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Comparison of two commercial kits and an in-house ELISA for the detection of equine rotavirus in foal feces

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A B S T R A C T

Group A rotaviruses (RVA) are important infectious agents associated with diarrhea in the young of several animal species including foals. Currently, a variety of diagnosis methods are commercially available, like ELISA, latex agglutination and immunochromatographic assays. These commercial tests are mainly designed for the detection of human RVA; its applicability in veterinary diagnosis has been poorly studied. The aim of this study was to compare the sensitivity and specificity of two commercial diagnostic kits, Pathfinder™ Rotavirus and FASTest Rota® strip, with an in-house KERI ELISA, for the detection of equine RVA.

A total of 172 stool samples from Thoroughbred foals with diarrhea were analyzed. The presence of equine RVA in samples in which only one of the three methods showed positive results was confirmed by RT-PCR. A sample was considered "true positive" when RVA was detected by at least two of the methods, and "true negative" when it tested negative by the three assays. Following these criteria, 50 samples were found positive and 122 were found negative, and were handled as reference population for the assay validation.

Pathfinder™ Rotavirus assay showed 32% sensitivity and 97% specificity, FASTest Rota® strip, 92% sensitivity and 97% specificity, and KERI ELISA, 76% sensitivity and 93% specificity. Pathfinder™ Rotavirus showed 77%, FASTest Rota® strip 95%, and KERI ELISA 88% accuracy to correctly classify the samples as equine RVA positive or negative. Pathfinder failed specifically to detect equine RVA G3P12I6 genotype; such performance might be related to the specificity of the monoclonal antibody included in this kit. According to our results, differences among VP6 genotypes could influence the sensitivity to detect equine RVA in foal feces, and thus assay validation of diagnostic kits for each species is necessary. In conclusion, FASTest Rota® strip is more suitable than ELISA Pathfinder™ Rotavirus for the screening of rotavirus infection in foals. The KERI ELISA showed an acceptable performance, and could be considered a proper economic alternative for equine RVA diagnosis.

1. Introduction

Group A rotaviruses (RVA) are associated with diarrhea in infants and in the young of many animal species including foals (Kapikian, 1994). Equine RVA, the cause of severe outbreaks of diarrhea in foals younger than 3 months old, is recognized as a very important sanitary problem for the horse breeding industry worldwide (Browning et al., 1991; Dwyer, 2007; Frederick et al., 2009; Kapikian, 1994; Slovis et al., 2013).

Rotaviruses (RV) are icosahedral non-enveloped viruses possessing a genome of 11 segments of dsRNA, and are classified into 7 groups, A–H, according to a common group antigen present in the intermediate capsid protein VP6. Within RV group A (RVA), 16 variants or genotypes of VP6 have been described; based on its nucleotide diversity, each variant is identified as an I-genotype (Inner capsid), from I1 to I18, (Estes et al., 2001; Ghosh et al., 2011; Matthijnssens et al., 2012b).

Differences in the two outer capsid proteins, VP7 and VP4, allow classifying RVA into G-types (*Glycoprotein*) and P-types (*Protease-sensitive*), respectively. Currently, 27 G and 37 P-types are recognized among RVA of all species (Matthijnssens and Heylen,

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2013). Genotypes G3 and G14 are the most common VP7 variants circulating in horses worldwide, and are associated with P[12], a VP4 variant only found in horses (Ciarlet et al., 1994; Collins et al., 2008; Elschner et al., 2005; Garaicoechea et al., 2011; Isa et al., 1996; Monini et al., 2011; Nemoto et al., 2011; Tsunemitsu et al., 2001; van der Heide et al., 2005). Equine RVA show a great level of conservation in their genome constellations worldwide, with eight gene segments possessing the same genotype. Only in the VP7, VP6 and NSP4 gene segments, the presence of two different genotypes (G3/G14, I2/I6 and E2/E12, respectively) has been found (Matthijnssens et al., 2012a). Accurate and rapid detection of RVA in diseased foals, early during the diarrhea outbreaks, represents a helpful tool, allowing the quick implementation of appropriate management measures to minimize the spread of the infection (Cho et al., 2012; Dennehy et al., 1994; McGuirk, 2008).

The diagnosis of RVA infection relies on the finding of viral particles, RVA antigens, or viral genomic RNA segments, in stool samples obtained early in the course of clinical signs. Several approaches have been developed for this purpose, including enzyme immunosorbent assays (EIAs), immune chromatography tests, latex agglutination, dot hybridization, polyacrilamide gel electrophoresis, RT-PCR, and direct and immune electron microscopy assays (Brooks et al., 1989; Cicek et al., 2007; Cornaglia et al., 1989; Cho et al., 2012; Dennehy et al., 1988, 1999, 1994; Eing et al., 2001; Klein et al., 2009; Lipson et al., 2001; Maes et al., 2003; Marchlewicz et al., 1988). Apart from a few exceptions, the diagnostic methods that are commercially available are mainly designed to detect human RVA strains. Therefore, we focused our attention in the validation of rapid antigen detection kits that could be used for routine diagnosis of equine RVA; if so, a treatment and management decision could be quickly applied (Nemoto et al., 2010).

The aim of this study was to evaluate the sensitivity, specificity and accuracy of two commercial kits and an in-house KERI ELISA, for equine RVA detection in stool samples of foals with diarrhea.

2. Materials and methods

2.1. Samples

A total of 172 stool samples from foals (1 day to 10 months old) with acute diarrhea were included in the study. The samples had been selected from the diarrhea cases submitted to the equine viral diseases diagnostic laboratory of the Virology Institute, INTA, Argentina, from 2009 to 2011.

2.2. Experimental design

All the samples were tested by the two commercial kits and an in-house double sandwich ELISA. All samples which tested positive in at least one of the three assays were further re-tested by RT-PCR to confirm the presence of RVA and to determine their G, P and I genotype. Electron microscopy (EM) was performed in samples showing discordant results. A sample was considered "true positive" when two or more assays detected rotavirus in it, and "true negative" when RVA was not detected by any of the three assays first mentioned. Taking into account this criterion, the "true positive" and "true negative" samples were assigned as reference population and used for the assays' validation. The sensitivity, specificity and accuracy of the assays were estimated by Receiver-Operating Characteristic (ROC) analysis, and the concordance of each assay to properly classify a sample was calculated by means of the weighted kappa statistic (κ test).

2.3. Rotavirus A detection

A commercial ELISA kit (Pathfinder™ Rotavirus EIA; BioRad, Marnes-la-Coquette, France), designed for the detection of RVA in human feces, was used. This kit includes plastic tubes coated with polyclonal antibodies to simian SA-11 (G3P[1]I2) rotavirus as the capture antibody, and a peroxidase-labeled monoclonal antibody directed to a VP6 epitope of murine rotavirus as the detection antibody. The assay was performed as specified by the manufacturer.

The other commercial test used was the FASTest Rota® strip (MEGACOR, Diagnostk GmbH, A-6912 Hörbranz, Austria), an immunochromatographic lateral flow assay for the qualitative detection of RVA in animal feces. In this test, RVA particles react with mobile anti-RVA monoclonal antibodies bound to red latex particles. When an antibody–antigen complex is formed, it migrates along the nitrocellulose membrane to the test zone producing a red line of variable intensity. The assay was performed according to the manufacturer's instructions.

All fecal samples were also tested using an in-house antigen capture enzyme-linked immunosorbent assay named KERI ELISA (KERI: Kit ELISA Rotavirus INTA, Cornaglia et al., 1989) to detect RVA antigen. Briefly, 96 well ELISA plates (Maxisorp, NUNC, Thermo Fisher Scientific, 75 Panorama Creek Drive, Rochester, NY, USA) were coated with appropriate dilution of a polyclonal guinea pig IgG against bovine RVA/Cow-xx/USA/INDIANA/XXXX/G6P[5]-I2 (Indiana) as positive capture (R1) antibodies, and control guinea pig IgG as negative capture (R2) antibodies (Ab), and incubated at 4 °C overnight. A ten-fold dilution (1:10) of each fecal sample was added to both R1 and R2 wells, and incubated at 37 °C for 1 h. A purified polyclonal bovine IgG against RVA Indiana strain was used as a secondary Ab. The plates were later incubated for 1 h at 37 °C with commercial HRP-labeled goat polyclonal Ab to bovine IgG (KPL, Kirkegaard & Perry Laboratories Inc., USA). Hydrogen peroxide and ABTS were used as substrate/cromogen system (KPL, Kirkegaard & Perry Laboratories Inc., USA). This assay has been routinely used in our Institute for RVA detection in bovine, equine, camelid and pig fecal samples, using a strict cut-off (0.200) in order to ensure 100% specificity (Marcoppido et al., 2011; Parreno et al., 2010). In these previous studies, a sample was considered RVA positive if the difference between the optical density (OD) measured at 405 nm in the positive (R1) and the negative (R2) antigen capture wells was greater than 0.200 absorbance units (corrected OD, **Δ**OD), and the OD in R1 was greater than the average OD of the wells with PBS (blank of reaction) in R1 plus 2 standard deviations. In the present study, the cut-off of the assay was re-calculated in order to increase its sensibility, retaining the high specificity (>90%), for the detection of RVA in foal stool samples.

2.4. Confirmative RVA RT-PCR and VP6, VP7 and VP4 typing

The presence of RVA in all samples resulting positive by at least one of the three mentioned assays was confirmed by RT-PCR of the VP6 gene (I type). In addition, RT-PCR to VP7 and VP4 genes were carried out to determine their G and P type, as described elsewhere (Garaicoechea et al., 2011; Matthijnssens et al., 2012a; Miño et al., 2013).

The PCR products were purified and sent to the "Unidad de Genómica, Instituto de Biotecnología, CICVyA, INTA-Castelar" for sequencing. The genotypes of VP6, VP7 and VP4 genome segments were determined according to the recommendations of the Rotavirus Classification Working Group (RCWG) (Matthijnssens et al., 2008) using the RotaC online classification tool (http://rotac.regatools.be/) (Maes et al., 2009).

Multiple sequence alignments were constructed in CLUSTALX 2 (Larkin et al., 2007) and subsequently edited in Bioedit (Hall, 1999). Phylogenetic and molecular evolutionary analyses were

conducted using MEGA5 (Tamura et al., 2011). Genetic distances were calculated using the Kimura-2 correction parameter at the nucleotide level, and the phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates.

2.5. Electron microscopy

Four samples showing discordant or peculiar results were tested by electron microscopy: a sample which was negative by Pathfinder (#33); a negative sample by the two commercial kits but positive by KERI and RT-PCR, yet untypable (#35); a positive sample by FASTest® Rota strip and KERI, but negative by Pathfinder and RT-PCR (#37); a positive sample by the three methods showing a mixed infection (#38). Briefly, the stool samples were diluted one in five in PBS and centrifuged at $1300 \times g$ at 4 °C for 20 min, and the supernatants were collected and centrifuged at $20{,}000 \times g$ for 30 min. The obtained pellets were suspended in 100 μl of PBS. A drop of the suspension was applied to a copper grid and negatively stained with 1% uranyl acetate. Viral particles were examined using a JEOL-1200EX-II transmission electron microscope.

2.6. RVA detection limit of the assays

The detection limit of Pathfinder™ Rotavirus kit, FASTest® Rota strip and KERI ELISA was estimated by testing serial ten-fold dilutions of H-2 (RVA/Horse-tc/GRB/H-2/1976/G3P[12]-I6) and Indiana (RVA/Cow-xx/USA/INDIANA/XXXX/G6P[5]-I2) RVA strain suspensions, both containing 4×10^5 and 4.64×10^5 fluorescent focus forming units per millilitre (FFFU/ml), respectively were previously diluted to start the assay at 1×105 FFFU/ml of infectious virus particles (Saif et al., 1997).

2.7. Statistical analysis (ROC analysis and κ test)

In order to assess the ROC analysis using the MedCalc® version 11.1.1.0 statistical software (Mariakerke, Belgium), number 0 was assigned to the "true negative" and number 1 to the "true positive" samples. The area under the ROC-curve (AUC index) was then calculated for each of the three assays. High values (close to 1) indicate a highly accurate test (Greiner et al., 2000). Additionally, the ROC analysis using the OD of each sample in R1 and the ΔOD – difference between the OD in R1 minus the one in R2 ($\Delta R1 - R2$) – was used to re-calculate the cut-off value of KERI ELISA.

The concordance of each assay to properly classify the samples as positive or negative for RVA was estimated by the calculation of the weighted kappa statistic (κ test). Values of κ test from 0.41 to 0.60 indicate moderate agreement; values from 0.61 to 0.80, substantial agreement, and values from 0.81 to 0.99, almost perfect agreements (Viera and Garrett, 2005).

3. Results

3.1. Equine RVA detection

From the total of 172 stool samples analyzed by Pathfinder™ Rotavirus, FASTest® Rota strip, KERI ELISA and RT PCR, 50 (29%) met the criteria to be considered RVE positives and 122 (71%) RVE negatives. These samples were taken as reference population for the validation of the assays. Regarding the concordance between the EIAs tests, the three assays concurred in testing 13 samples as positives and 105 as negatives. Discordant results were found in 54 samples, of which 25 were found RVE positive by two methods and were hence considered true positives. A total of 29 samples were positive only by one EIA method, of which 12 were positive by RT-PCR and were thus included in the study as true positives, and 17 were negative by RT-PCR and were considered true negatives (Table 1, supplementary material A).

3.2. Rotavirus typing

In 42 out of 50 (86%) RVA positive samples, the G, P and I-type could be determined as follows: 30 (72%) were G3P[12], 6 (14%) G14P[12] and 6 (14%) showed mixed (G3 + G14) infection. All equine RVA G3 were associated with the I6 genotype, whereas all G14 were with the I2 genotype; the phylogenetic analysis is shown in Fig. 1. The individual results are shown in supplementary material Table A.

3.3. Electron microscopy

The four stool samples showing discordant or peculiar results were confirmed positive by electron microscopy. Virus particles compatible in morphology with rotavirus were seen in all the samples (Fig. 2). The equine RVA in sample #33 was genotyped as G3P[12]I6; sample #35 was positive by RT-PCR but untypeable; sample #37 was positive by two EIAs but negative by RT-PCR; and sample #38 was positive by all the methods and presented a G3 + G14 as a mixed infection.

3.4. Detection limit

The detection limit of Pathfinder™ Rotavirus kit, FASTest® Rota strip and KERI ELISA was estimated by testing serial ten-fold dilutions of H-2 and Indiana RVA strain suspensions containing 1×10^5 fluorescent focus forming units per milliliters (FFFU/ml) of infectious virus particles. Pathfinder™ Rotavirus detected a minimum of 1000 FFFU/ml of both RVA strains; FASTest® Rota strip was more sensitive and detected up to 100 FFFU/ml of the equine RVA H2 strain and 100 FFFU/ml of the bovine RVA Indiana; and KERI ELISA was able to detect up to 100 FFFU/ml of bovine RVA Indiana and up to 1000 FFFU/ml of equine RVA H-2 (Table 1).

3.5. Assays performance: sensitivity and specificity for the diagnosis of equine rotavirus in field stool samples

The performances of the three methods were compared (Fig. 3A).

Pathfinder™ Rotavirus detected equine RVA in 17 (34%) out of 50 positive stool samples, indicating a very low sensitivity, but maintaining a high specificity (96%). The AUC index was 65%, and then only 77% of the samples were correctly classified (Table 2). This kit was able to detect all equine RVA genotype G14P[12]-I2, even when present in mixed infections, but seems not to be able to detect equine RVA genotype G3P[12]-I6 (Fig. 3C and D). From the 33 true positive samples in which RVA was not detected by this method, 85% (28/33) belong to G3P[12]-I6 genotype and 15% (5/33) were positive by FASTest® Rota strip and KERI ELISA, but could not be genotyped by RT-PCR. The κ value for this test was 0.358, indicating a low agreement. When only G14P[12]I2 RVA positive samples were considered in the analysis, Pathfinder ™'s specificity was retained (95%), its sensitivity was increased up to 65% (Fig. 2C), and the κ value was improved to 0.723 indicating substantial agreement to properly classify the reference samples.

FASTest® Rota strip was able to detect equine RVA with 92% (46/50) sensitivity and 97% (118/122) specificity. According to the AUC index, 95% of the samples were correctly classified (Fig. 3B). This commercial kit was able to detect both equine RVA genotypes: G3P[12]-I6 and G14P[12]-I2. RVA could not be detected by this method in four true positive samples. These samples were positive by KERI ELISA and RT-PCR; two of them were genotyped as G3P[12]-I6, and in the other two, the sequence could not be established due

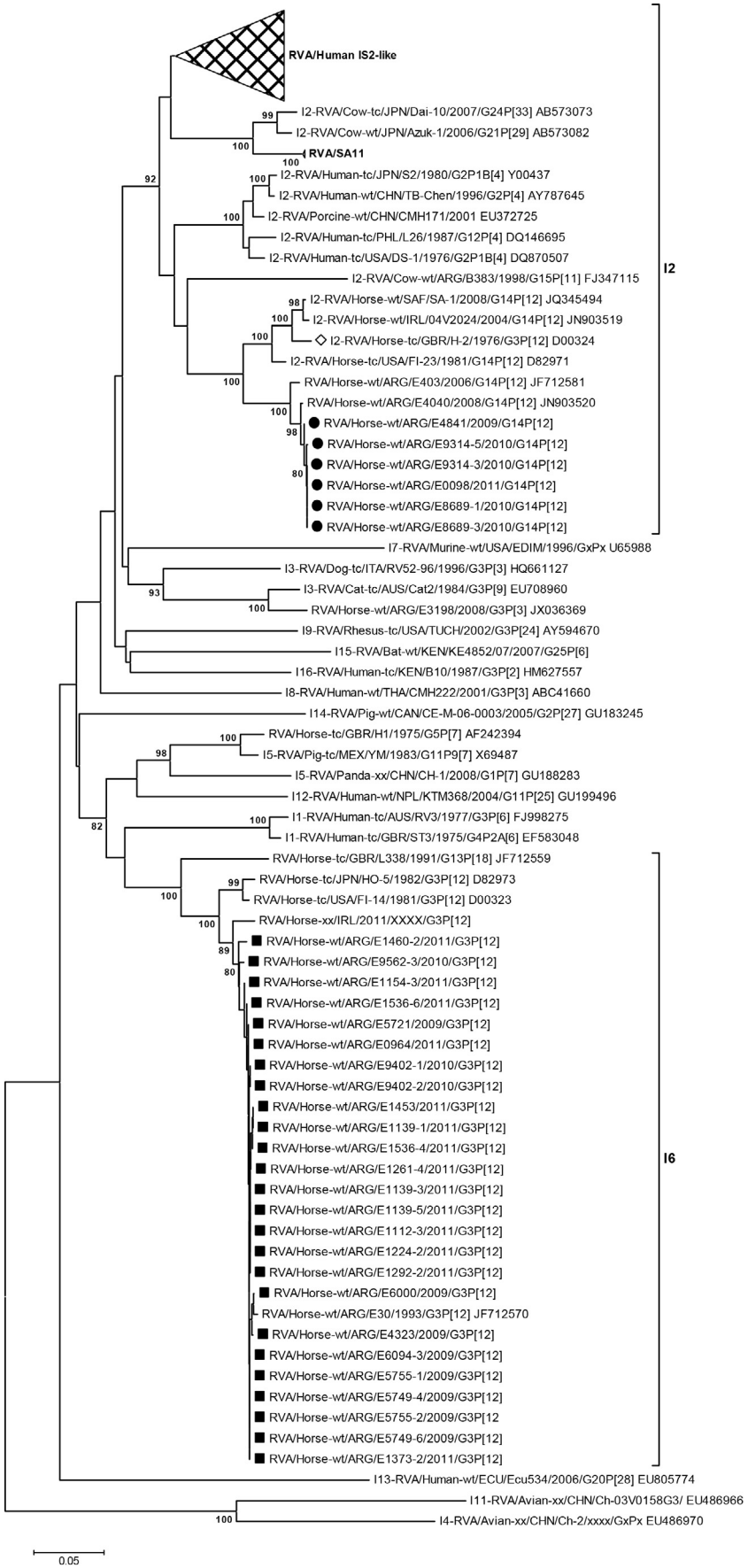

Fig. 1. Phylogenetic tree based on the ORF sequences of the VP6 inner capsid protein from equine RVA. Phylogenetic trees were constructed using the neighbor-joining method with the kimura-2-parameter. Bootstrap values (1000 replicates) above 80% are shown.

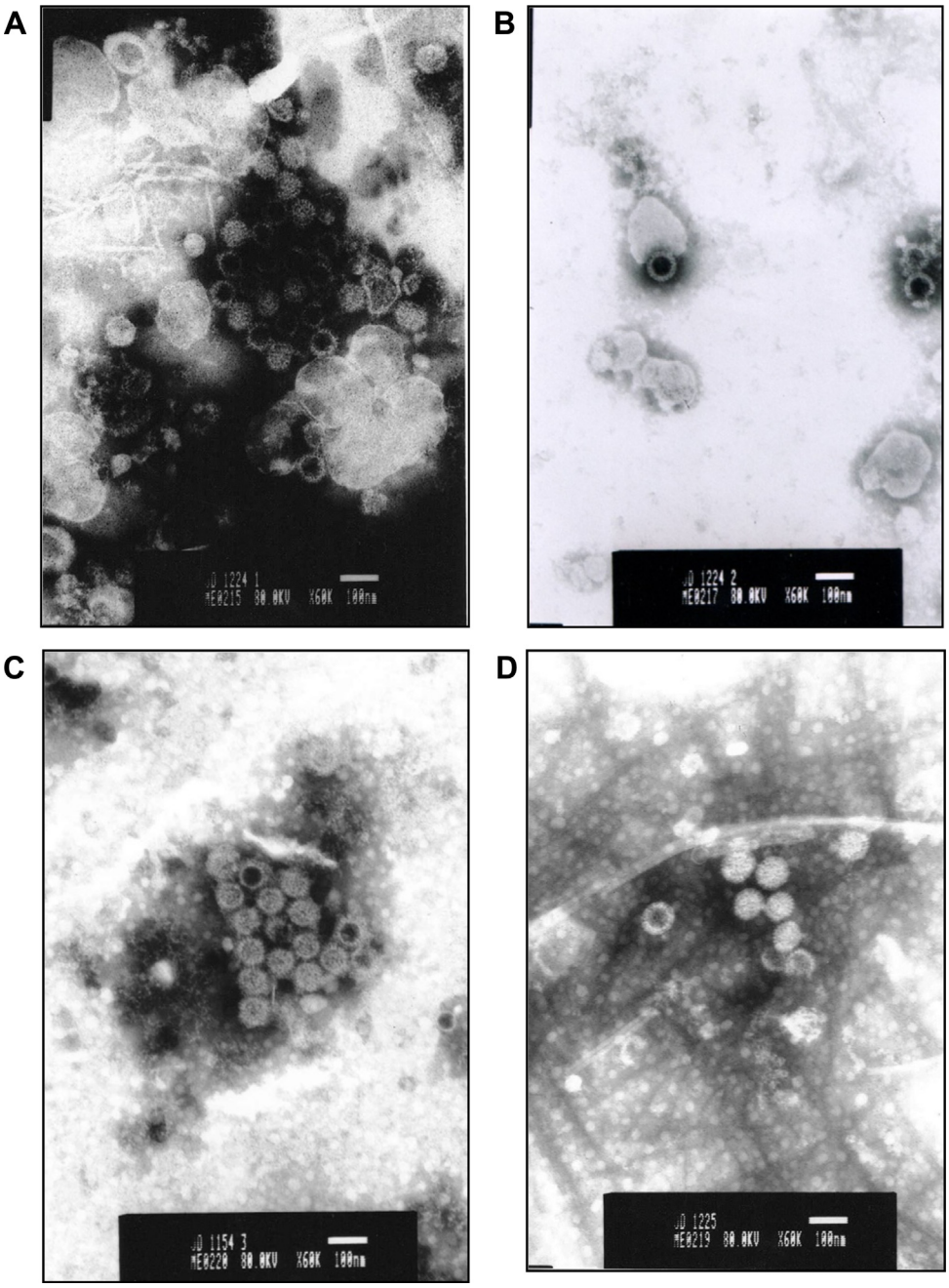

Fig. 2. Electron microscopy of the four stool samples showing discordant or peculiar results. Virus-like particles with the typical morphology of RV were seen in all the samples. (A) sample #33, genotyped as G3P[12]I6; (B) sample #35, untipable; (C) sample #37, positive by two EIAs but negative by RT-PCR and; (D) sample #38, positive by all the methods and presented a G3 + G14 as a mixed infection.

Table 1
Detection limit of the commercial antigen detection kits and KERI ELISA.

Assay	Reference RVA strain	Viral titer in FFFU/ml				
		1×10^5	1×10^4	1×10^3	1×10^2	1×10^1
Pathfinder™ Rotavirus	H2	+	+	+	–	–
	Indiana	+	+	+	–	–
FASTest Rota® Strip	H2	+	+	+	+	–
	Indiana	+	+	+	+	–
KERI ELISA	H2	+	+	+	–	–
	Indiana	+	+	+	+	–

Serial ten-fold dilutions of the virus stock H2 (G3P[12]) 1×10^5 FFFU/ml and Indiana (G6P[5]) 1×10^5 FFFU/ml.

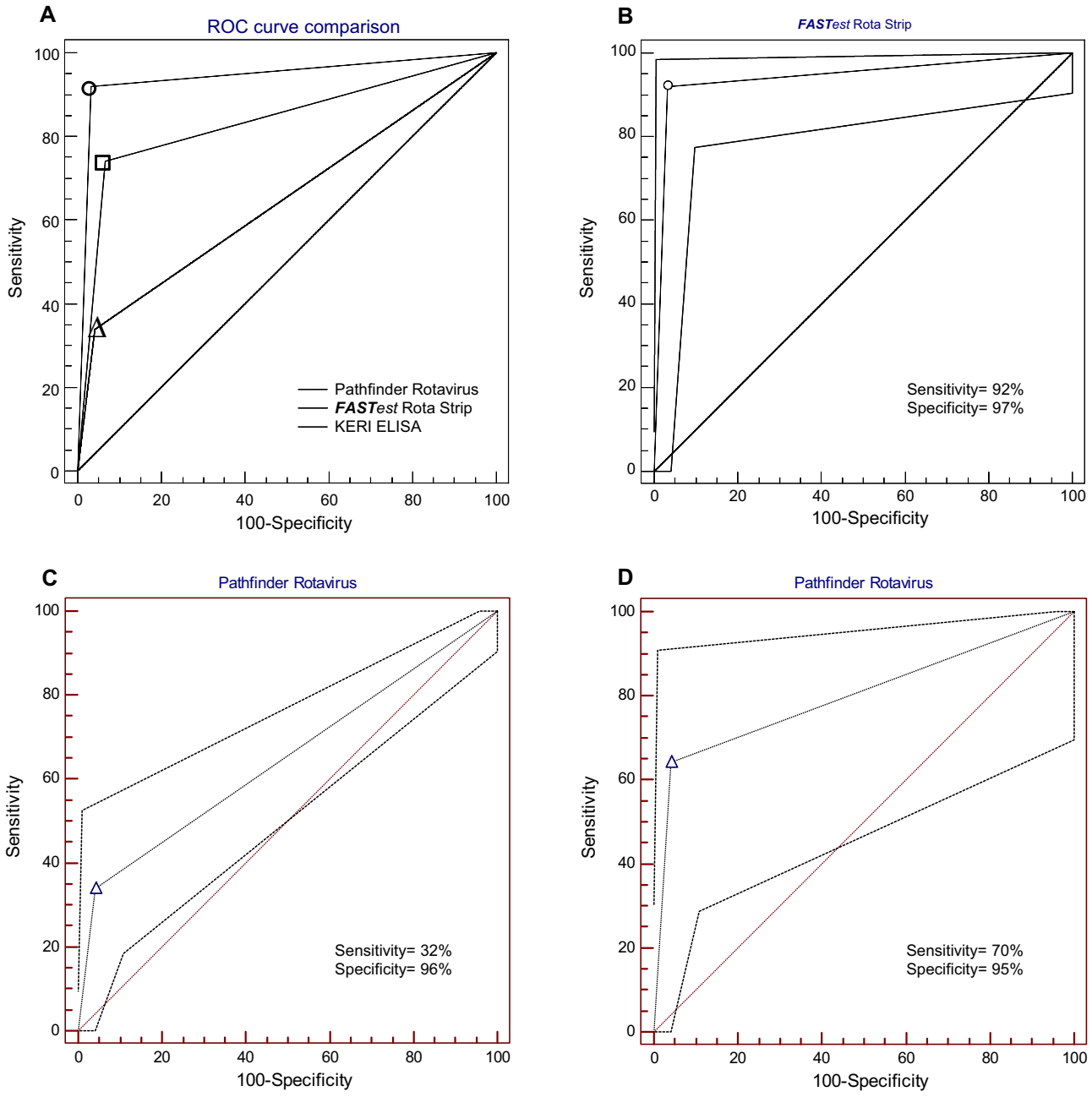

Fig. 3. (A) Overlay of all results of the ROC (receiver operating characteristics) analysis for all samples (overall) and for the three methods (Δ Pathfinder™ Rotavirus; ○ FASTest Rota® STRIP; □ KERI ELISA) separately, with the corresponding area under the ROC curve (AUC). The true positives ($n = 50$) and true negatives ($n = 122$) were used as reference populations; (B) results of the ROC analysis for FASTest Rota® STRIP; (C) results of the ROC analysis for Pathfinder™ Rotavirus for all population and; (D) results of the ROC analysis for Pathfinder™ Rotavirus for a population without the G3P[12]I6 samples. The ROC plots the true positive rate (sensitivity) against the false positives (100-specificity). The diagonal indicates no discriminatory power.

Table 2
Results of the three methods used to detect equine RVA in stool samples from diarrheic foals.

Test		True positives (50)	True negatives (122)	Sensitivity	Specificity	κ test	ROC analyses		
							AUC	95% CI	Correctly classified
Pathfinder™ Rotavirus	+	17	5	34% (17/50)	96% (117/122)	0.358[a]	65%	57–73%	77%
	–	33	117			0.723[b]			
*FAST*est Rota® Strip	+	46	4	92% (46/50)	97% (118/122)	0.887	95%	90–98%	95%
	–	4	118						
KERI ELISA	+	38	8	76% (38/50)	93% (114/122)	0.695	88%	79–91%	88%
	–	12	114						

A non-parametric analysis was performed. κ test, Kappa value; AUC, area under the ROC curve; CI, confidence interval.

[a] Kappa index for complete population.

[b] Kappa index without G3P[12]I6 equine RVA samples. Corrected classified index was calculated as both ELISA assay and true positives + both ELISA assay and true negatives/Total × 100.

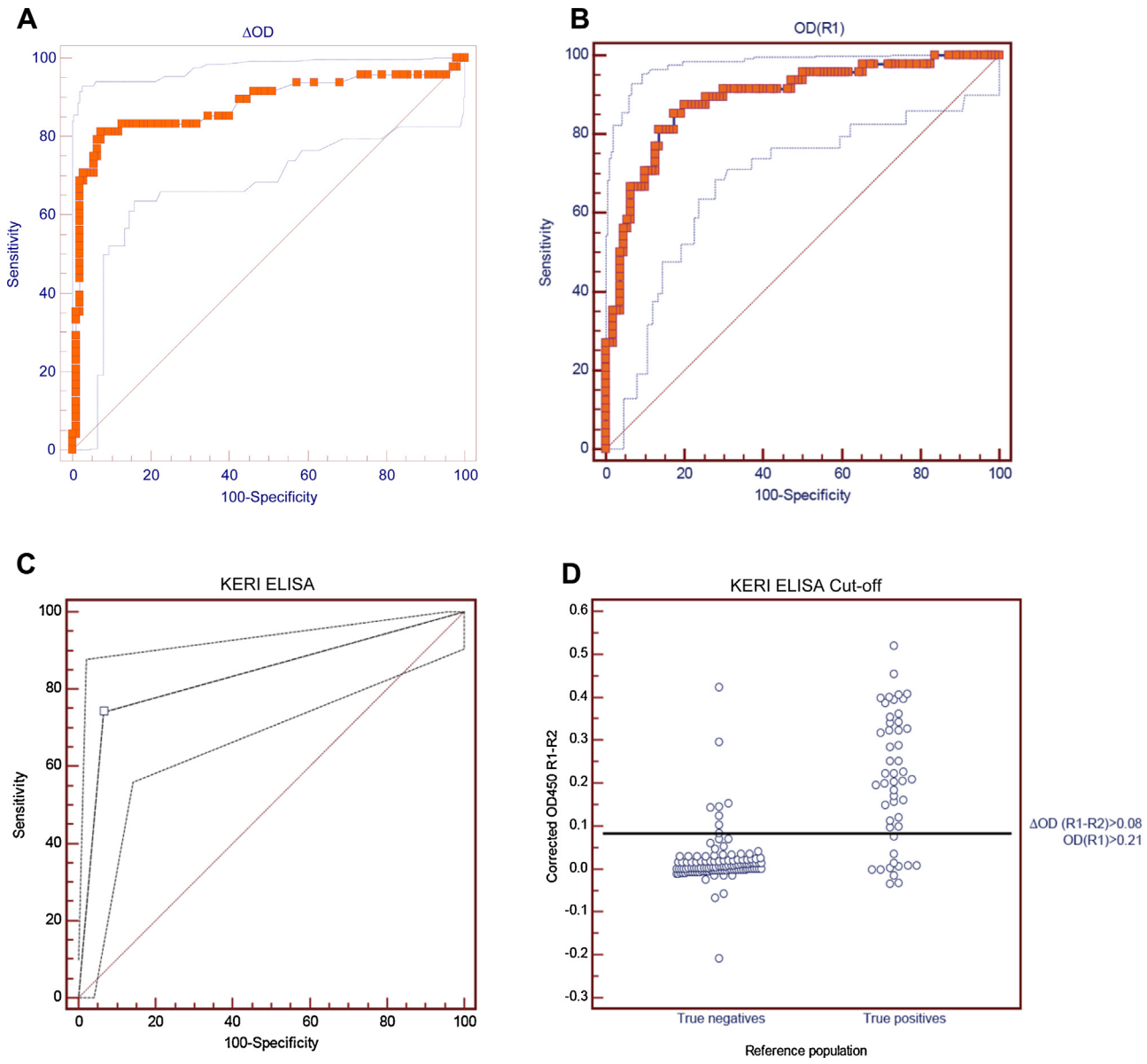

Fig. 4. ROC curve analysis of KERI ELISA including all samples ($n = 172$) for validation of the detection of equine RVA in stool samples of diarrheic foals. (A) The cut-off was defined as OD in R1 minus R2 (ΔOD). If the ΔOD ≥ 0.08 then, the sample is considered positive ($n = 46$); and negatives samples were defined as ΔOD < 0.08 ($n = 126$). The dotted line shows a confidence interval of 95%. (B) The positive samples were defined as R1 absorbance (R1OD) ≥ 0.213 ($n = 77$) and negative samples were defined as (R1 absorbance) R1OD < 0.213 ($n = 95$). Dotted line shows a confidence interval of 95%. (C) (□) ROC curve analysis to KERI ELISA. The reference population was defined using both R1OD ≥ 0.213 and ΔOD ≥ 0.08 criterions. The dotted line shows a confidence interval of 95%. (D) Distribution of samples using a proposed cut-off line.

to the small amount of virus load in the samples. FASTest® Rota strip κ value was 0.887 indicating an almost perfect agreement to correctly classify the reference samples (Table 2).

Regarding the result obtained with ELISA KERI, we must first take into account that the cut-off was re-calculated. Samples were considered RVA positive if the OD in R1 was equal or higher than 0.213, and if the ΔOD (R1 – R2) was higher than 0.082 (Fig. 4A and B). With this new cut-off, the assay showed 76% (38/50) sensitivity and 93% (114/122) specificity. The AUC index was 88%, meaning that samples can be correctly classified by KERI ELISA as RVA positive or negative in 88% of the times tested (Fig. 4C). The κ value for KERI ELISA was 0.695; indicating substantial agreement to correctly classify the reference samples (Table 3). Of the equine RVA detected by KERI ELISA, 47% (18/38) were genotyped as G3P[12]I6, 16% (6/38) as G14P[12]I2 and 13% (5/38) as mixed infection (G3P[12]I6/G14P[12]I2). Four samples, even if positive by VP6 RT-PCR, could not be sequenced, and five samples were not amplified by the RT-PCR. The 12 true positive samples undetected by this method were classified as follows: seven as G3P[12]-I6 genotype; two mixed infection G3/G14; one positive by RT-PCR but unable to be sequenced; and two positive by FASTest® Rota strip and Pathfinder™ Rotavirus, but negative by RT-PCR (supplementary material A).

4. Discussion

Group A rotavirus are important pathogens associated with diarrhea in foals in Argentina, with a detection rate around 21% (Garaicoechea et al., 2011), similar to the incidences reported worldwide (Papp et al., 2013). Hence, even when vaccination to prevent rotavirus diarrhea in foals is routinely used (Barrandeguy et al., 1998), the syndrome is still of concern in our country (Garaicoechea et al., 2011; Miño et al., 2013).

Table 3
KERI ELISA analysis.

	Cut-off	AUC	Sensitivity	Specificity	Correctly classified
OD(R1)	0.213	89%	87.5%	80%	89%
ΔOD (R1-R2)	0.082	88%	79%	94%	88%

AUC, area under the ROC-curve; OD(R1) = absorbance of R1; ΔOD (R1 − R2), difference in absorbance R1 minus R2; Corrected classified index was calculated as (both OD(R1) or ΔOD cut off and true positives + both OD(R1) or ΔOD cut off and true negatives/Total × 100).

Currently, a variety of methods are commercially available for rapid laboratory diagnosis of RVA infection; yet, most of these methods are validated for diagnosis of human RVA, and only a few assays were designed and/or properly validated for veterinary use on samples from different animal species, like calves, foals and piglets (Eing et al., 2001; Greiner et al., 2000; Matthijnssens et al., 2008; Monini et al., 2011; Slovis et al., 2013). The present study was conducted to compare the performance of two commercially available RVA diagnostic kits and one in-house ELISA, for the detection of rotavirus in stool samples from diarrheic foals.

The best performance to detect RVA was obtained by FASTest® Rota strip. This diagnostic kit has been designed and approved for the diagnosis of animal RVAs, and has shown 72% sensitivity and 95% specificity to detect bovine RVA in calves (Klein et al., 2009). In the present study, the assay was able to detect both genotypes (G3P[12]I6 and G14P[12]I2) (Fig. 3B) of equine RVA in foal feces, with 92% sensitivity and 97% specificity. Furthermore, this test properly classified the equine samples in 95% of the runs, with an excellent agreement. The antibody used in FASTest® Rota strip test was able to recognize both I2 and I6 VP6 variants circulating in horses.

Pathfinder™ Rotavirus is a kit designed for human RVA diagnosis utilizing a monoclonal antibody directed at VP6 I2 genotype. The sensitivity and specificity of the assay for RVA detection in stool samples from children with acute gastroenteritis was 100% and 92–98%, respectively (Dennehy et al., 1988, 1994). Regarding its use in the veterinary field, it was reported to have good sensitivity and specificity to detect bovine RVA carrying I2 genotype (Badaracco et al., 2014). However, in the present study this assay only detected 34% of the equine RVA positive samples tested. This is a particularly remarkable result, since there is a lack of universal acceptance of the usefulness of those diagnostic tools that have been designed for human RVA diagnosis, in veterinary medicine (Fushuku and Fukuda, 2006; Maes et al., 2003; Nemoto et al., 2010).

The type of antibodies (polyclonal versus monoclonal) used in the EIAs assays strongly affects their detection efficiency. The use of MAbs is usually associated to highly specific assays. This represents an advantage in complex matrices as feces, where specific enteropathogens must be detected in the context of gut microbiota and other proteic antigens that increase the probability of cross-reactions and false positive results. However, in the present study the specificity of the MAb included in Pathfinder™ Rotavirus kit led to a reduced performance to detect equine RVA. The assay was able to detect all RVA genotyped as G14P[12]I2, even in foals with co-infection (G3I6/G14I2) and reduce load of G14 strain in the sample, but seems not to be able to detect the equine RVA strains carrying I6-genotype, suggesting that this variant of VP6 suffers a mutation in the epitope recognized by this antibody. In fact, when conducting an alignment of the ERV strains under study, the strains carrying a VP6-I6 shown 36 aminoacid changes and 2 deletions compared with equine VP6-I2. Mapping these differences onto the atomic structure indicates that the majority of them are surface exposed and that many are situated in domain H (residues 151–331) at the top of the VP6 trimmer, in or close to antigenic sites. Three aminoacid changes (A244, M295 and N307) and 2 aminoacid deletion (P299, P300) are situated in sites interacting with VP7 (supplementary material, table C and D). These aminoacid positions might be involved in the putative site of the Pathfinder MAb recognition.

Equine VP6-I6 tent to associate most frequently to VP7(G3) while VP6-I2 tent to G14, with the exception of ERV/H-2/1976 strain (Acc number D00324). Further phylogenetic and structural studies are under progress in order to elucidate the differential epitopes between I2 and I6 genotypes, and their structural relationship with the G3 and G14 VP7 (Miño unpublished data).

It is important to highlight that up to date, at least 18 different I-genotypes have been reported among animal and human RVAs (Matthijnssens and Heylen, 2013; Matthijnssens et al., 2012b). Thus, all the EIAs assays based on MAbs directed to VP6 need to be strictly validated before their use in each animal species.

Finally, our in-house KERI ELISA was the third method evaluated for the detection of equine RVA in feces from diarrheic foals. KERI ELISA was designed based on polyclonal antibodies against bovine RVA Indiana (G6P[5]I2), and was routinely used for bovine RVA detection carrying a I2 genotype. The original cut-off of the assay was very strict, in order to have 100% specificity in RVA detection. The proposed cut-off established in the present study indicates that the sensitivity and specificity of the assay for equine RVA detection in samples from diarrheic foals were 76% and 93% respectively, with 88% of correctly classified samples. The use of two polyclonal antibodies as positive capture and as secondary antibody seem to give a proper VP6 cross-reactivity, granting the detection of different genotypes of RVA, including equine VP6 I6 genotype. However, the use of polyclonal antibodies may explain the low sensitivity thereof.

The detection limit of FASTest® Rota strip and KERI ELISA were higher than Pathfinder™ Rotavirus. The reason why this might have happened is not clear, but it may be due to the difference in the principles (the specificity and type of antibodies) used in these kits, which could affect the detection limits for equine RVA.

Some samples were reactive to Pathfinder™ Rotavirus kit, FASTest® Rota strip or KERI ELISA methods, but not to RT-PCR assay. Similar results were obtained by Adlhoch et al. (2011), and a possible explanation might be a higher amount of viral antigen in some stool samples compared to viral RNA load, and the RNA lability compared with proteins (Adlhoch et al., 2011). Alike, some fecal samples could contain inhibitors for RT-PCR reaction. The presence of virus like particles compatible in size and morphology with rotavirus in these samples was confirmed by EM, thus supporting the specificity of these detection methods.

Rapid diagnosis assays have the advantage of facilitating the process of large amount of samples in a very short period of time, allowing fast management measure and treatment to reduce disease severity and infection spread. In addition, highly-trained personnel or specialized laboratory equipment is not crucial to perform this test. The results of this study indicate that FASTest® Rota strip was the most accurate, sensitive and specific assay for equine RVA detection in foal feces, and that is can be also used as a pen-side diagnostic tool at horse farms. According to our results, Pathfinder™ Rotavirus is not appropriate for the diagnosis of RVA since it failed to detect equine RVA G3P[12]I6 in the field samples. Regarding our in-house KERI ELISA, it showed acceptable accuracy, sensitivity and specificity. Additionally, the cost of the KERI ELISA

assay produced in our laboratory is 10 times less expensive than the commercial ones, thus viable to analyze a large number of samples simultaneously under standard laboratory conditions.

5. Conflict of interest

Except for Kern A., staff of the MEGACOR Diagnostk company, none of the other authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper. Samples were processed and results evaluated by independent and uninfluenced people (S. Miño, M. Barrandeguy). Dr Parreño is a member of the "*Consejo Nacional de Investigaciones Científicas y Técnicas*" (CONICET).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.05.002

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