Pilot Study

Prevalence of 60 kDa and 52 kDa Ro/SS-A Autoantibodies in Anti-Ro Positive Thai Sera in Siriraj Hospital

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Background: *Anti-Ro antibody may directly react against either Ro60 or Ro52 or both antigens. To be more applicable for routine laboratory practice, the specific antigen type for antibody detection should be identified before test application.*
Objective: *Investigate the prevalence of 60 kDa and 52 kDa Ro/SS-A antibodies in Thai patients' sera in Siriraj Hospital.*
Material and Method: *Specimens for anti-Ro were requested between June and December 2005. They were tested with EUROLINE test kit for prevalence determination. The principle of the test is a qualitative in-vitro-assay that contains test strips coated with parallel lines of 14 highly purified antigens. Of 84 specimens requested for anti-Ro antibody, 76 were collected and tested with the EUROLINE test kits and eight were excluded due to inadequacy.*
Results: *The prevalence of anti-Ro60 and anti-Ro52 of all sera tested for anti-Ro by EUROLINE test kit were 30% (95%CI: 20-40%) and 26% (95%CI: 16-36%), respectively; and, those in anti-Ro positive Thai sera were 82% (95%CI: 68-96%) and 71% (95%CI: 54-88%), respectively. The prevalence of anti-Ro52 alone in anti-Ro positive Thai sera and all specimens requested for anti-Ro was about 18% (95%CI: 4-32%) and 7% (95%CI: 1-13%), respectively. The agreement and Kappa value between the two methods were 0.9 and 0.77, respectively. The study suggests that the test for anti-Ro detection should provide both Ro 60 and Ro 52 antigens.*
Conclusion: *The prevalence of both anti-Ro 60 and anti-Ro 52 were quite common, therefore, the test for this specific antibody should provide both antigens for antibody detection.*

Keywords: *Prevalence, Anti-Ro, Anti-Ro 60, Anti-Ro 52*

J Med Assoc Thai 2007; 90 (4): 782-7
Full text. e-Journal: *http://www.medassocthai.org/journal*

Anti-Ro (SS-A) autoantibodies are found in 10 to 50% of systemic lupus erythematosis (SLE) and 60 to 80% of primary Sjogren's syndrome sera[1] and are also present in serum samples from patients with other autoimmune diseases[2-4]. Anti-Ro/SSA antibodies recognize RNA-protein complexes. These complexes are composed of at least two proteins, the Ro 60 kDa and Ro 52 kDa[5,6] in association with any of four related small hY (human cytoplasmic) RNAs[7]. It remains uncertain, however, whether the Ro 52 protein is also a component of the native SS-A/Ro complex. Evidence has been presented that Ro 60 and Ro 52 are structurally unrelated[8-11]. The anti-Ro antibody may direct against either Ro 60 or Ro 52 antigen or both. Two studies by Peene and Bizzaro reported that the prevalence of anti-Ro 52 in anti-Ro positive patients with connective tissue disease was 88% and 95% while the prevalence of anti-Ro 60 was 75% and 66%, respectively[12,13]. To the authors' knowledge, no data about the prevalence of these two antibodies in Thai patients' sera are now available. If the prevalence of 60 kDa and 52 kDa Ro/SS-A antibodies is known, the

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preparation of the test for anti-Ro will be more applicable. Therefore, the aim of the present study was to investigate the prevalence of 60 kDa and 52 kDa Ro/SS-A auto-antibodies in Thai patients' sera at Siriraj Hospital.

Material and Method

Specimens

Sera routinely requested for anti-Ro antibody between June and December 2005 were collected and kept at -20° or -70°C. Specimens, which were hemolytic, lipaemic and inadequate for the present study, were excluded.

Method

The specimens from all sera requested for anti-Ro and tested with EUROLINE test kit were recruited for prevalence determination. The principle of the test was a qualitative *in vitro* assay that contains test strips coated with parallel lines of 14 highly purified antigens: nRNP/Sm, Sm, SS-A (SS-A native and Ro-52), SS-B, Scl-70, PM-Scl, Jo-1, CENP-B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-protein and AMA-M2. In the case of positive samples, the specific IgG antibodies also IgA and IgM will bind to the corresponding antigenic site. To detect the bound antibodies, an enzyme labeled anti-human IgG, which is capable of promoting a color reaction, was used in a second incubation. The detail and interpretation of the procedure were done according to the recommendation of manufacturer. Briefly, the test strip from the package was removed and placed in an empty channel. Each channel of the incubation tray was filled with 1.5 ml sample buffer according to the number of serum samples, and was incubated for 5 minutes at room temperature on a rocking shaker. Afterwards, all the liquid was aspirated off. Each channel was filled with 1.5 ml of the diluted serum samples and was incubated at room temperature for 30 minutes on a rocking shaker. Then each channel was aspirated off the liquid and washed three times for 5 minutes with 1.5 ml diluted wash buffer on a rocking shaker. One and a half milliliters of diluted enzyme conjugate (alkaline phosphatase-labeled anti-human IgG) was added into each channel and incubated for 30 minutes at room temperature on a rocking shaker. Each channel was aspirated off the liquid and washed three times for 5 minutes with 1.5 ml diluted wash buffer on a rocking shaker. The channels of the incubation tray were filled in with 1.5 milliliters substrate solution and incubated for 10 minutes at room temperature on a rocking shaker. Each channel was aspirated off the liquid and washed three times for 1 minute with distilled water. The test strip was placed on the evaluation protocol, air dried and evaluated. According to the manufacturer's instructions (Euroimmun, Germany 2005) and depending on the study population and reference methods, the sensitivity and specificity of each antigen ranged from 94% to 100% except histones and ribosomal P-protein. Inter and intra-assay reproducibility was excellent. As for the ELISA method, the principle of the test provides a simiquantitative or quantitative *in vitro* assay for human auto-antibodies of the IgG class against SS-A in serum or plasma. Each test kit contains microtiter strips with 8 break-off reagent wells coated with SS-A. In the first reaction step, diluted patient samples are incubated in the wells. In the case of positive samples, specific IgG antibodies (also IgA and IgM) will bind to the antigens. To detect the bound antibodies, a second incubation is carried out using an enzyme-labeled anti-human IgG (enzyme conjugate), which is capable of promoting a color. The detail and interpretation of the procedure were done according to the manufacturer's instructions (Euroimmun, Germany 2003).

Sample size

The prevalence of anti-Ro60 was assumed 60%, which was the lowest number among those of anti-Ro 60 and anti-Ro 52 and estimated to fall within 20% percentage points of the true value with 95% confidence interval. The number of positive samples with anti-Ro required was 24 specimens. The prevalence of positive specimens requested for anti-Ro was about 35% (by observation). Therefore, the total number of samples collected was about 69 specimens.

Statistical analysis

The prevalence of 60 kDa and 52 kDa Ro/SS-A autoantibodies in anti-Ro positive sera was determined and reported as a percentage of positive sera from total positive specimens with 95% confidence intervals.

Results

Of the 84 specimens requested for anti-Ro antibody, 76 were collected and tested with the EUROLINE test kits; eight were excluded due to the inadequacy of specimens. Of the total specimens, 25, including the excluded sera, were reactive with Ro antigens by ELISA. Therefore, the prevalence of anti-Ro of total specimens is about 30% (25 positive specimens/84 total specimens). The numbers of enrolled

sera reactive with Ro antigen by ELISA and EUROLINE test kits were 22 (29%) and 28 (37%) specimens respectively, when four specimens with borderline results by EUROLINE test kits were considered as negative. Of the 76 tested specimens, eight had discrepant results between these two methods. Eight specimens with discrepant results comprised seven negative and one positive sera by ELISA method. Of the seven negative results by ELISA, one was reactive with Ro 60 antigen, five were reactive with Ro 52 antigen, and one was reactive with Ro 60 and 52 antigens.

The prevalence of anti-Ro 60 and anti-Ro 52 in all tested sera (76 specimens) at Siriraj Hospital by EUROLINE test kit were 30% (95%CI: 20-40%) and 26% (95%CI: 16-36%), respectively; and those in anti-Ro positive Thai sera were 82% (95%CI: 68-96%) and 71% (95%CI: 54-88%), respectively. The prevalence of anti-Ro52 alone in anti-Ro positive Thai sera and all tested specimens were about 18% (95%CI: 4-32%) and 7% (95%CI: 1-13%), respectively. The agreement between the two methods was 0.9. Kappa value was 0.77, which is relatively good. There were 32 specimens unexpectedly reactive with different other autoantigens other than anti-Ro and anti-La as follows: 1 RNP/Sm, 6 Sm, 5 Scl-70, 2 PM-Scl, 3 centromere-B, 2 PCNA, 8 dsDNA, 8 nucleosome, 9 histone, 3 ribosomal-P, and 5 mitochondrial antigens. Of these, 15 specimens were also reactive to Ro antigens. The request record forms of these 32 specimens were reviewed in the period between March and December 2005 and it was found that 27 specimens were reactive with either one or more antigens other than those requested autoantibodies. The antibodies that were not requested were as follows: 1 RNP/Sm, 5 Sm, 5 Scl-70, 2 PM-Scl, 2 PCNA, 3 centromere-B, 2 dsDNA, 8 nucleosome, 9 histone, and 3 ribosomal-P.

Discussion

The prevalence of anti-Ro in Thai specimens requested for this test is quite common, whereas the prevalence of anti-Ro 60 and 52 are also high similar to others[12,13]. However, the present results had specimens reactive with Ro 60 more than Ro 52 antigens, whereas others had opposite results[12,13]. These discrepant results may be related to the difference of sample size[12] (15937 *vs* 76 serum specimens), detection technique[13] (Light-scattering assay *vs*. line immunoassay) and race. As for anti-Ro52 alone, the prevalence in Japanese patients with polymyositis/dermatomyositis and in anti-Ro positive sera from Italy were 6%[14] and 34%[13], respectively. In comparison with the present study, the anti-Ro positive sera was 18% (95%CI: 4-32%). It is suggested that this specific antibody alone is common in Thai patient's sera. Comparing the results of routine test (ELISA) with EUROLINE test kit, it was found that 9% (7 cases out of 76 all tested specimens) were not detected by the routine test method, whereas one case was missed by the EUROLINE test kit. Of the seven cases missed by ELISA method, five were reactive to Ro52 antigen alone. All of the five specimens were tested with another ELISA commercial kit from laboratories of other hospitals, and the results were also negative, this finding supports other studies that the anti-Ro52 reactivity was not detected by ELISA technique[15,16]. The authors did not know whether the specimens reactive with anti-Ro52 were worth for routine practice. Therefore, the clinical data of these five specimens were reviewed and found that no clinical associations were observed in four specimens, whereas the other one was from the patient with highly suspected primary Sjogren syndrome. Even though the clinical associations of anti-Ro52 auto-antibodies are less well established but anti-Ro 52 alone is commonly associated with primary Sjogren's syndrome whereas anti-Ro 60 is usually associated with SLE[17]. Association of anti-Ro 52 without anti-Ro 60 with polymyositis has also been reported[18,19]. There is a very strong association of neonatal lupus syndrome with maternal anti-Ro antibodies[20]. The presence of Anti-Ro 52 is associated with a higher risk of developing congenital complete heart block than anti-Ro 60[21,22]. Anti-Ro 52 antibodies are concentrated in the heart tissue of infants with complete heart block[23] and may cause damage and ultimately lead to the development of cardiomyopathy and complete heart block secondary to fibrosis of the conduction system[24-26]. These may help explain why the detection of anti-Ro52 alone should also be included in routine practices. In recent prospective studies of women with anti-Ro/SSA antibodies, the risk of complete congenital heart block was found to be 1-2%[27,28] and of transient cutaneous neonatal lupus about 5%[28]. Is it reasonable to change from an ELISA method to the test that provides both Ro 60 and Ro 52 antigens? Of those 76 specimens, four specimens were from an antenatal care unit and one out of those was reactive to Ro52 antigen alone. If the authors apply these figures for additional detection of a newborn with complete congenital heart block, it needs 7600 specimens requested for anti-Ro from routine practice. That is too high for one laboratory to gain additional benefit within a short period. Other factors to be concerned are about turnaround time

and cost. This depends on the test kit used to compare. As for the turnaround time, the EUROLINE test kit was more convenient to perform the tests for case requested than the ELISA method. Furthermore, the ELISA method needs more cases for cost saving. The direct material cost of anti-Ro and anti-La is 290 baht and 280 baht per test by ELISA method respectively compared with 500 baht per test of the EUROLINE test kit (less antigen test kit containing nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, Jo-1). This direct material cost was determined in conditions of an ELISA method that needs three wells for a standard curve and two wells for controls (positive and negative controls). The number of sera required for each run ranged between three and nine specimens with an average of 2.2 wells per specimen. Each well costs 135.14 baht. The data collection from August 2005 to March 2006 showed that there were 92 out of 107 cases simultaneously requested for both anti-Ro and anti-La. Accordingly, more than 80% of specimens in the authors' laboratory unit were simul-taneously requested for both anti-Ro and anti-La. Therefore, ELISA method will cost slightly more than the EUROLINE test kit [{(290+280) x 0.8 + (290 x 0.2)} *vs* 500]. An additional benefit of the EUROLINE test kit is that it shows some unexpected autoantibodies, some of which are specific for autoimmune diseases such anti-Sm[29], anti-dsDNA[30], anti-Scl-70[31] etc. Even though the less antigens test kit will be used for routine practices, those are still specific and useful for some connective tissue diseases. Although the present study has several limitations such as sample size and no gold standard for method comparison with several other studies, the benefits are as mentioned above. Therefore, EUROLINE test kits or other similar test kits should be considered in routine practices for anti-Ro detection.

In conclusion, these data are consistent with other studies that show the prevalence of both anti-Ro 60 and 52 antibodies are quite common[12,13]. Due to the common presence of anti-Ro 52 alone in anti-Ro positive Thai sera and the routine specimens requested, a more cost-effective and shorter turnaround time solution providing more information about other specific antibodies is required. This may call for a change from the routine method (ELISA) to a tests that provides both antigens of Ro 60 and Ro 52 test such as the EUROLINE test kits should be considered.

Acknowledgements

The authors wish to thank the Department of Clinical Pathology of Siriraj Hospital for its general support of the study and Hausen Bernstein Co., Ltd for the partial support of test kits.

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การศึกษานำร่องหาความชุกของออโตแอนติบอดีต่อ Ro 60 และ Ro 52 ในน้ำเหลืองที่มีผลบวก Anti-Ro ของผู้ป่วยไทยในโรงพยาบาลศิริราช

รณชัย วิริยะทวีกุล, สายขวัญ ศรีจำรูญ, ชัยเจริญ ตันธเนศ, ธนวรรณ กุมมาลือ, นิศารัตน์ โอภาสเกียรติกุล

แอนติเจนของ anti-Ro อาจเป็นชนิด Ro 60 หรือ Ro 52 หรือทั้ง 2 ชนิด ในการตรวจหาแอนติบอดีควรทราบว่าแอนติเจนชนิดใดเหมาะสม ได้ทำการศึกษาเพื่อหาความชุกของแอนติบอดีต่อ Ro แอนติเจน ชนิด 60 kDa และ 52 kDa ในน้ำเหลืองผู้ป่วยคนไทยในโรงพยาบาลศิริราช ตัวอย่างน้ำเหลืองที่เก็บได้ระหว่างมิถุนายน ถึง ธันวาคม พ.ศ. 2548 จะถูกนำมาตรวจด้วย EUROLINE test kit ชุดทดสอบที่ประกอบด้วยแอนติเจน 14 ชนิด เพื่อศึกษาความชุกในจำนวนตัวอย่างน้ำเหลืองทั้งหมด 84 ราย สามารถทำการทดสอบได้ 76 ราย อีก 8 ราย มีปัญหาด้านปริมาณน้ำเหลืองไม่เพียงพอ ผลการศึกษาพบว่าความชุกของแอนติบอดีต่อ Ro 60 และ Ro 52 ในตัวอย่างน้ำเหลืองที่ส่งตรวจหาแอนติบอดีในโรงพยาบาลศิริราช คือ 30% (95%CI: 20-40%) และ 26% (95%CI: 16-36%) ตามลำดับ และความชุกในตัวอย่างน้ำเหลืองที่ให้ผลบวกต่อแอนติเจน Ro คือ 82% (95%CI: 68-96%) และ 71% (95%CI: 54-88%) ตามลำดับ ความชุกของแอนติบอดีต่อ Ro 52 อย่างเดียว ในน้ำเหลืองที่ให้ ผลบวกต่อ Ro และในน้ำเหลืองทั้งหมดที่ส่งตรวจคือ 18% (95%CI: 4-32%) และ 7% (95%CI: 1-13%) ตามลำดับ ค่าของ agreement และ kappa ระหว่าง 2 วิธีเป็น 0.9 และ 0.77 ตามลำดับ การศึกษานี้แสดงว่า การตรวจหาแอนติบอดีต่อ Ro แอนติเจนควรมี ทั้งชนิด Ro 60 และ Ro 52

สรุปว่าความชุกของแอนติบอดี ต่อ Ro 60 และ Ro 52 แอนติเจนพบได้บ่อย ดังนั้นควรเลือกการทดสอบที่สามารถตรวจแอนติเจนได้ทั้ง 2 ชนิด