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PURIFICATION AND EXTRACTION OF POLYSACCHARIDE SPECIFIC OF *STREPTOCOCCUS PYOGENES* OF GROUP A FIXING ON LATEX PARTICLES

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Abstract

The ***Streptococci*** are widely distributed in nature and frequently form part of the normal human flora which are members. Approximately 5-15% of humans carry ***Streptococcus pyogenes***. Cytoplasmic membrane has antigens similar to those of human cardiac and heart valve fibroblasts. Patients may also develop immune- mediated such as acute rheumatic fever skin infections and endocarditis. ***S. pyogenes*** evolved an enzyme that specifically target IgG use as biochemical reactions, and serologic specificity. Serologic grouping is based on antigenic differences in cell wall carbohydrates (groupA). A technique for detection of antibodies against ***S. pyogenes*** group antigen / type antigens is being devised as one of the procedures possibly useable in prevention of infections and manufacturers in development of diagnostic and identification tools. Method used for detection of antibodies (latex agglutination) are being standardised with antibody levels has been introduced. The detection of antibodies induced by conjugated is under development for antibiotic resistance of ***Streptococcus pyogenes*** prepared and provided freeze-dried reference type cultures of ***Streptococci.*** The stability of the obtained component subjected at *4 °C and 37 °C* over one period to reveal several groups of serum representing healthy population (N = 20) and patients (N = 40) as a significant correlation for the diagnostic of acute rheumatic arthritis. We have shown that the total amount of specific antibodies does not necessary correlate with the level of neutralizing antibodies, and antibody quality might be clinically more important than antibody quantity

Introduction

Streptococcus pyogenes is an important human bacterial pathogen that causes a variety of diseases **Beall**, (2002).The survival of ***S. pyogenes*** depends on its ability to avoid the various actions of the human immune system. Immunoglobulin G (IgG) plays a key role in the immune defense by specifically recognizing invading microorganisms. **Cunningham**, (2000).

Microbiological diagnosis and identification of agents are based on a wide range of characteristics recommended. ***Streptococci*** are Gram-positive, facultative anaerobes, require enriched media (blood agar). Group A ***Streptococci*** have a hyaluronic acid capsule, hemolysis. The preliminary species identification by general test and technique (latex agglutination method) were detected of the group

antigen from isolated colonies and clinical material including swabs **Lei** (2001). The species that contain group antigens of more sero groups are identified by biochemical and physiological tests which allowing the detection of constitutive enzymes.For the reasons of standardisation the primarily uses commercial diagnostic kits with high identification **Facklam** (2002).

In order to persist, pathogenic bacteria have to find ways to avoid recognition by immunoglobulins. ***S. pyogenes*** has evolved a specific enzyme to deal with opsonizing IgG antibodies. This enzyme is a secreted proteinase that specifically cleaves the heavy chain of IgG , is the rsle of sole substrate in plasma samples , due to its early and sustained expression during growth and its highly specific proteolytic activity. **Agniswamy,** (2004).

Other analyses of antistreptococcal antibodies, however, have shown that the total amount of specific antibodies does not necessary correlate with the level of neutralizing antibodies, and antibody quality might be clinically more important than antibody quantity **Norrby-Teglund,** (1996). The fact that ***S. pyogenes*** has evolved an enzyme that specifically targets IgG, the presence of neutralizing antibodies correlates with manifestations of ***S. pyogenes*** infection or affects the severity of ***S. pyogenes***-induced disease **Sriprakash,** (2002).

Patient and Methods

- Fourty patients with ages ranged from 1 to up 12 years from Abu el rich hospital clinic .
- Twenty Healthy normal subjects with matched ages and sex as controls.
- Type-specific precipitation sera against all described polysaccharide and protein antigens are prepared for ***S. pyogens*** typing identified by the Quellung technique with 164 sera (polyvalent, group, type, factor) provided by the Statens Seruminstitut, Denmark .

- ELISA technique: Microtiter plates were coated with enzymatically inactive **Lukomski,** (2000). Nonpolar inactivation of the hypervariable streptococcal inhibitor of complement gene in serotype M1 ***Streptococcus pyogenes*** significantly decreases mouse mucosal colonization. Infect. Immun. at 0.4 µg/ml in coating buffer (0.05 M $NaHCO_3$, pH 9.6). Plates were washed with PBST (0.05% Tween in phosphate-buffered saline and blocked with 2% bovine serum albumin (Sigma) in PBST (PBSTA). Dilutions of each patient and control serum sample in PBSTA were added to the wells. Antigen concentrations and serum dilutions were judged as optimal by checkerboard titration. Bound antibodies were detected by incubation

with peroxidase-conjugated antibody against human IgG 1/3000 (Sigma), and enzyme-linked immunosorbent assay (ELISA) plates were developed as previously described **Baxter, (**2000). Blank samples without serum were included in quadreplicate on each plate. Optical density at 420 nm (OD_{420}) was determined as described previously. An ELISA index was calculated by subtracting OD_{420} values of blank control samples from the values obtained with serum samples and by dividing the mean OD_{420} value for each sample by the mean OD_{420} values for a standard positive serum sample; these were determined in quadreplicate on each plate.

- SDS-PAGE activity assays: SDS – PAGE was carried out in 0.75 mm thick , 12% vertical slab gels (E 64; Sigma) according to the method described by **Hollm-Delgado** (2004). Equal protein content of bacterial extracts (50µg / lane) was applied to the gel wells after mixing and boiling for 3 min with an equal volume of sample buffer [0.125 M trisma base , 4% (w /v) SDS , 20% (v/v) glycerol, 10 % (v/v) mercaptoethanol and 0.1% (w/v) bromophenol blue as a tracking dye]. A prestained molecular weight standard mixture [β-galactosidase Mw 117 Kda, fructose-6-phosphate kinase Mw 91.8 kda, pyruvate kinase Mw 72.7 Kda, fumarase Mw 57.8 , lactic dehydrogenase Mw 40.8 kda, and trio phosphate isomerase Mw 34.1 kda,] was prepared in parallel . Cleavage of IgG was determined by analyzing supernatant samples on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis which was carried out with constant volt of 200 V. Activity was determined by the presence of a diagnostic 31-kDa IgG cleavage product. The run was terminated when the bromophenol blue marker reached to the bottom of the gel. The gel was silver stained according to the method described by ***Morrissey et al,*** (1986).

A) Bacterial stock :

The used bacterium is the stock of ***Streptococcus pyogenes*** which secretes fusion protein directly into the medium, isolated and identified according to the instructions of ***Api*** (bioMerieux) National Committee for Clinical Laboratory Standard *(NCCLS 1999).*

B) Production and Extraction of polysaccharide :

Takes of culture of ***Streptococcus pyogenes*** for centrifugation, the precipitation is washing for several times by distilled water and then for purification added an enzymatic treatment as trypsine (0.02 %).A volume of nine liters of culture , the weight of polysaccharide obtained 120 mg extraction % (17.2), the total of PSA / volume of culture was 13.3 mg .

Extaction by formamide :

At 120 °C in water bath for 60 hrs then we added acetone and then in resin ion exchange , we take the extract of polysaccharide of group A , the composition of polysaccharide and there percentage by extraction of formamide and purification are:

Rhamnose (60 %) , where the chromatography indicates the absence of these amino acids: glucose amine(-),acetyl muramic acid(-),alanine(-),lysine(-), acetyl. glutamic acid(-) ,glycine(-). To control the quality of PSA obtained we have effected the dosage of rhamnose which considered as a good indicator to the quantity of polysaccharide which considered as 60 % for rhamnose extracted by formamide which liberate certain amino acids : 500ug of rhamnose in 0.5mg/ ml of aquous solution of polysaccharide of ***Streptococci*** of group *A* (PSA) then 0.5 ml in a distilled water , an addition of sulfuric acid 4.5 ml in a water bath for 10 minutes, after that we stopped the reaction with cold water, an aquous solution of 3 % cysteine hydrochloride were added , we take 0.1 ml with agitation , then we read the O.D at 415 nm by spectrophotometer .

-The latex :

The characteristic of preparation of latex uses : as Bacto – latex from Difco and the nature was PS (polysterine) with diameter 0.80 um and 5 % solidity , function of surface -COOH being free . The resins were anion and cation exchange:

Make washing by distilled water then washing by methanol and then 3 times by distilled water , addition of Na OH 3 N as washing , then we repeat 3 times by distilled water and we added Hcl 3 N then 3 times daily of distilled water , then we take the stokage of resins in a distilled water at 4 °C . The resins were purified before, than one ml of resin of anion exchange and 1ml of resin of cation exchange were mixed , addition of 0.5 ml of latex and 5 ml distilled water .

The suspension was mixed for 1 hour at room temperature .The latex was shown and stoked at 4 °C , this will take place for fixing the antigen on the latex particles .

The conjugation of active polysaccharide plus the cyanuric chloride (2-4-6-trichloro-1, 3, 5-triazine), ie.activates cellulose to immobilize DNA; gives a conjugate protein -polysaccharide. Series of plaques were being stoked at 4° C for 15 days O.D 415 and than the evolution of plaques was compared .

Amino acids act as stabilizers used in the range 20- 500 mM , amino acids and derivatives occur as osmolytes in nature. Sugars are being the best stabilizers but reducing sugars can react with protein amino groups leading to inactivation .

Gel filtration sample application: the technique was used for the detection of the polysaccharide antigen by using column chromatography packed by cation exchange resin, by means of the automatic analyzer 500 unit. Hundred ul of resin 1 mg / ml out of plug citrate of lithium 0.2 N is injected on the column. A S70, 150 x 4 x 0.1 mm, diameter 7 um. The elution was conducted by using different buffer solutions. The addition of ninhydrin can be used to detect the contaminated peptides or proteins, the proteins have been detected by electrophoresis on agarose gel. The duration of the separation was about 2-3 hrs.for developing patterns, the gel were washed with distilled water several times **Vlaminckx** (1996).

Conjugation of the poly-L-lysine:

These methods used both the activation of polysaccharide by which take part and the remainder present between the polysaccharide and the protein.

Results

Table 1

The result indicate the good degree of purity of PSA product , to control the quality of PSA obtain , we effected the dosage of rhamnose as a good indicator for the quantity of polysaccharide.

A 1st phase in the extraction and purification, using *column chromatography* (Fig.1)

Rhamnose (1, 3) N-acetylglucosamine (13,15,17,19)

Table2: Reactivity of different polysaccharides by technique Elisa

Serum (anti-***Streptococci***)	OD of serums Utilise (*)	PSA(**)	PSA- SBA	PSA-PLL
A	0.010	0.080	0.070	0.130
B	0.100	0.120	0.110	0.195
C	0.365	0.450	0.400	0.390
D	0.365	0.706	0.700	0.695
E	0.955	0.950	0. 980	0.860
F	1.100	1.060	1.010	1.125

(*) serums of known optical density , determined by technique ELISA
(**) optical density of PSA polysaccharide of ***streptococci*** of group A purified.
SBA serum bovine albumine PLL poly -L-lysine

After 5 min., we observe that the ppt. of PSA purified and the conjugation of PSA-SBA and PSA-PLL gives an identical antigenecity than the only one polysaccharide ; that means the immunological reactive is conserved . we find that the values with the PSA purified are identical with that known and obtained of the polysaccharide of reference. The conjugation made it possible to find the values of optical density of serums used to fix the rate of proteins on latex, confirms that PSA-PLL (1 mg/ml of PSA) remain fixed without loss of activity after 15 days of storage at 4 °C we observe a good stability of conjugation PSA-PLL than PSA-SBA during this work.

Table 3 : Result of normal adults

ELISA						ELISA					
Serum	IgG	IgM	Latex	Microa gglu	Hemag glu	Serum	IgG	IgM	Latex	Microagg.	Hemagg .
1	1.71	0.24	160	80	40	12	1.24	0.10	160	40	80
2	0.72	0.33	80	40	80	13	0.82	0.10	160	80	160
3	1.41	0.31	160	160	160	14	1.40	0.25	80	40	160
4	1.52	0.24	160	80	160	15	1.83	0.27	320	160	160
5	1.95	0.19	320	40	320	16	0.58	0.18	40	20	160
6	1.75	0.11	640	320	320	17	0.25	0.45	80	80	80
7	0.60	0.89	320	160	160	18	0.70	0.23	160	20	160
8	1.24	0.23	80	80	80	19	0.91	0.15	160	80	80
9	1.50	0.21	160	80	80	20	1.17	0.40	40	40	40
10	1.09	0.11	80	80	160	21	0.80	0.00	80	40	80
11	1.20	0.19	80	40	80	22	1.60	0.24	160	160	160

Microagglu. = Microagglutination Hemagglu = Hemagglutination N . of serum = 22

The adult normal values for the technique of microagglutination & heamagglutination the +ve for technique ELISA, experm. values due to O.D for adults are 1.20 for IgM and 0.47 for IgG .

Table 4: results of normal child

SERUMS	ELISA			LATEX
	IgG	IgM	IgT	
1.	0.96	0.20	1.05	40
2.	0.42	0.13	0.49	10
3.	0.72	0.07	0.75	80
4.	0.53	0.19	0. 55	20
5.	0.37	0.16	0.45	80
6.	0.15	0.07	1.15	80
7.	0.30	0.19	1.32	80
8.	0.24	0.50	1.40	160
9.	0.00	0.24	1.10	40
10.	0.20	0.40	1.30	80
11.	0.72	0.12	0.70	20
12.	0.34	0.10	0.35	40
13.	0.27	0.12	0.30	20
14.	0.53	0.21	0.55	20
15.	0.18	0. 21	0.25	10
16.	0.71	0.18	0.80	80
17.	0.30	0. 08	0.30	20
18.	0.21	0.35	0.35	80
19.	0.64	0.37	0.75	80
20.	0.54	0.25	0.60	40
21.	0.42	0.30	0.60	80
22.	0.27	0.47	0.45	80
23.	0.44	0.09	0.45	20
24.	0.21	0.86	0.85	160

Children before 12 the values are 0.90 for IgG and 0.40 for IgM . More than 60% (n = 40) of patients initially presented with skin or soft-tissue infections, and 27 of these met the criteria for NF.

Table 5 : Children suspecting by *Streptococci* infection

Serums	Elisa		Latex	Time
	IgG	IgM		
1	1.45	0.75	1780	4
2	0.10	0.30	80	3
3	0.15	0.25	80	3
4	1.50	0.15	320	6
5	0.50	0.30	160	5
6	0.15	0.20	20	1
7	0.10	0.70	320	Pediatric
8	0.40	0.25	20	5
9	0.80	0.55	640	9
10	0.20	0.35	160	13
11	0.40	0.15	80	13
12	1.25	0.20	160	17
13	0.60	0.45	320	17

Table 6 : children suspecting by *Streptococci* infections of age group Hospital Abu el rich .

H.Abu R		Microagglutination	Latex	Heamaglutination
1 to 3 years	N (effective)		22	
	X (medium)	92.73	374.55	83.18
	T (type)	177.36	474.17	92.02
	R (coeff correlation)	0.74		0.86
3 to 6 years	N (effective)		17	
	X (medium)	168.24	523.53	260.00
	T (type)	152.32	457.94	224.18
	R (coeff correlation)	0.78	x	0.80
6 to 12 years	N (effective)		28	
	X (medium)	130.71	621.79	260.00
	T (type)	102.40	496.20	224.18
	R (coeff correlation)	0.82	x	0.80
Up to 12 years	N (effective)		17	
	X (medium)	64.71	329.41	136.47
	T (type)	31.81	191.85	93.11
	R (coeff correlation)	0.44		0.48

H.Abu R= Hospital Abu el rich

Significant correlation with microagglutination and heamaglutination (1-3)

Significant correlation with microagglutination and heamaglutination (3 to 6)

Significant correlation with microagglutination and heamaglutination (6 to 12)

Non significant correlation with microagglutination and Sig.with heamaglutination (up to12)

Table 7 : Population atteind of acute rheumatic arthritis (ARA) .

	Microagglutination	Latex	Heamaglutination
N (effective)		21	*
X (medium)	172.86	519.05	423.81
T (type)	171.94	367.88	279.16
R(coeff correlation	0.19	x	0.78
N (effective)		16	**
X (medium)	162.50	605.00	525.00
T (type)	147.29	414.70	441.79
R(coeff correlation	0.75	x	0.96
N (effective)		25	***
X (medium)	246.80	696.40	588.80
T (type)	283.56	466.43	363.37
R(coeff correlation	0.65	x	0.71

* Non significant correlation with microagglutination, significant with heamaglutination

** Significant correlation with the 2 techniques. (without cardite)

*** Significant correlation with the 2 techniques. (with cardite)

The reactive latex prepared is sensitive, specific and has been utilized for the proportioning of the polysaccharide antibodies of ***Streptococcus*** of group *A*, comparatively with the ELISA.

Discussion

The ***Streptococci*** infections remain an important problem of health in the world not only for the industrialize countries but also for the developed countries. The selected method was used as to be of costless in the used techniques, the use of enteric bacteria is not possible and the ability to obtain a purified antigen and also secretes fusion protein directly into the media ***Abou El Enien*** *(1999).*

The extraction of polysaccharide by the method of ***Krause and MacCarty*** *(1996)* recently modified by using formamide, allowing to obtain polysaccharide in large quantity , without contaminant cellular substances after mechanical disintegration of the bacterial cell wall . The non reducing sugars can be recycled from buffers and is not a convenient food source for bacteria. The chromatographic and electrophoresis analyses reveal the absence of amino acids (alanine , glutamic acid etc.) as a constitution of peptidoglycan, indicates a certain purity of the antigenic preparation. For qualitative control of the polysaccharide the proportion of rhamnose concentration which is the main constituent with glucosamine was studied ***Pollard*** *(2000).*

Rhamnose concentration obtained in the sample under study is (48%), this concentration is close to that of the reference polysaccharide (45%) and comparable with the values of the data reported which obtained (60%) rhamnose.

PSA developed in 34%, renal dysfunction developed in 55%, hepatic dysfunction developed in 64%, and coagulopathy developed in 69% of patients. A total of 56% of patients were treated with IV polyspecific IgG , 81% were intubated and placed on mechanical ventilation, and 21% required renal replacement therapy. The median durations of ICU and hospital stay were 5.3 days and 15.0 days, respectively. The overall mortality was 40%. Mortality correlated directly with acute physiology and chronic health evaluation II score and the number of dysfunctional organs. Survivors were younger, had lower severity of illness scores, fewer dysfunctional organs, and were less likely to have shock or to receive treatment with vasopressors, mechanical ventilation, or pulmonary artery catheters **Kaul,** (1999).

There was no association between the use of vancomycim , surgical intervention, or clindamycin, and survival. Variables independently associated with mortality on multivariable analysis were the presence of coagulopathy ($p = 0.0005$) and liver

dysfunction (p = 0.0123). So , Patients with invasive *PSA* infection admitted to the ICU have a high mortality rate. In this group of patients, coagulopathy and liver failure were independently as sociated with mortality. We did not observe any association between the use of vancomycin, surgical intervention, or clindamycin, and survival **Hollm-Delgado**, (2005).

The presence of antipolysaccharide antibody *A* in the serum of healthy children using the latex was very high (4 to 10 times superior) compared to the normal value of the old aged. On the other hand the rates of *IgG* are inferior than the normal children less than 3 years. In adults suffering from acute rheumatoid arthritis latex are 3 to 4 times superior with the normal values. The proportion of the antibodies by *Elisa* give high values .The best coefficient of correlation between the two techniques are obtained with the *Ig* .

Patients with invasive GAS disease were admitted to the ICU in one hospital. More than 60% (n = 40) of patients initially presented with skin or soft-tissue infections, and 27 of these met the criteria for NF. APS was the second-most-common site of infection at presentation. Sixty-eight percent of patients had blood culture results positive for PSA, four of whom had no obvious source of infection. At presentation, approximately 50% of patients had a history of chronic disease, including coronary artery disease, congestive heart failure, and pulmonary, renal, or liver disease **Ben-Abraham**, (2002) .

The technique of fixation of the *PSA* to be purified on particles latex, after reduction and deacetylation, polysaccharides couldn't be use for the development of reaction of agglutination to latex. The immunological absence of reaction with respect to serum **Anti-*Streptococci*** of group *A* is a proof of important alteration of antigenecity The reported that polysaccharides has proteins by the action of Benzyl-azo-proteins . This solution act to fix a protein at the polysaccharide. The relating to the coupling of polysaccharide *A* had the albumin bovine serum ***(ABS)*** by the intermediate of cyanogens bromide. The used of cyano-chloride fixed polysaccharides poly-L-lysine {activates cellulose to immobilize DNA}.

The optimization of the covalent attachment on latex to function has to mix 50 ul of *PSA-PLL*. 20 ul *PSA* for the process of purification have been fixed at the end of 2 hours by carbodiimide concentration of 1mg/ ml of preparation by radioimmunological proportion with *group A* variable. The present reaction failed to show reactivity to cross with polysaccharides of other groups ***Streptococci*** . Use of a specific lectine of N-acetyl-glucosamine represent well but the best sensitivity is obtained by the immunoenzymatic technique was used to detect

antibodies up to 1/ 256000 ppm., carbodiimide on particles latex containing carboxylic functional groups lead to obtain a specific, significant reaction which is stable , also with low dose for storage and prevention from contamination where the result indicate the supplementation of irradiated performance. **Darenberg**, (2003).

The results of the present studies have a reproductivity technique, whatever the factor which can affect it. The stability of the reaction was maintained for one period of the study has 4°C and 37°C the action have been to be preserved for 4 weeks which is sensitive, specific and stability to use for proportion of antipolysaccharide antibodies of ***Streptococci*** of group *A* comparatively with the Elisa.

In general, at the time of reappearing of the rheumatic fever, the clono type is observed, indicating that the acquired immunity is to associate the persistence of the clone in children population it could be observe that the averages increase up to 6 years old , and down again up to 12 years old towards values close to the normal adult values for the techniques of microagglutination and heamagglutination . In the population having ***RAA***, values increase according to the cardiac attack. It is useful to compare agglutination latex with the two other techniques of agglutination using the red globules or the bacterial cell wall. The correlation between the techniques latex and heamagglutination is well in all cases; whereas between the microagglutination and agglutination latex isn't significant in the children of less than 12 years and in the cases of ***RAA***. The specificity of the persistence of the APSA among patients has been to be confirmed by studies, to detect a fall of rates of antibodies reached of RAA without cardiac **Mulla,** (2002).

The presence of the antipolysaccharide antibodies A has been to be observed in nearly 85% of the cases understudy of RAA confirms the assumptions of the role of these antibodies in the cardiac complications. The conjugation of polysaccharide has a protein (ASB or PLL) to develop for obtaining reaction latex, also to polyestyrine plates for the proportion of the ***APSA*** by Elisa technique. The adsorption of the polysaccharide antigen on the surface of the plates in the time course when it combines to PSA-PLL remain fixed without loss of activity after 15 days of storage at 4 °C and the protein coupled activated sepharose is usually more stable to denaturation than the protein in free solution, the choice of buffer solution depends on the properties of the particular coupled protein as storage **Basma,** (1999).

These systems could be interesting and should make it possible to improve the period of conservation and storage for a marketing of test proportion of the APSA, vancomycin320 µg once daily and 640 µg twice daily produced significantly greater

improvements in $PC_{20}FEV_1$ compared with placebo on Day 1 (within 2.5 hours), and on Days 3 and 7 (all $p<0.0001$). On Day 3, both ciclesonide doses significantly reduced exhaled NO levels by –17.7 ($p<0.0001$) and –15.4 parts per billion ($p<0.003$) versus placebo, respectively. Significant reductions were maintained during the study with both ciclesonide doses ($p<0.01$). Sputum interleukin-1α, -6, -8 and -12 were significantly ($p\leq0.01$) inhibited by ciclesonide 640 µg twice daily within 4 hours; effects were maintained during the study **Norrby-Teglund**, (2005).

The data obtained from the previous studies indicate the possibility of using a simple technique in diagnostic acute rheumatic arthritis.

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الـمـلـخص الـعـربـي

استخلاص وتنقية السكريات (عديدة الانتيجينيات) من الميكروب السبحي أ (ستربتوكوكس بيوجينز) المثبت على جزيئات لاصقة

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تعرف الأجسام المضادة بأنها تلك الموجهة ضد السكريات المتعددة (أ) ستريتوكوكس بيوجينز ذات الأهمية العظمى فى دراسة مسببات أمراض الاستربتوكوكس, وكذلك طرق الوقاية ضد انتشار مرض روماتيزم المفاصل الحاد والذي تصل درجة إصابته للقلب.

الغرض من البحث : لما انتشر هذا المرض فى البلدان النامية كان لزاما علينا نحن الباحثين أن نقوم بإجراء تجارب بسيطة وسريعة لقياس جرعات هذه المضادات.فقد تم استخراج هذه السكريات المتعددة (عديدة التسكر) وكذلك تنقيتها وتثبيتها بطريقة تسمى Covalent) (coupling على سطح (Latex) (التفاعل الارتباطي الوظيفي على سطح جزيئات اللاتكس) كعامل مساعد ورابط بين Poly-L-lysine.

وبعد الحصول على تثبيت التفاعل بدراسة التجارب عند درجة حراره 4 م° و 37 م° لمدة شهر (Period) كانت النتائج ايجابيه بالمقارنة بين المرضى المصابين بالروماتيزم الحاد وبين الذين تم اخذ عينات وهم الأصحاء بالتجربة التاكيديه .

تم تقييم التفاعل بطريقه ال Elisa (IgG + IgM) التي أظهرت مجموعات متعددة من السيرم بالمقارنة مع الإفراد الأصحاء وعددهم 20 والمرضى المصابين وكان عددهم 40 وقد اثبت التحليل صحة النتائج (باستخدام هذه الطريقة البسيطة الحديثة والتي إذا طبقت في الحياة العملية أعطت نتائج ايجابيه أينما و جد المرض .