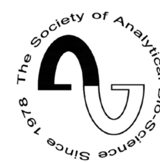
〈Research Article〉



Development of "Fluid Checker HAYASHI"-a staining solution for measuring cerebrospinal fluid cell counts

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Summary The cerebrospinal fluid (CSF) cell classification test is an important test for identifying central nervous system infections, such as bacterial and viral meningitis. Although Samson's solution has been widely used for CSF cell classification, it contains a single stain (fuchsin dye) that dyes mononuclear and polymorphonuclear cells red, thereby requiring skill for cell differentiation. Hence, CSF cytology testing is a burdensome task for inexperienced technologists. Herein, we developed a staining solution for CSF cell counting, Fluid Checker HAYASHI, which can be stained at a dilution factor similar to that of Samson's solution. It stains mononuclear cells red and polymorphonuclear cells indigo based on a Fuchs–Rosenthal calculator, rendering easier visual differentiation of CSF cells. The Fluid Checker HAYASHI stain showed good correlation for both polymorphonuclear and mononuclear cell fractions. Collectively, Fluid Checker HAYASHI enables quick and accurate CSF cytology tests and can improve clinical practice.

Key words: Cerebrospinal fluid, Samson's solution, Fluid Checker HAYASHI, Fuchs–Rosenthal calculator

1. Introduction

Cerebrospinal fluid (CSF) is produced by the choroid plexus, with the lateral ventricular choroid plexus playing a central role in CSF production. One gram of choroid plexus produces 0.4 mL/min of CSF at any given time, yielding approximately 1,000–1,500 mL of CSF per day[1].

The CSF has two main functions: (1) protecting the central nervous system (CNS) from external physical forces; and (2) maintaining homeostasis by regulating electrolytes and other biochemicals through the blood–brain barrier. Spinal fluid cell

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classification examination is an important test for diagnosing bacterial meningitis and viral meningitis, which are infectious diseases of the CNS. Bacterial meningitis is caused by bacterial or yeast infection of the spinal fluid, and the fatality rate is approximately 20% in adults and approximately 5% in children[2]. Treatment requires early detection and administration of appropriate antimicrobial agents, and rapid response substantially influences prognosis[3,4]. Immediate CSF cytology examinations are performed on duty at many facilities, but when the tests are performed by inexperienced technologists who are not regularly involved in CSF cytology tests, false reports or delays in reporting may occur. The reason for this discrepancy is that Samson's solution is a single stain containing only one dye and thus stains all leukocytes in the CSF red. Classification of leukocytes in the CSF into mononuclear and polymorphonuclear cells is difficult in some cases based on single-dye staining and cell morphology alone. Therefore, the development of a rapid and simple staining solution to replace Samson's solution is desired.

In this study, we developed a new staining solution for the classification of CSF cells, Fluid Checker HAYASHI, which uses a novel peroxidase reaction and dye instead of a single dye, such as Samson's solution. Fluid Checker HAYASHI can distinguish between mononuclear and polynuclear cells in a short period by color-coding.

2. Materials and Methods

Specimen preparation

Briefly, 5 mL of Polymorphprep™ (Axis-Shield, Oslo, Norway) was dispensed and layered with 5 mL of EDTA2K-added peripheral blood from healthy subjects. The mononuclear and polymorphonuclear layers were separated by centrifugation at 500 ×g for 30 min. The separated cells were diluted in RPMI 1640 medium and used as samples. This study was approved by the Research Institutional Ethics Committee of the Kagawa Prefectural University of Health Sciences (Kagawa, Japan) under approval number 340.

Fluid Checker HAYASHI reagent composition

The reagent comprised the following: 1-naphthol, methylene blue trihydrate, ethanol (Muto Pure Chemicals, Tokyo, Japan), safranin, hydrogen peroxide, and safranin with 30% acetic acid. All reagents except ethanol were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Fluid Checker HAYASHI substrate and dye concentration

The substrate, 1-naphthol, was dissolved in 100% ethanol and prepared at concentrations of 0.1, 0.3, and 0.6 g/L. For the dye, methylene blue was dissolved in 100% ethanol and prepared at concentrations of 10, 40, and 80 g/L.

Staining procedure and performance evaluation

Samson staining

The specimen (180 μL) was allowed to react with 20 μL of Samson's solution for 2 min, and leukocytes were classified into mononuclear cells and polymorphonuclear cells under a 200× microscope (OLYMPUS, BX40).

Fluid Checker HAYASHI staining

The 1-naphthol/ethanol and methylene blue/ethanol solutions were mixed in equal amounts. In addition, 5 μL of 3% hydrogen peroxide was added to the solution (reaction solutions) (Fig. 1).

Fluid Checker HAYASHI optimum concentration setting

Equal volumes of 10, 40, and 80 g/L of 1-naphthol and 0.1, 0.3, and 0.6 g/L of methylene blue in various combinations were mixed in 100% ethanol. and 5 μL of 3% hydrogen peroxide solution was added to the reaction solution. The sample and reaction solution were mixed and allowed to react for 1 min, and the cells were stained with 0.5% safranin acetic acid solution. The staining properties were compared using ImageJ version 1.52a (NIH, Bethesda, MD, USA).

The staining was evaluated by ImageJ; the difference in brightness before and after staining was quantified as ImageJ Cell Ratio.

Staining procedure with Cerebrospinal Fluid Checker HAYASHI

1. Add one drop of the two solutions into one ampoule and mix to form the reaction solution.
2. Add 10 μL of the reaction solution to 180 μL of spinal fluid sample, mix well, and allow to stand for 1 min.
3. Add another 10 μL of the three solutions, mix, and pour into the Fuchs-Rosenthal calculator.
   After mixing, pour the mixture into the Fuchs-Rosenthal calculator.
4. Calculated by classifying mononuclear cells and polymorphonuclear cells using a x200 microscope.

Fig. 1 Spinal fluid cell classification test procedure using Fluid Checker HAYASHI
For Fluid Checker HAYASHI staining, 10 μL of reaction solution and 10 μL of triplicate solution were added to 180 μLof CSF specimen. The dilution factor is same as that for Samson's solution.

Comparison of cell staining using Samson's solution and Fluid Checker HAYASHI staining

Each cell image was compared under the microscope (×400) after staining with Samson's solution and Fluid Checker HAYASHI stain.

In addition, neutrophils, monocytes, and lymphocytes were also comparatively assessed using Image J version 1.52a.

Correlation between Fluid Checker HAYASHI staining and classification values by the automated blood cell counter ADVIA2120i

We correlated the results of spinal fluid mode measurement of mononuclear cell and polymorphonuclear cell ratios calculated using ADVIA2120i (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) by injecting 25 identical samples into a Fuchs–Rosenthal calculator after staining with Fluid Checker HAYASHI.

Cell count evaluation of polymorphonuclear cells using Fluid Checker HAYASHI

The counts of polymorphonuclear cells were prepared at 5, 5×10, 5×10^2, and 5×10^3 cells/μL using samples isolated exclusively from peripheral blood. The effect of increased number of polymorphonuclear cells on staining by Fluid Checker HAYASHI was evaluated using ImageJ.

Effect of erythrocyte contamination on staining properties

For the undiluted solution, a sample containing 5×10^5 cells/μL of erythrocytes was used. The undiluted solution was diluted up to 32 times using saline to evaluate neutrophil staining using the Fluid Checker HAYASHI stain.

Statistics

The relationships between cytological staining and clinicopathological factors were examined using the $\chi 2$ test, with statistical significance set at $p<0.05$. All statistical analyses were performed using the SPSS 24.0 software (IBM SPSS, Armonk, NY, USA).

3. Results

Optimal concentration of Fluid Checker HAYASHI

There was no significant difference in the staining of neutrophils and monocytes between the different substrate concentrations at 10 g/L of methylene blue. In the case of 80 g/L methylene blue, the

leukocytes were stained well, but a pigment clot was observed in the background. This methylene blue clot also appeared with the combination of 0.6 g/L of 1-naphthol and 40 g/L of methylene blue. The combination of 0.1 g/L of 1-naphthol and 40 g/L of methylene blue showed a significant difference in staining between neutrophils and monocytes ($p<0.05$), whereas the combination of 0.3 g/L of 1-naphthol and 40 g/L of methylene blue showed a significant difference in staining among neutrophils, monocytes, and lymphocytes ($p<0.01$). Both mononuclear and polymorphonuclear cell populations were stained well (Fig. 2).

Comparison of Samson staining and Fluid Checker HAYASHI staining

In Samson's staining, lymphocytes, monocytes, and neutrophils were identified based on the morphological differences in nuclear shape and cytoplasm, making it difficult to differentiate mononuclear neutrophils. In Fluid Checker HAYASHI staining, lymphocytes and monocytes are stained red, and granulocytes such as neutrophils are stained indigo, making cell differentiation extremely easy (Fig. 3a and b).

Correlation between Fluid Checker HAYASHI staining and classification values using ADVIA2120i spinal fluid mode

The correlation of polymorphonuclear cell ratio was y = 0.961x+2.200 r = 0.996, and the correlation of mononuclear cell ratio was y= 0.961x+1.693 r = 0.996, indicating a very good correlation for both cell populations (Fig. 4a and b).

Evaluation of Fluid Checker HAYASHI staining ability for neutrophils with increasing cell number

No significant difference was observed in the staining ability using ImageJ Cell Ratio at the counts of 5, 5×10, 5×10^2, and 5×10^3 cells/μL of the sample multinucleated cells. However, microscopic evaluation showed that neutrophil staining was lower at a cell count of 5×10^3 cells/μL than at the other cell counts, suggesting that it is necessary to dilute the sample with saline before staining at cell counts higher than 5×10^3 cells/μL (Fig. 5a and b).

Effect of erythrocyte contamination on Fluid Checker HAYASHI staining

In the undiluted erythrocyte count, Fluid Checker HAYASHI staining showed poor staining of neutrophils. However, 16- and 32-fold diluted

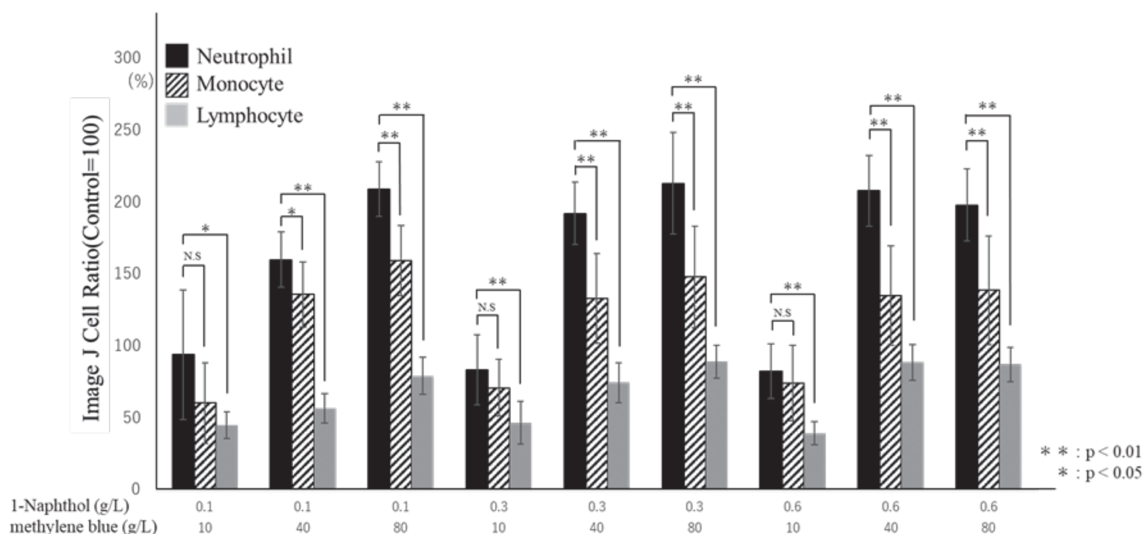

Fig. 2 Optimal concentrations of substrates and dyes in Fluid Checker HAYASHI
Fig. 2 shows the ImageJ evaluation of 10 neutrophils when stained with 1-naphthol (0.1 g/L) and methylene blue (10 g/L). This was Image J Cell Ratio 100% (control). This was compared with the staining of monocytes and lymphocytes the staining of neutrophils, monocytes, and lymphocytes when the reagent concentration was changed.

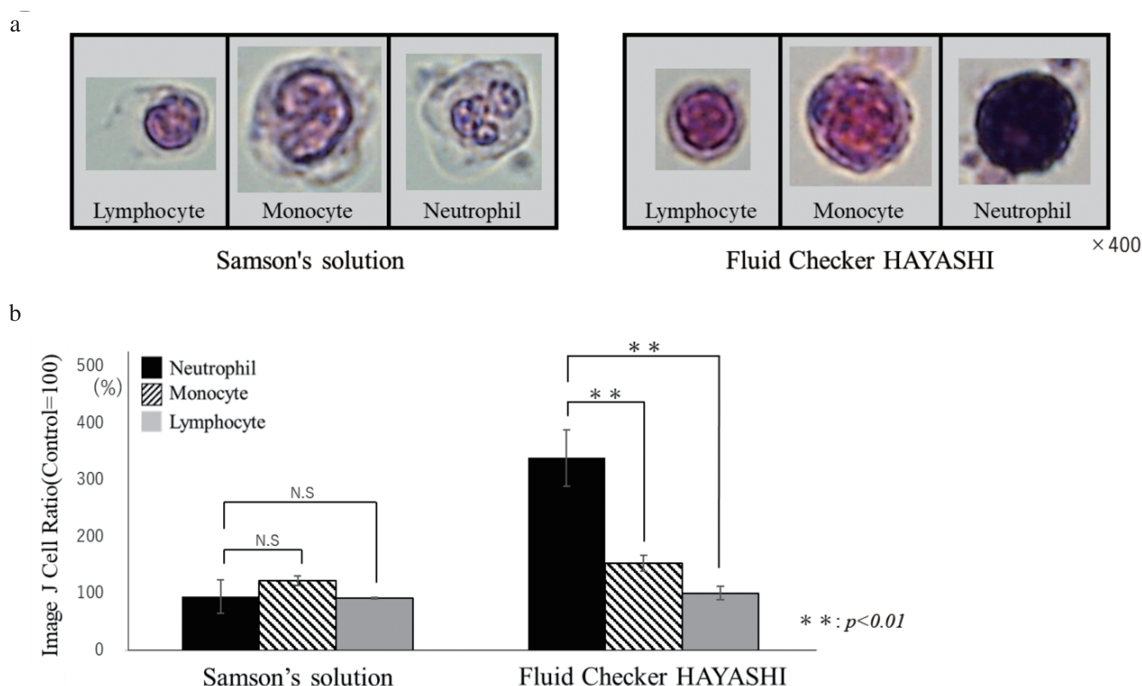

Fig. 3 Comparison between Samson's solution staining and Fluid Checker HAYASHI staining Fluid Checker HAYASHI stains lymphocytes red and neutrophils indigo. Staining (a), magnification ×400. In the graph, the cell ratio of 10 neutrophils stained with Samson's solution and measured using ImageJ is set to 100% (control). Lymphocytes, monocytes, and neutrophils were stained using Samson's solution stain and Fluid Checker HAYASHI stain. The mean and SD were calculated from the values using Image J Cell Ratio for 10 of each blood cell.

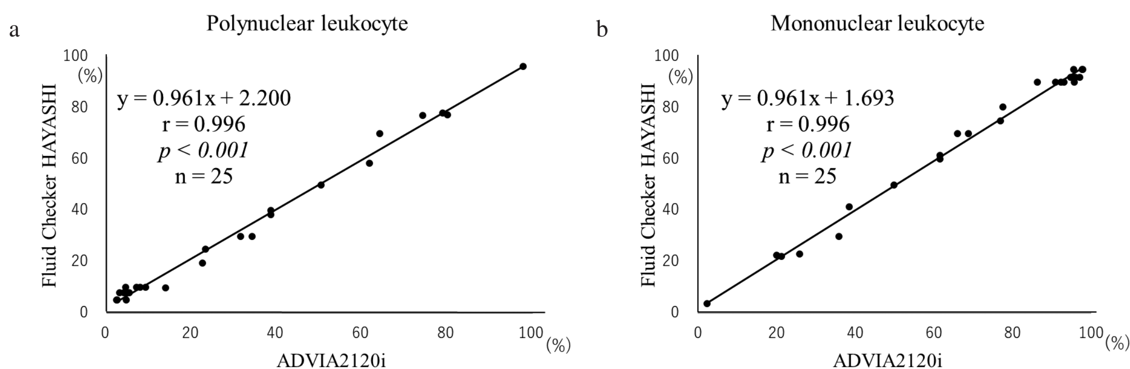

Fig. 4 Correlation using Fluid Checker HAYASHI and ADVIA2120i
Twenty-five samples containing a mixture of mononuclear and polymorphonuclear cells with different mixing ratios were used for evaluation.
The results of mononuclear and polymorphonuclear cell ratios following Fluid Checker HAYASHI staining and ADVIA2120i were correlated, respectively. $p<0.01$.

samples stained neutrophils (Fig. 6a and b).

4. Discussion

CSF cell examination is an important test in the differential diagnosis of CNS infections and for determining the efficacy of treatment. The mortality rate of bacterial meningitis is approximately 20%, and approximately 15% of the survivors are diagnosed with neurological disorders such as mental

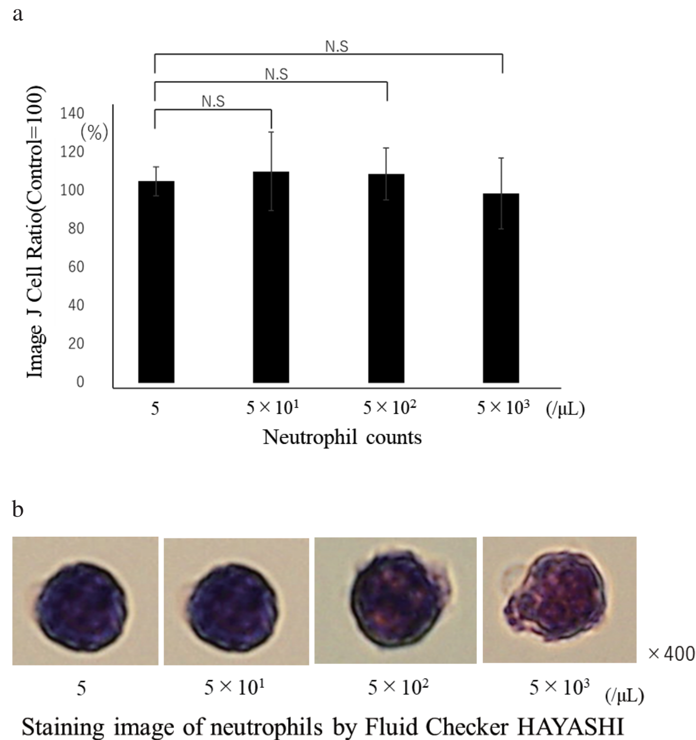

Fig. 5 Effect of cell count on neutrophil staining with Fluid Checker HAYASHI
Different cell counts (5, 5×10^1, 5×10^2, 5×10^3 cells/μL) were used for Fluid Checker HAYASHI staining.
At each cell count, three stained neutrophils were evaluated and compared using ImageJ (a).
Error bars indicate SD.
After Fluid Checker HAYASHI staining, the cell staining properties were confirmed under the microscope at ×400 (b).

retardation and sensorineural deafness as sequelae[5-7]. Rapid and accurate diagnosis is necessary because treatment requires prompt administration of antimicrobial agents. CSF cytology testing is also important for detecting CSF infiltration by tumor cells in hematopoietic tumors, such as acute leukemia and malignant lymphomas-infections of the CNS other than bacterial meningitis[8,9]. The automatic analyzers sold by some manufacturers now include a program for differentiating spinal fluid cells; it is possible to obtain polymorphonuclear and mononuclear cell ratios by measuring spinal fluid aspirated into the automatic analyzer[10-12]. However, although automated analyzers provide highly accurate results for mononuclear and polymorphonuclear cells that appear in meningitis, they fail to detect infiltration of epithelial diseases such as adenocarcinoma and hematopoietic tumors, and the equipment is usually expensive. Furthermore, this microscopic classification of CSF cells poses a challenge in differentiating cells. Since the Samson's solution is a single-stain dye containing fuchsin, all lymphocytes and monocytes, and other mononuclear and polymorphonuclear cells are stained red, requiring skill for accurate differentiation. This examination is often performed on spinal fluid specimens such as drainage fluid, in which the cell morphology is not preserved over time, making it difficult to differentiate cells using only a single dye solution. In this study, we developed a staining solution for CSF cell classification, called "Fluid Checker HAYASHI," which can be stained at a dilution factor similar to

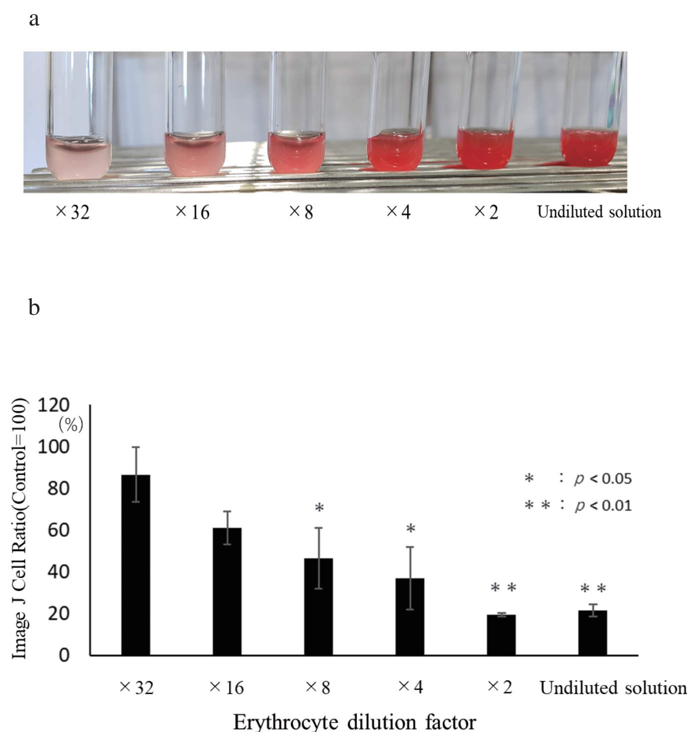

Fig. 6 Effect of erythrocyte count on neutrophil staining by Fluid Checker HAYASHI
The stock solution contains 5×10^5 cells/μL erythrocytes (a). CSF containing 5×10^5 cells/μL erythrocytes was diluted 32-fold to perform Fluid Checker HAYASHI staining, and the best-stained neutrophils were evaluated as controls (b).

that of Samson's solution for CSF cell classification. Fluid Checker HAYASHI uses 1-naphthol as the substrate and methylene blue as the dye and is based on the peroxidase reaction. Peroxidase is an enzyme that oxidizes its substrate, 1-naphthol, in the presence of hydrogen peroxide water. Methylene blue specifically adsorbs this oxidation product and stains the peroxidase-positive granules indigo. Fluid Checker HAYASHI stains polymorphonuclear cells indigo via a peroxidase reaction and mononuclear cells red by a post-staining safranin acetic acid solution. Fluid Checker HAYASHI solution I has a 1-naphthol concentration of 0.3 g/L. When the concentration of 1-naphthol was set to 0.3 g/L and the concentration of methylene blue was set to 40 g/L, the staining of polymorphonuclear cells and mononuclear cells was optimal.

Identifying polymorphonuclear cells following Samson staining can be difficult because the nuclei may appear mononuclear depending on the angle of the cell; however, Fluid Checker HAYASHI staining allows easy differentiation between polymorphonuclear cells and mononuclear cells owing to the enzymatic reaction involved. The correlation of polymorphonuclear and mononuclear cell ratios in Fluid Checker HAYASHI staining with the spinal fluid measurement mode of the ADVIA2120i automated hematology analyzer was excellent, and the reliability of the results was demonstrated to be high, as no deviations were observed in any of the samples.

Furthermore, we evaluated the effect of increased white blood cell count and erythrocyte contamination on polymorphonuclear staining using the developed stain. When we evaluated the ability of Fluid Checker HAYASHI for staining neutrophils with increasing cell numbers, both the ImageJ Cell Ratio and cell morphology results revealed that the staining was satisfactory up to a count of 5×10^2 cells. In most cases of central nervous system

infections, such as meningitis, the number of leukocytes in the spinal fluid increases only up to 5×10^2; therefore, Fluid Checker HAYASHI staining could be useful clinically.

The spinal fluid is usually clear, and contamination with red blood cells is unlikely; however, red blood cells may be present in the spinal fluid from cranial hemorrhage. The effect of erythrocyte contamination on Fluid Checker HAYASHI staining showed that for polymorphonuclear cells, the staining was satisfactory up to a count of 3×10^4 cells. Thus, if the staining is poor owing to extremely high white blood cell counts or red blood cell counts, the spinal fluid can be diluted tenfold with saline to achieve good staining.

From the results of this study, The Fluid Checker HAYASHI staining technique can differentiate leukocytes in spinal fluid into mononuclear cells and polymorphonuclear cells via a peroxidase reaction in a short period of time in liquid specimens without the need of a smear and can be used easily because the dilution factor is similar to that of the staining process using Samson's solution. Fluid Checker HAYASHI staining, which uses the peroxidase reaction, is useful not only for CSF examination in CNS infections but also for identifying CSF infiltration of peroxidase-positive acute myeloid leukemia cells. However, it may be difficult to detect the infiltration of peroxidase-negative tumor cells, such as malignant lymphoma cells.

In conclusion, the spinal fluid cell classification test using Fluid Checker HAYASHI stain is highly objective, simple, and rapid and can contribute to clinical practice in the differential diagnosis of CNS infections and the estimation of therapeutic effects. The developed stain can be useful in differentially diagnosing CNS infections and overall benefit inexperienced cytologists and lab technicians.

Conflicts of interest

The authors have no conflicts of interest. No funding was provided by Hayashi Pure Chemical Ind.

Acknowledgments

AI designed the study. TM performed sample preparation and staining. ST and KN conducted data compilation. SH edited and revised the manuscript. All authors have read and approved the final version of the manuscript.

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