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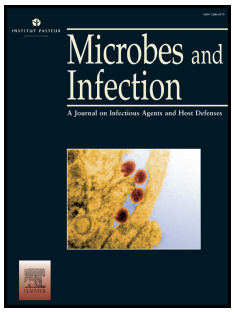

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Genetic features of verotoxigenic *Escherichia coli* O157:H7 isolated from clinical cases of Argentina and Chile

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Abstract

We aimed to compare the genetic diversity existing in VTEC O157:H7 strains isolated from cases of human disease from Argentina and Chile. For it, 76 strains were studied in relation to the distribution of genes encoding virulence factors and subtyped by lineage-specific polymorphisms (LSPA-6), and phylogroups assignment. Our results show the almost exclusive circulation of VTEC O157:H7 isolates belonging to lineage I/II, associated with hypervirulent strains, and to the phylogroup E and, on the other hand, genetic diversity present among Argentinean and Chilean strains analyzed, mainly in relation to putative virulence determinants and *nle* profiles.

Keywords

Verotoxigenic *Escherichia coli*; O157:H7; subtyping; virulence genes

1. Introduction

Verotoxigenic *Escherichia coli* (VTEC) O157:H7 is a group of emerging foodborne pathogens that cause severe diseases in humans, such as acute diarrhea (AD), bloody diarrhea (BD) and hemolytic uremic syndrome (HUS) [1]. An important geographical difference exists in the incidence and severity of VTEC O157:H7 infections and HUS of each country [2]. In Argentina HUS is endemic and reaches one of the highest incidence rates worldwide, between 7.8 and 17 per 100.000 children under five years [3]. In Chile, a HUS incidence of 3.2 cases per 100.000 inhabitants has been reported [4]. In both countries, the presentation of HUS is in the form of sporadic cases or diffuse outbreaks of difficult epidemiological definition [5]. VTEC O157:H7 has diverged evolutionarily in different parts of the world. The differences in the strain virulence of each geographical area could explain the differences in the incidence and severity of human diseases related to this microorganism [6].

Not all VTEC O157:H7 strains have the same ability to infect and cause disease in humans [7]. Wide variability has been observed regarding the clinical presentation of patients with O157:H7 infections and the factors that contribute to this variation are poorly understood [8]. The emergence, in recent decades, of hypervirulent VTEC O157:H7 clones with a worldwide distribution [5] is clear evidence of the genomic dynamics of this group of pathogens and their ability to transfer or acquire virulence factors [9]. Phylogenetic studies determined that these strains are part of globally dispersed subpopulations, which would differ in relation to their association with disease in humans, having different types and levels of expression of virulence factors [10].

Yang *et al*. [10] developed the lineage-specific polymorphism assay (LSPA-6). Based on polymorphisms presented in six genetic markers, this method separates VTEC O157:H7 in lineages (I, I/II and II). The frequencies in which these lineages are isolated from cattle and

humans suggest that LSPA-6 may be a useful indicator of virulence potential. By LSPA-6 different associations have been reported among VTEC O157:H7 from cattle and humans from within the same country and between different countries [6].

On the other hand, *E. coli* strains can be classified into 7 phylogroups (A, B1, B2, C, D, E and F) [11] that differ in phenotypic and genotypic characteristics, ecological niche, life-history traits and ability to cause disease [12]. Clermont *et al.* [11] developed this method which allows to assign phylogroups based on polymorphisms of four genetic markers.

Most of the virulence factors of VTEC O157:H7 are encoded in mobile genetic elements, such as prophages, islands of pathogenicity (PAIs) and plasmids [1]. The presence and expression of Vero toxins (*vtx* genes) in O157:H7 is considered essential to cause HUS [6]. Other virulence factors are also considered risk factors for developing HUS, as intimin and its receptor Tir (*eae* and *tir* genes), both encoded in the PAI named "*locus* of enterocyte effacement" (LEE), and an enterohemolysin, encoded by *ehxA* in the pO157 plasmid [1].

Several other virulence determinants have been described in O157:H7 strains, such as translocated effectors of the type III secretion system [13]. These proteins, encoded in non-LEE effector (*nle*) genes, contribute to the colonization and persistence of VTEC in cattle and interfere with the human inflammatory response [14].

Also, seven putative virulence determinants have been described in the TW14359 O157:H7, the strain associated with the raw spinach outbreak in the USA in 2006 [15]. These sequences, which could explain the high virulence of TW14359, include two putative types III secretion system effector proteins, candidate genes that could result in increased pathogenicity or adaptation to plants, and an anaerobic nitric oxide reductase gene.

Genomic comparison studies and evaluation of genes encoding virulence factors represent useful tools for analyzing genetic diversity and subtyping VTEC O157:H7. In this

study, we examined collections of human VTEC O157:H7 from Argentina and Chile by lineage-specific polymorphic assay (LSPA), phylogroup assignment, and virulence typing (including determinants of virulence, *nle* genes, and putative virulence determinants) to genetically characterize and compare VTEC O157:H7 strains from these two neighbor countries.

2. Materials and methods

2.1. Bacterial strains

A total of 76 VTEC O157:H7 strains obtained from patients of Argentina (n=38) and Chile (n=38) between 1999-2015 [16,17, Zotta, personal communication] were analyzed. They are only a selected portion of all the cases in these countries in this time frame and not a random selection of isolates. The Argentinean isolates were kindly provided by the National Institute of Epidemiology "Dr. Juan H. Jara" (INE-ANLIS).

The VTEC strains were isolated from 32 Argentinean and 13 Chilean cases of HUS, 16 Chilean cases of watery diarrhea (WD), 4 Argentinean and 8 Chilean cases of bloody diarrhea (BD), 2 Argentinean asymptomatic contacts of HUS cases (A), more 1 strain from Chile with a not identified kind of disease (Fig. 1).

2.2. Virulence typing

Genes *vtx*$_1$, *vtx*$_2$, *eae*, *ehxA* and *saa* were screened by a multiplex PCR [18]. Sixteen *nle* (non-LEE effector) genes: *nleB2*, *nleC*, *nleH1-1*, *nleD* (encoded in genomic island O-I 36), *nleG2-3*, *nleG5-2*, *nleG6-2* (O-I 57), *nleA*, *nleF*, *nleG*, *nleG2-1*, *nleG9*, *nleH1-2* (O-I 71), *ent/espL2*, *nleB*, *nleE* (O-I 122), were amplified according to Coombes *et al*. [13]. Also, genes encoding putative virulence determinants (PVD) (described in the TW14359 O157 strain associated with the raw spinach outbreak in the USA, 2006), ECSP_0242, ECSP_1773, ECSP_2687, ECSP_2870/2872, ECSP_3286 and ECSP_3620, were screened according to Kulasekara *et al*. [15].

2.3. LSPA-6

Amplification by PCR of six genomic *loci* was performed for LSPA-6 lineage typing. PCR products were run in 6% denaturing polyacrylamide gels and detected by silver staining.

The amplicon sizes were used to assign LSPA genotypes and reference strains, K12 and EDL933, were used to confirm the results. A number was assigned for each allele and each isolate and a lineage profile was formed by a six-digit binary code generated by assigning a number for each allele and each isolate. Strains were classified as lineage I, I/II or II according to the profile presented [10].

2.4. Phylogroups assignment

Phylogroups were determined according to Clermont *et al*. [11]. By a quadruplex PCR, which amplifies segments of *chuA*, *yjaA*, TspE4.C2, and *arpA* it is possible to identify 7 groups belonging to *E. coli sensu stricto* (A, B1, B2, C, D, E and F) and one corresponding to *Escherichia* Clado I. The phylogroups assignment was made according to the band patterns observed in electrophoresis gels. A subsequent simple PCR was performed to define between groups E and D. Each PCR amplification was repeated more than once.

2.5. Data analysis

The statistical association between virulence genes presence and source of isolates was analyzed by 2x2 contingency tables, chi-square test ($\chi 2$), and Fisher exact test, with a confidence level of 95%, using the software Epi InfoTM 7.1.5.2. The clustering analysis (UPGMA) and the Minimum Spanning Tree (MST) were performed using the software BioNumerics vs. 6.6 (AppliedMaths).

3. Results

The distribution of virulence-associated genes in the 76 VTEC O157:H7 isolates showed that the most frequent virulence profile was *vtx*$_2$, *eae*, *ehxA*, present in all VTEC O157:H7 strains from Chile and in 94.8% of the Argentinean strains. Only two Argentine strains, No. 60 and 65, presented different virulence profiles, *vtx*$_1$, *vtx*$_2$, *eae* and *vtx*$_1$, *vtx*$_2$, *ehxA*, respectively. As expected, neither strain was *saa*-positive (Fig. 1).

The distribution of 16 *nle* genes encoded in genomic PAIs was analyzed among VTEC O157:H7 strains from clinical cases of Argentina and Chile. A high proportion of *nle* genes was found. Most of the strains presented the complete *nle* profile (86.8%), regardless of the origin. Strain No. 65, obtained from a HUS case in Argentina, which strikingly was LEE-negative (*eae*-negative), presented the complete *nle* profile. The genes *nleH1-1*, *nleG2-3*, *nleB*, and *nleE* were present in all isolates analyzed (Fig. 1).

VTEC O157:H7 strains were grouped into ten *nle* profiles (Fig. 2). The most predominant *nle* profile was that positive for all *nle* genes, found in 32 Argentinean (84.2%) and 34 Chilean strains (89.5%). It was also the only *nle* profile shared by strains from both countries. The remaining ones were unique profiles except one, which was present in two Argentinean strains (Fig. 2). No significant association was found between *nle* genes or *nle* profiles and the origin of the VTEC O157:H7 strains ($P > 0.05$).

The distribution of PVD genes in clinical isolates of Argentina and Chile is shown in Fig. 1. ECSP_0242 and ECSP_3620 were the most prevalent virulence determinants in VTEC O157:H7 isolates from both countries. On the contrary, ECSP_1773 was the least prevalent gene in strains from both countries (18% in Argentina and 26% in Chile), while ECSP_2687 prevailed in the group of Argentinean strains (89%).

ECSP_2687, the gene encoding a protein that reduces the expression of cytokines, was significantly associated with Argentinean VTEC O157:H7 strains (OR = 99.17, $P < 0.05$). All VTEC O157:H7 isolates obtained from Argentinean HUS cases (n=32) presented this gene, meanwhile none of the isolates obtained from Chilean HUS cases (n=13) presented it (Fig. 1).

The 14 PVD gene profiles in which VTEC O157:H7 strains were grouped, are shown in Figure 3. Only four PVD profiles were shared by strains from both countries. No significant association was found between PVD profiles and the origin of the VTEC O157:H7 strains ($P > 0.05$).

Most VTEC O157:H7 strains from both countries (98.7%), showed the LSPA profile 211111, characteristic of lineage I/II, and were assigned to phylogroup E. Only two strains from Argentina were different, one isolate (No. 60) presented the LSPA profile 111111, belonging to the lineage I, and the other one (No. 48) was assigned to phylogroup D (Fig. 1). No association was found between the presence of a certain subtype and the origin of the VTEC O157:H7 strains.

4. Discussion

The incidences and severities of VTEC O157:H7 infections, particularly HUS cases, differ substantially worldwide [2], and the factors that contribute to this variation are poorly understood [8]. Food-handling practices, consumer preferences for foods, environmental factors and host susceptibility, may partly account for the regional discrepancies in disease characteristics [6]. However, this regional association suggests that O157:H7 strains have diverged evolutionary in different parts of the world through founder effects or genetic drift or by selective regional pressures [2]. It has been postulated that VTEC O157:H7 integrates subpopulations, which differ in relation to their association with disease in humans [10]. In this study, we performed a genetic characterization of VTEC O157:H7 strains isolated from clinical cases from Argentina and Chile. In both countries, this is the serotype most associated with infections and HUS [5,19]. However, there is a notable difference in the HUS incidence rates [3,4].

In relation to the LSPA6, the most frequent profile in both countries was 211111, characteristic of lineage I/II. These results are consistent with those reported for VTEC O157:H7 strains of human origin from different countries [6,20] and other regions of Argentina [20,21]. Only an Argentinean strain (No. 60) belonged to lineage I and, to our knowledge, this is the first report in relation to lineage for Chilean VTEC O157:H7 human strains. Many authors have associated VTEC O157:H7 strains belonging to lineage I/II with clade 8 [21,22], proposed as more virulent than the other clades [8].

In this study, VTEC O157:H7 isolates belonging to the phylogroup E were prevalent in both countries. Strikingly, one Argentinean strain (No. 48) belonged to group D. Most of the O157:H7 strains from different geographical regions and sources were also assigned to the

phylogroup E [12]. In relation to it, we found in a recent study a high prevalence (95%) of Argentinean O157:H7 strains of bovine and human origin belonging to group E [23].

Regarding the genetic diversity detected through the study of different virulence factors, most of the strains studied (97.4%) presented the virulence profile *vtx*$_2$, *eae*, *ehxA*, and only two Argentine strains (No. 60 and 65) had a different profile. The virulence genes which encode Vero toxins are essential for the development of HUS; however, most VTEC O157:H7 infections do not progress to HUS [6]. The 84% of the Argentinean O157:H7 strains analyzed in this study were isolated from HUS cases, while this occurred in only 34% of Chilean strains (Fig. 1). Although most of the analyzed strains from both countries presented the same virulence profile, the strains from Argentina were associated with more severe pathologies than those from Chile.

A high number of *nle* genes in VTEC isolated from human disease could be a virulence advantage [24]. We found a high proportion of *nle* genes, which encode effector proteins, in a majority of strains (87%), regardless of their origin. All the strains studied from both countries presented *nleB*. Recently, has been proposed that differences in the expression levels of the NleB effector could be important in the pathogenesis of VTEC by allowing the differentiation of HUS and non-HUS isolates of human origin [25]. In future studies, it would be interesting to determine the level of expression of this effector in VTEC O157:H7 strains from both countries.

Seven putative virulence factors were identified in TW14359 [15], a VTEC O157:H7 strain from the USA, associated with a severe outbreak occurred in 2006, representative of lineage I/II and clade 8 [8]. It was postulated that the presence of ECSP_3620 (encoding the anaerobic nitric oxidase, NorV), combined with any of the other virulence factors may contribute to the high virulence of these strains. However, only ECSP_3286 (encoding a

protein that binds with high affinity to heme) was related to the high incidence of HUS by Pianciola & Rivas [2]. On the other hand, in the present study, Argentinean and Chilean O157 strains presented common characteristics in relation to the presence of most of the putative virulence determinants, as well as Australian O157:H7 strains [20]. Only ECSP_2687 (encoding a protein that reduces the expression of cytokines, decreasing the immune response of the host) was shown to be more frequent in Argentinean isolates ($P < 0.05$). All the HUS-associated strains from Argentina had this gene while, surprisingly, none of the Chilean ones had it. In the future, it would be interesting to study the distribution of this gene and the association or not with the case of HUS in other geographic regions.

A series of characteristics related to the high incidence or severity of *E. coli* O157 infections have been postulated, such as belonging to the lineage I/II and the presence of the putative virulence factor ECSP_3286 [2]. However, these were not sufficient to differentiate the Argentinean and Chilean strains in this study. The only marker that was distributed significantly differently was ECSP_2687.

Our results show, i) the almost exclusive circulation of VTEC O157:H7 isolates belonging to lineage I/II, associated with hypervirulent strains, and phylogroup E and, ii) genetic diversity present among the analyzed strains of Argentina and Chile, mainly in relation to PVD and *nle* profiles.

The comparison of genetic characteristics considered in this study, with the exception of ECSP_2687, would not explain the hypervirulence associated to VTEC O157:H7 strains from Argentina. More studies are needed to understand the high incidence of HUS in this country.

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Conflict of interest

The authors declare no conflict of interest.

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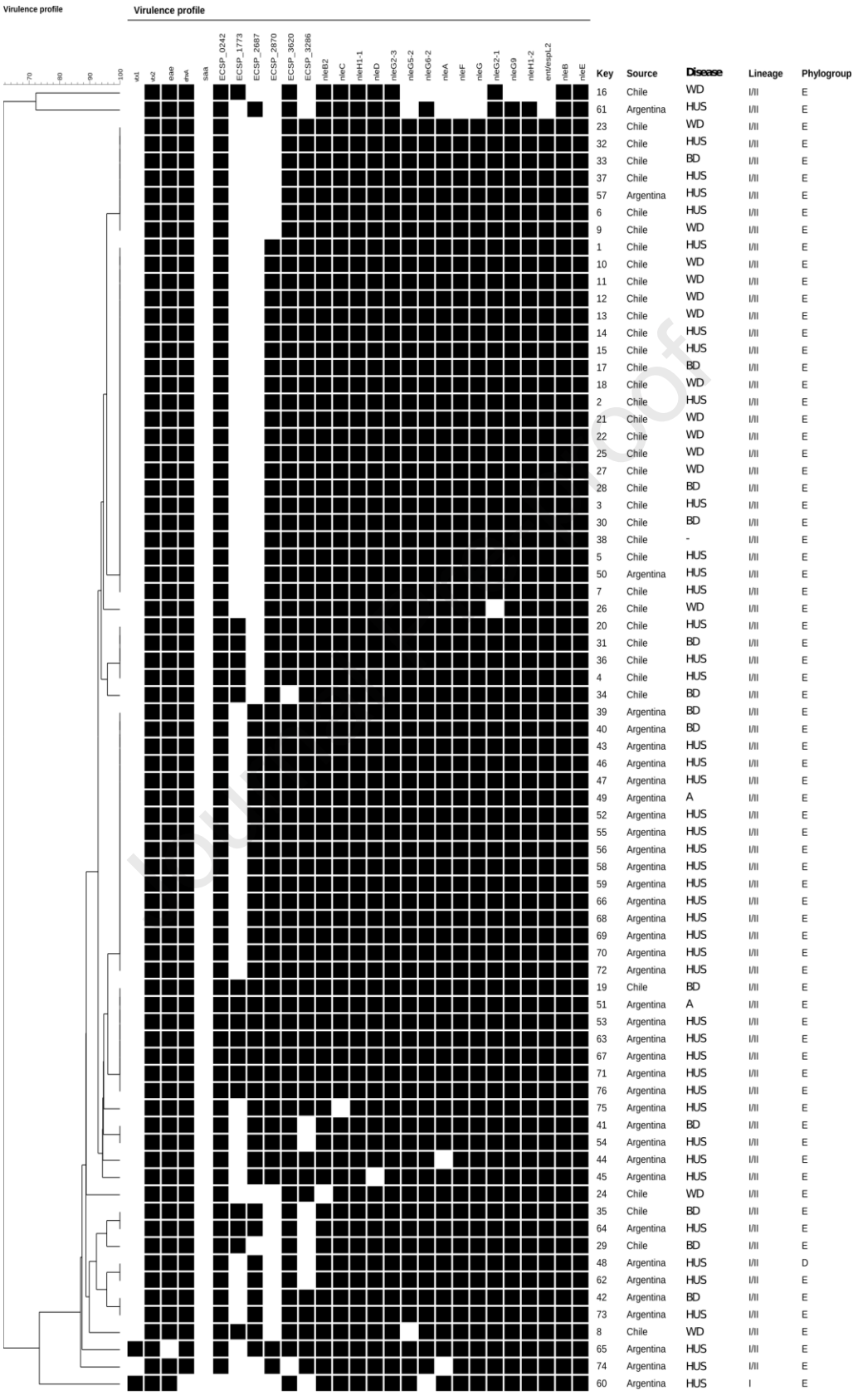
315 **Figure 1.** Cluster analysis of VTEC O157:H7 isolates from Argentina and Chile investigated
316 in this study based on virulence-associated genes. The presence of genes is indicated in black
317 and absence, in white. On the right, isolate number, source, kind of disease, lineage and
318 phylogroup are indicated. A, asymptomatic case; BD, bloody diarrhea; HUS, hemolytic-
319 uremic syndrome; *nle*: non-LEE effector (genes); WD, watery diarrhea.

320 **Figure 2.** Minimum spanning tree of the 76 verotoxigenic *Escherichia coli* (VTEC) O157:H7
321 isolates based on 16 *nle* genes (*nleB2, nleC, nleH1-1, nleD,* encoded in genomic island O-I
322 36, *nleG2-3, nleG5-2, nleG6-2,* O-I 57, *nleA, nleF, nleG, nleG2-1, nleG9, nleH1-2,* O-I 71,
323 and *ent/espL2, nleB, nleE,* O-I 122). The strains were grouped into ten profiles. Each circle
324 represents an *nle* profile; sizes of each circle corresponds to the number of isolates and the
325 partitions into each circle represent the number of isolates for each profile.

326 **Figure 3.** Minimum spanning tree of the 76 verotoxigenic *Escherichia coli* (VTEC) O157:H7
327 isolates based on six putative virulence determinants (PVD) genes (ECSP_0242, ECSP_1773,
328 ECSP_2687, ECSP_2870/2872, ECSP_3286 and ECSP_3620). The strains were grouped into
329 fourteen profiles. Each circle represents a PVD profile; sizes of each circle corresponds to the
330 number of isolates and the partitions into each circle represent the number of isolates for each
331 profile.

Figure 1.


Virulence profile
Virulence profile
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ECSP_2687
ECSP_2870
ECSP_3620
ECSP_3286
nleB2
nleC
nleH1-1
nleD
nleG2-3
nleG5-2
nleG6-2
nleA
nleF
nleG
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nleG9
nleH1-2
ent/espL2
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Key
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Disease
Lineage
Phylogroup
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61 Argentina HUS I/II E
23 Chile WD I/II E
32 Chile HUS I/II E
33 Chile BD I/II E
37 Chile HUS I/II E
57 Argentina HUS I/II E
6 Chile HUS I/II E
9 Chile WD I/II E
1 Chile HUS I/II E
10 Chile WD I/II E
11 Chile WD I/II E
12 Chile WD I/II E
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25 Chile WD I/II E
27 Chile WD I/II E
28 Chile BD I/II E
3 Chile HUS I/II E
30 Chile BD I/II E
38 Chile - I/II E
5 Chile HUS I/II E
50 Argentina HUS I/II E
7 Chile HUS I/II E
26 Chile WD I/II E
20 Chile HUS I/II E
31 Chile BD I/II E
36 Chile HUS I/II E
4 Chile HUS I/II E
34 Chile BD I/II E
39 Argentina BD I/II E
40 Argentina BD I/II E
43 Argentina HUS I/II E
46 Argentina HUS I/II E
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75 Argentina HUS I/II E
41 Argentina BD I/II E
54 Argentina HUS I/II E
44 Argentina HUS I/II E
45 Argentina HUS I/II E
24 Chile WD I/II E
35 Chile BD I/II E
64 Argentina HUS I/II E
29 Chile BD I/II E
48 Argentina HUS I/II D
62 Argentina HUS I/II E
42 Argentina BD I/II E
73 Argentina HUS I/II E
8 Chile WD I/II E
65 Argentina HUS I/II E
74 Argentina HUS I/II E
60 Argentina HUS I E

Figure 2.

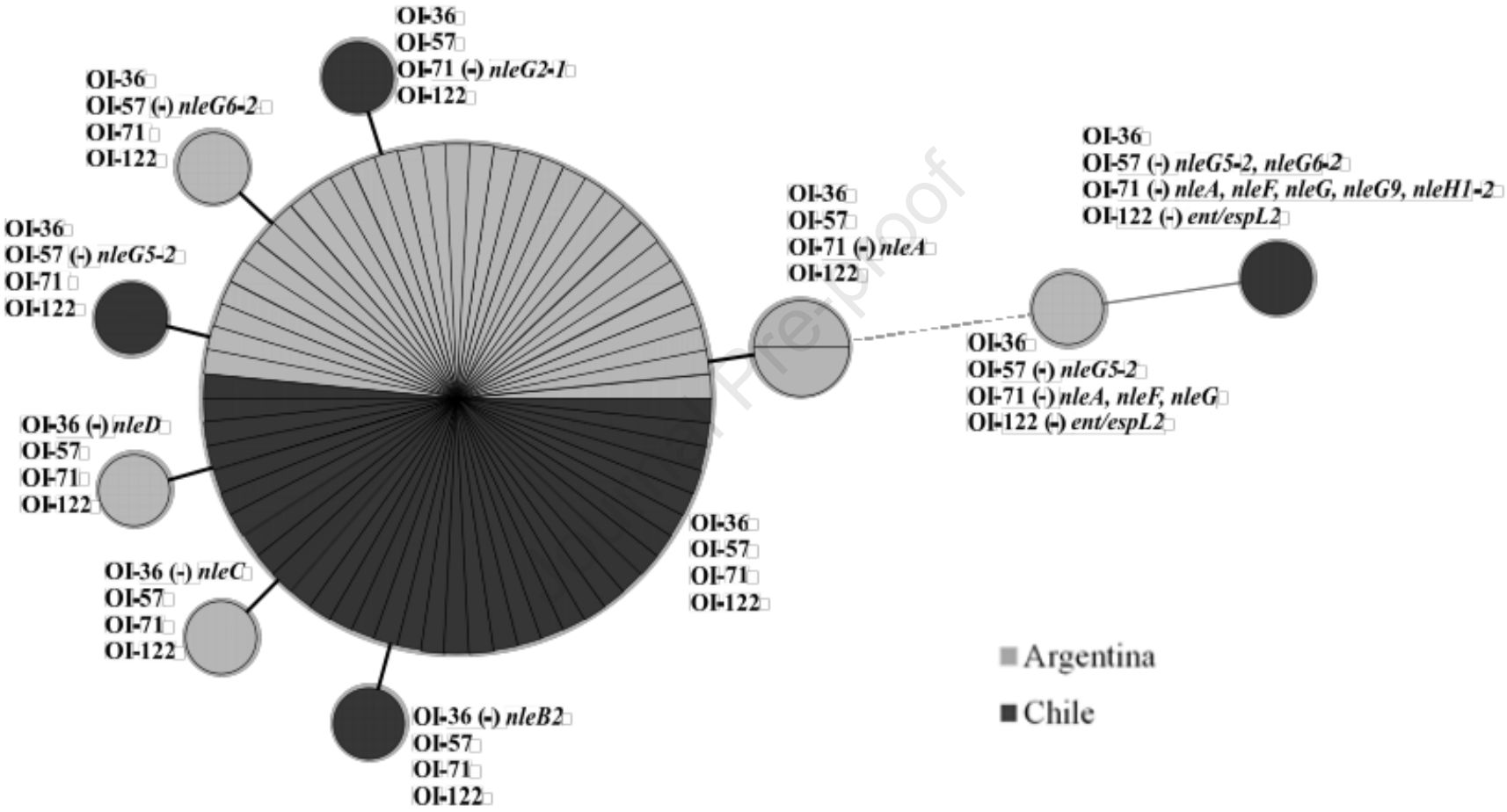
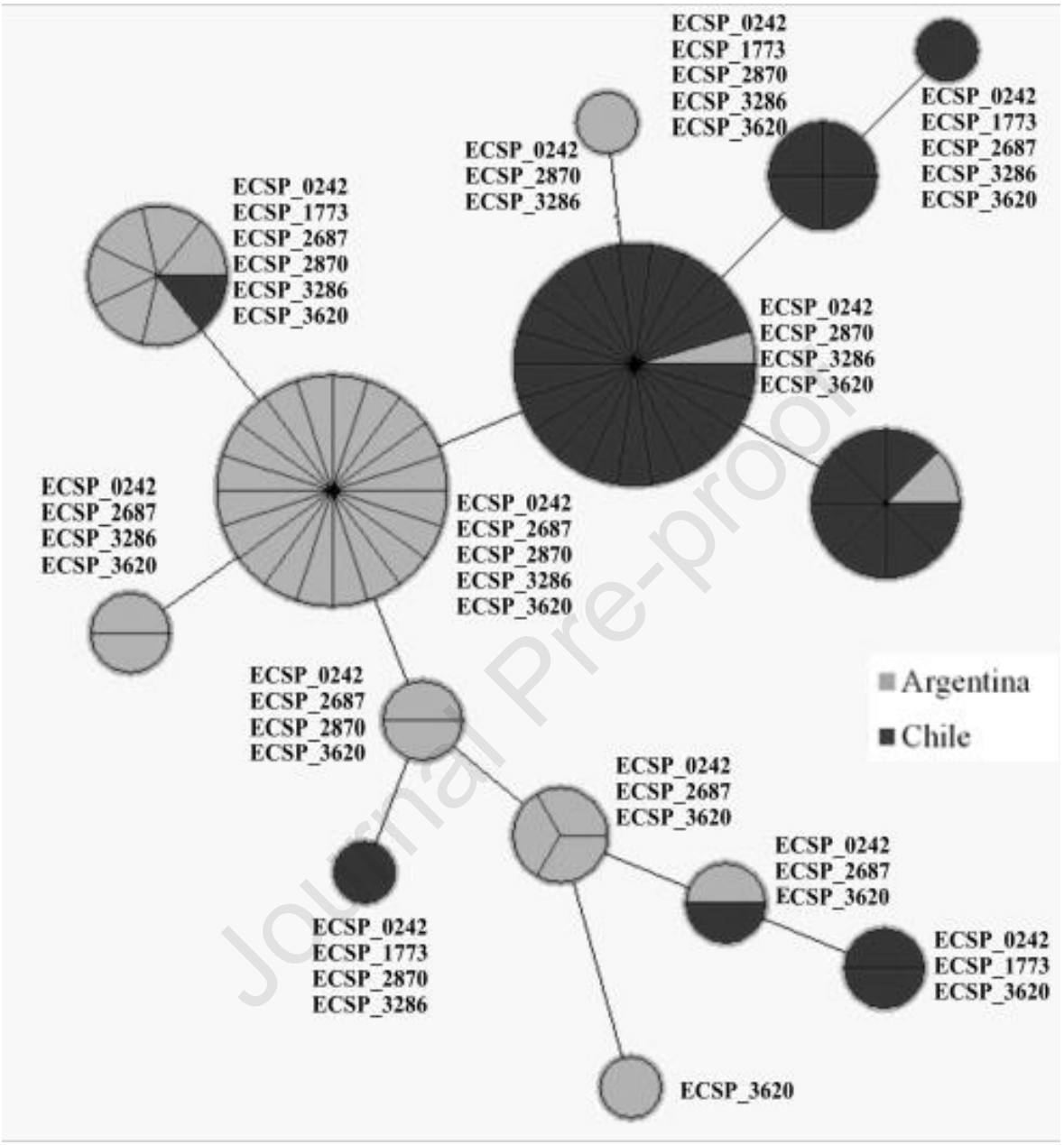

OI-36
OI-57
OI-71 (-) nleG2-1
OI-122
OI-36
OI-57 (-) nleG6-2
OI-71
OI-122
OI-36
OI-57 (-) nleG5-2
OI-71
OI-122
OI-36 (-) nleD
OI-57
OI-71
OI-122
OI-36 (-) nleC
OI-57
OI-71
OI-122
OI-36 (-) nleB2
OI-57
OI-71
OI-122
OI-36
OI-57
OI-71 (-) nleA
OI-122
OI-36
OI-57 (-) nleG5-2
OI-71 (-) nleA, nleF, nleG
OI-122 (-) ent/espL2
OI-36
OI-57 (-) nleG5-2, nleG6-2
OI-71 (-) nleA, nleF, nleG, nleG9, nleH1-2
OI-122 (-) ent/espL2
OI-36
OI-57
OI-71
OI-122
Argentina
Chile

Figure 3.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ECSP_1773
ECSP_2870
ECSP_3286
ECSP_3620
ECSP_0242
ECSP_1773
ECSP_2687
ECSP_3286
ECSP_3620
ECSP_0242
ECSP_2870
ECSP_3286
ECSP_0242
ECSP_1773
ECSP_2687
ECSP_2870
ECSP_3286
ECSP_3620
ECSP_0242
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ECSP_0242
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ECSP_0242
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ECSP_2870
ECSP_3620
Argentina
Chile
ECSP_0242
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ECSP_2687
ECSP_3620
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ECSP_1773
ECSP_2870
ECSP_3286
ECSP_0242
ECSP_1773
ECSP_3620
ECSP_3620