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ORIGINAL ARTICLE

Autoantibodies against dsDNA measured with nonradioactive Farr assay—an alternative for routine laboratories

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Abstract

Autoantibodies against dsDNA are utilized for the diagnosis and prognosis of SLE as they are highly specific and correlate with disease activity/renal involvement. However, different detection methods are used in routine diagnostic laboratories. Farr radio-immunoassay (Farr-RIA) has been designated as the preferred method, since it provides very specific and at the same time quantitative results, enabling follow-up of level variations over time. Using intercalating fluorescent dsDNA dye would enable all the benefits of Farr-RIA without the radioactive material and organic solvents. To develop a modified fluorescent Farr method (Farr-FIA) and compare it to the classical Farr-RIA in regard to laboratory parameters, as well as clinical utility. Assays were tested on sera of 70 SLE patients, 78 other autoimmune patients, and 145 healthy blood donors. DNA for Farr-FIA was isolated from healthy donor, for Farr-RIA, 14^{C}-labeled dsDNA from *E. coli* was used and mixed with sera in borate-buffered saline, followed by precipitation with saturated ammonium sulfate solution and centrifugation. The supernatant (S) was separated from the precipitate (P), and content of dsDNA was measured with PicoGreen (Invitrogen) in Farr-FIA or radioactive isotope in scintillation solution in Farr-RIA. The results were calculated as a ratio (P-S)/(P+S). Farr-FIA has a diagnostic sensitivity of 53% and diagnostic specificity of 100% (ROC AUC 0.781). Good correlation and agreement were shown between Farr-RIA and Farr-FIA. Also, there is good correlation between Farr-FIA and SLEDAI, comparable to that of Farr-RIA. Farr-FIA differs from Farr-RIA in the changed detection system yielding comparable results and thus could represent a nonradioactive replacement for Farr-RIA.

Keywords Autoantibodies · Biomarker · Diagnostic test · dsDNA antibodies · SLE

Rheumatology in Slovenia: Clinical practice and translational research

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease, characterized by a variety of pathological findings including inflammation, vasculitis, immune complex deposition, and vasculopathy. Among the classification criteria are malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, hematologic disorder, and immunological disorder, with anti-double-stranded DNA antibodies (anti-dsDNA), among others [1, 2]. Anti-dsDNA are highly specific for SLE diagnosis [3, 4], as well as disease monitoring [5, 6].

Anti-dsDNA were first reported and described in 1957 [7]. Since then, reports on their detection have been steadily increasing. Anti-dsDNA constitute classification criterion for SLE revised by the American College of Rheumatology (ACR) in the 1997 and validated by the Systemic Lupus International Collaborating Clinics (SLICC) in 2012 [2]. They are consistently associated with glomerulonephritis in SLE [3] and at least to some extent, also follow disease activity and predict flares [8, 9].

EULAR recommendations designate SLEDAI as one of three (in addition to BILAG and ECLAM) most appropriate SLE flare indexes [4], incorporating 24 clinical and laboratory parameters, including anti-dsDNA antibodies.

There are multiple assay formats routinely used to detect anti-dsDNA, and clinical utility of the results depends very much on the method used [10, 11].

Measurements have been widely performed with *Crithidia luciliae* indirect immunofluorescence test (CLIFT), enzyme-linked immunosorbent assay (ELISA), Farr radioimmunoassay (Farr-RIA) [12, 13], and more recently, also with multiplex bead assays, exploratory microarrays [14], while even taking advantage of surface plasmon resonance biochips [15] and electrophoretic mobility shift assays [16]. Farr-RIA is considered to be most diagnostically specific and at the same time quantitative, which gives it predictive power on activity of disease following SLEDAI/BILAG, as confirmed by several studies comparing methodologies [5, 17–20], but radioimmunoassay in general have been falling into disuse in routine practice based on several factors, including the radioactive nature of the assays.

In CLIFT, anti-dsDNAs are detected by binding to giant mitochondria-kinetoplast of the protozoan flagellate *Crithidia luciliae*. The kinetoplast does not contain any other nucleolar antigens or ssDNA that would facilitate false positive results; however, the method is only semiquantitative and thus not convenient for following disease activity. The second widely used test is ELISA, where dsDNA is bound to a solid surface (which might affect epitopes) and autoantibodies are detected using an enzyme-linked secondary antibody. ELISA detects both low and high avidity anti-dsDNA, thereby increasing its sensitivity and reducing its specificity. However, this test can produce false positive results, due to contamination of ssDNA from denatured dsDNA. Both methods, CLIFT and ELISA, use labeled secondary antibodies in their analyses.

Farr-RIA thus still remains the assay of choice, as a specific assay detecting only clinically important high avidity anti-dsDNA, following a sensitive screen [21]. The detection of high avidity, clinically important autoantibodies is enabled by the ionic strengths and pH of buffers used in the assay. The basic principle of the assay lies in the ability of ammonium sulfate precipitation to discriminate between free dsDNA and dsDNA-antibody immune complexes. The signal from radiolabeled dsDNA is then measured. However, in the last couple of decades, the search has been going on to find a nonradioactive alternative to Farr-RIA [17, 22, 23]. The problem is that the use of radioactive isotopes requires special handling, trained staff, and designated laboratory equipment, and the assay is also ecologically problematic. Recently, our group evaluated four different commercially available assays (two ELISA and two CLIFT) for anti-dsDNA detection [20] and compared them to Farr-RIA, using ^{14}C radioactive isotope-labeled *E. coli* DNA and scintillation fluid with organic solvents for enhancing the signal, as described by Pincus et al. [12].

Therefore, in the current report, we aimed to develop a nonradioactive Farr method and measure immune complex-bound DNA with intercalating dyes for measuring dsDNA, such as PicoGreen. The method precisely followed the Farr-RIA procedure till the final step of measuring, when cyanine fluorescent dye was added to detect dsDNA in the immune complexes. The analytical and diagnostic properties of the modified Farr fluorescent immunoassay (Farr-FIA) were analyzed and compared to the original Farr-RIA.

Methods

Patients and samples

The cross-sectional study compared sera of 70 SLE patients (3 men, 67 women, mean age 40.7 years) with fulfilled ACR criteria for SLE [24] and a patient control group consisting of 78 other systemic autoimmune diseases (14 men, 64 women, mean age 49.1), comprising of 25 antiphospholipid syndrome (APS), 25 Sjogren's syndrome (SjS), and 28 rheumatoid arthritis (RA) patients. Sera from 145 blood donors (89 men, 56 women, mean age 42.7 years) were obtained from the Blood Transfusion Center of Slovenia.

The Committee for Medical Ethics of Slovenia (#138/07/13) approved the study.

Blood withdrawal was performed in the Department of Rheumatology, University Medical Centre Ljubljana, after signing informed consent. Blood was allowed to clot and was centrifuged for 10 min at 1800*g* at room temperature, and sera were aliquoted and frozen at − 80 °C until used.

Farr-RIA

Farr-RIA assay was performed as previously described [20]. Briefly, in Farr-RIA assay 100 ng ^{14}C *E. coli* DNA (Amersham) was used as a source of DNA. After adding sera, borate-buffered saline, ammonium sulfate, and all the incubations and centrifugation in Farr-RIA assay, 100 μl of supernatant and 100 μl of precipitate were mixed with Bray scintillation solution and measured in a β-counter.

Farr-FIA

For Farr-FIA, dsDNA was isolated from human blood using QIAmp DNA Blood Maxi Kit (Qiagen) following manufacturer instructions. Briefly, proteinase K was mixed with 5 ml of whole blood and 5 ml of AL cell lysis buffer and incubated at 70 °C, 10 min. Then, 5 ml of absolute ethanol was added, mixed, and transferred to a membrane column, centrifuged at 1850*g* for 3 min and filtrate discarded. Membrane was washed with 5 ml of AW1, followed by 5 ml AW2 buffer and centrifuged in between. Finally, AE buffer was added to dissolve DNA, and after a short incubation and centrifugation, the concentration of dsDNA was measured using Nanodrop (Thermo Fisher Scientific).

The assay was performed with 100 ng of DNA, mixed with 5 μl of decomplemented sera (30 min, 56 °C) and volume adjusted to 100 μl with borate-buffered saline (0.15 M, pH 8.0). Following 1-h incubation at 37 °C and overnight at 4 °C, 100 μl of saturated ammonium sulfate solution was added and incubated at 4 °C for 1 h. Samples were centrifuged (1800*g*, 15 min, 4 °C) and 100 μl supernatant separated from 100 μl precipitate. Ten microliters of supernatant or precipitate was mixed with 90 μl PicoGreen reagent (diluted in assay buffer 1:200) from Quant-iT™ PicoGreen dsDNA assay kit (Invitrogen) and fluorescence measured in black 96-well plate (Nunc), using plate spectrophotometer Infinite F200 (Tecan). Results from both measurements were calculated as fluorescent signal (precipitate (P) − supernatant (S))/(P + S)).

Statistics

Comparison of Farr-RIA and Farr-FIA methods (Fig. 1) results was calculated using Passing Bablok, Bland Altman analysis, Wilcoxon-matched pair signed rank test, and ROC curve. An overall agreement (kappa coefficient) was estimated according to negative and positive results. The statistical software package SPSS version 22 (IBM, NY, USA), GraphPad version 6 (GraphPad software, San Diego, CA, USA) and Microsoft Excel 2010 were used for analyses. p values < 0.05 were considered significant.

Results

Analytical properties of Farr-FIA anti-dsDNA detection method

Precision of the method was tested with inter-assay and intra-assay variation. The test was performed with high and medium positive samples, according to the Clinical

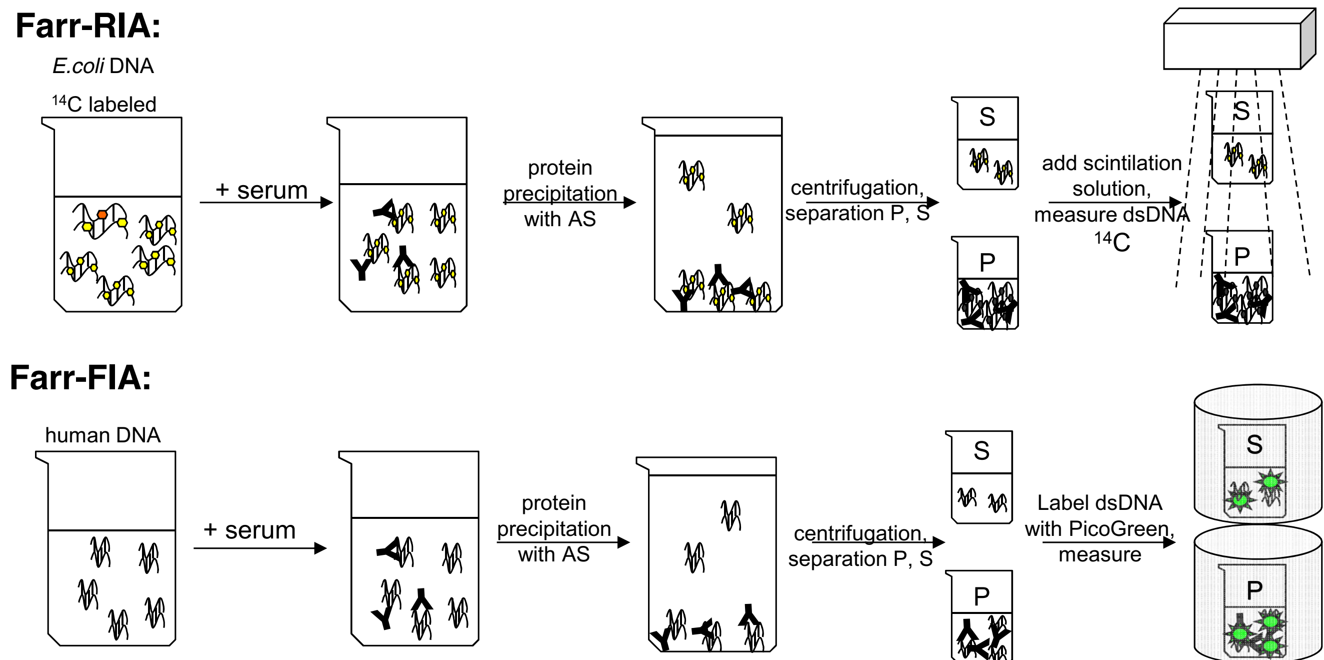

Fig. 1 Comparison of Farr-RIA and Farr-FIA methods

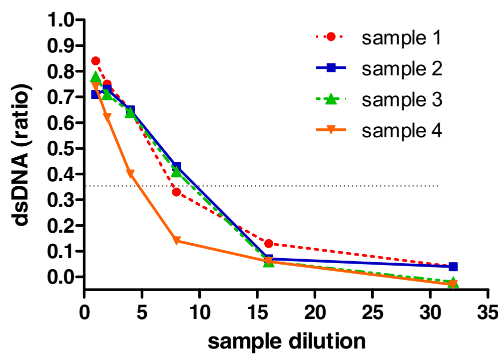

Fig. 2 Analytical sensitivity study result

and Laboratory Standards Institute (CLSI) guidelines, repeating two samples, five times for intra-assay and in five analyses for inter-assay variability [25]. Repeatability or within run imprecision varied between 6.1 and 12.3%, between run imprecision varied between 6.1 and 7.5%, and intra-laboratory imprecision varied between 8.6 and 14.4% for a high positive sample (mean result 0.75) and a medium positive sample (mean result 0.49), respectively. For the analytical sensitivity study, four positive serum samples were diluted (1:2, 1:4, 1:8, 1:16, 1:32), and in all samples, the 1:32 dilution was the point where the method could no longer distinguish that the serum was added (Fig. 2).

Agreements between anti-dsDNA antibody detection methods

An overall agreement (kappa coefficient) for 293 samples was estimated according to negative and positive results and was 0.87, which is regarded as good agreement. Measuring agreement between methods using linear regression Passing Bablok fit yielded an equation $y = 0.047 + 0.913x$, with the intercept 0.047 (95% CI 0.030–0.070) and slope 0.913 (95% CI 0.85–0.998) (Fig. 3a). As the intercept is at 0 and slope at 1, this means that the two methods yielded the same results. Correlation of Farr-RIA and Farr-FIA methods resulted in the Spearman correlation coefficient $r = 0.41$ (95% CI 0.31–0.50), $p < 0.001$; however, when analyzing only 70 SLE sera samples, the Spearman correlation coefficient was $r = 0.84$ (95% CI 0.75–0.90), $p < 0.0001$. We also performed Wilcoxon matched-pairs signed rank test on positive samples in both methods ($n = 32$; $p = 0.14$) which also showed that the H_o hypothesis was true, meaning the methods yielded the same results.

The Bland Altman comparison using an average of both measurements for samples (*x*-axis) and difference between methods (*y*-axis) also confirmed comparable results. The bias of methods was − 0.06 (with the SD of bias 0.11), because of higher Farr-FIA negative values; however, the limits of agreement were − 0.29 to 0.16, confirming the methods yielded comparable results (Fig. 3b).

Diagnostic utility, sensitivity, and specificity of results measured with Farr-RIA and Farr-FIA

In order to define the diagnostic utility of the results of both methods, area under the curve (AUC) for receiver operating characteristics (ROC) analysis was employed for 37 SLE and 223 non-SLE samples and AUC was found to be 0.887 (95% CI 0.83–0.94) for Farr-RIA, while 0.781 (95% CI 0.67–0.89) for Farr-FIA (Fig. 4a). Ninety-ninth percentile was 0.30, calculated based on 145 blood donor results. Using the cutoff value of 0.35 for both methods, diagnostic specificity was 100% for both methods and diagnostic sensitivity was 50% (Farr-RIA) and 53% (Farr-FIA) (Table 1) yielding comparable results. The diagnostic sensitivity was tested with the intention to compare results of both methods, but does not reflect general diagnostic sensitivity of results from both assays, as not all SLE in our cohort were treatment-naive.

Clinical performance of anti-dsDNA assays

When correlating the anti-dsDNA results with SLEDAI (SLE activity index), we observed even higher values for the newly developed Farr-FIA method ($r = 0.355$; $p = 0.003$), than for Farr-RIA ($r = 0.265$; $p = 0.027$) with the Spearman correlation coefficient (Fig. 4b).

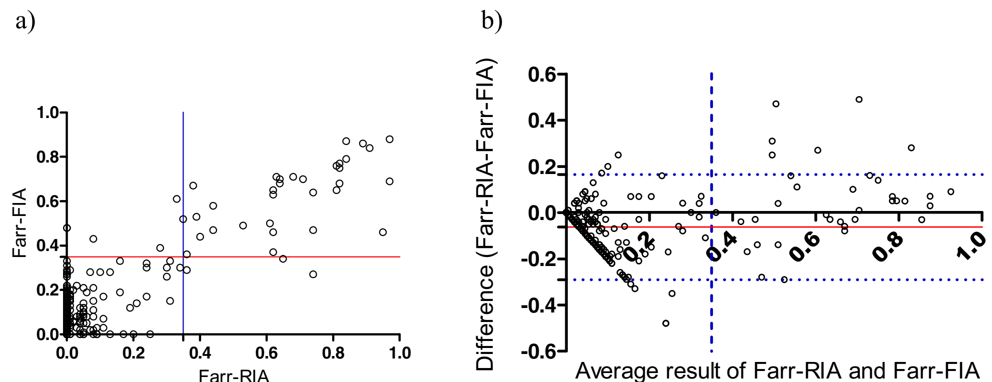

Fig. 3 Agreement results between methods

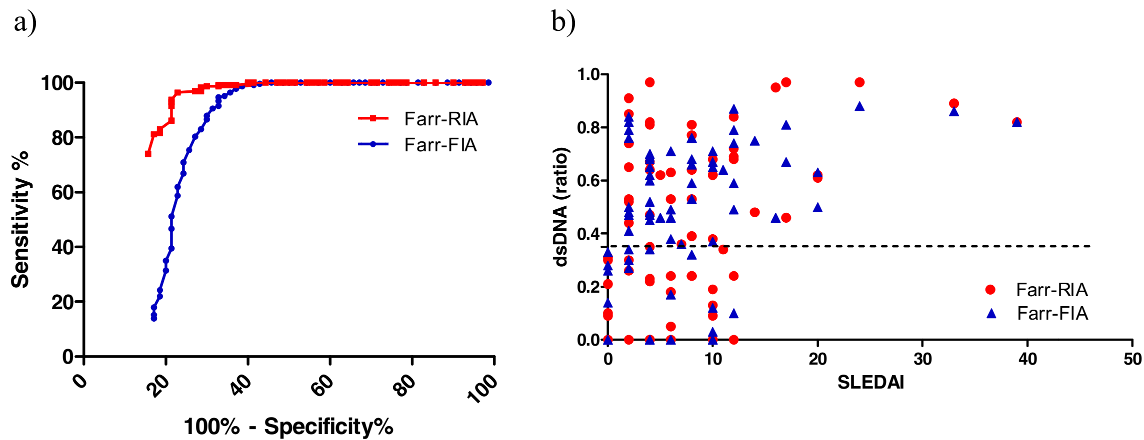

Fig. 4 Diagnostic utility of results measured with Farr-RIA and Farr-FIA and correlation of anti-dsDNA results with SLEDAI

To conclude, all our tests show that the change of the detection system is a viable and feasible option that could be clinically useful.

Discussion

In the current study, we found that the fluorescent intercalating dye PicoGreen yielded analytically and diagnostically comparable results to Farr-RIA, enabling the use of the preferred, highly specific method (Farr-RIA) for detection of clinically relevant high avidity anti-dsDNA antibodies without the need for a radioactive isotope.

Recently, another study by Duus et al. used the same principle of fluorescence sedimentation. However, they used plasmid DNA, instead of human DNA, as source material, a different intercalating dye (EvaGreen, instead of PicoGreen) for detection, and precipitation by polyethylene glycol, due to fluorescence of EvaGreen being reduced by ammonium sulfate. Thus, they precipitated both, high and low avidity antibodies (as opposed to just the clinically relevant high avidity antibodies precipitated by ammonium sulfate in our Farr-FIA) and expressed their results as absolute fluorescence units, rather than ratios between P-S/P+S. However, they also found a good correlation ($r = 0.62$, $p < 0.0001$) between Farr-RIA and their Fluoro-Farr assay from 57 SLE patients and high AUC in the ROC curve for Fluoro-Farr [23]. In the current study, we found an even better correlation between Farr-RIA and Farr-FIA, with $r = 0.84$ ($p < 0.007$) in 70 SLE patients. When we used SLE patients, combined with all controls ($n = 293$), the Spearman correlation coefficient was lower ($r = 0.41$, $p < 0.001$), due to higher negative sample results observed in Farr-FIA because of autofluorescence. Higher numbers in Farr-FIA for negative results are also observed in Bland Altman graph with overall bias −0.06 (Fig. 3b).

The advantage of reporting ratios in Farr-FIA between P-S/P+S is that you consider the background noise (represented by the free DNA that is subtracted) in each sample, leading to highly consistent and comparable results between samples, analyses, studies, and even different laboratories. Furthermore, in two fraction measurements, there is no need for extensive washing steps which optimizes the analysis process and reduces errors due to washing.

In other techniques, such as ELISA, false positives due to detection of nucleosomes or ssDNA autoantibodies were reported to be problematic. The DNA in our study was isolated using K proteinase and silica-based membrane purification, yielding highly purified DNA with a A260/A280 ratio > 1.9, appropriate also with DNA length up to 50 kb, so false positive results due to nucleosome immune complex are not an issue. Also, PicoGreen is a dsDNA-specific dye, with very low interference of ssDNA or RNA in equimolar concentrations. It was reported that some proteins [26], detergents [27], and

Table 1 Diagnostic specificity and sensitivity

Method (cutoff)		SLE		Diagnostic sensitivity	Diagnostic specificity
		Neg	Pos		
Farr-RIA (0.35)	Neg	223	35	50%	100%
	Pos	0	35		
Farr-FIA (0.35)	Neg	223	33	53%	100%
	Pos	0	37		

Two hundred twenty-three non-SLE sera comprised of 145 blood donors and other defined connective tissue disease sera (25 antiphospholipid syndrome, 25 Sjogren's syndrome, and 28 rheumatoid arthritis)

concentration of salts [28] could influence PicoGreen fluorescent signal. However, the isolated DNA was dissolved in water, further diluted with borate-buffered saline and ammonium sulfate, and the final dilution of serum samples was 1:400 for the measurements.

After testing 145 blood donors, a 99 percentile of 0.11 was obtained by Farr-RIA and 0.30 by Farr-FIA. This is most probably due to autofluorescence, since many organic substances autofluorescence at wavelengths between 400 and 500 nm. The cutoff for Farr-FIA remained the same as for the traditional Farr-RIA (0.35) yielding 100% specificity, meaning that the autofluorescent effect diminishes up to the level of the cutoff.

Currently, there is general consensus that monitoring anti-dsDNA is important, not only for diagnosis but also in the follow-up of SLE patients [17, 29]. Also, there is consensus that a combination of two assays, one sensitive and the other highly specific is recommended to be on the safe side [10]. In addition, caution is advised especially in serologically active and clinically quiescent SLE, adding a level of complexity for the prediction of flares in some patients [9]. In the current study, Farr-FIA correlated with the SLE disease activity index, SLEDAI $r = 0.355$ and Farr-RIA $r = 0.265$, indicating it could very well be used also for evaluating disease activity. Furthermore, there is an overarching presumption that if and when anti-dsDNA serum levels fall, they could very well be deposited in tissues, causing pathology [3]. It is now thought that anti-dsDNA antibodies could play more of an amplification role in the pathogenesis of lupus nephritis, rather than being the initiating factor or even the necessary/sufficient factor to cause lupus nephritis [30].

Conclusion

Taken together, Farr-FIA has the advantage of using the same procedure as Farr-RIA, with a dsDNA-specific dye but without the radioactive isotope. Results of Farr-FIA proved to have comparable diagnostic specificity and sensitivity to Farr-RIA results and assessment of precision met the criteria for assay validation. Farr-FIA targets high avidity, clinically relevant anti-dsDNA and correlates well with SLEDAI. Thus, Farr-FIA represents a good nonradioactive substitute for Farr-RIA.

Compliance with ethical standards

Disclosure The study is part of an approved national patent no. WO/2015/119582 (Fluorometric immunoassay for detection of anti-dsDNA antibodies, patent: Publication Date: 13.08.2015) and international patent pending (#PCT/SI2015/000003, International Filing Date: 03.02.2015. Geneva: World Intellectual Property Indicators, 2015).

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