Clinica Chimica Acta 462 (2016) 41–48

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

# Optimization of Cellular analysis of Synovial Fluids by optical microscopy and automated count using the Sysmex XN Body Fluid Mode

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ARTICLE INFO

*Article history:*
Received 29 July 2016
Received in revised form 25 August 2016
Accepted 26 August 2016
Available online 28 August 2016

*Keywords:*
Synovial fluid
Optical Microscopy
XN
Sysmex
Body Fluids

ABSTRACT

*Background:* This study was planned to assess the impact of pre-treating synovial fluid (SF) samples with hyaluronidase (HY), defining the best procedure for optical microscopy (OM) analysis and evaluating the performance of Sysmex XN-9000 Body Fluid module (XN-BF).
*Methods:* The cell count by OM was carried out both with and without HY pre-treatment, and using 3 different types of staining reagents. The evaluation of XN-BF included data comparison with OM (100 SFs), carryover, Limit of Blank (LoB), Limit of Detection (LoD), Limit of Quantitation (LoQ) and linearity.
*Results:* Unlike cell count in Burker's chamber and staining with Stromatol, pre-treatment with HY and staining with Methylene Blue and Turk's promoted cell clustering. The SF samples pre-treated with HY displayed excellent morphological quality, contrary to samples without HY pre-treatment. Excellent correlation was found between total cells counting with both OM and XN-BF. Satisfactory agreement was also observed between polymorphonuclear neutrophils compared to XN-BF parameter, whereas mononuclear cell count on XN-BF had suboptimal agreement with OM. The carryover was negligible. The LoB, LoD, LoQ and linearity were excellent.
*Conclusion:* XN-BF displays excellent performance, which makes it a reliable and practical alternative to OM for SF samples analysis in clinical laboratories.



## 1. Introduction

The synovial fluid (SF), also simply known as synovia, is a viscous liquid present in the cavities of synovial joints essentially produced by plasma filtration from the capillary network and enriched from local synthesis of hyaluronic acid from synovial cells [1]. The presence of hyaluronic acid confers a peculiar high viscosity to SF compared to other biological fluids such as ascites and pleural effusion [2]. The SF plays many important roles, such as nutritive function for the articular cartilage, joint lubrication and defence against host stresses [1].

Several articular diseases are characterized by fluid accumulation in joints, so that arthrocentesis and morphological analysis of SF are essential tools for differentiating a non-inflammatory joint disease (e.g. osteoarthritis and traumatic arthritis) from an inflammatory arthropathy (e.g., rheumatoid arthritis, or gout) or from an infectious disease (e.g., septic arthritis) [1–5]. Cell count and differentiation are hence important diagnostic aspects in patients with suspected infection of joint effusions, in prosthesis wearers who develop swelling, as well as in patients suspected of having peri-prosthetic fracture [6–9]. Despite the clinical

*Abbreviations:* BF, Body Fluid; CI, Confidence Interval; CV, Coefficient of variation; EO, Eosinophils; EO-BF, Eosinophil count by Body Fluid module of Sysmex XN-9000; FSC, Forward Scatter; HF-BF, Hight Fluorescent Cell count by Body Fluid module of Sysmex XN-9000; HY, hyaluronidase; In, Intercept; LoA, Limits of Agreement; LoB, Limit of Blank; LoD, Limit of Detection; LoQ, Limit of Quantitation; LY, Lymphocytes; LY-BF, Lymphocyte count by Body Fluid module of Sysmex XN-9000; MA, Macrophages; MN, Mononuclear cells; MO, Monocytes; MO-BF, Monocyte count by Body Fluid module of Sysmex XN-9000; NE, Neutrophils; NE-BF, Neutrophil count by Body Fluid module of Sysmex XN-9000; OM, Optical Microscopy; OTH, Other cells; PBS, Phosphate buffer saline; PMN, Polymorphonuclear cells; SF, Synovial fluid; SFL, Fluorescence Analysis; Sl, Slope; SSC, Laser Side Scatter; SY, Synoviocyte; TC, Total cells; TC-BF, Total cells count by Body Fluid module of Sysmex XN-9000; WBC, White blood cells; WBC, White blood cells count by Body Fluid module of Sysmex XN-9000; XN-BF, Body Fluid module on Sysmex XN-9000.

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http://dx.doi.org/10.1016/j.cca.2016.08.018
0009-8981/

usefulness of cell count and differentiation have been well established in all these pathological conditions, the lack of standardization and harmonization of cell enumeration remains a challenging issue and is regarded as a clear drawback for identification of disease-dependent diagnostic thresholds [4,9–12]. Despite these limitations, a discrete number of cut-offs have been defined. A number of white blood cells (WBC) $<200 \times 10^6$/L with up to 10% of polymorphonuclear leukocytes (PMN) is thought to be normal in SF [4,5,13], whereas a WBC count $>2000 \times 10^6$/L with >75% PMN is suggestive of an inflammatory disease such as the so-called crystalloid synovitis (gout, pseudogout) and autoimmune arthritis (e.g., rheumatoid arthritis, psoriatic arthritis). A WBC count of $>50000 \times 10^6$/L with >85% PMN is then suggestive of septic arthritis [6–14], whereas the identification of diagnostic thresholds for diagnosing peri-prosthetic infection remains controversial [6–13].

Despite optical microscopy (OM) analysis is still universally considered the reference technique for WBC enumeration and differentiation in SFs [13], many drawbacks have been emphasized including high cost, low throughput, long turnaround time (TAT), lack of inter-laboratory harmonization, high imprecision and need of specialized personnel for performing the analysis [14–24]. Although it is now undeniable that automation of SF analysis may generate major clinical and practical advantages (e.g., improving the TAT and enhancing the accuracy of cell counts) [17–24], automated cell enumeration in SF is still plagued by insufficient accuracy. This is mostly attributable to the risk of obtaining falsely increased values of WBC due to possible presence of fat globules, crystals, synoviocytes, chondrocytes and cartilage fragments in the sample [17–24]. In other cases, a matrix effect due to the presence of hyaluronic acid may lead to underestimating WBC number, since the increased viscosity may impair sample aspiration or prevent appropriate mixing [17–24].

The Sysmex XN-9000 (Sysmex, Inc. Kobe, Japan) is an automated hematological analyzer equipped with a module specifically designed for analysis of Body Fluids (XN-BF), displaying excellent performance for cell count and differentiation in cerebrospinal, pleural and ascetic effusions [17,20]. Since no reliable evidence has been published so far on SF to the best of our knowledge, this original study was planned to assess the impact of pre-treatment of SF samples with the enzyme hyaluronidase (HY), defining the best procedure for OM analysis and evaluating the performance of the XN-BF module for automated analysis of SF according to both the Clinical and Laboratory Standards Institute (CLSI) document H56- A [13] and the International Council for Standardization in Haematology (ICSH) guidelines [25].

## 2. Materials and methods

### 2.1. Synovial fluid samples

The study was originally planned by collecting 100 consecutive SF samples (30 from outpatients, 5 from patients hospitalized in the infectious diseases unit, 30 from orthopedic unit inpatients, 13 from patients acutely admitted to the local trauma center and 22 from patients hospitalized in the rheumatology ward) in $K_3$EDTA tubes (Becton Dickinson, Franklin Lakes, NJ). All samples were gently mixed immediately after collection.

All samples had been referred to the local clinical laboratory for routine analysis over a period of approximately 10 months. The analysis, always performed within 2 h from arrival of samples in the laboratory, was carried out according to the CLSI document H56-A [13]. The specimens were gently mixed immediately before testing and analyzed either before or after pre-treatment with HY (Sigma Chemical Co., St. Louis, Mo) at final concentration of 0.5 mg/mL. The solution was prepared by dissolving 2.5 mg of HY in 5 mL of 0.1 mol/L phosphate-buffered saline (pH 7.4). The final pre-treatment of SF samples consisted in mixing 20 μL of the HY solution with 1 mL of fluid and incubation at room temperature for 5 min, after which cell counts were simultaneously performed by OM and using the XN-BF module.

### 2.2. Optical microscopic cells count and differentiation

The cell count and differentiation by OM were performed according to the CLSI documents H56-A [13] and H20-A2 [26], as well as following the ICSH guidelines [25]. The total cell count by OM (TC-OM) was carried out both with and without HY pre-treatment, by 1:20 or 1:200 dilution with Turk's stain solution (Carlo Erba, Italy), Methylene Blue (Sigma Chemical Co., St.Louis, Mo), and Stromatol (Biolife, Masha Brunelli, Italy).

The pH of each solution or stain was evaluated using the pH meter Mettler-Toledo Titrator DL-15 (Metter-Toledo GmbH).

The slides for differential cell count were prepared by cytocentrifugation of SF samples at 100 × g for 3 min (Cytospin2 Thermo Scientific), followed by May-Grunwald-Giemsa staining (Carlo Erba, Italy). The chamber counting was carried out at ×400 magnification The slide review was carried out at ×400 magnification with ×40 oil-immersion objective (Objective Plan-Apochromat 40×/1.3 Oil DIC M27, D = 0.17 mm Carl Zeiss s.p.a, Italy) by two skilled operators on 200 cells. A third opinion was only required in the case of >5% disagreement [13,25,26]. When necessary, a more thorough cytomorphological evaluation by means of a second microscopic analysis was performed at ×1000 magnification with ×100 oil-immersion objective (Objective Plan-Apochromat).

### 2.3. The XN-BF module

The XN-BF module operates using fluorescent flow cytometry with hydrodynamic focusing, to generate total and differential counts in body fluid samples. The laser side scatter (SSC), forward scatter (FSC) and fluorescence analysis (SFL) enable the classification of nucleated cells according to internal complexity (x-axis), size (z-axis) and nucleic acid content (y-axis). The XN-BF module provides total cell (TC-BF), WBC (WBC-BF), PMN (PMN-BF) and mononuclear cell (MN-BF) counts. Additional research parameters include neutrophil (NE-BF), eosinophil (EO-BF), lymphocyte (LY-BF), monocyte (MO-BF) and high fluorescence cells (HF-BF) classification. Due to the use of a rinse cycle followed by a background check after each analysis, no problems of carryover or cross-contamination from blood samples have been reported.

### 2.4. Imprecision of XN-BF module analysis

The between-run imprecision of the XN-BF module was evaluated according to the CLSI document EP05-A3 [27], thus entailing the analysis of three levels (i.e., level 1, 2 and 3) of control material in duplicate for 40 consecutive working days.

**Table 1**
Analogy between XN-BF parameters and cells classes identified with optical microscopy (OM).

| OM differential morphology count | | XN-BF automated differential cells count |
|---|---|---|
| Cells Classes | OM type of cells counts | XN-BF parameters |
| LY-OM | Only lymphocytes | LY-BF |
| MN1-OM | Lymphocytes plus monocytes and macrophages | MN |
| MN2-OM | Lymphocytes plus monocytes, macrophages and synoviocytes | MN |
| MO1-OM | Only monocytes | MO-BF |
| MO2-OM | Monocytes plus macrophages | MO-BF |
| MO3-OM | Monocytes plus macrophages and synoviocite | MO-BF |
| NE-OM | Only neutrophils | NE-BF |
| PMN-OM | Neutrophils plus eosinophils and basophils | PMN |
| TC-OM | All nucleated cells present in BFs | TC-BF |
| WBC-OM | All nucleated cells present without other cells . | WBC-BF |