

Anti-cyclic citrullinated peptide antibodies: a comparison of different assays for the diagnosis of rheumatoid arthritis

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Objectives: Anti-cyclic citrullinated peptide (anti-CCP) antibodies are highly specific markers of rheumatoid arthritis (RA). Considering the heterogeneity of the target antigens involved, and the test platforms and conjugates proposed in commercial anti-CCP assays, we assessed the diagnostic performances of four fully automated anti-CCP assays in a cohort of patients with RA compared to patients with other autoimmune and inflammatory disorders. We also evaluated the agreement between the qualitative results of these immunoassays.

Method: We evaluated three anti-CCP2 assays [Eurodiagnostica enzyme-linked immunosorbent assay (ELISA), Elecsys electrochemiluminescence immunoassay (ECLIA) on the Modular E170 Analyzer, and Zenit chemiluminescence immunoassay (CLIA) on the Zenit RA Analyzer] and one anti-CCP3 assay (Inova ELISA). ELISAs were performed on an automated workstation. Samples from 112 patients with RA and a disease control group of 136 patients (53 with autoimmune diseases, 65 non-autoimmune disorders, and 18 infectious diseases) were studied (included 161 samples submitted consecutively to the laboratory).

Results: At a fixed specificity of 92%, the anti-CCP3 assay presented the highest sensitivity (75%) compared to the anti-CCP2 assays evaluated (63–72%). The Zenit anti-CCP2 assay gave the most false-positive results (especially in patients with viral infections and connective tissue diseases). The agreement between assays ranged from 86.3% to 95.2% and Kappa coefficients ranged from 0.724 to 0.899.

Conclusions: Recently released automated workstations provide a valuable alternative to ELISA to diagnose RA. However, differences in diagnostic performances are highlighted in our experience, especially for the Zenit assay. In our cohort, the anti-CCP3 assay gave slightly better performances than the anti-CCP2 assays (with the exception of the Zenit assay).

Historically, rheumatoid factor (RF) was the only laboratory diagnostic parameter included in the 1987 American College of Rheumatology (ACR) criteria for the classification of rheumatoid arthritis (RA) (1). Nowadays, anti-citrullinated peptide antibodies (ACPAs) are routinely used as serological markers for the diagnosis of RA. With the same diagnostic sensitivity (up to 75%) as RF and a higher specificity (94–99%) (2), ACPAs have been included in the criteria of the new 2010 RA classification. This classification have been developed to allow early management of the disease and to improve its outcome (3–6). ACPAs are useful prognostic markers because their presence is associated with greater radiographic progression and development of joint erosions (7, 8). However, the usefulness of measuring ACPA to monitor the clinical response to therapy is still controversial (8).

Several commercial immunoassays are available to detect ACPAs. Second-generation synthetic cyclic citrullinated proteins (CCP2) are the most widely used substrates in these tests. They have been selected from a screening of libraries of citrullinated peptides for their strong reactivity with sera of patients with RA. Beside anti-CCP2, a third-generation anti-CCP test (anti-CCP3) has been developed to increase sensitivity for the detection of patients with RA. Several automated immunoassay platforms are available as an alternative to enzyme-linked immunosorbent assays (ELISAs).

In this study we assessed the diagnostic performance of four fully automated anti-CCP assays including two random access immunoassay systems with chemiluminescence and electrochemiluminescence detection, Zenit and Elecsys anti-CCP2 assays respectively, and two ELISAs, Eurodiagnostica anti-CCP2 and Inova anti-CCP3 tests, performed on an automated workstation. Considering the heterogeneity of the antigens, the test platforms, and the conjugates used in these assays, we aimed: (1) to evaluate and compare the diagnostic

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accuracy of these tests to detect ACPAs in a cohort of patients with RA compared to patients with other autoimmune (AI) and inflammatory disorders; and (2) to evaluate the agreement between the results of the four immunoassays.

Method

Patients and samples

The study population included 248 serum samples. A first group of samples was obtained from 161 patients for whom anti-CCP antibody determination had been routinely prescribed for investigation of joint disease between December 2010 and August 2011. Clinical data were collected retrospectively by reviewing the electronic medical records. A second group of samples came from 87 individuals selected on the basis of their clinical diagnosis: 23 patients with RA and 64 patients with other AI or inflammatory disorders that may mimic RA and/or give false-positive anti-CCP results. All samples were stored at -20°C before analysis. The collection of sera and their use for this study were approved by our institutional ethical committee.

A total of 112 patients with RA [22 males, median age 57 (IQR 41–68) years; 90 females, median age 58 (IQR 46–69) years] were clinically diagnosed according to the 1987 revised ACR criteria (1). A total of 136 non-RA patients [40 males, median age 50 (IQR 32–60) years; 96 females, median age 50 (IQR 38–61) years] constituted the disease control group. Some of these patients had arthritis but did not fulfill the ACR criteria. The non-RA patients were classified into three groups: (1) AI diseases: patients were diagnosed with connective tissue disease ($n = 20$), juvenile idiopathic arthritis (JIA) ($n = 14$), organ-specific autoimmune disease ($n = 7$), vasculitis ($n = 5$), sarcoidosis ($n = 3$), psoriatic arthritis ($n = 2$), or

immune thrombocytopaenic purpura ($n = 2$); (2) non-AI diseases: patients were diagnosed with spondyloarthritis ($n = 21$), non-specific arthralgia ($n = 15$), degenerative arthropathy ($n = 11$), undifferentiated arthritis ($n = 9$), acute articular rheumatism ($n = 1$), tendinopathy ($n = 1$), epicondylitis ($n = 1$), or other ($n = 6$); (3) infections: patients were diagnosed with HIV ($n = 9$), hepatitis C ($n = 6$), chronic infection ($n = 2$), or hepatitis B ($n = 1$).

Anti-CCP antibody assays

Anti-CCP antibodies were determined using four fully automated immunoassays: (1) a second-generation ELISA (EDIA antiCCP, Eurodiagnostica, Sweden), (2) a third-generation ELISA (QUANTA Lite CCP3 IgG, Inova, San Diego, CA, USA) both carried out on an ELISA automate (DS2 Dynex, Magellan Biosciences, Chantilly, USA), (3) a second-generation electrochemiluminescence immunoassay, the Elecsys anti-CCP assay on the Modular E170 Analyzer (Roche Diagnostics, Mannheim, Germany), and (4) a second-generation chemiluminescence immunoassay, the Zenit anti-CCP assay on the Zenit RA Analyzer (Menarini, Florence, Italy). The procedures were conducted according to the manufacturers' recommendations. Table 1 compares the characteristics of each assay.

Statistical analysis

To evaluate the diagnostic performances of the anti-CCP assays for the diagnosis of RA, the sensitivity, specificity, and likelihood ratio (LR) of each assay were calculated using the manufacturer's cut-off value, the optimal cut-off value obtained by receiver operating characteristic (ROC) curve analysis, and a cut-off value set at a level of 92% specificity. The optimal cut-off value corresponds to the highest value of the sum of the sensitivity and specificity.

Table 1. Characteristics of the four anti-CCP assays evaluated.

	Eurodiagnostica	Inova	Elecsys	Zenit
Principle of the assay	ELISA	ELISA	ECLIA	CLIA
Citrullinated antigen	Second-generation synthetic CCPs	Third-generation synthetic CCPs	Second-generation synthetic CCPs	Second-generation synthetic CCPs
Conjugate	ALP-goat polyclonal anti-human IgG	HRP-goat anti-human IgG	$\text{Ru}(\text{bpy})_3^{2+}$ -mouse monoclonal anti-human IgG	Acridine ester derivative-mouse monoclonal anti-human IgG
Incubation time (min)	120	90	18	20
Sample dilution	1:101	1:101	–	1:50
No. of calibrators	6	5	2	2
Measuring range (U/mL)	0–300	15–250	7–500	0–320
Manufacturer's cut-off level (U/mL)	5	20	17	5

CCP, Cyclic citrullinated peptide; ALP, alkaline phosphatase; CLIA, chemiluminescence immunoassay; ECLIA, electrochemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IgG, immunoglobulin G; $\text{Ru}(\text{bpy})_3^{2+}$, tris(2,2'-bipyridyl)ruthenium(II) complex.

The LR indicates how many times more likely a test result in the given interval is obtained in a patient with RA than in the disease controls (9).

To evaluate the agreement between the qualitative results of the four anti-CCP assays, the overall percentage agreement and Kappa statistics were calculated. The results are expressed as positive or negative according to the manufacturers' cut-off values.

Data were analysed using GraphPad Prism 5 (GraphPad Software Inc, USA).

Results

The distribution of ACPA levels in patients with RA and in the disease control patients for the four immunoassays are shown in Figure 1.

Performances of the four immunoassays for diagnosis of patients with RA in our cohort

The diagnostic sensitivities and specificities of the four immunoassays, calculated according to the clinical diagnosis, are presented in Table 2. At the cut-offs proposed by the manufacturers, the sensitivity ranged from 77% to 79% and the specificity ranged from 79% to 91%. Considering the ROC curve analysis, the optimal cut-off values were determined, especially for the Zenit assay. We set the cut-off for the Eurodiagnostica assay at 3.7 U/mL to improve the sensitivity to 79% without loss of specificity. A cut-off at 24 U/mL for the Inova assay slightly increased the specificity while maintaining the same sensitivity. The cut-off for the Elecsys assay did not differ. The optimal cut-off for the Zenit assay was

Table 2. Diagnostic performances of the four anti-cyclic citrullinated peptide (anti-CCP) assays.

	Cut-off (U/mL)	Sensitivity (%)	Specificity (%)
Eurodiagnostica	5*	76.79	88.97
	3.7 [†]	78.57	88.24
	15 [‡]	70.54	91.91
Inova	20*	78.57	89.71
	24 [†]	78.57	90.44
	56 [‡]	75.00	91.91
Elecsys	17* [†]	76.79	91.18
	48 [‡]	72.32	91.91
Zenit	5*	78.57	79.41
	9 [†]	75.00	88.24
	29 [‡]	63.39	91.91

* Manufacturer's cut-off value.

[†] Optimal cut-off value obtained by receiver operating characteristic (ROC) curve analysis.

[‡] Cut-off at a level of 92% specificity.

increased to 9 U/mL and achieved a specificity of 88% with a sensitivity of 75%. The sensitivities calculated at a predefined specificity of 92% varied from 63% to 75%. The Inova anti-CCP3 assay, and the Eurodiagnostica and Elecsys anti-CCP2 assays exhibited a sensitivity of 75, 71, and 72% respectively, whereas the sensitivity of the Zenit anti-CCP2 assay was substantially lower (63%).

In addition, for each assay we calculated the LR for test result intervals based on cut-offs previously determined in patients with RA and in the disease control group (shown in Table 3). For anti-CCP values between the optimal cut-off value and the upper limit of the detection range, the LR for patients with RA ranged from 5 to 9 depending on the assay, which corresponds to a large increase in the likelihood of patients with RA compared to other diseases.

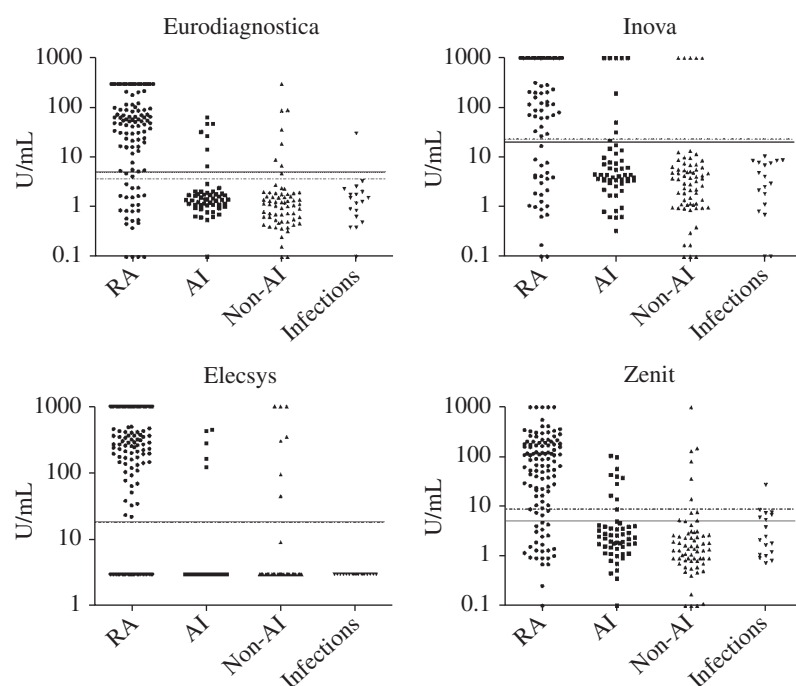


Figure 1. Distribution on a log scale of ACPA levels according to disease category. The solid lines indicate the manufacturers' cut-off values and the broken lines indicate the optimal cut-off. RA, Rheumatoid arthritis; AI, autoimmune diseases.

Table 3. Likelihood ratios (LRs) for test result intervals.

	RA (n = 112)	Non-RA (n = 136)
Eurodiagnostica		
CCP < 3.7 U/mL	0.2	6.7
CCP 3.7–300 U/mL	5.3	0.5
CCP > 300 U/mL	26.7	0.8
Inova		
CCP < 24 U/mL	0.3	8.2
CCP 24–250 U/mL	9.3	0.8
CCP > 250 U/mL	7.9	0.5
Elecsys		
CCP < 17 U/mL	0.3	8.7
CCP 17–500 U/mL	8.5	0.5
CCP > 500 U/mL	9.3	0.8
Zenit		
CCP < 9 U/mL	0.3	6.3
CCP 9–320 U/mL	5.8	0.4
CCP > 320 U/mL	14.6	0.9

RA, Rheumatoid arthritis; CCP, cyclic citrullinated peptide.

The LR increased with increasing antibody levels, which was especially true for the less specific assays, namely the Eurodiagnostica and Zenit assays.

Detailed information about false-positive and false-negative results is shown in Table 4. The number of anti-CCP-positive tests in patients with RA and in the disease control groups were determined at the manufacturers' and the optimal cut-off values. The Zenit assay gave the most false-positive results, especially in patients with viral infections and connective tissue diseases.

Agreement between the results of the four immunoassays

The overall percentage agreement and κ statistics for the four anti-CCP assays are shown in Table 5. The agreement between the qualitative results of the Eurodiagnostica, Inova, Elecsys, and Zenit assays ranged from 86.3% to 95.2%. The κ coefficients between the anti-CCP assays were all above 0.87 except for those between Zenit and the other assays (0.724, 0.768, and 0.748 between Zenit and Eurodiagnostica, Inova, and Elecsys assays, respectively).

Discrepant results among the four immunoassays

Good agreement was found between the assays, especially between the Eurodiagnostica, Inova and Elecsys assays, although we observed a few discordant results between the different assays (shown in Figure 2). The clinical diagnoses for these results are specified Figure 2. The number of discrepant results for the Zenit assay decreased considerably after adjustment of the cut-off value, clearly related to the false-positive results in the patients with HIV.

Discussion

In this study we evaluated and compared the RA diagnostic performance of four recently released fully automated anti-CCP assays, which has remained largely undetermined when tested in comparison with other AI and inflammatory disorders.

Table 4. Number of anti-CCP positive tests in patients with RA and control groups at different cut-off values.

	Total (n)	Eurodiagnostica		Inova		Elecsys	Zenit	
		Cut-off 5 U/mL	Cut-off 3.7 U/mL	Cut-off 20 U/mL	Cut-off 24 U/mL	Cut-off 17 U/mL	Cut-off 5 U/mL	Cut-off 9 U/mL
RA	112	86	88	88	88	86	88	84
AI disease								
CTD	20	3	3	5	5	3	7	6
JIA	14	3	3	3	3	2	3	2
Organ-specific AI disease	7	0	0	0	0	0	1	0
Vasculitis	5	0	0	0	0	0	0	0
Other	7	1	1	1	0	0	1	1
Non AI disease								
SPA	21	1	1	0	0	1	1	0
Non-specific arthralgia	15	2	3	1	1	2	2	1
Degenerative arthropathy	11	2	2	2	2	2	2	2
Undifferentiated arthritis	9	1	1	1	1	1	3	2
Other	9	1	1	1	1	1	3	1
Infection								
HIV	9	0	0	0	0	0	5	0
Hepatitis	7	0	0	0	0	0	2	1
Other	2	1	1	0	0	0	0	0

anti-CCP, Anti-cyclic citrullinated peptide; RA, rheumatoid arthritis; AI, autoimmune; CTD, connective tissue disease; JIA, juvenile idiopathic arthritis; SPA, spondyloarthropathy.

Table 5. Overall percentage agreement and κ statistics between the four anti-CCP assays evaluated.

Assay	κ statistics and percentage agreement between the anti-CCP assays (number of identical results/total)			
	Eurodiagnostica	Inova	Elecsys	Zenit
Eurodiagnostica	—	0.875	0.891	0.724
Inova	94.0 (233/248)	—	0.899	0.768
Elecsys	94.8 (235/248)	95.2 (236/248)	—	0.748
Zenit	86.3 (214/248)	87.5 (217/248)	87.5 (217/248)	—

The choice of automated assays is mandatory, especially for laboratories with a high volume activity, allowing a reduction in workload and improved reproducibility of the results. In this comparison, we included immunoassays that use different methods of detection (chemiluminescence, electrochemiluminescence, and ELISA) and different substrates (second- and third-generation synthetic CCPs) to provide an overview of the variety of commercially available assays. Our population, including both ACPA-positive and ACPA-negative patients, was selected from the requests for anti-CCP antibodies received in the laboratory, in order to give an accurate picture of the daily practice of rheumatology in our hospital. This may explain the slightly lower specificity (88–91%) obtained in this study compared to data reported in the literature (up to 96%) (10, 11).

To compare the sensitivities of the four immunoassays under equal conditions, we arbitrarily set the clinical specificity at 92%. From ROC curve analyses, we concluded that, in our cohort, the anti-CCP3 Inova assay achieved the highest sensitivity (75%) compared to the anti-CCP2 immunoassays tested (71, 72 and 63% for the Eurodiagnostica, Elecsys, and Zenit assays, respectively), although this difference was fairly small, with the exception of the Zenit assay. This observation is consistent with the results from other evaluations (12, 13) showing a better sensitivity of the anti-CCP3 assay (92%, 83%) compared with the anti-CCP2 assay (89%, 79%) with a similar specificity (88%, 93%). However, this superiority that is not found in other evaluations [reviewed in (10)], probably depends on the composition of the non-RA cohort.

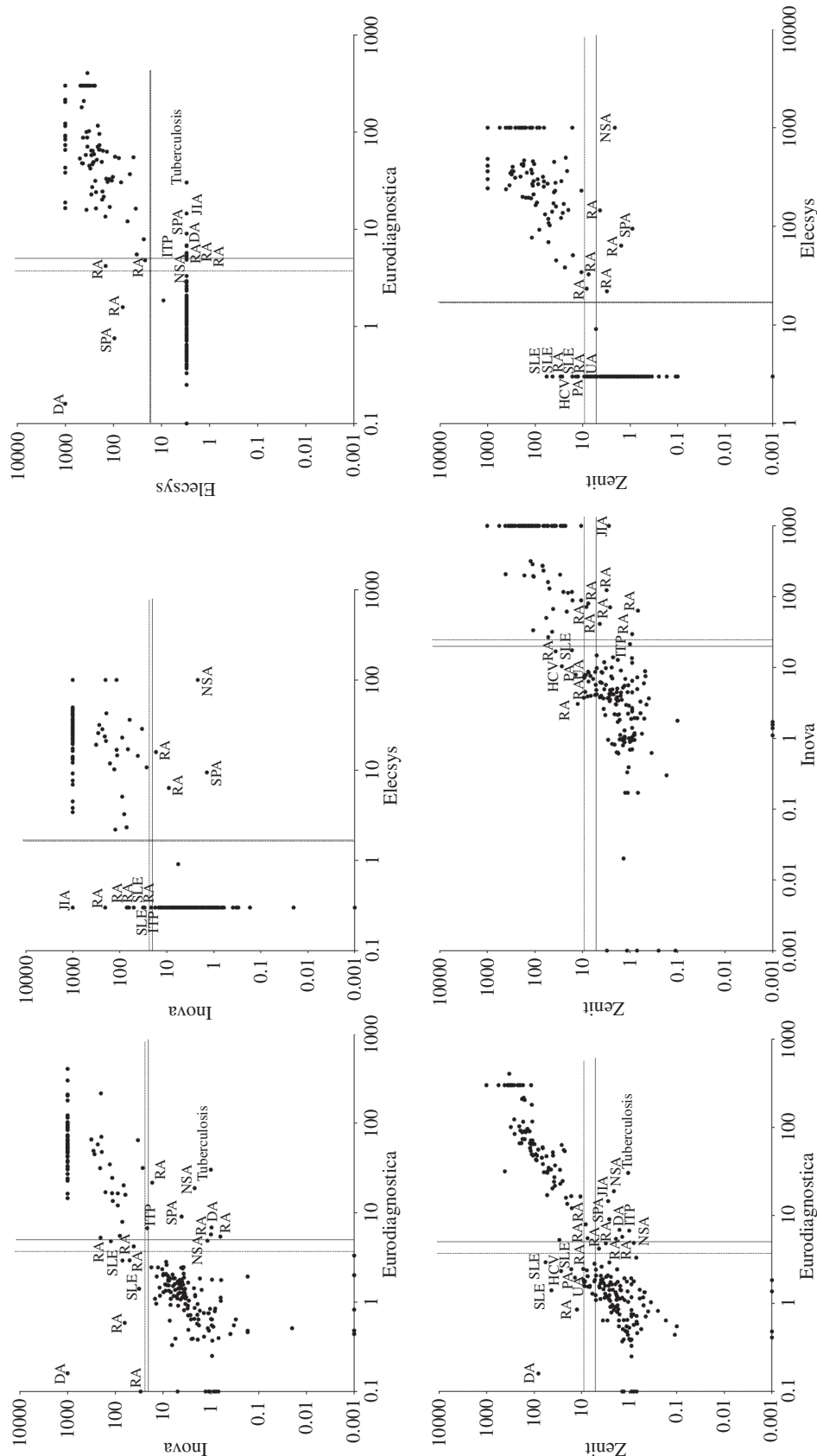
The optimal anti-CCP cut-off values, adjusted for our cohort, were determined for each test and the LR_s were determined for several levels of tests (shown in Table 3). In evidence-based medicine, the LR is used to assess the value of performing a diagnostic test, indicating whether the result of this test usefully alters the probability that the disease exists. In our case, it indicates how the odds of having RA (rather than an other AI or inflammatory disorder) increases when anti-CCP antibodies are positive. One of the main values of the LR is that it does not vary with the prevalence of the disease. Considering the patients with RA, the LR_s for a selected range, delimited by the optimal cut-off value and the upper limit of the detection range, were 5, 9, 9, and 6 for the Eurodiagnostica, Inova, Elecsys, and Zenit assays, respectively. We also observed that a significant increase

in the anti-CCP levels is more in favour of a true RA diagnosis, particularly for the less specific tests, namely the Eurodiagnostica and Zenit assays.

Good agreement was observed between the qualitative results of the Eurodiagnostica, Inova, and Elecsys assays (94–95%), with all κ coefficients > 0.87. However, the Zenit assay showed lower agreement compared to the other assays (86–88%), with κ coefficients between 0.724 and 0.768. A common reference material should allow quantitative comparisons and correlations between the results of the various anti-CCP immunoassays.

Figure 2 shows discrepant results among the four immunoassays, which illustrates again that the autoantibody repertoire of patients with RA is heterogeneous. The anti-CCP3 assay detects ACPAs that are not detected by the anti-CCP2 assays and vice versa. The discrepancies observed between the assays can be attributed not only to the nature of the citrullinated peptides but also to the specific analytical conditions of the assays (e.g. conjugate, sample dilution, solid phase).

The design of our evaluation allowed us to assess the diagnostic performances of the assays in the same context as their routine use. With regard to the false-positive results observed (shown in Table 4), as reported previously in the literature, ACPAs were detected in the samples of some patients (depending on the assay and on the cut-off used) with connective tissue disease (up to seven patients out 20) (e.g. systemic lupus erythematosus, Sjögren's syndrome), JIA (4/14), and infectious diseases (8/18), and also in patients with non-specific arthralgia (3/15), degenerative arthropathy (3/11), and undifferentiated arthritis (3/9). These latter might in fact be patients at risk of developing RA. It is interesting to note that, for the patients with spondyloarthropathy, one of the major differential diagnosis for RA, only two patients out 21 had positive anti-CCP results (using the optimal cut-off value). In addition, three patients out four with JIA with positive anti-CCP results were diagnosed as having polyarticular onset JIA with RF. The presence of ACPAs has been described in some of these cases as being associated with erosive forms of the disease (14). The manufacturer's cut-off value should be adjusted for each assay and laboratory depending on the local population for RA diagnosis. For example, in our cohort, the adjustment of the Zenit cut-off eliminated all of the false-positive results of the HIV-positive patients. The results should always be interpreted depending on the clinical context. The LR_s may help in the interpretation of the test results.



Conclusions

The results of this study indicate that recently released automated workstations provide a valuable alternative to ELISA to diagnose RA. Automation makes these immunoassays very attractive, especially for high-volume laboratories. However, differences in diagnostic performances are observed in our experience, especially for the Zenit assay. This highlights the importance of evaluating assays with an adequate control population before implementation in the laboratory. Proper selection of the control population and adjustment of the cut-off to the studied population have a major impact on the performances of assays for the diagnosis of RA. In our cohort, the anti-CCP3 assays gave slightly better performances than the anti-CCP2 assays (with the exception of the Zenit assay).

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