

Original Article

Determination of Allergenic Load and Pollen Count of *Cupressus arizonica* Pollen by Flow Cytometry Using *Cup a1* Polyclonal Antibody

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Background: There is an increase in the incidence of pollen related allergy, thus information on pollen schedules would be a great asset for physicians to improve the clinical care of patients. Like cypress pollen sensitization shows a high prevalence among the causes of allergic rhinitis, and therefore it is of interest to use it like a model of study, distinguishing cypress pollen, pollen count, and allergenic load level. In this work, we use a flow cytometry based technique to obtain both *Cupressus arizonica* pollen count and allergenic load, using specific rabbit polyclonal antibody Cup a1 and its comparison with optical microscopy technique measurement.

Methods: Airborne samples were collected from Burkard Spore-Trap and Burkard Cyclone *Cupressus arizonica* pollen was studied using specific rabbit polyclonal antibody Cup a1, labeled with AlexaFluor® 488 or 750 and analysed by Flow Cytometry in both an EPICS XL and Cyan ADP cytometers (Beckman Coulter®). Optical microscopy study was realized with a Leica optical microscope. Bland and Altman was used to determine agreement between both techniques measured.

Results: We can identify three different populations based on rabbit polyclonal antibody Cup a1 staining. The main region (44.5%) had 97.3% recognition, a second region (25%) with 28% and a third region (30.5%) with 68% respectively. Immunofluorescence and confocal microscopy showed that main region corresponds to whole pollen grains, the second region are pollen without exine and the third region is constituted by smaller particles with allergenic properties. Pollen schedule shows a higher correlation measured by optical microscopy and flow cytometry in the pollen count with a *P*-value: 0.0008 E⁻² and 0.0002 with regard to smaller particles, so the Bland and Altman measurement showed a good correlation between them, *P*-value: 0.0003.

Conclusion: Determination of pollen count and allergenic load by flow cytometry represents an important tool in the determination of airborne respiratory allergens. We showed that not only whole pollen but also smaller particles could induce allergic sensitization. This is the first study where flow cytometry is used for calculating pollen counts and allergenic load. © 2013 International Clinical Cytometry Society

Key terms: flow cytometry; allergenic load; pollen count; *Cupressus arizonica*; allergy

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In recent decades, a marked increase has been noted in patients suffering from allergic rhinitis, especially in developed countries. Prevalence in the general population is estimated between 10 and 25%. In the last epidemiologic study undertaken in Spain (Alergologica-2005), 4,991 patients were evaluated, of which 51% were sensitized to pollen allergens (35% allergic to gramineae, 20% to olive, 9% to cypress and 7% to Parietarie) (1). Pollens are the main causes of allergic diseases in the South of Europe, with allergens from food vegetables being the principal cause in the adult population (2).

Currently, the determination of pollen schedules in cities are based on the pollen count of whole pollen grains (with a well defined structure) through optical microscopy, without considering the relative allergenic load associated to whole pollen grains versus incomplete pollen structures which are also responsible for the symptoms shown by pollen-allergic patients. Knowledge of pollen schedules in each city and their associated allergenic loads considering both (pollens and incomplete particles with allergenic effect (expressed in particles/m³) would allow physicians to improve the diagnosis and treatment of pollen-allergy patients.

The most common species around the Mediterranean basin are *Cupressus sempervirens*, *Cup. arizonica*, *Cup. macrocarpa* and *Cup. lusitanica*. Among these, *Cupressus arizonica* has been the one which allergenic features have been best studied. Pollen of this species shows six immunoglobulin (Ig)E-binding protein bands, with the 43 kDa component being predominant both in its intensity and in the frequency of recognition by human IgE antibodies (3). This band was identified as the major allergen of *Cup. arizonica* (Cup a1). *Cupressus arizonica* and *C. sempervirens* extracts show a high cross-reactivity and share a number of common epitopes (4). Nevertheless extracts of *C. arizonica* pollen show a higher allergenic potential when compared with that of *C. sempervirens* and *C. lusitanica* (5). Besides, there is a high cross-reactivity (75–90% homology) between Cup a1 (major allergen of *C. arizonica*), Cha o1 (major allergen of *Chamaecyparis obtusa*, the Japanese Cypress) and Jun a1, the major allergen of *Juniperus ashei*, the mountain cedar (6,7).

The main objective of this study is to determine airborne pollen count and atmospheric allergenic load from pollens. As a model of study we will use *C. arizonica* pollen by means of a flow cytometry based technique using Cup a1 rabbit polyclonal antibody. We define allergenic load as a particle that can be recognised by a specific antibody; specifically, cypress pollen particles recognised by Cup a 1 rabbit polyclonal antibody in our study. We show daily qualitative data of the time-course of pollen counts. Although there is cross-reactivity toward other pollen species, we have considered *Cupressus arizonica*-like model because in our area between January and March, only the pollinization of *C. arizonica* species is present, without the presence of other cupressaceous species.

There are few studies determining allergenic load using RAST and ELISA techniques (8–10), but they give results after the pollenic season has already finished, which is their main drawback. To our knowledge, this is the first study that uses a similar approach, same technique (flow cytometry) for pollen count and allergenic load measurements.

MATERIAL AND METHODS

Burkard Spore-Trap and Burkard Cyclone Sampler

Atmospheric pollen was recorded using Burkard Spore-traps and counting through an optical microscope, following the standardized methodology proposed by Galan et al. (11) for the Spanish Aerobiology Network (REA) as the reliability of data is the prerequisite for successful monitoring (12).

Burkard Cyclone sampler consisted of a continuous volumetric sampler with wind orientation, using a single reverse-flow miniature cyclone with an air throughput of 16.5 L/min (23.7 m³/day). The instrument offers 100% efficiency sampling down to a 1.06-μm range (13). Particles were collected directly into a 1.5 mL Eppendorf vial. Our samples were collected in a phosphate-bisphosphate medium: Sørensen 2X (76 mM de NaH₂PO₄; 324 mM de Na₂HPO₄, pH 7.4). Samples correspond to daily periods. Samples were measured up to 24 h by flow cytometry.

These devices were in use from January to June 2012, although only data from February 23rd until March 23rd, 2012, when the presence of cypress pollen is higher. Both samplers were located in the same sampling station 1.5 m apart and 30 m above sea level.

Pollen Stability in Different Aqueous Buffers

We measured the stability of cypress pollen in different aqueous buffers, (considering its morphology and structure by optical microscopy): Sørensen, Sørensen 2X, water and PBS (137 mM NaCl, 2.7 mM KCl; 4.3 mM Na₂HPO₄; 1.47 mM KH₂PO₄, pH 7.4) for a time period of 148 h, in temperature ranges from 4 to 37°C, concluding that Sørensen 2X was the buffer that best conserved pollen structure.

Flow Cytometry Analyses

Flow cytometry analyses were performed in two different models: EPICS XL-MCL™ model and Dako CyAn ADP™ (both from Beckman Coulter, Brea, CA). For most experiments, forward scatter (FSC), side scatter (SSC), FL1 and FL9 fluorescence data was collected. Both flow cytometry models allowed us to confirm reproducibility of the results. In the EPICS XL-MCL™ model, the samples were labeled only with Alexa Fluor 488 in FL1 channel while in Dako CyAn ADP™ model, we could label such as Alexa Fluor 488 (channel FL1) and Alexa Fluor 750 (channel FL9). Samples were acquired for 1 min with a flow rate of 26.53 events/s used as control standardized counting beads. Purified *Cupressus arizonica* pollen was provided by Bial Pharmaceuticals™, Spain.

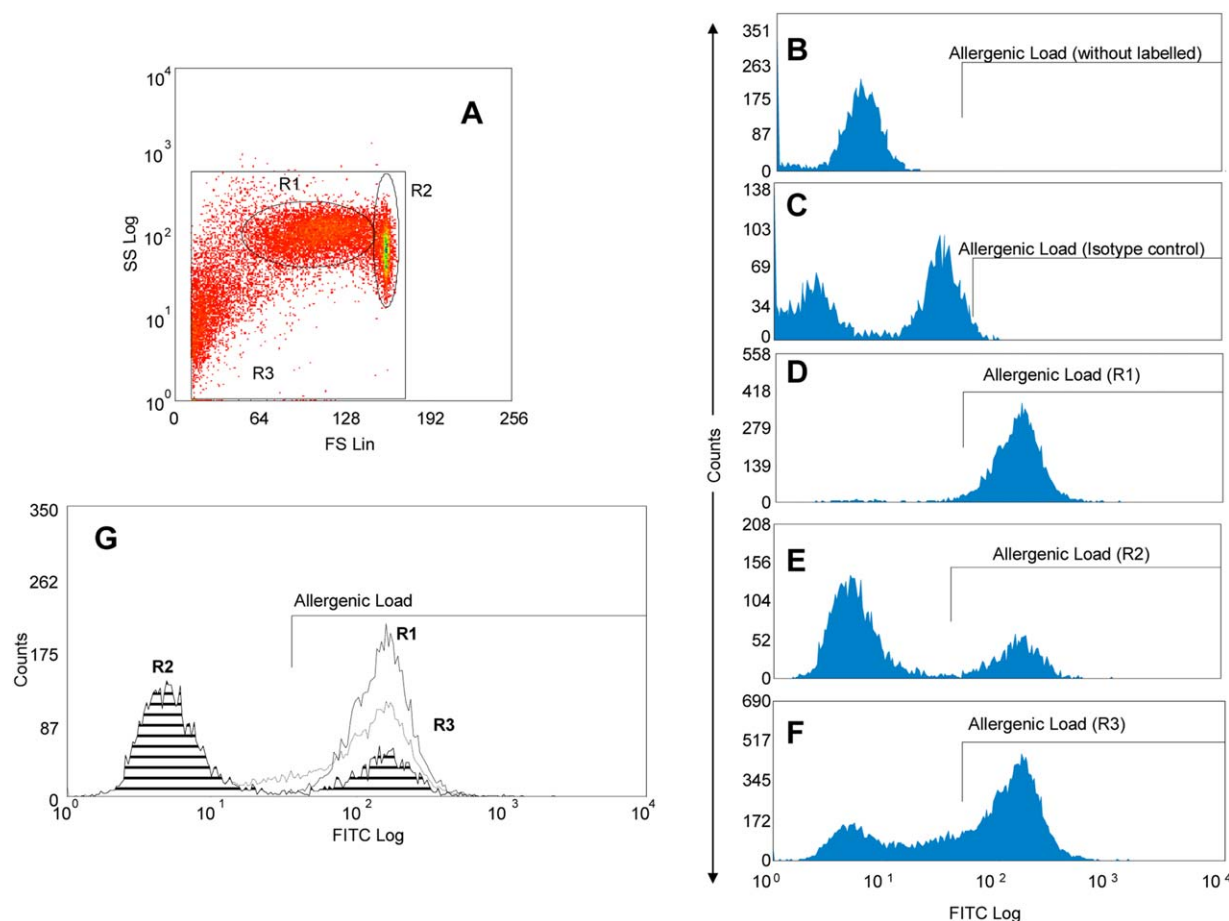


FIG. 1. Cytometric profile from *C. arizonica* pollen. (A) Histogram FSClin-SSLog. Three different regions: R1: 44.50%; R2: 25%; R3: 30.5%. Histogram FITC Log of *C. arizonica* pollen (B) without labeling, and (C) isotype control IgG rabbit. Different recognition of polyclonal antibody Cup a1: (D) R1: 97.28%, (E) R2: 28.28%, (F) R3: 37%, and (G) overlay of all regions in FITC. In this case, R3 involves only the allergenicity load of the smallest particles. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

Identification of Cypress Pollen Cytometric Profile

Airborne samples collected in 1.5 mL Eppendorf vials with Sørensen's 2X were resuspended in water. Then, 50 μ L aliquots were incubated with rabbit polyclonal antibody Cup a1 (concentration: 0.01 mg/mL) during 30 min at room temperature and subsequently, incubated with AlexaFluor 488 or 750 (dilution 1:20) for 30 min in the dark, at room temperature and immediately analysed using flow cytometry without washing. We used rabbit IgG like isotype control following manufacturer instructions.

To identify the cypress pollen profile through flow cytometry, we used the following gating procedure for Cup a1 labeled samples:

1. Events included in a gate in FSC-SSC histograms (R).
2. Events that emitted a positive signal ($>10^1$) in the FL9 channel (AlexaFluor 750) or $>10^2$ in the FL1 channel (FITC).

Pollen count was considered as events that accomplished both conditions.

Determination of Allergic Load Through Flow Cytometry

To determine allergic load, we selected all the events with emissions higher $>10^1$ in the FL9 channel (AlexaFluor 750) and in Gate (R) where there are full pollen particles and incomplete pollen that still show affinity for the antibody.

Optical, Fluorescence and Confocal Microscopy Study

For pollen count, optical microscopy was used as the reference technique and it was compared to the flow cytometry technique developed here. To avoid bias in the results, optical microscopy measurements were taken by a different center. Fluorescence and confocal microscopy were used to study different cypress regions (R), when they were separated by centrifugation (100 rpm for 15 min). Ten microliters labeled samples with AlexaFluor488 (see labeling protocols above) and EPA (1:1) were used in both microscopic techniques. Ten minutes after EPA addition, samples were measured.

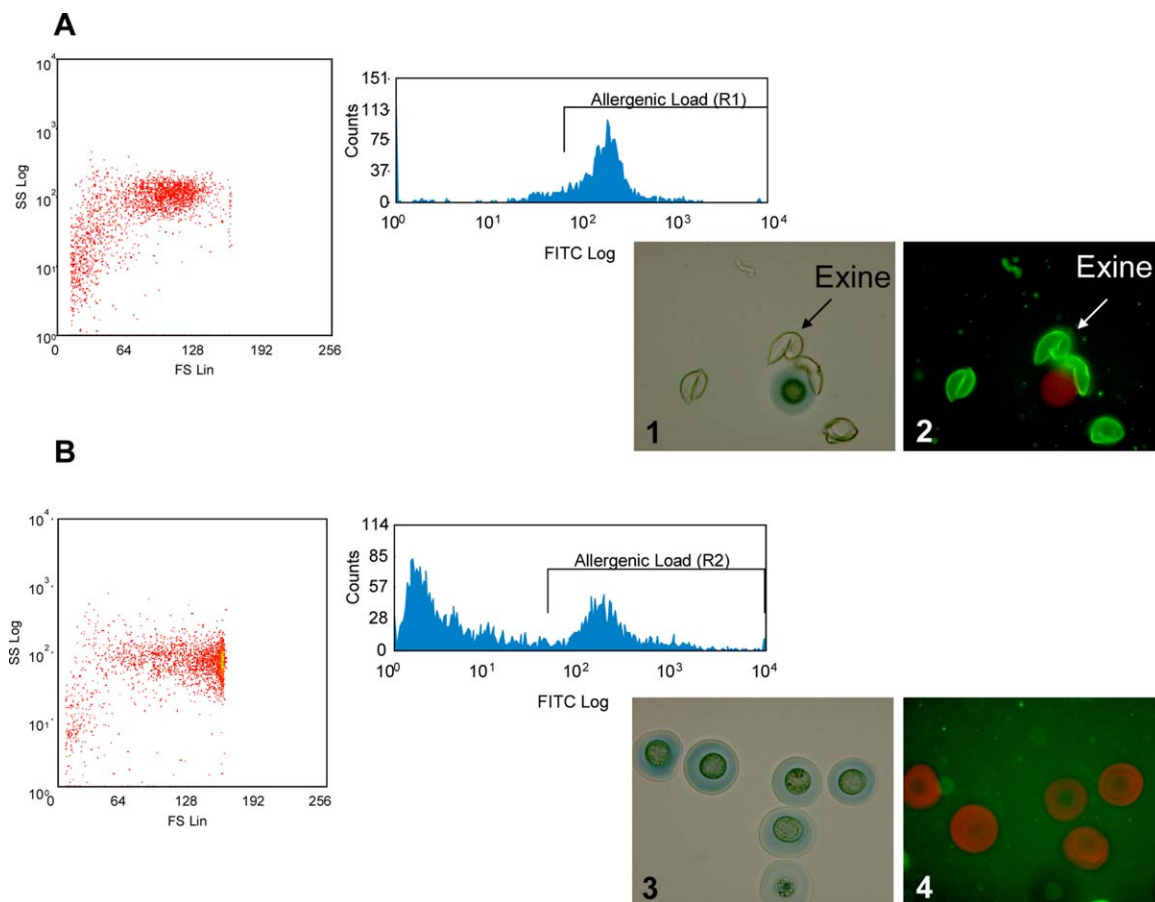


FIG. 2. Different regions were separated by centrifugation **A** (higher recognition of Cup a1) and **B** (lower recognition of Cup a1). Left images (1 and 3): Optical microscopy. We can see pollen cypress where the exine is detached from the grains. This belongs to R1 region (1) and R2 region (3) represented in flow cytometry. Right Images (2 and 4): fluorescence microscopy. We can see *Cupressus arizonica* pollen where the exine is detached from the grains. Polyclonal antibody Cup a1 recognises the epitopes of the Exine. This belongs to R1 region (2) and R2 region (4) represented in flow cytometry. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

Statistical Analysis

Pollen count between flow cytometry and optical microscopy techniques, and allergenic load analysis were compared for the same intervals of time. Data were analyzed using the Pearson correlation coefficient and linear regression to investigate the correlation between both methods. SPSS10 (Chicago, IL.) was used for all statistical analyses.

The definition of acceptable limit values is relevant for flow cytometry and optical microscopy techniques to be compared with limits of agreement (mean of difference $\pm$ 2 standard deviations) obtained from the Bland-Altman analysis.

RESULTS

Identification of *C. arizonica* Pollen Cytometry Profile

We observed three different groups in a reference sample of *C. arizonica* pollen when it was resuspended in Sørensen 2X buffer:

R1 is the main region, 97% of events in R1 region are recognized by polyclonal antibody Cup a1 (Figs. 1A and 1D).

R2, 28% of events in the R2 region is recognized by polyclonal antibody Cup a1 (Figs. 1A and 1E). This percentage increases with a concordant decrease in R1, without increasing the affinity of polyclonal antibody, when pollen is in contact with PBS or water (data not shown).

R3, 68% of events in the R3 region constituted by heterogeneous smaller particles, are recognized by antibody Cup a1 of 68%. (Figs. 1A and 1F).

When we included a washing step after labeling, smaller particles that were still recognized by the antibody and thus retain allergenic properties were lost; thus we did not include a washing step in the labeling process.

Fluorescence and Confocal Microscopy

Once we had obtained *C. arizonica* cytometric profile and we had separated different populations through centrifugation, we performed confocal and fluorescence microscopy studies to analyze the morphology of each

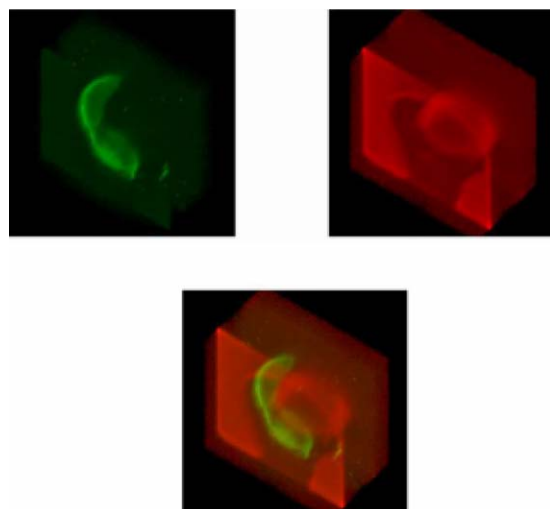


FIG. 3. Tridimensional conformation *C. arizonica* pollen. We can see the exine is separated from the rest of the pollen particle by confocal microscopy. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

population (R1 or R2). We can see different structures that show allergenicity, using fluorescence microscopy.

R1 region is full pollen. The exine is responsible for recognizing the allergen Cup a1 (Fig. 2A). R2 region corresponds to a mixture of pollen particles, but they don't contain exine. They reacted less strongly with polyclonal antibody Cup a1 (Fig. 2B).

To determine the conformational and tridimensional structure of less well-defined pollen and to study the different cypress populations, we analyzed the samples by means of a confocal microscope. We observed that antibody Cup a1 recognizes the exine. In this case, the exine is not completely separated from the internal pollen structure (Fig. 3).

Pollen Count and Allergenic Load Level in the Atmosphere

In 2012, the pollen count measured using Optical Microscopy showed a main pollination period (m.p.p.) from February 21st to March 24th (Fig. 4). On 14th of March there was the highest concentration of cypress pollen, 606 grains/m³ which was lower than previous years. This was due to a rigorous dry and cold autumn and winter. The pollen count with flow cytometry shows an m.p.p. from February 21st to March 24th. There was 2 days with a maximal concentration of cypress pollen, 14th and 20th of March with 600 and 572 grains/m³, respectively.

If we compare both techniques (flow cytometry and optical microscopy), we have a correlation in pollen

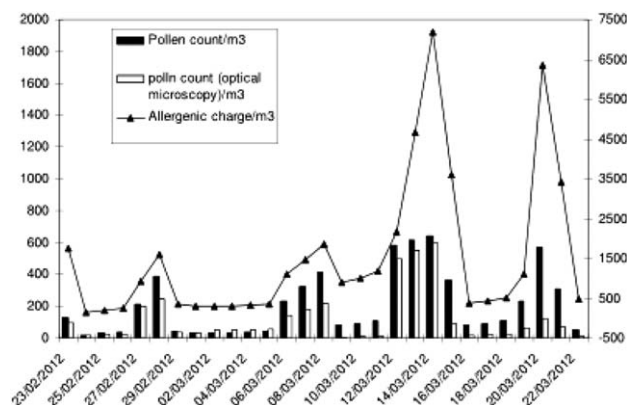


FIG. 4. Time-course of *C.* pollen count. Left y-axis: representation of pollen count by optical microscopy and flow cytometry. Right y-axis: representation of allergenic load by flow cytometry. Correlation between pollen count by optical microscopy and flow cytometry.

count with R^2 of 0.73; r-Pearson: 0.855; P -value of 0.0008 E^{-2} . The allergenic load level showed a correlation between pollen count using optical microscopy and flow cytometry with R^2 of 0.46; r-Pearson: 0.677; P -value of 0.0002 and R^2 of 0.75; r-Pearson: 0.868; P -value of 0.0002 E^{-12} , respectively.

Results obtained by the use of Bland and Altman technique showed a P -value 0.0003. (Table 1) with a IC of 95% (Fig. 5).

DISCUSSION

In this study, we characterized the *C. arizonica* pollen cytometric profile. When *C. arizonica* pollen was resuspended in Sørensen's 2X (pH 7.4) a single defined region in the histogram FS versus SS was recognized by rabbit polyclonal antibody Cup a1 including 94.7% of events, using standardized counting beads could test the measurement of events in each samples.

Our first observation was the low stability of this pollen in water, PBS (pH 7.4) or Sørensen's 1X (pH 7.4), where the exine is detached quickly from majority of allergen epitopes. When we treated *C. arizonica* pollen with PBS buffer, we observed two regions that showed different recognition profiles by rabbit polyclonal antibody Cup a1 (Fig. 1). R1 region showed the highest allergenic load level while R2 region displayed the lowest allergenic load level. When the sample remains in PBS or water for a longer period, the R2 region without allergenic load level increases while the homogeneity of the region decreased which explains the influence of physical factors, such as high humidity or rain, on the stability of cypress pollen.

In this study, we show that in addition to the population of full *C. arizonica* pollen, a population of smaller

Table 1
Data from Bland-Altman Study

Parameter	Coefficient	Std. error	<i>t</i> -value	<i>P</i>	95% CI
Intercept	96.3551	23.3740	4.1223	0.0003	48.3956–144.3145
Slope	−0.1220	0.1020	−1.1955	0.2423	−0.3313–0.08738

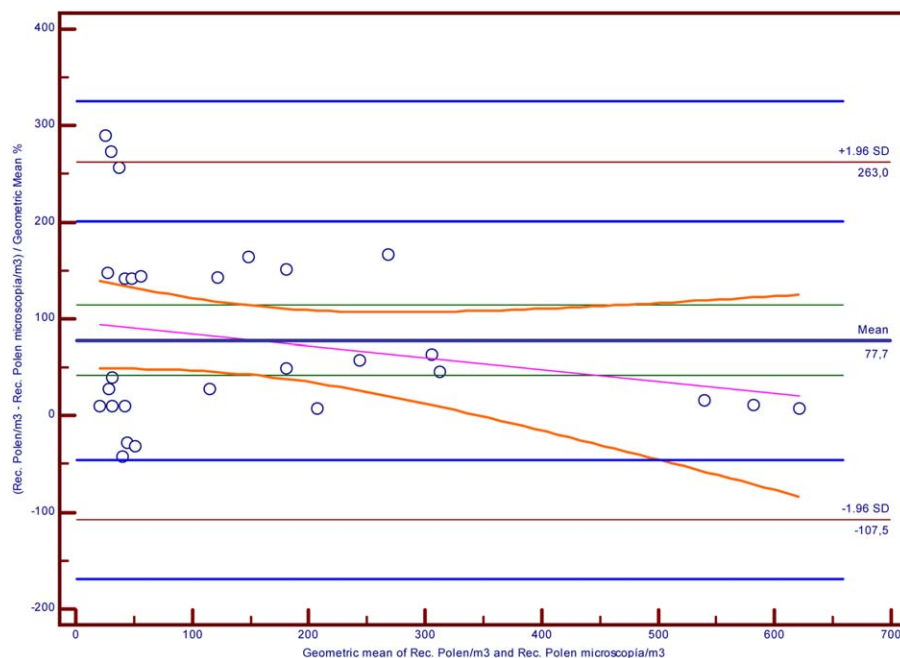


FIG. 5. Bland-Altman analysis. 95% CI was considered. [Color figure can be viewed in the online issue which is available at wileyonlinelibrary.com]

incomplete *C. arizonica* particles exists. These particles are not defined by optical microscopy but they are recognized by rabbit polyclonal antibody Cup a1 by means of a flow cytometry technique (37% of total allergenic load). Therefore, these particles can be responsible for symptoms in cypress-allergic patients. In agreement with our findings, the presence of sub-particles in the atmosphere and its association with an increase in asthma cases has been shown by other authors (14). Ubish Body particles have not been demonstrated in the *Cupressaceae* family but they have been shown to be present in *Peaceae*, *Betulaceae*, *chenopodiaceae*, *Fagaceae*, *Polygonaceae* and *Urticaceae*, but not in *Asteraceae* or *Olaceae* families. These particles have a size of around 2.5 μm , *C. arizonica* pollen is on average 10 μm in size as shown using flow cytometry.

When we studied both populations using optical and fluorescence microscopy, we observed that the R2 population without allergenic load was constituted by hydrated pollen fragments that were detached from the exine as demonstrated by confocal microscopy.

The pollen schedule obtained through optical microscopy shows a high correlation with pollen count through flow cytometry. However, the latter technique gives relatively less data in the pollen count (full pollen). This difference does not seem of great clinical significance, but this data justifies that flow cytometry can be used as a technique to count pollen. The performance of pollen counts by means of flow cytometry has the advantage that it can be performed by any technical personnel familiar with flow cytometry, while optical microscopy needs specific training to identify the pollen struc-

ture. An additional advantage is speed as one or several types of pollens can be analysed in less than 1 h.

The allergenic load found in cypress pollen shows a higher correlation with pollen count performed by flow cytometry. As an example, we can see a higher allergenic load after rainy days, although we cannot see a significant increase in pollen count identifiable by optical microscopy. A clinical trial will be necessary to determine the threshold allergenic load for the cypress-allergic patient to show allergic symptoms. Also, a trial with relevant types of pollen will be necessary.

In our study analyzing allergenic load, from June to July 2012, we can observe an increase in cypress allergenic load (not shown), indicating that particles which are recognized by polyclonal antibody Cup a1 exist. Interestingly, this feature is associated with lower allergic symptoms in cypress-allergic patients. Arilla et al. observed that polyclonal antibody Cup a1, used in this study recognized juniperus showing cross reactivity for both types of pollen (15). Thus, as a consequence of our study, we suggest considering juniper as a pollen with allergologic interest in our area, despite no pollen count by Optical Microscopy. This would justify an observational annual study of the allergenic load of main allergy-relevant species even in seasons outside their pollination period. Some pollens of interest in penology in our geographic area are: Olive, Parietarie, Gramineae and Cypress.

Good results obtained using Bland and Altman statistic allowed us to use this method to measure allergenic load in the air.

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AUTHOR CONTRIBUTIONS

F. Moreno and F. J. García-Cózar designed and conducted experiments, analyzed and interpreted results, and wrote the manuscript; M. Espinazo supervised the whole project, designed and conducted experiments, analyzed and interpreted results, and wrote the manuscript; A. Letrán wrote the manuscript; A. Cuvillo and P. Lobaton revised it critically.

All authors critically revised the manuscript and approved the final version. The opinions, results, and conclusions reported in this article are those of the authors. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

We have requested the protection of Utility Model, No. 201200760 of the experimental design from the Spanish Ministry of Industry, Energy and Tourism.

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