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Comparison of different immunoassays for the detection of antibodies against Intrinsic Factor and Parietal Cells

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ABSTRACT

Objectives: In the diagnostic work up of autoimmune gastritis several immunological methods are available for the detection of antibodies against Intrinsic Factor (IF) and Parietal Cells (PC). However, there are no recent reports directly comparing all the available assays and methods. The objective of this study was to compare the performance of several commercially available anti-IF and anti-PC antibody assays from different manufacturers in a multi-center multi-cohort setting.

Methods: Sera were used from 5 different cohorts consisting of samples from 25 healthy elderly, 20 HCV or HIV positive patients and 150 patients positive for anti-IF or anti-PC antibodies or in whom these antibodies were requested. These cohorts were tested for anti-IF antibodies with 6 different assays (IIF, ELISA, DIA and EliA) and for anti-PC antibodies with 7 different assays (IIF, ELISA, DIA and EliA). Performance was evaluated by calculating the concordance and relative sensitivity and specificity.

Results: Good concordance was found between the assays for both antibody specificities, ranging from 81 to 100% and 91–100% for anti-IF and anti-PC antibodies, respectively. Highest relative sensitivity was found with the (automated) ELISA based methods. However, all assays had a relative sensitivity between 85 and 100% for anti-IF antibodies and between 95 and 100% for anti-PC antibodies. The relative specificity ranged between 76 and 100% for anti-IF antibodies and between 96 and 100% for anti-PC antibodies.

Conclusions: We conclude that most assays perform well and are concordant to each other, despite the methodological differences and the different sources of antigen used. However, the method used affects the sensitivity and specificity. The (automated) ELISA based assays have the highest relative sensitivity and relative specificity. Care should be taken in the interpretation of positive results by IIF and negative results by the Blue Diver when testing for anti-IF antibodies.

1. Introduction

Chronic auto-immune atrophic gastritis (CAAG) is an auto-immune disease that affects the mucosa of the corpus-fundus of the stomach. CAAG is characterized by an infiltration of T and B cells in the mucosa leading to the destruction of gastric fundic glands, inducing metaplastic changes and replacement of parietal cells (PC) and zymogenic cells by intestinal epithelial cells. Subsequent loss of normal gastric cell composition leads to hypochlorhydria and reduced production of Intrinsic Factor (IF). Patients with CAAG can be asymptomatic or have several hematological, gastrointestinal or neurological symptoms related to the reduced uptake of iron, vitamin B12, and hypergastrinemia. Vitamin B12 is an essential, protein-bound nutrient only found in animal products or fortified food products. In the acidic milieu of the stomach vitamin B12 dissociates from these proteins. Intrinsic factor produced by the PC in the stomach is essential for the subsequent uptake of Vitamin B12 in the duodenum (REF (Minalyan et al., 2017).

Although the pathophysiology of CAAG is not yet known, antibodies against IF and/or PC are highly specific for CAAG especially in patient with pernicious anemia (REF (Lahner et al., 2009). Still, the diagnosis of CAAG is challenging due to the heterogeneity in clinical characteristics and laboratory features and usually requires a combination of clinical,

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serological and histological data (REF (Lenti et al., 2017; Lenti et al., 2019). Antibodies against PC have a sensitivity of 85–90% in patients with CAAG, however they are also found in healthy individuals and other auto-immune diseases, reducing their specificity (REF (Lahner et al., 2009; Rusak et al., 2016b). It has also been proposed that antibodies against PC may be detected before the onset of the symptoms (REF (Minalyan et al., 2017; Toh et al., 2012). The sensitivity of detection is related to the method used, with ELISA being reported to be more sensitive compared to indirect immune fluorescence (IIF) for the detection of anti-PC antibodies (REF (Toh et al., 2012). Antibodies against IF have a lower sensitivity of 60%, when measured with ELISA, however they are highly specific (98.5%) and seem to correlate very well with gastric atrophy (REF (Lahner et al., 2009). To allow a better selection of patients requiring further histopathological biopsy the term serological biopsy has been proposed, which includes a combination of markers to test deviant levels of gastrin and presence of antibodies against IF, PC and *H. pylori* (REF (Minalyan et al., 2017).

The patient population and characteristics in which anti-IF and anti-PC antibodies are requested is heterogeneous in terms of clinical symptoms, additional laboratory parameters but also requesting specialization. Furthermore, there is no gold standard for the assessment of antibodies against IF or PC and the sensitivity of the available diagnostic methods (IIF, RIA, ELISA etc.) differs. Therefore, data on the clinical performance of the individual assays remains unclear (REF (Toh et al., 2012; Bagnasco et al., 2018; Rusak et al., 2016a). Recently, a new automated fluorescence based random access assay has become commercially available (EliA IF and EliA PC). We therefore performed a multi-center and multi-assay comparison for antibodies against PC (7 assays) and IF (6 assays) based on 4 different methods (IIF, ELISA, LIA en FEIA). The study design was based on a recent guideline addressing multi-center validations of auto-immune assays by a group of Dutch medical immunology laboratory specialists (REF (Mulder et al., 2018). We obtained samples from 4 different hospitals and from 5 different cohorts of patients to minimize a selection-based bias and compared the results of the individual assays in relation to each other and the consensus of all the assays to get a better understanding of the performance of the different assays.

2. Materials and methods

2.1. Sample selection

Sera from five different cohorts were collected from existing biobanks from the University Medical Center Groningen (UMCG), the Maastricht University Medical Center (MUMC), the Meander Medical Center (MMC) and the Catharina Hospital Eindhoven (CZE) (Table 1). The first cohort comprised sera from apparently 25 healthy, elderly controls (HC), taken from an existing UMCG cohort of aged (> 65 years) healthy individuals, screened for past and present morbidities. The second cohort comprised 20 sera from patients with an active infection (10 HCV and 10 HIV) obtained from the UMCG. The third cohort comprised 50 sera obtained from the MUMC, derived from random patients for whom anti-PC and anti-IF antibodies were requested in the course of the selection period (spring 2017) irrespective of the final results. The fourth cohort comprised 50 biobank sera obtained from the UMCG, MUMC, MMC and CZE that had previously been reported to the clinic as anti-PC antibody positive. The fifth cohort comprised 50 biobank sera obtained from the MMC, MUMC and CZE that had previously been reported to the clinic as anti-IF antibody positive. Description of the cohorts are shown in Table 1.

2.2. Anti-Parietal cell and anti-Intrinsic Factor antibody detection by indirect immunofluorescence

All IIF assays were performed in the UMCG and according to the instructions provided by the manufacturer.

For anti-PC antibody detection by indirect immunofluorescence, rat liver/kidney/stomach section slides (NOVA lite, Inova Diagnostics, San Diego USA) or stomach (monkey/rat biochip slides (Euroimmun, Lübeck, Germany) were used. Incubation with patient sera was done at a dilution of 1:20 (NOVA lite) or multiple, 1:10–1:3200, dilutions (Euroimmun IIF).

For anti-IF antibody detection by IIF, slides coated with fine droplets of porcine stomach mucosa derived intrinsic factor (Euroimmun, Lübeck, Germany) were used. Incubation with patient sera was done at multiple, 1:10–1:3200, dilutions. Each slide was evaluated by two technicians and scored as positive or negative.

2.3. Anti-Parietal cell and anti-Intrinsic Factor antibody detection by ELISA

Antibody detection against PC and IF by ELISA was performed using commercially available ELISAs from Euroimmun (Lübeck, Germany), Inova Diagnostics, San Diego USA) and Orgentec (Mainz, Germany). For the detection of anti-PC antibodies, purified H^+/K^+ ATPase from porcine gastric mucosa was used as antigen in ELISAs from Euroimmun, Inova Diagnostics (QuantaLite), and Orgentec (Alegria). For the detection of anti-IF, the Euroimmun ELISA plates were coated with intrinsic factor purified from pig gastric mucosa. For the QuantaLite and Alegria IF purified full-length recombinant human intrinsic factor is used as antigen. The QuantaLite and Euroimmun are classic ELISAs, whereas the Alegria is an semi-automated ELISA based assay using strips. All assays were performed according to manufacturer's instructions and with QuantaLite performed at the Catharina Hospital Eindhoven and Alegria at the RH-MDC Delft).

2.4. Anti-Parietal cell and anti-Intrinsic Factor antibody detection by FEIA

Antibody detection against IF or PC by FEIA (Fluorescence Enzyme Immunosorbent Assay), Thermo Fisher, Freiburg, Germany) was performed on an Immunocap 250 with well technology with reagents (EliA IF and EliA PC) and equipment from Thermo Fisher (Freiburg, Germany), according the manufacturer's defined automated protocol. For detection, enzyme-labeled antibodies against human IgG antibodies (EliA IgG Conjugate) were used. The IF and H^+/K^+ ATPase used for coating was of human origin and isolated from porcine gastric cells respectively. Assays were performed at Meander Medical Center (cohort 4 en 5) and UMCG (cohort 1, 2, 3, 4 and 5).

Table 1
Samples characteristics used in the method and assay comparison of anti-IF and anti-PC antibodies.

Cohort	Number of samples	Center	Characteristics
1	25	UMCG	Healthy elderly
2	20	UMCG	Infection (10 HIV/10 HCV)
3	50	MUMC (PC with IIF and IF with ELISA)	Random PC/IF antibody request
4	50	UMCG (13 IIF), MUMC (13, IIF), MMC (12 IIF), CZE (13 ELISA)	anti-PC antibody positive
5	50	MMC (15 Blot), MUMC (17, ELISA), CZE (17 ELISA)	anti-IF antibody positive

UMCG; University Medical Center Groningen, MUMC; Maastricht University Medical Center, MMC; Meander Medical Center Amersfoort, CZE; Catherina Hospital Eindhoven. Between brackets the number of samples provided by each center and method of initial testing if applicable for the selection of samples.

2.5. Anti-Parietal cell and anti-Intrinsic Factor antibody detection by immunoblot

Antibodies against IF or PC were detected with the BlueDiver Gastritis IgG Immunodot kit (D-tek, Mons, Belgium) according to the manufacturer's instructions. Strips used in this assay were coated with IF (porcine stomach) and H^+/K^+ ATPase purified from porcine gastric mucosa. The intensity measured by densitometry (BlueScan and Dr. DOT software, D-tek, Mons, Belgium) is directly proportional to the antibody present. Assay was performed at Maastricht University Medical Center.

2.6. Statistical analysis

There is no gold standard in the measurement of anti-IF and anti-PC antibodies, nor were the sera clinically validated or derived from patients with a predefined clinical diagnosis. This means that no conclusion can be drawn on the clinical correctness of the results described in this study. However, since all results were interpreted as negative or positive according to the manufacturers' defined cut off, we calculated concordance of the individual assays with the consensus result. Consensus for anti-IF antibodies was defined as corresponding results for 5 of the 6 assays tested (either positive or negative, with equivocal results counted as positive). Consensus for reporting anti-PC antibodies was defined as corresponding results for 5 of the 7 assays tested (either positive or negative, with equivocal results counted as positive). We defined a Cohen's kappa coefficient of agreement with the consensus of ≥ 0.8 as a minimum acceptable performance value for a test used for clinical diagnostic purposes. Next, we calculated the relative sensitivity and specificity of the different assays. For the calculation of the relative sensitivity and relative specificity, we defined a reference result (positive or negative) based on unanimous test results. For anti-IF antibodies this implied that sera were included for which a unanimous test result (positive or negative, with equivocal results counted as positive) was obtained from 5 of the 6 assays. In practice, this definition was equal to of the consensus used to calculate the Cohen's kappa of agreement. For anti-PC antibodies this implied that sera were included for which a unanimous result (positive or negative, with equivocal results counted as positive) was obtained from 6 of the 7 assays. This resulted in the exclusion of 15 from a total of 195 sera used for anti-IF antibody characteristics and in the exclusion of 20 of 195 sera used for anti-PC antibody characteristics due to no consensus in determination of the reference result.

3. Results

3.1. Characteristics and method comparison of anti-IF antibodies assays

The assay characteristics and source of antigens used is described in Table 2. To assess the inter-assay variation of the quantitative assays included in this study (Alegria, BlueDiver, Euroimmun-ELISA, ELiA and QuantaLite), two samples with a high, and low concentration of anti-IF antibodies were measured five times. All the assays had an inter-assay variation lower than 15%, except for the samples measured with the BlueDiver (high sample 18.8%, low sample 28.3%) (Table 3). To check the linearity of the quantitative assays we used high anti-IF antibody samples ($> 10\times$ ULN) and made serial dilutions and calculated the r (Toh et al., 2012). The linearity of all tested assays was excellent with an $r^2 \geq 0.98$–1.00 for all assays (Table 3). Results of anti-IF antibody assays may be affected by high levels of e.g. vitamin B12 or rheumatoid factor (RF). Therefore we diluted a high vitamin B12 or RF sample (both $5\times$ ULN) in a normal serum (to a relative concentration of 0%, 25% and 50%) before adding to two different patient samples containing antibodies against IF. As a control, the high RF, high Vitamin B12 and the normal serum were tested negatively in the IF and PC antibody tests respectively. The high vitamin B12 and RF levels did not interfere with the final results of the Alegria, Euroimmun ELISA, EliA or Quantalite (data not shown). Testing the high vitamin B12 and RF samples some of the results with the BlueDiver were false positive, while testing the low vitamin B12 and RF samples, the BlueDiver results were equivocal (data not shown). Remarkably, the Euroimmun IIF results appeared false positive using the anti-IF antibody negative samples, also in the absence of interfering substances (data not shown).

For the assay comparison we used 195 samples from different cohorts (Table 1) and calculated the concordance, Cohen's Kappa and relative sensitivity/specificity for all the assays included in this study. Consensus for corresponding results in 5 of the 6 assays used to detect anti-IF antibodies was found in 180 of the 195 samples (92%).

The Cohen's kappa of the Euroimmun IIF assay was 0.6. The Cohen's kappa of the BlueDiver assay was 0.9. The Cohen's kappa of the other assays was 1.0. The lower Cohen's kappa for the BlueDiver assay was caused by 6 false negatives out of 180 assessed samples in the cohort that was selected based on positive results obtained in routine diagnostics. The lower Cohen's kappa for the Euroimmun IIF assay was caused by 34 false positives out of 180 assessed samples across the different cohorts (supplemental Table 1). These results were in line with the observation in the previous experiments assessing the interference of vitamin B12 and RF. Of note, the false positive results found by this assay were almost all consensus-positive for anti-PC antibodies.

We also calculated the relative sensitivity and relative specificity of the different assays. The relative sensitivity of all assays, except the BlueDiver assay (85%), was 98% - 100%. The relative specificity of all assays, except the Euroimmun IIF (76%), was 99% - 100% (Table 4).

Having performed all the assays with the same 195 sera allowed direct reciprocal comparison of the assays. In the 195 samples from all the cohorts, anti-IF antibodies were measured with the different assays and compared with each other using linear regression and Bland-Altman analysis. With the reciprocal comparisons, the Pearson correlation coefficient (r(Toh et al., 2012)) was > 0.8 with a P value < 0.01 for all the assays (Alegria, Euroimmun ELISA and QuantaLite) except for the Bluediver and EliA, which had a r^2 value of 0.74 and 0.73 respectively in comparison with the QuantaLite (data not shown). Being the newest assay commercially available, we made a primal comparison of the EliA assay versus the other assays. Bland Altman analyses revealed that despite the good correlation between the assays, for the quantitative assays there were significant differences in the test characteristics. In general, the results obtained with the Euroimmun ELISA had a negative bias compared to the other assays, while the results from EliA had a positive bias compared to the other assays (supplemental fig. 1). This means the results of EliA are expressed in higher units, while the Euroimmun ELISA are expressed in lower units compared to the other assays. Of note, this does not mean that the relative sensitivity of the EliA is higher than the Euroimmun ELISA.

3.2. Characteristics and method comparison of anti-PC antibodies assays

As with the anti-IF antibody assays, we also looked at inter-assay variation, linearity and interference of the anti-PC antibody detection assays. All the quantitative assays had an inter-assay variation lower than 15% except for the low anti-PC antibody sample measured with the BlueDiver. The high anti-PC antibody sample measured with the Alegria and EliA were above the upper limit of quantification and therefore, no CV was calculated for these combinations (Table 3). Of note, there was very little difference in U/ml between the intermediate and low anti-PC antibody samples measured with EliA (data not shown). The linearity was good for most of the tested assays with an $r^2 \geq 0.86$–1.00, but lower compared to the anti-IF antibody assays (Table 3). Dilution in sera containing high RF (> 5 ULN) or vitamin B12 levels (> 5 ULN), did not influence the measurement of anti-PC antibodies in any of the compared assays (data not shown).

The same 195 samples used for comparing the anti-IF antibody detection assay characteristics were also used for method comparison

Table 2
Characteristics of test used for anti-IF and anti-PC antibody assay comparison.

Anti-Intrinsic Factor antibodies						
Test	Source	Range and units	Cut-off	Positive	Equivocal	Negative
Alegria	Human	0–100 U/ml	6 U/ml	≥ 6 U/ml	NA	< 6 U/ml
BlueDiver	Porcine	0–100 AU	10 AU	> 10 AU	5–10 AU	< 5
Euroimmun ELISA	Porcine	Ratio	1	≥ 1	NA	< 1
Euroimmun IIF	Porcine	Titer	1:10	IF at 1:10		No IF at 1:10
EliA	Human	0,5–480 U/ml	10 U/ml	> 10 U/ml	7–10 U/ml	< 7 U/ml
QuantaLite	Human	U/ml	25 U/ml	≥ 25 U/ml	20,1–24,9 U/ml	≤ 20 U/ml

anti-Parietal cell antibodies						
Test	Source	Range and units	Cut-off	Positive	Equivocal	Negative
Alegria	Porcine	0–100 U/ml	10 U/ml	≥ 10 U/ml	NA	< 10 U/ml
BlueDiver	Porcine	0–100 AU	10 AU	> 10 AU	5–10 AU	< 5
Euroimmun ELISA	Porcine	Ratio	1	≥ 1	NA	< 1
Euroimmun IIF	Monkey stomach	Titer	1:10	IF at 1:10		No IF at 1:10
EliA	Porcine	0,2–192 U/ml	10 U/ml	> 10 U/ml	7–10 U/ml	< 7 U/ml
QuantaLite	Porcine	U/ml	25 U/ml	≥ 25 U/ml	20,1–24,9 U/ml	≤ 20 U/ml
NOVA lite	Rat stomach	Titer	1:20	IF at 1:20	Aspecific IF	No IF at 1:20

Characteristics as reported by the manufacturer, source indicates the origin of the respective antigen used. *NA Not Applicable, AU Arbitrary Units.*

Table 3
Assay variation and linearity.

Anti-Intrinsic Factor antibodies			
Test	CV Inter low	CV Inter high	Linearity (r^2)
Alegria	4.8	7.3	0.99
BlueDiver	28.3	18.8	0.99
Euroimmun ELISA	4.2	4.1	0.99
Euroimmun IIF	nd	nd	nd
EliA	10.7	11.2	1.00
QuantaLite	5.1	10.0	0.98

anti-Parietal cell antibodies			
Test	CV Inter low	CV Inter high	Linearity (r^2)
Alegria	10.7	No results	No results
BlueDiver	7.4	5.4	0.97
Euroimmun ELISA	3.7	1.9	0.86
Euroimmun IIF	nd	nd	nd
EliA	3.5	No results	1.00
QuantaLite	3.2	9.0	0.92
NOVA lite	nd	nd	nd

nd; Not Determined.

and characterization of the different anti-PC antibody assays (cohort 1–5 from Table 1). For anti-PC antibodies the consensus was defined as concordance in the results of 5 of the 7 assays, which rendered 175 of 195 samples (89%) to be included for the analysis. Because four results tested with the NOVA lite IIF assay could not be interpreted as positive or negative, due to non-specific staining, we used 171 of 195 samples (87%) in the analysis for this assay. For the calculation of the relative sensitivity and relative specificity, samples were used for which a unanimous result in 6 of the 7 assay was found. This latter was found for 164 of the 195 samples (85%).

The Cohen's kappa of the EliA, QuantaLite, Alegria, BlueDiver and Euroimmun ELISA was 1.0. The Cohen's kappa was 0.9 for the NOVA lite and 0.8 for the Euroimmun IIF, which was caused by a few false negative results across all cohorts (Table 4 and supplemental Table 2). The relative sensitivity and specificity of the different assays was good (> 95%). The relative sensitivity of 95% of the BlueDiver and the Euroimmun IIF was caused by 4 false negative results across all cohorts. The relative specificity of 96% of the Alegria was caused by 3 false positive results in samples from HIV or HCV infected patients (Table 4 and supplemental Table 2).

With the reciprocal comparisons for the quantitative assays (BlueDiver, Alegria, Euroimmun ELISA and the QuantaLite) Pearson correlation coefficients (r(Toh et al., 2012)) were all > 0.8 with a P value < 0.0001. The EliA had lower correlation coefficients with the Alegria assay (0.49), the Euroimmun ELISA (0.7) and the BlueDiver (0.74). Comparison of the assays by Bland Altman analysis showed remarkable differences in the assay characteristics. As with anti-IF antibodies, also with anti-PC antibodies we focused with Bland-Altman comparison on the new EliA assay versus the other assays. Compared to the Alegria the EliA appeared less sensitive with intermediate levels of anti-PC antibodies, while having an higher upper limit of detection. The same pattern was seen with the BlueDiver and to a lesser extent also with the QuantaLite. The Euroimmun ELISA results were characterized by a negative bias across the whole measuring range compared to the other assays. A pattern also seen with anti-IF antibodies (Supplemental fig. 2).

4. Discussion

Presence of antibodies against IF and PC play an important role in the diagnosis of CAAG, since patients can be asymptomatic or the symptoms of the resulting Vitamin B12 deficiency initially may be vague (REF (Minalyan et al., 2017). In 60% of the patients with a severe Vitamin B12 deficiency, antibodies against PC or IF are found and especially anti-PC antibodies may be present years before the clinical symptoms of CAAG (REF (Toh et al., 2012). Given the importance of their detection, it is important to note that the method used significantly affects the sensitivity and specificity. Sensitivity of ELISA has been reported to be superior to IIF for the detection of anti-PC antibodies (REF (Toh et al., 2012). Furthermore, when screening young and healthy male blood donors for anti-PC antibodies with ELISA, increased numbers were found compared to IIF. This is surprising, since in this population no antibodies against PC are expected and unfortunately, no further clinical or serological information was available to calculate the specificity of these low titer antibodies (REF (Bagnasco et al., 2018). In addition, RIA and recently an automated ELISA based fluorescence assay has become available for the detection of anti-PC antibodies. As with anti-PC antibodies, for anti-IF antibody detection several techniques are available with ELISA probably being the most sensitive (REF (Corcuff et al., 2008; Berth et al., 2016). There are no recent reports

Table 4
Concordance, relative sensitivity and specificity of anti-IF and anti-PC antibody assays.

	Anti-Intrinsic Factor antibodies					
Test	Concordance with positive results	Concordance with negative results	Overall concordance	Cohen's Kappa	Sensitivity	Specificity
Alegria	97.5	99.3	99	1.0	98	99
BlueDiver	85.0	100	97	0.9	85	100
Euroimmun ELISA	100	100	100	1.0	100	100
Euroimmun IIF	100	75.7	81	0.6	100	76
EliA	100	100	100	1.0	100	100
QuantaLite	100	100	100	1.0	100	100
	Anti-Parietal cell antibodies					
Test	Concordance with positive results	Concordance with negative results	Overall concordance	Cohen's Kappa	Sensitivity	Specificity
Alegria	100	96.3	95.3	0.9	100	96
BlueDiver	95.1	100	97.7	1.0	95	100
Euroimmun ELISA	100	98.8	98.9	1.0	100	99
Euroimmun IIF	95.1	98.8	91.4	0.8	95	99
EliA	100	100	98.9	1.0	100	100
QuantaLite	100	100	100.0	1.0	100	100
NOVA lite	98.7	98.8	95.3	0.9	99	99

comparing the different assays for the detection of anti-IF and anti-PC antibodies. In this study we therefore validated 7 different assays based on 4 different methods for detection of antibodies against PC and 6 different assays based on 4 different methods for detection of antibodies against IF.

In the Netherlands, the most widely used method for the detection of anti-IF antibody, according to the Dutch External Quality assessment scheme (SKML), is ELISA followed by IIF. The results from this study show that there is a good concordance for anti-IF antibody detection assays included in this study, with a consensus of 92% between all the assays with exception of the IIF. The IIF had a lower Cohen's Kappa of 0.6, mainly due to false positive results. Interestingly, these false positive IIF results were all found in the cohort of samples from anti-PC antibody positive patients. This may indicate interference by anti-PC antibodies in the anti-IF antibody assay or the IIF assay being more sensitive than the other assays, detecting lower levels of anti-IF antibodies. Indeed, seroconversion towards being positive for anti-IF antibodies later during the disease has been described in patients with pernicious anemia (REF (Minalyan et al., 2017), REF (Toh et al., 2012). However, given the reported sensitivity and specificity of the methods, this is not likely (REF (Corcuff et al., 2008; Berth et al., 2016). Furthermore, it has also been stated that positivity for anti-IF antibodies in the absence of anti-PC antibodies is very rare (REF (Berth et al., 2016; Khan et al., 2009). Indeed, in our entire cohort there was only one sample that tested positive for anti-IF antibodies in all assays and negative for anti-PC antibodies. Of note, in the cohort of 25 healthy elderly we found four subjects who tested negative for anti-PC antibodies and positive for anti-IF antibodies by IIF only. This is in line with the findings of Khan et al., who also found the same pattern of anti-PC negative, anti-IF positive antibodies in the older age group (REF (Khan et al., 2009). A possible explanation for the general high false positive anti-IF antibody detection rate with IIF might be that the IF isolated from porcine origin contains trace amounts of gastric H/K ATPase, the antigen recognized by anti-PC antibodies.

The concordance results are in line with a recent report were 5 different anti-IF antibody ELISAs were compared and good agreement between the different assays was found (REF (Berth et al., 2016). The good agreement between the different assays is in part remarkable, since concordance and harmonization in autoimmune testing remains difficult to achieve due to the heterogeneity of the autoimmune antibodies, as has been described e.g. for anti-phospholipid antibody tests and anti-dsDNA tests (REF (Mummert et al., 2018; Chayoua et al., 2019). In this light, the concordance found is especially interesting since the origin of antigens used in the tests studied here differs, indicating no apparent advantage of one specific source.

Since there is no gold standard for anti-IF and anti-PC antibody detection, the relative sensitivity and specificity was calculated by comparing the result to the consensus results. The good concordance between the different assays therefore also leads to excellent relative sensitivity and relative specificity for most of the assays, again with the exception of the Euroimmun IIF and the BlueDiver. The IIF had a lower specificity because of several false positives across the different cohorts. As described above most of these false positive anti-IF antibody results by the IIF were positive for anti-PC antibodies. Also interesting is the lower relative sensitivity of the assay based on immunoblot. The IF antigen source of the BlueDiver is the same as for both ELISAs (porcine). However, the method used for signal detection (densitometry on blot) is different, causing borderline samples to be incorrectly called negative by the BlueDiver.

Also for anti-PC antibodies there was a good concordance between the different assays, with excellent relative sensitivity and relative specificity for all the assays compared in this study. In 20 out of the 195 samples no consensus with a corresponding result for 5 of 7 assay was found. In contrast to anti-IF antibody testing, where only the immunoblot had a lower relative sensitivity, also one of the IIF assays for anti-PC antibodies had a slightly lower relative sensitivity. This is in line to what has been described in the literature, where ELISA or RIA assays perform better than IIF assays (REF (Toh et al., 2012), REF (Bagnasco et al., 2018). The difference in relative sensitivity between the two IIF assay included in our study could be related to the antigenic source, rat versus monkey stomach. Considering the relative specificity of the 7 assays, the only assay with a slightly lower relative specificity for anti-PC antibodies was the Alegria, with a relative specificity of 96% compared to the 99–100% of the other assays. This was caused by a few false positives in the cohort of HIV/HCV infected patients. Laboratories may therefore consider to implement reflex testing and confirm a positive anti-PC antibody result from Alegria with another assay. While ELISA is the method most used in the Netherlands for anti-IF antibody testing, anti-PC antibody detection is mostly done by IIF. However, in line with previously reported findings, we here show that ELISA (and FEIA) are more sensitive for the detection of anti-PC antibodies. This is especially important since anti-PC antibodies can already be present in asymptomatic CAAG patients (REF (Toh et al., 2012).

There are a few limitations to our study, the first limitation is that we do not have any clinical data and patient characteristics concerning CAAG or vitamin B12 deficiency. Thereby the true clinical sensitivity

and specificity could not be calculated. The second limitation concerns the lack of a gold standard in anti-IF and anti-PC antibody testing. Comparison of the assays was done by calculating a consensus result consisting of a unanimous result from 5 of the 6 anti-IF and 5 of the 7 anti-PC antibody assays. This method might introduce a slight selection bias by omitting the borderline positive/negative samples in which no consensus was obtained. However, a consensus was obtained for the vast majority of the samples: 92% for anti-IF antibodies and 89% for anti-PC antibodies.

The automated FEIA assay from Thermo Fisher for the detection of anti-IF and anti-PC antibodies, represents an immunoassay with a slightly different analytical principle to ELISA. These assays (EliA IF and EliA PC) showed good concordance with the other assays and also the CV and linearity were comparable to the other assays. Of note, intermediate and weak positive antibody sera were just borderline positive in these assays. This was also reflected by the lower Pearsons correlation calculated for this assay in relation to the other assays. Remarkably, the qualitative test results (positive or negative) of the assays were in good agreement with the other assays resulting in excellent relative sensitivity and specificity. However, these findings also imply that low, indeterminate or equivocal antibody results should be interpreted with caution in routine clinical practice.

In conclusion, irrespective of the source of antigen used, most assays tested perform well for the detection of antibodies against IF and PC and have acceptable analytical characteristics (sensitivity, specificity, reproducibility etc). However, results around the cut-off level should be evaluated with care and, as seen with previous studies, IIF and immunoblot based assays seem slightly less suitable for the detection of anti-IF and anti-PC antibodies. Further studies on the assay performances should include clinical, histological and laboratory parameters to evaluate the true characteristics of these assays.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2020.112867.

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