Production of Monoclonal Antibodies Against Human Cardiac Troponin I (cTnI) and Development of Antibodies Sandwich ELISA

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Abstract

Acute myocardial infarction (AMI) is a heart disease which causes unexpected death in human when it occurs. So early detection is very important for the patients. In the pass decades, the biomarkers used in the clinic to detection are poor in specificity, moreover, the concentration of those markers increase lately when AIM appears. Therefore, in this study, we choose cardic troponin I (cTnI) as biomarker which was demonstrated the best biomarker with most specific of AMI. However, cTnI exist in serum with the complex form of cTnI-T-C and cTnI-C. Additionally, cTnI is a subunit of the complex. In order to prepare monoclonal antibodies with higher affinity and specificity, we use online software to predict the dominant epitope of cTnI. Furthermore, Whether the epitope is reasonable is further evaluated by analyzing the structure and location of the epitope in the three dimensions structure of the complex which crystal structure has been resolved by Discovery Studio 4.0 software. In this study, we produced three high affinity and specificity cTnI monoclonal antibodies which can recognize both free and complex cTnI using the predicted dominant epitopes by hybridoma technique and cloning technique of limited dilution method. Besides, a pair antibodies of best match were used to developed a sandwich ELISA. The detection range of sandwich ELISA standard curve is 30ng/ml～500ng/ml. The affinity constant of Ab1 is 1.62×109L/mol and Ab2 is 2.60×108L/mol. 30 clinical serum samples were tested by this ELISA assay. Positive detection rate is 77.3% and negative detection rate is 100%. This makes the foundation for the establishment of rapid and easy detection of cTnI kit.

Keywords

Double sandwich ELISA assay; Human cardiac troponin; Monoclonal antibody.

List of Abbreviations:

Acute myocardial infarction: AMI;Aspartate transaminase: AST;cardiac troponin I: cTnI;Myoglobin: Mb;Lactate dehydrogenase: LDH;Phosphokinase: CK;Isoenzymes MB: CK-MB;Myoglobin: MYO;N-terminal pro-brain natriuretic peptide: NT-proBNP;cardiac troponin T: cTnT;troponin complex: Tn;troponin I: TnI;troponin C: TnC;Human cardiac troponin complex: cTnI-T-C;Fetal bovine serum: FBS;Horseradish peroxidase:HRP.

1. Introduction

Acute myocardial infarction (AMI) is a heart disease which causes unexpected death in human. So early detection is very important for the patients. Acute myocardial infarction (AMI) is one of the most common cardiovascular diseases. It is caused by blockage of one or more coronary arteries that supply the heart muscle with oxygen and nutrients[1,2], which results that the corresponding myocardium appears to be severe and persistent acute ischemia and becomes myocardial ischemic necrosis finally[3]. The condition of some patients is mild at the beginning, so they are not aware that some problem appear in the heart. Heart can not perform its pumping role anymore when condition becomes serious, which leads to death suddenly without any symptom. There for, early diagnosis of

AMI is essential for prevention and treatment. WHO stipulated three criterias for AMI diagnosis, that are, typical clinical symptoms, electrocardiographic and concentrations change of myocardial marker in the blood. However, the symptoms of AMI are always unspecific[4,5], and merely 40%-60% AMI patients can be identified if only depends on electrocardiographic diagnosis. Hence, it has become popular to search specific myocardial markers recently[6]. In the pass decades, the biomarkers used in clinic are Myoglobin(Mb), Lactate dehydrogenase(LDH), Aspartate transaminase(AST), Creatine phosphokinase(CK) and its Isoenzymes MB(CK-MB). However, some studies demonstrated that these biomarkers have poor specificity ,and concentration of those markers increase lately when AMI appears .In addition, diagnosis period of them is short , which are not favorable to early diagnosis of AMI [7] . Recently, researchers demonstrated that myoglobin(MYO), huamn N-terminal pro-brain natriuretic peptide(NT-proBNP) , cardic troponin I (cTnI) and cardic troponin T(cTnT) were considered as the best biomarkers of AMI, especially cTnI. CTnI in serum increases rapidly when myocardium gets injured. Therefore, it was considered as the most specific marker. Moreover, it keeps on increasing until eighth days and still can be tested in the tenth day, which is good for diagnosis. So, cTnI was determined as a gold standard to detect myocardial injury and to predict mortality and future cardiac events[8].

The troponin complex (Tn) which is constituted by troponin I(TnI) , troponin C(TnC) and troponin T (TnT) plays a role as regulator of heart contraction by binding to sarcomere thin filaments in skeletal and cardiac muscle tissue[9]. TnC binds Ca^{2+} ,which can mediate calcuium-dependent muscle contraction. TnT binds myosin to regulate the binding of troponin and thin filaments. Moreover, TnI is an inhibitor subunit of actin, which regulates the interaction of actin and myosin by inhibiting the activity of ATPase in the actin. The molecular weight of cTnI is 23876Da .it consists of 209 amino acids residues. The theoretical isoelectric point is 9.87. cTnI is 40% difference from sTnI. cTnI sequence is 31 amino acids residues more than sTnI. Moreover, cTnI only appears in myocardial cells with one isoform. Therefore, cTnI is specific in myocardial cells.

cTnI appears in myocardial cell with two kinds of form. Most of cTnI is bounded with the thin filaments of contractile apparatus, and rest of cTnI is free in the cytosol[10]. In the early period of heart injured , myocardial cells have not been damaged, but cell membrane is broken, and cTnI enters the tissue gap in a free form and through the lymphatic reflux into the blood**. Thus, cTnI is an accessible, specific and sensitive marker for early detection of damaged myocardial cell** [11]**.**Research shows that the boundary concentration of serum cTnI between a healthy person and a patient is below 50 pg/mL[12].In normal patients, the level of cTnI concentration is around 0.001 mg/L, but increased to100 mg/L in MI patients.

As we all known that most of cTnI exist in serum with the complex form of cTnI-T-C and cTnI-C. So it is necessary to produce antibodies which can recognize both free and complex cTnI[13] . Therefore, in this study, we took cTnI-T-C as detection antigen . As for immunogens, two epitopes (13-24 and 78-90 amino acid sequences) on cTnI subunit of cTnI-T-C were selected by Discovery Studio4.0 software and conjugated with BSA and KLH separately as BSA-1 and KLH-2. In addition, cTnI-T-C is another immunogen. We prepared three high affinity and specificity cTnI monoclonal antibodies, and selected the best pair antibodies to establish the double antibodies sandwich ELISA, which laid the foundation for the establishment of high sensitivity cTnI rapid detection kit.

2. Materials and methods

2.1 Materials

Human cardic tropnin complex(cTnI-T-C) were purchased from Hytest company. Two Antigen peptides were produced by Hangzhou Chinesepeptide company. Balb/c mice were purchased from experimental animal center of Southern Medical University. CBB G-250 was purchased from Shanghai biological technology co., LTD(China). BCA protein concentration assay kit was purchased from Thermo Fisher company. Tween-20 was purchased from Dingguo biology co.LTD(Guangzhou,China). freund's completed and imcompleted adjuvant **,**PEG2000 , HAT, HT

were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). TMB Color solution made by our lab. FetaL bovine serum(FBS) and RPMI-1640 Medium were purchased from Gibco company. HRP-Goat Anti Mouse IgG was purchased from ABCam company. HRP labeling kit (Type A) was purchased from Beijing Tai Tianhe biology technology company. SP2/0 myeloma cells were obtained from Shanghai Institute of Cell Biology.

2.2 Generation of monoclonal antibodies

2.2.1. Antigen selection and immunization

Through NCBI database we obtained the protein sequence of cTnI. Using this obtained protein sequence to predict its dominant epitope on IEDB website. Simultaneously, we obtained the crystal structure of the cTnI-T-C complex from the PDB database. The structure and location of the predicted epitopes in the cTnI-T-C complex are further analyzed by Discovery Studio 4.0 software. Select the two most favorable epitopes and couple them with BSA and KLH, respectively, and name them as BSA-1and KLH-2 separately.

In the initial immunization, BSA-1, KLH-2, cTnI-T-C were emulsified with same volume of Freund's complete adjuvant respectively. There were three groups 6 months female Balb/c mice immunized by three emulsified immuogen separately (every mouse was injected 40ug). From the second immunization, antigens were emulsified with same volume of Freund's imcomplete adjuvant. In the tenth day of the third immunization, blood was collected from the tail of mice and serum titer was tested by indirect ELISA assay.

2.2.2. Indirect ELISA assay

The 96-well enzyme plate was coated by antigen cTnI-T-C disssolved in coating solution(100ng/well) at 4 ° C for overnight, and the plates were washed three times with wash solution . Wells were blocked by 200ul blocking solution(5% slim milk in PST) at 37℃for 1h to decrease nonspecific binding. And washed it three times. Serum diluted with PBS was added into each wells and incubated at 37℃for 1h, and PBS is blank control. Wells were washed three times again . HRP-Goat Anti Mouse IgG (diluted 1:8000 by PBST)was added 100uL into wells, and wells were Incubated at 37℃for 1h. Washed it for five times. TMB solution (100ul/well) was added into wells and stored in the dark for 10min. Stop solution(50ul/well) was added into each wells. OD values of it were measured by enzyme-labeled instrument at the wavelengths of 450 nm.

2.2.3. Cell fusion and screening of monoclonal antibodies

The mice with good titers will be used for the preparation of monoclonal antibodies. The intraperitoneal boost injection had been done three days before cell fusion. The process of cell fusion follows to Kohler and Milstein[14] with a bit of modifications described by Lu et al[15]. The spleenocytes from immunization mice and logarithmical phase myeloma cells (SP2/0) were fused at the rate of 1:5～1:10 and were spun at 1000rap/min in 10min ,and threw supernatants . Myeloma cells and spleenocytes were fused in 1ml PEG. Fused cells were cultured in 2%HAT medium and cultivated at 37℃ 5% CO_2 cell incubator for two weeks.

The culture medium supernatant of hybridomas were collected and tested by indirect ELISA. Positive hybridomas cells were subcloned with limited dilution method. Positive monoclonal cells had been selected and was cultured in HT medium with same condition of culturing. Ascites-type monoclonal antibodies were prepared by injecting Balb/c mice with positive hybridomas cells intraperitoneally. Ascites was collected and purified.

2.3 Identification of antibodies characteristics

The purity of antibodies was tested by SDS-PAGE assay and the concentration of it was tested with BCA protein concentration assay kit. The subtype of mAbs were determined by Mouse monoclonal antibody isotyping ELISA kit. And the titer of antibodies was tested by indirect ELISA assay. Next we test whether three antibodies can bind to the others two forms of cTnI (free cTnI and cTnI-C complex) by indirect ELISA.

2.4 Establishment of double sandwich ELISA

2.4.1. Horseradish peroxidase(HRP) labeling of antibodies

Antibodies we acquired were labeled HRP by HRP labeling kit (Type A). The titer of HRP-Ab were tested by direct ELISA assay. The antigen cTnI-T-C was diluted to 0.5 μg / mL with coating solution and added 100uL into each well of 96-well enzyme plate. And incubated it at 4 ° C overnight. Washed it with PBST for three times. Wells were blocked by blocking solution(200ul 5% slim milk) and incubated at 37℃for 1h to decrease nonspecific binding. Washed it with PBST for three times. HRP-Abs diluted by PBST were added it into wells and incubated at 37℃for 1h. Washed it five times. TMB color solution (100ul/well) was added into well and stored in the dark for 10min.Stop solution(50ul/well) was added. OD values of it were measured by enzyme-labeled instrument at the wavelengths of 450 nm.

2.4.2. Selection of the best matched antibodies

The antibodies sandwich ELISA assay requires two or more antibodies that recognize different epitopes exposed to the surface of the protein. And it is better that different epitopes stay far away from each other. We tried to find out the best matched antibodies by sandwich ELISA assay.

The antibodies was diluted (2 μg / mL) by coating solution and added 100uL into each well of 96-well enzyme plate separately. Plates was incubated at 4 ° C overnight. Washed it with PBST for three times. Wells were blocked by blocking solution (200ul 5% slim milk) and incubated at 37℃for 1h to decrease nonspecific binding. Washed it with PBST for three times. Antigen diluted with PBS was added into wells and incubated at 37℃for 1h. Washed it with PBST for three times. HRP-Ab was diluted by PBST and added 100uL into wells. Incubated it at 37℃for 1h. Washed it with PBST for five times. TMB color solution (100ul/well) was added into well and stored without light for 10min.Stop solution(50ul/well) was added. OD values of it were measured by enzyme-labeled instrument at the wavelengths of 450 nm.

It is best pair of antibodies that value of OD_{450nm} of sandwich ELISA assay is higher when they recognize same concentration antigen and the best pairs of antibodies for double ELISA was expected with the widest detection range and the highest sensitivity.

2.4.3. Optimization of reaction conditions

As for antibodies sandwich ELISA assay, if the concentration of antibody is too high, it would be wasted, and it results in non-specific binding. If concentration is too low, its sensitivity would be low. So, it is necessary to optimize the reaction conditions of capture antibody and detective antibody.

It was known that the concentration of Ab1 is about 0.5μg/mL～4μg/mL and dilution ratio of HRP-Ab3 is about 1:2000 according to experiment before. Therefore, we set the concentration of Ab1 as 4μg/mL，2μg/mL，1μg/mL，0.5μg/mL and dilution ratio of HRP-Ab3 is 1:1000，1:2000，1:4000. Additionally, detection result was influenced by coloration time and temperature. During a certain range, the color of positive wells will be deeper with coloration time passed. So we tested the OD value at 450nm when coloration time is 5min，10min，15min，20min separately to find out which condition is the best for this antibodies sandwich ELISA.

2.4.4. Determination of linear range of double antibody sandwich ELISA and detection of clinical samples

There are different forms of cTnI in the serum of patients. It had been previously demonstrated that the Ab1 and Ab3 are capable of recognizing different forms of cTnI. And it is also necessary to verify that the antibody sandwich ELISA we built can recognize cTnI and cTnI-C. And built standard curve of sandwich ELISA in the best reaction condition. Furthermore, 30 samples were tested by this sandwich ELISA.

3. Results

3.1 Selection of immunogen and detection antigen

In order to produce antibodies which can identify both free cTnI and cTnI complex, we used cTnI-T-C complex as detection antigen. Moreover, cTnI-T-C is immunogens. The protein sequence of cTnI was analyzed in IEDB website. The prediction result of epitopes was shown in Fig 1. Epitope prediction as shown in Figure1B, with a Threshold of 0.290, five epitopes were predicted. Prediction of the β-turn position is shown in Figure 1C. With a Threshold of 0.941, the β-turn epitopes were predicted. Emini surface accessibility predictions are shown in Figure1D, with a Threshold of 1.000, epitopes were predicted. Karplus & Schulz Flexibility As shown in Figure 1E, with the Threshold of 1.011, the more flexible epitope were predicted. Karplus & Tongaonkar antigenicity prediction as shown in Figure1F, epitopes with high antigenicity were predicted with a Threshold of 1.002. Predicting Parker Hydrophilicity as shown in Figure1G, with Threshold of 2.339, more hydrophilic epitopes were predicted. Additionally, the structure of the predicted epitopes were analyzed on three dimensions structure of the cTnI-T-C complex by using Discovery Studio 4.0 software. Epitopes in which the α helix and the β folde structure are present are excluded, and an epitope located on the surface of the protein and having a corner structure is preferentially selected. As it shown in Fig1A. Two epitopes which are 13-24 and 78-90 amino acid sequence had been selected and conjucated BSA and KLH respectively as BSA-1 and KLH-2.

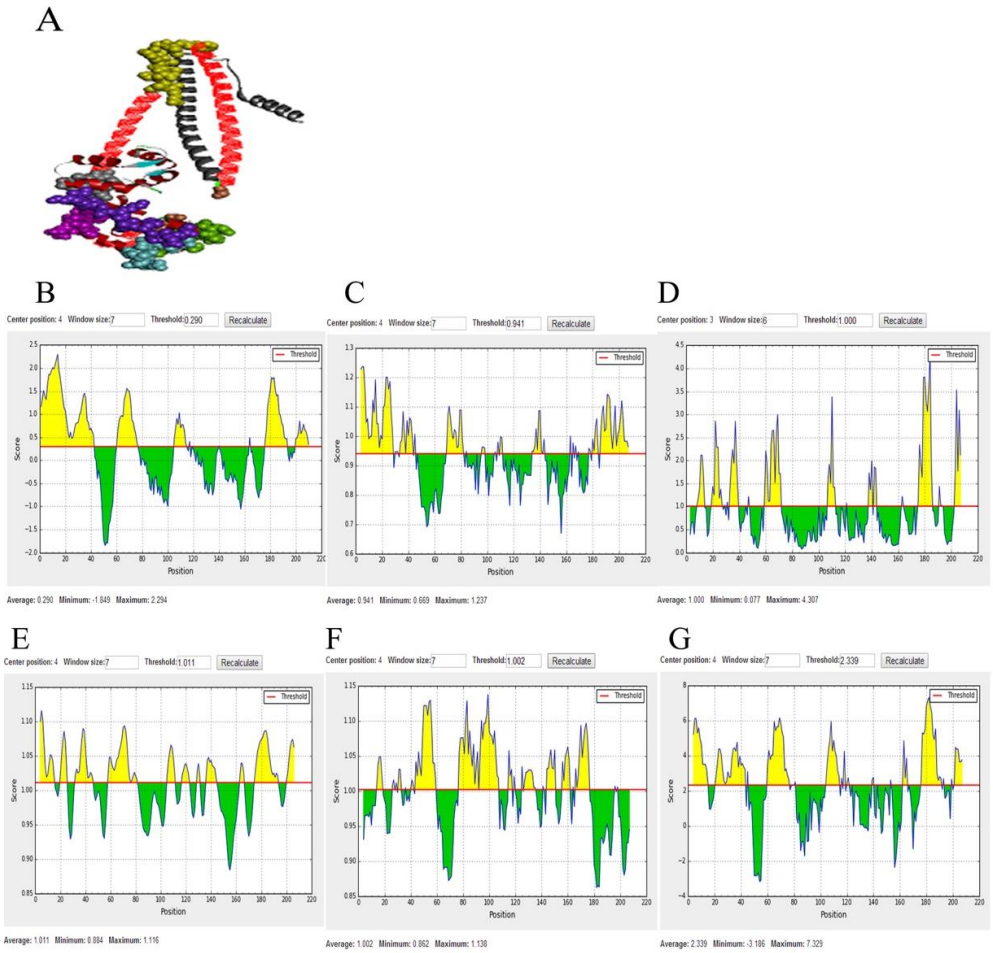

Fig 1 Prediction of epitopes. A: Three dimensional structure of cTn complex; B: Epitopes prediction ; C: Chou & Fasman Beta-turn prediction ; D: Emini surface accessibility prediction ; E: Karplus & Schulz flexibility prediction; F: Karplus & Tongaonkar antigenicity prediction; G: Parker Hydrophilicity prediction

3.2 Production of monoclonal antibodies specific to cTnI and cTnI-T-C

3.2.1. Mice immunization

Blood of mice had been collected from their tail in the tenth day of the fifth immunization. Serum was separated from blood and its titer was tasted by Indirect ELISA assay. For the mice immunized by BSA-1, the antibody serum titer of NO.3 mice is about 1:64000, which is higher than others. Thus NO.3 mice would be used for cell fusion(Fig.2 A). For the mice immunized by KLH-2, the antibody serum titer of NO.1 mice is about 1:1200, which is higher than others. Thus NO.1 mice would be used for fusion(Fig.2 B). For the mice immunized by cTnI-T-C, the antibody serum titer of NO.3 mice is about 1:32000, which is higher than others. So NO.4 mice would be used for fusion(Fig.2 C).

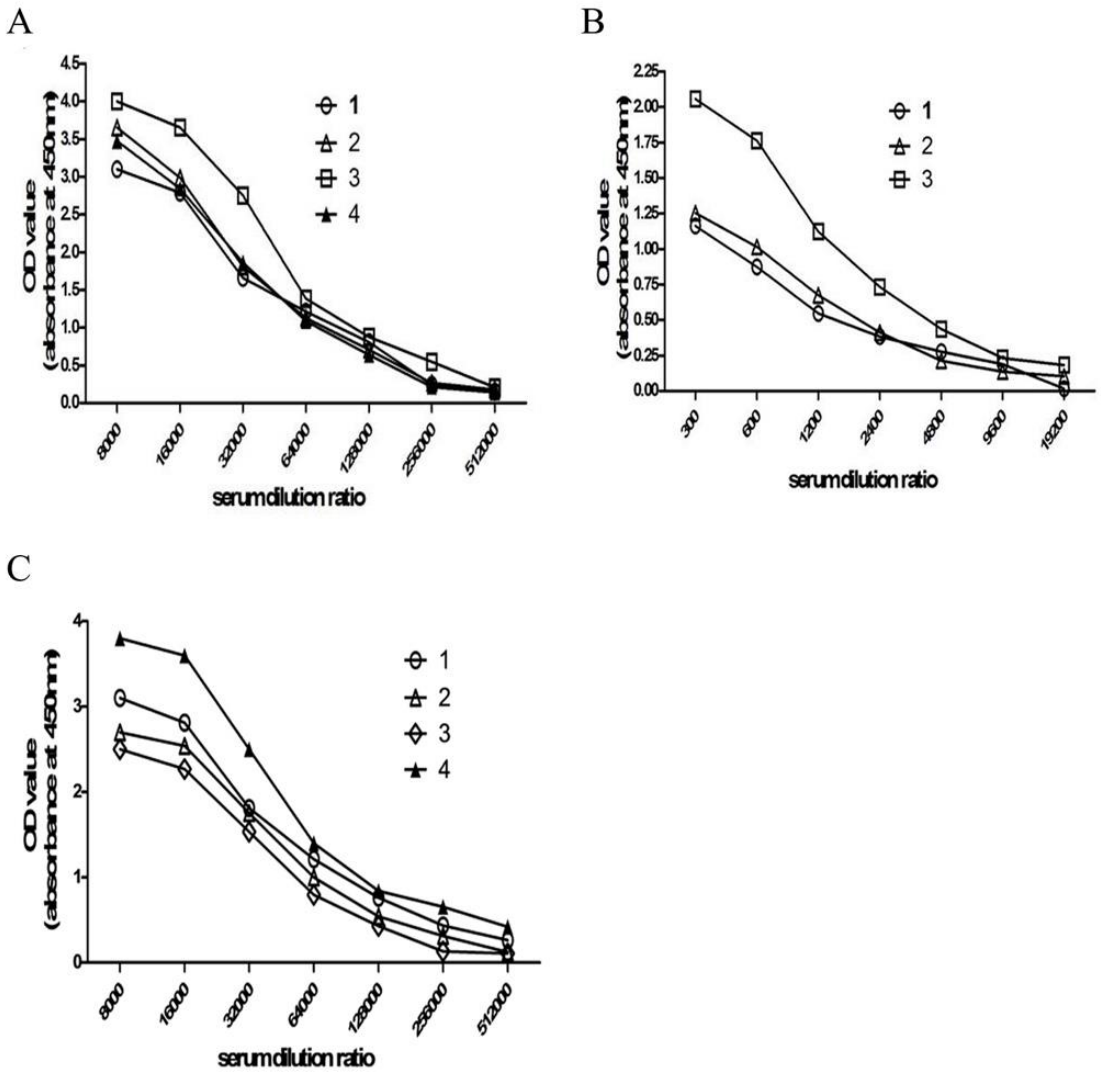

Fig.2 Polyclonal antibody serum titer of Mice. A: The mice immunized with BSA-1; B:The mice immunized with KLH-2; C:The mice immunized with cTnI-T-C

3.2.2. Cell fusion and screening of monoclonal antibodies

Three positive cells (7D2, 4C11, 7H4) which secret antibodies against for BSA-1, KLH-2, cTnI-T-C respectively were selected. Subtype of antibodies had been tested by antibody subtype ELISA kit. The subtypes of 7D2, 4C11, 7H4 were identified as IgG1, IgM, IgG1 separately and all subtype of light chains are κ.

3.2.3. Identification of antibodies characteristics

We named 7D2, 4C11, 7H4 as Ab1, Ab2 ,Ab3. Ab1 and Ab3 were purified with ammonium sulfate precipitation, deionization column and protein G column. However, Ab2 can't be purified with protein G column because IgM has no Fc fragment to connect construction of protein G. Therefore, Ab2 was purified only with ammonium sulfate precipitation and deionization column as initial purity. As shown in Fig.3 D, it is obvious that there are two bands at molecular weight of 50KD and 25KD for Ab1 and Ab3, which shows purification of Ab1 and Ab3 was done well. As for Ab2, there are more than two bands, which means there are some other proteins in Ab2. The concentration of antibodies had been determined by BCA protein concentration assay kit. The concentration of Ab1, Ab2, Ab3 are 13.558mg/mL; 4.236mg/mL; 8.7mg/mL respectively. The titer of antibodies were tasted by indirect ELISA. As it shown in Fig. 3(A-C), the titer of Ab1, Ab2, Ab3 are 1:1600000, 1:16000, 1:320000 respectively. As it was shown in Fig3 E, three antibodies can against with cTnI and cTnI-C. Moreover, Ab3 is an antibody immunized with cTnI-T-C, and it can identify cTnI, which means Ab3 identifies the epitope of cTnI. So, Ab3 is an antibody against cTnI. Three antibodies also can identify cTnI-C(Fig3 F), which means three antibodies can identify different forms of cTnI.

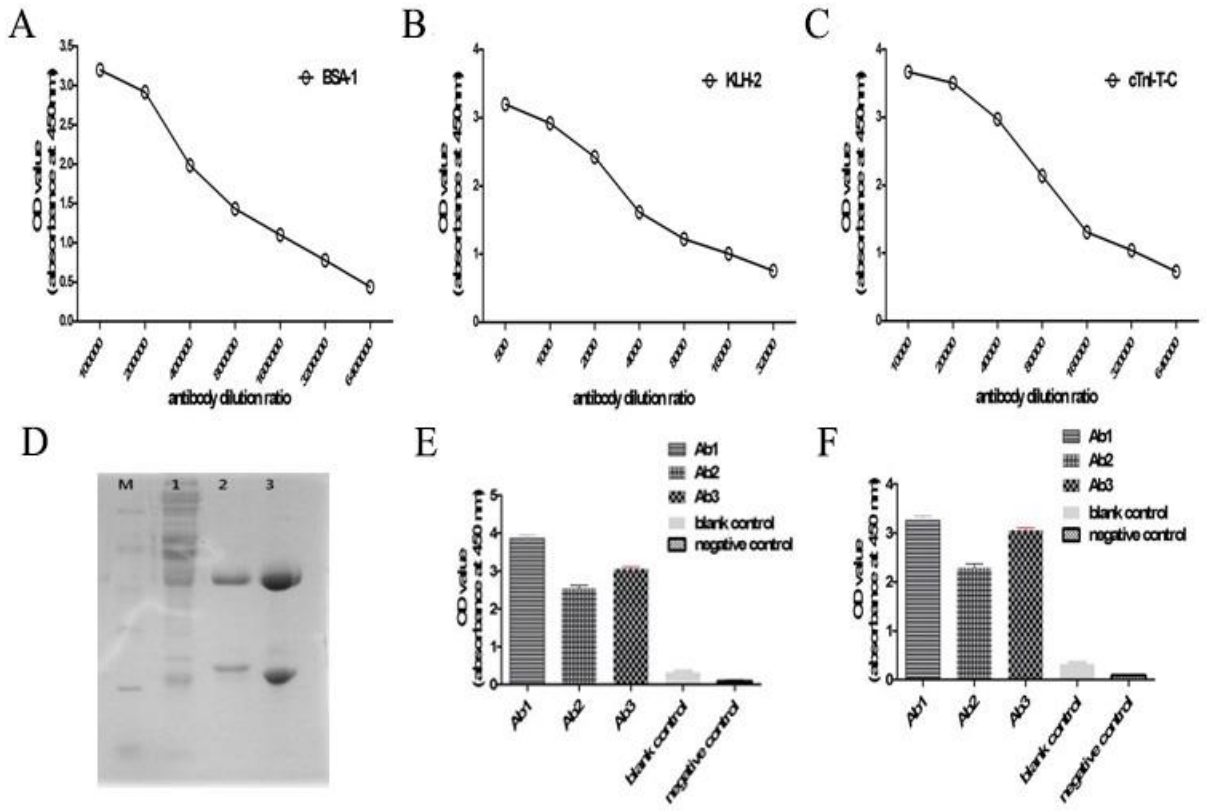

Fig. 3 Detection of purity antibodies. A -C: the titer of antibodies; D: SDS-PAGE tests the purity of the antibodies. Lane 1：Ab2; Lane 2:Ab1；Lane 3：Ab3； M：Marker，from up to down are 116, 66.2, 45.0, 35.0, 25.0, 18.0, 14.0kD; E: reaction between three antibodies and cTnI; F: reaction between three antibodies and cTnI-C.

3.3 Establishment of double sandwich ELISA

3.3.1 HRP labeling of antibodies

Three antibodies were labeled HRP to be detective antibodies. The result of HRP labeling was described in Fig. 4. It was considered as working ratio when OD_{450nm} value of corresponding ratio is about 2.0. Thus, working ratio of HRP-Ab1 is 1:10000 and HRP-Ab3 is 1:2500.

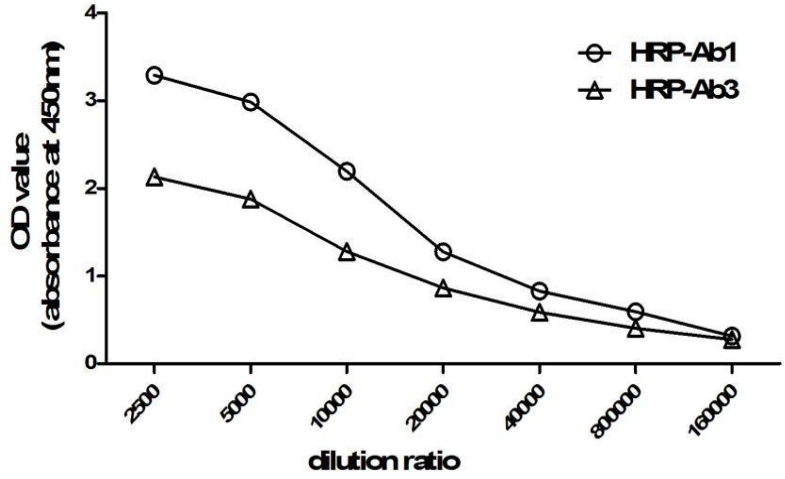

Fig. 4 Determination of HRP-Ab titer

3.3.2. Selection of the best matched antibodies

Ab1/HRP-Ab3 and Ab3/HRP-Ab1 are good matched antibodies and it shows that the detection curve of Ab1/HRP-Ab3 is steeper than the detection curve of Ab3/HRP-Ab1 and sensitivity of Ab1/HRP-Ab3 is higher than Ab3/HRP-Ab1 in Fig.5. Therefore, Ab1/HRP-Ab3 had been considered as the best pair of antibodies for sandwich ELISA.

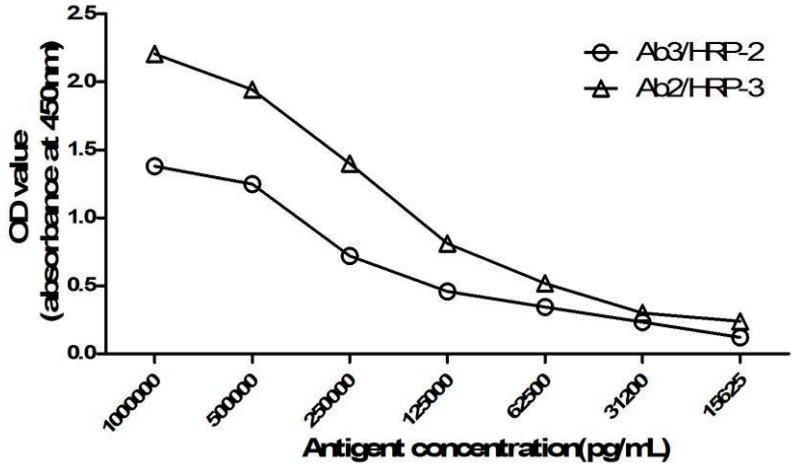

Fig. 5 Selection of the best pair of antibodies

3.3.3. Optimization of reaction conditions

As shown in Fig.6(A-D), when concentration of Ab1 is 0.5μg/mL and 1μg/mL, the change of curve is mild and the sensitivity is low which are not suitable for assay. When concentration of Ab1 is 2μg/mL and 4μg/mL, the change of curve is obvious and sensitivity is higher. Moreover, it is easy to find out that trend of these two curves are similar, which means it is saturated when Ab1 is 2μg/mL. There for, the best concentration of Ab1 is 2μg/mL.

As for HRP-Ab3, the sensitivity is lower when the dilution ratio of capture antibody is 1:4000, which cannot be selected. When the dilution ratio of it is 1:1000 and 1:2000, the trend of cure and range of detection are similar, which means it is saturated when dilution ratio of HRP-Ab3 is 1:2000. Thus, the best dilution ratio of HRP-Ab3 is 1:2000.

Additionally, it was shown in Fig.6E, when coloration time is 5min, OD value is lower, which means sensitivity is worse. And when coloration time is 20min, OD value was higher, but the value of

negative control is higher too, which will disturb the results. When coloration time is 10min and 15min, OD value is normal and sensitivity is good, which means 10min to 15min is best coloration time. Moreover, these two curves are almost similar, so 10min was considered as the best coloration time.

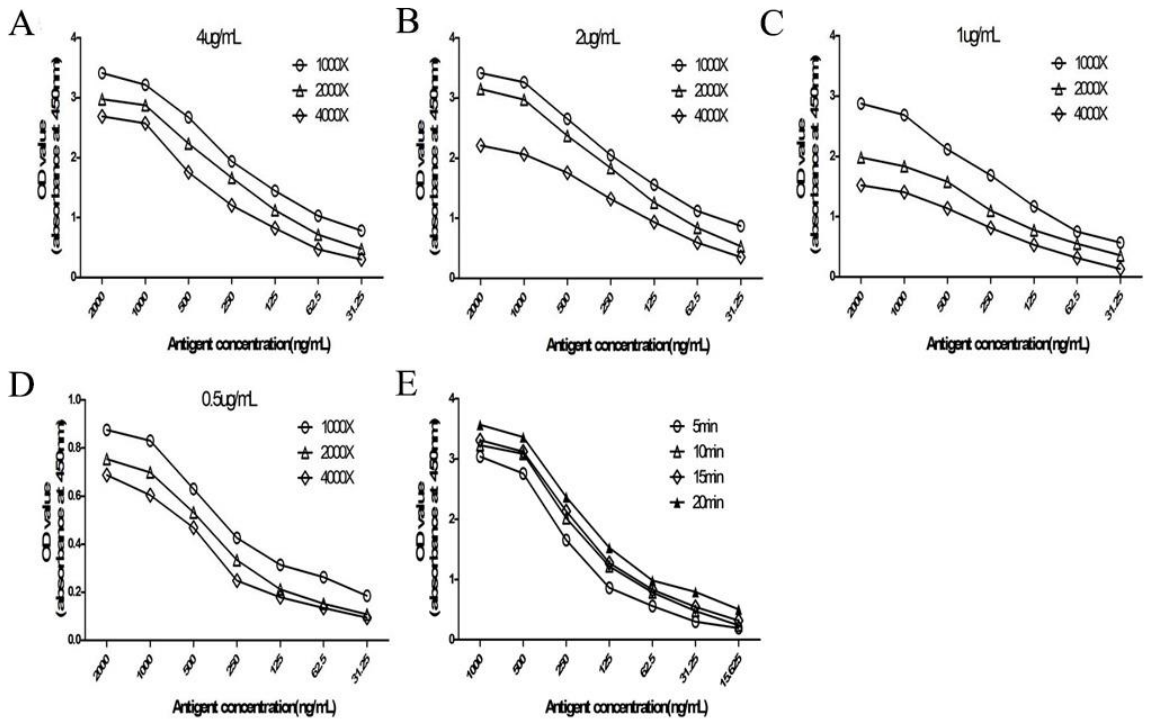

Fig .6 Optimizing reaction conditions. A-D: The concentration of Ab1 is 4ug/ml ; 2ug/ml ; 1ug/ml ; 0.5ug/ml; E: Optimization of reaction time

3.4 Determination of linear range of double antibody sandwich ELISA and detection of clinical samples

As shown in Fig7 A, the antibodies sandwich ELISA we made also can identify different forms of cTnI, so it can be used in detection clinical samples. Additionally as it shown in Fig.7 B, the linear range of detection is 30ng/mL～500ng/mL. The sensitivity of assay is lower, but range of detection is wide. 30 samples were tested by this sandwich ELISA. Positive sample detection rate is 77.3% and negative sample detection rate is 100%.

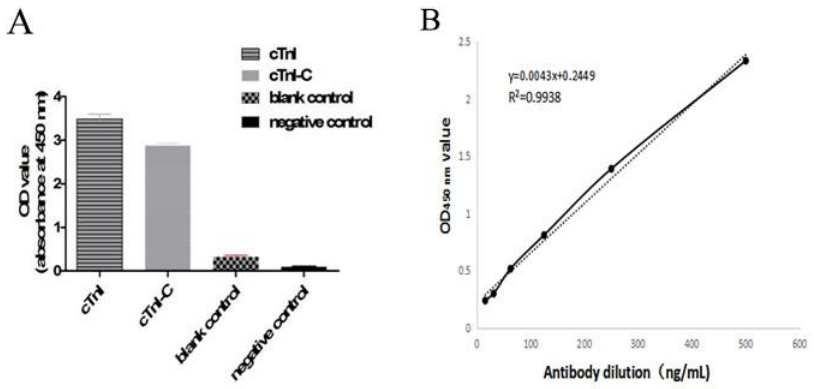

Fig 7. Antibodies sandwich ELISA. A: matched antibodies recognize cTnI and cTnI-C; B: Establishment of detection curve

4. Discussion

In this study, we produced a pair of high specificity and sensitivity MAbs which can recognize different forms of cTnI. The antibody titer of Ab1 is 1:1600000 and Ab3 is 1:320000. In addition,we developed a specific and sensitive antibodies sandwich ELISA assay for detection of cTnI-T-C. The antibodies sandwich ELISA we produced linear range of detection is 30ng/mL～500ng/mL. Clinical serum samples of patients had been tested by this sandwich ELISA, which shows this assay can be used in detection of samples and built a foundation for rapid detection of AMI.

Epitope is an area that can stimulate the body to produce immune response or sensitive lymphocytes to interact with epitope. Immune cells recognize one epitope of antigen instead of whole antigen [16]. There for, Specific epitopes can be selected from the antigen as antigenic peptides to produce specific antibodies. There are some principles for epitopes selection as follow. First, the peptide selected on the surface of the antigen are hydrophilic and flexibility. Because the hydrophilic areas are on the surface of the protein and antibodies only can recognize the corresponding region of epitope on the protein surface. When these antigenic epitopes are easily deformed and transferred to the position where the antibody is accessible, it will have high affinity to the antibody. Moreover we should select epitopes which are simple Loop structure to avoid of becoming α-helix structure. Additionally, peptides in N-terminal are generally selected because N-terminal and C-terminal are usually exposed to the surface of the protein. However, hydrophobicity of C-terminal peptides is too strong to be suitable as an antigen and the length of antigenic polypeptide sequence which was selected should be about 8-20 amino acid residues. If the length of sequence is too short, the affinity between antibody and native protein would be weaker. If the length of the sequence is too long, it would be possible to produce a secondary structure, which would cause that antibody is not specific for the predicted epitope. Furthermore, it would be more difficult to synthesis and purity when epitopes are secondary structure. Finally, high homology sequences cannot be selected to avoid cross-reactivity of antibodies.

Sequences of the antibody's heavy and light chain variable region gene were acquired. The models of antibodies were constructed ,and then were docked to antigen. It can be predicted whether the antigen could be matched to antibody with Discovery Studio 4.0 software. The result of prediction is Ab2 can match with Ab1, Ab3. However, the result of experiment showed that Ab2 was failed to match with Ab1, Ab3. The possible reason is that IgM is the largest molecular weight immunoglobulin, and it is a pentameric structure.So IgM binding to antigen molecules blocks the area of antigen where binds to another antibody, which results in unsuccessful pairing. The antibody can theoretically be matched with others antibodies, so we will re-prepared IgG antibody against for the 78-90 amino acid epitope.

Acknowledgments

This work was supported by the Guangdong province's core technology research project "immunofluorescence quantitative rapid detection and diagnosis", China (2012A080800007). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Disclosure Statement： none conflict of interest.

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