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Genotyping of *Leptospira interrogans* isolates from Mexican patients

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ABSTRACT

The aim of this study was genotypically characterize *Leptospira* sp. clinical isolates from Mexico which were previously identified as *Leptospira interrogans* serovar Pomona (POM) by phenotypic methods. The Random Amplified Polymorphic DNA (RAPD) method was used for DNA amplification with five oligonucleotides. A dendrogram was constructed using the Unweighted Pair Group Method Analysis (UPGMA). During the genotypic characterization, the studied isolates constituted a group which was associated with the reference strain *L. interrogans* serovar Pomona. The Minimum Spanning Networks (MST) analysis revealed the same cluster between Mexican isolates and the reference strain POM. Clinical isolates identified as *L. interrogans* serovar POM have a clonal reproduction type, suggesting that this clone is distributed in different regions of Mexico.

KEYWORDS: *L. interrogans* serovar POM. RAPD. UPGMA. Minimum Spanning Networks.

INTRODUCTION

Leptospirosis is caused by spirochetes that belong to the genus *Leptospira,* phylum Spirochaetes, order Spirochaetales and family *Leptospiraceae*[1]. Leptospirosis is a zoonosis of broad global distribution and is common in tropical and subtropical areas, although it is not rare in temperate zones[2]. Leptospirosis is transmitted to humans by contaminated mud and/or water or by direct contact with the urine of infected animals[1]. Cases of transmission from humans to animals have also been reported[3].

According to data reported by the World Health Organization (WHO), more than 500,000 cases of severe leptospirosis occur each year, with lethality rates higher than 10%. However, the burden of the disease is significantly underestimated due to limited epidemiological data and to the low sensitivity of standard diagnostic tests (culture and the microscopic agglutination test), which makes the diagnosis difficult[4]. In Mexico, according to the General Directorate of Epidemiology, the epidemiology of leptospirosis revealed a national rate of 0.65 cases per 100,000 inhabitants by the year 2000, and 45 by 2010, being stable over the last 10 years, according to the Handbook of standardized procedures for the epidemiological surveillance of leptospirosis[5].

The traditional taxonomic system, based on serology, divides the genus *Leptospira* into two species: *Leptospira interrogans* (pathogenic) and *L. biflexa* (non-pathogenic). These species are further divided into 26 serogroups, over

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300 serovars and strains, based on shared antigens[4,6,7]. Although this system has great epidemiological value, nowadays, molecular methods are needed for identifying and classifying the genus *Leptospira*[8]. The analysis based on DNA has identified 22 *Leptospira* species with nine main pathogenic species (*L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. kirschneri*, *L. alexanderi*, *L. alstonii*, and *L. kmetyi*) and six non-pathogenic species[9,10].

Thus, the characterization of *Leptospira* strains has evolved to more reliable and robust modern methods, including RFLP (Restriction Fragment Length Polymorphism)[11], PFGE (Pulsed-Field Gel Electrophoresis)[11,12], REA (Restriction Enzyme Analysis)[13], RAPD (Random Amplified Polymorphic DNA)[13,14], 16S rRNA sequencing[15], VNTR (Variable Number of Tandem Repeats) analysis, and MLST (Multilocus Sequence Typing), making it possible to obtain information about the molecular epidemiology of leptospirosis[12,16-21]. Among these techniques, RAPD has been used for identifying and typing *Leptospira* isolates. RAPD is a rapid, sensitive, safe and relatively simple technique; furthermore, the products obtained can be used in studies of phylogeny and population structure[8,14,18,19,22-24]. There are few genotypic characterization studies of *Leptospira* clinical isolates in Mexico (MX); therefore, some aspects of epidemiological importance remain unknown, such as the distribution of circulating clones in different regions of the country which could allow the understanding of the pathogen's transmission dynamics and hence, the implementation of adequate prevention and control measures. The aim of this study was to genotype clinical isolates of *Leptospira* sp. obtained from Mexican patients, using the RAPD method.

MATERIAL AND METHODS

Reference strains

Eleven reference strains (Table 1) were used. Strains were maintained in Ellinghausen and McCullough liquid culture medium modified by Johnson and Harris (EMJH) (Difco Laboratories, Detroit, USA) supplemented with SAVAT (Tween 80-bovine serum albumin, Difco Laboratories, Detroit, USA), at 28-30 °C.

Clinical isolates

In total, 89 primary cultures of *Leptospira* sp. obtained from Mexican patients with diagnosis of chronic leptospirosis were used. The isolates were phenotypically identified as *L. interrogans* serovar Pomona[25]. These primary cultures were sub-cultured in 3 mL of EMJH liquid culture medium (Difco), supplemented with SAVAT and de-complemented rabbit serum[3]. A 1:10 dilution of sample-culture medium was used and then incubated at 28-30 °C; samples were checked weekly, over six months, under a dark field microscope to ensure the development and adaptation to the culture medium[3,26,27]. The morphological study of *Leptospira* sp. was performed by a video recording apparatus using dark field microscopy (Carl Zeiss, Jena, Germany), with an immersion dark field condenser, at 400X magnification, connected to a high resolution video camera (Samsung, South Korea) and a screen (Sony, Japan)[3,28].

Table 1 - Reference strains of *Leptospira interrogans*, corresponding species and serovar.

Reference strains	Species	Serovar
ICT	*Leptospira interrogans*	Icterohaemorrhagiae
CAN	*Leptospira interrogans*	Canicola
POM	*Leptospira interrogans*	Pomona
AUT	*Leptospira interrogans*	Autumnalis
BRA	*Leptospira interrogans*	Bratislava
TA	*Leptospira interrogans*	Tarassoni
LAI	*Leptospira interrogans*	Icterohaemorrhagiae lai lai
PYR	*Leptospira interrogans*	Pyrogenes
BAL	*Leptospira interrogans*	Balum
SHER	*Leptospira interrogans*	Shermani
PTC	*Leptospira interrogans*	Ptc Patoc

Extraction of genomic DNA

The isolates and reference strains were cultured in 50 mL of EMJH for 7-10 days and centrifuged at 5600 g for 20 min at 4 °C, discarding the supernatant. Subsequently, the pellet was washed with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Immediately thereafter, cellular packages were heated at 80 °C for 10 min to inactivate cells; the recovered material was placed in 1.5-mL vials, resuspended in 200 µL of isotonic saline solution, and centrifuged at 10000 g for 5 min. The FastDNA® SPIN Kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions for DNA extraction. DNA concentration was determined by spectrophotometry and by 1% ethidium bromide-stained agarose gel electrophoresis (10 µg/mL) (Sigma-Aldrich, St. Louis, Missouri, USA). Different concentrations of λ phage (Invitrogen, Carlsbad, California, USA) were used as a reference. The isolated DNA was stored at 4 °C.

RAPD

For these assays, the O5 (5'-AGGGGTCTTG-3') oligonucleotide[19], the combination of B11 (5'-CCGGAAGAAGGGGCGCCAT-3') and B12 (5'-CGATTTAGAAGGACTTGCACAC-3') oligonucleotides[24], the M16 (5'-AAAGAAGGACTCA GCGACTGCG-3') oligonucleotide[14], and the PB1 (5'-GCGCTGGCTCAG-3') oligonucleotide[14] were used as described in Table 2.

Data analysis

RAPD resulting bands on different gels were statistically analyzed. RAPD markers were visually recorded, manually coded and translated into binary data that indicated either their presence (1) or absence (0). The genetic similarity between isolates was calculated with the Jaccard index. Genetic relationships among isolates were assessed using the mean of the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Distortion of the inferred tree was assessed with the cophenetic correlation coefficient (*CCCr*), which was calculated using the Mantel test[29]. A multidimensional analysis of minimum spanning networks (MST) was performed based on the original similarity matrix. Multivariate statistical methods were carried out using the NTSYS-PC program (version 2.0, Exeter Software, New York, USA)[30]. To distinguish clonal and recombinant structures in *Leptospira* isolates, the Index of Association (I_A), was used, which is a statistical test that measures the degree of non-random association between alleles at different loci (linkage disequilibrium)[31]. Therefore, I_A is zero in strictly recombining populations and 1 in strictly clonal populations. I_A was calculated using the LIAN 3.5 software[32].

RESULTS

Clinical isolates

Only 12 of the 87 primary isolates were adapted to the culture medium and showed characteristic spirochete morphology with one or two hooks, closed spirals, translational, helical and rotational movements, compatible with genus *Leptospira* (Table 3). This morphology was evident through video recording in dark field and with silver staining.

Genotypic characterization

DNA samples were obtained in a concentration range of 50 to 200 ng/μL and were adjusted to a concentration of 20 ng/μL for RAPD assays.

The number of markers obtained for each oligonucleotide

Table 2 - RAPD conditions used with each oligonucleotide.

Oligonucleotide	05	B11-B12	M16	PB1
Reaction volume	25 μL	25 μL	25 μL	25 μL
DNA	20 ng	20 ng	20 ng	20 ng
Taq buffer 10X (Tris-HCl 100 mM, KCl 500 mM, pH 9.0)	1X	1X	1X	1X
dNTPs	0.1 mM of each	0.1 mM of each	250 μM of each	250 μM of each
$MgCl_2$	3.5 mM	4.5 mM	1.5 mM	3.5 mM
Oligonucleotide	300 pmol	300 pmol	200 pmol	300 pmol
Taq DNA polimerase	0.5 U	0.5 U	0.5 U	0.5 U
Amplification program	One initial cycle of one min at 94 ºC, followed by 40 cycles of 1 min at 94 ºC, 1 min at 36 ºC, and 2 min at 72 ºC, with a final extension cycle of 3 min at 72 ºC	Two cycles of 5 min at 95 °C, 5 min at 40 °C, and 5 min at 72 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C, and 3 min at 72 °C, with one final extension cycle of 72 °C for 10 min	One cycle of 3 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, followed by 38 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C, with a final extension cycle of 1 min at 94 °C, 1 min at 55 °C, and 9 min at 72 °C	One cycle of 7 min at 94 °C, 1 min at 40 °C, and 1 min at 72 °C, and four cycles of 1 min at 94 °C, 1 min at 40 °C, and 1 min at 72 °C, which continued with 24 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final extension cycle of 1 min at 94 °C, 1 min at 55 °C, and 7 min at 72 °C

Table 3 - *Leptospira* isolates obtained from Mexican patients.

Isolate	Abbrevation
Rivepal	RIV
Verimol	SM
Veriluma	LMJT
Verimer	GGMC
Beribéri	BRS
Rivemar	LMFA
Verimat	MGR
Beriveca	BCB
Vecorisa	VECO
Verijua	HCHJ
Verichan	CHAN
Veritsa	TSAB

was 26 for the O5 oligonucleotide, 40 for the combination of B11-B12 oligonucleotides, 21 for the M16 oligonucleotide and 13 for the PB1 oligonucleotide, yielding 100 markers in total. Furthermore, the isolates from MX showed an identical band pattern, while reference strains corresponding to different serovars displayed different polymorphic patterns, as shown with the B11-B12 oligonucleotides (Figure 1).

The dendrogram constructed by UPGMA, based on the matrix of the presence and absence of bands with the 100 markers obtained by RAPD, showed six groups (Figure 2). Group I included three reference isolates (ICT, PYR, and CAM), with a similarity percentage among them of 48%. Group II included two reference strains (BALL and SHER), with a similarity percentage of 64%. Group III grouped all isolates of MX (RIV, SM, LMJT, TSAB, CHAN, HCHJ, VECO, BCB, MGR, MMFA, BRS, and GGMS), with a similarity percentage among them of 100% and a bootstrap of 100%; this group was associated with the reference strain POM at 80%. Group IV included the reference isolate PTC, with 28% similarity with the other groups. Group V included two reference isolates (AUT and TA) and was associated with the rest of the isolates at 22%. Finally, Group VI was composed of two reference strains (BRA and LAI), with 20% similarity with the rest of isolates. The cophenetic correlation coefficient ($CCCr$ = 0.99, $P < 0.0004$) showed that the tree was a good representation of the genetic relationship of the isolates and that different groups were consistent.

The multidimensional analysis with MST revealed the same grouping between the isolates from MX and the reference strains POM. The MST analysis showed a direct relationship between the MX isolates and the reference strain

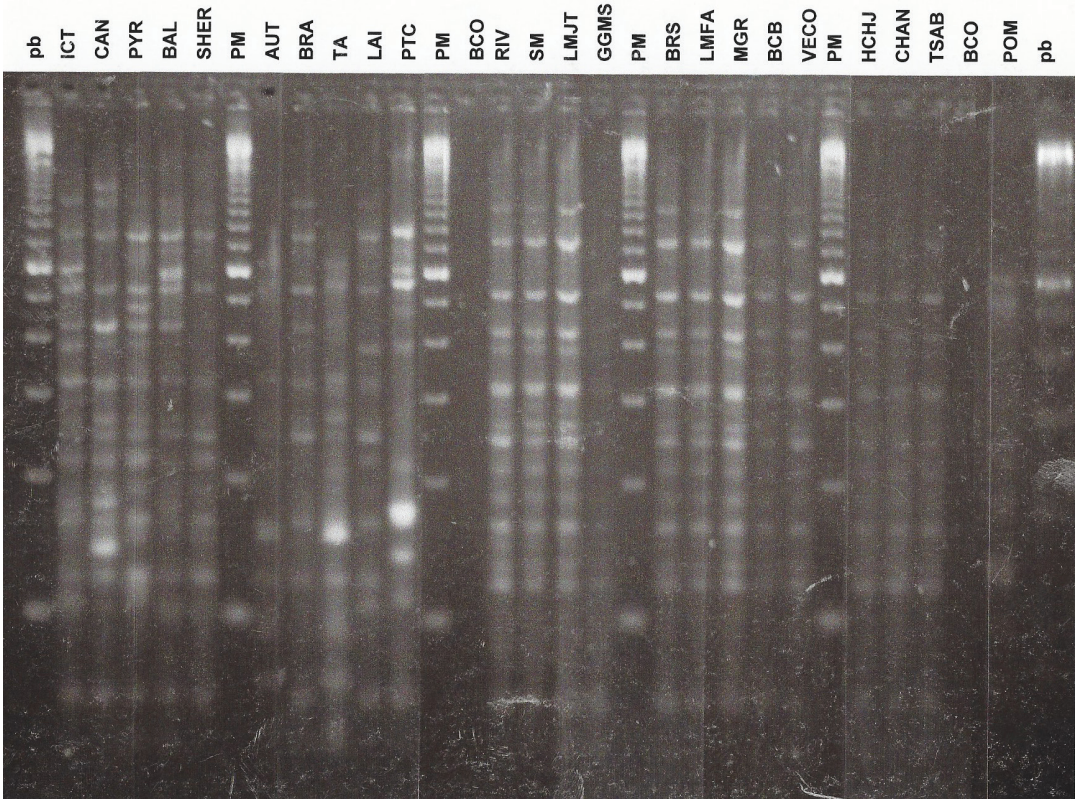

Figure 1 - Polymorphic patterns from the Mexican isolates and the reference strains obtained by RAPD using a combination of B11-B12 oligonucleotides.

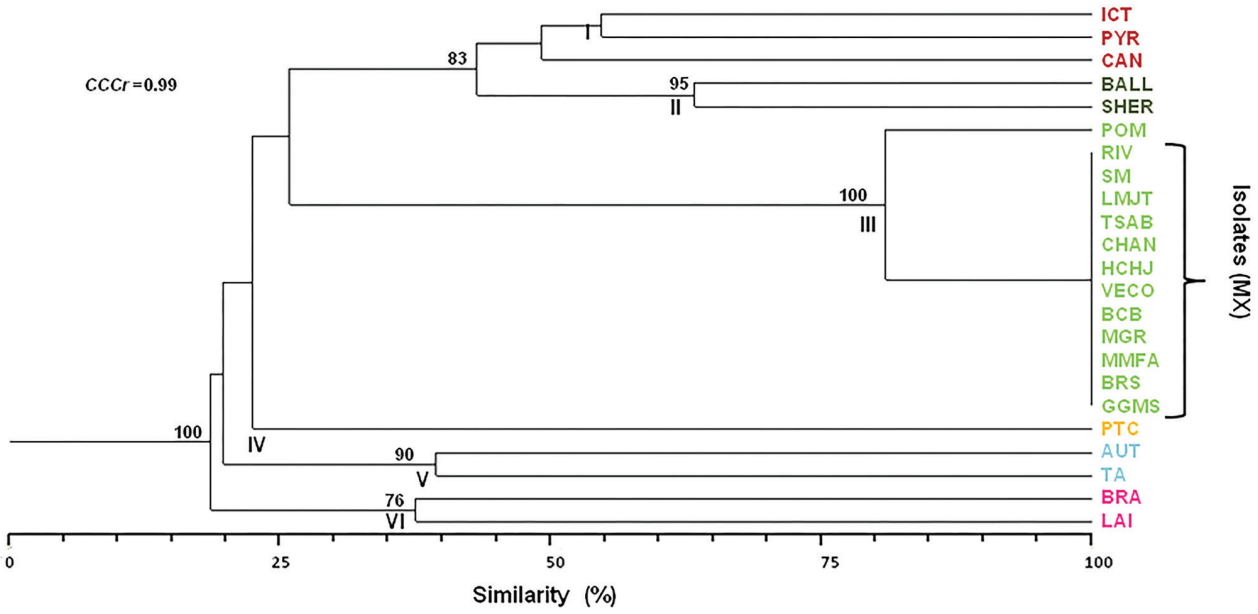

Figure 2 - Phenogram of *Leptospira* isolates obtained by RAPD. The phenogram was generated from genetic similarity coefficients obtained by determining the presence and absence of 100 DNA fragments from 12 Mexican *Leptospira* isolates and 11 reference strains, and is based on UPGMA. The numbers below the branches represent indices of support based on 1,000 bootstrap replications. **Group I**; **Group II**; **Group III**; **Group IV**; **Group V**; **Group VI**.

POM of *L. interrogans* serovar Pomona (Figure 3). A similar grouping was obtained in the dendrogram constructed with the oligonucleotides B11-B12 (Supplemental Figure).

The I_A value for the group of *L. interrogans* serovar Pomona isolated from MX was 1.0002 ($P < 0.001$), which confirmed that they exhibited clonal reproduction.

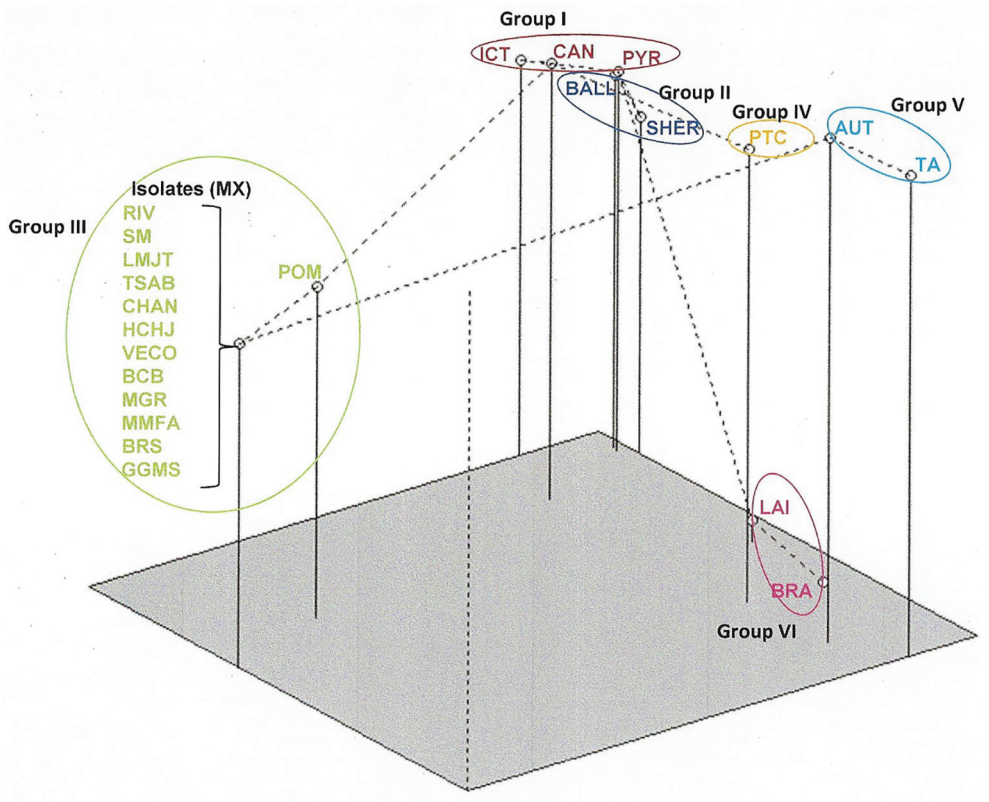

Figure 3 - Minimum spanning network (MST) of *Leptospira* isolates. All Mexican isolates form one group, directly related to the reference strain POM (*L. interrogans* serovar Pomona). **Group I**; **Group II**; **Group III**; **Group IV**; **Group V**; **Group VI**.

DISCUSSION

Infectious diseases cause approximately one-third of all deaths worldwide, in both children and adults. Earlier in this century, infectious diseases caused 5.7 million deaths, half of them in developing countries, where approximately 1,500 people died each hour. Most infectious diseases are zoonoses and among these is leptospirosis, which is considered a very important re-emerging disease in America, particularly in Latin America, with an incidence of 100 cases per 100,000 inhabitants/year during epidemics in tropical areas[26].

Leptospirosis usually presents with a wide range of clinical manifestations[33-36], sometimes similar to other diseases[33,37], therefore requiring the direct or indirect dentification of the causative agent[18,37,38].

Traditionally, the methods used in the identification and typing of different *L. interrogans* serovars are based on the study of their morphological characteristics, staining, structure, metabolic products and antigenic characteristics[25]. These procedures are slow and laborious, as has been widely mentioned in the literature[23,26]. A major disadvantage of these methods is that the phenotypic characteristics can change because of the technical procedures used, mutations or genetic exchange. The phenotypic characterization, despite its disadvantages, made it possible to identify *L. interrogans* serovar Pomona[25]. However, to confirm the identity of the studied isolates, molecular markers that have been used for decades to genotype members of the genus *Leptospira* were used. These molecular techniques make it possible to discriminate genetic differences among organisms, making it possible to identify strains from the same serovar in different geographical areas[16]. In this study, the RAPD was a useful tool for genotypically classifying clinical isolates of *Leptospira* from MX, phenotypically identified as *L. interrogans* serovar Pomona[25], showing a polymorphic pattern that was identical among them and different from the strains used as reference. The exception was the strain corresponding to *L. interrogans* serovar Pomona, which showed a similarity of 80%, as presented in Figure 2, confirmed by MST. In addition, this group showed a clonal reproduction evidenced by an I_A (1.0002) (Figure 3).

This work suggests the presence of *L interrogans* serovar Pomona in clinical isolates. The hosts of this microorganism are both marine and land animals; among the latter are domestic animals, a finding that has been demonstrated in dogs from the North of Mexico City by Rivera *et al*.[39]. Recently, it has been reported that South of Mexico City[40], humans can be infected by accidental contact with contaminated ground or water or by direct contact with animals carrying the causative agent of leptospirosis[20,41,42]. It has also been reported that the association of a particular serovar with an animal species acting as a carrier, is not absolute. *L. interrogans* serovar Pomona has previously had different types of carriers, such as horses, swine, dogs and other animals[39-42]. In this study, the isolates were from patients of different geographical regions in the Mexican Republic, such as Mexico City, Mexico State, Hidalgo, Veracruz and Yucatan. These are places where patients' contact with different animals and their excretions might have occurred, however, the source of infection is unknown.

CONCLUSION

The presence of *L. interrogans* serovar Pomona was confirmed in clinical isolates from different geographical regions of the Mexican Republic. In addition, the *L. interrogans* serovar Pomona strain isolated from patients has a clonal reproduction system, which means that this clone is spread throughout different Mexican regions.

CONFLICTS OF INTERESTS

The authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

RGG, MRRM, designed the study, wrote and revised the manuscript. BRS and OVC performed the recruitment for the collection of clinical isolates and carried out the phenotyping identification. ART performed the RAPD-PCR assays. EDE, MRRM and MGFDL performed the bioinformatics analysis. All authors contributed to and have approved the final manuscript.

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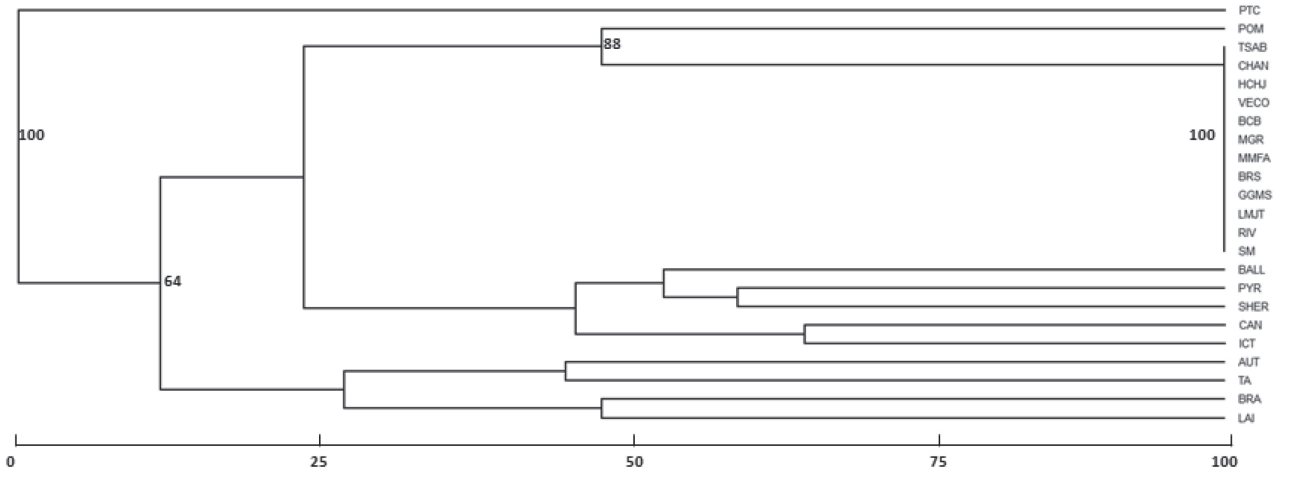
SUPPLEMENTARY MATERIAL



Supplemental Figure - UPGMA dendrogram obtained using the Jaccard's pairwise similarities index from the RAPD profiles generated with primers B11-B12.