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Isolation Of Mung Bean (*Vigna radiata* (L.) R. Wilczek) Proteins To Create A Skin Prick Test Reagent To The Diagnosis of Mung Bean Allergy

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Abstract: *Mung bean has been widely used by Indonesianan and world people for food and mung bean can caused an allergy reaction, particularly on children. The Major allergen of mung bean has been identified were protein Vig r 1, Vig r 2, Vig r 4, and Vig r 6. This study aimed to create a skin prick test reagent of mung bean that meets the requirements of the European Pharmacopoeia Monograph on Allergen Products 7 (2010:1063) to diagnosis of allergy. The protein contents of mung bean isolates was 87.98% with an extraction yield at 82.7% and recovery at 20,5 g/100g nut . The mung bean protein isolate was composed of 16 protein bands with the molecular weights of 11.1-127.4 kDa. The allergenic mung bean protein has the molecular weights of 10.9-142.2 kDa after analysis with immunoblotting method. The mung bean protein isolate was formulated into a skin prick test reagent. The mung bean reagent met the requirements of the European Pharmacopoeia for the parameters of moisture content, protein content, sterility and microbiology. A skin prick test to the respondents indicated that the sensitivity value of mung bean reagent was 75% with a negative error rate at 25%. The specificity of mung bean reagent was 100% with an error rate of positive diagnosis occurrence at 0%.*

Keywords - *allergy diagnosis, mung bean, protein isolate, skin prick test, sensitivity, specificity.*

I. INTRODUCTION

Mung bean has been known to trigger allergic reactions mediated by IgE antibodies, particularly in children. Major allergens contained in mung bean are Vig r 1, Vig r 2, Vig r 4, and Vig r 6 proteins [1]. Allergy data of mung bean in Indonesia are yet to be fully available. Several allergy clinics and hospitals have not been able to provide mung bean reagent for skin prick test (SPT). Most allergy clinics or hospitals provide peanut and soybean reagents for SPT. If someone is positively allergic to peanuts or soybeans, he/she will be advised not to consume nuts including mung beans. In fact, a person can be allergic to peanuts but not allergic to soybeans or vice versa, thus the diagnosis needs to be conducted.

Skin prick test (SPT) is a reliable method for diagnosing allergies that are mediated by IgE antibodies in patients with rhinoconjunctivitis, asthma, urticaria, anapylaxis, atopic eczema, drug and food allergies [2]. SPT method was first published by Helmtraud Ebruster in 1959 and has been used as a primary diagnostic method for detection of type I hypersensitivity reactions [3]. This study aimed to create mung bean reagent for SPT in compliance with the requirements of the European Pharmacopoeia Monograph on Allergen Products 7 (2010: 1063) and measure its sensitivity and specificity in the diagnosis of allergies.

II. MATERIALS AND METHODS

2.1 Materials

The main material used in this study was mung bean (*Vigna radiata* (L.) R. Wilczek) obtained from the local market, Bogor. The main chemicals used were BSA (bovine serum albumin), acrylamide, glycine, 2-Mercaptoethanol, 0.05 M carbonate-bicarbonate buffer at pH 9.6. coomasie brilliant blue R-250, coomasie brilliant blue G-250, IgE antibody, anti-IgE human antibody labeled with HRP (Horseradish Peroxidase) enzyme, DAB (3,3'-Diaminobenzidine) substrate, TMB (3,3',5,5'-Tetramethylbenzidine) substrate, N,N'-methylenebisacrylamide, low molecular weight proteins (LMW) Fermentas® (containing 7 types of protein, which are β-galactosidase standards (MW: 116 kDa), bovine serum albumin (MW: 66.2 kDa), ovalbumin (MW: 45 kDa), lactase dehydrogenase (MW: 35 kDa), REase BSP 981 (MW: 25 kDa), β-lactoglobulin (MW: 18.4 kDa), and lysozyme (MW: 14.4 kDa). Other chemicals were purchased from Sigma.

2.2 Equipments

The equipments used were high speed microcentrifuge, SDS-PAGE Bio-Rad Mini-Protean II tool, immunoblotting Mini Trans-Blot® Electrophoretic Transfer Cell Bio-Rad tool, Costar® 96-well ELISA microplates, LabSystem Multiskan EX ELISA reader, UV-VIS spectrophotometer, freeze dryer, nitrocellulose membranes for blotting pore size 0.45 µm, size 15 cm x 15 cm (Sigma N8267), pH meter, sonicator, vortex mixer, stirrer, 0.5 µL to 1000 µL micropipettes, 0.2 µm SFCA syringe filter, Whatman #1 filter paper, and other glasswares.

2.3 Isolation of Mung Bean Proteins

Mung beans were wetly ground and then sundried until dry. Dried mung beans were then pulverized with the flour mill machine and screened through 60-mesh sieve to obtain mung bean flour. The mung bean flour was then defatted using hexane solution. 100 g of mung bean flour was put in a beaker glass, added with 750 mL of hexane, then stirred using a magnetic stirrer at 38-40 °C for 1 hour. After that, allowed it to stand for 1 hour thus the mung bean flour was separated to the bottom of a beaker glass, then the upper hexane solution was discarded. 750 mL of hexane was added back into a beaker glass and then stirred using a magnetic stirrer at 38-40 °C for 1 hour. This step was repeated 3 times. Mung bean flour from the third repetition was stored in an open container for 1 night at room temperature to remove residual hexane. This treatment would obtain free-fat mung bean flour [4].

Fat-free mung bean flour was added with distilled water (1:10 w/v) in a beaker glass. Its pH was then increased to 8-8.5 by adding 1N NaOH while stirring. The solution was continuously stirred with a magnetic stirrer for 1 h at 40 °C. The solution was then centrifuged at 10,000 g for 30 min at 4 °C. The supernatant was collected in a beaker glass and the pH was lowered to 4.5 by adding 1 N HCl and centrifuged again at 10,000 g for 30 min at 4 °C. The obtained supernatant was discarded and the protein precipitates were collected and dried with a freeze drier to obtain mung bean protein isolates flour [5].

2.4 Serum Preparation

Serum collection was conducted in two steps. The first step was conducted toward 25 respondents who have food allergy (discovered from interviews). This serum was later used to conduct the allergenicity test of mung bean proteins using ELISA and immunoblotting methods prior to the SPT. The second step of serum collection was conducted toward 8 respondents with mung bean allergy and 12 respondents without mung bean allergy which later used to conduct allergenicity test of SPT reagent. Second-step respondents attended SPT in allergy clinic. Serum collection and SPT were conducted by medical personnel and allergologist physician who have obtained permission from the Health Research Ethics Committee, Faculty of Medicine Indonesia University, Cipto Mangunkusumo Hospital with number of ethical approval is 199/H2.F1/ETIK/2014. 10 mL of each respondent's blood was withdrawn then placed in a bottle containing no EDTA. The withdrawn blood was allowed to stand for 1 hour, then centrifuged for 20 minutes at 2500 rpm (1250 g). The obtained supernatant was a serum containing IgE antibodies and stored at -20 ° C [6].

2.5 SDS-PAGE Electrophoresis and Immunoblotting

Electrophoresis was conducted according to Laemmli [7] with 12% concentration of separating gel and stained with coomasie brilliant blue R-250. The unstained gel from electrophoresis result was transferred to 0,45 µm nitrocellulose membrane using transblotting equipment with 90 V voltage for 90 minutes. After being transferred, the membrane was removed from equipment and soaked with 50% methanol for 2 minutes, then blocked with 5% skim milk in PBST (Phosphate Buffered Saline with 0.05 % Tween® 20) for 1 hour at room temperature. The membrane was washed with PBST 3 times, each for 5 minutes. After being washed, the membrane was added with the allergic patient serum with 1:10 dilution in PBST, then incubated for 2 hours at room temperature. Washing was conducted again (3 times) with PBST, each for 5 minutes, then added with HRP conjugated monoclonal mouse anti-human IgE antibody (1:3000 dilution in PBST) and incubated for 1 hour while shaken. After that, the membrane was re-washed with PBST (3 times) each for 5 minutes, and added with DAB substrate. Positive detection results were marked with the formation of brown-colored band on nitrocellulose membrane [8].

2.6 Determination of Total IgE with ELISA Method

100 µL serum/well with a 1:10 dilution (in 0.05 M carbonate-bicarbonate buffer at pH 9.6) was attached to the microtiter plates and incubated for 1 night at 4 °C. Normal human sera (NHS) were used as control. The remaining sample was removed and the microtiter plates were washed 5 times with PBST (250 µL/well). After that, 200 µL/well of 5% skim milk in PBST was added and incubated for 1 hour at 37 °C. Furthermore, it was washed 5 times with PBST (250 µL/well) and added with 100 µL/well of HRP conjugated

monoclonal mouse anti-human IgE antibody (with 1:6000 dilution in PBST), then incubated at 37 °C for 1 hour. The microtiter plates were washed with PBST (250 µL/well) for 10 times, then added with 100 µL/well of TMB substrate. Positive results were marked when blue color developed. After 5 minutes, the reaction was stopped by adding 100 µL/well of 2 M H_2SO_4, and the solution would turn bright yellow. Optical density (OD) was measured using ELISA reader at 450 nm. Mean ± 2SD of normal controls was taken as cut-off for ELISA positive results [6]; [9].

2.7 Determination of Specific IgE with ELISA Method

100 µL/well of each mung bean proteins 10 µg/mL (in 0.05 M carbonate-bicarbonate buffer, pH 9.6) was attached to the microtiter plates, then incubated at 4 °C for 1 night. The remaining sample was removed and the microtiter plates were washed for 5 times with PBST (250 µL/well). After that, 200 µL/well of 5% skim milk in PBST was added and incubated for 1 hour at 37 °C. Furthermore, it was washed 5 times with PBST (250 µL/well). 100 µL/well of allergic patient serum with 1:10 dilution in PBST was added and incubated for 1 hour at 37 °C. Normal human sera (NHS) were used as control. After incubation, the microtiter plates were washed with PBST (250 µL/well) for 5 times, then added 100 µL/well of HRP conjugated monoclonal mouse anti-human IgE antibody (diluted 1:6000 in PBST), then incubated for 1 hour at 37 °C. Microtiter plates were washed with PBST (250 µL/well) for 10 times, and 100 µL/well of TMB substrate was added. Positive results were marked when blue color developed. After 5 minutes, the reaction was stopped by adding 100 µL/well of 2 M H_2SO_4, and the solution would turn bright yellow. Optical density (OD) was measured using ELISA reader at 450 nm. Mean ± 2SD of normal controls was taken as cut-off for ELISA positive results [6]; [9].

2.8 Creating Skin Prick Test Reagent

Each 0.2 g of mung bean proteins isolates was dissolved in 2 mL of phosphate buffer saline (PBS) at pH 7.4, sonicated 5 x @ 1 minute in cold condition and then centrifuged at 11780 g for 15 minutes. The obtained supernatant was filtered using 0,2 µm syringe filter for sterilization in order to obtain a sterile protein stock solution. The procedure was conducted in a sterile and aseptic room. The protein content of stock solution was determined using the Bradford method [10]. Stock solution was then dissolved in 50% glycerol-saline solution containing 0.4% sterile phenol, thus the concentration became 1 mg/mL. The obtained solution was then called "mung bean SPT reagent" which then tested chemically, sterilitically and microbiologically. If it was qualified from chemical, sterility and microbiological tests, the reagent would be used for SPT in humans [11].

2.9 Skin Prick Test

The SPT was conducted by allergologist physician who has received permission from the Health Research Ethics Committee with number of ethical approval is 199/H2.F1/ETIK/2014. SPT was conducted on the volar forearm. The area that dropped with reagent was marked with a ballpoint and disinfected with alcohol. 1 mg/ml histamine was used as a positive control and 50% glycerol-saline solution was used as a negative control. Positive and negative controls were dropped on the opposite area then other reagents were dropped. Skin prick that has been dropped with histamine, control buffer, and allergen extracts using the brown marrow needle. The pricking process was conducted slowly with 45° slope the penetrated the epidermal layer without causing bleeding. The diameter of the wheal (urtica) on the skin was measured after 15-20 minutes by measuring the developed wheal (urtica). The test results were transferred to the millimeter block paper by making line surrounding the wheal border with marker pen (size 0.2), taped with masking tape and masking tape was affixed to the millimeter paper block. Each circle diameter on the tape was then measured. The result expressed as "0" when the wheal size is equal to the negative control (no wheal formed), "+1" if the wheal size is 25% -50% greater than the negative control (<3 mm), "+2" if the wheal size is 50%-75% greater than the negative control (3 mm-5 mm) and "+3" if the wheal size is equal to the histamine (5 mm-7 mm), and "+4" if the wheal size is 25%-50% greater than the histamine and ">+4" when the wheal size is more than 50% greater than the histamine. From the SPT results, sensitivity and selectivity of each reagent were measured [11].

III. RESULTS AND DISCUSSION

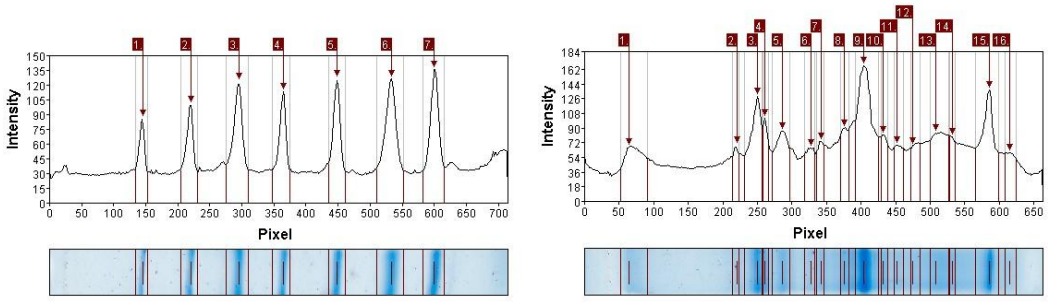
3.1 Protein Isolation

Defatting process was conducted before protein isolation using hexane solution [12]. Analysis result showed that there was 62% of fat decrease. Fat concentrations before and after defatting were 1.60 ± 0.13% and 0.61 ± 0.09%, respectively. High fat content may interfere protein identification by electrophoresis method and fat can cover the epitopes of allergens, thereby reducing the allergenicity of allergens during diagnosis. Isolation of mung bean proteins using isoelectric precipitation method produced 87.98% protein content. The yield and recovery of mung bean protein isolates were 82.7% of 20.5 g/100g, respectively. Study by [13] using the same technique with chickpeas, lentils, broad beans and kidney beans resulted in 52.83±3.36% pea proteins yield to 80.47±5.71% pea proteins yield, while the recoveries in each bean were 20.70±0.97; 29.58±1.49; 19.68±1.08; and 14.60±0.38 g/100 beans.

3.2 Analysis of Total IgE and Specific IgE Antibodies

Analysis of total IgE was conducted on 25 respondents (Respondent No. 1-25) who have allergy histories to seafood and nuts. Respondents' allergy history was revealed from the interviews and questionnaires. The analysis results by ELISA method showed that 25 respondents were people with allergies and 10 (40%) were allergic to mung beans. Respondents who have mung bean allergy were respondent no. 2-5, 7-8, 12-13, 16 and 23. Respondents who showed highest allergic reactions to mung beans were respondent no. 1, 12, 15 and 20 in which their serums would be used in the immunoblotting method.

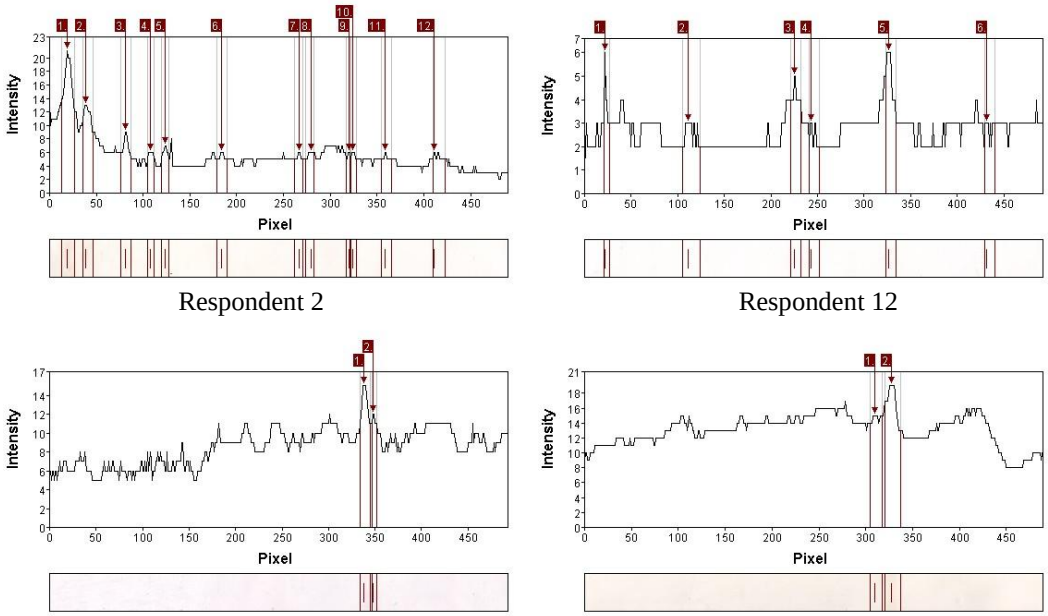
3.3 Protein and Allergen Profiles of Mung Bean Isolates



(A) Marker (B) Mung bean protein isolates

Figure 1. SDS-Page Electrophoresis results (A) marker and (B) Mung bean protein isolates

Figure 1 shows the marker profile and protein isolates of mung bean. Band identification results using the GelAnalyzer 2010a software showed that protein isolates of mung bean consisted of 16 bands with different intensities. The detected molecular weights of mung bean protein isolates were 127.4 kDa, 63.9 kDa, 55.8 kDa, 53.3 kDa, 47.6 kDa, 39.6 kDa, 37.1 kDa, 31.9 kDa, 28.2 kDa, 24.9 kDa, 22.8 kDa, 20.7 kDa, 17.8 kDa, 16.0 kDa, 12.6 kDa, and 11.1 kDa with the highest intensity was protein with molecular weight of 28.2 kDa at 17.1% followed by 12.6 kDa protein at 12.0% and 55.8 kDa protein at 9.7%. According to WHO/IUIS database [1], the main allergens found in mung beans are Vig r 1 protein with a molecular weight of 16 kDa, Vig r 2 protein with a molecular weight of 52 kDa, Vig r 4 protein with a molecular weight of 30 kDa, and Vig r 6 protein with molecular weight 18 kDa. Based on Figure 1, the 14th band with 2.8% intensity was suspected as Vig r 1 protein and the 13th band with 9.1% intensity was Vig r 6 protein. The 8th band with a molecular weight of 31.9 kDa was suspected as Vig r 4 protein and the 4th band with a molecular weight of 53.3 kDa was suspected as Vig r 2 protein. Among Vig r 1, Vig r 2, Vig r 4, and Vig r 6 proteins, Vig r 4 protein was suspected to have the highest intensity, followed by Vig r 4, Vig r 2 and Vig r 1 proteins, respectively.



Respondent 2 Respondent 12

Respondent 16 Respondent 23

Figure 2. Immunoblotting results of respondents 2, 12, 16 and 23.

Figure 2 shows the profile of mung bean protein isolates that could bind to specific IgE antibodies in the serum of mung bean allergy patients. The serum of allergy patients was selected based on the high allergenicity to mung beans which was priorly determined by ELISA. Proteins allergens that could bind to the specific IgE antibodies in serum 2, 12, 16 and 23 were 12 bands, 6 bands, 2 bands and 2 bands, respectively. Protein isolates of mung bean that could bind to specific IgE of serum 2 were 142.2 kDa, 125.7 kDa, 96.9 kDa, 81.8 kDa, 74.6 kDa, 51.0 kDa, 30.3 kDa, 28.0 kDa, 21.8 kDa, 21.3 kDa, 18.2 kDa, and 12.4 kDa, while in serum 12 were 139.6 kDa, 80.3 kDa, 39.5 kDa, 35.3 kDa, 21.1 kDa, and 10.9 kDa. Specific IgE antibodies of serum 16 could bind with the mung bean proteins of 19.5 kDa and 18.3 kDa, while serum 23 with 22.9 kDa and 20.5 kDa proteins. Protein with a molecular weight of 18 kDa was suspected as Vig r 6 which could be bound by IgE antibodies of serum 1 and 3. IgE antibodies of serum 1 could also bind to protein with a molecular weight of 30 kDa that was suspected as Vig r 4.

3.4 Formulation and Quality Requirements of Allergen Products for SPT

Allergen products or reagents made in this study was a product of glycerinated extract containing 50% glycerol. Figure 3 shows the sterile mung beans reagent for skin prick test.

Figure 3. A sterile mung bean reagent for skin prick test

Table 1 shows the requirements from European Pharmacopoeia 7 Monograph on Allergen Product (2010:1063) and the analysis results of allergen products of mung bean reagent. The analysis results showed that allergen products of mung bean used for the diagnosis of mung bean allergy with SPT method have met the requirements set by the European Pharmacopoeia 7 Monograph on Allergen Products (2010:1063) [14].

Table 1. Requirements of European Pharmacopoeia 7 Monograph on Allergen Product (2010: 1063) and the analysis results of mung bean reagent.

No	Parameters	Requirements	Analysis Results
1	Moisture content (%)	Maximum 5% for freeze-dried products and can be more than 5% for liquid products	63,00 ± 0,24 (liquid products)
2	Protein content (µg/µl)	80-120% from the stated concentration (1 µg/µl)	1,10
3	Sterility	Sterile, if it is not sterile then refers to chapter *5.1.4.* on *European Pharmacopoeia 7 01/2011:50104*	Sterile
4	Total plate count*	Max 10^2 CFU/g or CFU/mL	0
5	*Staphylococcus aureus**	Not detected in 1g or 1 mL	0
6	*Pseudomonas aeruginosa**	Not detected in 1g or 1 mL	0
7	Fungi*	Max 10^1 CFU/g or CFU/mL	0
8	Yeast*		0

*According to European Pharmacopoeia 7 01/2011:50104)

3.5 Skin Prick Test Results

Table 2 shows that all respondents were allergy patients based on total IgE and all respondents were allergic to mung beans based on the specific IgE result. SPT results showed that there were 6 respondents who demonstrated positive diagnosis results, in accordance with the specific IgE result. It shows that the sensitivity value of mung bean reagent was 75% (6/8) (positive allergy = 6 and the number of respondents = 8). Table 2 also shows that two respondents of SPT diagnosis showed negative result in which it should be positive. It shows that the negative error of mung bean reagent in the diagnosis of allergy was 25% (2/8) (negative allergy = 2 and the number of respondents = 8).

Table 2. Total IgE (tIgE), specific IgE (sIgE) and skin prick test (SPT) results of mung bean on the respondents with mung bean allergy

No	Resp. code	tIgE Result	sIgE Result	SPT Result
1	04	+	+	0
2	06	+	+	+2
3	08	+	+	+2
4	09	+	+	+1
5	14	+	+	+1
6	21	+	+	+1
7	28	+	+	0
8	31	+	+	+3

+: Positive, - and 0: Negative, Resp. code: respondent code

Table 3. Total IgE (tIgE), specific IgE (sIgE) and skin prick test results of mung bean on the respondents without mung bean allergy

No	Resp. code	tIgE Result	sIgE Result	SPT Result
1	07	+	-	-
2	11	-	-	-
3	12	-	-	-
4	17	+	-	-
5	18	+	-	-
6	19	+	-	-
7	20	+	-	-
8	23	+	-	-
9	24	+	-	-
10	26	+	-	-
11	30	+	-	-
12	34	-	-	-

+: Positive, - and 0: Negative, Resp. code: respondent code

Table 3 shows that 9 respondents were allergy patients and 3 respondents were not allergy patients based on total IgE result. According to the IgE specific analysis, all respondents in Table 3 were not allergic to mung beans, although there were 9 respondents who showed positive allergies. These 9 respondents were possibly allergic to other foods but did not allergic to mung beans. SPT results showed that all respondents have negative allergies. It means that the specificity of the mung bean reagent in diagnosing allergies was 100% with 0% false positives.

IV. CONCLUSION

Mung bean protein isolates could be used to make SPT reagent in compliance with the requirements of the European Pharmacopoeia 7 Monograph on Allergen Product (2010: 1063). Isolation of mung bean proteins with isoelectric method, suspected to contain the major allergens such as Vig r 1, Vig r 2, Vig r 4, and Vig r 6 and minor allergens. A skin prick test to the respondents indicates the sensitivity value of mung bean reagent was 75% with a negative error rate at 25%. The specificity of mung bean reagent was 100% with an error rate of positive diagnosis occurrence at 0%.

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