Herpes simplex virus (HSV) and varicella zoster virus (VZV) are the most common causes of viral vesicular dermatoses of the skin and mucous membranes (1). Tzanck smear is widely used as a bedside test for herpetic skin infections (1–3). It is performed using skin scrapings and traditionally involves Giemsa stain. Depending on the protocol, the multiple steps of staining, fixation, and washing usually require 15–60 min (4–7). Therefore, it is not always practical to perform this very useful test due to time constraints.

Methylene blue (MB) is an alternative and available blue dye (4). There are few reports regarding its use for Tzanck testing as a rapid technique (4,8,9); however, to our knowledge, no clinical studies have examined the diagnostic value of MB in the Tzanck smear. In this study, we evaluated the utility of MB in terms of its potential non-inferiority to the conventional Giemsa stain in the diagnosis of herpesvirus skin infections.

The study was designed as a cross-sectional analytical study. The protocol was approved by the local institutional review board (No. 674/59) and the trial was registered with clinicaltrials.gov (Identifier: NCT03178747). Written informed consent was obtained from each patient prior to participation in the study. The inclusion criteria were patients over 18 years of age with suspected HSV and VZV skin lesions. Negative control cases comprised patients with other vesiculobullous eruptions (e.g., acute eczema). Specimens obtained from the lesions were smeared onto 2 different glass slides and allowed to air-dry. Subsequently, 1 slide was stained with MB and the other with Giemsa. MB staining was achieved by putting 2–3 drops of MB solution (Merck KGaA, Darmstadt, Germany) onto the glass slide. No washing was needed and the cover slip was then placed. Final concentration of MB was 1.4 mg/ml (10). The entire staining process lasted approximately 10–15 s. In contrast, the Giemsa stain was performed using Giemsa solution (Merck KGaA) (11), but the specimen was not fixed. The slide was immersed in the solution and left for 15–20 min, then washed with distilled water for 3–5 s and left to air-dry. This process lasted approximately 25 min. The specimens stained by the 2 different methods were examined independently by a dermatologist (PR) and a highly experienced technician (TS) under a light microscope (magnification 100 × ). When there was disagreement in the diagnosis, the 2 examiners met to discuss each case and arrived at a consensus. Both were blinded to the final diagnosis and polymerase chain reaction (PCR) results. Diagnosis of herpesvirus skin infections was based on the presence of multinucleated giant cells (2,5). Fig. 1 shows the multinucleated giant cells identified by the 2 staining methods. As a gold

Fig. 1. (Color online) (A) Tzanck smear with methylene blue and (B) Giemsa stain demonstrate multinucleated giant cells (arrow heads), magnification 400 × .
standard for diagnosis of herpesvirus skin infection, we used medical history, clinical presentations, and patient treatment responses (clinical gold standard).

A subset of patients was randomly selected using a random number table to conduct further analysis for the presence of HSV-1, HSV-2, and VZV with PCR. The specimens were extracted and analyzed using Artus® Qiagen QS-RGQ HSV-1/2 and Artus® Qiagen QS-RGQ VZV (Qiagen, Hilden, Germany) according to the manufacturer’s protocols.

Sample size for the current study was adapted from the sample size calculation for the McNemar test for incidence discordance between 2 paired samples. Using a significance level of 0.05 and 80% power, we estimated that 120 patients would be required for a non-inferiority margin of 5%.

One hundred and fifty-six patients were recruited (case = 125, control = 31, prevalence of 0.8), and PCR analysis was carried out in 72 (46.2%) patients. There was no difference between subjects with and without PCR regarding duration and morphology of the skin lesions, and type of previous treatments with antiviral therapies. However, the number of negative control cases was significantly fewer in the group with PCR analysis ($p = 0.008$). Baseline demographic data and clinical characteristics are shown in Table 1.

An analysis with McNemar’s Chi-squared test with continuity correction provided no evidence that either type of staining method was inferior, in both the full sample ($\chi^2_{Mc} = 0.64286$, df = 1, $p$-value = 0.4227) or in patients who were diagnosed with PCR ($\chi^2_{Mc} = 0.16667$, df = 1, $p$-value = 0.6831). The Exact mid-p method estimates of the difference in proportions of positive cases between the MB and Giemsa methods was 0.027 (95% CI: −0.011, 0.041). Since the lower 95% limit falls within the 0.05 non-inferiority margin, we demonstrate the non-inferiority of the MB method compared to the Giemsa method. Compared to the clinical gold standard, the sensitivity of MB staining was 0.81(0.73–0.87), while Giemsa staining revealed a value of 0.78 (0.69–0.85). Specificity was 1.00 (0.89–1.00) for both staining methods. The positive and negative predictive values of the tests are shown in Table 2.

When the results of PCR were set as a gold standard ($N = 72$), the sensitivity of MB staining was 0.75 (0.63–0.85) versus 0.72 (0.60–0.83) of that of Giemsa staining and, as with the clinical gold standard, we can demonstrate that MB is not inferior to the Giemsa method (Diff = 0.037, 95% CI: −0.043, 0.104; $p < 0.05$). The specificity of both tests was 1.00 (0.59–1.00) and 1.00 (0.63–1.00), respectively.

Next, to evaluate the diagnostic accuracy of the clinical gold standard itself, we compared clinical diagnoses against the results of PCR analysis. The sensitivity, specificity, negative predictive value, and positive predictive value of the clinical diagnoses (against PCR) were computed and all 4 statistics yielded a value of 1 (perfect agreement).

The sensitivity of Tzanck smear for overall herpes infections has been reported as ranging from 76.9 to 86.3, and its specificity is reported as ranging from

### Table 1. Baseline demographic data, diagnosis, clinical features, and previous therapies of the patients in the study

|                  | All subjects | Subjects without PCR | Subjects with PCR done | $p$-value |
|------------------|--------------|----------------------|------------------------|-----------|
| $N$              | 156          | 84                   | 72                     |           |
| Female N(%)      | 94 (60.3)    | 53 (63.1)            | 41 (56.9)              | 0.536     |
| Age (Years)      | 53.63 ± 19.66 | 51.71 ± 19.86       | 55.88 ± 19.33          | 0.189     |
| Diagnosis N(%)   |              |                      |                        | 0.008     |
| - Herpes simplex | 32 (20.5)    | 11 (13.1)            | 21 (29.2)              |           |
| - Herpes zoster  | 80 (51.3)    | 42 (50.0)            | 38 (52.8)              |           |
| - Varicella zoster | 13 (8.3)  | 7 ( 8.3)             | 6 ( 8.3)               |           |
| - Negative control | 31 (19.9) | 24 (28.6)            | 7 ( 9.7)               |           |
| Duration (days)  | 5.22 ± 5.56  | 5.79 ± 6.06          | 5.40 ± 5.63            | 0.685     |
| Morphology N(%)  |              |                      |                        | 0.079     |
| - Vesicle        | 104 (66.7)   | 63 (75.0)            | 41 (56.9)              |           |
| - Bullae         | 14 ( 9.0)    | 7 ( 8.3)             | 7 ( 9.7)               |           |
| - Pustule        | 5 ( 3.2)     | 3 ( 3.6)             | 2 ( 2.8)               |           |
| - Macule/papule  | 18 (11.5)    | 8 ( 9.5)             | 10 (13.9)              |           |
| - Erosion/ulcer  | 12 ( 7.7)    | 2 ( 2.4)             | 10 (13.9)              |           |
| - Crusted lesion | 3 ( 1.9)     | 1 ( 1.2)             | 2 ( 2.8)               |           |
| Previous treatment with antiviral therapy | | | | |
| - Systemic       | 20 (12.8)    | 9 (10.7)             | 11 (15.3)              | 0.542     |
| - Topical        | 10 ( 6.4)    | 3 ( 3.6)             | 7 ( 9.7)               | 0.217     |
From our study, the sensitivity and specificity of the MB test is 81 and 100, respectively. Thus, our proposed staining method provides results similar to those obtained in previous studies. The main advantages of staining with MB are that it is readily available, inexpensive, quick, and simple, involving a single-step process without the need for fixation or washing. Based on our current study, the staining process for MB lasts 10 to 15 seconds, whereas the Giemsa process lasts 25 min. Therefore, MB may be an option for clinicians as a diagnostic test that can be performed at the points of care of different departments. Although not all our patients have been tested with PCR analysis, we demonstrated that our clinical diagnosis, which had sufficient controls, agreed perfectly with the PCR results. In addition, in this study, we used a single concentration of MB solution (1.4 mg/ml) to stain the specimens. Further studies to look into the optimal concentration of MB are of interest, as this may provide better resolution and contrast of the cells. This may be achieved with a serially diluted MB solution (12). In conclusion, we propose that the staining protocol with MB will serve as an easy and simple bedside test for clinicians and will allow a quick and reliable diagnosis of herpes infections.

Acknowledgments The authors heartfully thank Mr. Tinnakorn Siriaram, Ms. Porntepin Champaphan, Ms. Ruangrong Klinhom, and Ms. Prathanporn Kaewpreedee, the technicians and scientists that tremendously contributed to the laboratory works for this study.

Conflict of interest None to declare.

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