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ORIGINAL ARTICLES

Immunodot Technique for Early Detection Rheumatoid Arthritis Patient in Indonesia Based on Autoimmune Marker Matrix Metalloproteinase-3 (MMP-3)

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ABSTRACT

Rheumatoid arthritis (RA) is a rheumatic disease characterized by inflammation that causes pain, swelling, stiffness, and decreased of joint function. Prevalence of RA in Indonesia is estimated between 23.6% -31.3%. Inflammation and cartilage degradation are major parts in the pathogenesis of RA. The cartilage degradation is closely related with increasing activity of MMP-3. Detection of joint damage in RA is usually performed using radiological photo technique (X-ray), but the sensitivity is low. Therefore, it required more sensitive and specific diagnostic tool for detecting joint damage in RA patients. This research were conducted to determine the sensitivity and specificity of antibody agant to MMP-3, by immunodot technique, sharp score assessment of metacarpo phalangeal joint and density of MMP-3 in RA and no RA sera patients. Total patients used in this research are 18 RA patients (age 42.2 ± 12.1) years and 12 non-RA patients (age 38.3 ± 7.3) years. MMP-3 protein is used as immunogenic agent for inducing antibody againts to MMP-3 in New Zeland white rabbit. The results showed that MMP-3 protein has molecular weight of 54 kDa as immunogenic molecules that induced MMP-3 antibody production. Antibody against to MMP-3 was confirmed to recognize MMP-3 in sera patients by immunodot, western blot and ELISA Sensitivity of antibody against to MMP-3 is 1:120 sera dilution. There are significant differences between the MMP-3 concentration, MMP-3 density and sharp score both of RA and non RA patients ($p < 0.05$). Pearson correlation showed a very strong correlation between MMP-3 density with MMP-3 concentration and MMP-3 density with sharp score ($p < 0.01$), whereas correlation between MMP-3 concentration and sharp score (X-ray) showed a strong correlation ($p < 0.01$). This result showed that there are strong correlation between level of joint damage and level of MMP-3 in RA patients. ROC (Receiver Operating Characteristic) results showed that sensitivity and specificity of detection based on MMP-3 is much better than radiological assesment ($p < 0.001$) with cut off points of MMP-3 density is 10.48, where the density value ≥ 10.48 would be diagnosed as RA patients.

Key words: Rheumatoid Arthritis, Joint Disruption, antibody against to MMP-3 and Sharp Score.

Introduction

Rheumatoid Arthritis(RA) is one of the rheumatic diseases characterized by inflammation that cause spain, swelling, stiffness, and decreased joint function (Mackay and Rosen, 2001). There are currently more than 80 different autoimmune diseases, affecting approximately 100 million people worldwide. The etiology of most autoimmune diseases is unknown. The highest incidence of these diseases is in the developed countries and they are more common in women than in men. The study by Zeng, *et al.* (2008) showed the prevalence of RA in Indonesia is quite high at between 23.6% - 31.3%. Radiological examination of the RA will appear connective tissue swelling, narrowing the gap joints, and the erosion/degradation of cartilage (Koopman, 1997). Inflammation and degradation of cartilage is a major event in the pathogenesis of RA, but the patogenesis of RA remains unclear. The occurrence of cartilage degradation is closely associated with increased activity of matrix metalloproteinase enzymes. Based on Ribbens's (2002) research results, notice that RA patients have elevated levels of matrix metalloproteinase-3 (MMP-3).

Matrix Metalloproteinases (MMPs) are a family of zinc metallo endopeptidases secreted by cells, and are responsible for much of the turnover of matrix components. The matrix metalloproteinases are major degradative enzymes. The MMPs are involved in a wide range of proteolytic events, in normal and pathological

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circumstances. Normal physiological roles for the MMPs include neurite growth, cell migration, bone elongation, wound healing and angiogenesis,

Detection of joint damage in RA is usually done using a technique radiology photo (x-ray) and severity can be determined by using the sharp score. However, the sensitivity of x-ray is only about 67% (Heijde, *etal.,* 1999). The importance of early diagnosis performed on RA cases was an attempt to prevent the occurrence of a continuing joint destruction in patients with RA. MMP-3 is released in the early stages of RA pathomechanism. It is believed that is responsible for the production of auto-antibodies and in consequence attack of the immune system against host tissues and organ. Thus MMP-3 can be used for early detection of RA-based autoMMP-3 in response toMMP-3 excess.

Materials and Methods

Blood Collection:

Collecting samples (Sera and retrieval X-ray results of RA and Non-RA Patients) obtained from Reumathology Polyclinics of Syaiful Anwar hospital, Malang., Indonesia. Samples were completed with informed consent. The number of RA patients (18 men; age 42.2±12.1 [mean ± SD]) and non-RA patients (12 men; age 38.3±7.3). The diagnosis of RA is based on the criteria of the American College of Rheumatology (ACR) in 1987. For the interpretation of X-ray used assessment based on the Modified Sharp Score 1985. The use of patient sera have been certified ethical acceptance form ethics committee, School oMedical Faculty, Brawijaya University, Malang, Indonesia.

Isolation dan Confirmation of MMP3:

The MMP3 were collected from sera patients by electro elution technique. To ensure that the protein sample contains MMP-3, it was confirmed using immunodot dan Western blot technique. Protein concentration performed using the Nanodrop technique.

Immunodot Analysis:

Sera sample from both RA and Non RA patients were coated in nitrocellulose membranes (NC). Each dot of 10 µL of MMP3 from sera patients (10 µL/dot) was coated in NC. The NC were incubated 2 h in blocking buffer containing 5% (w/v) skim milk powder in PBS and followed by washing membrane three times in PBS-T and incubated with primary antibody (antibody against to MMP3) for 1 h. The membrane were washed three times using PBS-T, and incubated with 1/750 dilution rabbit anti-mouse alkaline phosphatase conjugate for 1 h. After further washes, they were exposed to alkaline phosphatase substrate consisting of 0.21 mg/mL Nitro Blue Tetrazolium and 0.42 mg/mL Bromo- 4-chloro-3-indoxyl-phosphate in Tris buffer. The reaction was terminated by rinsing with tap water

SDS-PAGE and Western blotting:

Standard methods of SDS-PAGE and Western blotting were used based on Aulanni'am (2005). The resolving gel was set at 12% (v/v) concentration, and sera patients of 35 µL/lane from both RA and Non RA patients were loaded and run at a constant current of 10 mA. The transfer was attained at a constant current of 150 mA for overnight to NC membrane. After transfer the membrane was washed in PBS + NaN_3 and immersed in 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) substrate buffer to develop the band patterns.

Production and Purification of antibody againts to MMP-3:

Production of Anti-MMP-3 were conducted in rabbits. The use of rabbit has received ethical clearance certificate from the UB team of Medical Faculty Ethics Committee .Purification of Antibody againts to MMP-3 from rabbit serum that induced by MMP3 were collected by 50 % of SAS (Saturated Ammonium Sulphate)

Result and Discussion

Results Assessment of Modified Sharp Score:

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The results of X-ray images in Figure 1A visible presence Juxta Articularerosis and narrowing the gap on MCPJ joints (metacarpo phalangeal joint). In contrast to that seen in Figure 1B which shows a clear trabeculatin and may not find the narrowing gap in MCPJ joints.

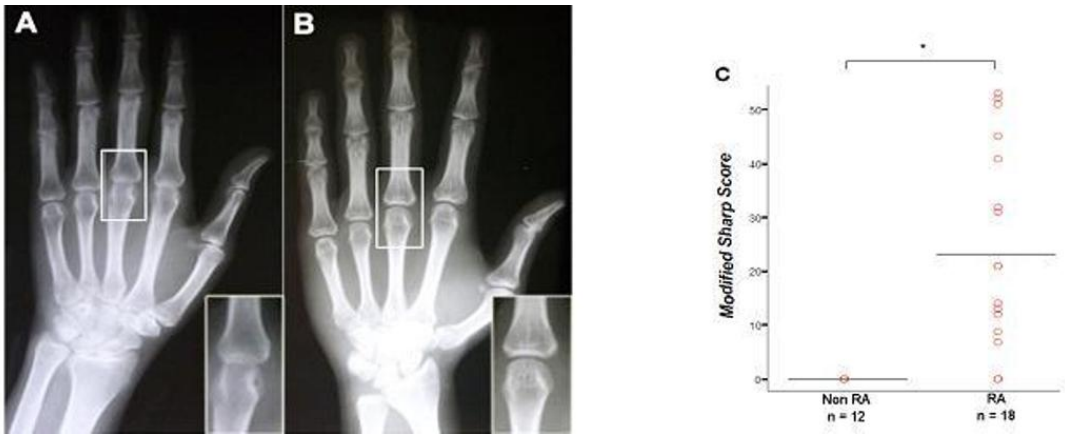

Fig. 1: The results of X-Ray Photo position (A) RA patients, (B) non-RA patients, (C) Distribution of Modified Sharp Score Interpretation between RA and non-RA patients (* $p<0.05$)

Figure1 Cx-ray examination of patients can be seen that the results of Sharp scores among RA patients was significantly higher (22.33 ±19.32 [mean ± SD]) compared with non-RA patients (0.00 ±0.00) ($p<0.05$). In RA patients still found the score zero in some patients. It is indeed quite low due to the sensitivity of radiology and it ranged 67% within 0.5 to 1 year in patients with suspected RA (Heijde, *et al.,* 1999).

Protein levels of RA and non-RA Patients:

Protein levels between RA patients showed no significant difference (82494.44 ±20416.35 [mean ± SD]) compared with non-RA patients (96733.33 ±24864.53) ($p>0.05$), although levels protein non-RA patients have a higher tendency compared with RA patients (Figure 2). Yamasaki, *et al.* (2005) explained that during RA is a process of oxidative stress in patients that lead to increase the activity of RE (hyper-endoplasmic reticulum (ER)-associated degradation [hyper-ERAD]). This has led to increase the degradation of unfolded proteins.

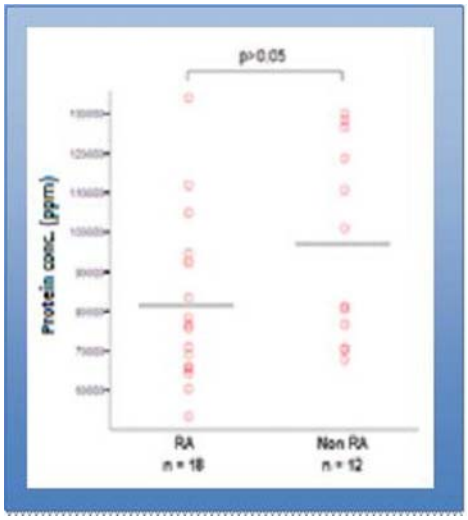

Fig. 2: Results Levels of Protein Sample Distribution of RA and non-RA patients.

Based on Densitometry technique, it resulted that protein band of 54 kDa has a higher density than any other protein. In RA patients the protein density of 54 kDa (39.89%) is higher than in non-RA patients (38.21%).

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Spesifity and SensitivityTestAntibody againts to MMP-3:

To prove that the protein with molecular weight of 54 kDa found in people with RA serum MMP-3 was then performed with Western Blot confirmation and immuno Blot (Fig 3. A, B, C).

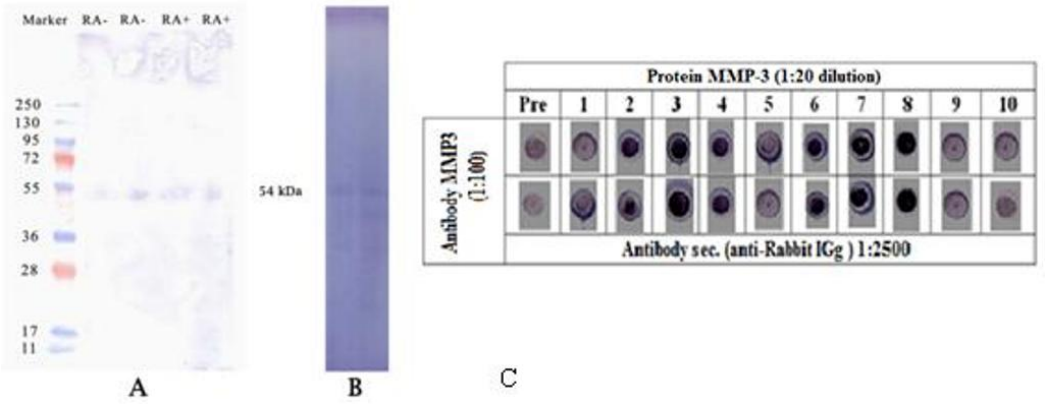

Fig. 3:(A) The results of Western blot of MMP-3 and (B) MMP-3 protein collected by elecrtro elution technique, (C) Specificity MMP-3 antibody to MMP3 isolated from sera RA patients by immunodot technique

MMP-3 with a molecular weight of 54 kDa found in both of RA and non-RA patients. The MMP-3 bands showed on non-RA sera patients due to normal, because MMP-3 was released for maintaining hemostasis (Tchetverikov, *et al.,* 2003). Specificity test of MMP-3 antibody was performed by 3methods were immunodot, ELISA and western blot. The results of immunodot confirmed that there is a specific reaction between an MMP3 (protein with molecular weight of 54 kDa and it's antibody, which is seen as purplish blue stain as shown in Figure 3 A.

The ability of the protein 54 kDa (MMP-3) to induce 54 kDa protein polyclonal antibody (Anti-MMP-3) can be measured quantitatively using indirect ELISA. The presence of 54 kDa protein polyclonal antibody (Anti-MMP-3) measured by the absorbance at λ= 504 nm (Fig.3D). The highest value of anti-MMP-3 obtained at the bleeding-8, which is also shown qualitatively by dot blot produce the darkest color (Figure 3C week 8). This is because of the formation of memory cells in the immune system of rabbits produced by lymphocyte cells (Abbas, *et al.*, 2004). In the next process of antibody at week-8 isused as an anti-MMP-3 polyclonal in detecting damage in RA patients. The protein 54 kDa (MMP-3) is immunogenic protein that confirmed by production of antibody againts to MMP-3 (Fig. 4).

Sensitivity of MMP3 Antibody:

The sensitifity of MMP-3 antibody was confirmed by the indirect dot blot (Fig.4) showed that the antigen could be detected by dilution of 1:40, but still detected until the dilution1:120. These results indicate that the sensitivity of MMP-3 antibody up to 120 times dilution still able to recognize with MMP-3 as an antigen.

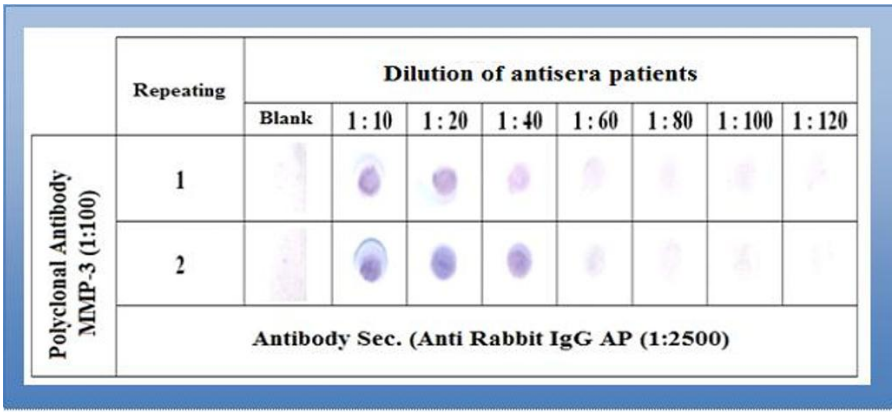

Fig. 4: Sensitivity antibody against to MMP-3 o detect serum RA patient by immunodot technique.

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The Correlation between The Density of MMP-3, MMP-3 Concentration and The Modified Sharp Score:

The results of Figure 5A showed a significant difference ($p < 0.05$) between the density of MMP-3 RA patients (16.97 ±3.93%) compared with non-RA patients (8.47 ±1.13%), as well as concentrations of MMP-3 (Figure 5B) between RA patients (147.38 ±12.88 ng/mL) with non-RA (107.42 ±9.07 ng/mL) ($p < 0.05$). While the Modified Sharp Score (Fig.5C) between RA patients (21.33 ±19.32) with non-RA (0.00 ±0.00) ($p<0.05$).

Based on Pearson correlation analysis, it was obtained a very strong relationship between the density of MMP-3 with a Modified Sharp Score ($r = 0.913$, $p < 0.01$) and the concentration of MMP-3 ($r = 0.915$, $p < 0.01$). While the concentrations of MMP-3 with a Modified Sharp Score showed a strong relationship with $r = 0.794$ ($p<0.01$).

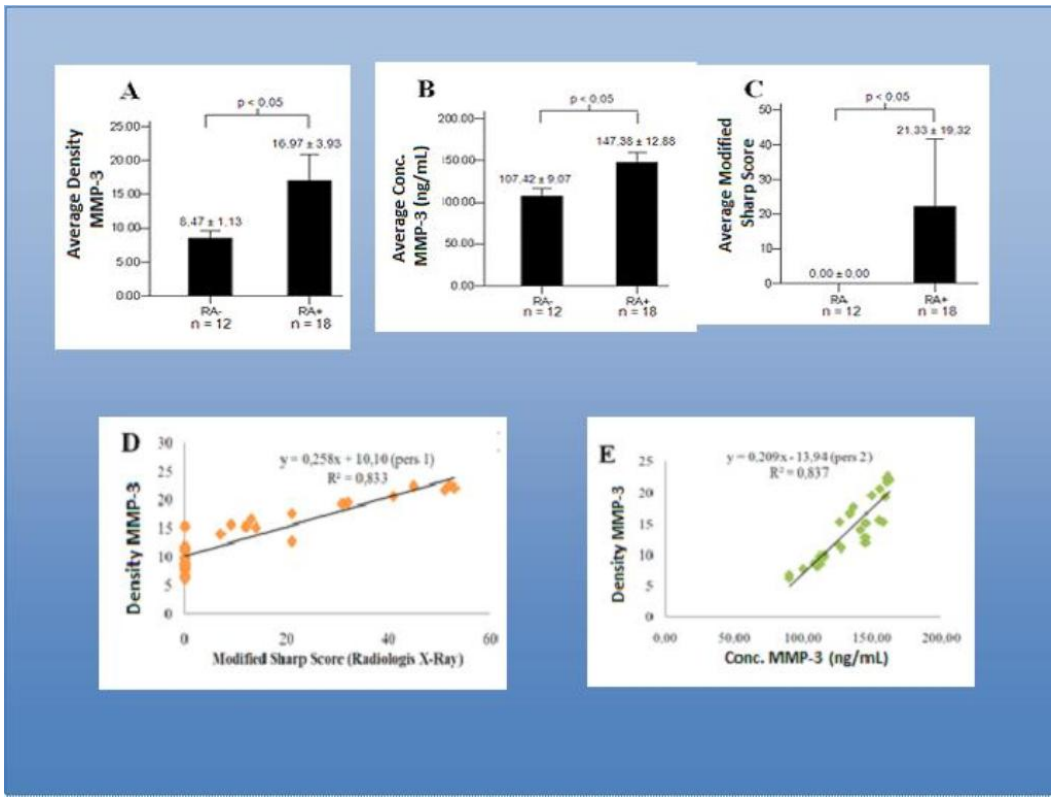

Fig. 5: (A) Diagram of stem density of MMP-3, (B) Diagram Trunk Concentrations of MMP-3, (C) Diagram Trunk Modified Sharp Score, (D) Linear equations between density of MMP-3 with a Modified Shrap Score, (E) Linear equations between density of MMP-3 with MMP-3 concentration.

Findings of Joint Damage on Early Detection Kit (ARTDECT) in Patients:

Direct results of dot blot in serum of patients (Figure 6A) as basis for determining the density scale MMP-3 (Figure 6B), and obtained the following formula:

RA+ = value of the density of MMP-3 ≥10.48

RA- = The density of MMP-3 <10.48

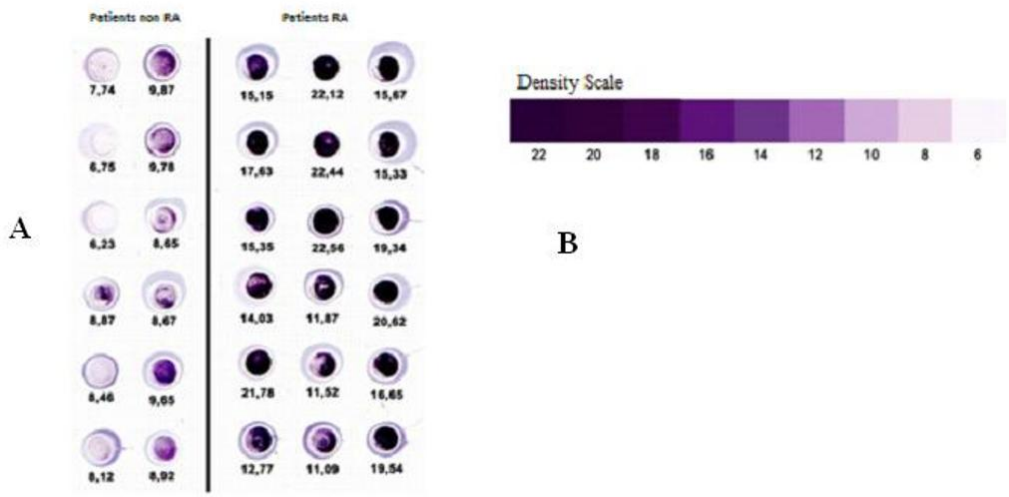

Fig. 6: (A) The Density Value of MMP-3 RA and Non-RA Patients, (B) Scale Density by ARTDECT

This formula was obtained by determining the cut of point between the specificity and sensitivity. While the cut off density of MMP-3 is for patients with RA is a ≥ 10.48. This means patients who have a density of MMP-3 ≥ 10.48 will be diagnosed as a patient who has undergone RA. Thus, screening of patients with RA is expected to be faster, and with the known density of MMP-3 patients, it can be predicted well Modified Sharp Score and concentrations of MMP-3 through equations 1 and 2 (Figure 5D, E).

Comparison of Sensitivity and Specificity Between ARTDECT with other devices (Radiological [x-ray]):

The results of the ROC (Receiver Operating Characteristic) analysis note that the RA subjects was 18 of 30 patients. Thus, the prevalence of RA in rheumatology polyclinic of RSSA was by 60% compared with other rheumatic diseases. Value of AUC (Area Under the Curve) is a value that describes the sensitivity and specificity of the device used. AUC values obtained for examination by using radiological (x-ray) were 88.9% ($p<0.001$). While the AUC for the concentration and density of MMP-3 was 100% ($p<0.001$). This is because a small number of subjects and is a grouping of patients according to inclusion and exclusion criteria.

However, the results of this study indicate that detection using the density has a better value compared with radiological (x-ray). AUC values between the concentration and density of MMP-3 has the same value and is able to predict 100% of sufferers, this is because the same principle of measuring the concentration of MMP-3 with a density assessment of MMP-3 is based on antigen-antibody binding is highly specific (lock and key) (Abbas, etal., 2004).

Conclusions :

1. MMP-3 isolated from sera patients had molecular weight of 54 kDa, a density of 39.89% and an immunonogenic molecules.
2. Antibody against to MMP-3 specifically recognizes with MMP-3 of sera RA patients that confirmed by dot immunodot, ELISA and Western blot techniques, whereas the sensitivity of antibody against to MMP-3 were known until dilution of 1:120.
3. The concentration ofMMP-3, antibody against to MMP-3 and density Sharp radiologic score(x-ray) has a proportional relationship, as the higher of MMP-3 the higher of joint destruction in RA patients.
4. The cut off point density of MMP-3 between patients with RA and nonRA was to be 10.48, which values ≥ 10.48 will be diagnosed as a patient who has undergone to RA.
5. Examination based on MMP-3 has a better sensitivity and specificity compared with radiological examinations (x-ray).

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