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Evolution of Canine Parvovirus in Argentina between years 2003 and 2010: CPV2c has become the predominant variant affecting the domestic dog population

Marina Gallo Calderón^a, Carina Romanutti^a, Alejandra D' Antuono^a, Leticia Keller^b, Nora Mattion^a, Jose La Torre^{a,*}

^a Centro de Virología Animal, Instituto de Ciencia y Tecnología Dr. Cesar Milstein, CONICET, Saladillo 2468, C1440FFX, Buenos Aires, Argentina

^b Fundación de Estudios en Virología Animal (FEVAN), Guaminí 1682, Ciudad de Buenos Aires, C1440ESD, Argentina

ARTICLE INFO

Article history:

Received 17 December 2010
Received in revised form 17 February 2011
Accepted 17 February 2011
Available online 24 February 2011

Keywords:

Canine Parvovirus
VP2 gene
CPV2c variants
Sequence analysis

ABSTRACT

The current frequency of Canine Parvovirus variants (CPV2a, CPV2b and CPV2c) in the Argentine dog population was investigated by PCR amplification of a 583 bp fragment in the VP2 gene. From a total of 79 rectal swab samples that have been submitted to our laboratory since 2008, 55 (69.6%) resulted positive and were further analyzed by direct DNA sequencing. Fifty positives samples (91%) were characterized as CPV2c variant, which appeared in Argentina in the year 2003 and has been the prevalent type since 2008, whereas CPV2a and CPV2b, still found in Argentine dogs, were represented in 3.6% and 5.4% of the population, respectively. Considering that CPV2c is spreading worldwide, and that this variant is also affecting vaccinated dogs, efforts should be made towards the development of new matched CPV vaccines.

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1. Introduction

Four different families of animal viruses have been identified as the etiological agents of severe enteritis in dogs: Canine Parvovirus (CPV), Canine Coronavirus, Canine Rotavirus and Canine Distemper Virus (Mochizuki et al., 1993; Pollock and Carmichael, 1983; Decaro et al., 2009a). Because of the severity of the disease and its rapid spread throughout the canine population, CPV has arisen a great deal of public interest.

CPV is a non-enveloped virus with a single-stranded DNA genome of approximately 5000 bases which encodes two structural (VP1 and VP2) and two non-structural (NS1 and NS2) proteins (Cotmore and Tattersall, 1987).

CPV was first identified in May 1978 as a new virus infecting dogs (Parrish and Kawaoka, 2005; Kapil et al., 2007). In the late 1970s and early 1980s both live and inactivated Feline Panleukopenia (FPL) vaccines were used to protect dogs against CPV disease. However the level of protection they afforded was poor and the duration of immunity was short. These vaccines were replaced by live attenuated CPV vaccines, which provided good protection and longer lasting immunity (Spibey et al., 2008).

Some biological properties of the virus, particularly its rate of nucleotide substitution which is closer to that of RNA viruses

(aprox. $10^{-3}/10^{-4}$) than to that of double-stranded DNA viruses (Shackelton et al., 2005), determined the continuous appearance of new variants with high sanitary impact in the vaccinated and unvaccinated dog populations. Two antigenic variants, named CPV2a and CPV2b, appeared a few years after the discovery of the first characterized CPV2.

Currently, the original CPV2 strain is not circulating in dog populations, although it is still present in vaccine formulations, whereas the variants CPV2a and CPV2b are distributed worldwide (Parrish et al., 1991; Martella et al., 2005a; Decaro et al., 2006b; Mochizuki et al., 1993; Buonavoglia et al., 2000; de Ybanez et al., 1995; Greenwood et al., 1996; Sagazio et al., 1998; Steinel et al., 1998; Truyen et al., 1996; Pereira et al., 2000; Cavalli et al., 2008; Buonavoglia et al., 2001; Martella et al., 2004; Martella et al., 2005b; Truyen et al., 2000; Martella et al., 2006).

CPV2a, differs from the original type 2 strain in the presence of five amino acid (aa) changes in the VP2 coat protein, while the antigenic differences observed in CPV2b are the consequence of only one aa substitution (Asn426Asp), located in the major antigenic site of the capsid (epitope A) (Parrish et al., 1991).

A new antigenic variant, carrying the aa substitution Asp426Glu, in the major antigenic site of the viral capsid of the VP2 protein, was reported in Italy in the year 2001 (Buonavoglia et al., 2001; Desario et al., 2005). This newest variant -designated CPV2c- has already been detected in other European countries, as well as in Asia, Africa and America (Decaro et al., 2009a,b; Nakamura et al., 2004; Kapil et al., 2007; Martella et al., 2004; Decaro et al., 2007; Hong et al.,

* Corresponding author. Tel.: +54 11 4686 6225; fax: +54 11 4686 6225.
E-mail address: jlatorrecevan@centromilstein.org.ar (J. La Torre).

2007; Perez et al., 2007; Joao Vieira et al., 2008; Calderon et al., 2009; Nandi et al., 2010).

It is important to emphasize that the updating of methodologies by incorporation of the Minor Groove Binder probe technology, as well as PCR amplification followed by sequencing, allowed a precise identification of emerging CPV variants, therefore becoming important tools for the study of CPV epidemiology (Decaro et al., 2006a; Decaro et al., 2005c; Decaro et al., 2005b).

In the present study, we report an update of the identification and characterization of different CPV variants in Argentina.

2. Materials and methods

2.1. Clinical specimens

A total of 79 rectal swabs samples were obtained from domestic dogs from the Argentine cities of Buenos Aires ($n=50$), Dolores ($n=2$), Tandil ($n=4$), Pehuajó ($n=1$), Mar del Plata ($n=1$), Bahía Blanca ($n=12$), Córdoba ($n=2$), Misiones ($n=1$) and Río Negro ($n=3$), located 216, 350, 370, 400, 690, 710, 1060, and 1500 Km from Buenos Aires, respectively. A sample from Paraguay was also analyzed while the origin of two other samples was unknown.

Clinical specimens were submitted to our laboratory for diagnostic purposes between the years 2008 and 2010. Information about the animals whose samples tested positive for CPV, such as clinical symptoms, age, gender, breed and vaccination status, is provided in Table 1.

2.2. Preparation of samples for PCR analysis

CPV genomic DNA was extracted directly from rectal swabs and from a commercial vaccine (Vanguard[®] Plus CPV, Pfizer), in a lysis buffer containing 50 mM Tris–HCl pH 8, 100 mM NaCl, 25 mM sucrose, 10 mM EDTA and 1% SDS. After lysis, the extracts were digested with proteinase K (Invitrogen[®], USA) at 56 °C for 30 min and the DNAs were extracted with phenol–chloroform.

2.3. Primers and PCR amplification

For the amplification of a large fragment of the VP2 capsid protein-encoding gene of CPV, the 555for/555rev primer set (5'-CAGGAAGATATCCAGAAGGA-3'/5'-GGTGCTAGTTGATATGTAATAAACA-3'), was used. The resulting product (583 bp), comprises at least six or seven informative aa responsible for important biological properties of the virus (Buonavoglia et al., 2001).

PCR amplification was performed using Taq recombinant polymerase (Invitrogen, USA) in an MJ Research cycler (PTC-100, Sierra Point, CA), with an initial denaturation step at 94 °C for 10 min, followed by 40 cycles of PCR (94 °C for 30 s, 50 °C for 60 s, and 72 °C for 60 s). The final concentration of MgCl₂ was 1.5 mM.

2.4. Sequencing of the amplified DNA

The DNA fragments amplified from clinical samples were directly sequenced by the dideoxy-mediated chain-termination method (MacroGen Inc., Korea). Multiple sequence alignment analysis was performed using ClustalW.

The GenBank accession numbers of the aa and nucleotide sequences of reference CPV strains are: CPV-b strain (CPV2, M38245); CPV-15 strain (CPV2a, M24003); CPV-39 strain (CPV2b, M74849) and 56/00 (CPV2c, FJ222821).

The GenBank accession numbers of the nucleotide sequences of the DNA fragments amplified from local variants are: HQ589341, HQ589342, HQ445626, HQ450025, HQ450027, HQ45002, and

HQ450029 (sample from Paraguay). For practical purposes, identical sequences were grouped, and only one of them was registered in the database.

3. Results

3.1. CPV detection by PCR

During this study, 79 rectal swabs samples from animals suspected of CPV infection were tested. CPV specific DNA was detected by PCR amplification of a 583 bp fragment, in 55 out of 79 (69.6%) analyzed rectal swabs. The DNA extracted from a commercial vaccine was used as positive control. In all cases, the obtained PCR products were analyzed electrophoretically, and no differences were found in the size of the amplicons (data not shown).

Negative samples were assayed twice to confirm the results. Forty-nine out of 55 positive animals (96%) showed clinical symptoms compatible with CPV, while no records were available for the other six dogs. Interestingly, most of the positive samples (78.2%) were recovered from 1 to 5 months old puppies (Table 1). Forty-three of them (78%) were obtained from puppies which had been vaccinated against CPV at least once, whereas eleven (20%) were obtained from unvaccinated dogs, and the vaccination status of the remaining dog was unknown.

No relationship could be established regarding sex or breed (Table 1).

3.2. Identification of CPV variants by DNA sequencing

In order to identify and differentiate CPV variants in local clinical samples, the PCR products from all the positive samples were sequenced and the deduced aa sequences were aligned.

Fig. 1 shows the results of the sequence alignment from the position 407–456 of VP2, derived from seven representative local field samples identified as CPV2a, CPV2b and CPV2c, along with the sequences of the vaccine strain (CPV2) and three international reference strains. At the 426 position CPV2a variants showed an Asn (N), CPV2b an Asp (D) whereas the CPV2c variants showed a Glu (E) residue. Similar analysis performed with all the CPV positive samples, revealed that 50 out of 55 (91%) carried the Asp426Glu mutation, characteristic of the CPV2c variant, whereas two samples were characterized as CPV2a, and the remaining three, were defined as CPV2b (data not shown). In addition, the aa alignments performed with the complete 583 bp, amplification products, showed a low degree of variation ($\leq 1\%$) among the Argentine CPV2c variants. This observation is in accordance with previous reports from other authors (Decaro et al., 2009b; Kapil et al., 2007; Pereira et al., 2007).

From the results shown in Fig. 2 and Table 1, it is clear that all CPV2 variants are at present affecting the Argentine dog population. However since the year 2008 CPV2c has become the predominant variant considering that 91% of the positive samples belonged to this variant, whereas CPV2a and CPV2b were represented only in 3.6% and 5.4% of the clinical samples, respectively.

3.3. A potentially relevant aminoacidic change

Besides the aa substitution at the 426 position, analysis of the sequences of Argentine CPV variants, showed the appearance of a new mutation, T440A, which is restricted to the CPV2c local variants, and whose worldwide presence has been recently reported (Decaro et al., 2009b; Kapil et al., 2007; Hong et al., 2007; Chinchkar et al., 2006; Kang et al., 2008).

The analysis performed, clearly demonstrated that 100% of the samples characterized as CPV2c obtained in 2008, presented the typical aa T at the 440 position, whereas 58% and 50% of the CPV2c

Table 1
Age, gender, vaccination records and clinical signs of dogs infected with CPV.

Strain	Year	Age (months)	Sex	Breed	Vaccination status	Clinical signs	CPV strain	Origin	aa at 440
Arg19	2008	2	M	R	V (C)	+	CPV2c	Buenos Aires	T
Arg20	2008	2	NA	AD	V (C)	+	CPV2c	Bahía Blanca	T
Arg21	2008	2	NA	AD	V (C)	+	CPV2c	Bahía Blanca	T
Arg22	2008	2	NA	AD	V (C)	+	CPV2c	Bahía Blanca	T
Arg23	2008	2	NA	AD	V (C)	+	CPV2c	Bahía Blanca	T
Arg24	2008	5	M	MB	V (C)	+	CPV2c	Buenos Aires	T
Arg25	2008	2	F	MS	V (C)	+	CPV2c	Tandil	T
Arg26	2008	2	M	GS	V (C)	+	CPV2c	Río Negro	T
Arg27	2008	2	M	GS	V (C)	+	CPV2c	Río Negro	T
Arg28	2008	4	F	MP	V (C)	+	CPV2c	Buenos Aires	T
Arg29	2008	3	M	GP	V (C)	+	CPV2c	Buenos Aires	T
Arg30	2008	19	M	GP	V (C)	+	CPV2a	Buenos Aires	T
Arg31	2008	4	M	SFT	V (C)	+	CPV2c	Bahía Blanca	T
Arg32	2008	1	M	P	NV	+	CPV2c	Buenos Aires	T
Arg33	2008	3	F	AD	V (C)	+	CPV2c	CórdobaT	T
Arg34	2008	NA	NA	MS	NA	NA	CPV2c	Buenos Aires	T
Arg35	2008	4	M	FT	V (C)	+	CPV2c	Tucuman	T
Arg36	2008	NA	NA	Pi	V (NA)	NA	CPV2a	Buenos Aires	T
Arg37	2009	3	F	BD	V (NA)	NA	CPV2c	Buenos Aires	T
Arg38	2009	3	F	MB	NV	+	CPV2c	Buenos Aires	T
Arg39	2009	3	F	MB	NV	+	CPV2c	Buenos Aires	T
Arg40	2009	1	M	D	NV	+	CPV2c	Buenos Aires	A
Arg41	2009	2	F	MS	V (C)	+	CPV2c	Buenos Aires	A
Arg42	2009	12	M	MP	V (C)	+	CPV2c	Buenos Aires	T
Arg43	2009	3	F	C	V (C)	+	CPV2c	Buenos Aires	A
Arg44	2009	3	M	B	V (C)	+	CPV2c	Buenos Aires	T
Arg45	2009	NA	F	R	NV	+	CPV2c	Buenos Aires	T
Arg46	2009	3	M	BD	V (C)	+	CPV2b	Buenos Aires	T
Arg47	2009	4	F	PB	NV	+	CPV2c	Buenos Aires	T
Arg48	2009	6	F	LR	V (C)	+	CPV2c	Buenos Aires	A
Arg49	2009	3	F	MB	NV	+	CPV2c	Buenos Aires	A
Arg50	2009	1	M	G	V (C)	+	CPV2b	Dolores	T
Arg51	2009	1	F	G	V (C)	+	CPV2b	Dolores	T
Arg52	2009	2	M	LR	NV	+	CPV2c	Buenos Aires	A
Arg53	2009	2	F	MP	V (C)	+	CPV2c	Buenos Aires	A
Arg54	2009	8	M	MP	V (C)	+	CPV2c	Buenos Aires	A
Arg55	2009	NA	NA	YT	V (NA)	NA	CPV2c	Buenos Aires	A
Arg56	2009	2	M	B	V (C)	+	CPV2c	Buenos Aires	A
Arg57	2009	7	M	GS	V (C)	+	CPV2c	Buenos Aires	A
Arg58	2009	2	F	SFT	V (C)	+	CPV2c	Bahía Blanca	T
Arg59	2009	1	M	MP	V (C)	+	CPV2c	NA	A
Arg60	2009	2	F	Bea	NV	+	CPV2c	Buenos Aires	A
Arg61	2009	5	M	MB	V (C)	+	CPV2c	CórdobaT	T
Arg62	2009	4	M	RR	V (C)	+	CPV2c	Misiones	T
Py1	2009	4	M	NM	V (NA)	+	CPV2c	Paraguay	A
Arg63	2010	2	M	GR	V (C)	+	CPV2c	NA	T
Arg64	2010	NA	NA	SFT	NA	NA	CPV2c	Bahía Blanca	T
Arg65	2010	NA	NA	SFT	NA	NA	CPV2c	Bahía Blanca	T
Arg 66	2010	5	F	Bea	V (C)	+	CPV2c	Buenos Aires	A
Arg67	2010	NA	F	MS	V (C)	+	CPV2c	Buenos Aires	A
Arg 68	2010	3	M	MS	V (C)	+	CPV2c	Mar del Plata	T
Arg 69	2010	2	M	Pug	V (C)	+	CPV2c	Buenos Aires	A
Arg 70	2010	2	F	GR	V (C)	+	CPV2c	Buenos Aires	A
Arg 71	2010	2	M	P	V (C)	+	CPV2c	Buenos Aires	A
Arg 72	2010	4	M	SFT	V (C)	+	CPV2c	Bahía Blanca	T

F: female; M: male; MB: Mixed Breed; GR: Golden Retriever; LR, Labrador Retriever; GS: German Shepherd; R: Rottweiler; D: Doberman; AD: Argentine Dogo; MS: Miniature Schnauzer; FT: Fox Terrier; YT: Yorkshire Terrier; SFT: Smooth Fox Terrier; C: Cocker; P: Poodle; MP: Miniature Poodle; B: Boxer; Bea: Beagle; BD: Bulldog; Pi: Pinscher; PB: Pitbull; G: Greyhound; NM: Neapolitan Mastiff; RR: Rhodesian Ridgeback; GP: Giant Poodle. V: vaccinated; NV: no vaccinated; NA: no information available. C: complete vaccination according to its age.

samples obtained in 2009 and 2010, respectively, showed the presence of a new aa substitution (T440A), indicating that the variant CPV2cA440 has been introduced recently in the Argentine dog population.

3.4. Age distribution of CPV2c variant

As can be seen in Fig. 3, the distribution of the CPV2c variant according to age clearly shows that the majority of the puppies (86%) affected by this variant were between 1 and 5-month-old, with most of the samples belonging to 2 month-old puppies.

4. Discussion

The present work represents an updating of the incidence of different CPV variants in Argentina, including the cases characterized in a previous report (Calderon et al., 2009). Although CPV2c was first detected in 2003, the results presented herein clearly show that CPV2c has become the predominant variant since the year 2008, as 91% of the samples analyzed were found to carry the typical aa substitution Glu426 (Table 1 and Fig. 2). An additional mutation located at an exposed domain of the VP2 capsid protein (Glu426-Ala440) was also found in some of the CPV2c samples (Fig. 1), which is in agreement with previous reports (Decaro et al., 2009b; Kapil et al.,

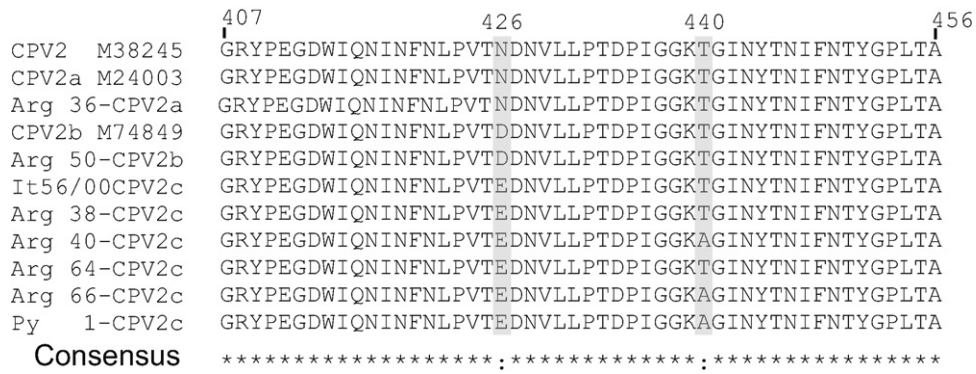


Fig. 1. Alignment of the deduced amino acid sequences of a fragment of the 583 bp amplicon of the VP2 gene amplified by PCR from clinical samples (aa 407–456). The aligned sequences correspond to a vaccine strain (CPV2, M38245), a CPV2a reference strain (CPV2a, M24003), a CPV2b reference strain (CPV2b M74849), an Italian CPV2c strain (FJ222821.1), and Argentine CPV2a, CPV2b and CPV2c variants. The positions 426 and 440 are highlighted.

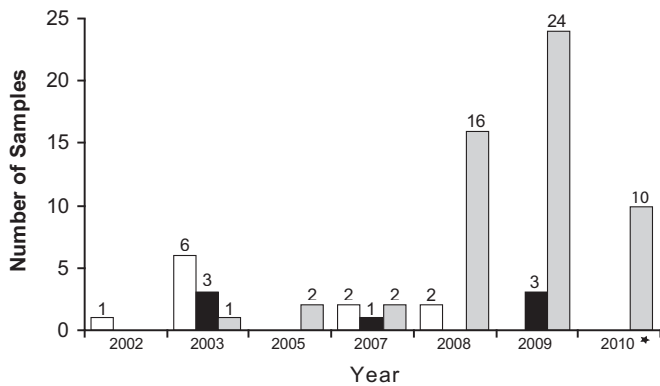


Fig. 2. Updating of the evolution of CPV variants in Argentina. The number of cases detected in the years 2002–2010 belonging to each CPV variant are shown on top of the bars (previous study, Calderon et al., 2009). *Up to October 2010. White bars, CPV2a variants; black bars, CPV2b variants; grey bars, CPV2c variants.

2007; Hong et al., 2007; Chinchkar et al., 2006; Kang et al., 2008). It is interesting to point out that this mutation was found exclusively in samples collected during the years 2009 and 2010, indicating its recent appearance in the Argentine dog population.

The correlation of the T440A substitution with biological properties of the virus is still unknown, but since it is located at an important VP2 domain, exposed to selective pressure of vaccine

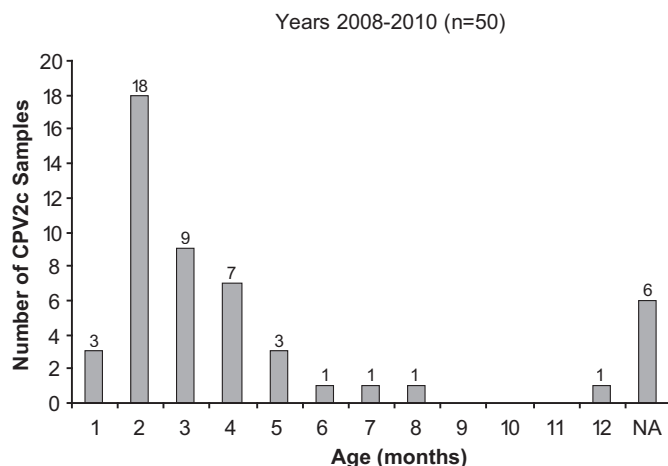


Fig. 3. Distribution of the CPV2c variants in Argentine dogs, according to age.

or colostrum antibodies, epidemiological surveillance is required to investigate the evolution, behavior and eventual sanitary impact of the viruses carrying this mutation (Hoelzer and Parrish, 2010; Hoskins, 2009).

Except for the two relevant aa substitutions mentioned, a low degree of variability ($\leq 1\%$) was found in the VP2 sequences of CPV2c Argentine field variants. This observation is in agreement with previous reports from other regions of the world (Decaro et al., 2009b).

It has been reported that dogs infected with the newest CPV2c variant showed frequently atypical clinical symptoms, thus making the diagnosis of CPV disease difficult (Decaro et al., 2005a). In addition, classical diagnostic procedures are not suitable for precise CPV strain identification. The traditional PCR amplification of a 583 bp fragment showed to be effective in the detection of CPV DNA, but as the size PCR products derived from CPV2, CPV2a, CPV2b and CPV2c are indistinguishable from each other; sequencing is mandatory to allow the precise identification of each variant.

The clinical samples analyzed in this work were obtained from a population composed predominantly by young puppies, and were submitted to our laboratory by associated dog breeder's veterinarians. Our results clearly showed that the higher incidence of CPV2c was in 1–5-month-old puppies, with a peak in 2-month-old animals (Fig. 3). The vaccination status of this particular population was variable, with animals who had received only one or up to three doses of vaccine.

Although these findings need further confirmation by analyzing a larger number of animals, it may be speculated that at an early age there is a weakness or even a gap in the protective status of the puppies, probably due to low levels of maternal antibodies, or to the presence of an immature immune system, unable to respond properly to vaccination. Similar results were reported by our laboratory in the case of calf neonatal diarrhea caused by rotavirus (Bellinzoni et al., 1989). A strong increase in the level of colostrum antibodies was demonstrated in this case through immunization of pregnant dams 40 days prior to delivery or by feeding exposed calves with hyperimmune colostrum. Therefore, vaccination of pregnant bitches might also increase the specific colostrum and milk antibody levels in dogs, which can help dealing with the CPV2c infections in young puppies. Another possibility might be the development of improved vaccines or the updating of the vaccination schedules, in order to induce effective early protective responses.

Canine Parvovirus evolution and its rapid spreading through dog populations, raises concerns among breeders, owners and veterinary practitioners about the need of updating current CPV vaccines (Truyen, 2006). In addition, complementary sanitary measures, such as increasing the biosecurity levels of the breeding premises

and the implementation of stronger sanitary regulations in the trade or exhibitions of live dogs, could also be relevant.

Acknowledgements

This work was supported with funds from FEVAN, CONICET and Tecnovax S.A. We thank Drs. Marena, Leonardo Mauro and Ruben Somoza for providing the clinical specimens. We also thank Drs. Maximiliano Wilda and Blanca Robiolo for the critical revision and helpful discussion of the manuscript and Miss Silvia Rojana for her technical help.

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