Introduction

*H. pylori* infects about 50% of the human population, colonizing the gastric epithelial mucosa for decades and in most cases causing an asymptomatic mucosal inflammatory response. However, a fraction of infected people develop peptic ulcer, gastric carcinoma or MALT-lymphoma. Epidemiological studies suggest that *H. pylori* infection is acquired early in life and although transmission occurs preferentially within families, some studies suggest transmission from other households and even environmental sources in developing country settings. This very mode of transmission suggests that *H. pylori* may have evolved mechanisms to adapt to its different hosts in various human ethnic groups. In fact, considerably evidence shows that *H. pylori* has co-evolved with humans for thousand of years and has been intimately associated with modern humans, even before migration out of Africa some 50 to 70,000 years ago [1]. *H. pylori* displays a genetic diversity that reflects geographic and ethnic separation between the human groups it colonizes. Analyses of genetic diversity and genotyping of *H. pylori* isolates is often accomplished by comparing sequences of several housekeeping or virulence associated genes, or by analyzing total gene content with whole genome-microarrays. In addition, different predominant allele types of *H. pylori* virulence genes such as *vacA*, *cagA*, *hspA* and *opiA* are associated with different human ethnic groups [3], [4], [5]. For instance, *vacA* signal
sequence $sle$ type is found mainly in *H. pylori* from East Asia but not Africa, Europe, or the Americas, whereas $sfb$ is commonly found in *H. pylori* from Spain, Portugal and Latin-America. Likewise, the $3'$ region of the cagA gene is highly polymorphic, and this translates in distinct patterns at the carboxy terminal of the protein. The term “ABD” has been used to define the amino acid sequences flanking EPIYA (tyrosine phosphorylation) motifs that are prevalent in East-Asian isolates; whereas an ABC pattern is typical of most *H. pylori* from Western regions. In addition, an inserted segment, ins180, is more frequent in African and African-American strains than in others [6], especially East Asian strains. Studies on the population genetics of *H. pylori* have improved considerable human typing.

Previous reports studying *H. pylori* phylogeny generally have not characterized their human host [2], [3], which is particularly relevant when studying indigenous groups, whose exact ancestries may not be known. There is also a need to extend these studies to more diverse indigenous communities across the world to better define adaption, evolution, and disease associated with this infection. Thus, the aim of our study was to genetically characterize both human individuals and their *H. pylori* isolates from different indigenous groups of Mexico and to compare their virulence and housekeeping genes with those of *H. pylori* strains from other human populations.

**Results**

A total of 208 volunteers who still spoke their native language (Table 1) were screened for this study. Of these only individuals with an O-Rh+ blood group phenotype were included to reduce the probability of including outmixed (mestizo) individuals. In the end 191 individuals (91.8%), each from a different family were selected for analyses. One individual sampled in the Huichol community turned out to be native from the Otomi group and was included in the study (23O).

**Amerindian mtDNA, Y-chromosome, and STRs genotypes are highly frequent in the three Native groups**

Typical Amerindian mtDNA haplogroups (A, B, C, and D) were found in the Mexican Natives studied, with some differences among groups. Haplogroup A was at higher (35%) frequency in Nahau, than in Huichols and Tarahumaras, whereas haplogroups B and C were more common among Huichols and Tarahumaras. Haplogroup D has a generally low prevalence (Table 1). In addition, we found one individual from the Tarahumara group with haplogroup X, which is common in some natives from North America, especially Alaska [8–9]. Table 1 also describes frequencies of mtDNA haplogroups in Mexican and other groups reported previously and the previous data are in accord with ours, and emphasizes that Mestizo, Caucasian and Asian groups tend to differ in these mtDNA markers [10,11,12,13].

Among Y-chromosome markers a higher frequency of DYS19T (characteristic of Amerindians) was observed in the Huichol and Nahau than in the Tarahumara group (Table 2). Previous studies have also reported higher frequency of this allele in Huichol and Nahau than in Tarahumaras and also shown it to be even lower in Mestizo groups (Table 2) [14,15]. The DYS19T allele frequency is very low in Europe, and moderately low in North Asia (Table 2) [16]. Thus, our results document Amerindian markers in all three populations, with the Huichol showing evidence of higher genetic isolation than the Tarahumara and Nahau groups.

To further characterize these groups, we also studied 15 informative STRs alleles. The alleles most frequent in our three Mexican groups also are typical Amerindian populations (Table 3). The frequencies of the STR alleles found in this study are in agreement with previous studies in Mexican Native communities [17,18], this is further detailed in Table S1. Of special interest to us were STR markers of persons colonized with Asian-related *H.

### Table 1. Native language and frequency of mtDNA haplogroups in the Mexican Indigenous populations of this study, contrasted with previous studies in Mexican and other populations.

| Population         | Language | mtDNA Haplogroups, percent |
|--------------------|----------|---------------------------|
|                    |          | A  | B  | C  | D  | other |
| **This study:**    |          |    |    |    |    |       |
| Nahua              | 105      |    |    |    |    |       |
| Huichol            | 14       |    |    |    |    |       |
| Tarahumara         | 68       |    |    |    |    |       |
| **Previous studies:** |          |    |    |    |    |       |
| Nahua Atoconap $^a$ | 59       |    |    |    |    |       |
| Otomi SnAnt $^b$   | 38       |    |    |    |    |       |
| Mexican Mestizo $^c$ | 270     |    |    |    |    |       |
| **Other populations $^d$** |        |    |    |    |    |       |
| Caucasus           | 1        | 0.5 | 4  | 4  |    | 90.5  |
| East Asia          | 7        | 16  | 5  | 26 | 46  |
| Africa             | 0        | 0   | 0  | 0  | 100 |

*a, Peñaloza-Espinosa et al. [11]; b, Sánchez-Rojo et al. [12]; c, Guardado-Estrada et al. [13]; d, http://www.mitomap.org/bin/view.pl/MITOMAP/HaplogroupMarkers [14].

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Table 2. Frequency of polymorphisms in DYS19T of the Y-chromosome in Native and Mestizo Mexican Populations and other populations.

| Population          | Num. studied | DYS19T (%) |
|---------------------|--------------|------------|
| This study:         |              |            |
| Nahua               | 31           | 74.2       |
| Huichol             | 4            | 100        |
| Tarahumara          | 21           | 33.3       |
| Previous studies:   |              |            |
| Huichol             | 34           | 100        |
| Tarahumara          | 20           | 55.0       |
| Nahua               | 34           | 79.5       |
| Guerrero/Mestizos   | 4            | 25.0       |
| Western/Mestizos    | 191          | 17.3       |
| Other populations   |              |            |
| Native Americans    | 588          | 76.4       |
| Europe              | 237          | 0.4        |
| North Asia          | 669          | 17.9       |

* Rangel-Villalobos et al. [15];
* Bonilla C. et al. [16];
* Zegura et al. [17].

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Phylogenetic analyses suggest the existence of specific cagA and vacA Amerindian clusters

Phylogenetic analyses of vacA confirmed the grouping of 368H Huichol strain outside the West s1a and s1b clusters, and placed it in a cluster related to, but not within the East-Asian group (Figure 2). Interestingly, this cluster included also isolates from indigenous communities of Alaska, Colombia and Peru, indicating the existence of a novel vacA Amerindian cluster. In contrast, strain 23O, which also seemed Asian like in its cagA D motif, contained a Western-like vacA s region allele, as did several Colombian isolates (NA1764, NA1766 and NA1768 in Figure 2).

In the phylogenetic analyses of cagA, most Mexican indigenous isolates were in the Western group, except for the Huichol 368H and Otomi 23O strains, which clustered in a group related to the East-Asian group (Figure 3). This was expected since the cagA sequence of these strains presented a mixture of Western and East-Asian motifs. It is noteworthy that indigenous isolates from Colombia, Peru and Venezuela, clustered with this two unusual isolates forming two subgroups (Figure 3), like those recently described and designated as AM-I and AM-II, for Amerindian subgroups [20].

Phylogenetic analyses of hspA show evidence of Asian or African ancestry for some Mexican isolates

Phylogenetic analysis of hspA showed that isolates from the three Mexican indigenous groups were present in all, Asian, European and African groups (Figure 4). Still, Nahua were more prevalent in the Europe group and Tarahumara in the Asian group. In addition, there were strains from native groups of Canada and Venezuela, which also grouped within the Asian cluster. A few isolates from the three Mexican communities clustered within the African group, together with isolates from aboriginals of Venezuela, Guatemala and Brazil.

Consistent with the phylogeography observed with cagA and vacA, the indigenous strains 368H and 23O clustered within the Asia group of hspA (Figure 4).

Results with the MLST analyses confirmed the existence of an Asian-related Amerindian H. pylori group

As depicted in Figure 3, the MLST analyses of concatenated six housekeeping genes showed that many of the Mexican isolates clustered within the European group, as did isolates of mestizo population from other Latin-American countries. However, the analyses also showed that two strains, one from the Otomi (23O), and one from the Tarahumara (590T) isolates were in the Western group, except for the Huichol 368H and Otomi 23O strains, which clustered in a group related to the East-Asia group of hspA (Figure 2c). In the individual analyses of the six housekeeping genes 390T presented the Amerindian allele in the six genes whereas 23O presented Amerindian allele in three genes for the Asian in one.

Five other strains, two Huichols, two Tarahumaras, and one Nahual located in a position between the European and the Asian groups (Figure 5), at a unique intermediate genetic distance not observed in any strain from all the other population groups. Of these, the 368H had the cagA Amerindian type and an Amerindian allele in two housekeeping genes, whereas strains 602T and 383T presented Asian allele in four and three housekeeping genes respectively.

Pylostrains marked in grey in Table 3, one Huichol (368H), one Otomi (23O) and one Tarahumara (590T). Table S1 shows that their STR alleles were not among the most frequent in these groups, and confirms that these three individuals have Amerindian markers uncommon in the rest of the aborigines studied.

vacA and cagA alleles of H. pylori from native Mexican communities

In total we recovered and genetically analyzed H. pylori from 35 study participants: 18 Nahua, 11 Tarahumaras, 5 Huichols and one Otomi (living in a Huichol community) (Table 4), and the GenBank accession numbers of vacA and cagA sequence are in Table S2. At the vacA locus, most strains contained European-type s1b signal sequence and m1 mid region alleles. However, four of the five Huichol isolates were s1b m2, and a few Nahua and Tarahumara isolates (4/18, 3/11, respectively) were of the putatively less virulent vacA s1b m2 type (Table 4). We also note, that one exceptional Huichol isolate (368H) contained an s1c-type allele.

All but four of our 35 isolates contained a cagA virulence gene, which, in most cases seemed to be Western-like in sequence at the critical carboxy terminal region, although it was generally smaller than that reported by others in Mexican-Mestizo isolates [19]: 492 to 615 codons vs. 500 to 850 codons in ref 20. In fact, most of the CagA proteins encoded in our strains contained just three EPIYA-type repeats and one CM motif (Table 5). Several strains [ten] contained variant EPIYT B-type (Figure 1) and/or variant CM motifs (most often, K, not R, at position 5, Figure 1). A few strains, especially from Tarahumara people, contained two CM motifs (Figure 1). Of particular note were two isolates with a variant GISYD B motif, a distinctive D-like motif and a partially deleted CM motif – strains 23O and also 368H, which also contains vacA s1c-type allele (Figure 1). This type of peculiar CagA sequence has also been reported in strain NA1692 from an indigenous Colombian (Figure 1).
Table 3. Frequencies of STRs alleles in three Mexican Native populations*.

| Allele | D8S1179 | D21S11 | D7S820 | CSF1PO | D3S1358 | D13S317 | D16S539 | D2S1338 | D19S433 | D18S51 | D5S818 | FGA |
|--------|---------|--------|--------|--------|---------|---------|---------|---------|---------|---------|---------|-----|
| 6.3    | --      | --     | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 7      | --      | --     | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 8      | --      | --     | --     | --     | 0.0172  | 0.0333  | --      | --      | --      | --      | --      | 0.1333|
| 9.3    | --      | --     | --     | --     | --      | 0.3000  | 0.1207  | --      | --      | --      | --      | 0.0167|
| 10     | --      | --     | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 11     | --      | 0.1552 | 0.1964 | --     | 0.2167  | 0.2241  | --      | --      | --      | --      | --      | 0.0333|
| 12     | 0.0476  | --     | 0.3276 | 0.5714 | --      | 0.0833  | 0.3793  | --      | --      | --      | 0.0345  | 0.1500|
| 12.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 13     | 0.3810  | --     | 0.0600 | 0.0357 | 0.1167  | 0.1379  | --      | --      | 0.1667  | 0.2069  | 0.0833  | --  |
| 13.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | --      | 0.2000  | --  |
| 14     | 0.2540  | --     | 0.0357 | 0.0167 | 0.0500  | --      | --      | --      | --      | 0.1500  | 0.2069  | --  |
| 14.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.0500  | --      | --  |
| 15     | 0.1111  | --     | --     | --     | 0.5500  | --      | --      | --      | --      | 0.1500  | 0.1552  | --  |
| 15.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.1000  | --      | --  |
| 16     | --      | --     | --     | 0.3333 | --      | --      | --      | --      | --      | 0.0690  | --      | --  |
| 16.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.1167  | --      | --  |
| 17     | 0.0159  | --     | 0.1000 | --     | --      | 0.0500  | --      | --      | --      | 0.2241  | --      | --  |
| 17.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.0667  | --      | --  |
| 18     | --      | --     | --     | --     | --      | --      | --      | 0.1000  | --      | 0.0690  | --      | --  |
| 18.2   | --      | --     | --     | --     | --      | --      | --      | 0.1667  | --      | 0.0172  | --      | 0.2414|
| 19     | --      | --     | --     | --     | --      | --      | --      | 0.1667  | --      | 0.0172  | --      | 0.2414|
| 20     | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.1833  | --      | 0.0345|
| 21     | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.0167  | --      | 0.0862|
| 22     | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.1667  | --      | 0.1207|
| 23     | --      | --     | --     | --     | --      | --      | --      | 0.2167  | --      | --      | --      | 0.0517|
| 23.2   | --      | --     | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 24     | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.0833  | --      | 0.1897|
| 25     | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.0167  | --      | 0.0862|
| 26     | --      | --     | --     | --     | --      | --      | --      | --      | --      | 0.0167  | --      | 0.1379|
| 27     | --      | 0.0161 | --     | --     | --      | --      | --      | --      | --      | --      | --      | 0.0345|
| 28     | --      | 0.0161 | --     | --     | --      | --      | --      | --      | --      | --      | --      | 0.0172|
| 29     | --      | 0.2097 | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 30     | --      | 0.2742 | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 31     | --      | 0.0161 | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |
| 32     | --      | 0.0161 | --     | --     | --      | --      | --      | --      | --      | --      | --      | --  |

*Letters in bold mark the more frequent alleles; in italics are the alleles distinguishing the three Native strains, 368H, 23O, and 590T.

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Activity of indigenous strains on AGS cells

Because of the peculiar sequence in virulence genes, we explored the genomic content of the *H. pylori* Huichol 368H isolate using microarray based comparative genomic hybridization (a-CGH). Of the 1,660 genes analyzed, 1,339 were present in the isolate (genome core) and 117 (7.04%) genes were absent and were considered as variable genes. Of these 117 variable genes, 41 (35%) were distributed within the previously described plasticity zones (PZs) [22], 21 within the PZ1 and 20 within the PZ2; the remaining 76 genes were scattered along the genome (Figure 6A). Results with the 368H strain were compared with those previously reported in Mexican mestizo groups (Figure 6B). Important differences were found in the variable genes, and the total number of genes absent in 368H was significantly lower than in the mestizo groups (Table S3) and the difference was mostly observed in the PZs and cag PAI (Figure 6B). In fact, as much as 225 genes reported as variable in mestizo groups were more conserved, and a concordance analyses showed that PZ1 was the zone with higher disagreement between the mestizo strains and 368H.

Activity of indigenous strains on AGS cells

Because of the particular sequence of *cagA* in the 368H and the 23O strain, as well as the difference in gene content observed in microarrays with the 368H strain, we tested the activity of these isolates on AGS cells (Figure 7). Since both strains have a highly modified EPIYA-B motif (GSIYD) and a single CM motif with deletions and partial homology to the Asian motif (Figure 1), we expected a reduced activity on cells. Indeed, whereas 368H and 23O caused a poor elongation after 24 hs of incubation (Figure 7a and 7b), the strain from a Mexican mestizo caused a marked cell elongation (Figure 7c). The two strains were able to adhere to the cell surface after 6 hs of co-culture, although the pattern of adherence of both strains differed from that observed in isolates from a Mestizo individuals; 368H and 23O strains formed microcolonies around the cell (Figure 7d and 7e), whereas bacteria of the other Mestizo strain scattered across the AGS surface (Figure 7f). Both 368H and 23O strains caused a marked induction of IL-8 (666 and 681 pg/ml, respectively). We tested two Tarahumara strains that were cagPAI negative (594T and 582T), both adhered to the cell with a scattered pattern, but none were positive for hummingbird effect or IL-8 induction (results not shown).

**Discussion**

Evidences of mtDNA and Y-chromosome confirm a mixture of Asian-Amerindian and African ancestry in the native groups studied

In this work we aimed to learn about ancestry in the native groups studied for *H. pylori* infection in order to genetically characterize both, the host and the bacteria. It is known that the Native American groups are usually characterized by different proportions of mtDNA haplogroups A, B, C, D and X [23, 24]. In the present work, we found the presence of all mtDNA Amerindian haplogroups reported in both, North and South America [9], which confirms a Native-American maternal
ancestry in the three Mexican groups studied. We found the haplogroup B as the most frequent, followed by haplogroup C, haplogroup A and haplogroup D, respectively. We found the haplogroup X only in one Tarahumara individual, an haplogroup which has been reported in some native groups of North America [10]. This finding confirms previous reports on the presence of this uncommon haplogroup in a few individuals of the same Tarahumara groups, which live in the North part of Mexico [25].

For the polymorphisms in the Y-chromosome, the DYS19T marker was the most frequent in the Mexican groups studied, similar to reports in other Native American communities [17]. This polymorphism was present in all Huichol individuals tested (West of Mexico), in most of the Nahua population (Center of Mexico), and to a lesser extent in the Tarahumara group (North of Mexico). These frequencies are similar to previous studies in Mexican native and mestizo groups, and confirm the admixture of pre-Spaniard native Mexican communities with European population, and to a much lesser extent with African markers [26]. The frequency of STRs alleles in the populations studied also demonstrated similarities with previous studies in Mexican Natives [18,19]. Thus, genotyping of individuals from the three Mexican native groups studied document that although all have Amerindian markers, each population is different based on the frequency of mtDNA, Y-chromosome and STRs markers. Although these are not novel findings, the relevant fact in our study is that we confirmed that the indigenous Mexican groups studied conserved many Amerindian alleles. Still, we should note that no significant differences are observed when contrast with Mexican mestizo population, particularly with the STRs alleles, a finding which has been reported previously [18] and which suggest that Mexican mestizo population still conserve many Amerindian genetic markers. A few strains from the Tarahumara and Huichol groups had Amerindian STRs alleles that were uncommon in the rest of the population studied, which is in agreement with the fact that these strains presented also Amerindian types in housekeeping and/or virulence genes (see below).

**Polymorphisms in virulence genes vacA and cagA point to a discrete Amerindian type**

Similar to results with the human genotypes, polymorphisms in virulence genes of the *H. pylori* infecting strains also differed among the populations. Thus, in the Nahua group the most prevalent vacA allele was s1bm1, which is similar to what we previously observed in Mexican mestizo groups [27] and it is also the genotype more often observed in populations of Latin America. This observation is also in accordance with the fact that in the Nahua group we observed the higher frequency of the mtDNA haplogroup A, which is also the most prevalent in Mexican-mestizo groups and reflects the outmix of Spaniards and Native Americans [26]. In contrast, the vacA haplogroup s1m2 was highly prevalent in the Huichol group, which also presented the higher frequency of the mtDNA haplogroup B. In addition, the 368H Huichol strain presented an s1c-related allele, characteristic of East-Asian *H. pylori*. These results were confirmed with the phylogenetic analyses of the s region sequence, where we observed that 368H grouped in a cluster closely related to the East-Asian group. Furthermore, in this Asian-like group were included strains from Natives of Alaska, Mexico, Peru and Colombia and represent a novel vacA Amerindian group not previously described.

Concerning CagA, most of the indigenous Mexican isolates shared homology with a Western type sequence, with most strains having an ABC-2CM motifs pattern; which contrasts with the ABCG-3CM pattern which we previously observed in several isolates from Mexican-mestizos living in Mexico City [20].

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**Figure 1. Alignment of amino acid sequences of the CagA 3’ region of *H. pylori* strains from Mexican native groups.** a) Most strains presented a Western-type sequence, with an EPIYA ABC pattern and two CM motifs. The sequence of strain 26695 was used as reference. b) One Huichol strain, 368H and one Otomi strain, 23O presented a chimeric sequence with some homology with Asian-type sequences, with insertions, deletions and substitutions and similar to the Colombian strain NA1692. Sequences are compared with the Japanese isolate OK113. doi:10.1371/journal.pone.0027212.g001
However, two isolates, one from Huichol (368H) and one from Otomi (23O) groups presented a chimeric CagA 3' region with partial homology with the East-Asian D motif and with one partially deleted CM motif, which showed segments of partial homology with both, the Western and the East-Asian CM motif. Of note, some *H. pylori* isolates from indigenous Colombia, Venezuela and Peru groups [4,28] also presented a chimeric 3’region with high homology with the 368H and 23O Mexican isolates, showing evidences of similar Western-Asian recombination in *H. pylori* Amerindian groups of both, North America and South America. In the phylogenetic analyses of CagA, 368H and 23O clustered together with indigenous isolates from Colombia, Venezuela and Peru and formed a group related to, but not intermingled with the East-Asian group, which might represent a novel Amerindian group of CagA, similarly to what we observed in the case of VacA (see above). Independently Suzuki et al. [21] documented specific CagA sequences in strains from the remote Peruvian Amazon that are similar to those reported here, and placed their sequences in two sub-groups designated as AM-I and AM-II. The cagA genes of our Mexican Native strains 368H and 23O are of the AM-I sub-group. This supports the view that these unusual alleles were likely widespread in Amerindian populations. Thus, our results confirm the important report by Suzuki et al [21] and extend the finding to isolates from indigenous groups of Mexico and Colombia.

In addition, as indicated above, we also suggest the existence of an Amerindian type of vacA, formed by the same strains included in the cagA Amerindian group. This would represent a novel group of cagA and vacA present in strains colonizing indigenous Amerindian groups across America (Amerindian cagA and vacA). These observations in *H. pylori* strains encompassing North and South America (as exemplified with isolates from Alaska, Mexico,
Venezuela, Peru and Colombia) suggest the presence of a discrete group of Amerindian strains with frequent recombination events in \textit{vacA} and \textit{cagA}, most probably the result of adaption to the human groups who populated the Americas some 30,000 years ago [29]. Thus, our results represent current traces of the Asian-Alaska-North America-South America population wave of the Americas.

Analyses of HspA show admixture of African, Asian and European genes in American Indigenous isolates. HspA is informative about African ancestry [5,6] and is a gene with a vital role as co-chaperone and as a stress response protein [30,31]. We analyzed this gene and identified traces of African ancestry among a few of the Mexican indigenous strains, and in Native strains from Central and South America, and may represent remnants of the primary African origin of \textit{H. pylori}, documented in previous studies [1], or traces of the migrant African groups which came as slaves. The analyses also showed Asian ancestry for the Amerindian strains 368H, 23O and 590T and for isolates from other Indigenous groups from South America and Canada, which is consistent with our findings in the 3′ region of \textit{cagA}, and provides an independent support for Asian ancestry in Native groups across America. By looking at the phylogenetic pattern of \textit{hspA} it might be suggested that this gene has been more conserved than \textit{vacA} or \textit{cagA} across human migrations, probably because less selective pressure, or because of differences in recombination rates.

The MLST analyses further support an Asian-related Amerindian group with indigenous strains from Mexico and Peru. The MLST analyses of housekeeping genes have shown to be a robust and consistent test to study ancestry and evolution of \textit{H. pylori} populations [2]. Applied to the Mexican native isolates,
although most strains from the three native groups studied were placed within the European group, we also confirmed the Asian ancestry of one Tarahumara and one Otomi strains. Recently, Kersulyte et al. [32] reported that strains from remote Amazon were related to those from Asia, suggesting they descend from *H. pylori* infecting Asian people who migrated to America some 30,000 years ago [29]. Still, isolates from this Shimaa community clustered in a discrete separated group from East-Asia isolates, documenting a separate evolutive adaption to this geographically distant region. Of particular interest is the observation that the two Mexican isolates in the Asian branch also clustered with the discrete group formed by the Shimaa isolates, suggesting a common ancestry for these Amerindian groups across America, and the presence of similar selective forces in these indigenous
Latin-American communities located in North America (Mexico) and South America (Peru).

Some Mexican isolates did not cluster with either, the European or the Asian groups, but localized at an intermediate genetic distance, suggesting that they represent Western-Asian recombinant strains, probably in the process to evolve from an Amerindian to a European genetic type. This localization was exclusive for native Mexican strains, not observed in any strain from the other populations studied, and might represent living evidence that Amerindian strains are being displaced by European strains [32].

It should be noticed that the three Mexican strains with Amerindian type in housekeeping genes and virulence cagA and vacA genes, 590T, 368H and 23O were isolated from

Figure 5. Phylogenetic analyses of concatenated six housekeeping genes (Multilocus sequence typing). Results show that although many of the Mexican isolates clustered within the European group, two strains (23O and 590T) clustered within a group closely related to the East Asian, together with other H. pylori isolates from Indigenous Amazon people from Peru which is now identified as a novel Amerindian group.

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indigenous people with Amerindian STRs alleles uncommon in the rest of the Native people studied, which would be in agreement with a selection of strains based on the genetics of the host, leading to the suggested co-evolution of \textit{H. pylori} with human groups.

Gene content and activity on cells of the Huichol 368H strains differ from Mexican-mestizo strains

We analyzed the gene content of the 368H strains with the same microarray system we used previously to study Mexican mestizo strains [33], with the main finding being that the variable genes...
present in mestizo strains were significantly less present in the Huichol strain, mostly in the PZs and cag PAI, which would suggest a modified activity of this strain on epithelial cells. Our results did confirm a reduced elongation activity of the 368H and 230 strain on AGS cells, which would be in agreement with the fact that these strains have a modified EPIYA-B motif [20]. Still, they were able to induce IL-8 with values similar to those observed in strains from mestizo patients with duodenal ulcer or gastric cancer [20]; similar results were recently reported for the Venezuelan strain v225 d, which presents homology with 368H in the 3’ region of CagA [28]. Both strains were able to bind and reproduce on epithelial cells, although using an adherence pattern different from that observed in other strains from Native or Mestizo Mexican individuals, suggesting important differences in the way they interact with epithelial cells. Thus, in spite of having a modified EPIYA and CM motifs, these strains still displayed activity on AGS cells.

Conclusions

This study describes genotyping of virulence and housekeeping genes in $H. pylori$ strains from Mexican indigenous groups and shows that Mexican Natives with Amerindian genes are infected with $H. pylori$ strains with traces of Asian or African ancestors. The study shows novel alleles in cagA and vacA virulence and in housekeeping genes, particularly in communities genetically more isolated. In addition these Amerindian types were found in strains of indigenous groups from North to South America. These results call for more studies on $H. pylori$ strains from Amerindian groups to better understand their co-evolution in the new world races and eventually learn more about this adaption and its consequences for disease.

Materials and Methods

Ethics statement

This work was approved by the National Ethics Committee for Research of the Instituto Mexicano del Seguro Social, Mexico. Volunteers were included in the study after they were informed of the nature of the study and signed a consent form.

Population studied

One hundred and eighty nine individuals from different Mexican indigenous groups were studied, none of the groups were accessible by public transport: 105 Nahua from San Pedro Tlacotenco and Santa Ana communities nearby Mexico City, reached by not paved roads, but with access to public transportation; 32 Huichol from Sierra de Guadalajara in the West states of Jalisco and Nayarit, reached only by foot trail and no public transportation; and 52 Tarahumaras from Bahuichivo and Bocina in the North sate of Chihuahua, located in a large canyon reached by uneasy foot trails (Figure S1). In addition, two Otomi native individuals living in the Huichol community were included. People were informed about the nature of the study and those willing to participate signed an informed consent.

Biological Samples

All biological samples were taken from volunteers at their respective community sites. Four ml of peripheral blood were drawn in a tube with EDTA and kept on ice during transportation to the central lab. The string test was applied to obtain gastric juice for isolation of $H. pylori$ as previously described,[34]. In brief: once extracted from the volunteers, the string was immediately inoculated on blood agar plates with antimicrobials, packed in Jars with Campy-pack CO2 generators (Beckton Dickinson Co., Sparks MD USA) and transported to the central lab for isolation of $H. pylori$.

Genotyping of native individuals in blood samples

ABO and Rh(D) blood groups were determined in blood samples using commercial antisera (Ortho Diagnostics, Raritan NJ). DNA was isolated from white blood cells and stored at −20°C until tested for mtDNA types and in the case of male volunteers for polymorphisms in the Y chromosome. Four different mtDNA regions were amplified by PCR using primers and conditions previously described [35], and cut with restriction enzymes: HstIII for haplotype A, HincII for haplotype C, and AluI for haplotype D, and the resulting restriction fragments were analyzed by electrophoresis in 2% agarose gels. The haplogroup B was analyzed in 8% polyacrylamide gels electrophoresis. In order to study polymorphisms in Y-chromosome, two loci, DYS19 and DYS199 were analyzed for a polymorphic tetranucleotide microsatellite, and for a biallelic marker (defined as M3) using primers and conditions reported previously [36]. The M3 PCR product was digested using MstI, and the resulting restriction fragments were analyzed by electrophoresis on polyacrylamide gels. In addition, DNA samples were studied for STRs markers, and D8S1179, D21S11, D7S820, CSFIP1O, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA loci along with amelogenin gene fragment were co-amplified in a multiplex PCR reaction using the AmpFISTR Identifiler TM Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocols. The amplified products, together with reference allelic ladders were analyzed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Capillary electrophoresis results, as well as allele determination, were analyzed using GeneMapper1 software Version 3.5.

$H. pylori$ isolation and genotyping

The blood-agar plates with the string-extracted gastric juice were incubated at 37°C in a 10% CO2 atmosphere. From the primary growth, a single colony was picked up and propagated; and the 24 h growth was swept for DNA isolation. Primers used for amplification and sequencing of vacA, cagA and hspA are presented in Table 6. For sequencing, the vacA, hspA, and cagA PCR products were purified (Rapid Gel extraction systems, Marligen Bioscience, U.S.A.) and sequencing was performed by the dyeoxyxynucleotide chain termination method with a BigDye Terminator Cycle Sequencing kit (Version 3.1, Applied Biosystems, Rockland, USA) in an ABI PRISM 377 automated DNA sequencer (Applied Biosystems) as previously described [37]. The nucleotide sequences were analyzed by Chromas software (version 1.62; Tecnelysium) and aligned with DNAMAN program (Version 3.0, Lonnyn BioSoft). All sequences were registered in GenBank and accession numbers are detailed in Table S2. Phylogenetic trees were constructed using the Neighbour-joining method with Kimura two-parameter distances, using MEGA V2.0 and 500 bootstrap samplings. PCR of representative $H. pylori$ housekeeping genes, sequencing of PCR products and phylogenetic analyses of sequences obtained (MLST; multilocus sequence typing) were carried out as described [32]. The housekeeping gene sequences from strains of countries other than Mexico, included here for comparison, are the same as those used in ref 32.

Microarray Experiments

The microarray used represents the superset of genes present in $H. pylori$ strains 26695 and J99 whole genome sequences, as
previously described [38]. For the test, 0.5 μg of each of 26695 and J99 genomic DNA were denatured and five μl of 10X Buffer (400 μg/ml random octamers, 0.5 M Tris-HCl, 100 mM MgSO4 and 10 mM DTT), 5 μl dNTP/dUTP mix (0.5 mM dGTP, dATP, dCTP, 0.2 mM aminoallyl dUTP and 0.3 mM dTTP) and 1 μl Klenow were added and the reaction incubated overnight. Free amines were removed and the probe (mixed J99 genomic DNA were denatured and five μl yeast tRNA, 1.5 μM (400 G) was labelled with Cy5 dye (Amersham). One μg of genomic DNA from each test strain was labeled with Cy5 dye (Amersham). One μg of genomic DNA from each test strain was labeled with Cy5 dye (Amersham). One μg of genomic DNA from each test strain was labeled with Cy5 dye (Amersham). Labeled probe and test DNA were combined and unincorporated dye removed, and 1 μl of 10 mg/ml yeast tRNA, 1.5 μl of 20X SSC and 1.5 μl of 1% SDS were added. The mixture was denatured, applied to the microarray slide and incubated overnight. The microarray slide was washed with 2X SSC and 0.1% SDS and then with 1X SSC for 5 minutes, three times. The microarray was scanned using an Axon scanner with GENEPIX 3.0 software (Axon Instruments, Redwood City, CA) and data were normalized and processed as previously described [38]. The mean of the normalized red/green (R/G) ratio was calculated using data from two arrays per isolate, yielding four readings for each gene. The cut off for absence of a gene was defined as a log2 (red/green) of < -1.0 based on test hybridizations using the sequenced strain J99 against the 26695/J99 mixed reference. The false positive and false negative rates were determined to be 3.5% and 0.34%, respectively [39]. All data is MIAME compliant and the raw data has been deposited in GEO (number GSM609344).

Activity on AGS cells

For the cell assay 1×10⁵ AGS cells/ml were grown in 6-wells plates with F12 medium and 10% fetal bovine serum during 48 h. H. pylori strains to be tested were grown for 48 h in blood agar plates and a single colony was re-seeded on agar plate and incubated for growth during 24 h. H. pylori growth was harvested, and suspended in serum free F-12 medium, to reach an optical density of 0.1 at 550 nm (1.2×10⁸ bacteria/ml) before addition to AGS cells at a multiplicity of infection (MOI) of 1:100 (cell:bacteria). H. pylori strain 26695 was used as positive control [20]. To monitor morphologic effects, co-cultures in 6-wells plates was incubated for 24 hs, and observed for morphological changes in a microscope. The pattern of adhesion of H. pylori to cells was observed after 3 hs of co-culture, and induction of IL-8 was determined after incubating co-cultures for 6 hs, after this time concentration of IL-8 in the culture media was estimated using a commercial ELISA assay (BD Biosciences, San Diego, CA).

Supporting Information

Figure S1 A map of Mexico with the location of the three Native groups studied, and location of the closest city for reference. (TIFF)

Table S1 Describes the STR alleles present in thed individuals from the three Native Mexican groups studied, Nahuat, Tarahumara and Huichol. (DOC)

Table S2 Describes the GenBank accession numbers of the sequences from Mexican Native strains reported in this study. (XLSX)

Table S3 Concordance analyses in the content of variable genes in the plasticity zones between the 368H Huichol and mestizo strains from patients with gastric cancer, gastritis and duodenal ulcer. (DOC)

Author Contributions

Conceived and designed the experiments: JT MCP GPP GGV. Performed the experiments: GGV MCP IM RPE CR IR LM DK JG. Analyzed the data: ARL GPP JT DB JG MCP. Wrote the paper: JT GPP. Contributed in writing the paper: MCP RPE DB.

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