

IgE antibodies to alpha-gal in foresters and forest workers from La Rioja, North of Spain

Running title: Alpha-gal sIgE in foresters

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Abstract

Objective: To investigate the prevalence and associated factors to the presence of alpha-gal-specific IgE in a risk group of foresters and forest workers from La Rioja and in a control group.

Methods: A total of 169 workers and 100 individuals who did not recall tick-bites were selected. A questionnaire including demographic data and number of tick bites per year was administered by a physician. Alpha-gal sIgE was assessed with serum samples that had been taken in 2010 using ImmunoCAP®.

In 2015, second serum specimens were taken from all the workers but one who had positive specific IgE to alpha-gal in 2010. These new samples were tested for IgE to the alpha-gal epitope and to mammalian meat.

Results: The prevalence of positive sIgE to alpha-gal in the risk population was 15% vs. 4% in the control population. Alpha-gal sIgE positivity was associated with the number of tick bites per year and with the seniority in the workplace. Thirteen out of 21 patients sensitized to alpha-gal in 2010 showed positive specific IgE to alpha-gal with serum samples from 2015. Eleven of them had specific IgE to mammalian meat but none reported symptoms of meat allergy.

Conclusion: The prevalence of alpha-gal sIgE antibodies in this Spanish risk population was higher than in the control group and it was associated with the number of tick-bites per year and with the seniority in the workplace. None of worker sensitized to mammalian meat developed meat allergy, possibly due to the discrete levels of sIgE to alpha-gal.

Key words: Allergens and epitopes, IgE, anaphylaxis, tick bites, alpha-gal

Resumen

Objetivo: Estudiar la prevalencia de IgE específica a alfa-gal y los factores asociados en el grupo de riesgo de agentes forestales y retenes de incendios de La Rioja respecto a un grupo control.

Métodos: Se incluyeron 169 trabajadores y 100 personas que referían no haber sufrido picaduras de garrapata. Cumplimentaron un cuestionario dirigido en el que se recogieron datos demográficos y número de picaduras de garrapata al año. Se realizó determinación de IgE específica a alfa-gal mediante la técnica InmunoCAP® utilizando muestras serológicas extraídas en 2010.

En 2015 se realizó nueva extracción serológica a todos los trabajadores sensibilizados a alfa-gal en muestra de 2010 excepto a uno. Se realizó nueva determinación de IgE específica a alfa-gal y a carne de mamíferos.

Resultados: La prevalencia de IgE específica a alfa-gal en la población de riesgo fue del 15% vs. 4% en el grupo control. La positividad de la IgE específica a alfa-gal se asoció con el número de picaduras al año y con la antigüedad en el puesto de trabajo. Trece de los pacientes sensibilizados en 2010 seguían sensibilizados en 2015. Once de ellos mostraban IgE específica frente a carnes sin manifestar síntomas de alergia.

Conclusión: La prevalencia de IgE específica a alfa-gal en población de riesgo Española fue superior a la del grupo control y se asoció al número de picaduras-año y a la antigüedad en el puesto de trabajo. Ninguno de los trabajadores sensibilizados a carnes, desarrolló alergia, probablemente por los niveles discretos de IgE específica a alfa-gal.

Palabras clave: Alérgenos y epítomos, IgE, anafilaxia, picaduras de garrapata, alfa-gal

Introduction

Antibodies to alpha-gal are present in all non-immunocompromised human subjects and some early studies suggested that the IgG antibodies to alpha-gal constituted about 1% of circulating immunoglobulins in human subjects, apes, and Old World monkeys (1). These antibodies are continuously produced throughout life as an immunological response to antigenic stimulation by bacteria of the normal flora. However, the production of IgE antibodies to alpha-gal, only occurs in a variable percentage of the population depending on different geographical areas (2-9). They are the cause of distinct forms of anaphylaxis: Delayed-onset anaphylaxis 3 to 6 hours after ingestion of red meat (3,7,10-20) and immediate-onset anaphylaxis after exposure to the monoclonal antibody cetuximab (2), Crotalidae polyvalent immune Fab antivenom (21), zoster vaccine (22), vaginal capsules (23), bovine derived gelatine colloids (13), bioprosthetic aortic valve (24), bovine amniotic fluid (25) and gelatin-containing sweets (26).

To date, tick bites are the only proven cause of IgE antibodies to alpha-gal (27). This association was first detected in the US by Commins *et al.* They reported strong correlation between IgE antibodies to alpha-gal and history of tick bites, and found that these antibodies were not produced in regions where tick bites were not common. The same authors also reported the correlation between IgE antibodies to proteins derived from *Amblyomma americanum* ticks and IgE antibodies to alpha-gal (4). Afterwards, in other areas of the world, several studies showed association between IgE antibodies to alpha-gal and tick bites of *Ixodes holocyclus* in Australia (10) or *Ixodes ricinus* in Europe (7,8). Lastly, Hamsten *et al* demonstrated that the alpha-gal epitope was present in the gastrointestinal tract of *I. ricinus* using immunohistochemistry (28).

La Rioja is a land from the North of Spain very rich in flora and fauna that constitutes an ideal habitat for ticks. In this region, ticks that most frequently parasitize humans belong to the genera: *Ixodes*, *Rhipicephalus* and *Dermacentor* (29)

We performed this study to investigate the prevalence and associated factors to the presence of alpha-gal-specific IgE in a risk group of foresters and forest workers from La Rioja and in people without knowledge of having been bitten by ticks (control group).

We also studied the changes in the level of specific IgE to alpha-gal in sensitized patients past five years to investigate the relationship with the number of bites suffered over these five years.

Methods

Patients

All the workers from the list of 169 foresters, forest workers and drivers provided by the Consejería de Medio Ambiente del Gobierno de La Rioja in 2010 (equivalent to the Environment Ministry), ~~foresters and forest workers~~ with high probability of having suffered multiple tick bites and who agreed to participate in the study were selected as sample population. Individuals who attended to the Allergy Unit at Hospital San Pedro (Logroño, Spain) for allergy studies to beta-lactam antibiotics from February to April 2015 and reported that they never had been bitten by ticks were included as control group.

Ethical Issues

All individuals consented to participate in the study, which was approved by the Institutional Review Boards (Ethics Committee, La Rioja, Spain, code PI-175), conformed to the current Helsinki Declaration.

Questionnaires

An structured questionnaire including basic demographic data (age, gender, seniority in the workplace and geographical working area) was administered in 2015 by a physician. Geographical areas (High Rioja, Medium Rioja and Low Rioja) were established and people were grouped according to their living areas (in the valley or in the mountains), since these facts could be interesting for the different distribution of ticks.

Clinical data obtained from patients included information about atopy, history of tick bites, number of bites/year, and localized or generalized allergic reactions to tick bites. In order to involve the tick species causing the bites, we asked about the month of the year when tick-bites occurred and the area of the body affected. We also asked about

food allergies (specifically, red meat allergy) and about a history of present or past cancer that would have required treatment with cetuximab.

Finally, we asked if the number of tick bites/year suffered during the last five years had been greater, same or lower than before 2010.

Serum specific IgE to the alpha-gal epitope and to beef, pork and chicken meats

Serum samples from the foresters and forest workers and from the control group individuals were tested in 2015 for IgE to alfa-gal (o215) using the ImmunoCAP® 250 automated platform (Thermo Fisher Scientific, Uppsala, Sweden). Serum samples from 2010 stored in the serum bank of the Department of Infectious Diseases at the Hospital San Pedro-Center of Biomedical Research of La Rioja (CIBIR) were used to analyze the professional risk group and the remaining of sera samples that were left of the drug allergy study carried out in 2015 were used for the control group. All but one of the foresters and forest workers with positive specific IgE to alpha-gal were tested again for IgE to the alfa-gal epitope, to beef meat (f27), pork meat (f26), and chicken meat (f87) using serum samples taken in 2015. The cut-off values for IgE were ≥ 0.1 kU/L, as suggested by the manufacturer.

Statistical analyses

Statistical were performed using the SPSS software, version 21.

The variables that follow a normal distribution were described by the mean and standard deviation and those that do not follow a normal distribution by the median and interquartile range (IQR).

A 2-sided p value of less than 0.05 was considered to indicate statistical significance.

The chi-square was used to investigate the association between categorical variables.

The Student's t-test and the Wilcoxon test were, when appropriate, used to compare

numerical variables between groups. We compared quantitative continuous variables by using the Spearman's rank correlation coefficient. Logistic regression was used for multivariate analysis of factors associated with positivity to alpha-gal IgE antibodies.

Results

A total of 147/169 workers at risk agreed to participate in the study: 65/71 forest officials (91.5%), 75/91 forest workers (82.4%) and 7/7 drivers (100%). The questionnaire and the serum specific IgE test to the alpha-gal epitope was performed for all 147 participants (mean age=40.5 years; 145 male and 2 female) with serum samples from 2010. A total of 100 sera samples were also analysed as controls.

The prevalence of alpha-gal sIgE in the population of foresters and forest workers was 15% (22/147) and it proved to be significantly higher than the prevalence in the control population (4%; 4/100) (odds ratio: 4.2; confidence interval 95: 1.4 to 12.6). Individual data of cases with alpha-gal sIgE ≥ 0.1 kU/L are represented in the table 1. Titres were generally low. In the professional group median was 0.22 kU/L, with IQR 0.19 kU/L. The control group showed lower titres (minimum 0.1 and maximum 0.15 kU/L). Only five individuals from the foresters and forest workers population (3.4%) and none (0%) from the control group showed alpha-gal sIgE levels ≥ 0.35 kU/L (the classical threshold for positivity), and only one forester had alpha-gal sIgE level ≥ 3.5 kU/L (class 3 or higher).

One hundred and twenty four (84.4%) individuals belonging to the sample population reported a history of tick bites while 23 (15.6%) were not aware of having suffered tick bites. A statistically significant between the number of bites per year and the alpha-gal sensitization was found using Spearman test (0.22; $p < 0,008$). The percentage of alpha-gal sensitized for the professional group ranged from 4% for workers who were

unaware of having suffered tick bites, 12% having 0-5 bites per year, 13% having 5-10 bites per year, 28% having 10-20 bites per year to 33% for those reporting more than 20 tick bites per year.).

The mean age in the group of non-sensitized workers was 39.89 years with a standard deviation of 8.99, and in the group of sensitized workers it was 43.95 years with standard deviation of 8.92 ($p=0,052$). Regarding years in the workplace, the mean was 16.95 years with standard deviation of 8.96 for the group of sensitized patients and 12.92 with standard deviation of 8.38 for the group of non-sensitized workers. These differences were statistically significant ($p=0.041$). In logistic regression studies, the seniority in the workplace and the number of tick bites were both associated with an increased risk of sensitization to alpha-gal, regardless of other analyzed variables. Thus, workers with more than ten tick bites per year showed a statistically significant risk of sensitization to alpha-gal vs. workers who had not suffered bites. The age lost association with sensitization to alpha-gal when other variables (number of tick bites and seniority in the workplace) were considered as confounders (table 2).

In our country, tick-bites occurring during the winter or affecting the scalp are probably caused by ticks of the genus *Dermacentor*. No significant differences between alpha-gal sensitization and data suggesting bites by *Dermacentor* spp., atopy, local reaction to tick bites and geographical working areas of foresters and forest workers were found (table 3).

Twenty-one out of the 22 patients sensitized to alpha-gal in serum samples from 2010 were tested again for IgE to the alfa-gal epitope with sera taken in 2015 (table 1). In this determination, 13 patients still showed a specific IgE to alpha-gal >0.1 kU/L, while in 8 patients specific IgE to alpha-gal had become negative. In these 13 patients median was 0.64 kU/L (IQR= 0.75 kU/L). Nine of them showed alpha-gal sIgE levels $\geq 0,35$ kU/L. The mean difference of specific IgE to alpha-gal samples between 2010

and 2015 was -0.31 kU/L, with slight decrease in the IgE value in 2015. These differences were not significant.

When we comparing the changes in the specific IgE in relation to having suffered a greater or lesser number of bites from 2010 to 2015, we did not find significant differences, since all the patients but one reported the same number of bites-year before and after 2010. Only a patient reported having suffered fewer bites during last five years and in this patient the IgE to alpha-gal had become negative.

Six patients had specific IgE to pork meat > 0.1 kU/ L (median 0.84 kU/L; IQR: 1.23 kU/L) and 11 cases to beef meat (median 0.31 kU/L; IQR: 0.37 kU/L) .

Therefore, the prevalence of sensitization to red meat in the sample of 13 patients sensitized to alpha-gal with serum taken in 2015 was 77% with 95% confidence interval ranging from 55% to 99%. High correlation between the specific IgE values to alpha-gal and specific IgE values to pork was found, with a value of Pearson correlation coefficient of 0.76 ($p < 0.01$) and very high correlation with beef with Pearson correlation coefficient of 0.93 ($p < 0.01$). Specific IgE values to chicken were found in two patients (0.28 and 0.15 kU/L, respectively). No patients suffered clinical allergy to mammalian meat.

Discussion

The prevalence of sensitization to alpha-gal in foresters and forest workers exposed to multiple tick bites in La Rioja was higher than in people who were not aware of having been bitten by ticks (15% vs. 4%). This fact supports that tick bites are the unique proven cause of IgE antibodies to alpha-gal (27). These results are not comparable to the 29% of sensitization to alpha-gal found in our preliminary study carried out in La Rioja (30). In the previous study people who reported multiple tick bites were included, whereas herein 15% of forest workers reported that they had never suffered from tick bites. When our findings were compared to those of the related literature performed in the general population the results showed disparities. Thus, the values varied from 15-20% in south-eastern US (2-5); 25 % in a rural area of the south-western Italy (9); 10% in Stockholm (7); 8% in north-western Spain and 5% in Denmark (8). Very high percentages in areas with tropical climates such as Kenya (76%) and the Province of Esmeralda (37%) had also been reported (4). Disparities can be attributed to different criteria to establish the cut-off value of specific IgE to alpha-gal. Herein, the cut-off value =0.1 kU/L was considered, as recommended by the manufacturer of technical Termofisher-ImmunoCAP. Reports carried out in the US considered a specific IgE value to alpha-gal > 0.35 kU/L as positive (2-5), whereas the same criteria as ours was used in Sweden (7), Denmark, Spain (8) and Italy (9). In two of these works the prevalence of sensitization to alpha-gal halved (9), and even decreased four times (8) when the criteria of positivity was changed to 0.35 kU/L since most sensitized cases showed specific IgE in this range (0.1-0.35 kU/L). In our study, when the positivity threshold was increased to 0.35 kU/L, the prevalence of sensitization decreased to 3.4% (only five workers showed specific IgE value to alpha-gal > 0.35 kU/L in the sample from 2010). Nevertheless, 9 patients had specific IgE value to alpha-gal > 0.35 kU/L in 2015, suggesting that the prevalence of sensitization would be higher although

we could not calculate it because we only repeated this determination in patients sensitized in 2010.

Since the prevalence of sensitization to alpha-gal in our group risk was lower than that of the general population from South-East of US, we can deduce that the prevalence in the general population of La Rioja would be even lower than that of the general population in the South-East of US. This could be explained because tick species parasitizing humans in our area (mainly *Ixodes*, *Dermacentor* and *Rhipicephalus*) are not as aggressive as *Amblyoma americanum*, the predominant species in the US, or perhaps because their ability to induce sensitization to alpha-gal could be lower. Currently, the presence of alpha-gal in the digestive tract of *I. ricinus* nymphs has been detected by immunohistochemistry (28), but the origin of this carbohydrate determinant which subsequently induces the IgE production is still unknown. There are different theories that support that it would be due to a component of saliva, or part of a glycoprotein or glycolipid from the tick through the intake of blood in a previous feeding, or microorganisms such as *Rickettsia* or *Borrelia* that are present in ticks (27).

In our work, the association between a history of tick bites and sensitization to alpha-gal is demonstrated. In addition, linear correlation between the number of tick bites/year and the probability of being sensitized to alpha-gal was found. Using logistic regression studies, the fact of suffering from 10 bites per year represented a risk factor for alpha-gal sensitized regardless of the number of years working and the age, with respect to people who did not recall tick bites. Anyway, the tick species that had bitten our patients were not determined, although we assumed that tick-bites in winter were caused by *Dermacentor* spp. since this is the active species during cold months. Thus, with our data, we can only state that there was no relationship between having suffered *Dermacentor* spp. bites and sensitization to alpha-gal. These findings are consistent with those that found correlation between specific IgE to alpha-gal against *A. americanum*, which did not occur against *Dermacentor variabilis* (4).

Four people from the control group (4%) were sensitized to alpha-gal, with values ranging from 0.1 and 0.15 kU/L. This could be explained because the possibility of suffering unnoticed tick bites is feasible. Only 50-70% patients diagnosed with Lyme disease recalled having been bitten by ticks (31-33). Moreover, 1/23 (4%) foresters and forest workers who believed that they had never suffered from tick bites was sensitized to alpha-gal.

Another finding of our study is the association between sensitization to alpha-gal and the variables time worked and age. This association was less intense with age and it is lost in the logistic regression analysis, which is logical, since age itself is not a risk factor, if the patient is not at risk of bites.

The workers who carried out their professional activities in mountain areas and Medium Rioja (Cameros mountains) had about twice probability to be sensitive vs. workers from the valley and Low and High Rioja, although differences were not significant. This finding supports the fact that ticks are more abundant in mountain areas with supra-Mediterranean climate, suitable for livestock and forestry activities. In addition, *I. ricinus* ticks are more frequent in mountain areas and this is the species that most frequently bites humans vs. other species such as *Haemaphysalis*, more commonly found in the valley with mesomediterranean climate.

A total of 77% foresters and forest workers sensitized to alpha-gal, were also sensitized to mammalian meat but none of them showed symptoms of allergy to meat. This could be explained by discrete values of specific IgE to alpha-gal (in the class 1 or 2) for all but two patients (whose values belonged to class 3). In our experience and also according to findings of other research groups, patients allergic to red meat typically showed very high levels of specific IgE to alpha-gal, although sometimes they developed clinical symptoms with IgE values in class 1 or 2 (2, 7, 12, 14, 17, 34).

In conclusion, we found that the prevalence of alpha-gal sIgE in the risk population of foresters and forest workers was higher than in the control population. In addition, the prevalence of sIgE to alfa-gal and the number of reported tick bites per year and seniority in the workplace were related. It has been demonstrated that tick species parasitizing humans in the North of Spain are able to induce alpha-gal sIgE. Many patients sensitized to alpha-gal were also sensitized to mammalian meat. Since successive tick bites may induce an increased level of sensitization to alpha-gal and the development of meat allergy, we recommend to this risk group to take precautionary measures to prevent further tick bites (35).

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Addendum

While we were reviewing this paper, Fisher et al reported a cross-sectional study about prevalence of alpha-gal-specific IgE in 300 forest service employees and hunters from southwest Germany (36).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Table 1. Specific IgE values to alpha-gal and meats (kU_A / L) in the twenty-two sensitized professionals.

Patient	ImmunoCAP α-Gal (2010) KUA/l	ImmunoCAP α-Gal (2015) KUA/l	ImmunoCAP pork meat (2015) KUA/l	ImmunoCAP beef meat (2015) KUA/l	ImmunoCAP chicken meat (2015) KUA/l
1	0,10	0,09	0,05	0,06	0,01
2	0,95	0,71	0,11	0,31	0,02
3	0,19	0,10	0,04	0,11	0,15
4	0,54	5,48	1,29	1,29	0,03
5	0,72	0,66	0,23	0,29	0,02
6	0,14	0,02	0,03	0,03	0,02
7	18,20	6,19	1,54	2,31	0,03
8	0,39	0,09	1,39	0,52	0,04
9	0,21	0,64	0,38	0,36	0,05
10	0,25	0,23	0,04	0,05	0,06
11	0,32	0,04	0,04	0,06	0,01
12	0,32	0,05	0,06	0,06	0,06
13	0,29	0,40	0,00	0,43	0,28
14	0,10	0,46	0,08	0,15	0,01
15	0,14	0,01	0,02	0,04	0,01
16	0,19	0,04	0,02	0,03	0,02
17	0,10	0,28	0,08	0,12	0,05
18	0,25	1,21	0,07	0,17	0,03
19	0,14	0,04	0,02	0,03	0,02
20	0,15	0,80	0,06	0,09	0,02
21	0,23	0,11	0,07	0,09	0,03
22	0,11	NP	NP	NP	NP

NP: Not performed.

Table 2. Results of the logistic regression model variable alpha-gal sensitization regarding the variables age, seniority in the workplace and number of bites per year

	Coef	O.R.	Confidence interval OR
Seniority in the workplace	0,068	1,07	1,01 a 1,13
Bites-year			
From <5 bites with regard to 0 bites	1,26	3,54	0,40 a 30,8
Between 5 and 10 bites with regard to 0 bites	1,61	5,03	0,49 a 51,4
Eleven to 20 bites with regard to 0 bites	2,37	10,71	1,01 a 113,13
Over 20 bites with regard to 0 bites	2,76	15,89	1,51 a 166,95
Age	0,017	1,01	0,92 a 1,11

Table 3. Relationship between α -Gal IgE response and the variables atopy, local reaction to tick bites, geographical working area of foresters and fire seals and data suggesting bites by *Dermacentor* spp.

Variable	α -gal-IgE response		p-value
	no	yes	
Atopy			
No	93/110 (84.5%)	17/110 (15.5%)	0.8
Yes	32/37 (86.5%)	5/37 (13.5%)	
Local reaction			
No	76/87 (87.4%)	11/87 (12.6%)	0.34
Yes	49/60 (81.7%)	11/60 (18.3%)	
Geographical area			
High Rioja	53/61 (86.9%)	8/61 (13.1%)	0.44
Medium Rioja	43/54 (79%)	11/54 (20.4%)	
Low Rioja	23/26 (88.5%)	3/26 (11.5%)	
All over Rioja	6/6 (100%)	0/6 (0%)	
Mountains	96/116 (82.8%)	20/116 (17.2%)	0.28
Valley	23/25 (92%)	2/25 (8%)	
Mountains and valley	6/6 (100%)	0/6 (0%)	
Data suggesting bites by <i>Dermacentor</i> spp			
Bites during Winter			
No	99/120 (82.5%)	21/120 (17.5%)	0.18
Yes	4/4(100%)	0/4 (0%)	
Bites affecting the head			
No	87/105 (82.9%)	18/105 (17.2%)	0.29
Yes	16/19 (84.2%)	3/19 (15.8%)	