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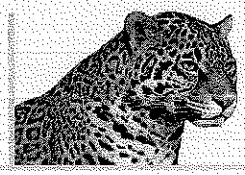
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Preclinical efficacy against toxic activities of medically relevant *Bothrops* sp. (Serpentes: Viperidae) snake venoms by a polyspecific antivenom produced in Mexico

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Abstract: The assessment of the preclinical neutralizing ability of antivenoms in Latin America is necessary to determine their scope of efficacy. This study was aimed at analyzing the neutralizing efficacy of a polyspecific bothropic-crotalic antivenom manufactured by BIRMEX in Mexico against lethal, hemorrhagic, defibrinogenating and *in vitro* coagulant activities of the venoms of *Bothrops jararaca* (Brazil), *B. atrox* (Perú and Colombia), *B. diporus* (Argentina), *B. mattogrossensis* (Bolivia), and *B. asper* (Costa Rica). Standard laboratory tests to determine these activities were used. In agreement with previous studies with bothropic antivenoms in Latin America, a pattern of cross-neutralization of heterologous venoms was observed. However, the antivenom had low neutralizing potency against defibrinogenating effect of the venoms of *B. atrox* (Colombia) and *B. asper* (Costa Rica), and failed to neutralize the *in vitro* coagulant activity of the venom of *B. asper* (Costa Rica) at the highest antivenom/venom ratio tested. It is concluded that, with the exception of coagulant and defibrinogenating activities of *B. asper* (Costa Rica) venom, this antivenom neutralizes toxic effects of various *Bothrops* sp venoms. Future studies are necessary to assess the efficacy of this antivenom against other viperid venoms.

Key words: snake venom, antivenom, *Bothrops*, *Crotalus*, neutralization.

The vast majority of snakebite envenomings occurring in Latin America are inflicted by species of the genus *Bothrops* (Fan & Cardoso, 1995; Warrell, 2004; Gutiérrez, 2010), which are distributed from Southern Mexico to Argentina (Campbell & Lamar, 2004). Depending on their severity, these envenomings are characterized by local tissue damage, i.e. edema, myonecrosis, hemorrhage, blistering, and by systemic alterations, i.e. hemorrhage, coagulopathies, acute kidney injury and cardiovascular shock (Warrell, 2004; Otero-Patiño, 2009; França & Málaque, 2009). Timely parenteral administration of antivenom is the only validated treatment for these envenomings. Diverse manufacturing laboratories in the region produce either monospecific or polyspecific bothropic antivenoms (Gutiérrez, Higashi, Wen, & Burnouf, 2007). The immunization

mixtures used to generate these antivenoms greatly vary between laboratories. For instance, a polyspecific bothropic antivenom is manufactured by several laboratories in Brazil, using a mixture of the venoms of *Bothrops jararaca*, *B. jararacussu*, *B. moojeni*, *B. neuwiedii*, and *B. alternatus* as antigen (Cardoso, Yamaguchi, & Moura da Silva, 2009). On the other hand, a polyspecific antivenom produced in Costa Rica is generated by immunizing horses with a mixture of venoms of *Bothrops asper*, *Crotalus simus* and *Lachesis stenophrys* (Segura et al., 2010).

There are situations in which antivenoms have to be imported from other countries in Latin America, either because there is no local production or because the national stocks of these immunobiologicals are insufficient. In these circumstances, it is highly relevant to ensure that antivenoms being imported are indeed effective in the neutralization of venoms of the most important snakes of the country. Hence, the preclinical assessment of the ability of antivenoms to neutralize venoms from species distributed in other countries in the region is important in order to have a knowledge-based platform for the distribution of antivenoms between countries in Latin America, often under the coordination of the Pan American Health Organization (PAHO) (Gutiérrez, 2014). Several studies have been performed in the past for assessing the preclinical efficacy of antivenoms in Latin America (see for example Otero et al., 1995; Gutiérrez, Rojas, Bogarín, & Lomonte, 1996; de Roodt, Dolab, Fernández, Segre, & Hajos, 1998; Bogarín et al., 2000). One of the most comprehensive analysis of the preclinical efficacy of antivenoms against venoms of various species of *Bothrops* sp. evaluated seven polyspecific antivenoms produced in Argentina, Brazil, Peru, Bolivia, Colombia and Costa Rica against the venoms of five species of *Bothrops* from different countries (Segura et al., 2010). This and other studies have underscored a widespread pattern of cross-neutralization of antivenoms against heterologous *Bothrops* sp. venoms. The present report extends these observations by investigating the preclinical efficacy of an antivenom manufactured in Mexico when confronted with the venoms of species of *Bothrops* from Brazil, Peru, Colombia, Argentina, Bolivia and Costa Rica.

MATERIALS AND METHODS

Venoms: The venoms of the following species were utilized: (a) *Bothrops diporus* (previously classified as *B. neuwiedi*) (Argentina), provided by Centro Nacional de Control de Calidad de Biológicos (CNCCB)-ANLIS "Dr Carlos G. Malbrán", Buenos Aires; (b) *B. mattogrossensis*) (previously classified as *B. neuwiedi*) (Bolivia), provided by the Instituto Nacional de Laboratorios de Salud (INLASA), La Paz; (c) *B. jararaca* (Brazil), provided by Instituto Butantan, Sao Paulo; (d) *B. atrox* (Peru), provided by Instituto Nacional de Salud (INS), Lima; (e) *B. atrox* (Colombia), provided by Instituto Nacional de Salud (INS), Bogotá; and (f) *B. asper* (Costa Rica), provided by Instituto Clodomiro Picado, Universidad de Costa Rica, San José. These venoms corresponded to the same pooled samples utilized in the study of Segura et al. (2010).

Antivenom: The antivenom tested is manufactured in Mexico by Laboratorios de Biológicos y Reactivos de México S.A. (BIRMEX; batch sv-162; in some experiments two additional batches were used: SV-165 and SV-187). This antivenom is produced from the plasma of horses immunized with a mixture of the venoms of *B. asper* and *Crotalus basiliscus* obtained from specimens collected in Mexico and kept at the serpentarium of BIRMEX. Fractionation of hyperimmune plasma is achieved by digestion of plasma proteins with pepsin at acid pH (Pope, 1939) and ammonium sulfate precipitation. It is a freeze-dried preparation composed of $F(ab')_2$ antibody fragments.

Neutralization of toxic activities: The following toxic activities of venoms were investigated: lethal (by the intraperitoneal route), hemorrhagic, defibrinogenating, and *in vitro* coagulant activities. The methods used for the characterization of the toxic activities of these venoms were described in a previous publication (Segura et al., 2010), and the same procedures were followed in the present work. For the analysis of the neutralization of these effects, a fixed amount of venom, which varies according to the effect to be studied, were incubated with various dilutions of the antivenom. Incubations were performed for 30 min at 37 °C. Venoms were dissolved in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS). Controls included venom incubated with PBS without antivenom. After incubation, aliquots of the mixtures, containing a 'challenge dose' of venom, were tested in the corresponding assay systems described by Segura et al. (2010). The 'challenge doses' used were: For lethality, four Median Lethal Doses (LD_{50}s); for hemorrhage, five Minimum Hemorrhagic Doses (MHDs); for defibrinogenation, two Minimum Defibrinogenating Doses (MDDs); and for *in vitro* coagulation, two Minimum Coagulant Doses (MCDs). For lethal and hemorrhagic effects, the neutralizing efficacy was expressed as Median Effective Dose (ED_{50}), i.e. the ratio μL antivenom/mg venom in which the effect of the venom alone was reduced by 50 % (Gutiérrez et al., 1990). In the case of neutralization of lethality, results were also expressed as the ratio mg venom/mL antivenom. ED_{50} for lethality was estimated by probits (Finney, 1971). For defibrinogenating and *in vitro* coagulant activities, neutralization was expressed as Effective Dose (ED), as defined by Gené, Roy, Rojas, Gutiérrez and Cerdas (1989) and Segura et al. (2010).

RESULTS

The six venoms studied induced lethal, hemorrhagic, defibrinogenating and *in vitro* coagulant activities, as previously described (Segura et al., 2010). Since the venom pools used were the same as those utilized by Segura et al. (2010), the results of these toxicity tests are not reported here, and the reader is referred to this previous study. Regarding neutralization, the polyspecific antivenom of BIRMEX neutralized the lethal activity of all venoms tested, albeit with varying ED_{50}s depending on the venom (Table 1). Highest neutralization was achieved against the venoms of *B. diporus* (Argentina) and *B. mattogrossensis* (Bolivia), both of which were previously classified as *B. neuwiedi*, whereas the lowest neutralization was against the venom of *B. atrox* from Peru (Table 1). Antivenom was also effective in the neutralization of hemorrhagic activity of all venoms tested. Highest neutralization was achieved against the venoms of *B. asper* (Costa Rica) and *B. atrox* (Colombia), whereas the lowest neutralization was against the venom of *B. atrox* (Peru) (Table 1). Antivenom neutralized defibrinogenating activity of the venoms of *B. diporus* (Argentina), *B. atrox* (Peru), *B. mattogrossensis* (Bolivia) and *B. jararaca* (Brasil); in contrast, it required a high antivenom/venom ratio (4 000 μL antivenom/mg venom) to neutralize defibrinogenating activity of the venoms of *B. atrox* (Colombia) and *B. asper* (Costa Rica) (Table 1). Regarding *in vitro* coagulant activity, antivenom was effective in neutralizing the venoms of *B. atrox* (Peru and Colombia), *B. diporus* (Argentina), *B. mattogrossensis* (Bolivia), and *B. jararaca* (Brasil), being ineffective, at the highest antivenom/venom ratio tested (4 000 μL antivenom/mg venom), to neutralize coagulant activity of the venom of *B. asper* from Costa Rica (Table 1). In order to corroborate this finding observed with the antivenom batch used (batch SV-162), two additional batches (SV-165 and SV-187) were tested for their ability to neutralize coagulant activity of Costa Rican *B. asper* venom. These two batches also failed to neutralize this effect at the highest antivenom/venom ratio tested.

DISCUSSION

Our results corroborate, for the polyspecific crotaline antivenom produced in Mexico by BIRMEX, the extensive cross-reactivity described for bothropic antivenoms in Latin America (Bogarín et al., 2000; Segura et al., 2010). In this case, when using the venoms of *B. asper* and *C. basiliscus* from Mexico in the immunizing mixture, the antivenom generated is effective for the neutralization of the four activities in the venoms of the South American species *B. jararaca*, *B. diporus*, *B. mattogrossensis* and *B. atrox* (Peru). The neutralization of lethality is the gold standard in the preclinical assessment of antivenom potency; in this regard, it is noteworthy that BIRMEX antivenom has a higher neutralizing ability of lethal effect of the venoms of *B. asper* (Mexico) (Segura et al., 2012), *B. diporus*, *B. jararaca*, *B. atrox* (Colombia), and *B. mattogrossensis* than against the venoms of *B. asper* (Costa Rica) and *B. atrox* (Peru). In contrast with other bothropic polyspecific antivenoms analyzed in previous works, BIRMEX antivenom has a relatively low neutralizing ability of defibrinogenating activity of the venoms of *B. asper* from Costa Rica and *B. atrox* from Colombia, and failed to neutralize *in vitro* coagulant activity of Costa Rican *B. asper* venom, at the highest antivenom/venom ratio used in this study (4 000 µL antivenom/mg venom).

A previous study showed that BIRMEX antivenom was highly effective in the neutralization of the venom of *B. asper* from Mexico, which is used in the immunizing mixture (Segura et al., 2012). The values of ED_{50} were 188 ± 27 µL antivenom/mg venom and 129 ± 4 µL antivenom/mg venom for lethal and hemorrhagic activities, respectively, and the values of ED were 1 135 ± 19 µL antivenom/mg venom and 2 000 µL antivenom/mg venom for *in vitro* coagulant and defibrinogenating actities, respectively (Segura et al., 2012). Moreover, in this study it was shown that BIRMEX antivenom was more effective in the neutralization of *in vitro* coagulant activity of the venom of *B. asper* from Mexico, as compared to the venom of *B. asper* from Costa Rica (Segura et al., 2012). This is likely to reveal differences in the immunological properties of procoagulant toxins in the venoms of *B. asper* from Mexico and Costa Rica, an issue that deserves further investigations. Owing to the relevance of coagulant and defibrinogenating activities in the overall pathophysiology of *Bothrops* sp. envenoming, these results suggest that BIRMEX antivenom may not be effective for the control of coagulopathies in envenomings by *B. asper* from Costa Rica, and may have a low efficacy for controlling defibrinogenation in envenomings by *B. atrox* from Colombia, probably requiring high volumes of antivenom to achieve therapeutic success. As a way to confront this problem, the venom mixtures used for horse immunization could be enriched with venoms of specimens of *B. asper* collected in countries other than Mexico.

In conclusion, the polyspecific bothropic-crotalic antivenom manufactured by BIRMEX in Mexico presents a pattern of cross-neutralization when confronted with heterologous *Bothrops* sp. venoms from various South American countries. However, this antivenom shows a low neutralizing ability against defibrinogenating and *in vitro* coagulant activities of the venom of *B. asper* from Costa Rica.

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this investigation. This study was supported by Vicerrectoría de Investigación, Universidad de Costa Rica (project 741-B2-091).

RESUMEN

Eficacia de un antiveneno poliespecífico producido en México para neutralizar, a nivel preclínico, las actividades tóxicas de venenos de *Bothrops* sp (Serpentes: Viperidae) de importancia médica. Es necesario estudiar a nivel preclínico la capacidad neutralizante de los antivenenos producidos en América Latina, para conocer su espectro de cobertura. En este estudio se analizó la eficacia preclínica de un antiveneno poliespecífico botrópico-crotálico producido por BIRMEX, en México, para neutralizar los efectos letal, hemorrágico, desfibrinogenante y coagulante *in vitro* de los venenos de *Bothrops jararaca* (Brasil), *B. atrox* (Perú y Colombia), *B. diporus* (Argentina), *B. mattogrossensis* (Bolivia) y *B. asper* (Costa Rica). Se emplearon metodologías de laboratorio estándar en los análisis. En consonancia con estudios anteriores con diversos antivenenos botrópicos en América Latina, se observó un amplio patrón de neutralización de estos venenos heterólogos en la mayoría de los efectos estudiados. Sin embargo, el antiveneno mostró una baja capacidad neutralizante contra el efecto desfibrinogenante de los venenos de *B. atrox* (Colombia) y *B. asper* (Costa Rica) y no neutralizó la actividad coagulante *in vitro* del veneno de *B. asper* (Costa Rica) a la máxima razón antiveneno/veneno empleada.

Palabras clave: veneno de serpiente, antiveneno, *Bothrops*, *Crotalus*, neutralización.

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TABLE 1

Neutralization of toxic activities of *Bothrops* sp venoms by BIRMEX polyspecific antivenom[a]

Venom	Lethality (mg V/mL AV)[b]	Lethality (µL AV/mg V)[b]	Hemorrhagic (µL AV/mg V)	Defibrinogenating (µL AV/mg V)	Coagulant (µL AV/mg V)
B. atrox (Peru)	1.70 (1.28-2.16)	588 (463-781)	825 ± 93	2 000	425 ± 18
B. atrox (Colombia)	3.12 (2.31-4.22)	321 (237-433)	125 ± 18	4 000	1 096 ± 49
B. asper (Costa Rica)	2.34 (1.77-2.29)	427 (437-565)	136 ± 6	4 000	>4 000
B. diporus (Argentina)	6.75 (5.16-8.82)	148 (113-194)	151 ± 1	1 000	385 ± 7
B. jararaca (Brazil)	3.48 (2.36-4.62)	287 (216-424)	279 ± 12	1 000	438 ± 22
B. mattogrossensis (Bolivia)	4.44 (2.56-7.70)	225 (130-391)	210 ± 7	1 000	854 ± 35

[a]Neutralization of lethal, hemorrhagic and coagulant activities is expressed as Median Effective Dose (ED_{50}), whereas neutralization of defibrinogenating activity is expressed as Effective Dose (ED) (see the text for details). Results are presented as mean ± S.D. (n = 4), except in lethality where the 95 % confidence limits are included in parentheses.

[b]Neutralization of lethality is presented in two different ways: mg venom per mL antivenom, and µL antivenom per mg venom.

REVISTA DE Biología Tropical

Relevance of the ancestry for the variability of the Drug-Metabolizing Enzymes *CYP2C9, CYP2C19* and *CYP2D6* polymorphisms in a multiethnic Costa Rican population

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Abstract: CYP2C9, CYP2C19 and CYP2D6 metabolize around 40 % of drugs and their genes vary across populations. The Costa Rican population has a trihybrid ancestry and its key geographic location turns it into a suitable scenario to evaluate interethnic differences across populations. This study aims to describe the diversity of *CYP2C9*, *CYP2C19* and *CYP2D6* polymorphisms in Costa Rican populations in the context of their ancestry. A total of 448 healthy individuals were included in the study: Bribri (n= 47), Cabécar (n= 27), Maleku (n= 16), Guaymí (n= 30), Huetar (n= 48), Chorotega (n= 41), Admixed/Mestizos from the Central Valley/Guanacaste (n= 189), and Afro-Caribbeans (n= 50) from Limón. *CYP2C9* (alleles **2, *3, *6*) and *CYP2C19* (**2, *3, *4, *5, *17*) genotypes were determined by Real-Time PCR. African, European and Native American ancestry were inferred using 87 ancestry informative markers. The frequency of the decreased activity allele *CYP2C9*2* is lower in the self-reported Amerindian groups compared to the admixed population, and the highest frequencies of *CYP2C19*2* (null activity) and the *CYP2C19*17* (increased activity) were found in the self-reported Afro-Caribbean population. Moreover, a frequency of 0.7 % *CYP2C9* gPMs in the Admixed population and a variable frequency of *CYP2C19* gUMs (0.0-32.6 %, more prevalent in Afro-Caribbeans) in Costa Rican populations, was found. Finally, the following alleles were positively correlated with genomic African ancestry and negatively correlated with genomic Native American ancestry: *CYP2D6*5* (null activity), *CYP2D6*17* (decreased activity), *CYP2D6*29* (decreased activity) and *CYP2C19*17* (increased activity). No correlation for *CYP2C9* polymorphisms and genomic ancestry was found. Further studies assessing the *CYP2C9* and *CYP2C19* sequence in these populations, preferentially by sequencing these genes, are warranted. Rev. Biol. Trop. 64 (3): 1067-1076. Epub 2016 September 01.

Key words: *CYP2C9*, *CYP2C19*, *CYP2D6*, Costa Rica, Amerindian, Afro-Caribbean, genomic ancestry.

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Cytochrome P450 enzymes (CYPs) are involved in the phase I metabolism of endobiotics and xenobiotics (i.e. drugs). Thus, the activity of these enzymes is related to the plasmatic levels of active drug in patients, as well as to their therapeutic effect. In the CYP2C subfamily of drug-metabolizing enzymes (DMEs), CYP2C9 and CYP2C19 are encoded by the *CYP2C* gene cluster in 10q24, and are polymorphic, presenting interethnic variability (CYP2C19 allele nomenclature, 2015; Sistonen et al., 2009).

CYP2C9 is involved in the metabolism of drugs such as warfarin, losartan, fluoxetine and non-steroidal anti-inflammatory drugs. Around 60 *CYP2C9* gene variants have been described (CYP2C9 allele nomenclature, 2015), which explain a considerable proportion of variability in the drug metabolism. Some *CYP2C9* alleles have been related to a null or decreased hydroxylation capacity, such as *CYP2C9*3* allele that dramatically reduces the enzyme activity (Ingelman-Sundberg, Sim, Gomez, & Rodriguez-Antona, 2007). Thus, the carriers of two *CYP2C9*3*, as well as, alleles with null capacity are predicted to be poor metabolizers (gPMs) and to suffer adverse drug reactions (ADRs) (Yang et al., 2013).

CYP2C19 is responsible for the metabolism of antidepressants and proton pump inhibitors, among other drugs. A total of 34 *CYP2C19* allelic variants that affect enzyme activity have been described, from null (i.e. *CYP2C19*2, *3, *4, *5*) to increased activity (i.e. *CYP2C19*17*) (CYP2C19 allele nomenclature, 2015). Individuals carriers of two inactive *CYP2C19* alleles are predicted to be gPMs, while carriers of **1/*17* or **17/*17* genotypes are predicted ultrarapid metabolizers (gUMs). In pharmacological treatment, ADRs for both metabolic groups have been shown. Drug Regulatory Agencies report *CYP2C19* as a pharmacogenetic biomarker for 16 drugs (Center for Drug Evaluation and Research, 2015) and that *CYP2C19* status of patients might predict clinical outcomes (Altar et al., 2015; Niu et al., 2015; Tabata et al., 2015).

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CYP2D6 metabolizes a wide range of drugs such as antidepressants, antiarrythmics, antipsychotics, and antihistamines. More than 100 allelic variants have been described for this gene, some of which have been related to null, decreased, normal and increased enzyme activity (CYP2D6 allele nomenclature, 2015). CYP2D6 gPMs and gUMs have been related to clinical outcomes in pharmacological therapy (Rolla et al., 2014; Seripa et al., 2015; Youngster et al., 2014) and Drug Regulatory Agencies report *CYP2D6* as a pharmacogenetic biomarker for forty drugs (Center for Drug Evaluation and Research, 2015).

Interethnic differences in such cytochrome P450 genetic polymorphisms are partially responsible for the variations among populations in drug disposition. The trihybrid ancestry of the Costa Rican population (Segura-Wang, Raventós, Escamilla, & Barrantes, 2010), and the key geographic location of the country makes Costa Ricans fairly representative of the human genetic diversity in Central America.

The CEIBA.FP Consortium of the Ibero-American Network of Pharmacogenetics & Pharmacogenomics (RIBEF) has carried out studies in different Latin American populations (Dorado et al., 2012a; Dorado, Gallego, Peñas-Lledó, Terán, & Llerena, 2014), contributing to increase the pharmacogenetic knowledge of these neglected populations. Nevertheless, this is the first report of the *CYP2C* subfamily in a Costa Rican population including groups from different ethnic backgrounds. The present study aims to estimate the allele frequencies of *CYP2C9, CYP2C19* and *CYP2D6* polymorphisms in Costa Rican populations with different ancestry backgrounds.

MATERIALS AND METHODS

Subjects: The study comprised 448 healthy individuals, of which 385 were previously studied for the *CYP2D6* gene (Céspedes-Garro, Jiménez-Arce, Naranjo, Barrantes, & Llerena, 2014). The following Native American Chibchan populations were analyzed: Bribri (n= 47), Cabécar (n= 27), Maleku (n= 16), Guaymí (n= 30) and Huetar (n= 48). An Oto-Manguean Mesoamerican Amerindian group: Chorotega (n= 41) was also included. Moreover, Admixed/Mestizos from the Central Valley and Guanacaste (n= 189), and Afro-Caribbeans (n= 50) from Limón were included (Céspedes-Garro et al., 2014a). The number of analyzed subjects varied according to the *CYP2C* gene and genomic ancestry analyses (Table 1, Table 2 and Table 3).

All DNA samples were obtained from a DNA biobank of the School of Biology of the University of Costa Rica. The samples were collected and stored after approval from review boards of the University of Costa Rica. Further information of collection and demographic data is available elsewhere (Azofeifa et al., 2004; Barrantes et al., 1990; Barrantes, Smouse, Neel, Mohrenweiser, & Gershowitz, 1982). The inclusion criteria of individuals in each group were previously defined (Céspedes-Garro et al., 2014a).

Methods: The strategy followed in the study has been designed by the CEIBA-MESTIFAR Project (Sosa-Macias et al., 2015).

Genomic ancestry: A total of 87 ancestry informative markers (AIMs) selected from

TABLE I
Mean of the genomic ancestry for different Costa Rican ethnic groups

Population	n	European ancestry	African ancestry	Native American ancestry
Admixed	32	0.429	0.168	0.403
Bribri	12	0.182	0.072	0.745
Chorotega	26	0.220	0.090	0.690
Guaymí	18	0.030	0.027	0.943
Afro-Caribbean	11	0.093	0.863	0.044

n: number of subjects.

TABLE 2

Frequencies (%) of *CYP2C9* alleles and phenotypes predicted from genotype in different Costa Rican ethnic groups

Self-reported Ancestry	n	*1	*2	*3	*6	gPMs
Admixed	137	88.7	7.7	3.6	0.0	0.7
Bribri	46	97.8	1.1[a]	1.1	0.0	0.0
Cabécar	27	98.1	0.0[a]	1.9	0.0	0.0
Chorotega	31	95.1	3.2	1.6	0.0	0.0
Guaymí	27	100.0	0.0[a]	0.0	0.0	0.0
Huetar	48	86.5	8.3	5.2	0.0	0.0
Maleku	15	100.0	0.0	0.0	0.0	0.0
Afro-Caribbean	45	95.6	1.1[a]	3.3	0.0	0.0

n: number of subjects; gPMs: predicted poor metabolizers from genotype.

[a]$P < 0.05$ compared to the admixed and Huetar populations.

TABLE 3

Frequencies (%) of *CYP2C19* alleles and phenotypes predicted from genotype in different Costa Rican ethnic groups

Self-reported Ancestry	n	*1	*2	*3	*4	*5	*17	gPMs	gUMs
Admixed	141	81.9	7.1	0.0	0.7	0.0	10.3	0.0	17.7
Bribri	23	91.3	4.3	0.0	0.0	0.0	4.3	0.0	8.7
Chorotega	36	84.7	12.5	0.0	0.0	0.0	2.8	0.0	5.6
Guaymí	24	98.0	0.0	0.0	0.0	0.0	2.0	0.0	4.0
Maleku	12	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Afro-Caribbean	46	58.7	19.6[a]	0.0	0.0	0.0	21.7[b]	0.0	32.6[b]

n: number of subjects; gPMs: predicted poor metabolizers from genotype; gUMs: predicted ultra-rapid metabolizers from genotype.

[a] $P < 0.05$ compared to the rest of populations with the exception of the Chorotega tribe; [b] $P < 0.05$ compared to the rest of populations.

103 previously proposed (Yaeger et al., 2008) were genotyped to infer African, European and Native American ancestry at individual and population levels. The SequenomiPLEX platform (San Diego, CA, USA) genotyping (Pereira et al., 2012) was performed at the Centro Nacional de Genotipado (CEGEN, Santiago de Compostela, Spain). In the analyses, data from 119 Yoruba unrelated individuals from Ibadan, Nigeria (YRI) and 60 Utah residents with European ancestry from the CEPH collection (CEU) from The International HapMap Consortium (2010) were included as parental populations. The Expectation Maximization method implemented in the software Admixture (Alexander, Novembre, & Lange, 2009) was used to estimate ancestry, assuming three parental populations (k= 3).

***CYP2C9* and *CYP2C19* genotyping:** Genotyping for the *CYP2C9*2* (rs1799853), **3* (rs1057910), **6* (rs9332131) and *CYP2C19*2* (rs4244285), **3* (rs4986893), **4* (rs28399504), **5* (rs28399504) and **17* (rs12248560) alleles was carried out on genomic DNA using TaqMan assays as previously described (Dorado et al., 2012b; Llerena et al., 2014a; Peñas-Lledó et al., 2014). Chromosomes lacking the above-mentioned alleles/SNPs were classified as *CYP2C9*1* and *CYP2C19*1*.

***CYP2D6* genotyping:** Data on *CYP2D6*2* (rs1080985), **3* (rs35742686), **4* (rs1065852, rs3892097), **6* (rs5030655), **10* (rs1065852), **17* (rs28371706), **29* (rs59421388), **35* (rs1080985 and rs769258), **41* (rs28371725), *CYP2D6*5*, *CYP2D6*1xN*, **2xN*, **4xN* and

10xN alleles were published for the Costa Rican population (Céspedes-Garro et al., 2014a; Céspedes-Garro et al., 2014b).

Predicted hydroxylation capacity group: To infer metabolic phenotype from the genotypes, zero value was assigned to *CYP2C9*3* and **6* and *CYP2C19*2, *3, *4* and **5* variants, 0.5 value to *CYP2C9*2*, one to *CYP2C9/19*1*, and two to *CYP2C19*17* (Peñas-Lledó et al., 2014). Individuals with activity score values equal to zero were classified as gPMs, and individuals with activity score higher than two were classified as gUMs (for *CYP2C19*) (Peñas-Lledó et al., 2014). An activity score was adapted for CYP2D6 (Gaedigk et al., 2008; Llerena et al., 2012). Previously reported data on *CYP2D6* have been analyzed together with original ancestry information of the subjects.

The differences in *CYP2C9* and *CYP2C19* allele frequencies among populations were compared using the Fisher's exact test (alpha = 0.05). Hardy-Weinberg equilibrium for alleles was determined using a contingency table X^2 statistic with Yate's correction. Statistical analyses were performed using the STATISTICA 4.3 (StatSoft, Tulsa, OK, USA) and GraphPad Prism 3.02 (GraphPad Software, San Diego, CA, USA).

The correlation between individual ancestry and the number of copies of a specific allele in each individual was estimated using the Spearman's rank correlation, with the R cor.test command (R Foundation, 2015).

RESULTS

Genomic ancestry: The studied individuals and populations from Costa Rica encompass a wide spectrum of continental ancestry and self-reported admixed individuals from the Central Valley and Guanacaste showed many of the possible combinations of European, African and Native American admixture (Fig. 1). Moreover, the three self-reported

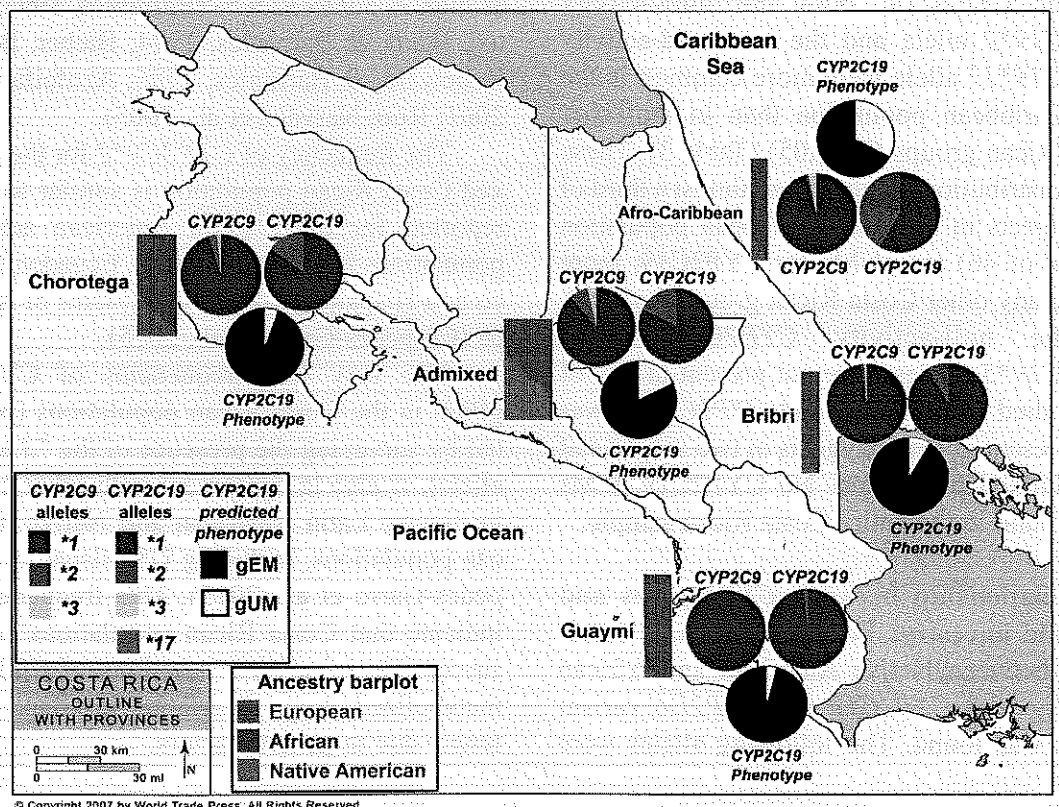

Fig. 1. Barplots of individual continental ancestry, main *CYP2C9* and *CYP2C19* alleles and CYP2C19 predicted phenotypes frequency distributions in Costa Rican populations. The approximate location of populations is shown in the chart.

Native groups mostly have Native American ancestry, with low African ancestry (<9 %) and European ancestry that range from 3 % (in Guaymí) to 22 % in the Chorotega. The Afro-Caribbean population has a very high African ancestry (86 %), with all the individuals showing more than 76 % of African ancestry (Table 1 and Fig. 1).

***CYP2C9* and *CYP2C19* alleles and predicted metabolic phenotypes:** *CYP2C9* and *CYP2C19* genotype frequencies fit the Hardy-Weinberg equilibrium for all the studied populations.

Consistently with previous studies, the wild-type *CYP2C9*1* is modal in all populations (Table 2). The decreased-activity *CYP2C9*2* allele frequency was higher in the Admixed and Huetar populations (7-8 %) than in the Afro-Caribbean, Bribri, Cabécar, Maleku and Guaymí (<1.1 %; P <0.05). No differences were found in the frequency of the decreased-activity *CYP2C9*3* allele across the different groups. The null-activity *CYP2C9*6* variant was not detected in the Costa Rican populations. Moreover, only one admixed subject was a CYP2C9 predicted poor metabolizer (gPMs).

For *CYP2C19,* both the null-activity *CYP2C19*2* allele and the increased-activity *CYP2C19*17* allele were more common in the Afro-Caribbean population than in the other Costa Rican groups (P <0.05) (Table 3). These allele distributions suggest that almost a third of the subjects in the Afro-Caribbean population (15 out of 46) are gUMs for CYP2C19, more than in any other Costa Rican group (P <0.05).

The null-activity *CYP2C19*3* and *CYP2C19*5* alleles were not present in any of the studied populations, and *CYP2C19*4* was only present in heterozygosis in two subjects of the admixed population. No CYP2C19 gPMs were found in the entire Costa Rican sample.

Relationship between *CYP2* genes and genomic ancestry: For Costa Rica, a correlation between Native American and African ancestry with *CYP2C19* and *CYP2D6* variant alleles was found. The following alleles were positively correlated with African ancestry and negatively correlated with Native American ancestry: *CYP2D6*5* (P<0.01), *CYP2D6*17* (P<0.01), *CYP2D6*29* (P= 0.027 for African and P= 0.045 for Native American) and *CYP2C19*17* (P<0.01). We found no correlation for *CYP2C9* polymorphisms and ancestry.

DISCUSSION

This is the first study on *CYP2C9* and *CYP2C19* in a multiethnic Costa Rican population, and it has been contextualized estimating the genomic ancestry of these populations.

The observed low frequency of the decreased-activity allele *CYP2C9*2* in the most Amerindian populations from Costa Rica (Bribri, Cabécar, Maleku and Guaymí) (Azofeifa, Ruiz, & Barrantes, 2001), is consistent with other studies on North- and South- Amerindians, which reported frequencies from 0 to 4.8 % for this allele (Céspedes-Garro et al., 2015). Noteworthy, the high *CYP2C9*2* frequency in the Huetar (8.3 %) is similar to that of the Costa Rican admixed population (7.7 %), in agreement with reports that estimate that the Huetar groups have European and African admixture as high as 3.9 to 32.9 % (Barrantes, 1993; Santos, Ward, & Barrantes, 1994; Bieber, Bieber, Rodewald, & Barrantes, 1996; Azofeifa et al., 2001; Ruiz-Narváez et al., 2005).

The frequency of *CYP2C9*2* for the admixed Costa Rican population is similar to those reported for other Latin American admixed populations from Brazil, Chile, Ecuador, Mexico and Hispanics from United States of America (Céspedes-Garro et al., 2015).

The very low frequencies of CYP2C9 gPMs in the Costa Rican populations (predicted by surveying the presence of the **3* and **6* allele), is also in agreement with other studies in diverse Latin American and Native American populations, including US-Hispanics (Céspedes-Garro et al., 2015). This frequency can indicate that Costa Rican populations are not susceptible to adverse reactions of CYP2C9-metabolized drugs (warfarin, losartan, diclofenac) due to genetic factors.

Regarding *CYP2C19* in Native Costa Ricans, the frequency of the null-activity allele *CYP2C19*2* varies from 0 to 12.5 %. The *CYP2C19*2* frequency in the Chorotega tribe (12.5 %) was similar to that reported for Ameridian populations from Brazil (10.4 and 11.1 %) (Santos et al., 2011; Vargens, Petzl-Erler, & Suarez-Kurtz, 2012). The low frequencies for this allele in Bribri, Maleku and Guaymí populations (4.3, 0 and 0 %, respectively) are similar to those from the Purépechas, Tzotziles, Tojolabales and Tzeltales Mexican Amerindian tribes (5.4, 5.6, 3.6 and 0 %, respectively) (Salazar-Flores et al., 2012).

As previously reported, *CYP2C19*3* frequency is rare outside Eastern Asia and Melanesia (Sistonen et al., 2009); for this reason, the lack of this allele in Costa Rican populations was expected.

The increased-activity *CYP2C19*17* allele frequency of the Afro-Caribbean population is consistent with its high frequency in the West African Gambia population (23.0 %) (Janha et al., 2014), which is in accordance to the predominant Western African origin of the African diaspora to the Americas (Madrigal, 2006). The ascertainment of CYP2C19 gUMs by genotyping of the *CYP2C19*17* allele has only been performed in other two Latin American admixed populations from Brazil and Ecuador (26.8 and 41.4 %, respectively) (Santos et al., 2011; Vicente et al., 2014), and both frequencies are higher than that of the Costa Rican admixed population (17.7 %; $P<0.05$). This result suggest that Costa Rican populations are less susceptible to therapeutic failure or adverse reactions in therapies based on drugs metabolized by CYP2C19, such as omeprazole and clopidogrel.

A correlation among *CYP2D6*5*, **17* and **29* alleles with ancestry was also found in this study, consistently with higher frequencies of **17* and **29* in African populations (Llerena et al., 2014b). The null-activity *CYP2D6*4* allele is associated to European ancestry (Llerena et al., 2014b), and is supposed to be a marker of this ancestry. However, in our multiethnic sample of individuals, the *CYP2D6*4* allele is also present in 23 individuals with more than 30 % of Native American ancestry and less than 60 % of European ancestry. Furthermore, the highest frequencies of *CYP2D6*4* worldwide are in the Chibchan groups (e.g. Bari from Venezuela - 42.5 % - and Bribri and Cabécar from Costa Rica - 31.9 and 26.8 %, respectively) (Céspedes-Garro et al., 2014a; Céspedes-Garro et al., 2014b). Altogether, our results suggest that the **4* allele may be also common in Native American populations.

A limitation of this study is the low number of individuals in some of the populations, mainly Amerindians. However, considering that Amerindians are under-represented in pharmacogenetics surveys, important information is provided. Another limitation, shared with most pharmacogenetics studies, is that we genotyped specific SNPs that define specific alleles (haplotypes) and classified as wild type (*CYP2C9*1* or *CYP2C19*1*) the individuals that do not carry these alleles. However, it is unknown if populations that are under-represented in pharmacogenetics studies, such as Native Americans, may present variants that were not genotyped, or that were even unknown and that may alter enzymatic activity. Thus, further studies assessing the *CYP2C9* and *CYP2C19* sequence in an unbiased fashion, preferentially by sequencing these genes, would be necessary.

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RESUMEN

Relevancia de la ancestría para la variabilidad de polimorfismos de las enzimas metabolizadoras de fármacos CYP2C9, CYP2C19 y CYP2D6 en una población multiétnica de Costa Rica. CYP2C9, CYP2C19 y CYP2D6 metabolizan aproximadamente el 40 % de los fármacos y los genes que las codifican varían en las distintas poblaciones humanas. La población costarricense posee ancestría trihíbrida y su posición geográfica estratégica la convierten en un escenario idóneo para evaluar la variabilidad interétnica en sus poblaciones multiétnicas. El presente estudio tiene como objetivo describir la diversidad de los polimorfismos *CYP2C9*, *CYP2C19* y *CYP2D6* en las poblaciones costarricenses en el contexto de su ancestría. Un total de 448 individuos sanos fueron incluidos: Bribri (n= 47), Cabécar (n= 27), Maleku (n= 16), Guaymí (n= 30), Huetar (n= 48), Chorotega (n= 41), mestizos del Valle Central y Guanacaste (n= 189) y afrocaribeños de Limón (n= 50). Los genotipos *CYP2C9* (alelos **2, *3, *6*) y *CYP2C19* (**2, *3, *4, *5* y **17*) fueron determinados mediante PCR tiempo real. Las ancestrías africana, europea y nativa americana fueron inferidas usando 87 marcadores informativos de ancestría. La frecuencia del alelo de actividad disminuida *CYP2C9*2* fue menor en los grupos autodefinidos de amerindios que en la población mestiza y las frecuencias más altas de *CYP2C19*2* (actividad nula) y *CYP2C19*17* (actividad incrementada) se encontraron en la población autodefinida afrocaribeña. Asimismo, se encontró una frecuencia de gPMs *CYP2C9* de 0.7 % en la población mestiza y una frecuencia variable de gUMs *CYP2C19* (0.0 a 32.6 %, más prevalente en afrocaribeños) en las poblaciones costarricenses. Por último, los siguientes alelos fueron positivamente correlacionados con la ancestría africana y negativamente con la ancestría nativa americana: *CYP2D6*5* (actividad nula), *CYP2D6*17*, *CYP2D6*29* (ambos de actividad disminuida) y *CYP2C19*17* (actividad incrementada). No se encontró correlación entre los polimorfismos *CYP2C9* y la ancestría. Se requieren estudios posteriores que evalúen la secuencia de *CYP2C9* y *CYP2C19* en estas poblaciones, preferiblemente mediante la secuenciación de estos genes.

Palabras clave: *CYP2C9*, *CYP2C19*, *CYP2D6*, Costa Rica, amerindios, afrocaribeños, ancestría genética.

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