

ALLERGY TO POLLEN OF SIX COMMON GRASSES IN THE HIGHLANDS OF MADAGASCAR

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ABSTRACT

Aim: Few studies have investigated allergy to tropical grass pollen in the highlands of Madagascar where savanna and grassland cover extensive areas. The aim of this work was to study the prevalence of allergy to common grass pollen from these areas. **Method:** We studied the prevalence of allergy to IgE-binding proteins from six common highland grass pollens and cross-reactivity between the pollen allergens, using the skin-prick test (SPT) and isoelectric focusing (IEF) immunoblot. Sixty-five grass-pollen-allergic patients from the Antananarivo region were selected on the basis of their seasonal atopic symptoms. **Results:** When comparing SPT and immunoblot results, it was clear that, with the exception of one species, *Cynodon dactylon*, cross-reactivity of patient serum IgEs with pollen proteins was more visible in SPT responses than in immunoblots. Pollen allergens from either tropical or temperate grass, like *Dactylis glomerata*, inhibited the IgE reactivity of sera from *Rhynchelytrum repens* pollen-sensitive patients. **Conclusion:** This is the first report of cross-reactivity between these six pollen allergen sources. Among them, *R. repens* pollen was the dominant source of allergens. The allergens of the tropical grass pollens studied have a high impact on Madagascan grass-pollen-allergic patients and could also have a real impact on patients from other regions worldwide where some of these grasses are present, mainly as invasive weeds.

INTRODUCTION

Madagascar is the largest island in the South-West Indian Ocean. It is situated around 400 km from the East Africa coast at latitude of 12-25° south. The highlands are in the central part of the island and have a subtropical oceanic climate known as 'Cwb' in the Köppen climate classification, with mean temperature higher than 15°C for the coldest month of the year¹⁻² and large variations in monthly rainfall during the year (Fig. 1). This type of climate also exists in parts of East, South and South-Eastern Africa, in some mountainous areas across southern Europe, North, Central and South America and across South-East Asia.³

Populated by humans from different parts of the world during the last three millennia, many regions of the island still have very high biodiversity with, for instance, from north to south, evergreen forests in the eastern part of the

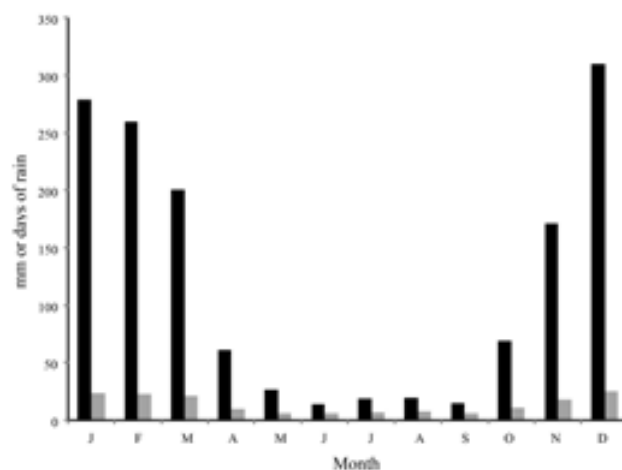


Fig. 1. Rainfall in the Antananarivo region's subtropical highlands: millimeters (mm) of rain per month (black) and number of rainy days per month (grey).

highlands.^{4,5} The western part of these mountain regions is, in contrast, covered by savanna, either natural or resulting mainly from agricultural activity.⁶⁻¹² Large areas of these territories, and more specifically those around the capital (Antananarivo; latitude 18°53' south, longitude 47°31' east; 425 km²; ±10% of the total island population) and around cities like Antsirabe (Vakinankaratra region) and Tsiroanomandidy (Bongolava region), are used for rice production and peri-urban agriculture. Vast areas on the plateau have vegetation that can almost completely be characterised as open grass cover with many eroded or impoverished soil pastures. Bosser¹¹ listed more than 450 grass species in Madagascar, including ubiquitous and ruderal species – mostly wind-pollinated – which are very dominant even close to the towns. These grasses shape the current landscape of these regions. The six grasses chosen for the present study are among the commonest currently growing in the highlands. There are two main periods of pollen emission in the highlands, from November to May (warm and rainy season) with a predominance of pollen concentrations from herbaceous taxa, and from June to October (cool and dry season) with a predominance of atmospheric grains from woody species.¹³ In the first period, grass pollen is by far the most abundant (84-88%). On an annual basis, grass pollen represents 26-27% of the total airborne pollen in Antananarivo. The main peak is in March (data from 2004 to 2006).^{14,15}

In densely inhabited regions of the central part of Madagascar, the strong presence of several species of grass is of importance in terms of allergy risk. The effect of grass pollen on allergic patients is well documented throughout the world.^{16,17} Although some progress has been made in understanding grass-pollen allergy in Central and South Africa,¹⁸⁻²⁶ and in Madagascar itself,^{13-15,27} many aspects of this disease are less well understood in these Southern Hemisphere regions.

The aim of this work was to study the prevalence of allergy to common grass pollen from the highlands of Madagascar, initially assuming possible cross-reactivity: (i) between the pollen allergens of tropical grass species, and (ii) between these tropical grass pollen allergens and pollen allergens from temperate grasses like *Dactylis glomerata*. We used both clinical and immunobiological approaches to study the incidence of the pollen of 6 selected grass species.

MATERIAL AND METHODS

PATIENT RECRUITMENT AND PATIENT SERA

In collaboration with the lung and allergy department of the Institut d'Hygiène Social and the Infirmerie de la

Gendarmerie du Toby Ratsimandrava (Antananarivo), 65 pollen-allergic patients were selected on the basis of their seasonal atopic symptoms. All patients – 28 males and 37 females – participated voluntarily in the study. They were given a standardised questionnaire by physicians with extensive experience in allergic diseases. Patients' main symptoms were rhinitis, conjunctivitis and/or asthma during the flowering season.

Blood samples were obtained from all Malagasy individuals. Two other sera, noted A and B, from Caucasian/European patients born in the Strasbourg area (France) were used.²⁸ They had never been to Madagascar. They were sensitive to grass and birch pollen and their sera were used in an inhibition experiment. Serum samples were stored at 4°C in sodium azide (0.02%) until use. The difference in serum volume obtained from patients had a direct impact on the immunoblot experiments because in some cases all six selected grass pollens could be tested, whereas in others this was not possible.

GRASS POLLEN MATERIAL

Six grass species were harvested in savanna or grassland vegetation from the highlands of Madagascar: *Imperata cylindrica*, *Rhynchelytrum repens*, *Panicum maximum*, *Pennisetum polystachion* (subfamily Panicoideae), *Cynodon dactylon* (subfamily Chloridoideae) and *Aristida rufescens* (subfamily Arundinoideae).^{9,29}

The pollen was collected directly from panicles throughout the year according to the different flowerings: the warm and rainy season for *I. cylindrica* (December), *C. dactylon* (January), *R. repens* and *P. maximum* (March-April), and *P. polystachion* (April-May), and the cool and dry season for *A. rufescens* (August-September)^{7,8,30} (Fig. 1). *D. glomerata* (Pooideae subfamily) pollen was from Allergon AB (Ängelholm, Sweden).

POLLEN EXTRACTS FOR SKIN-PRICK TESTS

Pollen extracts were prepared³¹ with 100 mg of each grass pollen in 1 ml of 0.9% NaCl, 0.3% NaH(CO₃)₂. The mixture was homogenised, stirred gently for 1 hour at ambient temperature and then centrifuged for 10 minutes at 9 000 g and 4°C. The supernatant was again centrifuged and filtered through a sterile 0.22 µm filter. The final product was diluted with aqueous glycerol (50%) and phenol (4.5%). Skin-prick tests (SPTs) were performed using these pollen extracts and tested on 60 patients for *R. repens*, *P. maximum* and *P. polystachion*, 45 for *A. rufescens*, 41 for *I. cylindrica* and 25 for *C. dactylon* (see Table I below). Because the number of patients was not the same, the results were

recalibrated by extrapolation from the total number of patients studied (65). For sera A and B, the pollen extracts used for SPTs were provided by Stallergènes Laboratories (Antony, France).²⁸

Positive SPT responses were defined by a wheal of at least 75% of the mean diameter of that induced by a histamine control. One negative control test with saline solution and one positive control test with histamine (1 mg/ml) were also included in each set of SPTs.

was detected by chemoluminescence according to the manufacturer's instructions (Amersham ECL western blot detection kit, GE Healthcare, Uppsala, Sweden). IEF has the advantage over other electrophoretic separation techniques in providing very accurate separations of proteins in their native conformation, allowing better characterisation of IgE reactivity.³²

Table I. Skin-prick test results for different aqueous grass-pollen extracts

SPT	Rhy r	Pan m	Pen p	Imp c	Cyn d	Ari r
positive	33	32	27	19	12	24
negative	27	28	33	22	13	21
not done	5	5	5	24	40	20
tested	60	60	60	41	25	45

Rhy r – *Rhynchelytrum repens*; Pan m – *Panicum maximum*; Pen p – *Pennisetum polystachion*; Imp c – *Imperata cylindrica*; Cyn d – *Cynodon dactylon*; Ari r – *Aristida rufescens*

POLLEN EXTRACTS FOR ISOELECTRIC FOCUSING AND IMMUNODETECTION

In order to obtain water-soluble extracts, for each taxon 30 mg of fresh pollen was incubated for 1 hour at 22°C in 150 µl sterile water and then centrifuged at 4°C for 15 minutes at 10 000 g. The supernatants were kept at -20°C. These extracts were used to perform isoelectric focusing (IEF).

IEF SEPARATION AND IMMUNODETECTION

IEF was performed in a 0.8% agarose gel (Isogel, FMC Rockland, Maine, USA)³² containing 12% (w/v) sorbitol and 2% (v/v) of a mixture of carrier ampholytes (Servalyt pH 3-10, Serva, Heidelberg, Germany). After IEF, one part of the gel was stained with Coomassie blue. The other part of the gel was blotted on a nitrocellulose (NC) sheet (Optitran BA-S 83, Schleicher & Schuell, Dassel, Germany).³² The NC was then dried and cut into strips (3 mm wide).

Each strip was blocked with Tris buffer saline pH 7.4 (TBS) in 137 mmol.l⁻¹ NaCl containing 5% (w/v) defatted milk and 0.2% (v/v) Tween 20 (TBS-M-Tw). All the strips were incubated overnight at 22°C with individual sera diluted 1:10. After washing (5 minutes) in TBS-M-Tw, the strips were incubated (2 hours at 20°C) with peroxidase (PO)-conjugated goat anti-human IgE (Sigma-Aldrich, Saint Louis, USA) diluted 1:1 000 in TBS-M-Tw. After 3 washes with TBS-Tw and 3 washes with TBS, IgE binding

The immunoblots were performed with 64 patient sera for *P. maximum*, 63 for *A. rufescens*, 58 for *P. polystachion*, 51 for *R. repens*, 39 for *C. dactylon* and 30 for *I. cylindrica*.

IgE INHIBITION EXPERIMENT

The NC sheet activated by cyanogen bromide³³ (NCA) was saturated (1 hour) either with *D. glomerata* or with *R. repens* pollen extracts at 20 µg protein per strip, in order to use it as an immunoabsorbent. It was dried (10 minutes at 30°C) and saturated in TBS-M-Tw (20 minutes). Then the NCA sheet was cut into 3x10 mm strips that were incubated (18 hours) with either sera from patients sensitive to *R. repens* pollen allergens or sera A and B from European patients at a 1:10 dilution or TBS-M-Tw solution as control. The set of sera and the control solutions were then collected and incubated with NC strips obtained after IEF separation of *R. repens* pollen extract, under the same conditions as described above for immunodetection.

RESULTS

EVALUATION OF GRASS POLLEN SENSITIVITY BY SPT

Patient sensitivity and potential patient cosensitivity for several taxa are depicted in Tables I and II, respectively. The SPT results indicate that more than 50% of the 65 patients in the study showed sensitivity to *R. repens* and *P. maximum* pollen extracts. In addition, 40-50% of the patients were sensitive to 1 of the 4 other taxa. Clearly the allergic patients studied seem to react more commonly to

Table II. Positive skin-prick test results for different aqueous grass-pollen extracts: evaluation of cosensitivity

Species	Rhy r	Pan m	Pen p	Imp c	Cyn d	Ari r
Rhy r	36 (55%)	23	23	12	7	17
Pan m		35 (53%)	20	15	10	16
Pen p			29 (45%)	13	7	13
Imp c				30 (46%)	11	14
Cyn d					31 (48%)	7
Ari r						35 (53%)

Rhy r – *Rhynchelytrum repens*; Pan m – *Panicum maximum*; Pen p – *Pennisetum polystachion*;

Imp c – *Imperata cylindrica*; Cyn d – *Cynodon dactylon*; Ari r – *Aristida rufescens*

Results for the different taxa were not obtained in the same number of patients, but extrapolated from the total patients studied (65)

Table III. Number of patient sera showing IgE positivity on IEF immunoblots according to different aqueous grass pollen extracts: evaluation of cosensitivity

Species	Rhy r	Pan m	Pen p	Imp c	Cyn d	Ari r
Rhy r	34 (53%)	11	7	13	18	15
Pan m		13 (20%)	5	9	5	7
Pen p			11 (17%)	2	3	6
Imp c				17 (27%)	8	6
Cyn d					27 (41%)	12
Ari r						26 (40%)

Rhy r – *Rhynchelytrum repens*; Pan m – *Panicum maximum*; Pen p – *Pennisetum polystachion*;

Imp c – *Imperata cylindrica*; Cyn d – *Cynodon dactylon*; Ari r – *Aristida rufescens*

Results for the different taxa were not obtained in the same number of patients, but extrapolated from the total number of patients studied (65).

R. repens and *P. maximum* pollen on SPT, and there is cosensitivity with the other grasses. The highest cosensitivity was detected for *R. repens*, *P. maximum* and *P. polystachion*; cosensitivity for the other 3 taxa was lower. When comparing the total number of SPTs, the fact that the number of tested patients is not the same for each pollen extract has, of course, to be taken into consideration (see 'Material and methods').

IMMUNOBLOT ANALYSIS

As was the case with the SPT analysis, the total number of sera tested was not the same for each taxon and the results were extrapolated from the total number of patients tested (Table III). The highest number tested (64) was for the *P. maximum* pollen extract and the lowest (30) for *I. cylindrica*. The highest percentages for IgE positivity were for *R. repens*, *C. dactylon* and *A. rufescens*.

When zoning the immunoblots from the most acidic parts to the basic ones, four main pI groups can be delimited – A, B, C, D in Figure 2 – after excluding the pI <4.5, for which no IgE signal was detectable in most of the extracts. For the six studied extracts, the main IgE signal was visible in the A, B and C zones. From the description and analysis of the four zones, it was clear that, for the *R. repens* and *P. maximum* pollen extracts, the pI range of the IgE signal was wider, from 4.5 to 9, than for *I. cylindrica* and *C. dactylon* (pI 5-8.5). In the *R. repens* and *P. maximum* pollen extracts, the basic (pI 8-9) proteins (D zone) were strongly recognised by IgE from patient sera. For instance, some IgE from sera 25 and 65 recognised several basic proteins in *R. repens*, *P. maximum* and *I. cylindrica* extracts, but not in *C. dactylon* extract. For *P. polystachion* pollen extract, only 3 sera showed heterogeneous IgE responses, mainly in zone A and also – for just one serum (No. 65) – in zones A, C and D (data not shown). For *A. rufescens* pollen extract, most sera showed

an IgE response against pollen proteins from pI 4.0 to 9.0 (Fig. 3). Nine of the 30 sera from this figure showed a very strong IgE response and 3 of them (46, 54 and 55) IgE reactivity in a very acidic zone (pI <3.5). Some patient sera showed a very restricted specific IgE response in almost a single pI band: 6.4 for the serum 2 on *C. dactylon* extract, 7.3 for serum B and 7.4 for sera 4, 11, 44 and 48 on *R. repens* extract and 8.7 for serum 36 and serum B for *P. maximum* extract. Nevertheless, most of the patient sera gave very heterogeneous responses from acidic to basic pI zones.

The IgE of the 2 European patient sera were strongly sensitive to *D. glomerata* pollen extract and recognised proteins present in the *R. repens* and *P. maximum* pollen extracts, albeit with different profiles than in the Malagasy patients (Fig. 2), but not in the two other tested pollen extracts: *A. rufescens* and *P. polystachion* (data not shown).

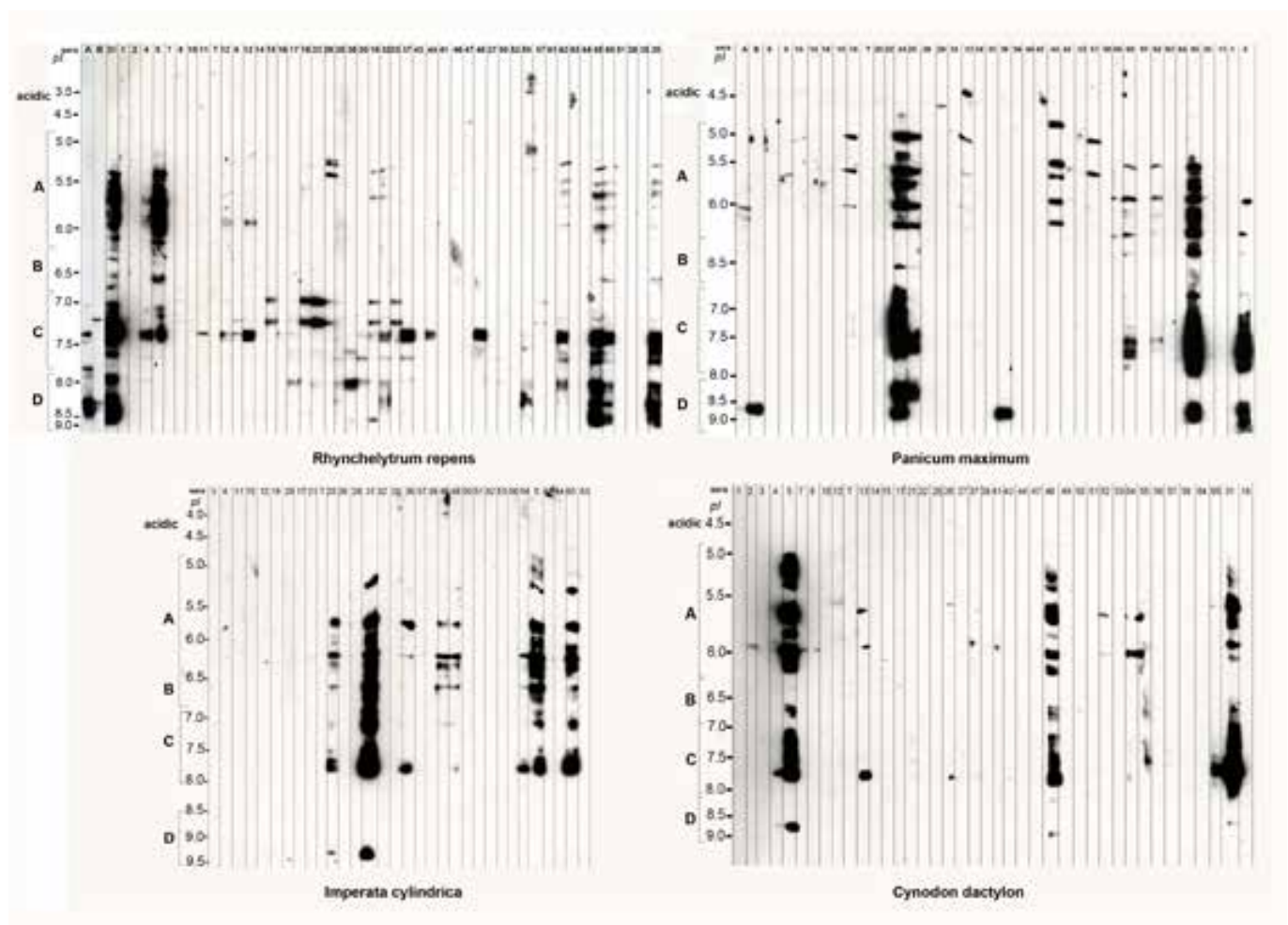


Fig. 2. Most representative patterns from 1D IEF immunoblots for 4 of the 6 studied grass pollens. Each lane corresponds to IgE reactivity from individual allergic patient sera (47 sera shown for *R. repens*, 39 for *P. maximum*, 31 for *I. cylindrica*, 39 for *C. dactylon*). At the top: serum number; on the left: pI values and pI zones (A, B, C, D).

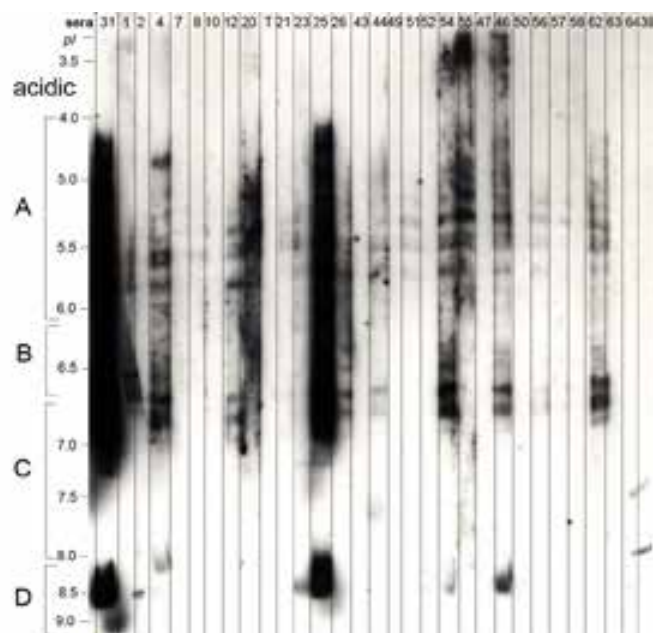


Fig. 3. Most representative patterns from 1D IEF immunoblots for *A. rufescens* grass pollen. Each lane corresponds to IgE reactivity from individual allergic patient sera (30 sera shown). At the top: serum number; on the left: pI values and pI zones (A, B, C, D).

As a complement to this last result showing cross-reactivity between *R. repens*, *P. maximum* and *D. glomerata* pollen extracts, the inhibition experiment (Fig. 4), based on *R. repens* extract IEF immunoblots incubated with 3 patients' sera, tested positive for *R. repens* extract (lane a), clearly showing that the IgE signal was non-existent or very weak (lanes b and c) after immunoadsorption. Similar results were obtained using the sera from patients A and B allergic to European *D. glomerata* pollen (data not shown). Such results suggest that allergens from *R. repens* or *D. glomerata* pollen inhibit the IgE reactivity of sera from patients sensitised to *R. repens* pollen.

DISCUSSION

The different patient sera showed a great heterogeneity in their IgE responses, particularly against proteins from *R. repens* and *P. maximum* pollen extracts. Such IgE response heterogeneity has been described in other studies, for instance, for 15 different grass-pollen extracts, including pollen from *D. glomerata*, *Phleum pratense* and *Pennisetum typhoides*.³⁴ In the present study, IgE responses were less heterogeneous for *I. cylindrica* and *C. dactylon* protein extracts.

When SPT and immunoblot results were compared, with the exception of *C. dactylon* (7 v. 18 patients), cosensitivity evaluated by SPT was clearer, for at least two taxa,

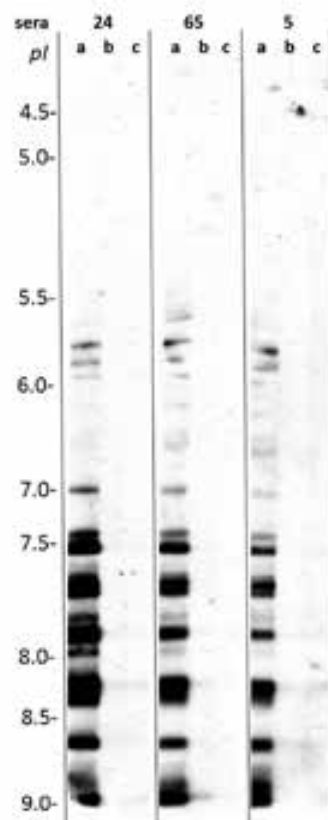


Fig. 4. Inhibition experiment for three patients' sera (5, 24 and 65). Lane a corresponds to an IEF blot of the *R. repens* pollen extract incubated with each of the three patients' sera that tested strongly positive with *R. repens* pollen extract. Lane b corresponds to an IEF blot of the *R. repens* pollen extract incubated with each serum on saturated NCa strips containing adsorbed *R. repens* pollen extract. Lane c corresponds to an IEF blot of the *R. repens* pollen extract preincubated with each serum on saturated NCa strips containing adsorbed *D. glomerata* pollen extract. No protein band was visible on TBS-M-Tw control strips (data not shown).

than cross-reactivity measured by immunoblots with the same pollen extracts. But the distribution is more or less similar between these two methods, except for *P. maximum* which shows weak IgE reactivity with the immunoblot method. In terms of cross-reactivity, *R. repens* was the dominant pollen of the 6 tested, and also, more specifically, among the 4 pollens from the subfamily Panicoideae. The immunoblot results gave a first idea of the allergenic proteins recognised by the IgE for both sensitisation and cosensitisation. Qualitative observation of different immunoblots using a set of sera is very informative when no IgE tests for pollen taxa are commercially available, as is the case for 5 of the 6 grasses of our study, the exception being *C. dactylon* (g2 in ImmunoCap, Phadia). It is possible

that the differential genetic evolution of Malagasy patients of Austronesian genetic heritage³⁵ and European patients²⁸ plays a role in the IgE response visualised on immunoblots. Normally, it would have been unlikely that the Caucasian/European patients had been in contact with the pollen from the 6 tropical grasses studied here. Our inhibition experiment results, which provide information on the existence of a potential cross-reactivity between tropical grass (*R. repens*) and temperate grass (*D. glomerata*) pollen, suggest that allergic patients, whether from Madagascar or Europe, react to each of the grass pollens studied here. As already asserted by Suphioglu,¹⁷ 'despite the difference in geographical dominance of certain grasses, there are extensive studies highlighting a high degree of allergenic similarity or cross-reactivity between the different grasses'.

From the pollen material from the four subfamilies Chloridoideae, Arundinoideae, Panicoideae and Pooideae that we studied, no very clear specific IEF pattern can be distinguished in terms of IgE response from one extract to another, even if allergenic differences have already been demonstrated between Chloridoideae and Pooideae subfamilies.³⁶ It is known that some pollen allergens of *C. dactylon*, one of the most important allergen sources in tropical and subtropical climates,³⁷ lacks major allergen groups (e.g. groups 2 and 5) which are abundant in the Pooideae like *D. glomerata*.³⁸ Westritschnig *et al.*²³ have shown, in a very large cohort of allergic individuals from Zimbabwe, that *C. dactylon* pollen allergens can play a larger role than, for instance, *P. pratense* in allergy in Central Africa.

Because at least 3 of the 6 studied tropical grasses, *R. repens*, *P. polystachion* and *I. cylindrica*, have the status of invasive weeds (<http://plants.usda.gov>), their allergenic potential is in no way restricted to the highlands of Madagascar. Indeed, *R. repens* is now naturalised throughout the tropics from Hawaii and other tropical Pacific regions. It is also present in several southern states of the USA.³⁹ *I. cylindrica* is considered as one of the 10 worst weeds in the world and its spread is of concern to ecologists because it competes with and often displaces native plants.⁴⁰ Even *A. rufescens*, listed by the web site <http://www.pnae.mg> (dedicated to biodiversity in Madagascar) among the endemic Poaceae growing mainly in the west ecofloristic zone at low altitude (0-800 m), has now to be considered as a grass with a large distribution from Africa to the Western Indian Ocean (Online World Grass Flora: <http://www.kew.org>).

In conclusion, from the results of this study in Madagascar, it can be stated that the pollen of the six tropical grasses *I.*

cylindrica, *R. repens*, *P. maximum*, *P. polystachion*, *C. dactylon* and *A. rufescens* has a high impact on Malagasy grass-pollen-allergic patients and can also have an impact on allergic patients moving to Madagascar,⁴¹ for instance, when grass pollen dispersion is at its peak (around March-April). These pollens can also be of importance in other areas worldwide where these grasses are abundant.

Cross-reactions have been highlighted for each of the grass species showing, at the same time, that tropical and temperate grass pollen can be of importance for very different potential allergic patients throughout the world. In immunochemical terms, two-dimensional electrophoretic separations could improve characterisation of the allergen repertoire of the six different grass pollens. Some other as yet untested grass pollens found locally should be compared with pollen from southern African grasses. Prescott and Potter, for instance, have noted the importance of *Pennisetum clandestinum*, *Stenotaphrum secundatum* and *Eragrostis curvula* pollen in different parts of South Africa.⁴² In clinical terms, we followed what Van Ree and Yazdanbakhsh¹⁸ proposed: 'it is essential to perform studies from a "non-Eurocentric" angle (e.g. local allergens, locally validated questionnaires and diagnostic procedures)'. In terms of diagnostic and immunotherapy tools, tests and vaccines using *R. repens* pollen extracts should be envisaged, because they will be better adapted at the regional scale than, for instance, the European SPT, IgE tests or current immunotherapy products. Such studies combining complementary clinical and allergomic approaches to grass-pollen allergy could help prevent, and treat more effectively, allergic diseases in developing countries, like those of the western Indian Ocean region, but also in other regions worldwide.

Declaration of conflict of interest

The authors declare no conflict of interest.

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