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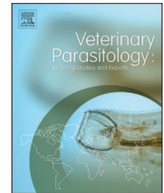

Original Article

Development of a PCR assay for identification of *Neobalantidium coli* (Pomajbíková et al., 2013) in Argentina

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ABSTRACT

Neobalantidium coli (Pomajbíková et al., 2013) is a cosmopolitan ciliate which colonizes the intestine of humans and animals. Pigs are the most important host and reservoir for this parasite, although others mammals have been described. Humans can acquire the disease through the ingestion of water and food contaminated with cysts and even from person to person contact. Farmers and slaughterhouse workers from rural areas of developing countries have an increased incidence of balantidiosis. In Argentina, despite swine production on family farms covers 70% of domestic consumption requirements; there is a lack of veterinary animal health planning which result in high rate of animal mortality, as well as environmental risk due to inefficient facilities and mismanagement of manure and effluents. At present there are no epidemiological data on balantidiosis in Argentina, except for isolated reports. Therefore, the aims of this study were to establish the frequency of *N. coli* in pigs raised under different conditions and to explore the zoonotic potential. In order to confirm the identity of *Neobalantidium coli* like-cysts founded in the feces, a set of *N. coli* specific primers based on 18S rRNA gene sequences was designed. The molecular identification of *N. coli* was performed in 88.9% (16 out of 18) of swine stool samples in which cysts had been visualized. The fecal samples obtained from pigs raised on more open farmland showed a lower percentage of *N. coli* than those obtained from animals raised in swine pens. On the other hand, molecular identification of *N. coli* was also performed in human feces. Pairwise comparison of sequences obtained from pigs and human fecal samples from the NW Region of Argentina showed a high percentage of similarity, indicating a possible zoonotic transmission.

1. Introduction

Neobalantidium coli (Pomajbíková et al., 2013) is a cosmopolitan ciliate that is common in tropical and subtropical regions which colonizes the intestine of mammals (Nakauchi, 1999). Pigs are the most important host and reservoir for this parasite (Goldberg, 2007). Even though parasitized pigs remain mostly asymptomatic, on occasions animals may experience mild to severe diarrhea (Schuster and Ramirez-Avila, 2008).

Humans can acquire the disease through the ingestion of water and food contaminated with cysts and even from person to person contact (Fletcher et al., 2012). *N. coli* is ciliate protozoan that infects humans and non humans primates causing from asymptomatic balantidiosis to acute fulminant colitis (Schuster and Ramirez-Avila, 2008). Trophozoites invade the mucosal lining of the ileum and colon producing mucosal ulceration and inflammation which, in the acute presentation of the disease, account for the symptoms including diarrhea, hematochezia, abdominal pain and weight loss. Untreated infections may progress to toxic colitis with fever, peritonitis, intestinal perforation leading to a death rate of about 30%, or fulminating dysentery associated with hemorrhage and shock (Goldberg, 2007; Fletcher et al., 2012).

The most important factors in the spread of human disease are the presence of infected pigs and the improper waste disposal. Humans living in close contact with swine such as farmers and slaughterhouse workers from rural areas of developing countries have an increased incidence of infection (Goldberg, 2007; Schuster and Ramirez-Avila, 2008). Notwithstanding, opportunistic intestinal infections (Ferry et al., 2004) and very rare invasions of extra-intestinal organs were described occasionally in immunosuppressed hosts living in urban environments,

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where pigs are not a factor in infection (Anargyrou et al., 2003; Verweij and Stensvold, 2014).

The worldwide prevalence of balantidiosis was estimated to be in the range 0.02–1% although wide variations were reported according to the geographic location surveyed (Schuster and Ramirez-Avila, 2008). Although the worldwide human incidence of this neglected parasitosis remains very low, the highest prevalence rates are reported in tropical and subtropical regions (Solaymani-Mohammadi and Petri, 2006). Unfortunately, epidemiological studies are scarce; some of them involving small rural communities and small sample size. A very interesting study covering 22 communities in the Bolivian Altiplano included the stool testing for more than two thousand children and fifty pigs reared on family farms from the same area. The overall prevalence was found to be 1.2% for school children; 54% of swine stool were positive for the ciliate (Esteban et al., 1998). Moreover, *N. coli* was reported as responsible for at least one water-associated outbreak (Karanis et al., 2007).

Despite swine production on family farms in Argentina covers 70% of domestic consumption requirements, the activity is influenced by technological, productive, commercial, social and environmental issues which reduce their performance, i.e., unskilled or untrained workers, lack of veterinary animal health planning which result in high rate of animal mortality, inefficient facilities, and environmental risk due to mismanagement of manure and effluents (Beyli et al., 2012). These issues foster the cycle of infection among animals and, probably are involved in the dynamics of zoonotic and anthroponotic transmission of the disease. *N. coli-like* cysts were the most frequent protozoan in ratites commercially raised on farms throughout the country (Martínez-Díaz et al., 2013), a finding with sanitary implications for wild birds as rural establishments release certain proportion of captive animals for their reintroduction into the wild (Martella and Navarro, 2006). Recently, *N. coli*-like cysts were identified in stool samples from wild Lesser Rhea (*Rhea pennata*) collected in Peninsula Valdés, a protected area from Patagonia, Argentina (Frixione et al., 2014). At present there are no epidemiological data on balantidiosis in Argentina, except for isolated reports (Borda et al., 1996).

This scenario shows that the application of more stringent diagnostic tools that allow the proper identification of species is crucial to highlight the actual impact of pathogenic species on human and animal health. Therefore, the aims of this study were to establish the frequency of *N. coli* in pigs raised under different conditions, by microscopy and molecular techniques, and to explore the zoonotic potential.

2. Methods

2.1. Sample collection and study population

In 2011 fresh stool samples were obtained from pigs (*Sus scrofa domestica*) reared on small family farms from the NW Region of Argentina (n = 18, Misión Nueva Pompeya (MNP), Province of Chaco) and on an Agricultural Experimental Station located in the Central Region of the country (n = 10, Marcos Juárez (MJ), Province of Córdoba). Samples were collected in plastic bottles containing 70% alcohol as preservative solution.

Individual human samples (n = 15) had been collected, in the occasion of conducting an epidemiological survey in the same region, to determine the prevalence of intestinal parasites in the population living in rural places of the “El Impenetrable Chaqueño” forest (appropriate verbal consent from all participants had been obtained) (Buyayisqui et al., 2013). Typically, fecal samples are preserved in SAF at 4 °C in our laboratory for several years in the dark in order to be used in future research.

2.2. Microscopic diagnosis

Samples were processed according to a centrifugation method previously reported (Garbossa et al., 2013). Briefly, fecal suspensions were washed only with saline solution in order to recover undamaged trophozoites. At least two fresh preparations (one of them lugol iodine-stained) were examined by light microscopy (400 × magnification).

2.3. DNA extraction

Feces were placed in a mortar with liquid nitrogen, crushed with a pestle three times and, thereafter, the DNA extraction procedure of a commercial kit (QIAmp™ DNA Stool Mini Kit, Qiagen) was applied to the whole sample according to the manufacturer's instructions. DNA was kept at − 20 °C until used as template for PCR amplification reactions.

In the case of human feces, due to scarcity, each stool sample consisted of a pool of five, chosen at random the same rural location (Pozo del Sapo (PS1H), Pozo del Toba (PT2H) and Laguna Araujo (LA3H) from Province of Chaco) where fecal specimens were taken from pigs.

2.4. Molecular identification of Neobalantidium coli

In order to confirm the identity of the cysts morphologically similar to *Neobalantidium coli*, a set of *N. coli* specific primers was designed, based on the 18S rRNA gene sequences deposited at the GenBank database (GQ903678.1, AM982722.1, AM982723.1, and AF029763). The primers targeted a conserved region of the gene and match a polymorphic region: Bal_F 5′-TTGTGATTGTAGTGAGGGTATTCC-3′ and Bal_R 5′-CCAAGATTCTAGACACATTCG-3′. The amplification was performed in 50 μl reaction mixture containing 0.4 μM of each primer, 0.2 mM of each deoxyribonucleotide triphosphate (Promega, USA), 1.25 U of Taq DNA polymerase (Qiagen), 1 × PCR buffer and 250–300 ng of genomic DNA. Conditions used for the amplification of nucleic acids by PCR were: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 51 °C, 1 min at 72 °C and finally, an extension for 1 min at 72 °C. The amplification product was fractionated by electrophoresis in agarose gel and visualized in a UV transilluminator. The 271 bp amplicon was extracted from the PCR mix (QIAquick PCR Purification Kit, Qiagen). Sequencing by capillary electrophoresis was performed at Unidad Genómica of Instituto de Biotecnología, CICVyA, INTA, using Bid Dye Terminator v3 and automatic sequencers xl 3500 Genetic Analyzer (Applied Biosystems) using the same primers set used to obtain the original PCR product.

Contigs of 269 bp were edited and assembled using the Software Vector NTI10.3.0. The Basic Local Alignment Search Tool (BLAST) was employed to confirm the identity of the sequences. Subsequently, the Clustal W multiple alignment (Larkin et al., 2007) was employed to find regions of similarity. To compare pairwise distances from DNA sequences and calculate the percentage of similarity between different isolates the maximum composite likelihood model was applied employing the Software Mega 5.1 (Tamura et al., 2011).

2.5. Data analysis

Agresti–Coull binomial 95% confidence intervals (CI) were used for proportions (Brown et al., 2001).

3. Results

Microscopic inspection of swine feces allowed detecting cysts morphologically compatible with *Neobalantidium coli* (Fig. 1) in 55.5% (95% CI: 34–75; 10 out of 18) of samples collected in family farms (MNP; NW Region) and in 80% (95% CI: 48–95; 8 out of 10) of samples from the Agricultural Experimental Station (MJ; Central Region).

Amplification of the 18S rRNA gene and subsequent sequences alignment allowed the unequivocal identification of *N. coli* in 80% (8 out of 10) of samples from MNP and in 100% (8 out of 8) samples from MJ in which cysts had been visualized (Table 1).

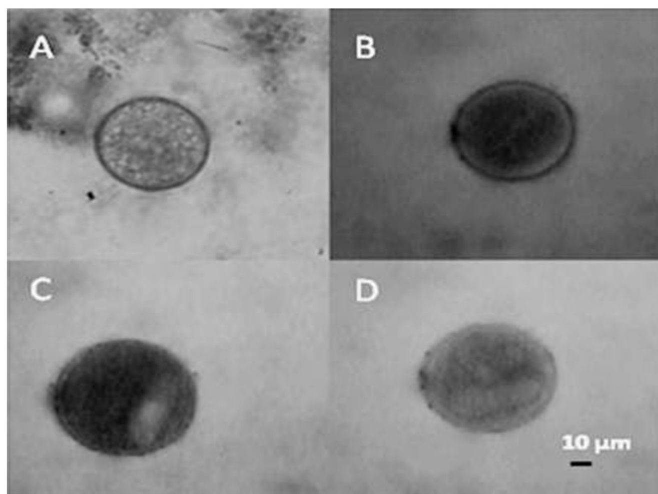

Fig. 1. Cysts of *Balantidium coli* (55–60 μm). A. Cyst iodine-stained, many vacuoles are observed inside of the cell. B, C y D. Cyst in fresh samples, the macronucleus is visible in some cysts. 400 × magnification.

Table 1
Neobalantidium coli frequency in samples from MNP and MJ swine stools.

	M[a]	95% CI	PCR[b]	95% CI
MNP (Chaco)	55.5% (10/18)	55 (34–75)	80% (8/10)	72 (48–95)
MJ (Córdoba)	80% (8/10)	72 (48–95)	100% (8/8)	84 (63–100)

CI: Confidence Interval.
[a] Microscopy.
[b] *N. coli*-specific fragment of DNA.

Although the differences were not statistically significant the prevalence of *N. coli* (confirmed by molecular methods) was greater in pigs reared in the Agricultural Experimental Station than in those reared in freedom in Chaco (80%; 95% CI: 48–95 vs. 44%; 95% CI: 63–100).

On the other hand, sixteen sequences from swine fecal samples described herein (GenBank accession numbers listed in Table 2), 3 sequences from human fecal samples (KR349515, KR349516, KR349517) and those ones available from different hosts (*Sus scrofa domestica*: JQ073304.1, GQ903678.1, JQ073335.1, JQ073334.1, JQ073333.1, JQ073326.1, JQ073324.1, JQ073323.1, JQ073322.1, JQ073321.1, JQ073320.1; *Sus scrofa*: JQ073319.1; *Pan troglodytes*: JQ073332.1, JQ073329.1, JQ073318.1; *Papio hamadryas*: JQ073305.1; *Gorilla gorilla*: EU680309.1 and *Elephas maximus indicus*: AB794980.1) were used to perform the alignment. The global alignment of sequences confirmed that the cysts detected in both pigs and humans samples correspond to *N. coli* (Supplementary data).

Pairwise comparison showed similarity between sequences. 100% similarity was used as the criterion to cluster and to assign the same letter (a, b, c, d, e and f groups) to similar sequences. Four isolates from swine feces from MNP (NF_1, NF_2, NF_8 and NF_9) were identical to each other and also identical to four isolates from MJ (MJ_1, MJ_3, MJ_6 and MJ_9) (Table 2, group c).

Even though Neobalantidium cysts had not been detected previously in human feces by optical microscopy, to explore the zoonotic potential each of all three stool pool (PS1H, PT2H and LA3H) was subjected to nucleic acid extraction and PCR amplification. Remarkably, the amplicon of expected size was found in samples and their subsequent sequencing allowed establishing that the fragments fitted in the 18S rRNA subunit gene of *N. coli*. Pairwise comparison showed that these isolates were 100% identical. In addition, they were identical to one swine isolate from Misión Nueva Pompeya (PS_57) and other swine isolate from Marcos Juárez (MJ_2) (Table 2, group a).

Table 2
Microscopic and molecular diagnosis of *Neobalantidium coli* in swine feces.

Geographical location	Sample ID	Microscopy	PCR	100% Parwise similarity	GenBank accession nr
Misión Nueva	PS_6	−	−		
Pompeya	PS_54	−	−		
	PS_57	+	+	a	KR349506
24°55′36″S	PT_16	+	+	d	KR349504
61°28′48″W	PT_17	+	+	b	KR349505
	PT_20	+	ND	ND	
	PT_21	+	ND	ND	
	LA_24	−	−		
	LA_25	−	−		
	LA_26	−	−		
	LA_27	−	−		
	NF_1	+	+	c	KR349499
	NF_2	+	+	c	KR349500
	NF_3	−	−		
	NF_4	−	−		
	NF_8	+	+	c	KR349501
	NF_9	+	+	c	KR349502
	NF_11	+	+	b	KR349511
Marcos Juárez	MJ_1	+	+	c	KR349507
32°42′00″S	MJ_2	+	+	a	KR349514
62°06′00″W	MJ_3	+	+	c	KR349512
	MJ_4	+	+	e	KR349513
	MJ_5	−	−		
	MJ_6	+	+	c	KR349508
	MJ_7	−	−		
	MJ_8	+	+	d	KR349509
	MJ_9	+	+	c	KR349510
	MJ_10	+	+	f	KR349511

ND: Both methods were applied to all samples except for two that could not be amplified.
a, b, c, d: 100% Pairwise similarity among sequences is represented by the same lowercase letter.
e, f: No similar sequences were found.

4. Discussion

Although *N. coli* cysts were previously detected in stool samples of children living in the northeast region of Argentina (Borda et al., 1996) and more recently in environmental samples from the northwest region of the country (Poma et al., 2012), this is the first study that confirm its presence in pig and human feces by the use of molecular techniques.

Nowadays, light microscopy is not always an adequate method for genus and species identification. In our hands, it only allowed detecting cysts with morphological features similar to those of *N. coli*. Moreover, the large sized cysts and trophozoites may be prone to render iodine-overstained artifacts when lugol is selected as the stain for wet mounts (Verweij and Stensvold, 2014). Furthermore, the potential presence in stool of *N. coli*-like microorganisms could lead to misdiagnosis. Though rarely found to infect swine, *Buxtonella sulcata* is also a protozoan parasite and is similar to *N. coli* (Tomczuk et al., 2005). The similarity between both parasites makes it essential to unambiguously identify cysts found in stool samples. At this point the question arises about how reliable are the molecular methods to yield real results. For this reason, we designed a primer set targeting the V4 hypervariable region of the 18S rRNA and a PCR assay which proved to be a valuable tool for the detection and unambiguous identification of *N. coli* either in isolate from swine and human feces.

Even though the 18S rRNA allowed the identification at genera and species level it is not sensitive enough to distinguishing intraspecific genetic variants that could be geographically associated or linked to different hosts. The few DNA sequences available in the GenBank have been obtained mainly from ribosomal genes 5.8s, 18s and 28s and their internal transcribed spacers ITS1 and ITS2 (Verweij and Stensvold, 2014). However, all isolates from human feces in this work were found to be 100% identical not only among them but also to two swine fecal

isolate (PS_57 and MJ_2). This finding would suggest a transmission between humans and pigs reared on family farms from the NW Region of Argentina. Notwithstanding, it is necessary to find new target genes and to use other approaches to better describe the genetic diversity and to identify *N. coli* subpopulations. Studies will have to further analyze the secondary structure of the V4 and the ITS2 regions in an attempt to contribute to a better understanding of intra- and intergenomic variability and understanding some epidemiological aspects of transmission of *N. coli*.

On the other hand, an interesting aspect to highlight about the results of this work is that the frequency of balantidiosis in animals raised under controlled production conditions was higher than in those raised in rural areas. Therefore, it is possible to suggest that even though animals are given feed and corn and are subject to regular sanitary controls in the Agricultural Experimental Station, the overcrowding would favor fecal accumulation in pens and consequently would result in direct contact with feces. A similar study conducted in Italy showed a markedly higher prevalence of balantidiosis in swine raised in pens and fed commercial feed, as compared to those raised "en plein air", since the latter farming system could have a "dilution" effect on protozoa fecal excretion (Giarratana et al., 2012).

It is essential to point out the key role of swine acting as transmitters and as reservoirs of other zoonotic microorganisms in both the domestic and wild transmission cycles (Solaymani-Mohammadi et al., 2004). In agreement with this premise, some animals in the present study were found to be infected also with the protozoan *Entamoeba polecki* which may infect humans (data not shown). Furthermore, it has been suggested that wild boars (*Sus scrofa*) could constitute a reservoir for Balantidium in the wild transmission cycles (Solaymani-Mohammadi et al., 2004). This hypothesis cannot be ruled out as the rural area where sampling was conducted is immersed in an ecosystem whose prevailing vegetal species constitute a dense and thorny forest — known as the "El impenetrable chaqueño"— where the residents usually hunt wild animals for their livelihood (Buyayisqui et al., 2013) and where the vulnerability of the inhabitants (the close contact of the people with parasitized animals and the absence of sanitary controls) constitute the largest risk to the perpetuation of the parasitic life cycle.

Bearing in mind that these findings suggest the zoonotic transmission of balantidiosis, we deem it is necessary to develop a comprehensive program of health education and health promotion to protect vulnerable families who live in rural communities as those described herein. Such educational program should be coordinated between animal health agencies and public health institutions with the aim of improving the health requirements for small family farms and to control the spread of this and other zoonotic diseases.

5. Conclusions

1. A set of specific primers for *N. coli* was developed, based on 18S rRNA gene sequences deposited in the GenBank database, and which allowed the unequivocal identification of the parasite.
2. The fecal samples obtained from pigs raised on more open farmland showed a lower percentage of *N. coli* than those obtained from animals raised in swine pens.
3. Pairwise comparison of sequences obtained from pig and human fecal samples showed a high percentage of similarity, deducing the zoonotic potential of *N. coli*.

6. Recommendation

To promote the networking between universities, research institutes, professional associations and NGOs to involve them in advocating for global, regional or local health with the goal sharing best practices and to develop concrete proposals to promote health. And finally, to encourage a closer cooperation between the scientific community and policymakers to further promote future evidence-based health policies.

Conflict of interest

The authors declare that they have no competing interest. The authors also affirm that they have no financial or personal interest that could affect their objectivity, or inappropriately influence their actions while they were conducting this research.

Ethical statement

The experiments carried out in swine reported in this manuscript were conducted following the Guide for the care and use of animals-INTA (Approved by resolution CICVyA No. 14/07) and internationally recognized guidelines of "Care and Use of Experimental Animals" as Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition, 2010.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.vprsr.2017.08.010.

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