form extraction was better than both the uncorrected delta 450 and the delta 410 correction *(6)*. Interestingly, they also reported that chloroform extraction caused a decrease (mean of 20%) in the delta 450 measurement in 90% (93 of 103) of specimens. However, they attributed this to the presence of blood that is not detected by visual inspection in the majority of amniotic fluid samples. Hochberg et al. *(5)* also reported that results for 11 of 21 (52%) samples were significantly different after chloroform extraction. Of these 11 samples, the results for 8 were decreased with a mean difference of −43%. Regardless of whether the cause of the decrease is bilirubin trapped in the protein layer, as we speculate, or visually undetectable hemoglobin, it is clear that the post-chloroform extraction delta 450 measurement is lower than the pre-chloroform extraction measurement in the majority of samples. However, the Liley plot was created using unextracted delta 450 bilirubin measurements. Therefore, if chloroform is used, a new so-called "Liley curve" needs to be derived.

Recently, Egberts et al. *(8)* reported an iterative spectrophotometric method for the determination of bilirubin in amniotic fluid. This method corrects for contamination with oxy- and methemoglobin. Furthermore, the authors were able to extrapolate their data to a Liley chart and showed excellent correlation. Further studies correlating outcomes are needed, but this method looks promising for assessing erythroblastosis fetalis.

Our data were generated from in vitro experiments that may not duplicate all in vivo conditions. The amount of met- and oxyhemoglobin may be different in vivo. Clinical outcome studies are required to determine the effect of hemolyzed blood contamination on patient management.

These data suggest that the delta 450 bilirubin measurement, delta 410 correction, and chloroform extraction can all lead to false interpretations of fetal hemolytic status if the amniotic fluid sample is contaminated with hemolyzed blood. The clinical impact of these findings is not clear, but on the basis of these data, we recommend that amniotic fluid samples containing hemolyzed blood not be used to assess fetal hemolysis.

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Interassay and Interobserver Variability in the Detection of Anti-neutrophil Cytoplasmic Antibodies in Patients with Ulcerative Colitis, *Sofie Joossens,[1] Marco Daperno,[2] Zakera Shums,[3] Kristel Van Steen,[4,5] James A. Goeken,[6] Claudio Trapani,[7] Gary L. Norman,[3] Godelieve Godefridis,[8] Greet Claessens,[1] Angelo Pera,[2] Marie Pierik,[1] Severine Vermeire,[1] Paul Rutgeerts,[1] and Xavier Bossuyt[8*]* ([1] Department of Gastroenterology and [8] Laboratory Medicine, University Hospital Gasthuisberg, KULeuven, Leuven, Belgium; [2] Department of Gastroenterology and [7] Immunology Laboratory, Ospedale Mauriziano Umberto I, Torino, Italy; [3] INOVA Diagnostics Inc., San Diego, CA; [4] Center for Statistics, Limburgs Universitair Centrum, Diepenbeek, Belgium; [5] Harvard School of Public Health, Department of Biostatistics, Boston, MA; [6] Department of Pathology, University of Iowa, Iowa City, IA; * address correspondence to this author at: Laboratory Medicine, University Hospital Gasthuisberg, KULeuven, 3000 Leuven, Belgium; fax 32-16-347931, e-mail Xavier.Bossuyt@uz.kuleuven.ac.be)

Inflammatory bowel disease (IBD) represents a spectrum of disorders that affect the gastrointestinal tract *(1)*. IBD includes two major entities, Crohn disease and ulcerative colitis (UC). Although the etiology of IBD is unknown at present, it is believed to be an immunologically mediated disease *(2)*. Over the last 40 years, various (auto)antibodies have been described in IBD *(3)*. Anti-*Saccharomyces cerevisiae* antibodies *(4)* and perinuclear anti-neutrophil cytoplasmic antibodies (pANCAs) *(5)* have relatively high prevalence in patients with Crohn disease and UC, respectively. Unlike ANCAs present in vasculitis *(6)* and in Wegener granulomatosis *(7)*, the exact target antigen of UC-associated pANCAs has not been identified *(5, 8)*.

As a consequence, immunofluorescence microscopy is the only widely available technique for screening for these antibodies. Commercially available substrates, however, are not standardized, and part of the discrepancy in results could be attributable to differences among the substrates/assays used, as reported recently *(9)*. Moreover, because specific microscopic criteria to distinguish UC-associated pANCAs from pANCAs seen in vasculitis vary among laboratories, discrepant results could also be attributable to an investigator's interpretation of the fluorescence pattern.

Despite these methodologic problems, it has been suggested that the determination of pANCAs in UC could serve as an adjunct to conventional tools in the diagnosis of IBD and could be used for better phenotypic classification of the disease. Therefore, pANCA analysis is

widely performed in the context of laboratory evaluation of IBD.

The aim of this study was to assess the interassay and interobserver variability in the detection of UC-associated ANCAs.

Sera obtained from 50 patients with UC (23 females and 27 males; mean age, 40.7 years; range, 19–75 years), defined according to the Lennard-Jones criteria *(10)*, were studied. The clinical data for this study population are shown in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue8/. The same patient cohort was tested by all assays and by all laboratories, and serum from each patient originated from the same blood sampling.

Ethanol- and formalin-fixed human neutrophil substrates were used. Specimens with pANCA reactivity associated with vasculitis, as well as with UC, exhibit perinuclear staining in ethanol-fixed neutrophils. In formalin-treated neutrophils, however, the vasculitis-associated pANCA reactivity converts to a granular cytoplasmic (cANCA) pattern. Formalin fixes and prevents the artifactual migration of positively charged cytoplasmic proteins such as myeloperoxidase to the negatively charged nuclear membrane in ethanol-fixed neutrophils, thus leading to the apparent conversion of a vasculitis-associated pANCA pattern to a cANCA pattern. Recently, it has been suggested that, particularly for UC-associated pANCAs, use of both fixatives could be helpful *(11)*.

For evaluation of the interassay variability, the patient cohort was tested by one experienced observer from one laboratory (Laboratory Medicine, University Hospital Gasthuisberg, Catholic University Leuven, Leuven, Belgium), using substrates obtained from four different commercial sources: INOVA Diagnostics, Immunoconcepts, Bio-Rad, and The Binding Site. Each assay was performed according to the manufacturer's instructions. Manufacturers' control samples were tested in each run. Sera were diluted in the manufacturer's specified diluent and incubated for 30 min at room temperature. After a washing step, fluorescein isothiocyanate-labeled goat (INOVA, Immunoconcepts, Bio-Rad) or sheep (The Binding Site) anti-human IgG was added to each slide. The conjugates provided by INOVA, Immunoconcepts, and Bio-Rad were IgG specific, whereas the conjugate provided by The Binding Site was IgG heavy- and light-chain specific. After incubation for 30 min at room temperature, the slides were washed, covered with mounting medium, and covered with a coverslip. Slides were examined under ultraviolet light, and sera that had fluorescence on indirect immunofluorescence microscopy were carefully classified.

For evaluation of interobserver variability, we assayed sera from the same patient cohort as described above for the different ANCA patterns in four geographically distinct laboratories—INOVA Diagnostics (San Diego, CA); Immunopathology Laboratory, University of Iowa Hospitals and Clinics (Iowa City. IA); Immunology Laboratory, Ospedale Mauriziano Umberto I (Torino, Italy); and Laboratory Medicine, University Hospital Gasthuisberg (Leuven, Belgium)—with one company's method (INOVA). All observers were experienced with reading the results indirect immunofluorescence microscopy. The microscopes used were a Leitz Ariston microscope (Iowa), an Olympus BX40 (Torino), a Nikon Labphot-2 (San Diego), and a Leitz Wetzlar Orthoplan (Leuven).

The prevalences of the ANCA patterns on ethanol-fixed substrate and/or on formalin-fixed substrate were calculated. The κ for nominal data as concordance between multiple rates was used to evaluate agreement *(12)*: $\kappa < 0$ indicates poor agreement; 0–0.2 indicates slight agreement; 0.2–0.4 indicates fair agreement; 0.4–0.6 indicates moderate agreement; 0.6–0.8 indicates substantial agreement; and 0.8–1 indicates almost perfect agreement *(13)*.

In the intermethod study, ANCA was assayed with substrates from, respectively, The Binding Site, Bio-Rad, INOVA, and Immunoconcepts (Table 1A). The prevalence of ANCAs varied for the respective ANCA patterns; i.e., p- and cANCA, pANCA on ethanol-fixed substrates, and pANCA on ethanol-fixed substrates combined with a negative finding on formalin-fixed substrate. Seven samples were identified to be pANCA-positive based on the ethanol-fixed substrate and cANCA-positive based on formalin-fixed substrates. This phenomenon was observed with three of the four substrates tested. However, the finding was not consistent among these assays, except for one sample in which the phenomenon was observed with two assays (INOVA and Immunoconcepts; see Table 1A).

The κ values for agreement among the methods were 0–0.32 for p- and cANCA, 0.07–0.47 for pANCA on ethanol-fixed substrates, and 0.04–0.38 for pANCA on ethanol-fixed substrates combined with a negative finding on formalin-fixed substrate (see Table 2a in the online Data Supplement). Cytoplasmic staining (cANCA) was found in 1 of 50 with The Binding Site method and in 6 of 50 of the samples with the INOVA method. For all but one assay, a relatively high number of noninterpretable results [in which no (clear) pattern could be distinguished] was observed, ranging from 3 of 50 with the INOVA assay to 5 of 50 with the Immunoconcepts assay and 16 of 50 with The Binding Site assay.

For the interobserver study, the same assay was used by different readers. The prevalences of the respective patterns ANCA, pANCA (ethanol+), and pANCA (ethanol+/formalin−) are shown in Table 1B and varied between 56% and 70%, between 42% and 64%, and between 40% and 64%. Five samples were reported to be pANCA-positive on the ethanol-fixed substrate and cANCA-positive on formalin-fixed substrates by three laboratories. For one sample, this finding was reported by all three laboratories, whereas for the remaining samples, this observation was unique for each laboratory (see Table 1B). The κ values in the interobserver study were 0.36–0.65, 0.35–0.72, and 0.28–0.71 for p- and cANCA, pANCA on ethanol-fixed substrates, and pANCA on ethanol-fixed substrates combined with a negative finding on formalin-fixed substrate (see Table 2a in the online Data Supplement). cANCA was found by all observers: 3 of 50

Table 1. Results of interassay and interobserver variability studies.

A. ANCA patterns observed in the interassay study (n = 50)

	Prevalence, n (%)			
	Bio-Rad	**The Binding Site**	**Immunoconcepts**	**INOVA**
pANCA+				
Ethanol+/formalin+	1	0	3	3
Ethanol+/formalin−	19 (38)	8 (16)	31 (62)	22 (44)
Total	20 (40)	4 (18)	34 (68)	25 (50)
cANCA+(ethanol fixation)	0	1	0	6
ANCA+(pANCA+/cANCA+)	20 (40)	9 (18)	34 (68)	31 (62)
Noninterpretable patterns	0	16	5	3

B. ANCA patterns observed in the interobserver study (n = 50)

	Prevalence, n (%)			
	San Diego	**Iowa City**	**Leuven**	**Torino**
pANCA+				
Ethanol+/formalin+	1	1	3	0
Ethanol+/formalin−	23 (46)	20 (40)	22 (44)	32 (64)
Total	24 (48)	21 (42)	25 (50)	32 (64)
cANCA (ethanol fixation)	4	10	6	3
ANCA+(pANCA+/cANCA+)	28 (56)	31 (62)	31 (62)	35 (70)
Noninterpretable patterns	7	6	3	0

samples in Torino, 6 of 50 in Leuven, 4 of 50 in INOVA, and 10 of 50 in Iowa. Except for Torino, noninterpretable results were observed in 3 of the 50 samples in Leuven, 7 of 50 in San Diego, and 6 of 50 in Iowa City.

The differences among the commercially available assays for UC-associated pANCA detection were remarkable. These results confirm earlier data of Sandborn et al. *(9)*. We were unable to identify the exact nature of the differences observed. To clarify this issue, a more extensive study is needed in which only one variable is examined at a time (e.g., substrates from different manufacturers with the same conjugate and buffer). This, however, was beyond the scope of the present study.

Better agreement in UC-associated pANCA detection was found when we looked at the interobserver study, although there were differences in the interpretation of results. It has been suggested that differences among assays could be explained by the fact that different assays preferentially detect different antigens *(9)*. Because low κ values were also seen in the present interobserver variability study, this suggestion is probably only a partial explanation. Immunofluorescence microscopy implies semiquantitative results. Determinations are thus dependent on the expertise of the technician, the variable quality of test reagents, and the equipment used.

cANCA staining patterns were found in this UC study population by all observers and by two of four assays. This observation suggests that sera from a subgroup of UC patients also reacts against a cytoplasmic antigen. This finding confirms previously reported data *(5)*.

Despite the various observations of UC-associated pANCAs, these antibodies have been suggested to be clinically useful, and the controversies on such issues as their predictive value for pouchitis *(14–17)* and the appearance of these antibodies in unaffected first-degree relatives of patients with UC *(18, 19)* may be partially explained by the large interassay and interobserver variability.

Strict guidelines for immunofluorescence detection of UC-associated pANCAs are needed to standardize this currently used technique until a solid-phase assay is available. Since 1993, an international cooperative study group has reported twice the development of a standardized methodology for the detection of cANCAs and pANCAs by indirect immunofluorescence and solid-phase assays *(20, 21)*. These recommendations are widely accepted and serve as the gold standard for detection of Wegener granulomatosis and small-vessel vasculitis. This is not the case for UC-associated pANCAs. Recently, Terjung et al. *(11)* attempted to define reliable microscopic criteria by use of indirect immunofluorescence microscopy and confocal laser scanning microscopy as well as various fixatives to be able to distinguish, in particular, UC-associated pANCAs from other pANCAs. However, the criteria they defined have not yet been validated internationally. In our study, sera obtained from UC patients produced different patterns on ANCA substrates: pANCA, cANCA, noninterpretable, and negative results on ethanol-fixed substrates and negative or positive (cANCA pattern) results on formalin-fixed substrates.

A 50-kDa myeloid cell-specific nuclear envelope protein has been reported as the target antigen of UC-associated pANCAs. Reactivity to this new identified antigen was found in 92% of sera containing UC-associated pANCAs *(22)*. Identification of the target antigen may not only lead to better understanding of the possible role of these antibodies in the immunopathogenesis of

IBD, but may hopefully lead to the development of highly sensitive, specific, and reproducible assays.

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Survivin mRNA Copy Number in Bladder Washings Predicts Tumor Recurrence in Patients with Superficial Urothelial Cell Carcinomas, *Iman J. Schultz,*[1*] *Lambertus A. Kiemeney,*[2,3] *Herbert F.M. Karthaus,*[4] *J. Alfred Witjes,*[3] *Johannes L. Willems,*[1] *Dorine W. Swinkels,*[1] *Jacqueline M.T. Klein Gunnewiek,*[1] *Jacques B. de Kok*[1] (Departments of [1] Clinical Chemistry, [2] Epidemiology and Biostatistics, and [3] Urology, University Medical Center Nijmegen, Nijmegen, The Netherlands; [4] Department of Urology, Canisius Wilhelmina Hospital, Nijmegen, The Netherlands; * address correspondence to this author at: AKC/564, University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands; fax 31-243541743, e-mail i.schultz@akc.umcn.nl)

Urothelial cell carcinoma (UCC) is the most common form of bladder cancer *(1)*. Approximately 70% of patients diagnosed with UCC have superficial tumors (designated Ta or T1) and are treated by transurethral resection of the tumor (TURT). Most of these patients develop recurrences after TURT and therefore need to be followed intensively. Cystoscopy is the gold standard to check a patient's bladder for recurrences. Unfortunately, cystoscopy is invasive, labor-intensive, and costly. Accurate prediction of UCC recurrence could significantly reduce the number of cystoscopies performed during patient follow-up.

Currently, the major determinants of a patient's follow-up scheme and treatment procedures are pathologic tumor stage and grade. However, pathology-based assessment of bladder tumor stage and grade is subject to variability *(2, 3)*. Furthermore, it is important to address other disease characteristics, such as tumor multiplicity and tumor size, to carefully assess the risk of recurrence in patients with superficial UCC *(4–6)*. As a possibly better and more standardized estimation of the risk of recurrence in patients with UCC, molecular biological alterations have been studied *(7–10)*. An important molecular tumor marker that has emerged is survivin *(11)*. The concentrations of both survivin protein and mRNA in tumor tissue from patients with superficial UCC are indicative of the risk of tumor recurrence *(12, 13)*.

Assessment of prognosis in UCC is usually restricted to analysis of the resected tumor tissue. However, routine cystoscopy frequently overlooks carcinoma in situ and