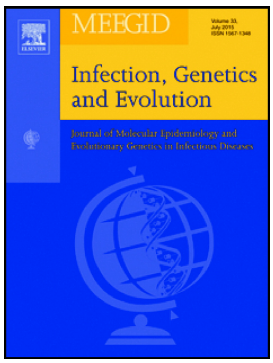

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Genotyping of *Ascaris* spp. infecting humans and pigs in Italy, Slovakia and Colombia

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ABSTRACT

Background: The systematics and taxonomy of *Ascaris lumbricoides* and *Ascaris suum*, two of the world's most widespread nematodes, still represent a highly debated scientific issue. Two different transmission scenarios have been described according to endemicity: separated host-specific transmission cycles in endemic regions, and a single pool of infection shared by humans and pigs in non-endemic regions. The swine roundworm *A. suum* is now recognized as an important cause of human ascariasis also in endemic areas such as China, where cross-infections and hybridization have also been reported, as well as in non-endemic regions like Italy. This study aimed to investigate the molecular epidemiology of human and pig ascariasis in three countries representing different epidemiological scenarios: Italy as a non-endemic country, Colombia as an endemic country, and Slovakia as a non-endemic country, but with a poor socio-economic context linked to some focal populations of Roma settlements.

***Materials and methods*:** A total of 237 nematodes were analysed: 46 from Colombia (13 from humans, 33 from pigs), 114 from Slovakia (20 from humans, 94 from pigs) and 77 from Italy (17 from humans and 60 from pigs). Genotyping by PCR-RFLP of nuclear (ITS) and sequencing of mitochondrial (*cox1*) target regions were performed. ITS genotypes were used to estimate the Hardy-Weinberg (HW) equilibrium according to hosts and country of origin. The partial *cox1* sequences were used to analyse genetic polymorphisms according to hosts and country of origin, as well as to infer the network of haplotypes, their evolutionary relationships and geographical distribution.

***Results*:** 110 quality *cox1* sequences were obtained. Haplotype network revealed three main groups corresponding to clade A, B and C. Clade C included most of the human cases from Italy, while those from Slovakia and Colombia were grouped in clade B. *Ascaris* from Italian and Colombian pigs showed HW equilibrium at the ITS marker, while disequilibrium was

found in *A. lumbricoides* from Slovak pigs, which suggest a high unexpected amount of roundworms of human origin circulating also in pigs.

Conclusions: This study updates and extends the current understanding of *Ascaris* species and genotypes circulating in different epidemiological scenarios, with particular attention to the inclusion of human-derived *Ascaris* in the phylogenetic cluster C. Despite the evidence of HW equilibrium in the ITS in pig-derived Italian samples, the amount of genetic variation seems to support the existence of two closely related species.

***Keywords*:** *Ascaris lumbricoides, Ascaris suum,* ITS, cox1, genotyping

1. INTRODUCTION

Ascariasis caused by roundworms of *Ascaris* spp. is a public health issue (Hadush and Pal, 2016), and it is still considered a neglected tropical disease (World Health Organization, 2020a). *Ascaris lumbricoides* and *Ascaris suum* are mostly found to infect humans and pigs, respectively (Zhou et al., 2020). *A. lumbricoides* is one of the most widespread soil-transmitted helminths, and is responsible for approximately 60.000 deaths in humans each year (World Health Organization, 2020b), while *A. suum* may cause production losses in outdoor pig farming systems, where it is reported as the cause of the most common nematode infection (Katakam et al., 2016).

The two roundworms are very closely related (Centers for Disease Control and Prevention, 2019) and undistinguishable by morphology, and consequently their taxonomy has been debated for decades. Some authors suggested that *A. lumbricoides* and *A. suum* are different

species (Betson et al., 2014; Jensen et al., 2016), while other authors stated that they could be considered as a single species (Leles et al., 2012; Shao et al., 2014), depending on the species definition adopted (Betson et al., 2013). Although this is an issue currently unresolved, two different scenarios of transmission patterns, according to endemicity, have been proposed: separated host-specific transmission cycles in endemic regions, and a single pool of infection shared by humans and pigs in non-endemic regions (Anderson, 2001; Peng et al., 1998). Therefore, several studies have been conducted in countries such as Italy, Honduras, Brazil, Ecuador, Zanzibar, Myanmar, Thailand, and China (Cavallero et al., 2013; Monteiro et al., 2019; Palma et al., 2019; Sadaow et al., 2018; Sparks et al., 2015; Zhou et al., 2018), aiming to evaluate the amount and nature of genetic diversity of *Ascaris* spp. circulating in humans and pigs, and to explore their zoonotic potential and cross-transmission. Those studies used different techniques with variable discriminatory power and molecular markers. The nuclear ribosomal ITS allows the identification of the two species and their hybrid at banding pattern analyses, by the mean of a fast and relatively cheap method widely used by several research groups (among several: Peng et al., 2003; Leles et al., 2010; Cavallero et al., 2013; Sparks et al., 2015), while mitochondrial *cox*1 and *nad*1 are suitable for haplotype-based studies of genetic structuring according to different geographical regions or hosts. Interesting findings have been reported, such as the high number of infections with *A. suum* in humans in endemic areas as China (Zhou et al., 2012), and cross-infections and hybridization in non-endemic countries as Italy (Cavallero et al., 2013).

The zoonotic potential of *Ascaris* spp. has important implications for evaluating and applying effective prevention and control strategies for ascariasis, as zoonotic transmission could lead to greater morbidity (Betson et al., 2014). Additional studies involving the molecular epidemiology of *Ascaris* spp. in different countries and scenarios being considered, taking into

account epidemiologically relevant variables such as socio-economic factors which may affect the prevalence of roundworm infection, are thus needed (Gunawardena et al., 2004). Similarly, the estimation of hybrid occurrence is of great interest, since hybridization is recognized as a significant process in the evolution of free-living organisms (Arnold, 2004; Criscione et al., 2007), however, its role in parasitic organisms is still underinvestigated (King et al., 2015). Some hybridization studies in parasitic living organisms have been conducted, e.g. in the species pairs of *Schistosoma mansoni* Sambon, 1907, and *S. rodhaini* Brumpt, 1931; as well as in *Anopheles gambiae* s.s. Giles, 1902, and *A. coluzzii* Coetzee and Wilkerson, n. sp. (King et al., 2015; Steinauer et al., 2010). Knowledge of hybridization events is critical from an epidemiological perspective as they may be related to changes in key pathogenic and transmission traits, such as invasive and allergenic potential, which have been demonstrated in cryptic species (Llorens et al., 2018; Vicente et al., 2017).

In this context, this study aimed to increase our knowledge of *Ascaris* species and genotypes circulating in three different countries and respective epidemiological scenarios (according to Pullan et al., 2014): Italy, considered a non-endemic country, Colombia considered an endemic country, and Slovakia generally considered not endemic for ascariasis but with a focus on poor socio-economic context concerning the samples here analysed. Genetic variation was investigated using two molecular markers commonly employed in this genus, the nuclear ribosomal non-coding region (ITS) and a portion of the mitochondrial *cox1* gene within and among *Ascaris* populations of human and pig origin. Molecular data were used to genotype samples and estimate frequency of hybrids in the two host species, in order to infer phylogeographic relationships and population genetic indices among samples.

2. MATERIALS AND METHODS

2.1 Samples

A total of 237 adult nematodes were collected between 2015 and 2020: 46 from Colombia (13 from humans, 33 from pigs) representative of the North Caribbean region of the country (Department of Bolivar); 114 from Slovakia (20 from humans, 94 from pigs), from districts representative of the entire country (western: Dunajská Streda district; central: Rimavská Sobota district; eastern: Košice, Rožňava, Michalovce, Trebišov, Svidník districts), and 77 from Italy (17 from humans, 60 from pigs) collected from representative regions spanning Italy from north to south (Piedmont, Lombardy, Umbria, Latium, Calabria and Campania). Specimens were washed with saline solution (0.9% NaCl) and stored in 70% ethanol at room temperature.

2.2 Molecular analyses: genotyping, phylogenetic network and population genetics

DNA extraction from nematodes was performed individually using the Isolate II Genomic DNA kit (Bioline, UK), according to the manufacturer´s protocol.

PCR-RFLP of the nuclear ITS segment using the endonuclease *Hae*III and PCR amplification of the mitochondrial *cox1* gene were performed following published methods (Zhu et al 1999; Cavallero et al., 2013). Good quality *cox1* sequences were used to obtain the alignment of the partial mitochondrial region using ClustalW in MEGA7, and to analyse the genetic polymorphism indices as number of variable sites, number of haplotypes and heterozygosity according to host and country of origin, as well as to infer the network of haplotypes, their evolutionary relationships and geographical distribution. The following haplotypes representative of *Ascaris* clades were included in the analysis, in order to clearly trace the clade affiliation (KC455933 clade A1, KC455934 clade A2, AB591800 clade B, KC455923

clade C). Haplotype polymorphism analysis and population genetic data such as allelic frequencies, gene diversity, mean number of pairwise differences as nucleotide diversity, expected heterozygosity, sample differentiation (pairwise comparison using Fst approach and the Exact test of sample differentiation for significance test), and AMOVA were evaluated using DnaSP v5 (Librado and Rozas, 2009) and Arlequin 3.11 (Excoffier et al., 2005). AMOVA groups and populations corresponded to the three countries of origin and according to hosts, respectively; evolutionary distances were estimated using MEGA 7 by the mean of genetic *p-distance* over sequence pairs within and between groups, developed based on geography and host affiliation. Sequences were also tested for haplotype correspondence with retrievable homologous GenBank sequences. New haplotypes are available in GenBank under the following accession numbers: MZ008278-MZ008308.

ITS genotypes were used to estimate the Hardy-Weinberg (HW) equilibrium according to pig host alone and country of origin. The Yates´ correction was performed for Colombian nematodes of pig origin due to the low number of samples. Human-derived samples were not considered as a population, since all isolates were collected separately, originating from single cases of infections.

2.3 Ethical statement

Colombian samples of human origin were obtained from naturally infected subjects in deprived communities participating in the FRAAT birth cohort study in Cartagena, after the study participants or their parents notified the finding of worms in the faeces. Scientist staff provided information for worm storage and then collected the specimens at the donor's houses, within 6 hours. Alternatively, some individuals were contacted after being found infected during faecal examination and after receiving an anti-helminth treatment either in a

local health care unit or at schools. Collection of samples of pig origin was done from a slaughterhouse and farms in two rural municipalities nearby Cartagena (Ballestas, María la Baja). Ethical approval for the collection of samples in Colombia was obtained by the ethics committee of the University of Cartagena. All Colombian samples were collected in compliance with Nagoya protocol (https://www.cbd.int/abs/about).

Italian samples of human origin were obtained from existing collections at the Sapienza University of Rome and from private laboratories. Data collection includes only the geographical origin of patients and no reference to personal data was recorded, thus guaranteeing the absolute anonymity of the infected individuals.

Sample collection at the Polyclinics or laboratories that provided the nematodes from humans was performed in concordance with the WMA Helsinki Declaration (Edinburgh 2000) and its subsequent modification, as well as with the Italian National Law n. GDPR 2016/679 on the protection of personal data.

For Slovak samples, specimens of human origin were obtained from segregated villages and settlements within a survey aimed at investigating the current status of intestinal parasitic infections among the Roma communities living in five eastern districts of the country and to compare the results with data from the late 1970s. The proposal to conduct the survey on individuals aged until 18 years was reviewed and approved by the Ethical Commission of the Košice Self-governing Region (document no. 5436/2019/ODDZ-25820). Samples were collected in pediatric departments in municipal hospitals of regional cities. Adult *Ascaris* worms from infected children were collected into plastic containers with physiological saline solution. Children parents (legal representatives) agreed with all investigations and signed the informed consent prior to examinations.

3. RESULTS

3.1 Molecular analyses on nuclear marker: genotyping and HW equilibrium

In total, 207 positive identifications through PCR-RFLP of the ITS target region were obtained (Table 1).

Table 1. Results obtained from the PCR-RFLP analysis of the ITS target region, per host origin and country. Number of specimens per host and number of positives are listed.

Country	Host	Number of hosts	Number of nematodes obtained	PCR-RFLP ITS genotyping			
				Positive	*Ascaris suum*	Hybrids	*Ascaris lumbricoides*
Colombia	Human	13	13	13	0	0	13
	Pig	14	33	31	22	8	1
Slovakia	Human	20	20	15	0	0	15
	Pig	35	94	73	58	8	7
Italy	Human	20	20	16	9	3	4
	Pig	20	60	59	50	9	0

The observed genotypes were the following (Figure 1): in Colombia, all samples from humans were *A. lumbricoides* and most of the pig samples were *A. suum*, with 8 hybrids and only one *A. lumbricoides* reported in this host species; in Slovakia, all human samples were *A. lumbricoides* as observed in Colombia, while pig-derived samples showed most of the specimens identified as *A. suum* and lower occurrence of hybrids and *A. lumbricoides*; in Italy, more than half of the human-derived samples were identified as *A. suum*, while four specimens were *A. lumbricoides* and other 3 showed the hybrid pattern.

HW analyses performed only for pig-derived specimens have shown significant results demonstrating *Ascaris* populations from the three countries as being in HW equilibrium, with an excess of *A. lumbricoides* genotype in pigs from Slovakia.

3.2 Molecular analyses on mitochondrial marker: variability, population differentiation and network analyses

A total of 110 quality sequences of 353bp were obtained through the sequencing of the *cox1* target region: 28 from Colombia (10 from humans, 18 from pigs), 32 from Slovakia (5 from humans, 27 from pigs), and 49 from Italy (16 from humans, 34 from pigs). Analyses of haplotypes revealed the presence of 37 haplotypes: 22 in pig specimens, 12 in human specimens, and 3 found both in humans and pigs (Table 2). Four haplotypes were shared among specimens from different countries and no haplotype was shared by all the three countries, while thirty-three haplotypes have not been reported before. Some haplotypes showed high frequencies, such as Hap1 in human-derived samples from Colombia and Slovakia (50% and 60%), and being completely absent from the Italian dataset, and Hap2 reported in Italian human-derived samples and in both human and pig-derived samples from Colombia. Interestingly, only three haplotypes were observed in human samples from Slovakia and Colombia, while in Italy a total of 11 haplotypes were observed circulating in humans. The sequence comparison through NCBI BLAST revealed five haplotypes showing 100% identity with already described haplotypes: Hap1 was identical to *A. lumbricoides* isolated from a Japanese patient (Accession number AB591800, Arizono et al., 2010); Hap2 and Hap9 to *A. lumbricoides* isolated in Brazilian human-derived samples (Accession number MK143390 and MH800275, respectively); Hap4 to *A. suum* isolated from a Brazilian pig (Accession number MK143381) and Hap11 to *A. lumbricoides* isolated from a pig in the

Czech Republic (Accession Number LN600400). Hap2 and Hap4 were previously reported also from pigs from USA (KY200855 and KY200852, respectively). The remaining 32 haplotypes are newly described. The complete list of the BLAST results is available as supplementary material (Table S1).

Human-derived samples from Italy showed one private haplotype at a higher frequency (Hap23; around 19%), and three less frequent haplotypes (Hap2, Hap4, Hap11) shared with Colombian samples from both hosts and with pig-derived samples from Slovakia. Several low-frequency haplotypes were also found in the sample set (Table 2).

Table 2. Haplotype frequencies obtained through *cox1* sequences analysis of *Ascaris* spp., per host and country of origin.

Haplotype	Colombia		Slovakia		Italy	
	Human origin (N=10)	Pig origin (N=18)	Human origin (N=5)	Pig origin (N=27)	Human origin (N=16)	Pig origin (N=34)
Hap1	0.50		0.60			
Hap2	0.30	0.22			0.12	
Hap3	0.20					
Hap4		0.22			0.06	
Hap5		0.11				
Hap6		0.06				
Hap7		0.06				
Hap8		0.17				
Hap9		0.11				
Hap10		0.06				
Hap11				0.33	0.12	0.06
Hap12				0.07		
Hap13				0.11		
Hap14				0.26		
Hap15				0.04		
Hap16				0.07		
Hap17				0.04		
Hap18				0.04		
Hap19				0.04		
Hap20			0.20			
Hap21			0.20			
Hap22					0.12	
Hap23					0.19	
Hap24					0.06	
Hap25					0.06	
Hap26					0.06	

Hap27	0.06	
Hap28	0.06	
Hap29	0.06	
Hap30		0.03
Hap31		0.03
Hap32		0.59
Hap33		0.09
Hap34		0.12
Hap35		0.03
Hap36		0.03
Hap37		0.03

Estimation of genetic variability indexes are available in Table 3. Gene diversity ranged from 0.07 to 0.95: the highest level of gene diversity and nucleotide diversity was recorded for Italian human-derived samples, while the lowest level was observed in human-derived samples from Slovakia, which represent also the sample set with the lowest number of the analysed specimens (5). When samples were pairwise compared, the Fst ranged from 0.004 to 0.629. The most distantly related populations appeared to be *Ascaris* isolated from pigs in Italy and *Ascaris* isolated from humans in Slovakia, while the most similar populations were the *Ascaris* specimens isolated from pigs in Colombia and those isolated from humans in Italy (Table 4). The analysis of molecular variance described a significant variation among populations between groups defined according to geography and within populations, whereas variance among groups was not statistically relevant.

Table 3: Results from genetic variability estimations of *A. suum* and *A. lumbricoides*, per host and country of origin.

Country	Host	Number of Haplotype	Gene Diversity	Nucleotide Diversity	Expected Heterozygosity
Colombia	Pig	8	0.89 +/-0.04	0.012 +/- 0.009	0.29
	human	3	0.69 +/-0.10	0.015 +/-0.009	0.53
Slovakia	Pig	9	0.82 +/-0.05	0.023 +/-0.012	0.31
	human	3	0.07 +/- 0.22	0.002 +/-0.002	0.40
Italy	Pig	9	0.64 +/- 0.09	0.011 +/- 0.006	0.14
	Human	11	0.95 +/- 0.04	0.028 +/- 0.015	0.26

Table 4: Results from pairwise comparison of *Ascaris* spp. according to host and country of origin, estimated using the Fst approach and the exact test of sample differentiation, including the significance (* p<0.05). *Ascaris* specimens analysed: HCol and PCol = Humans and pigs from Colombia; HSlo and PSlo = Humans and pigs from Slovakia; HIta and PIta= Humans and pigs from Italy.

	HCol	**PCol**	**PSlo**	**HSlo**	**HIta**	**PIta**
HCol	0					
PCol	0.049	0				
PSlo	0.378*	0.355*	0			
HSlo	0.310*	0.420*	0.448*	0		
HIta	0.035	0.004	0.226*	0.291*	0	
PIta	0.429*	0.325*	0.365*	0.629*	0.285*	0

Within-host species levels of evolutionary distances were 2.3% and 2.6% in human- and pig-derived *Ascaris*, respectively, while between-host species distance was 2.8%. Taking into account also geographical criteria, the highest percentage of genetic distance was obtained between pig-derived *Ascaris* from Slovakia and human-derived *Ascaris* from Italy (3.3%), while the lowest value was obtained between human roundworms from Colombia and Slovakia (1.4%).

Results from haplotype network analyses have shown a reticulate relationship among specimens, distributed in apparently separated clusters (Figure 2). Specifically, the three main clusters, analogous to clusters named A, B and C described in previous works (Anderson and Jaenike, 1997; Cavallero et al., 2013; Easton et al., 2020; Iñiguez et al., 2012; Šnábel et al., 2012), were also observed in the present study, according to the affiliation of the selected reference sequences representative of clades. None of these groups was exclusive for specific host or country of origin. Considering also the already described haplotypes, Cluster A includes samples from both pigs and humans collected from endemic and non-endemic zones; it showed further slight internal subdivision extended to two-branches, with a star-like subcluster A1 indicated in black including several specimens collected from Italian pigs (28 of

34 total sequences analysed). This network pattern suggests a structured population. All these specimens showed *A. suum* or hybrid genotypes at RFLP analyses. The second branch of Cluster A, namely A2, is linearly shaped and it included few pig-derived specimens from Slovakia and Colombia, indicated in green and red respectively, and several samples of human origin from Colombia and Italy. In these specimens, all three detectable genotypes through the RFLP analyses were recorded. The remaining two groups, assigned as Cluster B and C, were both closely related to Cluster A, and showed an internal reticulate relationship among haplotypes. Specifically, Cluster B was represented by pig-derived samples from all countries that formed the first lines of the branch, followed by human-derived specimens. All these samples showed *A. lumbricoides* genotypes at RFLP analysis. The last group, Cluster C, included pig derived samples from the three countries, and human derived samples from Italy, mostly manifesting the *A. suum* genotype at RFLP analysis.

4. DISCUSSION

The present study contributes to updating and extending information on the genetic diversity of the etiological agent of the NTD ascariasis and add knowledge on the distribution of *Ascaris* genotypes circulating in pigs and humans from three countries in two continents, namely Italy, Slovakia, and Colombia, where alternative epidemiological scenarios are considered to occur for human ascariasis.

Assuming that proximity between humans and pigs can result in cross-transmission, some human-derived samples are expected to show worms with *A. suum* and/or hybrid pattern. On the contrary, separated cycles are frequently found in endemic areas, while hybrids or cross-transmission events are usually observed only in pigs.

Previous studies focused on endemic countries reported diverse patterns of genotypes, such as high level of cross-infections with *A. suum* in humans in China (Zhou et al., 2012); 9% of hybrids in pig-derived worms in Honduras (Palma et al., 2019); the absence of cross-transmission and the presence of *A. lumbricoides* in people and *A. suum* in pigs in Ecuador and Honduras (Sparks et al., 2015); very low percentage of *A. suum* in human samples from Zanzibar (Sparks et al., 2015), and pig-associated genotype in all samples from pigs and mainly human-associated genotype in samples from humans in Thailand, the Lao PDR, and Myanmar (Sadaow et al., 2018). The latter pattern is similar to the genotype distribution found in the present study in Colombia, here representative for the endemic countries.

On the other hand, evidences from non-endemic countries revealed, for instance, cross-infections and hybridization in Italy (Cavallero et al., 2013), cross-infections with *Ascaris* from pigs in Denmark (Nejsum et al., 2005) and Japan (Arizono et al., 2010), and the presence of human genotypes in pigs in the United States (Chelladurai et al., 2017). Similar evidences were obtained in the present study for Italian and Slovak samples.

Parameters of population differentiation obtained using the HW approach and the Fst index revealed no fixed differences between human and pig *Ascaris*, describing the two taxonomic entities as closely related and therefore likely to experience gene flow, in particular where low circulation of one taxa (i.e. *A. lumbricoides* in Italy) increases the probability of acquiring infections from the zoonotic source (i.e. *A. suum*) in humans. *Ascaris* populations from the three countries were mostly in HW equilibrium, which suggests the existence of panmictic populations. A different pattern was found in China, where worm populations significantly deviated from HW equilibrium (Zhou et al., 2018). Hardy-Weinberg disequilibrium was herein found in Slovak pigs, and was due to an unexpected excess of *A. lumbricoides* homozygotes. This could be explained by i) a potential high level of *A. lumbricoides* in

humans in Slovakia and subsequent environmental contamination facilitating the cross-species infections or by ii) a partial reliability of nuclear marker to assign species identity, and the possibility to observe the F2 backcrosses hybrids or by iii) the retention of an ancestral polymorphism which complicates genotype assignment. However, it must be taken into account that the use of a single marker as ITS provides limited information on the genetic variation of *Ascaris*, and may be of relative support for diagnostic properties. The retention of ancestral polymorphisms and introgression can lead to the segregation of divergent alleles in the same populations, and to the occurrence of identical alleles in genetically distinct populations, thus, misleading conclusions on the taxonomic status or epidemiology, or incorrect species identification can occur when analysing a single locus (Anderson, 2001). Moreover, ITS is a multicopy genomic region and intraindividual variation may occur as a result of incomplete concerted evolution (Celes et al., 2010). Given the potential limitation using a single marker, further studies with more sensitive approaches (e.g. microsatellites) appear needed to confirm this finding.

Although Slovakia is globally considered an area of non-endemicity, there are several foci of hyperendemicity associated with Romany settlements, a community of more than 215,000 people, which built the majority of their settlements in the 18th century (Mušinka et al., 2014). It is well known that these groups present high levels of infection risk, as infections are often transmitted at places with high population density, poor hygiene conditions and low socio-economic status. More than 80% of children living in distressed socio-economic conditions in focal regions of eastern Slovakia were found infected with parasites (Solovič et al., 2011). Studies involving Roma children from separated settlements found an average rate of *Ascaris* infection from 20.7% to 52.2% (Bystrianska et al., 2019; Pipiková et al., 2017a, 2017b; Rudohradská et al., 2012; Štrkolcová et al., 2019). On the contrary, the infection rate in non-

Roma children ranged from 0 to 2.6% (Bystrianska et al., 2019; Königová et al., 2010; Pipiková et al., 2017a; Štrkolcová et al., 2019).

In the present study, a surprisingly high occurrence of cross-infections with *A. lumbricoides* was found in pigs from several locations of eastern Slovakia. Since the majority of Slovak sampling sites were in proximity to Roma settlements, we must consider the substantial environmental contamination by *Ascaris* eggs in these areas. Romani communities often own dogs freely circulating around settlements, and a high prevalence of *Ascaris* (40.9%) has been found in dog feces from Roma settlements (Pipiková et al., 2017b). This represents a risk factor for soil contamination and dispersal of roundworm eggs in the proximity of pig farms and breeding, thus creating a likely scenario for the occasional cross-infections observed in pig hosts. Additionally, coprophagous insects, birds through adhering the eggs to their feet in wet soil, and the dry dust through the wind can mechanically spread *Ascaris* eggs into adjacent areas (Bidinger et al., 1981).

Assuming the measure of the evolutionary distances (calculated as the total percentage of sequence differences for each pair) as useful for defining cryptic species boundaries (*sensu* Blouin, 2002), a low level of differentiation was observed. A genetic distance of around 2-3% was estimated comparing the *Ascaris* populations according to the geographical origin as well as according to the host affiliation. Usually, values around 2%–6% are thought to reflect the existence of cryptic species for mtDNA markers, while values exceeding 10% are suggestive of different morphospecies, as observed in other parasitic nematodes (Blouin, 2002; Blouin et al., 1998; Cavallero et al., 2019).

Regarding the evolutionary relationships of haplotypes obtained using a phylogenetic network approach, clusters A-B-C appeared to be quite equally distant (two lost ancestral states are visible in both branches), suggesting a complex scenario. However, the internal branches

revealed a partially different pattern in comparison to the previous data: cluster A appeared to be apparently equally distant from both clusters B and C, while it has been originally described using the same molecular marker as closely related to cluster B, and cluster C was even separated (Betson et al., 2014; Cavallero et al., 2019). High levels of genetic divergence between clades using both *Ascaris* spp. *cox*1 alone and the complete mitochondrial genomes were observed by Easton et al. (2020), in which clades A and B were closer to each other while C was more distinct and closely related to clade B. An additional study by Nejsum et al. (2017), based on the entire mtDNA, suggested slight differences compared to the results obtained through the use of single or multiple markers. The genetic distance between clusters A/B and cluster C was similar using data for all mt genes in this recent study, whereas the partial *cox1* sequencing suggested that cluster B is more closely related to cluster C. Here, the *Ascaris* samples partitioned into the three clusters revealing different degree of host affiliation. Accordingly, most of the worms found in pigs in Italy belonging to cluster A and to less extent to cluster B, while worms from pigs of Slovakia are more widely represented in cluster C but showing a wider distribution in all clusters. In Slovakia, humans are expected to poorly contribute to transmission. However, Slovak samples originated from a hyperendemic Roma settlement, thus cross-transmission may have determined at least a part of human-like patterns in pigs from abattoirs in close locations. The frequencies of haplotypes varied along with locations: the majority of human worms from Italy (62%) and pig worms from Italy (82%) and Colombia (72%) belonged to cluster A; whereas all the human worms from Slovakia, half of the human worms from Colombia and to a lesser extent from Italy (19%) belonged to cluster B. These Italian human cases showed *A. lumbricoides* at nuclear ITS genotype analyses. Cluster C had unique composition, as it included most of the pig worms from Slovakia (59%) and few pig worms from Italy and Colombia (6% and 5.5%, respectively), and around 20% of

human worms from Italy. All these Italian human cases showed *A. suum* at nuclear ITS genotype analyses. It is worth to underline that the cluster C was not identified in human worms from Slovakia nor Colombia.

An allele commonly found in one host species in one location may be infrequent in the same host species in a different location, as evidenced e.g. in pig and human populations in China and Uganda (Peng et al., 2005; Betson et al., 2011). Coupled with these data, Peng and Criscione (2012) summarized population genetics data from previous studies and stated that due to the recurrent pattern of colonization, it is not possible to infer local contemporary cross-transmission based on global allele frequency data. In the present study, significant differences in Fst indices were recorded in all pairs of host-associated populations including those originating from the same continent (Italy, Slovakia), except for human-derived populations from Italy and Colombia (Fst = 0.035). Results from both mtDNA (a lack of consistency of human- and pig-derived samples in A-C clades at a global scale) and nuclear microsatellite markers (Anderson and Jaenike, 1997, Criscione et al., 2007) suggested that genetic differentiation between populations was primarily driven by geography, with secondary differentiation resulting multiple host colonization events. Moreover, Easton et al (2020) suggested a lack of differentiation into distinct species and a potentially large interbreeding population analysing complete mitochondrial genomes, also, after constructing a reference-quality *Ascaris* genome from a single worm collected from a person in Kenya, it was found to be highly similar to the *A. suum* genome from worms collected in the United States from pig hosts.

5. CONCLUSIONS

This study contributes to the updating of *Ascaris* molecular epidemiology by determining genotypes circulating in humans and pigs living in different epidemiological scenarios. It must be highlighted the presence of human-derived *Ascaris* into the phylogenetic cluster C and the strong zoonotic relevance of *Ascaris suum* in non-endemic regions. The finding of extensive genetic variation is supporting the existence of two closely related *Ascaris* taxonomic entities, with residual ability of interbreeding. *Ascaris* from both pigs and humans may be important in human disease, requiring a one-health approach to avoid the spread of human ascariasis.

FIGURE LEGEND

Figure 1: *Ascaris* genotypes frequency. Genotypes occurrence according to hosts (human-derived *Ascaris* in dark grey and pig-derived *Ascaris* in dark yellow) and country of origin (Colombia; Slovakia; Italy). Number of positive specimens are reported in vertical bars.

Figure 2. Haplotype network of *Ascaris* spp. Haplotype network of *Ascaris* spp. according to *cox*1 sequences, with indication of host and country and clade affiliation. Circles diameters are proportional to haplotype frequency in individuals. Small dots at nodes represents extinct haplotypes and transversal bars indicates polymorphisms occurring among haplotypes. Haplotype identity (e.g. Hap1) is indicated as number next to each circle. Host and country of origin are indicated with colours.

DECLARATION OF INTEREST

Authors have no competing interests to declare.

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Declaration of Interest

on behalf of all authors of the research article entitled "Genotyping of *Ascaris* spp. infecting humans and pigs in Italy, Slovakia and Colombia" I declare we all have no conflict of interest.

Serena Cavallero: Conceptualization, Methodology, Software, Data curation, Writing- Original draft preparation

Silvia Rondón: data curation and reviewing

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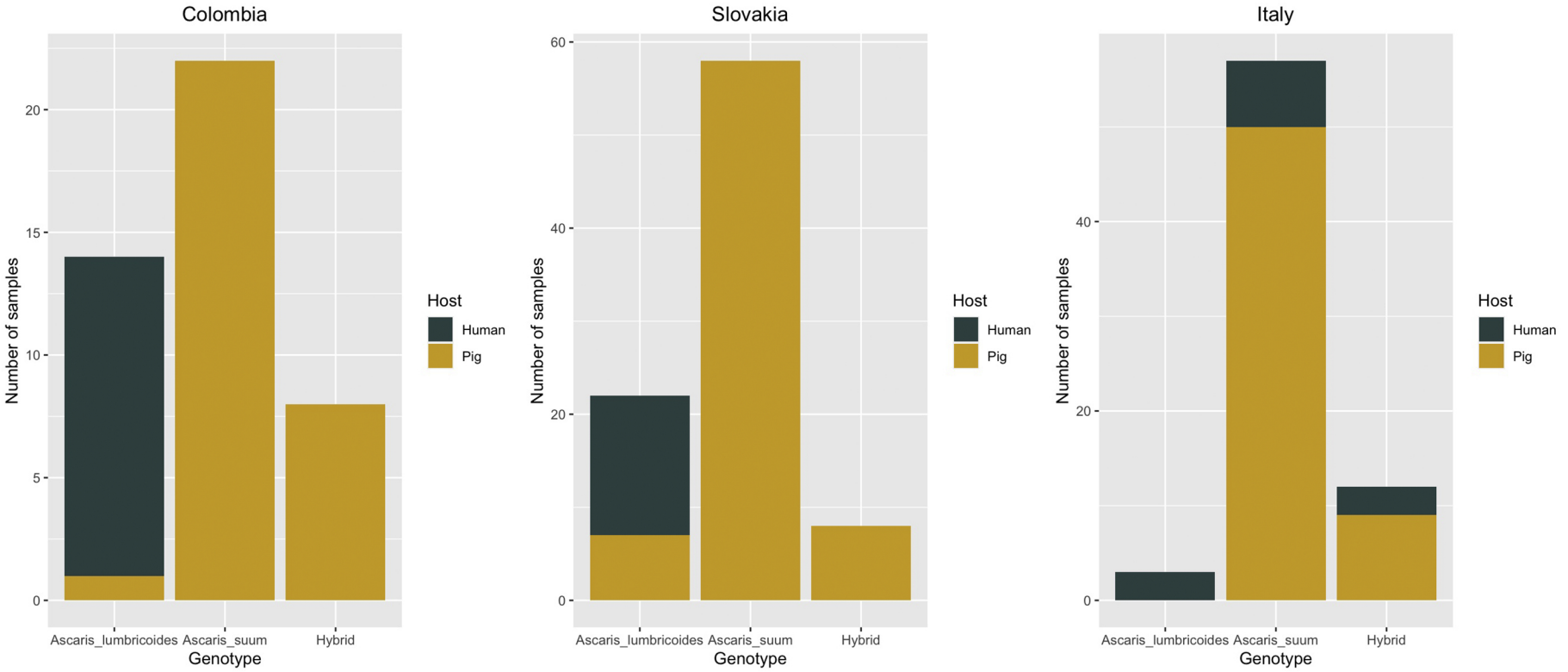
Highlights

- Haplotype network of *Ascaris cox*1 confirmed the presence of three clades.
- *Ascaris* from Italian and Colombian pigs showed to be in Hardy-Weinberg equilibrium.
- Hardy-Weinberg disequilibrium was found in *Ascaris* from Slovak pigs.
- Significant genetic variation within populations *Ascaris* based on geography was found.


Colombia
Slovakia
Italy
Number of samples
Genotype
Ascaris_lumbricoides
Ascaris_suum
Hybrid
Host
Human
Pig
0
5
10
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20
20
40
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40

Figure 1

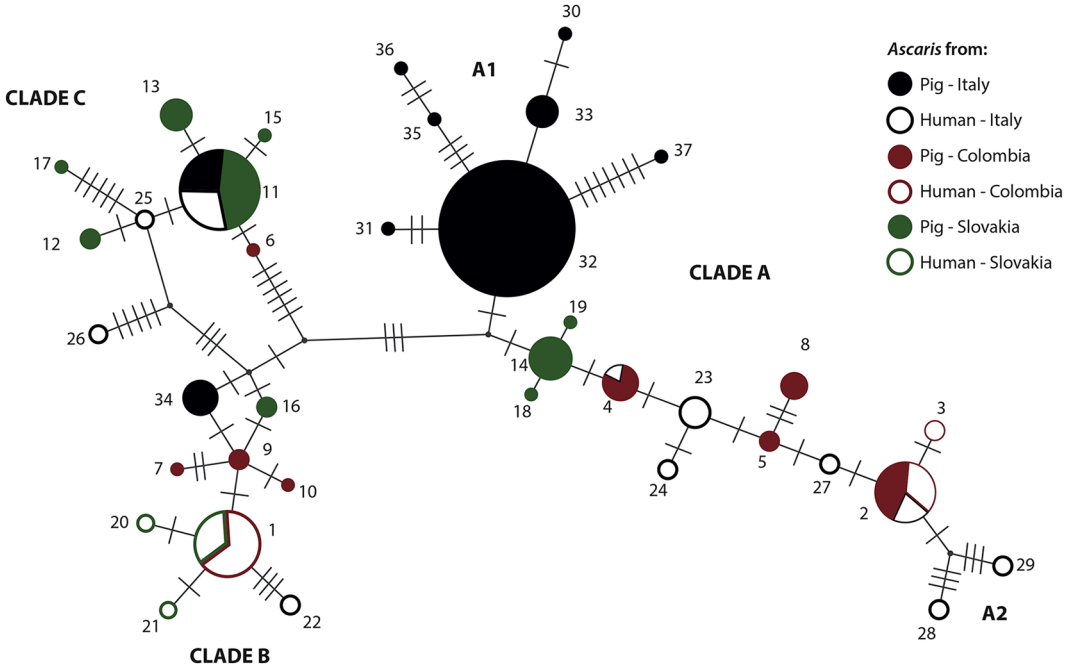
CLADE C
A1
CLADE A
CLADE B
A2
Ascaris from:
Pig - Italy
Human - Italy
Pig - Colombia
Human - Colombia
Pig - Slovakia
Human - Slovakia
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Figure 2