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MAESTRÍA EN CIENCIAS BIOMÉDICAS

Asociación de los polimorfismos 89 G>A, 90 T>C y 92 G>C del gen *PADI4* con anticuerpos contra péptidos citrulinados cíclicos (anti-CCP) en pacientes con artritis reumatoide del Sur de México

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QUE PARA OBTENER EL GRADO DE

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En el periodo que cursó la Maestría en Ciencias Biomédicas, la QBP. Christian Johana Baños Hernández (CVU: 560787) fue beneficiada con la beca del CONACYT No.370920.

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En la ciudad de Chilpancingo, Guerrero, siendo los 03 días del mes de julio de dos mil quince, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Asociación de los polimorfismos 89 G>A, 90 T>C y 92 G>C del gen *PADI4* con anticuerpos contra peptidos citrulinados cíclicos (anti-CCP) en pacientes con artritis reumatoide del sur de México"**, presentada por la alumna Christian Johana Baños Hernández, para obtener el Grado de Maestría en Ciencias Biomédicas. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

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I. TITLE

Peptidyl arginine deiminase type IV polymorphisms (*PADI4*_89, 90 and 92) and the functional haplotype are associated with rheumatoid arthritis susceptibility in a southern Mexican population

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II. ABSTRACT

Rheumatoid arthritis (RA) is a common autoimmune disease with a complex genetic background. The peptidyl arginine deiminase type IV (*PADI4*) gene has been associated with RA due to several single nucleotide polymorphisms (SNPs) and their haplotypes have been associated with RA susceptibility in several populations. This study explores the relationship between *PADI4* gene SNPs and their haplotypes in 200 RA patients and 200 control subjects from southern Mexico. The SNPs were genotyped using the PCR-RFLP technique and antibodies to cyclic citrullinated peptides (anti-CCP) were measured by enzyme-linked immunosorbent assay (ELISA). In addition, we performed a haplotype analysis with data of each of the *PADI4* gene SNPs (*PADI4*_89, *PADI4*_90, *PADI4*_92). *PADI4* gene polymorphisms and the GTG haplotype were individually associated with RA susceptibility. Likewise, we confirmed that patients who were seropositive for anti-CCP antibodies and carriers of the susceptibility genotypes of the three *PADI4* gene SNPs, had an increased risk to develop RA. In conclusion, our replication study from a southern Mexican population suggests that *PADI4* individual polymorphisms and the susceptibility haplotype (GTG) are also genetic risk markers for RA and confirm that in combination with anti-CCP (+) antibodies, these SNPs influence the autoimmune processes towards the development of RA in this population. Based on these results, we acknowledge the importance of the replication of this study in other regions of Mexico in order to further understand the influence of ethnicity on RA susceptibility. **Keywords:** Rheumatoid arthritis; Peptidylarginine deiminase 4 (*PADI4*) gene; Single nucleotide polymorphism; Genetic susceptibility; Anti-CCP antibodies.

III. INTRODUCTION

Rheumatoid arthritis (RA) is a common autoimmune disease that is associated with progressive disability, systemic complications, early death, and socioeconomic costs, affecting approximately 0.5-1 % of the adult population worldwide (1) and 1.6% of the Mexican population (2). It is characterized by synovial inflammation, hyperplasia, autoantibody production (rheumatoid factor and anti–citrullinated protein antibody [ACPA]), cartilage and bone destruction ("deformity"), and systemic features, including cardiovascular, pulmonary, psychological, and skeletal disorders (3). The etiology of RA to date is unknown, however, according to the main hypothesis, both genetic and environmental factors contribute to RA susceptibility (4).

The genetic component of RA pathogenesis may account for up to 60% (5), and the locus believed conclusively to be associated with RA is the Human Leukocyte Antigen-DRB1 (*HLA-DRB)*, which accounts for about one-third of the genetic component (6). To date, additional candidate genes outside of the HLA region have been investigated to identify new RA susceptibility loci (7). The peptidylarginine deiminase 4 (*PADI4*) gene was the first non-HLA genetic risk factor known to be associated with RA, especially in Japanese population (8,9). Nevertheless, an association has also been observed in Korean and North American populations (10,11), and similarly, our research group recently found an association in a western Mexican population (12).

The *PADI4* gene is located on chromosome 1 (1p36) and encodes the type 4 peptidylarginine deiminase enzyme which catalyzes the post-translational modifications of proteins by conversion of arginine to citrulline producing citrullinated proteins (13) which are a target of anti-citrullinated peptide antibodies (ACPA) (14), currently the most

sensitive (≈80%) and specific (>95%) serological markers for RA (12). The first association of *PADI4* gene with RA, was reported by Suzuki et al. in a Japanese population; they observed that four *PADI4* gene single nucleotide polymorphisms (SNPs: *PADI4*_89, *PADI4*_90, *PADI4*_92 and *PADI4*_104) were in linkage disequilibrium and thus two main haplotypes were identified and defined as "susceptible" or "non-susceptible" for RA (8). Since this first positive association, several studies have been performed in order to replicate the association between *PADI4* and RA (5). Considering these facts, the aim of this study was to analyze the association of the *PADI4*_89, *PADI4*_90 and *PADI4*_92 *PADI4* gene SNPs and haplotypes with RA and evaluate the relationship between anti-CCP status and *PADI4* gene SNPs and haplotypes for RA susceptibility in a population from southern Mexico.

IV. MATERIALS AND METHODS

Study population

This case-control study involved 200 RA patients and 200 control subjects (CS). RA patients were recruited from the Rheumatology Department at the Hospital General of Chilpancingo "Dr. Raymundo Abarca Alarcón", Chilpancingo de los Bravo, State of Guerrero, Mexico and all fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (15). Spanish HAQ-DI (Spanish version of the health assessment questionnaire disability index) (16) and DAS28 (disease activity score using 28 joint counts) scores (17) were applied to RA patients in order to measure the functional disability and clinical activity, respectively. The CS group comprised healthy individuals (identified by self-report) recruited from the general population. RA patients and CS were unrelated individuals from the same Mexican population. To prevent population heterogeneity, only Mexican Mestizo subjects with Mexican ancestors at least back to the third generation were enrolled from southern Mexico. Informed written consent was obtained from all subjects before their enrollment to the study. The investigation was performed according to the ethical guidelines stated on the Declaration of Helsinki and was approved by the Ethics Committee of the Hospital General of Chilpancingo "Dr. Raymundo Abarca Alarcón", Chilpancingo de los Bravo, State of Guerrero, Mexico.

Laboratory assessment and quantification of antibodies

Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), were quantified by the Wintrobe method and by a turbidimetric assay (Mindray, BS-120, Chemistry

Analyzer, Shenzhen, China). Rheumatoid factor (RF) was measured by a turbidimetric assay (Mindray, BS-120, Chemistry Analyzer, Shenzhen, China) according to the manufacturer's instructions. Individuals with values >30 IU/mL were considered as RF positive. Anti-Cyclic citrullinated peptide (anti-CCP) antibodies (IgG) were measured with a second-generation enzyme-linked inmunosorbent assay (ELISA) according to the manufacturer´s recommendations (Anti-CCP, DIASTAT™, Axis-Shield Diagnostics Limited, Dundee, Scotland, UK). Samples with values above the calibration curve were diluted and measured again. A cut off of >5 U/mL was used as a stringent criterion for positive anti-CCP values.

PADI4 Gene Polymorphisms Genotyping

The *PADI4*_89 (rs11203366), *PADI4*_90 (rs11203367) and *PADI4*_92 (rs874881) *PADI4* gene SNPs were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) system. The primers used in this study were those reported by Cantaert et al. (18). Since the *PADI4* 89G>A and 90T>C polymorphisms are in proximate sequences, a single PCR reaction was performed for the amplification of both polymorphic fragments using the following primers: 5´TTGTCCACAGCTCTGCCC3´ (Sense) and 5´ACACTGCCACCCCACAG3´ (Antisense); the reaction was carried out in a final volume of 25 µl containing 0.12 µM of each primer, 100 ng of gDNA, 1.5 U of *Taq* DNA polymerase (Invitrogen™ life technologies, USA), 1X enzyme supplied buffer, 4.5 mM $MgCl_2$, 1.4 M Betaine (SIGMA Life Science) and 0.1 mM of dNTPs (Invitrogen™ life technologies, USA) under the following conditions: initial denaturation at 94◦C for 3 m, followed by 35 cycles of 30 s at

94◦C, 30 s at 68◦C and 30 s at 72◦C each, with an ending extension step of 3 m at 72 °C. The resultant 221 bp fragment was later digested in separate reactions with the *BtgI* enzyme or with the *MscI* enzyme (New England, BioLabs, Inc.) for the identification of the *PADI4* 89G>A and 90T>C polymorphisms, respectively. The digested products were then electrophoresed on a 6% polyacrylamide gel (29 acrylamide: 1 bisacrylamide) and stained with 2% $AgNO_3$. In the *PADI4* 89G>A polymorphism, the G allele was identified by 2 fragments (144 and 77 bp) and the A allele was recognized as a 221 bp fragment, whereas in the *PADI4* 90T>C polymorphism, the T allele was represented by 2 fragments (165 and 56 bp) and a single 221 bp fragment represented the C allele.

Considering the *PADI4* 92G>C genotyping, PCR was carried out using the following primers (18): 5´GTTCAGATTTCATACTACGGACC3´ (Sense) and 5´GGGATGAGACGGCACTC3´ (Antisense) in a final volume of 15 µl containing 0.12 µM of each primer, 100 ng of gDNA, 0.65 U Taq DNA polymerase (Invitrogen™ life technologies, USA), 1X enzyme supplied buffer, 2.5 mM $MgCl_2$ and 0.18 mM dNTPs (Invitrogen™ life technologies, USA) under the following conditions: initial denaturation at 94◦C for 3 m, followed by 30 cycles of 30 s at 94°C, 30 s at 66°C and 30 s at 72°C, then a final extension of 3 m at 72°C. The PCR product resulted in a 106 bp fragment that was later digested with the *MspI* restriction endonuclease (New England BioLabs, Inc.). The products were then electrophoresed on a 6% polyacrylamide gel and stained with $AgNO_3$. The G allele was represented as two fragments (59 and 47 bp) while the C allele resulted in single fragment of 106 bp. A 50 bp molecular weight standard (Invitrogen™ life technologies, USA) and the corresponding positive and negative controls for each *PADI4* gene SNPs were loaded on polyacrylamide gels.

Statistical analysis

For the descriptive analysis, nominal variables were expressed as frequencies, and continuous variables not normally distributed were expressed as medians and 5 to 95 percentiles. Hardy-Weinberg equilibrium and comparisons of allele and genotype distributions between groups were evaluated with the χ^2 test or Fisher exact test, as appropriate. Haplotypes were reconstructed using the SHEsis software (a powerful platform for linkage disequilibrium analyses, haplotype construction and genetic association at polymorphism loci) (19). The odds ratio (OR) and 95% confidence interval (95% CI) were estimated to analyze the risk of *PADI4* genotypes and haplotypes associated with RA. Differences in clinical parameters among groups were evaluated by the Wilcoxon-Mann-Whitney test, as appropriate. Statistical analysis was performed using STATA Software v11.0. A probability (*p*) value of less than 0.05 was considered significant.

V. RESULTS

Demographic and clinical features

The demographic and clinical data of RA patients and CS are shown in Table 1. The median age in both groups, RA patients and CS was 46.5 and 47 years, respectively, and 94% were female in both groups, since they were matched by age and sex. For RA patients at the time of the inclusion, the median of disease evolution was 6 years with moderate disease activity (DAS28: median=2.8) as well as they presented some functional disability to perform any daily activity (HAQ-DI: median=0.23), and they were treated mainly with disease-modifying antirheumatic drugs (DMARDs) and non-steroidal anti-inflammatory drugs (NSAIDs). RF and anti-CCP antibodies were positive in 87% and 88% of RA patients, while only 4% and 2.7% of the CS were positive for RF and anti-CCP antibodies, respectively. In regards to the laboratory assessment, RA patients showed significantly higher levels of acute phase reactants (ESR and CRP), anti-CCP antibodies, and RF in comparison with CS ($p < 0.001$; Table 1).

Frequency of PADI4 gene polymorphisms and haplotype analysis in both study groups

The distribution of genotypic and allelic frequencies of the *PADI4*_89 (rs11203366), *PADI4*_90 (rs11203367) and *PADI4*_92 (rs874881) gene SNPs in RA patients and CS is shown in Table 2. The 3 exonic *PADI4* gene SNPs were in Hardy-Weinberg Equilibrium in the CS group ($p > 0.05$; data not shown). We found significant differences in genotype and allele frequencies between RA patients and CS for each of the 3 *PADI4* gene SNPs analyzed, which confirmed an association of these SNPs in the *PADI4* gene with an

increased risk of RA in our study population from southern Mexico. In addition, a strong linkage disequilibrium among the 3 *PADI4* gene SNPs was identified (LD values 0.93, 0.91 and 0.97; p<0.0001) (Figure 1).

Based on this finding, it was of our interest to perform a haplotype analysis with data of these polymorphisms in RA patients and CS (Table 3). We identified six different haplotypes in our population, where the ACC and GTG haplotypes were the most frequent, representing 94.9% and 96% in RA patients and CS, respectively. Therefore, we carried out the distribution analysis of these two common haplotypes, and the GTG haplotype was found more frequently in RA patients than CS, thus confirming its association as a “susceptible” haplotype in our study population (OR 1.36, 95% CI 1.02-1.80; p=0.03). In addition, when we analyzed the homozygous haplotype carriers (GTG/GTG *vs* ACC/ACC), we found that the magnitude of the association increased (OR 2.27, 95% CI 1.18-4.41; p= 0.008) (Table 3).

Demographic and clinical characteristic and laboratorial assessment according to the polymorphisms and haplotype of the PADI4 gene in RA patients

Considering the demographic and clinical characteristics as well as the laboratorial assessment with the 3 *PADI4* gene SNPs, we did not find any associations between the *PADI4*_92 SNP and the different characteristics evaluated (data not shown). Nevertheless, a significant and marginal association in the Spanish HAQ-DI score was observed for the *PADI4*_89 and *PADI4*_90 SNPs respectively (p= 0.04 and p= 0.05, respectively; data not shown). In addition, we stratified RA patients according to anti-CCP antibody status, and in the group of seropositive patients for anti-CCP antibodies

we observed an increased risk for RA in each of the *PADI4* gene SNPs (*PADI4*_89: OR 1.80, 95% CI 1.01-3.20, *p*= 0.03; *PADI4*_90: OR 1.80, 95% CI 1.01-3.20, *p*= 0.03 and *PADI4*_92: OR 1.89, 95% CI 1.05-3.41, *p*= 0.02) (Table 4). In regards to the possible association of the "susceptible" haplotype of *PADI4* (GTG) with the demographic, clinical and laboratorial assessment characteristics in RA patients, we analyzed the homozygous haplotype carriers (ACC/ACC *vs* GTG/GTG), and we did not find significant differences in this Southern Mexico population, however, a slight tendency to a significant association with the functional disability (Spanish HAQ-DI score) was observed (p= 0.05; data not shown).

VI. DISCUSSION

Rheumatoid arthritis is characterized by persistent synovitis, systemic inflammation, and presence of autoantibodies. To date, we know that approximately 60% of the risk for developing RA is attributable to genetic factors (20). Within the past years, genetic studies have produced a rapidly growing list of risk loci for RA. The HLA region, considered the most important region which contributes approximately to half of the genetic susceptibility for RA. Nevertheless, others variants in potentially pathogenic genes located in non-HLA regions have been implicated; these genes include *PTPN22*, *STAT4* and *PADI4* (21). The *PADI4* gene, has been of particular interest for the pathogenesis of RA, since the PADI4 enzyme catalyzes the change from peptidylarginine to peptidylcitrulline, a target of anti-CCP antibodies through a post-translational modification process referred to as citrullination (14).

The association of *PADI4* gene with RA susceptibility was first reported in a Japanese population (8) and this association has been replicated in several populations including Korean (11,22), Chinese (23), German (24) and North American populations (10); while such an association has not been replicated in other populations, among them England (25-27), French (28), Spanish (29) and Indian populations (30); some studies revealed low magnitude associations in populations of European descent in comparison to populations of Asian descent according to a meta-analysis by Iwamoto (31).

The genetic heterogeneity observed between different populations could be partly explained by the difference of disease severity between the study populations. Therefore, there is a need to further investigate the impact of genetic variations in *PADI*-associated genes in additional populations. Since ethnic differences are likely to go

hand in hand with various environmental exposures, it is useful to study distinctive populations with certain similarities within their genetic background and contribute to a better understanding of the complex etiology and genetic factors involved in RA (14,32).

Recently, in a study by our research group *PADI4* gene SNPs were found to be associated with susceptibility to RA in a western Mexican population (12), where the previously reported ancestry in this population was mainly European (60-64%), followed by Amerindian (25-21%), and African (~15%) (33). Based on the above, in the present study it was in our interest to evaluate whether the *PADI4*_89, *PADI4*_90 and *PADI4*_92 gene SNPs are associated with RA susceptibility in a southern Mexican population, since the ancestry of this population has been described as being mainly Amerindian (48%), European (38%), Euroasian (10%) and African (4%) (34). Therefore, we considered of primary importance to test if the genetic contribution of *PADI4* to RA susceptibility is also replicated in other Mexican populations with similar genetic background. Additionally, the relationship of the *PADI4* gene SNPs and haplotypes with anti-CCP levels and clinical characteristics was also analyzed; and in accordance with other authors and our recent research, we demonstrated a strong significant association between *PADI4*_89, *PADI4*_90 and *PADI4*_92 *PADI4* gene SNPs with RA susceptibility, since both the risk allele and the genotype of the 3 different SNPs were significantly more frequent in RA patients than controls, confirming that these polymorphisms provide an increased risk for RA.

The genotypic and allelic frequencies reported for Japanese (8) and western Mexican populations (12) were similar in the present study. We also observed that the three *PADI4* gene polymorphisms studied are in strong linkage disequilibrium for this

population (LD > 0.9), which suggests that these three genetic markers can be analyzed as a panel and not only by their single allele or genotype frequencies. Thus, when the haplotype analysis was performed, we found that the ACC and GTG haplotypes were the most frequent in our population representing approximately 95.5% of the total population. In addition, we observed a significant association between the susceptibility haplotype (GTG) with RA. This same association was previously reported in Japanese, Korean and western Mexican populations (8,11,12,22).

Regarding the association of *PADI4* gene SNPs and haplotypes with clinical, demographic and laboratorial data of RA patients in our study, no significant differences were observed. However, a significant and marginal association in the functional disability (Spanish HAQ-DI score) was detected for the *PADI4*_89 and *PADI4*_90 SNPs respectively, similar for the susceptible haplotype (GTG) where we found a slight tendency towards a significant association with the functional disability. In contrast with the previous report by our research group, where an association between *PADI4* haplotypes with disease onset (≤ 40 years) of RA from a western Mexican population was observed, a higher percentage in GTG haplotype carriers, than non-susceptibility carriers (ACC), was shown (12).

In addition, we evaluated the relationship between anti-CCP (+) status and *PADI4* individual SNPs for RA susceptibility in a population from southern Mexico and confirmed that in patients who were seropositive for anti-CCP antibodies, the presence of the susceptibility genotypes of the three *PADI4* gene SNPs increases the risk for RA development, but not in RA patients who were seronegative for this autoantibody. Nevertheless, we did not find a significant association between anti-CCP serum levels

with susceptibility genotypes and haplotypes of the *PADI4* gene polymorphisms. These results are in disagreement with the previously reported in a western Mexico population, where an important finding was the association between anti-CCP serum levels and the *PADI4* polymorphisms and the susceptibility haplotype (GTG) (12). These findings suggest that these differences could be influenced by the genetic background of populations with different ancestry.

Suzuki et al. reported for the first time that the susceptibility haplotype carriers are more likely to be positive for antibodies to citrullinated filaggrin in comparison to non-susceptibility haplotype carriers (8); a similar association was reported with anti-CCP levels and *PADI4* susceptibility haplotype, specifically at early stages of disease (<34 months) (35). These findings support the hypothesis that *PADI4* susceptibility haplotype, which leads to three amino acid substitutions at the protein level, could enhance citrullination of proteins, creating neo-epitopes that subsequently in a predisposed individual, will lead to the production of anti-CCP antibodies and the development of RA (12).

In conclusion, our replication study from a southern Mexican population suggests that *PADI4* individual polymorphisms and the susceptibility haplotype are also genetic risk markers for RA and confirm that in combination with anti-CCP (+) antibodies, these SNPs influence the autoimmune processes towards RA development in this population. Moreover, we hope to encourage other research groups to replicate this study in different regions and ethnic groups in Mexico in order to acquire a broader understanding of the influence of ethnicity on RA susceptibility.

Conflict of interest

The authors declare no conflicts of interest related to this study

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VIII. TABLES AND FIGURE

Table 1. Clinical and demographic characteristics of the RA patients and CS

Variables	RA (*n*=200)	CS (*n*=200)	*P*
Demographics			
Age (yrs)[a]	46.5 (26.5-69)	47 (28-70)	0.98
Gender % (n)			1.0
Male	6 (12)	6 (12)	
Female	94 (188)	94 (188)	
Wood smoke exposure % (n)	53 (106)	31.5 (63)	<0.001
Smoking % (n)	10 (20)	18.5 (37)	0.01
Clinical assessment			
Disease evolution (yrs)[a]	6 (1-22.5)	-	
DAS28 score[a]	2.8 (1.85-6.64)	-	
DAS28 % (n)			
Remission (<2.6)	37 (74)	-	
Low activity (≥2.6 <3.2)	23.5 (47)	-	
Moderate activity (≥3.2 <5.1)	27.5 (55)	-	
High activity (≥5.1)	12 (24)	-	
Spanish HAQ-DI, 0-3 scale[a]	0.23 (0-1.73)	-	
ESR (mm/h)[a]	34 (12-56)	27 (6.5-46.5)	<0.001
CRP (mg/L)[a]	15.8 (3.45-101.95)	10.7 (2.5-46.5)	<0.001
RF (UI/mL)[a]	173.87 (3.5-300)	0 (0-14.9))	<0.001
RF % (n)			<0.001
Negative (<20 UI/mL)	13 (26)	96 (192)	
Positive (≥20 UI/mL)	87 (174)	4 (8)	
anti-CCP (U/mL)[a]	107 (0-900)	0 (0-3)	<0.001
anti-CCP % (n)			<0.001
Negative (≤5 U/mL)	11.6 (23)	95.3 (142)	
Low positive (5.1-14.9 U/mL)	8 (16)	2 (3)	
High positive (≥15 U/mL)	80.4 (160)	2.7 (4)	
Drug treatment % (n)			
NSAIDs	57 (114)	-	
Steroids (Prednisone)	41 (82)	-	
DMARDs			
Azulfidine (Sulfasalazine)	18 (36)	-	
Chloroquine	39 (77)	-	
Methotrexate	70 (139)	-	

[a] Data provided in median (p5-p95). The *p* values were calculated by X^2 test or Wilconxon-Mann-Whitney test, as appropriate. RA, rheumatoid arthritis; CS, control subjects; DAS28, disease activity score 28; Spanish HAQ-DI, Spanish version of health assessment questionnaire disability index; ESR, erythrocyte sedimentation rate; CRP, C reactive protein; RF, rheumatoid factor; anti-CCP, anti-cyclic citrullinated peptide antibody; NSAIDs, non-steroidal anti-inflammatory drugs; DMARDs, disease modifying anti-rheumatic drugs.

Table 2. Genotypic and allelic frequencies of the three *PADI4* SNPs among RA patients and CS.

	RA *n*= 200 % (*n*)	CS *n*= 200 % (*n*)	OR (95% CI)	*p*[b]
PADI4 89G>A				
GG	28.0 (56)	24.0 (48)	1.89 (1.02-3.50)	0.02
GA	55.0 (110)	48.5 (97)	1.83 (1.07-3.15)	0.01
AA[a]	17.0 (34)	27.5 (55)	1	-
Allele				
G	55.5(222)	48.2 (193)	1.34 (1.00-1.78)	0.04
A	44.5 (178)	51.8 (207)	1	-
PADI4 90T>C				
TT	28.5 (57)	24.0 (48)	1.92 (1.04-3.56)	0.02
TC	54.5 (109)	48.5 (97)	1.82 (1.06-3.13)	0.02
CC[a]	17.0 (34)	27.5 (55)	1	-
Allele				
T	55.8 (223)	48.2 (193)	1.35 (1.01-1.80)	0.03
C[a]	44.2 (177)	51.8 (207)	1	-
PADI4 92G>C				
GG	28.5 (57)	24.5 (49)	1.90 (1.03-3.54)	0.02
GC	55.0 (110)	48.5 (97)	1.86 (1.08-3.20)	0.01
CC[a]	16.5 (33)	27.0 (54)	1	-
Allele				
G	56.0 (224)	48.8 (195)	1.34 (1.00-1.78)	0.04
C[a]	44.0 (176)	51.2 (205)	1	-

RA, rheumatoid arthritis; CS, control subjects; OR, odds ratio; 95% CI, 95% confidence interval.

[a] Reference genotype.

[b] *p* Values were calculated by logistic regression comparisons with the reference category.

Table 3. Haplotype frequencies of the three *PADI4* SNPs in RA and CS.

	Haplotypes			AR *n* = 400 % (*n*)	CS *n* = 400 % (*n*)	OR (95% CI)	*P*
	89G>A	***90T>C***	***92G>C***				
H1	A	C	C	41.7 (167)	49.5 (198)	1	-
H2	G	T	G	53.2 (213)	46.5 (186)	1.36 (1.02-1.80)	0.03
H3	A	C	G	2.5 (10)	1.3 (5)	-	-
H4	A	T	G	0.3 (1)	1.0 (4)	-	-
H5	G	C	C	0	1.0 (4)	-	-
H6	G	T	C	2.3 (9)	0.7 (3)	-	-
	Homozygous haplotype						
H1[a]	A-C-C/A-C-C			33.7 (27)	53.7 (51)	1	-
H2	G-T-G/G-T-G			66.3 (53)	46.3 (44)	2.27 (1.18-4.41)	0.008

RA, rheumatoid arthritis; CS, control subjects; H, haplotype; OR, odds ratio; 95% CI, 95% confidence interval; NA, not applicable.

[a] The *p* value for the GTG haplotype and the GTG/GTG homozygous haplotype were calculated by logistic regression comparison with the reference haplotype ACC and ACC/ACC homozygous haplotype, respectively.

Table 4. Frequencies of the *PADI4* genotypes in RA stratified according to anti-CCP antibodies status.

	anti-CCP (+)* (*n*= 176)	CS (*n*= 149)	OR (95% IC)	*p*	anti-CCP (-)* (*n*= 23)	CS (*n*= 149)	OR (95% IC)	*p*
PADI4* *89G>A								
GG+GA % (*n*)	83.5 (147)	73.8 (110)	1.80 (1.01-3.20)	*p*= 0.03	78.3 (18)	73.8 (110)	1.27 (0.41-4.69)	*p*= 0.65
[a]AA % (*n*)	16.5 (29)	26.2 (39)	1		21.7 (5)	26.2 (39)	1	
PADI4 90T>C								
TT+TC % (*n*)	83.5 (147)	73.8 (110)	1.80 (1.01-3.20)	*p*= 0.03	78.3 (18)	73.8 (110)	1.27 (0.41-4.69)	*p*= 065
[a]CC % (*n*)	16.5 (29)	26.2 (39)	1		21.7 (5)	26.2 (39)	1	
PADI4* *92G>C								
GG+GC % (*n*)	84.7 (149)	74.5 (111)	1.89 (1.05-3.41)	*p*= 0.02	74.0 (17)	74.5 (111)	0.97 (0.33-3.22)	*p*= 0.95
[a]CC % (*n*)	15.3 (27)	25.5 (38)	1		26.0 (6)	25.5 (38)	1	

RA, rheumatoid arthritis; CS, control subjects; OR, odds ratio; 95% CI, 95% confidence interval.

[*] Referred as subgroups of rheumatoid arthritis patients

[a] Reference genotype

[b] *p* Values were calculated by logistic regression comparisons with the reference category

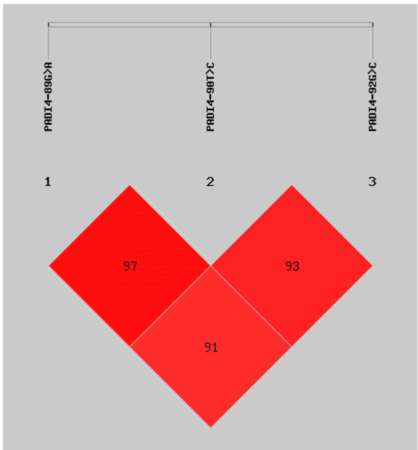


Figure 1. Linkage disequilibrium test of *PADI4* SNPs. Haplotyoe frequencies and linkage disequilibrium (LD) were calculated using SHEsis software. A D´ value of 100 indicates a complete LD between two markers and a D´ value of 0 indicates complete linkage equilibrium. The darker the cell, the greater the linkage disequilibrium between the SNPs. rs11203366: PADI4 89G>A; rs11203367 PADI4 90T>C; rs874881 PADI4 92G>C.

IX. ANNEXES

Anexo 1.

Criterios para el diagnóstico y la clasificación de AR (ACR/EULAR 2010)

Población objetivo que debe examinarse, pacientes que:
1) Tengan al menos una articulación inflamada
2) Siempre y cuando la sinovitis no tenga otra explicación

Se necesita una puntación ›6/10 para la clasificación de un paciente con AR definida

CRITERIO	PUNTAJE
A. Articulaciones involucradas	
1 articulación grande	0
2-3 articulaciones grandes	1
1-3 articulaciones pequeñas(con o sin involucro de articulaciones grandes)	2
4-10 articulaciones pequeñas(con o sin involucro de articulaciones grandes)	3
> 10 articulaciones (por lo menos una articulación pequeña)	5
B. Serología	
FR negativo y ACPA negativo	0
FR bajo positivo o ACPA bajo positivo	2
FR alto positivo o ACPA alto positivo	3
C. Reactantes de fase aguda	
pCr y VSG normales	0
pCr o VSG anormales	1
D. Duración de los síntomas	
< 6 semanas	0
≥ 6 semanas	1

FR, factor reumatoide; ACPA, anticuerpos contra proteína citrulinada; pCr, proteína C reactiva; VSG, velocidad de sedimentación globular.

(Modificado de Aletaha, 2010)

Anexo 2. Carta de consentimiento informado

UNIVERSIDAD AUTÓNOMA DE GUERRERO
UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD ACADÉMICA DE MEDICINA-UIEM
MAESTRÍA EN CIENCIAS BIOMÉDICAS

CARTA DE CONSENTIMIENTO INFORMADO

Por medio de la presente acepto participar en el proyecto de investigación titulado: Asociación de los polimorfismos 89G/A, 90T/C y 92G/C del gen *PADI4* con anticuerpos anti-CCP en artritis reumatoide del Sur de México.

Se me ha explicado que mi participación consistirá en **responder un cuestionario y una toma de sangre periférica por punción venosa.** Declaro que se me ha informado ampliamente sobre los posibles riesgos, inconvenientes y molestias derivados de mi participación en el estudio, como **la posible formación de un hematoma en la zona de punción.**

El investigador principal se ha comprometido a darme información oportuna sobre cualquier procedimiento alternativo adecuado que pudiera ser ventajoso para mi tratamiento, así como a responder mis preguntas y aclarar cualquier duda que tenga acerca de los procedimientos que se llevarán a cabo o cualquier otro asunto relacionado con la investigación. Además de que me ha dado seguridades de que mi identidad no será divulgada en las presentaciones o publicaciones que deriven de este estudio y de que los datos personales que proporcione serán manejados en forma confidencial.

NOTA: El número telefónico al que se podrá comunicarse para cualquier emergencia o dudas: (044) 7471405468.

Lugar y fecha: ____

Paciente	**Responsable**
______________________________	______________________________
Nombre y firma del paciente	Q.B.P. Christian Johana Baños Hernández C. P. 8251355

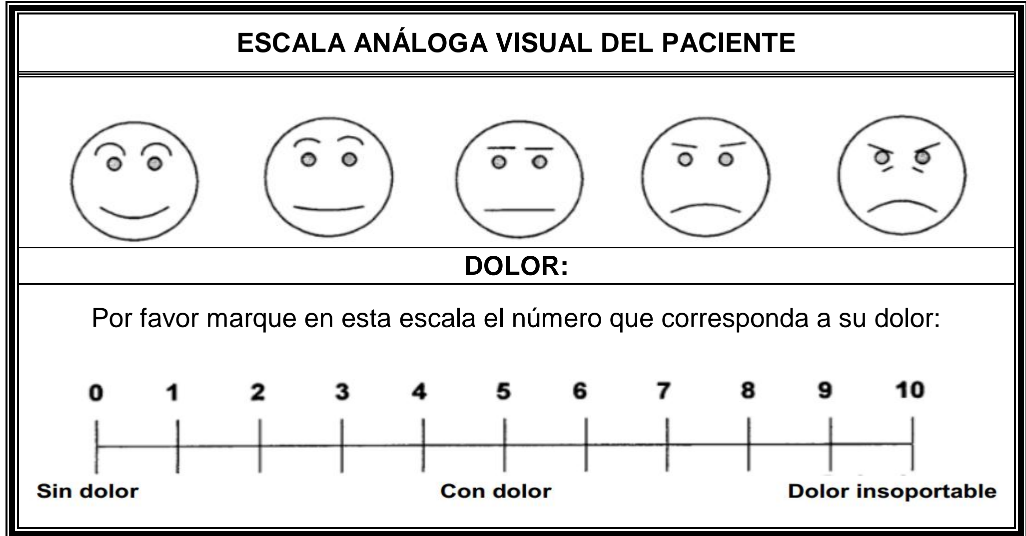
Investigadores Principales

______________________________	______________________________
Dr. en C. José Francisco Muñoz Valle Doctor en Biología Molecular en Medicina	Dr. José Eduardo Navarro Zarza Medicina Interna – Reumatología

Anexo 3.

DAS28 (Disease Activity Score 28-joint counts)

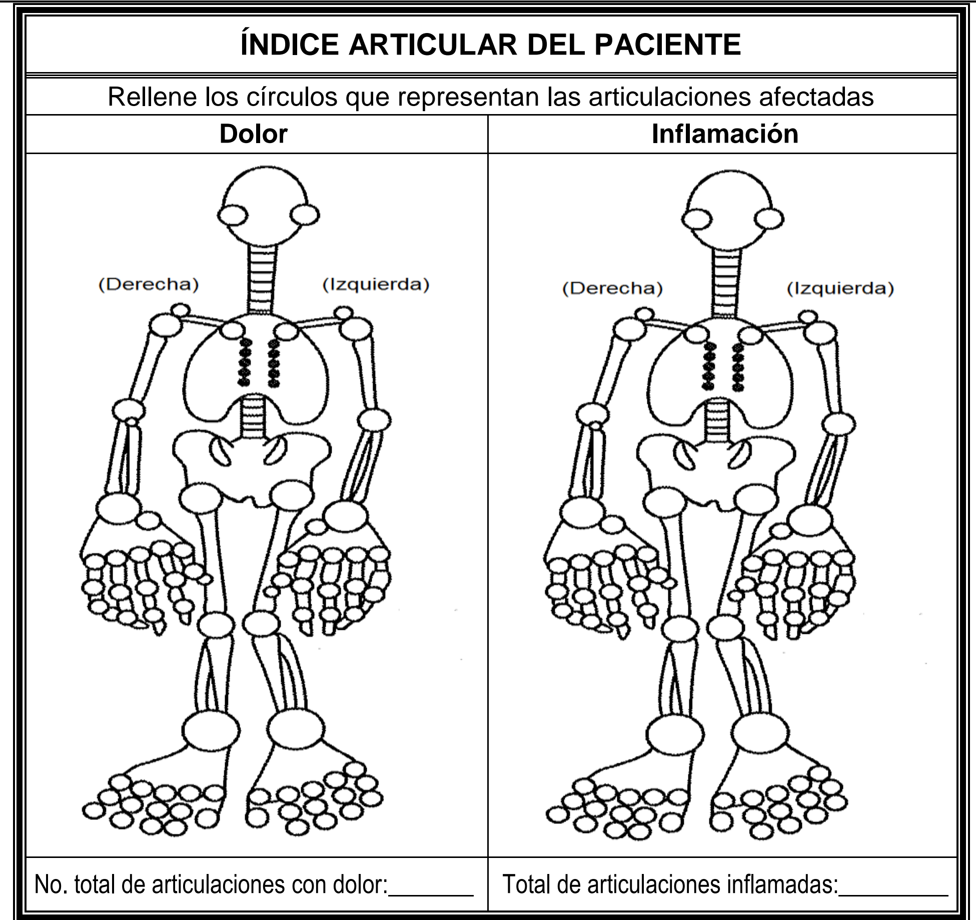
Nombre del paciente:____ **Fecha:**____________



ESCALA ANÁLOGA VISUAL DEL PACIENTE

DOLOR:

Por favor marque en esta escala el número que corresponda a su dolor:



ÍNDICE ARTICULAR DEL PACIENTE

Rellene los círculos que representan las articulaciones afectadas

Dolor	**Inflamación**
(Derecha) (Izquierda)	(Derecha) (Izquierda)
No. total de articulaciones con dolor:_______	Total de articulaciones inflamadas:_________

Anexo 4.

Health Assessment Questionnaire (HAQ)

Nombre del paciente:______________________________________ **Folio:**_______

INSTRUCCIONES: Por favor marque con una “X” la respuesta que mejor describa su capacidad para hacer las cosas o sus habilidades usuales.

	Durante la última semana, ¿ha sido usted capaz de…	Sin dificultad	Con alguna dificultad	Con mucha dificultad	Incapaz de hacerlo
Vestirse y asearse	1) Vestirse solo, incluyendo abrocharse los botones y atarse los cordones de los zapatos?				
	2) Enjabonarse la cabeza?				
Levantarse	3) Levantarse de una silla sin brazos?				
	4) Acostarse y levantarse de la cama?				
Comer	5) Cortar un filete de carne?				
	6) Abrir un cartón de leche nuevo?				
	7) Servirse la bebida?				
Caminar	8) Caminar fuera de casa por un terreno llano?				
	9) Subir cinco escalones?				
Higiene	10) Lavarse y secarse todo el cuerpo?				
	11) Sentarse y levantarse del retrete?				
	12) Ducharse?				
Alcanzar	13) Cargar un paquete de azúcar de 1 Kg de un estante colocado por encima de su cabeza?				
	14) Agacharse y recoger ropa del suelo?				
Prensión	15) Abrir la puerta de un coche?				
	16) Abrir tarros cerrados que ya antes habían sido abiertos?				
	17) Abrir y cerrar los grifos?				
Otros	18) Hacer los recados y las compras?				
	19) Entrar y salir de un coche?				
	20) Hacer tareas de casa como barrer o lavar los platos?				

Anexo 5. Procedimiento para la cuantificación de anti-CCP

La prueba anti-CCP de Axis-Shield es un análisis de inmunoabsorción ligado a enzimas (ELISA). Las instrucciones para su uso son los siguientes:

1. Establecer referencias en los pocillos para su identificación.
2. Pipetear 100 µl de control de referencia/calibradores por duplicado, controles positivos y negativos prediluidos (1:100) por duplicado y muestras de paciente prediluidas (1:100) por duplicado en los pocillos pertinentes. Este paso no debería superar los 10 minutospara cada uno de los conjuntos de calibradores/controles/muestras.
3. Incubar durante 60 ± 10 minutos a 18°C-25°C.
4. Decantar el contenido de la tira mediante inversión rápida sobre una pila adecuada para desechar materiales biológicos, teniendo en cuenta el potencial peligro infeccioso de las muestras. Secar las tiras invertidas bien con toallas de papel.
5. Lavar los pocillos cuatro vecescon un mínimo de 300 µl de tampón de lavado diluido. Decantar y secar después de cada paso de lavado.
6. Añadir 100 µl de conjugado a cada pocillo.
7. Incubar durante 30 ± 5 minutos a 18°C-25°C.
8. Repetir los pasos 4 y 5.
9. Añadir 100 µl de sustrato a cada pocillo.
10. Incubar durante 30 ± 10 minutos a 18°C-25°C. No decantar.
11. Añadir 100 µl de solución de parada a cada pocillo, en el mismo orden y velocidad que la adición del sustrato. Golpear suavemente los pocillos para mezclar y asegurarse de que no hay burbujas visibles.
12. Leer las tiras a 450 nm.
13. Leer la prueba 60 minutos después de finalizar el análisis.

Anexo 6. Método de extracción y purificación de ADN con CTAB (Bromuro de hexadeciltrimetilamonio)

1. Se colocan 300 µl de sangre periférica con EDTA (ácido etilendiaminotetraacético) en un tubo de 1.5 mL y agregar 500 µL de buffer de lisis e incubar a 68°C durante 8 minutos.

2. Después de la incubación agregar 500 µL de cloroformo al 100%, agitar vigorosamente durante 5 minutos, y centrifugar a 10 000 rpm por 10 minutos.

3. Recuperar cuidadosamente la fase acuosa a otro tubo de 1.5 mL estéril.

4. Adicionar 200 µL de buffer CTAB y 600 µL de agua desionizada, agitar suavemente por 20 minutos y centrifugar a 10 000 rpm durante 10 min.

5. Decantar el sobrenadante y resuspender la pastilla de ADN en 300 mL de NaCl 1.2 M y 1 mL de etanol al 100% frío y mezclar suavemente por inversión. Centrifugar a 10 000 rpm por 5 minutos.

6. Decantar el sobrenadante y agregar 1 mL de etanol frío al 70%, agitar vigorosamente y centrifugar a 10 000 rpm por 5 minutos.

7. Repetir el paso 6 al menos 2 veces. Decantar el sobrenadante y secar lapastilla de ADN hasta que el etanol se evapore completamente.

8. Resuspender la pastilla de ADN en 100 µL de buffer TE e incubar a 55°C por 5 minutos.

9. Almacenar el ADN a 4°C.

10. Etiquetar correctamente.

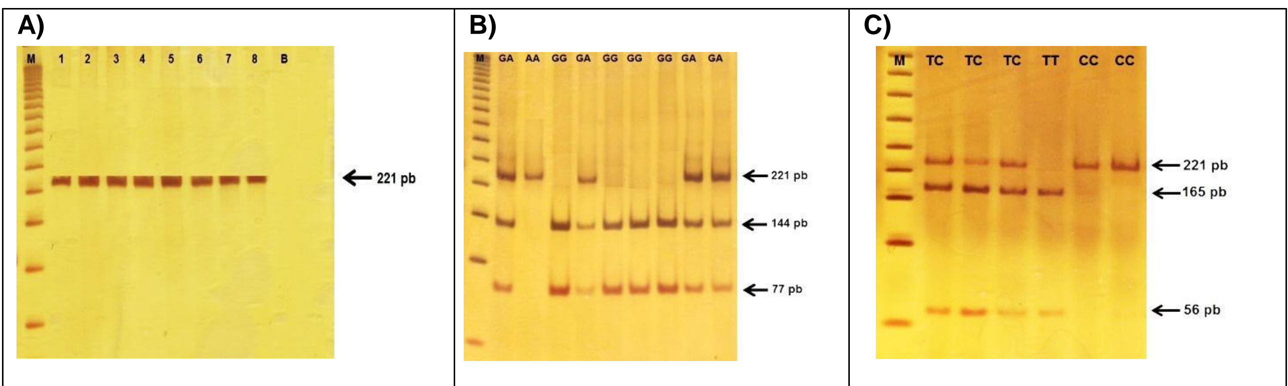
Anexo 7. Condiciones y mezcla para las reacciones de PCR de los polimorfismos 89G>A, 90T>C y 92G>C del gen *PADI4*

Condiciones para la amplificación del DNA				
Polimorfismo	**Etapa**	**Temperatura y tiempo**	**Ciclos**	**Tamaño del fragmento**
	Inicio	94 °C por 3’		
	Desnaturalización	94 °C por 30’’		
89G>A	Alineamiento	70 °C por 30’’	30	**221 pb**
	Extensión	72 °C por 30’’		
	Elongación final	72 °C por 1’		
Mezcla: Solución amortiguadora 1X, iniciadores 0.12 μM, dNTPs 0.1 mM, $MgCl_2$ 4.5 mM, *Taq* polimerasa 1.5 U, Betaine 1.4 μM, gDNA 100 ng.				
	Inicio	94 °C por 3’		
	Desnaturalización	94 °C por 30’’		
90T>C	Alineamiento	70 °C por 30’’	30	**221 pb**
	Extensión	72 °C por 30’’		
	Elongación final	72 °C por 1’		
Mezcla: Solución amortiguadora 1X, iniciadores 0.12 μM, dNTPs 0.1 mM, $MgCl_2$ 4.5 mM, *Taq* polimerasa 1.5 U, Betaine 1.4 μM, gDNA 100 ng.				
	Inicio	94 °C por 3’		
	Desnaturalización	94 °C por 30’’		
92G>C	Alineamiento	66 °C por 30’’	30	**106 pb**
	Extensión	72 °C por 30’’		
	Elongación final	72 °C por 1’		
Mezcla: Solución amortiguadora 1X, iniciadores 0.12 μM, dNTPs 0.18 mM, $MgCl_2$ 2.5 mM, *Taq* polimerasa 0.65 U gDNA 100 ng.				

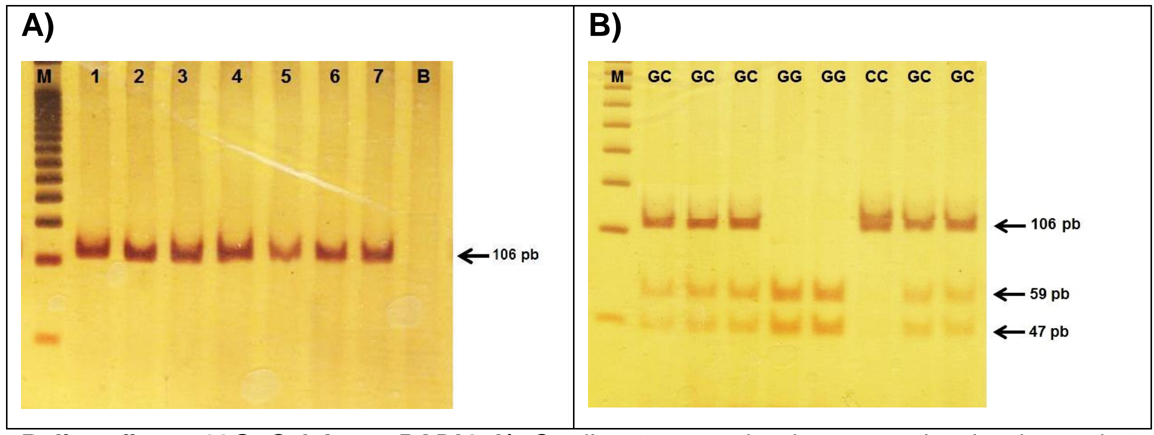
Anexo 8. Condiciones de restricción para la identificación de los polimorfismos 89G>A, 90T>C y 92G>C del gen *PADI4*

Polimorfismos	Enzima y secuencia de reconocimiento	Condiciones	Patrón de restricción
89G>A	*BtgI* C▼CACGG	5 μL de producto de PCR 1 μL de buffer 3 3.3 μL de agua estéril 0.7 μL de enzima (7 U) Incubar durante 3 hrs. a 37 °C	GG: 144 y 77 pb GA: 221, 144 y 77 pb AA: 221 pb
90T>C	*MscI* TGG▼CCA	5 μL de producto de PCR 1 μL de buffer 4 3.8 μL de agua estéril 0.2 μL de enzima (5 U) Incubar durante 3 hrs. a 37 °C	TT: 165 y 56 pb TC: 221, 165 y 56 pb CC: 221 pb
92G>C	*MspI* C▼CGG	5 μL de producto de PCR 1 μL de buffer 4 3.6 μL de agua estéril 0.4 μL de enzima (5 U) Incubar durante 2 hrs. a 37 °C	GG: 59 y 47 pb GC: 106, 59 y 47 pb CC: 106 pb

Anexo 8. Geles de poliacrilamida para la identificación de los polimorfismos 89G>A, 90T>C y 92G>C del gen *PADI4*



Polimorfismo *89G>A y 90T>C* del gen *PADI4* . A). Carriles 1: marcador de peso molecular de 50 pb; 1, 2, 3, 4, 5, 6, 7 y 8: producto de PCR; 9: blanco. **B).** Carril 1: marcador de peso molecular de 50 pb; 4, 6, 7 y 8: genotipo GG;2, 4, 9 y 10: genotipo GA; 3: genotipo AA. **C)** Carril 1: marcador de peso molecular de 50 pb; 5: genotipo TT; 2, 3 y 4: genotipo TC; 6 y 7: genotipo CC.



Polimorfismo *92G>C* del gen *PADI4.* A). Carriles 1: marcador de peso molecular de 50 pb;1 - 7: producto de PCR; Carril 8: blanco. **B).** Carriles 1: marcador de peso molecular de 50 pb; 6: genotipo CC; Carril 1, 2, 3, 7 y 8: Genotipos GC; Carril 4 y 5: Genotipo GG