Modification and optimization of a microtiter plate (in-house plate coating) for immunoglobulin M (IgM) measles detection by indirect enzyme-linked immunosorbent assay (ELISA)

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Abstract. Cases of measles infection in Indonesia are still widespread. Clinical measles infections can only be confirmed at the National Laboratory. The routinely used commercial testing kits are limited. This paper reports the development of a specific in-house plate coating with indirect ELISA. This method involves modifying and optimizing a microtiter plate with measles virus culture. Viral measles culture was obtained by growing it on the cell culture vero/hSLAM. The plate coating was optimized using a culture of measles virus with MO/2007/Jakarta and J/2010/Riau isolates in dilution of 1:1–1:2.048. The virus was inactivated at the time of coating. The indirect ELISA examination for the in-house plate coating was optimized for conjugate concentrations of 1:10, 1:25 and 1:50. The coating showed optimum results for detecting IgM measles at 1:16 dilution with MO/38/V/07 isolates in inactivation and examination of indirect ELISA using a 1:25 conjugate concentration.

Keywords: Indirect ELISA, measles, plate coating, vero/hSLAM cell

1. Introduction
Measles is an acute infectious disease caused by a virus of the genus morbillivirus, family Paramyxoviridae. The Ministry of Health recorded 12,222 individual cases and 2,104 measles outbreaks in 2014. The cases recorded by the South East Asia Region Organization (SEARO) from 2000–2006 increased from 78,574 to 94,562. Measles infections can lead to complications such as diarrhea, gastroenteritis, otitis media, pneumonia, encephalitis and even death [1].

Measles is diagnosed from clinical symptoms, and the diagnosis is confirmed by laboratory testing with serology techniques. Serology is the gold standard for clinical tests and checks for the IgM antibodies of measles in serum. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) specific to IgM measles antibodies is recommended by the World Health Organization (WHO) for the routine examination of measles cases [2, 3].

Serum specimens from patients with clinical symptoms of measles infection are taken and are then examined using indirect ELISA with a commercial kit. The SIEMENS kit is used by many national laboratories to test for measles-rubella in Southeast Asia. The kit offers sensitivity and specificity of 100 % but is expensive, at 12 million to inspect a maximum of 90 specimens, and the kit has a long delivery lag of about 3 months.
Plate coating is used in ELISA by coating a microtiter plate using an antigen or antibody. Development of ELISA techniques and devices for the diagnosis of infectious diseases is urgently needed as the successful monitoring and control of infectious diseases is determined by the efficacy and efficiency of diagnostic tools. By developing a less-expensive ELISA technique, we intend to optimize the use of available resources, reduce operational costs by utilizing local products, reduce dependence on imported products, provide domestic employment opportunities, and support the monitoring and control of infectious diseases in Indonesia [4].

The in-house plate-coating ELISA method is applied by modifying the microtiter plate using measles virus culture as well as by optimizing this in-house plate coating so that it reacts optimally to measles IgM. This alternative ELISA kit may simplify measles diagnosis and public-health monitoring in Indonesia, offering a shorter turnaround time while utilizing facilities already available at the National Laboratory.

2. Materials and method

2.1. Growth vero/hSLAM cell
The growth of vero/hSLAM cells begins with culture and subculture in a Dulbecco’s Modified Eagle Medium (DMEM) containing FBS 10% as preparation for the growth of the measles virus. The growth of Vero/hSLAM was observed under inverted microscope every day until 80–90% confluence.

2.2. Propagation of measles virus in vero/hSLAM cell culture
The measles virus was propagated with the aim of obtaining measles virus culture in large quantities. A total of 500 mL of suspension measles virus isolates was put into a flask of vero/hSLAM cell culture that reached 80–90% confluence. 5–6 mL of DMEM containing FBS 5% was added to the flask, and then, growth was observed under the inverted microscope to check for the cytopathic effect (CPE).

2.3. Harvest measles virus
Viral-culture measles with CPE +4 were stored in a freezer at -81 °C and then thawed so that the monolayer layer on the flask was dispersed into a suspension. Centrifugation was applied at a rate of 3,000 rpm for 10 min with a temperature of 4 °C to separate the supernatant containing the measles virus particles from pellets containing dead vero/hSLAM cells.

2.4. Coating of the microtiter plate with measles virus culture
Measles virus culture (@100 mL/well) is diluted several times (1:1–1:2,048) with a coating solution buffer (@100 mL/well) and is then coated into each well of a microtiter plate [Nunc MaxiSorp] with odd-number strips. Vero/hSLAM cell culture was diluted with the same dilution of 1:1–1:2,048 and then coated into the even strips of the microtiter plate. The microtiter plate was covered with aluminum foil and then incubated at 4 °C for 18 h. 300 mL of blocking buffer was added into the wells on a microtiter plate, and the plate was again covered with aluminum foil and then incubated at 37 °C with 3.5% CO2 for 1 h. The plate was then washed with the buffer with four repetitions of 300 mL into each well on the microtiter plate. The plate with measles virus cultures modified was then tested with reagents from kits Enzygnost® anti-measles virus/Ig-M [SIEMENS] and specimens in the form of positive control (P/P) and negative control (P/N) from the SIEMENS kit.

2.5. Indirect ELISA IgM procedure
The developed procedures for indirect ELISA refer to the protocol published by SIEMENS Healthcare [5].
2.6. Optimization of in-house plate coating
The coating is optimized by testing the positive control specimens from the SIEMENS commercial kit. Coatings were made with isolate MO/2007/Jakarta and J/2010/Riau at various dilutions (1:1–1:2,048). Coatings were prepared with active or inactive viruses at conjugate concentrations of 1:10, 1:25, or 1:50.

2.7. Validation, evaluation of sensitivity, specificity, and agreement in-house plate coating
The study was conducted by comparing the results of the in-house plate-coating modifications with the microtiter plate from the SIEMENS kit that has sensitivity and specificity of 100%. Indirect ELISA was performed using 45 serum specimens from patients with suspected measles infection coming from several regions in Indonesia.

3. Results and discussion
The microscopic results of the vero/hSLAM cell culture indicate that a monolayer formed on the substrate with cells in an irregular polygonal formation (figure 1). The cell culture of vero/hSLAM takes about 5–7 days to reach confluence. The results of the measles virus growth on day 2 show morphological changes in the vero/hSLAM cell culture. Several cells fused (figure 2) and then began to form holes or plaque that enlarges steadily. This morphological change is called the CPE and indicates syncytium, or multinucleated-cell, formation. Growth of the measles virus on days 5 and 6 shows that the vero/hSLAM cells had died and lifted from the substrate.

The results of the indirect ELISA testing are qualified in terms of the intensity of the resulting color change, and quantitative results were obtained from the value of optical density (OD). Wells with positive results changed color to yellow and produced a value of OD of more than 0.2, whereas wells with negative results retained the same color and produced a value of OD of less than 0.1. On the basis of the OD values and interpretation of the SIEMENS kit results, in-house plate coating with isolates of MO/2007/Jakarta shows OD values higher than the isolates of J/2010//Riau (table 1 and table 2).

The optimum concentration of the virus is determined from the results of the value of OD near the nominal value (0.54) (table 3). On the basis of the OD value with different viral conditions (inactive and active), inactive viruses have higher OD values when compared to active viruses. On the basis of the value of the OD produced, the optimum dilution is 1:16 for virus isolates MO/38/V/07. The conjugate concentration of 1:25 was selected because the value of OD produced is sufficient based on the value of the average OD positive control used in the laboratory (0.3–0.5).

Of the 45 serum specimens tested with in-house plate-coating, 21 specimens tested positive and 24 specimens tested negative, whereas with the microtiter plate from SIEMENS kit (table 4), 21 specimens tested positive, 23 tested negative, and 1 result was equivocal.

![Figure 1. Vero/hSLAM cell culture (day-6).](image1)

![Figure 2. Cytopathic effect (syncytium formation) (day-2).](image2)
Only one of the 45 specimens showed different results from the two kits; the proposed test returned a negative result (0.058), and the Siemens kit result was equivocal (0.182).

Calculation of sensitivity, specificity, and agreement was performed only using 44 specimens. According to the Food and Drug Administration (2003), the determination of the sensitivity, specificity, and agreement of a new test should include equivocal results. The results of calculations with 44 specimens showed that the sensitivity, specificity, and agreement from the in-house plate coating are 100% [6]. An ideal instrument or tool has a sensitivity value and specificity close to 100%:

\[
\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Negative}} \times 100\% \\
= \frac{21}{21 + 0} \times 100\% = 100\% 
\]  

(1)

\[
\text{Specificity} = \frac{\text{True Negative}}{\text{False Positive} + \text{True Negative}} \times 100\% \\
= \frac{23}{0 + 23} \times 100\% = 100\% 
\]  

(2)

\[
\text{Agreement} = \frac{\text{True Positive} + \text{True Negative}}{\text{True Positive} + \text{False Negative} + \text{False Positive} + \text{True Negative}} \times 100\% \\
= \frac{21 + 23}{21 + 0 + 0 + 23} \times 100\% = 100\% 
\]  

(3)

Table 1. The value of OD in-house plate-coating modification with isolates MO/2007/Jakarta.

|        | 1:1  | 1:2  | 1:4  | 1:8  | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1,024 | 1:2,048 |
|--------|------|------|------|------|------|------|------|-------|-------|-------|---------|---------|
| Blank  | 0.003| 0.002| 0    | 0    | 0    | 0.001| 0.002| 0.001 | 0.002 | 0    | -0.001  | 0.001   |
| Positive Control | 0.215 | 0.253 | 0.301 | 0.356 | 0.449 | 0.433 | 0.442 | 0.342 | 0.286 | 0.158 | 0.09    | 0.064   |
| Negative Control | 0.001 | 0.009 | 0.009 | 0.009 | 0.004 | 0.004 | 0    | 0.01  | 0.003 | 0.003 | -0.007  | 0.001   |

Table 2. The value of OD in-house plate-coating modification with isolates J/2010/Riau.

|        | 1:1  | 1:2  | 1:4  | 1:8  | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1,024 | 1:2,048 |
|--------|------|------|------|------|------|------|------|-------|-------|-------|---------|---------|
| Blank  | 0.004| 0.003| 0.001| 0.005| 0    | 0.001| 0.002| 0.001 | 0.002 | 0    | -0.001  | 0.002   |
| Positive Control | 0.15  | 0.205 | 0.291 | 0.241 | 0.247 | 0.259 | 0.252 | 0.201 | 0.18  | 0.179 | 0.056   | 0.025   |
| Negative Control | 0.001 | 0    | -0.005| 0.0105| 0.01  | 0.018 | 0.019 | 0.012 | 0.007 | 0.008 | 0       | 0.001   |
Table 3. The value of OD in-house plate-coating optimization and modification results.

|                | Conjugate 1:10 |          | Conjugate 1:25 |          |
|----------------|---------------|----------|---------------|----------|
|                | MO Active     | MO Inactive | J Active      | J Inactive |
| 1:1            | 0.28          | 0.345     | 0.192         | 0.304     | 0.121 | 0.213 | 0.092 | 0.112 |
| 1:2            | 0.377         | 0.479     | 0.229         | 0.378     | 0.159 | 0.239 | 0.129 | 0.133 |
| 1:4            | 0.382         | 0.656     | 0.413         | 0.474     | 0.188 | 0.337 | 0.188 | 0.154 |
| 1:8            | 0.447         | 0.72      | 0.455         | 0.566     | 0.291 | 0.351 | 0.155 | 0.169 |
| 1:16           | 0.659         | 0.852     | 0.526         | 0.547     | 0.358 | 0.392 | 0.218 | 0.126 |
| 1:32           | 0.639         | 0.829     | 0.39          | 0.526     | 0.332 | 0.295 | 0.14   | 0.145 |
| 1:64           | 0.612         | 0.712     | 0.302         | 0.395     | 0.332 | 0.264 | 0.152 | 0.224 |
| 1:128          | 0.528         | 0.782     | 0.233         | 0.388     | 0.272 | 0.219 | 0.133 | 0.112 |
| 1:256          | 0.483         | 0.773     | 0.23          | 0.233     | 0.226 | 0.198 | 0.142 | 0.12  |
| 1:512          | 0.366         | 0.689     | 0.18          | 0.198     | 0.149 | 0.108 | 0.08  | 0.043 |
| 1:1024         | 0.281         | 0.393     | 0.171         | 0.084     | 0.094 | 0.062 | 0.305 | 0.006 |
| 1:2048         | 0.183         | 0.195     | 0.058         | 0.038     | 0.047 | 0.004 | 0.058 | 0.003 |

Table 3 (continued). The value of OD in-house plate-coating optimization and modification results.

|                | Conjugate 1:50 |          |
|----------------|---------------|----------|
|                | MO Active     | MO Inactive | J Active | J Inactive |
| 1:1            | 0.08          | 0.105     | 0.056     | 0.066     |
| 1:2            | 0.107         | 0.149     | 0.073     | 0.056     |
| 1:4            | 0.114         | 0.153     | 0.094     | 0.095     |
| 1:8            | 0.132         | 0.169     | 0.106     | 0.13      |
| 1:16           | 0.14          | 0.18      | 0.084     | 0.127     |
| 1:32           | 0.122         | 0.147     | 0.07      | 0.118     |
| 1:64           | 0.11          | 0.145     | 0.049     | 0.099     |
| 1:128          | 0.087         | 0.144     | 0.045     | 0.07      |
| 1:256          | 0.091         | 0.118     | 0.041     | 0.048     |
| 1:512          | 0.054         | 0.101     | 0.028     | 0.026     |
| 1:1024         | 0.03          | 0.048     | 0.013     | 0.018     |
| 1:2048         | -0.008        | 0.005     | 0.004     | -0.003    |

Table 4. Specimens tested with in-house plate-coating.

| In-house plate coating | SIEMENS Kit | Total |
|------------------------|-------------|-------|
| Positive               | 21          | 21    |
| Negative               | 0           | 23    |
| Total                  | 21          | 44    |
4. Conclusion
Microtiter plates were successfully modified in-house with coatings using virus measles cultures. The optimization results showed 1:16 dilution for MO/2007/Jakarta isolates in inactive and indirect ELISA testing using a conjugate concentration of 1:25. This demonstrates that the IgM measles antibody can be detected efficiently and inexpensively.

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