

Coronavirus Pandemic

The efficiency of selective pooling strategy in a COVID-19 diagnostic laboratory

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Abstract

Introduction: Mass testing is essential in the surveillance strategy for fighting the COVID-19 pandemic. It allows early detection of suspected cases and subsequently early isolation to mitigate spread. However, the high cost and limited consumables and reagents hinder the mass testing strategy in developing countries such as Indonesia. The specimen pooling strategy is an option to perform mass screening with limited resources. This study aims to determine the positivity rate cut-off and to evaluate the efficiency of pooling strategy for the laboratory diagnosis of COVID-19.

Methodology: Between August 4th, 2020, and November 11th, 2020, a four-sample pooling strategy testing to detect SARS-CoV-2 was carried out at the Microbiology Diagnostic Laboratory of Diponegoro National Hospital, Semarang, Indonesia. Pools with positive results were subjected to individual specimen retesting. Spearman's correlation and linear regression analysis were used to determine the best positivity rate cut-off to apply pooling strategy.

Results: A total of 15,216 individual specimens were pooled into 3,804 four-sample pools. Among these pools, 1,007 (26.47%) were positive. Five hundred and ten (50.64%) were 1/4 positive. A maximum positivity rate of 22% is needed to save at least 50% extraction and qRT-PCR reactions in a four-sample pooling strategy. CT values between individual specimens and pools showed a good interval agreement.

Conclusions: Pooling strategy could reduce personnel workload and reagent cost, and increase laboratory capacity by up to 50% when the positivity rate is less than 22%.

Key words: COVID-19; SARS-CoV-2; pooling; qRT-PCR.

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Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection or Coronavirus Disease (COVID)-19 was declared a global pandemic by the World Health Organization on March 11th, 2020, after it was reported to cause clusters of pneumonia cases resembling SARS in Wuhan, China [1]. This recently discovered disease has been demonstrated to be highly contagious, with an unpredictable disease course, resulting in severe physiological and mental effects, as well as individual and social consequences, leading to healthcare system failure and economic stagnation in affected countries [2,3]. A short-range droplet transmission from the infected person was the main entry route to the human respiratory tract. Global transportation leads to the spread of the disease and increased transmission rate [4].

With its unique archipelagic geography and uneven population distribution, Indonesia has a huge challenge to combat COVID-19 [5]. In mid-April 2020, the total SARS-CoV-2 molecular testing capacity reached 35,000 but it still did not achieve the number recommended by the WHO, i.e. 1 per 1000 people each week [6,7]. By the end of July 2020, up to 24,000 samples were waiting to be processed. Meanwhile, up to 2,000 new reported cases of COVID-19 were reported per day. The latter number, however, was estimated to be much lower than the actual figures due to low screening and testing capacity.

Mass screening is one of the strategies to fight against COVID-19 [8]. Unfortunately, due to its high population and uneven distribution of Polymerase Chain Reaction (PCR) tests, Indonesia's mass screening was challenging in the first year of the

COVID-19 pandemic due to higher needs for consumables and reagents for diagnostic testing. Lack of human resources, high demands for consumable materials, Deoxyribonucleic Acid/ Ribonucleic Acid (DNA/RNA) purification kits, and PCR kits hinder the applicability of mass screening strategy in Indonesia.

A pooling strategy, which pools multiple nasopharyngeal swabs into one pooled specimen that undergoes one RNA extraction followed by one PCR reaction, can be applied to achieve mass screening. Pooling strategy was previously used in other pathogens. The specimen pooling strategy may significantly reduce personnel workload and cost for diagnostic tests while also increasing laboratory capacity [9]. However, there is a concern regarding the test sensitivity that may be reduced due to sample dilution [10]. Carrying out pooling testing without knowing the pre-test probability will lead to a high number of retests, which in turn increases workload, costs, and turnaround time [11]. Therefore, we carried out a selective pooling strategy for asymptomatic groups and samples from mass random screening. This article aims to determine the positivity rate cut-off and to evaluate the efficiency of pooling strategy for the laboratory diagnosis of COVID-19.

Methodology

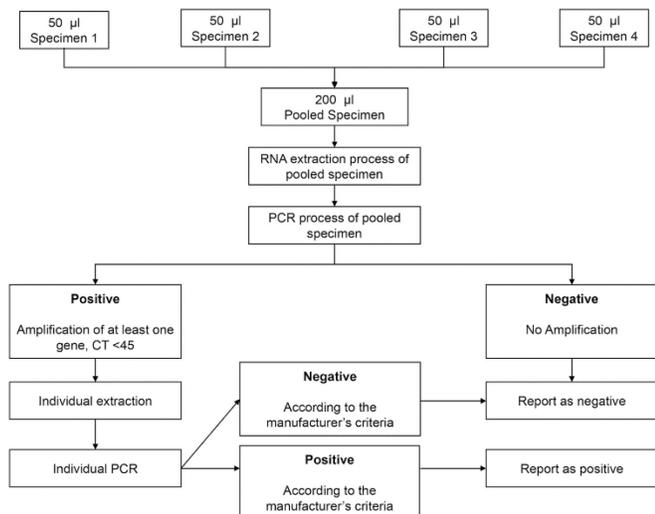
Between August 4th and November 11th, 2020, a four-sample pooling strategy testing to detect SARS-CoV-2 was carried out at the Microbiology Diagnostic Laboratory of Diponegoro National Hospital, Semarang. As one of the COVID-19 network laboratories in Indonesia, it received specimens from districts in Central Java Province, Indonesia.

Four different individual viral transport medium (VTM) swab specimens, 50 µL each, were pooled in a single tube in our pooling strategy. Not all specimens sent to our laboratory were pooled. Criteria for pool testing were swabs taken from asymptomatic individuals and the ones taken from mass screening regardless of the symptom. Two or more samples taken from the same individuals were grouped into one sample tube. Meanwhile, the swabs from symptomatic suspects and swabs for follow-up were not pooled. We did not pool samples from the regency or district which had more than half of pool specimen positive in the previous day until we saw a decrease of their daily positivity rate < 30%.

The pools then underwent RNA extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) using available SARS-CoV-2 RNA and qRT-PCR detection kits. For RNA extraction, we used DaAn Gene RNA/DNA Purification Kit Magnetic Bead and Spin Column (DaAn Gene Co. Ltd., Sun Yat-Sen University, Guangzhou, China), Liferiver Viral DNA/RNA isolation kit centrifuge column (ZJ Bio-Tech Co., Ltd., Shanghai, China), and QIAamp 96 virus QIAcube HT Kit (Qiagen, Valencia, CA). Meanwhile, for SARS-CoV-2 qRT-PCR, we used DaAn 2019-nCoV Diagnostic kit regular (DaAn Gene Co. Ltd., Sun Yat-Sen University, Guangzhou, China), Allplex 2019-nCoV assay (Seegene, Seoul, South Korea), mBioCoV-19 RT-PCR kit (Biofarma, Bandung, Indonesia), and Liferiver 2019-nCoV Real-Time Multiplex RT-PCR Kit (ZJ Bio-Tech Co., Ltd., Shanghai, China). The reagents were randomly used during the study based on the supply from the government and other donors. The PCR machine used was LightCycler 480 and LightCycler 96 (Roche, Penzberg, Germany), and Quantstudio5 (Applied Biosystems, Thermo Fisher Scientific, Singapore). We used three different PCR machine to avoid the delay in laboratory turnaround time.

A pool showed positive amplification by at least one target gene (RdRp, N, E, and Orf1ab) with a Cycle Threshold (CT) value < 45 was regarded as a positive pool. The respective individual specimens contributing to positive pools were examined individually. For individuals who has two or more specimens taken and sent on the same day, we mixed the specimens into one. Individual specimens were regarded as positive or negative based on the manufacturer’s criteria for each kit used. The diagram of our pooling examination is depicted in Figure 1.

Figure 1. Diagram of our pooling examination.



Statistical Analysis

Spearman’s correlation and linear regression analysis were used to determine the best positivity rate cut-off to apply the pooling strategy during the study period. A Bland-Altman plot was used to identify the interval of agreement of CT values between individual specimens and pools. SPSS 25 for Windows was used for all analyses. A Kruskal-Wallis’s test was used to evaluate whether CT value differences between pool and individual specimens tested with the three reagents were equal.

Ethics Statement

This study has been approved by the Institutional Review Board of the Faculty of Medicine, Universitas Diponegoro.

Results

During the study period, our lab received 42,032 nasopharyngeal/ oropharyngeal swab specimens for SARS-CoV-2 qRT-PCR. Of those, 15,216 (36.2% of the total specimen) met our criteria for pooled testing. Thus, we carried out 3,804 four-sample pooled testing. Among these, 1,007 pools (26.5%) were positive. In these positive pools, there were 4,028 specimens from 3,921 subjects. After individual examination of specimens from these 3,921 subjects, it was shown that most of the positive pools were 1/4 positive (50.6%) (Table 1).

In the present study, the number of positive pools strongly correlated with the number of positive specimens found daily during the study period (Figure 2, $r = 0.921$, and $p < 0.001$). A simple regression test showed an equation as $Y = 0.492 \times X - 2.426$.

Figure 2. A scatter plot showing a correlation between the number of positive pools and the number of positive specimens.

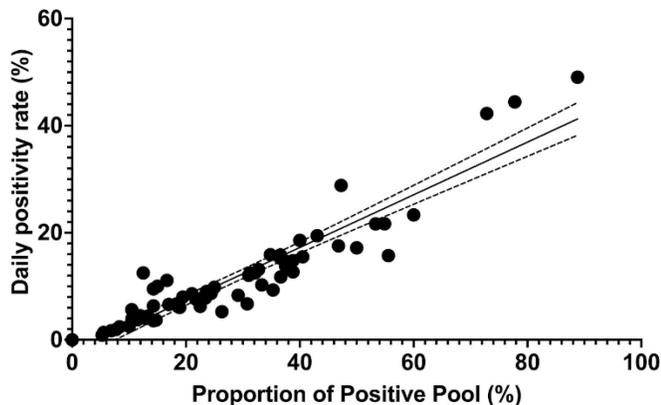


Table 1. Number of positive individual specimens among positive pools.

Number of positive individual specimens	Number of pools; n (%)
1	510 (50.64)
2	317 (31.48)
3	135 (13.41)
4	45 (4.47)
Total	1007 (100)

The Efficiency of Pooling Strategy

Instead of performing individual 15,216 extractions and qRT-PCR reactions, our pooling strategy performed 3,804 (number of pools) plus 3,921 (number of individual specimens from positive pools) reactions. By this, we saved 7,491 (49.2%) reactions.

The CT Value

We analysed the CT value difference between positive pools and individual positive specimens (only from 1/4 positive pool) of each qRT-PCR kit.

Liferiver 2019-nCoV Real-Time Multiplex RT-PCR Kit

A total of 337 pools were tested using Liferiver 2019-nCoV Real-Time Multiplex RT-PCR Kit, in which 62 pools were positive. Fifteen pools met the criteria for Bland-Altman analysis. The mean difference of CT values among E, Orflab, and N genes was under 2 (Table 2). The Bland-Altman plots showed good interval agreement and no proportional bias of pool specimens compared to individual specimens (Figure 3).

DaAn 2019-nCoV Diagnostic kit regular

A total of 2,378 pools were tested using DaAn 2019-nCoV Diagnostic kit regular, in which 557 pools tested using were positive. Three hundred and eleven pools met the criteria for Bland-Altman analysis. The mean difference of CT values among the Orflab and N genes was under 2 (Table 2). The Bland-Altman plots showed good interval agreement and no proportional bias of pool specimens compared to individual specimens (Figure 3).

Allplex 2019-nCoV assay

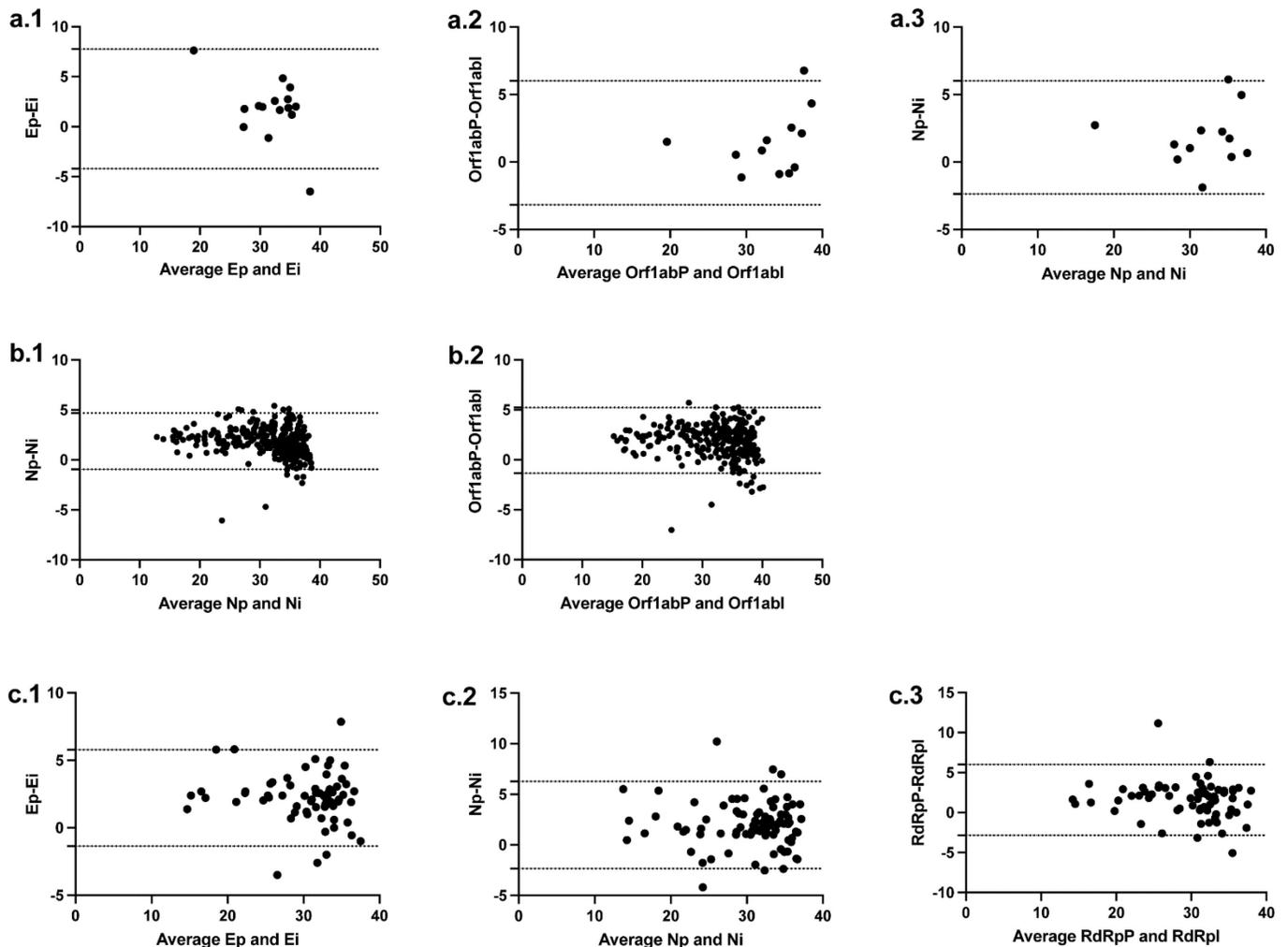
A total of 1,007 pools were tested using Allplex 2019-nCoV assay, in which 385 pools were positive. Ninety-eight pools met the criteria for Bland-Altman analysis. The mean difference of CT values among E, N, and RdRp genes was under 2.5 (Table 2). The Bland-Altman plots showed good interval agreement and no proportional bias of pool specimens compared to individual specimens (Figure 3).

Table 2. CT values of pools and individual specimens tested among reagents.

	E¹ (Mean ± SD)	Orf1ab² (Mean ± SD)	N³ (Mean ± SD)	RdRp⁴ (Mean ± SD)
Liferiver 2019-nCoV Real-Time Multiplex RT-PCR Kit				
CT values of pools	32.79 ± 4.17	33.88 ± 5.82	32.98 ± 5.59	NA
CT values of individual specimens	31.02 ± 5.69	33.83 ± 5.32	31.51 ± 5.35	NA
CT value differences between pools and individual specimens	1.77 ± 3.05	1.41 ± 2.34	1.812 ± 2.14	NA
DaAn 2019-nCoV Diagnostic kit regular				
CT values of pools	NA	33.24 ± 5.79	32.27 ± 5.73	NA
CT values of individual specimens	NA	31.85 ± 5.91	30.64 ± 6.02	NA
CT value differences between pools and individual specimens	NA	1.67 ± 1.68	1.86 ± 1.43	NA
Allplex 2019-nCoV assay				
CT values of pools	31.49 ± 5.41	NA	31.44 ± 5.53	30.86 ± 5.53
CT values of individual specimens	30.32 ± 5.60	NA	29.65 ± 5.69	30.12 ± 5.80
CT value differences between pools and individual specimens	2.21 ± 1.82	NA	1.96 ± 2.19	1.58 ± 2.26

E = E (envelope) gene; Orf1ab = Orf1ab (Open Reading Frame 1a and b) gene; N = N (nucleocapsid) gene; RdRp = RdRp (RNA-Dependent RNA Polymerase) gene.

Figure 3. Bland-Altman plot of (a) Liferiver 2019-nCoV Real-Time Multiplex RT-PCR Kit; (b) DaAn 2019-nCoV Diagnostic kit regular; (c) Allplex 2019-nCoV assay. Upper and lower dashed line indicate 95% confident interval. Ep: E gen in pool, Orf1abP: Orf1ab gene in pool, Np: N gene in pool, RdRpP: RdRp gene in pool, Ei: E gen in individual sample, Orf1abI: Orf1ab gene in an individual sample, Ni: N gene in individual sample, and RdRpI: RdRp gene in individual sample.



All three reagents provided similar CT value differences for all target genes ($p = 0.280$). The other three positive pools were tested using mBioCoV qRT-PCR kit (total 42 pools), in which none was qualified for the Bland-Altman analysis.

Discussion

The pooling strategy is used to expand the laboratory capacity in mass screening, reduce the time required for testing, preserve testing reagents and resources, and reduce the testing cost [12]. This strategy offered benefits, especially in low resource countries and in periods where distribution and availability of resources are minimal, such as during the COVID-19 pandemic. The pooling strategy has been applied in clinical examinations to detect other pathogens, such as human immunodeficiency virus, hepatitis B, hepatitis C, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Mycoplasma genitalium* [13-15].

Although 5-10 pool size had no sensitivity issue [16], we decided to use a four-size specimen pool instead of a larger pool size because we want to minimize the number of inhibitors that might be present in samples that can decrease PCR efficiency, which in turn might reduce the sensitivity of the assay. Nasopharyngeal swabs can contain potential inhibitors, such as blood, collagen, immunoglobulin, and proteinases, and food that can hamper PCR reaction [17]. Furthermore, the prevalence of COVID-19 during the study period was dynamic, and a four-size pool would conserve more resources when the prevalence suddenly increased.

In our study, approximately 36.2% of total specimens were carried out using a pooling strategy during the study period. We did not pool all specimens. Specimens from hospitalized patients and evaluation swabs were not pooled because the positivity rate was high, and the time needed for retesting can be detrimental in terms of transmission prevention in hospitals.

During the study period, there was a sudden increase in COVID-19 daily positivity rate in some regencies (up to 50%). This condition made us retest up to 70% of pools individually, which led to additional workload, cost, and turn-around time.

The proportion of positive pools was directly proportional to the daily positivity rate (Figure 2). Our study estimates a 50% positivity rate among pools with a prevalence of 22.2%. Fifty percent positivity rate among pools is the highest limit if we want to benefit from the pooling strategy. Thus, we recommend

running the pooling strategy with a four-size pool if the prevalence is under 22%.

During the study period, the number of positive individual specimens among pools was 11.4% compared to 26.8% among total specimens tested in our laboratory. With our selective pooling strategy, 7,491 (49.2%) reactions were saved, equivalent to 149,820 USD (20 USD/reaction). The workload decreased by 17.8%, which seems to be a low number. However, this low number was due to a sudden increase in prevalence during our study period. This increase in prevalence was dynamic and not expected beforehand. Another study has documented that a five-size pool effectively saved resources, reduced personnel workload, and increased testing capability by at least 69% when the incidence rate was under 10% [18]. Another study also reported that a five-size pool at a positivity rate of 9.8% could save 43.4% of reagents and 23.7% of work [19]. Compared to those studies, we had a higher positivity rate (up to 2.5 times), but our selective strategy plus smaller size pool led to equivalent savings in cost and workload. A pooling strategy with a smaller size pool is more beneficial for areas with higher positivity rates.

During our study period, RNA extraction and qRT-PCR were done by different operators, using different reagents and systems, which may lead to CT value difference [20,21]. However, based on the Kruskal-Wallis's test, there was no significant difference in Δ CT value between pools and individual specimens among all reagents used (ranged from 1.41-2.21). Furthermore, the Bland-Altman plots showed good interval agreement with minimum biases. It is important to note that all operators were adequately informed and trained before executing the pooling strategy.

The application of pooling strategy might introduce false negative results. We tried to minimize this by retesting individual specimens from pools with CT value < 45 and showed even a small amplification in at least one target gene. With this approach, it is very unlikely to misdiagnose samples with CT value ≤ 35 .

The SARS-CoV-2 pandemic is estimated to gradually shift into an endemic disease in the future [22]. Therefore, we assume that applying a pooling strategy in the future has several advantages in various sectors, whether it is in a pandemic or hyperendemic state. In the economic sector, many countries were significantly affected by the COVID-19 pandemic. Economic growth slowed down, and most of the expenditure was incurred for COVID-19 testing [23]. The end of the SARS-CoV-2 pandemic does not seem near despite the development of SARS-CoV-2 vaccines, antiviral drugs, and transmission control

guidelines. The virus itself has mutated and produced new variants that have had significant global public health repercussions, as seen with the delta and omicron variants [24]. It is proven that public health measures such as mask-wearing, social distancing, mass testing, isolation, and vaccination must be implemented together to achieve COVID-19 control. The pooling strategy can reduce the country's expenditure on testing which can be shifted to the vaccination program.

During the peak wave of COVID-19, there is an increased human resource demand in the laboratory and many laboratory technicians are shifted from their original work to COVID-19 diagnostics. The pooling strategy will reduce this demand and allow many technicians to focus on their original work.

The educational sector also suffers significantly from the pandemic. Online learning has been implemented since March 2020 and is still going on in many areas in Indonesia. Based on the SMERU study, the COVID-19 pandemic has caused a negative impact, especially on children from lower economic backgrounds. A pooling strategy can be implemented to regularly screen students and school staff to ensure a safe offline learning environment [25].

Conclusions

In summary, our study demonstrated that a selective pooling strategy could reduce personnel workload and reagent cost, and increase laboratory capacity by up to 50% when the positivity rate is less than 22%. Furthermore, different reagents and PCR systems used in our study did not affect the result.

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Authors' contributions

RH designed the study, performed laboratory work, analysed data, and wrote the manuscript. IK carried out data collection, statistical analysis, and wrote the manuscript. NM, FC, and EM performed laboratory work and contributed to manuscript editing. SPJ designed the study and contributed to manuscript editing. All authors read and approved the final manuscript.

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