum, Stanley and Heidelberg were noted. Given, however, the very few number of isolates for respective NTS serotypes in this present study, the results should be interpreted with caution.

Antimicrobial resistance to ciprofloxacin (1.2%), third-generation cephalosporins (12.2%), ampicillin (68.2%) and TMP/SMX (17.0%) have been reported in the first population-based estimates of NTS from Southeast Asia. Similar rates have been found in a tertiary-care setting in Bangkok among children, where rates were 68.3%, 33.9%, 3% and 17.4% respectively.²⁶ Further, a 2009 multi-national study showed a high prevalence of reduced susceptibility to ciprofloxacin among non-typhoidal *Salmonella* strains from Taiwan (48.1%), Thailand (46.2%), Korea (36.5%), and Sri Lanka (8.0%).²⁷ In the present study, resistance to third generation cephalosporins (cefuroxime and ceftriaxone) were likewise high at 33.07% as was also observed in Taiwan and Thailand.²⁸ It is noteworthy that the present study herewith reports occurrence of invasive NTS (n=212) with isolates showing resistance to chloramphenicol, ampicillin and sulfamethoxazole-trimethoprim.

The combination of ampicillin and ciprofloxacin resistance were mostly seen in *Salmonella* Enteritidis and *Salmonella* Typhimurium isolates in this present study. In addition, *Salmonella* Typhimurium mostly possess ampicillin-ciprofloxacin-chloramphenicol resistance. Similarly, *Salmonella* Typhimurium and *Salmonella* Enteritidis were the two main NTS serotypes that showed multiple resistance to amoxicillin, ciprofloxacin, cefotaxime and gentamicin in Kenya.²⁹

CONCLUSION

The present study showed that prevailing *Salmonella* serotypes in the Philippines were similar with *Salmonella* serotypes reported from other Asian countries. There were 69 serotypes of *Salmonella* identified with the most common being *Salmonella* Typhi: n=1895 (79.39%), *Salmonella* Enteritidis: n=182 (7.62%), *Salmonella* Typhimurium: n=87 (3.64%), *Salmonella* Weltevreden: n=24 (1.00%), *Salmonella* Paratyphi A: n=17 (0.71%), *Salmonella* Stanley: n=17 (0.71%), *Salmonella* Anatum: n=13 (0.54%), *Salmonella* Heidelberg: n=12 (0.50%), *Salmonella* Choleraesuis var. Kunzendorf: n=9 (0.38%). Typhoidal isolates were high among 6-17 years old and were mostly from males.

Multidrug resistance for both *Salmonella* Typhi and NTS were relatively low. The multidrug resistant *Salmonella* serotypes reported in this study were mostly resistant to ampicillin, cefotaxime, ciprofloxacin combinations (Figure 8). Continued and enhanced surveillance is needed to monitor the rising levels of antimicrobial resistance, determine risk factors and exposures associated with *Salmonella* Typhi and iNTS infection to guide prevention and control measures.

STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

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Comparing Differential Gene Expression in Chronic Traumatic Encephalopathy, Parkinson's Disease, and Bipolar Disorder

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ABSTRACT

Introduction. Chronic traumatic encephalopathy (CTE) is a progressive neurodegenerative disorder that is defined, neuropathologically, by the presence of aggregated hyperphosphorylated tau in the neurons and astrocytes of the perivascular area that is located deep in the cerebral sulci. The lesion is associated with repetitive brain trauma, from the spectrum of asymptomatic subconcussive head injury to grossly identifiable features of concussion. Although the diagnostic neuropathology of CTE is well-characterized, the precise mechanism that causes this to occur in CTE is not yet clearly elucidated. The features of hyperphosphorylated tau in CTE is quite similar with Alzheimer's Disease (AD), as is the reduced expression of certain genes that are required to dephosphorylate tau, which is the putative culprit in the generation of amyloid aggregates and hyperphosphorylated tau.¹ In comparison, Parkinson's Disease (PD) is a neurodegenerative disease that is caused by accumulation of misfolded alpha-synuclein (α -syn) that causes the formation of intraneuronal Lewy Body aggregates. The pattern of accumulation for α -syn involves the olfactory bulb and the gut with progressive involvement of the posterior part of the brain.² Despite establishing the presence of two different intraneuronal inclusions for CTE and PD, contact sports associated with the clinical spectrum of CTE has been shown to present with Parkinsonian features along with dementia. Mood disorders has been reported to occur in patients with these neurologic conditions. Several studies have documented that patients had a previous experience of traumatic brain injury prior to the diagnosis of Bipolar Disorder (BD). A review of electronic literature suggested that having an earlier diagnosis of BD increased the likelihood of having a diagnosis of PD in the future.^{3,4}

Objectives. This research aimed to compare the over- and underexpressed genes in cases with Parkinson's Disease (PD), cases with Bipolar Disorder (BD), and cases with Chronic Traumatic Encephalopathy (CTE) versus normal controls. This was done to determine if parallel overexpression in certain genes may indicate the possible association at the level of gene expression. Identifying similar RNA sequence establishing gene expression may provide an insight to the relationship of the diseases in terms of pathobiological behavior. Determining the similar over- or underexpression pattern may provide an insight on the common pathobiologic mechanisms that may be the reason for the three disorders being associated by way of pre-morbid or co-morbid condition.

Methodology. Transcripts from the public domain archive of the NCBI SRA were identified for the RNA sequence (RNAseq) of interest using the search string "Chronic Traumatic Encephalopathy", "Bipolar Disorder", and "Parkinson". Only public domain transcriptome files of post-mortem brain samples labeled as RNAseq data extracted thru the Illumina platform that have a paired normal control were selected. A total of ten (10) cases for each disorder and thirty (30) normal subjects for control in the NCBI SRA RNAseq database with a whole exome sequence file that was available for public domain use was utilized for differential gene expression analysis.^{6,7,8}

Results and Discussion. Among 21,122 identified genes from the RNAseq, the analysis was able to identify 26 genes exhibiting increased expression of up to >15 log₂ fold change among cases with CTE, PD, and BD compared with normal controls. In contradistinction, only 6 well-described genes exhibited a decreased

expression among cases with CTE and BD compared to normal controls. However, there were no identified genes that exhibited underexpression in cases with PD compared with normal controls.

The identification of parallel gene overexpression among the CTE, BD, and PD groups with respect to structural integrity, cellular metabolism, homeostasis, and apoptosis may indicate a common pathway that have been initiated as part of the response to maintain tissue function or as a consequence of the underlying pathobiologic mechanism that caused the primary lesion.

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In comparison, the underexpressed genes detected in the CTE and BD cases compared to the normal controls and the PD cases may indicate the lack of genes that have a role in repressing the mRNA for protein coding.

Conclusion. The overexpression of genes responsible for homeostasis, regulation of inflammation, balance of apoptosis and anti-apoptosis, and maintenance of structural integrity among the CTE, BD, and PD groups indicate that there is an interrelated mechanism that serves converging pathways as part of the response to lesional- forming structures in the brain. The goal of studies such as this is to have a better understanding on the common pathways that explain the interrelatedness of brain disorders and the putative mechanism for being co-morbid and pre-morbid conditions of each other.

Key words: differential gene expression, RNAseq, Chronic Traumatic Encephalopathy, Bipolar Disorder, Parkinson's Disease

INTRODUCTION

Chronic traumatic encephalopathy (CTE) is a progressive neurodegenerative disorder that is defined, neuropathologically, by the presence of aggregated hyperphosphorylated tau in the neurons and astrocytes of the perivascular area that is located deep in the cerebral sulci. The lesion is associated with repetitive brain trauma, from the spectrum of asymptomatic subconcussive head injury to grossly identifiable features of concussion. Although the diagnostic neuropathology of CTE is well-characterized, the precise mechanism that causes this to occur in CTE is not yet clearly elucidated. The features of hyperphosphorylated tau in CTE is quite similar with Alzheimer's Disease (AD), as is the reduced expression of certain genes that are required to dephosphorylate tau, which is the putative culprit in the generation of amyloid aggregates and hyperphosphorylated tau.¹

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Despite establishing the presence of two different intraneuronal inclusions for CTE and PD, contact sports associated with the clinical spectrum of CTE has been shown to present with Parkinsonian features along with dementia. Mood disorders has been reported to occur in patients with these neurologic conditions. Several studies have documented that patients had a previous experience of traumatic brain injury prior to the diagnosis of Bipolar Disorder (BD). A review of electronic literature suggested that having an earlier diagnosis of BD increased the likelihood of having a diagnosis of PD in the future.^{3,4} Newer studies in chronic mental illness have shown that patients diagnosed with BD exhibited aberrant aggregation of Disrupted-In-Schizophrenia 1 (DISC1) in post-mortem brain tissue, particularly in the cingulate cortex.⁵

The association of intraneuronal aggregates with the clinical presentation of the lesions, the presence of BD as a co-morbid or pre-morbid condition for both CTE and PD patients, and the relationship of CTE and PD, may lead to a possible association between these three lesions. The association may be in terms of increased or decreased gene

expression of the patients compare to that of the normal population that provides an intra- and extracellular milieu to develop protein aggregates and manifest with the clinical symptoms.

OBJECTIVES

This research aimed to compare the over- and underexpressed genes in cases with Parkinson's Disease (PD), cases with Bipolar Disorder (BD), and cases with Chronic Traumatic Encephalopathy (CTE) versus normal controls. This was done to determine if parallel overexpression in certain genes may indicate the possible association at the level of gene expression. Identifying similar RNA sequence establishing gene expression may provide an insight to the relationship of the diseases in terms of pathobiological behavior. Determining the similar over- or underexpression pattern may provide an insight on the common pathobiologic mechanisms that may be the reason for the three disorders being associated by way of pre-morbid or co-morbid condition.

METHODOLOGY

Transcripts from the public domain archive of the NCBI SRA were identified for the RNA sequence (RNAseq) of interest using the search string "Chronic Traumatic Encephalopathy", "Bipolar Disorder", and "Parkinson's". Only public domain transcriptome files of post-mortem brain samples labeled as RNAseq data extracted thru the Illumina platform that have a paired normal control were selected. A total of ten (10) cases for each disorder and thirty (30) normal subjects for control in the NCBI SRA RNAseq database with a whole exome sequence file that was available for public domain use was utilized for differential gene expression analysis.⁶⁻⁸ Available transcriptome of post-mortem brain tissue from cases with CTE, cases with PD, cases with BD, and normal controls were analyzed separately. Analysis was done using the Galaxy platform⁹ to extract the RNAseq of the identified cases from the European Nucleotide Archive (ENA).¹⁰ The RNAseq were compared to the reference Homo sapiens (human) genome Genome Reference Consortium Human Build 38 (hg38).¹¹ Alignment of the RNAseq to the reference genome was done using HISAT2.¹²

The RNAseq was categorized per disease type with 10 PD, 10 CTE, 10 BD, and 30 normal subjects that were compiled separately to generate a table to compare the counted gene

expression per identified gene.^{13,14} Calculation of the log₂ fold change of all the identified genes for normalization were done to determine which genes were overexpressed or underexpressed with doubling of the original value equal to a log₂ fold change of 1 was done using R under RStudio with the assigned FDR adjusted p-value <0.05.^{15,16}

Comparison between the genes of patient groups with CTE, PD, and BD, with the cut off of overexpression assigned as ≥15 log₂ fold change across all patient categories, versus ≤0 log₂ fold change expression at FDR adjusted p-value <0.05 in normal controls identified the presence of overexpressed genes that significantly differed from normal controls. Comparison for gene underexpression in CTE, PD, and BD with normal controls with a difference of 1 log₂ fold change at FDR adjusted p-value <0.05 was also done. Calculation for statistically significant difference between the transcriptome of normal subjects versus CT, PD, and BD were done using Mann-Whitney U with a p-value of <0.01.

RESULTS AND DISCUSSION

Among 21,122 identified genes from the RNAseq, the analysis was able to identify 26 genes exhibiting increased expression of up to >15 log₂ fold change among cases with CTE, PD, and BD compared with normal controls. The genes have been observed to have an observable functional association based on worldwide gene co-expression database (Figure 1. Observed Co-expression of the Genes in Homo sapiens). It must be noted that, although not all genes have an outright direct connection in terms of their cellular processes, the network of overexpressed genes have a significantly greater interaction than expected (Figure 2. Network Interaction of the Genes in Homo sapiens). In contradistinction, only 6 well-described genes exhibited a decreased expression among cases with CTE and BD compared to normal controls. However, there were no identified genes that exhibited underexpression in cases with PD compared with normal controls.¹⁷

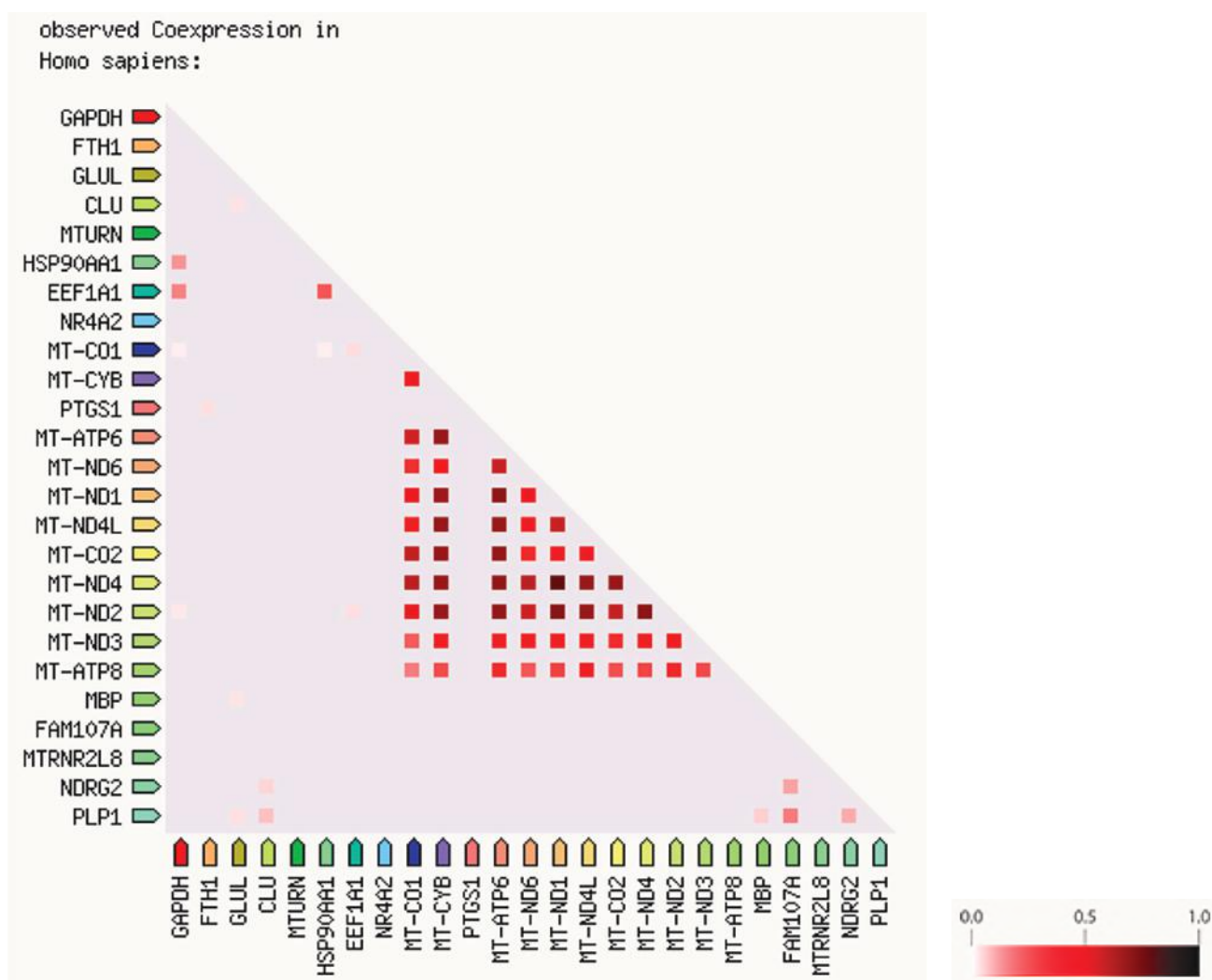


Figure 1. Co-expression predicts functional association: In the triangle matrix above, the intensity of color indicates the level of confidence that two proteins are functionally associated, given the overall expression data in the organism. *Generated using STRING v11.* <https://string-db.org/>

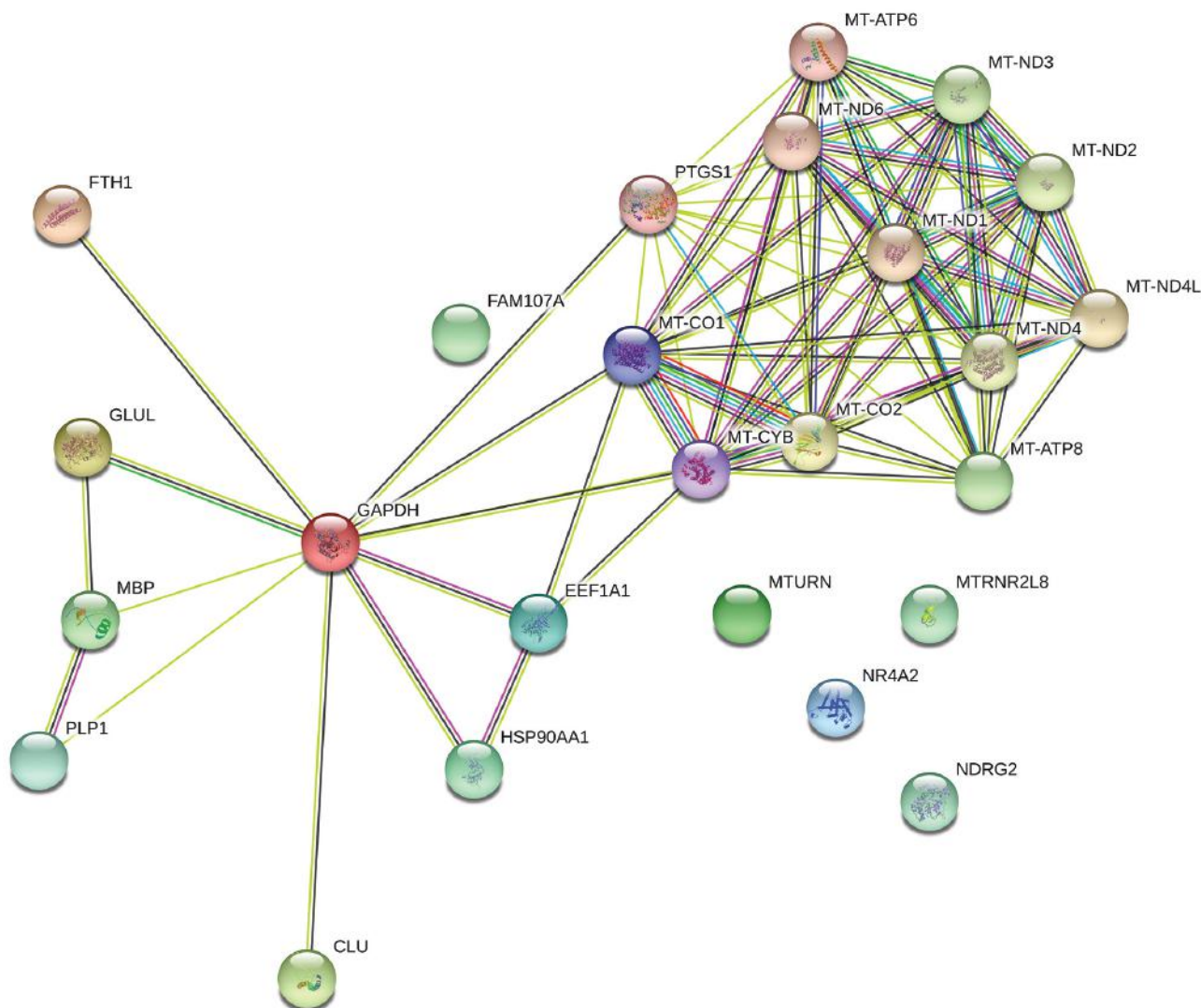


Figure 2. Each node (three-dimensional circle) represents the protein produced by a single, protein-coding gene locus identified in the study. The colored nodes indicate the query proteins which are products of the overexpressed genes. The filled nodes indicate that some of the protein structures are known or predicted. The connecting bars connote interactions that may be from predicted gene neighborhood, fusion, co-occurrence, co-expression, protein homology or textmining. *Generated using STRING v11. <https://string-db.org/>*

Among the genes with increased expression, FAM107A, or family with sequence similarity 107 member A, showed the most documented association with BD since high expression has been found in the dorsolateral prefrontal cortex of patients with BD and schizophrenia.¹⁸ The protein encoded by this gene has been responsible for the modulation of actin filamentous (F-actin) dynamics that contributes to synaptic and cognitive function at the level of the hippocampus.¹⁹

FTH1, or ferritin heavy chain 1, also has an increased expression, and this gene codes for the component of Ferritin protein. Ferritin functions as storage of iron in a soluble and nontoxic state. Previous studies have described an increase in Ferritin reactivity in the brains of PD patients.²⁰

As for the regulation of apoptosis and structural integrity, RNR2 has been noted to have an anti-apoptotic function,²¹ while GAPDH has been reported in previous studies as an apoptosis initiator.²²

CLU, or clusterin, has been described to encode for a protein that functions as a chaperone which is secreted in the cytosol under some stress conditions. Involvement has been described in processes such as cell death, tumor progression, and neurodegenerative disorders.²³

HSP90AA1, or heat shock protein 90 alpha family class A member 1, has a product that consists of an inducible molecular chaperone that aids in proper protein-folding by use of an ATPase activity.²⁴

Genes involved in neuronal development and growth regulation have also been identified in the overexpressed category, such as MTURN, NDRG2, GLUL, PLP1, RNR1/NR4A2, MBP, and EEF1A1.

MTURN, which stands for maturin, and known as neural progenitor differentiation regulator homolog, has been known to represses NF-kappa-B transcriptional activity and has been documented in early neuronal development.²⁵

NDRG2, from the NDRG family, was another overexpressed protein that was identified with neuronal growth involvement since the protein encoded by this gene is a cytoplasmic protein that may play a role in neurite outgrowth.²⁶

GLUL, or glutamate-ammonia ligase, codes for a protein that has a role in ammonia and glutamate detoxification, acid-base homeostasis, cell signaling, and cell proliferation.²⁷

PLP1, or proteolipid protein 1, encodes for a protein that has a function in ammonia and glutamate detoxification, acid-base homeostasis, cell signaling, and cell proliferation.²⁸

RNR1/NR4A2, or ribosomal 1/ nuclear receptor subfamily 4, group A, member 2, has been described as a regulator of insulin sensitivity and metabolic homeostasis.²⁹

MBP, or myelin basic protein has been documented to play a role in regulating blood-brain barrier function since antibodies specific to MBP have been shown to increase blood-brain barrier permeability.³⁰

EEF1A1, or eukaryotic translation elongation factor 1 alpha 1, is a subunit of a protein that has been described to enzymatically deliver aminoacyl tRNAs to the ribosome.³¹

In terms of cellular metabolism, three functioned as part of cytochrome c oxidase components (COX1/ PTGS1, COX2, COX3). However, it has been shown that the key enzyme produced by COX1/ PTGS1 play a role in neuroinflammatory processes and COX2 has been responsible for mediating neuroprotection;³² while an increase in COX3 has been shown to increase astrocytic aerobic metabolism.³³ Several other genes involved in oxidative phosphorylation and carbon handling have also been observed such as cytochrome c reductase complex (CYTB),³⁴ part of the mitochondrial membrane ATP synthase (ATP6, ATP8),³⁵ part of the NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6),³⁶ and part of the glycolytic pathway (GAPDH).³⁷

On the other hand, the underexpressed genes detected in the CTE and BD cases compared with normal controls included MIR3960, MIR149, UTF1, HSPA1A, IL10, and MYBL2. There is no distinct significant interaction between the group of genes compared with what can be seen in the normal population.

MIR3960 and MIR149 (microRNA 3960 and microRNA 149) have been known to be incorporated into RNA-induced silencing complex (RISC) and functioned as a translational inhibitor or destabilizer of the target mRNA.^{38,39}

UTF1, or undifferentiated embryonic cell transcription factor, although mostly reported in pluripotent cells, was described as encoding a transcriptional repressor protein.⁴⁰

HSPA1A, or heat shock protein family A (Hsp70) member 1A, has been noted to prevent existing proteins from

aggregation by stabilization and assist in protein folding in the cytosol and organelles.⁴¹

IL10, or interleukin 10, was characterized as encoding a cytokine that down-regulates Th1 cytokine and MHC class II antigen expression, while enhancing B cell survival, proliferation, and antibody production. It has been described to block NF-kappa B activity and has a co-stimulatory function on macrophages.⁴²

MYBL2, or MYB proto-oncogene like 2, has been shown to encode for a protein with an activator function for G2/M phase proteins such as Cyclin B1, CDK1, and Cyclin A2.⁴³

All of the overexpressed and underexpressed genes observed in the CTE and BD cases had a significantly increased and decreased expression, respectively, in comparison with the normal subjects ($p < 0.01$). In contrast, only the overexpressed genes in PD have shown a significant difference with normal controls ($p < 0.01$), and the genes that showed an increase in expression in the normal controls, as compared with the CTE and BD cases with a log₂ fold change of 0, were also increased in the cases with PD.

The identification of parallel gene overexpression among the CTE, BD, and PD groups with respect to structural integrity, cellular metabolism, homeostasis, and apoptosis may indicate a common pathway that have been initiated as part of the response to maintain tissue function or as a consequence of the underlying pathobiologic mechanism that caused the primary lesion.

The presence of FAM107A among all the groups may imply the presence of related pathways among CTE, BD, and PD that have an impact to the regulation of synaptic function that are responsible for learning and long-term memory. This may also have a role on the other cognitive function that have a connection to the hippocampus.

On the other hand, the increased expression of the ferritin heavy chain 1 gene among the three groups may indicate the attempt of the brain to optimize neuroprotection and prevent oxidative damage since the presence of free iron may contribute to the increase in reactive oxygen species thru redox reactions and lead to the increased burden of cellular oxidative stress that may cause cell death.⁴⁴

The presence of genes that are associated with mitochondrial function with respect to aerobic metabolism may indicate an increase in the general metabolic activity of the brain as a response to cellular injury and an attempt to facilitate repair. This is likewise seen in the context of an increased COX1/ PTGS1 and COX2 expression, which may be interpreted as a response to an inflammatory process and the need to increase the metabolic output to generate energy for the maintenance or restoration of homeostasis.

The similarities in structural integrity dysfunction that may be interpreted as a response to inflammation can be illustrated by the increase in MBP expression and such increase may indicate a response to a pathologic process that threatens the relative impermeability of the blood-brain barrier. The remaining genes such as CLU,

HSP90AA1, MTURN, NDRG2, GLUL, and RNRI, may all have been increased to enable the production of proteins that ensure the maintenance of the tissue structure despite the injury. This may be further supported by the increase in EEF1A1, which plays a role in amino acid formation.

The presence of an apoptosis initiator in the form of GAPDH may indicate a selective facilitation of structures that require the cessation of processes due to an injured state. As such, the apoptosis may prevent further worsening of tissue inflammation by disrupting improper cellular functions that may lead to necrosis.

In comparison, the underexpressed genes detected in the CTE and BD cases compared to the normal controls and the PD cases may indicate the lack of genes that have a role in repressing the mRNA for protein coding such as MIR3960, MIR 149, AND UTF1. Interestingly, there is a decreased expression of an aggregation inhibitor gene, HSPA1A, which may have a role in preventing abnormal protein folding. The absence of this gene may be related to accumulation of abnormal protein structures that cause cellular destabilization and subsequent inflammation.

In terms of IL10 expression, mild CTE cases have been documented to have an increase in IL-10 cytokine.⁴⁵ However, murine studies have shown that the IL10^{-/-} have higher superoxide production, are more susceptible to excitotoxicity, and neurotoxicity due to metabolic and hypoxic processes.^{46,47} Although there are contradictory statements regarding the role of IL-10 in BD, the decreased expression of the IL10 gene may explain the imbalance in the pro-inflammatory and the anti-inflammatory cytokines that are associated with the adverse effects of neuroinflammation. Furthermore, a study indicating the presence of IL-10 cytokine only in the early stage of BD with subsequent loss of IL-10 during the later stages of BD after 10 or more years with the disease may explain the unmitigated neuroinflammation that is associated with BD brain lesions.⁴⁸

On the other hand, MYBL2, or MYB proto-oncogene like 2, has been described in human gliomas as being associated with poorer prognosis.⁴⁹ The underexpression of MYBL2 compared to controls may indicate the absence of uncontrolled astrocytic proliferation driver in the setting of tissue injury.

CONCLUSION

The overexpression of genes responsible for homeostasis, regulation of inflammation, balance of apoptosis and anti-apoptosis, and maintenance of structural integrity among the CTE, BD, and PD groups indicate that there is an interrelated mechanism that serves converging pathways as part of the response to lesion-forming structures in the brain. Likewise, the underexpression of certain genes may indicate the lack of significant modifying proteins that may promote the silencing of genes that may contribute to the CNS pathology or increase modulating substances that can serve to mitigate the severity of the lesions. The role of studies such as this serves the primary purpose of paving the way for future large-scale studies that would correlate the clinical behavior of complex brain disorders with the

histopathologic features and the gene expression profile. Although the impact of the research may be limited by the number of post-mortem brain samples, the analysis may provide an initial point of evaluation for further studies in comparative analysis. The goal of studies such as this is to have a better understanding on the common pathways that explain the interrelatedness of brain disorders and the putative mechanism for being co-morbid and pre-morbid conditions of each other. Furthermore, the study posits the challenge that attenuating the pre-morbid mechanisms that trigger the cascade of neuroinflammatory response may provide a means to delay the neural lesions that are accumulated in the late stages of the disorders.

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Calcifying Fibrous Tumor of the Jejunum in a 27-year-old Primigravid: A Case Report*

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ABSTRACT

The most common mesenchymal tumors of the gastrointestinal tract are gastrointestinal stromal tumors (GIST) and smooth muscle neoplasms; however, other soft tissue tumors may also present in the intestines and cause diagnostic dilemmas. We report the case of a 27-year old primigravid, with no known complications, who underwent cesarean section for cephalopelvic disproportion. Intraoperatively, a well-demarcated, solid mass measuring 1.5 x 1.0 x 0.7 cm was noted at the jejunum. The patient underwent segmental resection of the mass. Microscopic examination of the mass reveals a non-encapsulated, solid mass composed of bland spindle cells and dense, hyalinized collagen in whorls and bundles. Dystrophic calcifications and a lymphoplasmacytic inflammatory infiltrate are seen within the collagen bundles. Immunohistochemical staining with desmin, CD117, and DOG-1 was done, which are all negative. The case was signed out as calcifying fibrous tumor (CFT). Inclusion of CFTs in the differential diagnoses for mesenchymal tumors of the gastrointestinal tract is important, as these neoplasms are benign and have an excellent prognosis.

Key words: Calcifying fibrous tumor, jejunum, neoplasms, fibrous tissue

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INTRODUCTION

Mesenchymal tumors, both benign and malignant, can arise at virtually any site. In the digestive system, benign soft tissue neoplasms are more common than soft tissue sarcomas, with an incidence of at least 100-fold higher than the latter.^{1,2} Common benign mesenchymal tumors of the gastrointestinal tract include small, clinically occult gastrointestinal stromal tumors (GIST), lipomas, leiomyomas, vascular lesions, nerve sheath tumors, and tumors of myofibroblastic origin.¹

A majority of these mesenchymal tumors arise spontaneously; however, a definitive etiology can be readily established in some cases, including viruses (e.g. Kaposi sarcoma), prior surgeries (e.g. angiosarcoma), genetic defects (e.g. desmoid fibromatosis), or as part of inherited syndromes (e.g. chondroid and vascular tumors in Maffucci syndrome). Somatic genetic mutations may also play a role in the pathogenesis of mesenchymal neoplasms, but the exact mechanisms of such molecular events remain to be elucidated.¹

Clinically, mesenchymal tumors typically present as painless masses. Unless they arise in the trunk and extremities, these neoplasms may go unnoticed for a long period of time. Occasionally, patients with intra-abdominal soft tissue masses may experience non-specific symptoms, such as abdominal discomfort or pain. The unremarkable presentation of these lesions may suggest a benign course; nevertheless, deep-seated lesions should be regarded as probably malignant and must be evaluated accordingly.²



One such tumor that is clinically benign but may raise suspicion for malignancy is calcifying fibrous tumor (CFT), a low-grade, soft tissue neoplasm that can arise anywhere within the gastrointestinal tract. CFT is a well-demarcated, hypocellular tumor characterized by the presence of hyalinized collagen, dystrophic calcifications and focal inflammatory infiltrates. It is usually encountered as an incidental finding in patients undergoing endoscopy or colonoscopy. In some cases, CFTs may be discovered in open abdominal procedures. Recognizing CFTs is important because surgery is curative and its prognosis is much better than other soft tissue tumors of the gastrointestinal tract.³

This paper reports the incidental finding of a jejunal CFT in a 27-year old primigravid who underwent cesarean section.

CASE

This is the case of a 27-year-old primigravid who was admitted at the labor room of a private hospital because of regular uterine contractions. The patient carried her pregnancy to term and had an unremarkable obstetric and maternal history. Due to dystocia secondary to cephalopelvic disproportion and premature rupture of membranes, the patient was scheduled for an elective cesarean section procedure.

Intraoperatively, an ovoid, firm, solid nodule measuring 1.5 x 1.0 x 0.7 cm was palpated incidentally at the jejunum. Located at the antimesenteric side, the mass does not appear to cause any degree of luminal obstruction. After the delivery of a live baby boy, the patient underwent segmental resection of the jejunum and the specimen was submitted for routine histopathologic examination.

Gross examination of the mass revealed an intramural, unencapsulated, cream white, solid mass that appeared to be centered at the muscularis propria on cut sections. Further sections of the mass revealed no hemorrhage or soft areas that might suggest necrosis. Occasionally, the mass was gritty to cut.

The case was signed out as a spindle cell neoplasm, with considerations of: 1) gastrointestinal stromal tumor and 2) leiomyoma. The case was referred to our institution for review.

MICROSCOPIC DESCRIPTION

There is a well-circumscribed, unencapsulated, paucicellular lesion located within the muscularis propria. The tumor distends the serosa and does not invade the mucosa and submucosa (Figure 1). Scanning the lesion reveals dense hyalinized collagenous tissues arranged in bundles and whorls, with scattered foci of dystrophic calcification (Figure 2). High-power views of the lesion reveal bland spindle cells with scant cytoplasm and occasional, scattered lymphoplasmacytic infiltrates (Figure 3). No prominent blood vessels are seen within the lesion. Myxoid changes are present in some areas; however, there is no necrosis, mitoses, atypia, and pleomorphism.

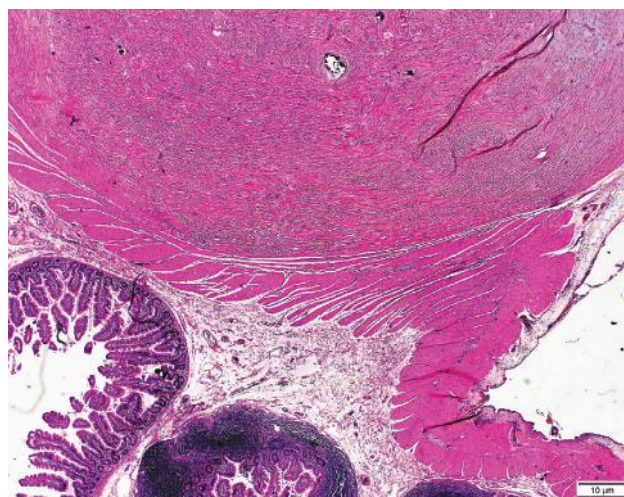


Figure 1. A well-demarcated, unencapsulated, hypocellular mass, composed of thick bundles of collagen and with scattered dystrophic calcifications, is seen distending the intestinal wall (H & E, 40X).

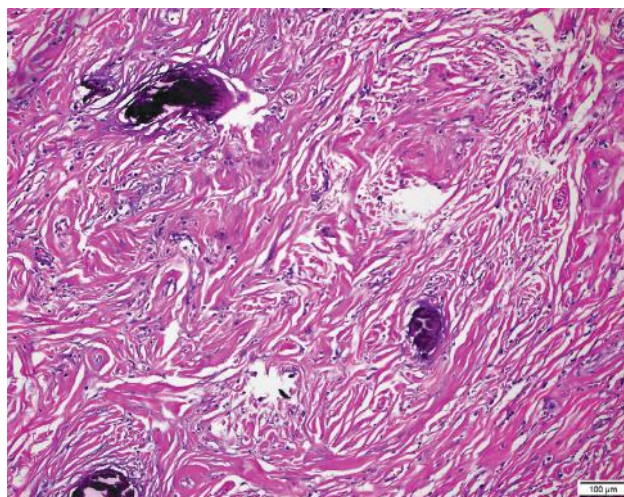


Figure 2. Dystrophic calcifications are a characteristic feature of the lesion (H & E, 100X).

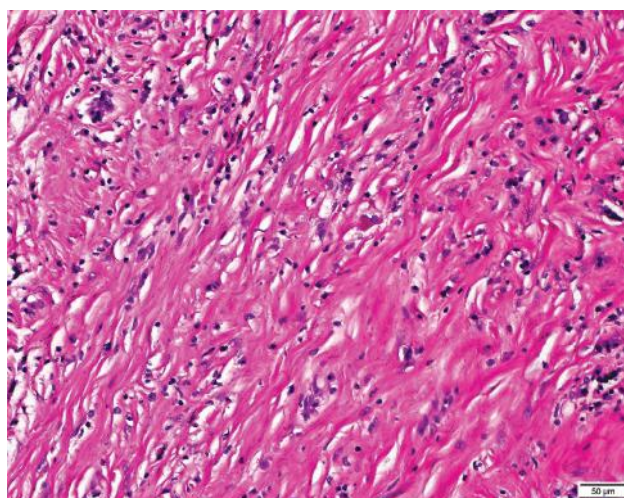


Figure 3. Hyalinized collagen are arranged in bundles, with bland, spindle cells and infiltrating inflammatory cells, composed mostly of lymphocytes and plasma cells (H & E, 400X).

Immunohistochemical stains for desmin, CD117, and DOG-1 were done (Figure 4). Both DOG-1 and desmin are negative in the spindle cells. The spindle cells are also negative for CD117, with only sporadic mast cells taking up the stain; thus, the initial considerations of GIST and leiomyoma are ruled out.

Given these microscopic findings, in correlation with the patient's unremarkable clinical and obstetric history, the case was signed out as calcifying fibrous tumor (CFT) of the jejunum.

DISCUSSION

The vast majority of soft tissue tumors in the digestive system (95%) are either GISTs or tumors derived from smooth muscle, i.e. leiomyoma or leiomyosarcoma. The usual dilemma presented by the more uncommon mesenchymal neoplasms is that they may be easily confused with GIST.⁴ Calcifying fibrous tumor (CFT) is one of the other tumor types that may be encountered in the gastrointestinal tract, albeit occurring at a lower incidence. It has a predilection for children and young adults, with a mean age of 34 years for patients with abdominal CFTs.^{5,6}

Also known by the term "childhood fibrous tumor with psammoma bodies" and "calcifying fibrous pseudotumor," CFTs were initially considered to represent a reactive process secondary to abnormal healing. In the consensus classification document by the World Health Organization in 2002, this entity was given the name calcifying fibrous tumor. CFTs have been reported in various anatomic sites, including the peritoneum, mediastinum, lungs, adrenal glands, etc.⁴ In the review by Chorti et al., less than 50 CFTs occurring in the gastrointestinal tract have been reported in the English literature, and are more common in the stomach than in the intestines.⁷

Clinically, most CFTs of the gastrointestinal tract are incidental findings, as seen in this case. If symptoms are present, they are usually non-specific, such as abdominal discomfort and bowel obstruction. CFTs may also result in complications that require more aggressive surgical management, e.g. ileocolic intussusception.^{7,8} Radiologic studies are non-specific and may reveal a well-defined lesion with microcalcifications on both ultrasound and CT scan.^{6,7}

Pathologic examination remains the most essential tool in the diagnosis of CFT. Grossly, CFTs present as a solitary, well-circumscribed, unencapsulated mass that has a wide range of sizes (up to 25 cm in some case reports). In the gastrointestinal tract, CFTs are reported to arise in the submucosal, intramural, or subserosal layers. Microscopic features include the presence of myofibroblastic spindle cells; paucicellular, hyalinized collagen in lamellar bundles and whorls; psammomatous or dystrophic calcifications; and mononuclear inflammatory infiltrates, which include lymphocytes, plasma cells, mast cells, and polymorphonuclear leukocytes.⁷

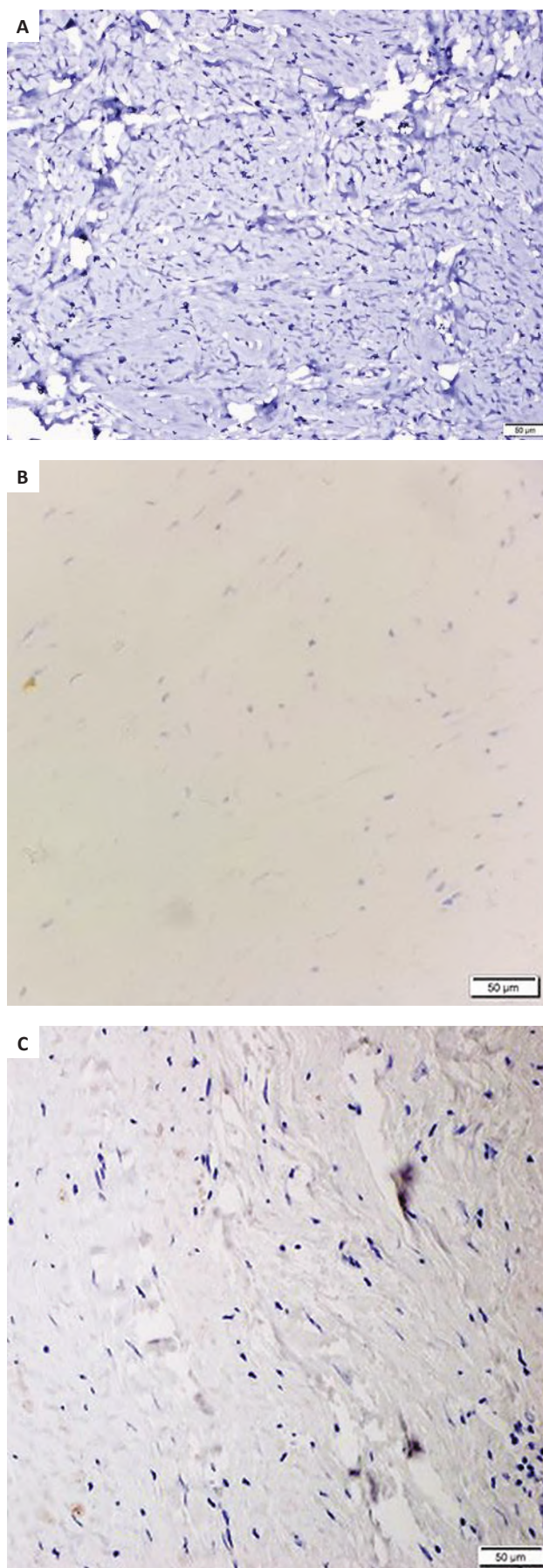


Figure 4. The lesion is negative for DOG-1 (A), Desmin (B), and CD117 (C) (IHC, 100X).

The differential diagnoses for CFT include GISTs, leiomyoma, and other soft tissue tumors such as schwannomas and desmoid tumors. Due to its highly collagenized nature, other entities that may also be considered include inflammatory myofibroblastic tumor, desmoid fibromatosis, and solitary fibrous tumor. In particular, inflammatory myofibroblastic tumor (IMT) shares several histopathologic features with CFT, including bland spindle cells, hyalinized stroma, whorling architecture, fibrosis, calcifications, and an inflammatory infiltrate. However, unlike CFT, there may be some moderate pleomorphism in IMT, with myoid cells showing prominent nucleoli. The spindle cells in IMT are also plump, in contrast to the spindle cells of CFT which have scant, pale cytoplasm. IMT also has a more prominent lymphoplasmacytic infiltrate, and appears much more cellular than CFT. One study hypothesized that CFT might be the late regressive stage of IMT due to the similar histologic features and overlapping methylation patterns of the two entities; however, ALK rearrangement, which is a hallmark of IMT, is not seen in CFT.⁹ Differentiating CFT from IMT is important because the former is benign, while the latter can persist locally, invade adjacent structures and, rarely, metastasize.¹⁰

Desmoid fibromatosis, another tumor that presents with zones of hyalinization and a myxoid background, must also be ruled out. This lesion arises more commonly in the mesentery, and is characterized microscopically by the presence of long sweeping fascicles of slender spindle or stellate cells and prominent, small vessels. Unlike CFT, desmoid fibromatosis is an infiltrative lesion that has a minimal inflammatory component. On immunohistochemistry, it is positive for actin, and at least 80% of tumors present with nuclear staining of β -catenin.^{1,10} Desmoid fibromatosis is known to recur, and infrequently may undergo spontaneous regression.¹

Solitary fibrous tumor (SFT), which is more common in adults, may present with striking hyalinization of its stroma and some degree of myxoid change, therefore mimicking CFT. However, SFTs have prominent thin-walled, dilated, and branching staghorn-shaped blood vessels, which may also present with perivascular hyalinization. CFTs, as demonstrated in this case, do not present with a prominent vascular network.¹

Therefore, routine evaluation of microscopic features on hematoxylin and eosin (H and E) slides may suffice to establish the diagnosis of CFT; however, immunochemical stains may prove helpful in resolving some cases. CFTs express vimentin, Factor XIIIa, and occasionally, CD34 and CD68. These tumors are negative for other commonly used markers such as CD117, desmin, S100, ALK-1, CD31, CD99, Bcl-2, β -catenin, and STAT6.⁷

Local excision is curative for CFTs, and the prognosis is excellent. CFTs do not have any metastatic potential, and, specifically for CFTs of the gastrointestinal tract, no cases of recurrence have been reported.^{4,7}

CONCLUSION

Calcifying fibrous tumors are solitary, non-encapsulated, well-demarcated masses that are characterized microscopically by bundles of hyalinized collagen with microcalcifications and lymphoplasmacytic infiltrates. Although rare, they should be considered in the differential diagnoses of gastrointestinal masses, as the surgical management is more conservative and the prognosis is excellent.

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Double Trouble: Establishing Synchronous Primary Tumors of the Urothelium and Prostate by Immunohistomorphology: A Report of Two Cases

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ABSTRACT

Synchronous primary tumors of the urothelium and prostate are a diagnostic challenge among pathologists. Differentiating carcinomas of urothelial and prostatic origin requires careful assessment of histomorphology coupled with ancillary studies such as immunohistochemistry stains (IHC) to support the diagnosis. We report two cases of adult patients who underwent transurethral resection of the prostate (TURP), with two distinct morphologies noted on routine H&E sections. After a panel of immunohistochemical stains (HMWCK, CK5/6, CK7, CK20, GATA-3, p63, NKX3.1, and PSA), both cases were signed out as papillary urothelial carcinoma and prostatic acinar adenocarcinoma. Correlation of histomorphology with an IHC panel consisting of cytokeratins (CK5/6, CK7, CK20), a urothelial marker (GATA-3), and at least two prostatic markers (PSA, NKX3.1) is recommended in such cases.

Key words: Immunohistochemistry, PSA, prostatic adenocarcinoma, urothelial carcinoma

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INTRODUCTION

Prostate cancer is the fifth most common malignancy and the seventh leading cause of death in the Philippines. Bladder cancer, meanwhile, has an incidence of 3.1 per 100,000 males in 2018.^{1,2} The epidemiology of a double primary lower genital tract malignancy is not uncommon. A retrospective analysis of a single center study of radical cystectomy specimens showed a 53.1% incidence of prostate cancer. At least half had a Gleason score of 7 and above with at least pT3a on tumor staging.³ In another study, a review of radical cysto-prostatectomy cases showed 45.7% incidence of prostatic adenocarcinoma in specimens with urothelial carcinoma, all of which with pN0.⁴ However, the reporting of double primary malignancies with at least an aggressive prostatic carcinoma is infrequent with only a number of published case reports abroad.^{5,6} After an exhaustive search on local literature (HERDIN plus and Philippine e-journals), no local published cases are available. Diagnosis of double primary prostatic and urothelial carcinomas pose a diagnostic challenge to pathologists because of likely morphologic overlap between the two tumors. The management differs for urothelial carcinomas and prostatic adenocarcinoma with different risk-stratified treatment algorithms.^{7,8} In such cases, additional tests such as IHC may be needed to document the presence of two primary tumors. We report two cases of double primary urothelial and prostatic carcinoma to give insights on the approach to their diagnosis and to address paucity of local data.

CASE 1

A 61-year-old male who came in for regular screening at another institution, had an elevated PSA (> 40 ng/mL). Ultrasound showed an intraluminal lobulated focus at the urinary bladder that is adherent to the bladder base, and an enlarged prostate with homogeneous



echopattern. Impression at this time was between a primary bladder mass and an intravesical extension of a primary prostatic mass. CT scan showed an additional finding of enlarged iliac nodes. The patient underwent TURP. Microscopic examination showed two distinct patterns: one consists of papillary fronds in an inverted growth pattern, lined by cells with enlarged, hyperchromatic nuclei; and the other consists of cribriform glands lined by cells with vesicular nuclei and prominent nucleoli. The tumor cells of the former stained positive for HMWCK, CK7, GATA-3, and p63, while the those of the latter stained positive for PSA and NKX3.1 (Figures 1 and 2).

This case was signed out as low-grade papillary urothelial carcinoma, and prostatic acinar adenocarcinoma (4+4), WHO-ISUP grade group 4.

CASE 2

A 71-year-old male with obstructive uropathy and elevated PSA (> 100 ng/mL) was diagnosed with low-grade papillary urothelial carcinoma after TURP and transurethral resection of bladder tumor (TURBT) at another institution. He presented a month after with recurrence of difficulty in voiding. Imaging showed a

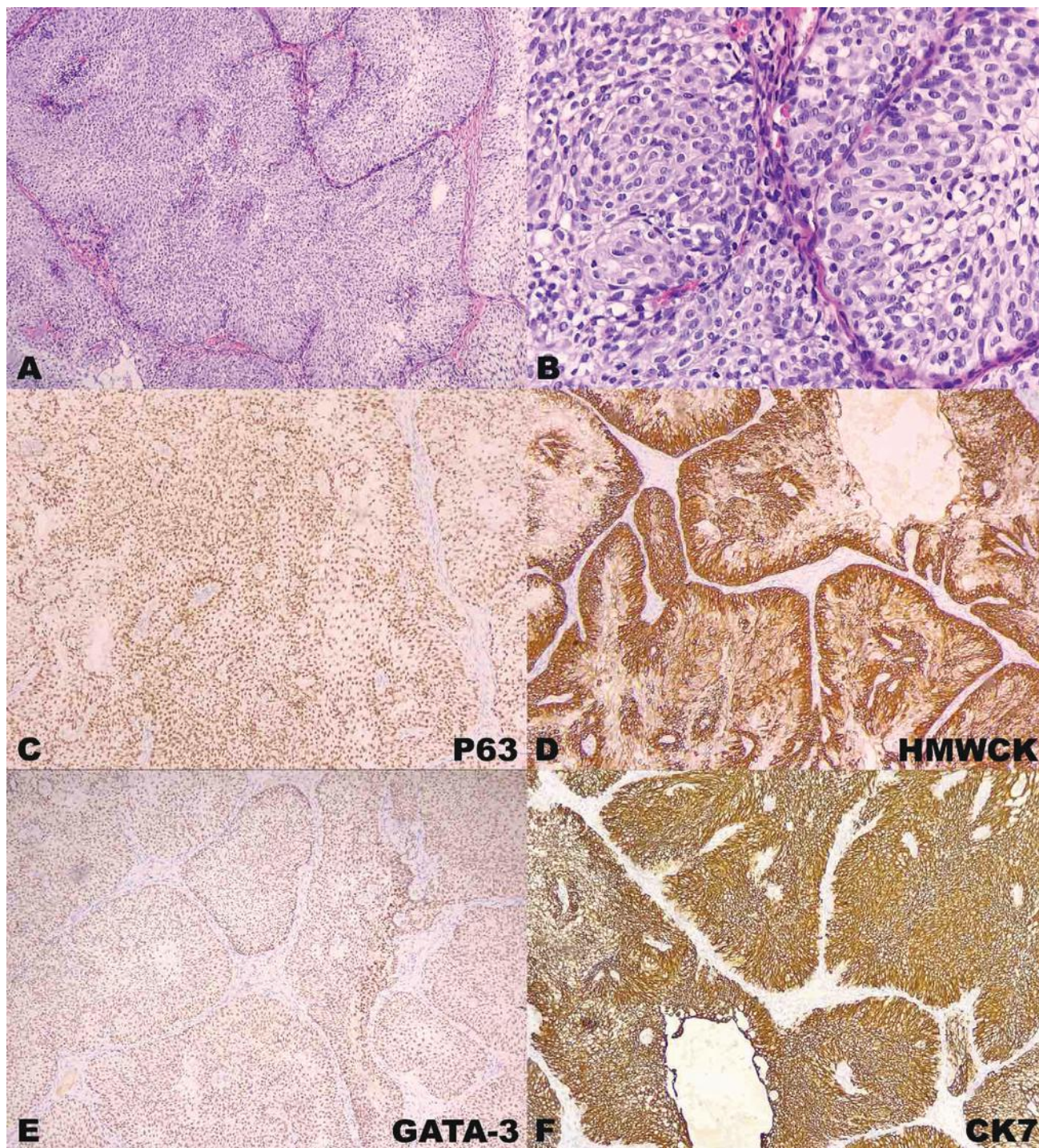


Figure 1. Case 1 – (A) One group of tumor cells show an inverted papillary architecture (H&E, 100X); **(B)** it is composed of cells with enlarged and hyperchromatic nuclei (H&E, 400X); **(C to F)** the IHCs show positivity for p63, HMWCK, GATA-3 and CK7 (100X).

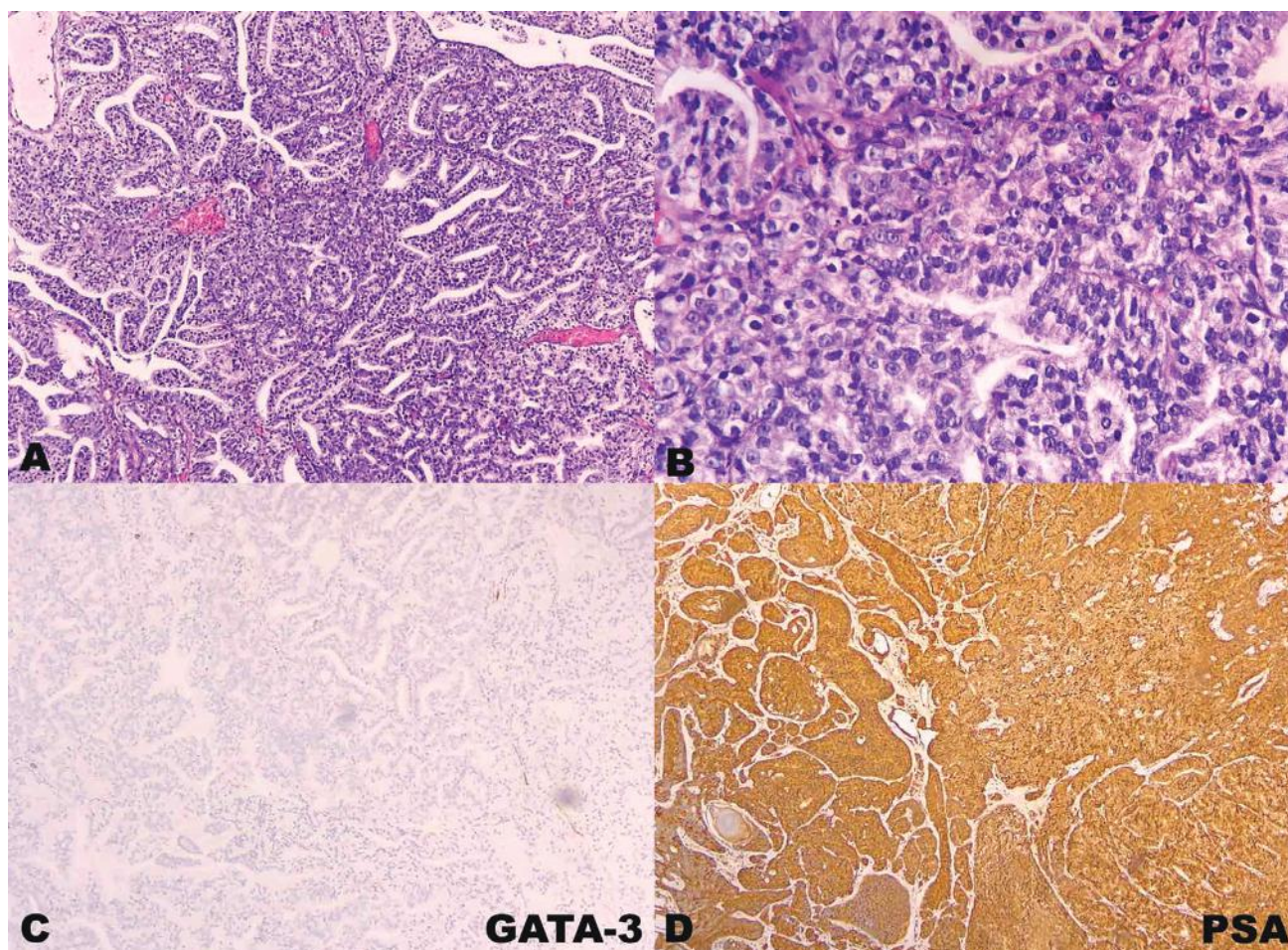


Figure 2. Case 1 – (A) The second group of cells shows a cribriform architecture (H&E, 100X); (B) the tumor cells have vesicular nuclei and prominent nucleoli (H&E, 400X). (C) IHCs stained negative for GATA-3 (100X); (D) but they were positive for PSA (100X) and NKX3.1 (not shown).

bladder mass and diffuse cystitis. He underwent repeat TURP and TURBT. Microscopic examination showed two distinct patterns: one group consists of papillary fronds that demonstrate marked architectural disarray, and are lined by cells that exhibit marked nuclear atypia; and the other group consists of nests and sheets of cells with hyperchromatic to vesicular nuclei with prominent nucleoli. IHC stains were done on the former group of tumor cells which showed diffuse positivity for GATA-3, focal positivity for CK 5/6 but negative for PSA and NKX3.1 (Figures 3 and 4). This case was signed out as high-grade papillary urothelial carcinoma, and prostatic acinar adenocarcinoma (5+4), WHO-ISUP grade group 5.

DISCUSSION

The WHO classification of urinary tract tumors distinguishes infiltrating and non-invasive urothelial carcinomas. Infiltrating urothelial carcinomas are usually high-grade, and exhibit varied morphology. Non-invasive urothelial carcinomas are graded based on degree of architectural disarray and nuclear atypia, and frequency of mitosis. Diagnosis of prostatic acinar adenocarcinoma depends on a constellation of features, which include glandular luminal contents, cytoplasmic appearance, and nuclear features with prominent nucleoli are the most widely recognized characteristics.⁹

The difference in architecture and cytomorphology allows distinction between non-invasive urothelial carcinomas and prostatic acinar adenocarcinomas in cases of their co-existence, such is what the two cases demonstrated. Non-invasive papillary urothelial carcinomas are evaluated based on order of architectural and cytological features on both low (100X) and medium-power magnification (200X). Heterogeneity of the histological features are typical of papillary urothelial carcinomas. They usually form papillary fronds with fibrovascular cores and exhibit moderate to marked pleomorphism with glassy, eosinophilic cytoplasm and enlarged nuclei.^{9,10} On the other hand, prostatic acinar adenocarcinomas do not have a single pathognomonic feature. Major and minor criteria have been developed to aid the pathologist in assessment of prostatic specimens.¹¹ Prostatic acinar adenocarcinomas usually exhibit infiltrative small or cribriform glands and the absence of basal cell layer. Though not present in all acinar adenocarcinomas, other helpful features include intraluminal wispy blue mucin, pink amorphous secretions, intraluminal crystalloids and adjacent high-grade prostatic intraepithelial neoplasia. Cytological features include absent to minimal pleomorphism with pale to foamy cytoplasm and enlarged nuclei with prominent nucleoli. Mitotic figures may or may not be present.⁹⁻¹¹

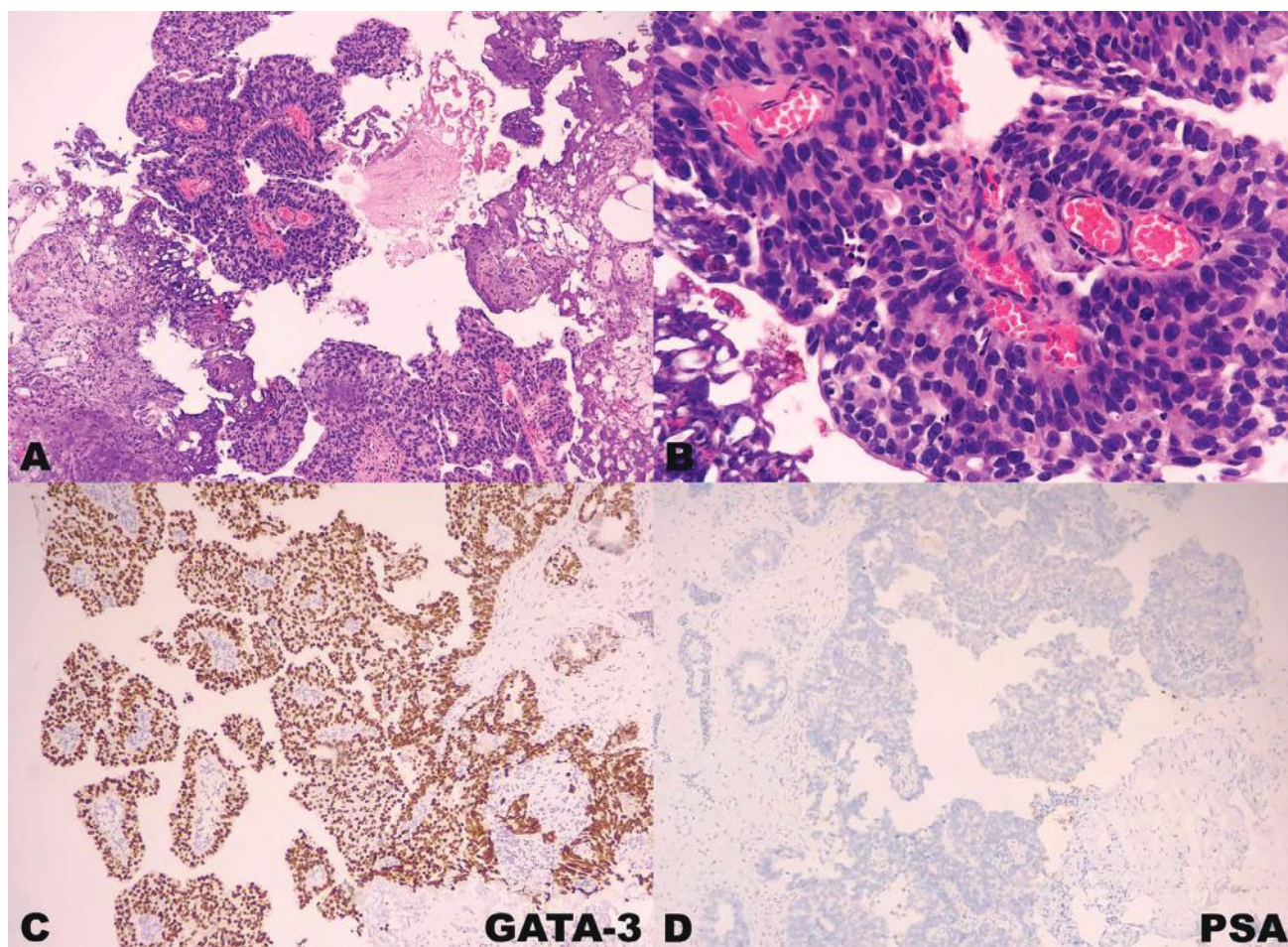


Figure 3. Case 2 – (A) One group of tumor cells exhibits papillary fronds with markedly distorted architecture (H&E, 100X); **(B)** the tumor cells have pleomorphic and hyperchromatic nuclei (H&E, 400X). **(C)** IHCs show positive cytoplasmic staining for GATA-3 (100X); **(D)** but negative for PSA (100X) and NKX3.1 (*not shown*).

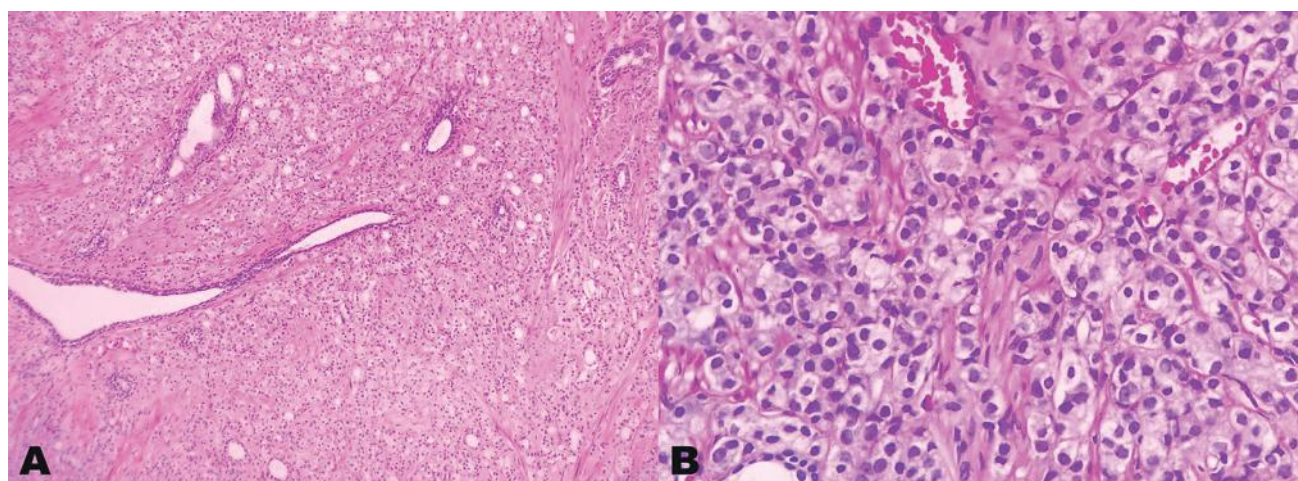


Figure 4. Case 2 – (A) Another group of tumor cells exhibits sheets, some forming acini (H&E, 100X); **(B)** they have rounded, hyperchromatic nuclei and prominent nucleoli (H&E, 400X).

The morphologies of the two tumors in both cases are distinct; however, one must exercise caution in signing out double primary tumors by H&E alone. In cases of morphologic overlap between the two tumors such as morphologic variants of infiltrating urothelial carcinomas, poorly-differentiated (Gleason score of ≥ 8) acinar adenocarcinomas and pseudopapillary features of

prostatic acinar adenocarcinomas mimicking urothelial carcinoma, IHC becomes crucial in documenting the presence of two tumors.

Urothelial and prostatic carcinomas have different classic immunophenotypes. By convention, urothelial carcinomas express cytokeratins (HMWCK, CK5/6,

CK7, CK20), and the opposite is true for prostatic carcinomas^{9,14}. However, aberrant expression of these markers may complicate diagnosis. There are some cases of prostatic carcinomas that stain positive for CK5/6, CK7, and CK20. Some poorly differentiated prostatic adenocarcinomas have overlapping CK7/CK20 profiles with urothelial carcinomas.¹⁵

To circumvent this problem, it is prudent to employ additional IHCs to distinguish between the two tumors. Suggested IHCs for urothelial carcinoma include the following: p63, thrombomodulin, and GATA-3.⁹ The most widely used among these urothelial markers is GATA-3, as it is widely available. Newer immunostains, such as uroplakin II, have shown high specificity (100%) but suffers from low sensitivity (65.6%), and it is still not widely available in local institutions.¹⁶ GATA-3 remains superior in terms of sensitivity (84.8%) when compared against other urothelial markers such as p63 (73.9%), CK34βE12 (75.4%) and thrombomodulin (45.7%) despite having comparable specificities (96.4-100%).¹⁷ Suggested IHCs for prostatic carcinomas include PSA, PSAP, NKX3.1, P501S, PSMA, and AR. The most established among the said immunostains is PSA, because of its high sensitivity (100%) and specificity (90.6%).¹⁷ However, problems encountered with PSA include decreased staining among poorly differentiated prostatic adenocarcinomas and nonspecific background staining. NKX3.1 has become more popular due to its high sensitivity (88.3%) and specificity (100%) as a prostatic marker. It is now being recommended as a prostatic marker of choice in some institutions.^{15,17}

Cytokeratin stains alone must not be interpreted independently since it is not sufficient in establishing the origin of both tumors. Though there is a caveat of aberrant expression, cytokeratin can still be used as part of a panel to document the tumor immunoprofile. In our monthly evaluation of IHC diagnostic utility, the PSA antibody has been a reliable marker for prostatic tumors while GATA-3 antibody has been widely used in bladder, prostate, pleural and peritoneal fluid aspirates. To give more credence to the diagnosis of a prostatic tumor, especially those with higher Gleason scores, a combination of two prostatic markers is needed. Hence, a panel approach is beneficial in documenting a double primary prostate and urothelial carcinoma with a cytokeratin, at least two prostatic markers (NKX3.1 and PSA) and a urothelial marker (GATA-3).

CONCLUSION

Correlation of histomorphologic and immunohistochemistry studies is crucial to the diagnosis of suspected double primary urothelial and prostatic carcinomas because management of the two tumors is completely different. Aberrant expression must be kept in mind when requesting for IHC stains, particularly in cytokeratin and PSA. An IHC panel with a cytokeratin (CK5/6, CK7, CK20) may still be performed with at least two prostatic markers (PSA, NKX3.1), and a urothelial marker (GATA-3) to demonstrate the presence of two primary tumors.

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ETHICAL CONSIDERATION

All attempts were made to obtain the consent of the patients; however, they were both lost to follow-up. This case report was written in accordance to the principles based on the Declaration of Helsinki.

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Acquired Platelet Dysfunction with Eosinophilia

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Table 1. Laboratory work-up done during initial consult and follow-up after two months

Parameter	Reference Range	Units	Initial Consult	Follow-up
Complete Blood Count				
RBC Count	4.00 - 5.20	x10 ⁶ /L	4.54	4.78
Hemoglobin	11.5 - 15.5	g/dL	12.0	12.4
Hematocrit	35 - 45	%	34.9	36.6
MCV	75 - 87	fL	76.9	76.6
MCH	25 - 33	pg	24.6	25.9
MCHC	32 - 36	%	34.4	33.9
RDW	11.5 - 15.0	%	13.8	12.8
Platelet Count	150 - 450	x10 ⁹ /L	179	338
PDW	9.5 - 16.0	%	13.3	9.0
MPV	6.5 - 10.0	fL	10.2	8.2
WBC Count	5.0 - 14.5	x10 ⁹ /L	8.46	8.46
Neutrophil	32 - 54	%	41.0	55.0
Lymphocyte	28 - 48	%	16.5	28.0
Monocyte	3 - 6	%	4.5	10.0
Eosinophil	0 - 3	%	36.0	6.0
Basophil	0 - 1	%	1.5	1.0
Absolute Eosinophil Count	0 - 0.6	x10 ⁹ /L	3.05	0.33
Coagulation Tests				
PT	8.7 - 11.5	sec	10.2	not done
APTT	31.8 - 43.7	sec	35.6	not done
Platelet Function Tests				
Bleeding Time (Ivy Method)	2 - 8	min	6.5	not done
Platelet Closure Time (PFA-100)				
Col/Epi	82 - 150	sec	>287	104
Col/ADP	62 - 100	sec	115	70

INTRODUCTION

A six-year-old male was brought in with 1-month history of recurrent spontaneous bruising which resolves without intervention. There was no history of trauma, other bleeding episodes, medication intake, nor recent viral infection. Birth, past medical, and family histories were unremarkable. Pertinent physical examination showed multiple, non-tender ecchymosis of varying chronicity and sizes on his upper and lower extremities and abdomen. The rest of the examination was essentially normal.

Initial laboratory work-up with complete blood count, prothrombin time, activated partial thromboplastin time, and bleeding time (Ivy Method) was done (Table 1). Peripheral blood smear examination showed eosinophilia and adequate qualitative platelet count. However, the platelet morphology shows numerous agranular and hypogranular grey platelets (Figure 1). Based on the history, physical examination, and initial work-up, acquired platelet dysfunction with eosinophilia (APDE) was suspected. Additional work-up with platelet closure time using Platelet Function Analyzer-100 (PFA-100) was done (Table 1) which provides evidence of platelet dysfunction. No intestinal parasites were seen in three consecutive fecalyses done. Nonetheless, the patient was started on mebendazole. Subsequent follow-up after two months showed resolution of ecchymosis, eosinophilia, and platelet dysfunction (Table 1), with no noted recurrence.

APDE is a syndrome with transient state of platelet dysfunction mostly reported from children in Southeast Asia.¹ APDE requires the following features for diagnosis: clinical manifestation of spontaneous ecchymosis on the trunk or extremities; hemogram showing eosinophilia; and evidence of platelet dysfunction.² The spontaneous ecchymosis seen clinically is indistinguishable from that of idiopathic thrombocytopenic purpura (ITP). APDE is differentiated from ITP by the absence of thrombocytopenia. The eosinophilia observed may be an epiphenomenon seen in areas with endemic parasitism, since intestinal parasitism is the evident cause of eosinophilia.^{3,4} The platelet dysfunction is demonstrated by light transmission aggregometry, with results consistent with a platelet storage pool disorder.^{3,5} Alternatively, PFA may be used especially in pediatric patients since it requires less blood.²

However, platelet aggregation studies are offered only in specialized laboratories and it may take a few days before results are released. A presumed diagnosis can be



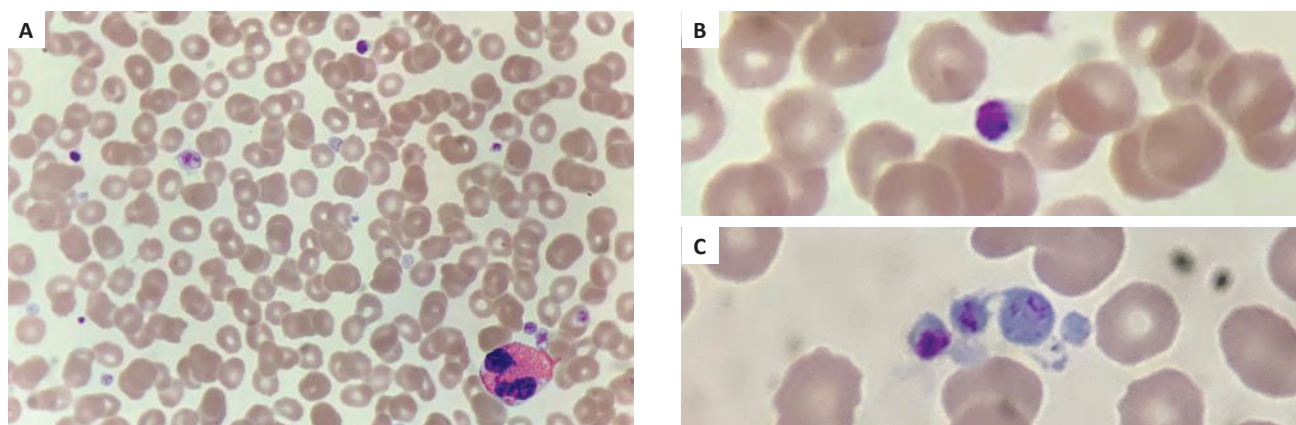


Figure 1. Peripheral blood smear photomicrograph showing an oil immersion field with an eosinophil, agranular grey platelets, hypogranular grey platelets, and a platelet with normal morphology (A) 1000X magnification; normal platelet (B) and three side-by-side platelets with decreasing granularity and a fourth agranular platelet (C), 3000X magnification.

made rapidly in any clinical laboratory offering routine hematology services, by microscopic examination of the platelet morphology.³ Wright stained blood smears show gray, pale staining platelets with smooth, round cell membrane contour. The cytoplasm may show fewer granules to none at all. Platelets with abnormal morphology may comprise about 30-80% of the platelets examined. This proportion correlates with the severity of the bleeding.⁶

The pathogenesis is still unknown. The platelet dysfunction has been thought to be due to the high production of IgE antibodies from a type I hypersensitivity reaction. The binding of immune complexes to platelets leads to in-vivo platelet activation. This activation promotes the release of adenine nucleotides, resulting in acquired storage pool deficiency.⁵⁻⁷

The prognosis is generally good, with a benign clinical course, and resolves spontaneously within 3 to 6 months to about a year.^{1,5}

STATEMENT OF AUTHORSHIP

All authors certified fulfillment of ICMJE authorship criteria.

AUTHOR DISCLOSURE

The authors declared no conflict of interest.

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None.

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At least three (3) keywords but no more than six (6), preferably using terms from the **Medical Subject Headings (MeSH) list of Index Medicus**, should be listed horizontally under the abstract for cross-indexing of the article.

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- All references, tables, figures and illustrations should be cited in the text, in numerical order.
- All abbreviations should be spelled out once (the first time they are mentioned in the text) followed by the abbreviation enclosed in parentheses. The same abbreviation may then be used subsequently instead of the full names.
- All measurements and weights should be in System International (SI) units.
- Under **Methodology**, information should be provided on institutional review board/ethics committee approval or informed consent taking (if appropriate).
- **Acknowledgements** to individuals/groups of persons, or institution/s who have contributed to the manuscript but *did not qualify as authors* based on the ICMJE criteria, should be included at the end of the text just before the references. Grants and subsidies from government or private institutions should also be acknowledged.

References

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- References should be numbered consecutively in the order by which they are mentioned in the text. They should not be alphabetized.
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More than Six Authors

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Authors Representing a Group

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Book

Byrne, DW. Publishing your medical research paper: What they don't teach in medical school. Baltimore: Williams & Wilkins, 1998.

World Wide Web

Barry JM. The site of origin of the 1918 influenza pandemic and its public health implications. [Commentary]. *JTranslational Med*. January 20, 2004;2(3):1-4. <http://www.translational-medicine.com/content/2/1/3>. Accessed November 18, 2005.

Tables

- Cite all tables consecutively in the text and number them accordingly.
- Create tables preferably using Microsoft Excel with one table per worksheet.
- Tables should not be saved as image files.
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- Each table must be self-explanatory, being a supplement rather than a duplicate of information in the text.
- Up to a maximum of five (5) tables are allowed.

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- Where appropriate, all illustrations/photographic images should be at least 800 x 600 dpi and submitted as image files (preferably as .png, .jpeg, .tif, .psd or .pdf files).
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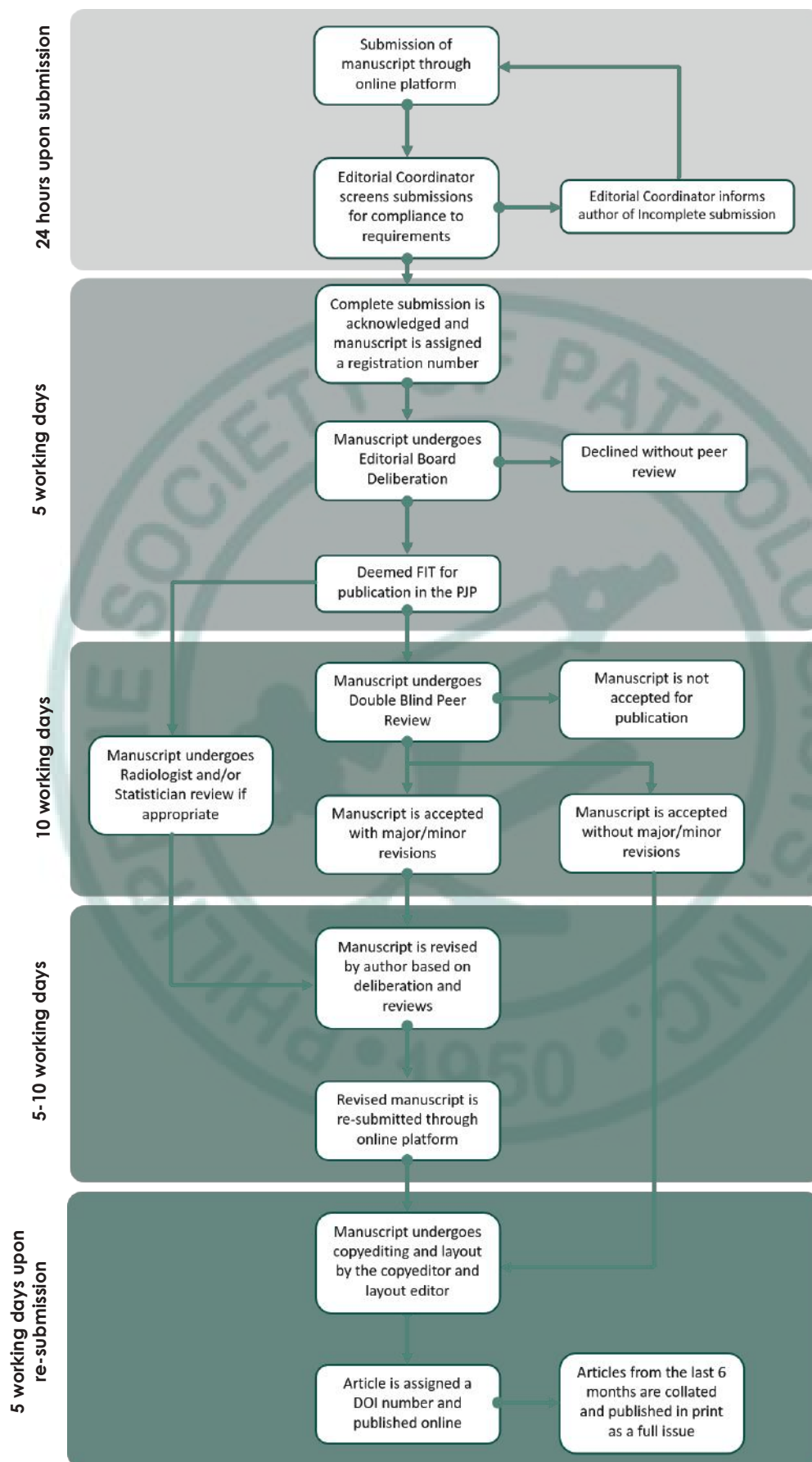


Figure 1. Editorial Process Flow.



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