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THE ROLE OF GOAT'S AND BUFFALO'S MILK ALLERGENS AS CAUSATIVE AGENTS OF TYPE I HYPERSENSITIVITY AND THEIR CROSS – REACTIVITY WITH COW'S MILK ALLERGENS

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ABSTRACT

An allergic extracts from cow's, goat's and buffalo's milk were prepared with extraction, followed by purification and fractionation by gel filtration, one major peak was obtained from cow, goat and buffalo milk with molecular weight of 23KD, 26 KD, 15KD respectively.

Total and specific IgE ELISA testing was performed on 137 patients serum samples. The rate of specific IgE positive ELISA results was 58%in case of patients tested with goat milk allergen and 57% in case of patients tested with buffalo milk allergen. There were significant differences $P<0.05$ among age groups, males and females regarding the rate of milk allergic patients who had positive specific IgE ELISA results.

In rural region ,the rate of patients who had goat's and buffalo's milk allergy was higher than that in urban region.

There was a cross-reaction among cow's milk extract protein , goat's and buffalo's milk protein extract and the IgE binding capacity of buffalo's milk protein extract was higher than that of goat's milk protein extract since lower concentration of this protein extract was needed to inhibit up to 50% the binding of specific IgE to cow's milk allergosorbent.

INTRODUCTION

Milk allergy is a protein problem and is not improved by changing the milk shugare , this protein is casein and tend to be stable , but milk shugare converted to dairy products in all types of milk[1] .

Cow ? goat and buffalo milk allergy caused by immunological mechanism against milk casein and patients are suffering from breathing problems, hives and rash, abdominal pain and serious weight loss[2].

Goat milk protein had many significant differences in their amino acid compositions in compare to milk of mammalian species especially in relative proportion of the various milk proteins and in their genetic polymer[3].

The major protein in cow milk is alph α-s-l casein , but goot milk differ genetically by having either none (alph – S – 1) or much alpha – S – 1 these types have shorter rennet coagulation time , less resistance to heat treatment and curd firmness is weaker. The protein content of goat milk is 3.1% and in buffalo 3.7% [4].

Skin testing and laboratory assay of specific antibody may be useful in allergy testing . Radio Allergosorbent Test (RAST), measurement of total and specific IgE by Enzyme Linked Immunosorbent Assay (ELISA) and ELISA inhibition are performed when skin testing is not available or in severe eczema or when a person is taking antihistamines that interfere with accurate testing[5].

The aim of this study was to define and characterize the local goat and buffalo milk allergens, to estimate the total and specific IgE antibody, and to determine the cross-reaction between cow milk allergen with goat and buffalo milk allergen.

MATERIALS AND METHODS

Antigen preparation

Fresh cow , goat , baffalo milk antigens were prepared according to methods of Dreborge and frew[6]

Briefly fresh milk was collected from local cow ,goat and buffalo, and defatted by cooling in refrigerater then mixed with phosphate buffer saline PBS , 0.15M , PH 7.2 at 5:100 V/V. The mixture was clarified by refrigerated centrifuge at 10.000 rpm for 1 hour at 4^0C. The supernatant was sterilized by milipore filter (0.22 μm) and stored at 4 ^{0}C .

The purification and fractionation of milk extract protein on G- 75 sephadex.

The gel chromatography was used for the isolation and purification of protein extract into different molecular size using G-75 sephadex according to the method of (Leslic and Frank)[7].

Determination of protein content

The protein content of each protein extract was estimated according to Whitaker and Granum method[8], 3 ml of each extract was piptted in a quartz cuvattes. The absorbance value was measured spectrophotometrically at 235 and 280 nm.

The protein content in mg/ml was calculated by the following equation:

Protein mg/ml=A 235-A280/ 2.51.

Determination of the sterility of milk extract

The sterily of milk extract was determined according to method of Macckie and Mccartney[9] ,by inculation of the extract into deplicate plates of nutrient and blood agar. Then these plates were incubated aerobically and anaerobically at 37 ^{0} C .

Inoculated plates were observed daily for 7 days after inoculation to determine the culture sterility .

Serum Samples

Serum Samples to estimate total and specific IgE were obtained from (137) Patients attending the center of asthma and allergic disease in Basrah . The negative control sera were obtained from fifty individual seen in Basrah hospital who did not suffer from allergic diseases.

Enzyme Linked Immunosorbent Assay (ELISA).

Total IgE ELISA technique:

Total IgE was quantitatively determined according to the method of Biomaghreb kit (Tunisia). Briefly kit assay buffer (100 µl) was added to each well of microtiter plates which was coated previously with mouse monoclonal anti – human IgE followed by the addition of (20 µl) of kit control to the first and second wells of the first vertical row. Then to other six wells of first vertica lrow and to the four wells of second vertical row standard IgE at concentrations (2,5,20,50,200 and 500µl) were added and patient sera (20µl) were added to the rest of wells .

Plates were then covered with plastic film , homogenized by shaking at 300rpm and incubated at 37C° for 90 minutes followed by washing with PBS- Tween 20 (0.05%). After washing ,(100µl) of goat anti – human IgE alkaline phosphatase conjugates was added to each well. The plates then covered with plastic film and incubated at 37^{0}C for 90 minutes . After that the plates were washed and freshly prepared para – nitro – phenyle – phosphate solution (100µl) was added to each well – then the plates were incubated at room temperature for 30 minutes in the dark and (100µl) of the stopping solution (2N NaOH) was added to each well . The absorbence of each well was read at (450nm) using microplate reader (Dynatchs microplate reader models MR 600 , U.S.A.).

Specific IgE ELISA technique:– specific IgE was determined according to the method of Biomaghreb Kit. Briefly the reference disc D allergen (Dermat pteron) were added to well of microtiter plate started with 3rd well of first vertical row to 8th well of second vertical row followed by the addition of referenc se,um calibrator (A-H) in which IgE concentration was (52.50, 17.50,3.50 , 0.70 and 35µl) to the reference D disc . fIlter paper discs were prepared sterilized by autoclaving at 121^0 C for 15 minutes and saturated with locally prepared goat and buffalo allergen extracts .

The protein content of these extracts was determined according to the protein content of the standard milk allergen discs (Biomaghreb , Tunisia) the locally prepared discs of goat and buffalo's allergen discs were added to the bottom of the rest of wells. The protein content of each goat and

buffalo disc allergen was 0.03 mg/ml and 0.02 mg/ml respectively. Serum samples (50µl)were added to all goat and buffalo allergen discs. Other steps of ELISA technique were performed as in the total IgE ELISA.

ELISA - inhibition

For competition experiments microtiter 96 well plates were coated with allergen extract discs at 0.03 (goat) and 0.02 (buffalo) for 1 hour at

37^0 C [10] After wards (25µl) of the serum pool and (25µl) of goat and buffalo allergen extract at 3 protein concentration 0.003 , 0.03 , 0.3 mg/ml (goat) and 0.002 , 0.02 , 0.2 mg/ml (buffalo) were added to the wells and incunbated for 2 hours at room temperature. Other steps of ELISA inhibition were performed as in the total IgE ELISA

Statistical methods

For the determination of statistical significance the qi-squer test was used.

RESULTS

Purification of protein extracts:

On fractionation one major peak was obtained from cow goat and buffalo milk protein with amolecular weight of 23 KD (cow), 26KDa (goat) and 15KDa (buffalo). Fig – 1,2,3.

ELISA results:

Estimation of total IgE value:

According to the IgE values there were three types of allergy , allergy not probable (< 20µg/ml) , allergy questionable (20 – 100 µg/ml) and allergy very probable (> 100µg/ml) table -1 .

Estimation of specific IgE ELISA value:

The highest rate of specific IgE ELISA results were observed in females tested with goats and buffalo's milk allergen (60.6% and 65.6% respectively).

Also the highest rate of positive specific IgE ELISA results were observed in males tested with goats milk allergen (35.8%) in comparison to other males tested with buffalo milk allergen .(Table 2).

According to age and sex , the highest rates of positive specific IgE ELISA observed in first and second age groups in both males and females (Table . 3,4.)

The mean and standard deviation (SD) of the optical density value (OD_{492}) in 137 patients tested with goat's and buffalo's milk allergen using specific IgE ELISA test were observed in table (5).

In rural region, the rate of goat and buffalo milk allergic patient is higher than that of urban region and the higher rates (51.7%) of positive IgE ELISA were absevred in patients tested with

goat's milk allergen. While in urban region the higher rate (49.1%) were obsereved in patients tested with buffalo's milk allergens. (Table 6)

The relationship between total and specific IgE values

The relationship between total and specific IgE value were estimated in 137 patients , the patients with allergy not probable show negative specific IgE ELISA when tested with goat and buffalo milk allergen . In case of patient's who had questionable allergy the higher rate of positive specific IgE ELISA results were observed in patients tested with goat milk (68.34%).Patients with very probable allergy the higher rate was observed in patients tested with goat milk 50.8% (table 1).

ELISA –inhibition

The cross-reactivity of cow, goat's and buffalo's milk protein extracts was evaluated by mean of competition ELISA . (Table 7) stated clearly that cow's milk extracts could inhibit to high extend at concentration (0.3 mg/ ml) the binding of specific IgE to the goat and buffalo milk protein extracts (81.8%, 85.1 %). Suggestion that these protein bear major allergenic determinants. Furthermore ,the IgE binding capacity of buffalo's milk protein was found to be higher than that of goat's milk protein since lower concentration (0.003 mg/ml) needed to inhibit 50% the binding of specific IgE to allergosorbent phase.

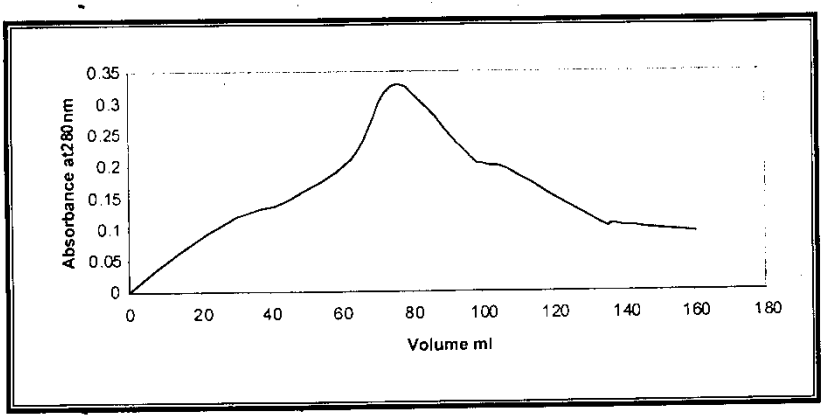
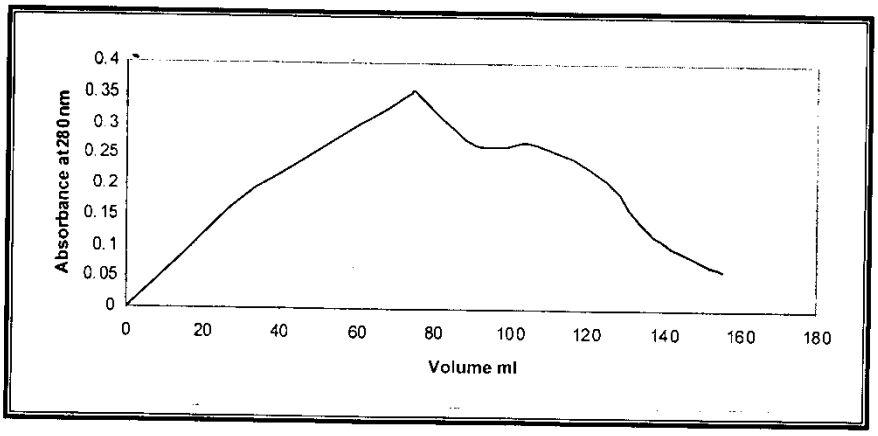


Fig . 1 : Evalution profile of fresh goat milk protein extract



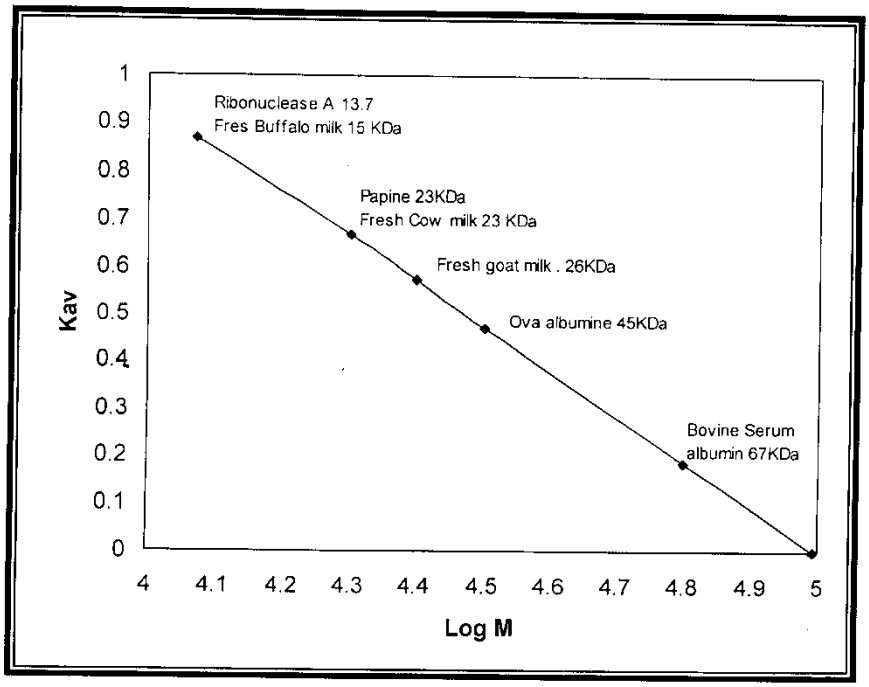


Fig 3; The calibration curve of protein extract using some standard proteins .

Table (1): - Relationship between total IgE value and specific IgE value in 137 patients examined with goat's, and buffalo's milk allergens .

Total IgE ELISA value	Exam no.%	Type of allergy	Patients No. examined with			
			Goat 's milk allergen		Buffalo 's milk allergen	
			+Ve No. %	-Ve No. %	+Ve No. %	- Ve No. %
<20 µg / ml	37(27.0)	Allergy not probable	Nil	Nil	Nil	Nil
20-100µg/ml	41(29.9)	Allergy questionable	28(68.3)	11(26.8)	28(68.3)	13(31.7)
>100µg/ml	59(43.0)	Allergy very probable	30(50.8)	31(52.5)	29(49.2)	30(50.8)
	137		58	42	57	43

Table (2) :- Rate of positive specific IgE ELISA results in 100 patients examined with goat's and buffalo milk allergens .

Protein extracts	Positive response No. %				Total	
	Males		Females			
	Exam No.	Positive specific IgE and percentage %	Exam No .	Positive specific IgE and percentage %	Exam No .	Positive specific IgE and percentage %
Goat's milk allergens	39	21 (53.8)	61	37 (60.6)	100	58 (58.0)
Buffalo's milk allergens	39	17 (43.6)	61	40 (65.6)	100	57 (57.0)

Table (3): - The rate of total and specific IgE ELISA results in 137 patients tested with fresh goat milk allergen

Age group (years)	Males			Females			Total
	Exam. No.	Total IgE No. %	Specific IgE No. %	Exam. No.	Total IgE No. %	Specific IgE No. %	
10 – 20	12	9(75.0)	9 (75 . 3)	19	16(84.2)	14 (73.6)	31
21 – 30	9	7(77.7)	6(66.6)	18	12(66.6)	12(66.6)	27
31 – 40	17	8(47.0)	5(29.4)	19	15(78.9)	7(36.8)	36
41-50	15	8(53.3)	1(6.6)	19	10(52.6)	4(21.05)	34
51– 60	2	1(50)	0 (0.0)	7	3(42.8)	0 (0.0)	9
	55	33	21	82	56	37	137

Table (4): - The rate of total and specific IgE ELISA results in 137 patients tested with fresh buffalo's milk allergen

Age group (years)	Males			Females			Total
	Exam. No.	Total IgE No. %	Specific IgE No. %	Exam . No .	Total IgE No. %	Specific IgE No. %	
10 – 20	12	9(75.0)	8 (66.6)	19	16(84.2)	12 (63.1)	31
21 – 30	9	7(77.7)	5 (55.5)	18	12(66.6)	11 (61.1)	27
31– 40	17	8(47.0)	3 (17.6)	19	15(78.9)	9 (47.3)	36
41-50	15	8(53.3)	1(6.6)	19	10(52.6)	8 (42.1)	34
51 - 60	2	1(50)	0(0.0)	7	3(42.8)	0 (0.0)	9
	55	33	17	82	56	40	137

Table (5): - ELISA results of patients with positive specific IgE responses to goat's and buffalo's milk allergen .

Age group (years)	Males		Females	
	Mean ± SD Of F. G. M. A.	Mean ±SD Of F. B. M. A.	Mean ± SD Of F. G. M. A.	Mean ± SD Of F. B. M.A.
10-20	2.5 ± 1.38	2.47 ± 1.22	0.99 ±0.6	1.04 ±0.59
21-30	1.73 ± 1.37	1.68 ±0.89	0.808 ±0.57	0.74 ±0.49
31-40	0.54 ±0.24	0.75 ± 0.32	0.54 ± 0.29	0.51 ± 2.51
41-50	0.44 ± 0.000	0.42 ± 0.000	0.5 ± 0.14	0.42 ± 0.21
51 – 60	Nil	Nil	Nil	Nil

SD = Standerd devasion
F. G.M.A. =Fresh goat's milk allergy
F.B.M.A. = Fresh buffalo's milk allergy

Table (6): - The distribution rate of goat's and buffalo's milk allergy in rural and urban region of Basrah.

	The distribution %		Total
	Rural no.	Urban no.	
Goat milk allergy	35(51.7)	23(39.6)	58
Buffalo milk allergy	29(50.8)	28(49.1)	57

Table (7): - The cross – inhibition of specific IgE binding of cow with goat's and buffalo's milk extracts using ELISA inhibition

Allergosorbent	Inhibitor mg / ml (%)		
Goat milk extract	0.003 / 47.05	0.03 /72.4	0.3 / 81.8
Buffalo milk extract	0.003 / 53.8	0.03 / 69.4	0.3 / 85.1

DISCUSSION

Purification and fractionation of protein extracts

Gel filtration analysis of goat's and buffalo's milk protein extracts demonstrated one major peak with a molecular weight 26KDa (goat), and 15KDa (buffalo). This finding was in line with Dreborge *et al*[11] . Marsh and Norman [12], Who reported that the allergen usually has a molecular weight of (5000-70000) KD . IgE recognition pattern of goat and buffalo milk protein extracts was determined by the ELISA technique, these proteins were predominant both in the intensity and frequency of their recognition by human allergic sera. Therefore; these component can be regarded as major allergic component of goat's and buffalo's milk extracts. This finding was in line with the finding of Dreborge *et al.*[11] who reported that the major allergen is very abundant protein in the source material and is most readily extracts from the source.

ELISA result

Estimation of totol IgE value

The rate of patients who had very probable allergy (>100 µ/ml) was (43%) and this results was higher than that reported by other studies as the study of Hattevig *et al*[13]. and Bock [14] who reported 25% and 7.2% respectively . The explanation of this discrepancy is based on differences in animal species, geographic areas, climates and genetic factor[15].

Estimation of specific IgE value

The rates of patients who had specific IgE positive ELISA results who were tested with goat's and buffalos milk allergen were (58% and 57%) respectively which are similar to the rates of Varjonen *et al.*[16] who reported that (58% and 55.5%) of patients tested with goat's and buffalo's milk allergen respectively had positive specific IgE ELISA. There is a significant differences $P< 0.05$ among age groups of patients tested with goat's and buffalo's milk allergen, regarding the rate of patients who had a positive specific IgE ELISA, these results are in agreement with that of (Hattevig *et al* [13], who reported that the immune responses was acquired progressively during childhood, peaking between 15 and 25 years and declining gradually. Also, the rate of positive specific IgE ELISA was higher in females tested with goat's and buffalo's milk allergens in compare to males and this result differ from the result of kanng *etal*[17],. who reported that the rate of positivity in patients up to 15 years of age is more frequent in males . The explanation of this discrepancy depend on differences in genetic factor and climate[15].

In rural region, the rate of goat's milk allergic patients (51.7%), and the rate of buffalo's milk allergic patients (50.8%). These results are in agreement with the results of Pepys [18], who reported that 51.5% of pateint allergic to goat milk allergy and (50.5 %) allergic to buffalo milk,. because in urban area people, as usually, use dried milk in which drying process is probably denaturated protein so decreasing the antigencity of milk .

ELISA – inhibition

As far as the allergenic activity of protein was concerned, data presented in this work clearly evidence that proteins of goat's and buffalo's milk extracts were the most clinically relevant inhalant allergens. Besides, allergens of these extracts independently, accounted for a high percentage, in (goat's milk extracts 81.8% and buffalo's milk extract 85.1%) ; demonstrating that they were the main allergens from the goat's and buffalo's milk extracts and consequently other allergens might be present in the extracts with a little allergenic importance

On the other hand, goat 's milk allergen and buffalo's milk allergen cross-reacted with cow's milk allergen in IgE binding inhibition,. completely inhibiting the binding of specific IgE to each other. It seems clear that these proteins bear the same allergenic epitopes. These results are in agreement with results of Hoffman [19], who reported that mammalian allergens of different species

had been shown to cross – react such as allergen extracts from goat's and buffalo's milk extracts cross – react with cow milk extract .

In conclusions, the protein extract of goat's and buffalo's milk have allergenic activitites and there were cross – reaction a mong cow's , goat's and buffalo's milk.

دور مستأرج حليب البقر والجاموس كمسبب للنوع الاول من فرط الحساسية وعلاقتهما التصالبية مع مستأرجات حليب الابقار

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الخلاصة

تم تحضير وأستخلاص المحاليل البروتينية من حليب الماعز والجاموس ومن ثم تنقيتها وتجزئتها بواسطة الترشيح الهلامي اذ تم الحصول على قمة واحدة من حليب الماعز والجاموس ذات وزن جزيئي (26 دالتون و 15 دالتون) على التوالي .

وقد اجري فحص ELISA على 137 عينة مصل لتقدير قيمة IgE المتخصص والكلي وكانت نسبة النتائج الموجبة في فحص ELISA المستخدم لتقدير IgE المتخصص (58%) من المرضى المفحوصين بمستأرجات حليب المعز و (57%) من المرضى المفحوصين بمستأرجات حليب الجاموس.

ولقد لوحظ فرق احصائي معنوي بين الفئات العمرية وبين الذكور والاناث فيما يخص نسبة الأشخاص الذين اظهروا نتائج موجبة مع حليب الماعز والجاموس.

في الريف كانت نسبة المرضى المتحسسين لحليب المعز والجاموس أعلى من النسبة في المدينة .

وأخيراً فان هناك تفاعل تصالبي بين مستخلصات حليب الابقار مع مستخلص حليب الماعز والجاموس وان قدرة ارتباط المستخلص البروتيني لحليب الجاموس مع IgE أعلى من قدرة ارتباط المستخلص البروتيني لحليب الماعز وذلك لان المستخلص البروتيني لحليب الجاموس ثبط ارتباط IgE مع مستأرج حليب الابقار الممتص بنسبة (50%) وبتركيز بروتيني قليل .

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