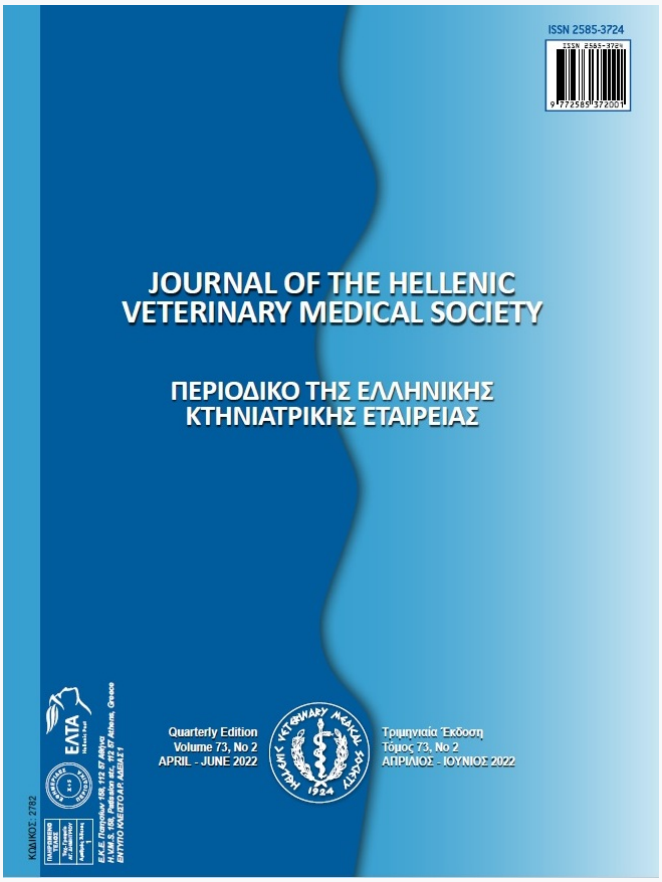
Journal of the Hellenic Veterinary Medical Society

Vol 73, No 2 (2022)

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doi: 10.12681/jhvms.23704



**To cite this article:**

Hamedian-Asl, M., Zakian, A., Azimpour, S., Davoodi, F., & Kahroba, H. (2022). Evaluation of diagnostic methods for the Detection of Bovine Coronavirus and Rotavirus in feces of diarrheic calves. *Journal of the Hellenic Veterinary Medical Society*, *73*(2), 3951–3960. https://doi.org/10.12681/jhvms.23704

J HELLENIC VET MED SOC 2022, 73 (2): 3951-3960
ΠΕΚΕ 2022, 73 (2): 3951-3960

Research article
Ερευνητικό άρθρο

# Evaluation of diagnostic methods for the detection of Bovine Coronavirus and Rotavirus in faeces of diarrhoeic calves

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**ABSTRACT:** The purpose of the present study was to evaluate the efficacy of Enzyme-Linked Immunosorbent Assay (ELISA), immunochromatographic (ICG), and reverse transcription-polymerase chain reaction (RT-PCR) methods for the detection of rotavirus (RV) and bovine coronavirus (BCV). Faeces samples were collected from 90 diarrhoeic calves (male and female) up to one month of age and the immune response against RV and BCV infection was assessed by using AgELISA, ICG, and RT-PCR. To determine the performance and accuracy of each diagnostic method in comparison to the diagnostic gold standard (RT-PCR) method, different statistical tests including receiver operating characteristic curve (ROC) and concordance correlation were used. Results revealed the prevalence of RV and BCV and RV+BCV according to RT-PCR were equal to 8.89 (95% CI: 6.64-10.07), 14.44 (95% CI: 11.23-6.90), and 2.22 (95% CI: 0.89-3.72), respectively. The best agreement and the highest sensitivity and specificity were obtained between the RT-PCR and AgELISA (100% and 94.3%), and also the ICG test (95% and 94.3%) was less accurate method in comparison to ELISA method for identifying RV and BCV, but a good correlation and concordance between ICG diagnostic techniques and RT-PCR were observed. To put it in a nutshell, our results demonstrate that the AgELISA is the most accurate technique in comparison to RT-PCR, however the ICG assay can help improve the speed of diagnosis RV and BCV infections in dairy field. New scientific strategies for promoting accuracy and transparency of ICG-based technique in early diagnosis of the cause of calf diarrhoea should be used. Altogether, we suggest that positive ICG samples should be tested by AgELISA or RT-PCR techniques to avoid false results in farm animals.

***Keywords:*** calf diarrhoea, rotavirus, bovine coronavirus, immunochromatography, RT-PCR.

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*Date of initial submission: 7-07-2020*
*Date of acceptance: 27-12-2021*

## INTRODUCTION

Diarrhoea is one of the major neonatal period diseases, which leads to economic losses due to increased mortality, stunted growth, longevity in the herd, productivity performance, and the cost of treatment (Uhde et al., 2008). The mortality rate of calves in the first weeks after birth is approximately 84% of the total mortality, with the highest mortality occurring in the third week after birth (Chauhan and Singh, 1996). Neonatal diarrhoea frequently occurs in calves less than two months of age; however, calves up to four months may be affected (Fremont et al., 2004).To increase livestock productivity, reduce mortality in the first weeks of life, and prevent calf diarrhoea, it is necessary to know the etiological factors (Lorino et al., 2005). Several infectious agents and non-infectious factors (nutrition, environment) cause diarrhoea in neonatal calves. Rotavirus (RV) and bovine coronavirus (BCV) are the most common pathogens associated with gastroenteritis and diarrhoea in young calves (Cho and Yoon, 2014). RV and BCV are ubiquitous and as a result, most of the animals, including pregnant cows originating from intensive livestock farms, have specific antibodies against these pathogens (Morshedi et al., 2010). BCV causes respiratory and gastrointestinal disease in calves and winter dysentery in adult cattle (Boileau and Kapil, 2010; Sunniva Oma et al., 2016). RV is another intestinal pathogen that is transmitted by the faecal-oral route. In calves, the onset of the disease is rapid, and depression, diarrhoea, and dehydration are observed (Dhama et al., 2009). One of the important factors involved in increasing calf diarrhoea, duration of diarrhoea, and the mortality rate is the failure of passive transfer of immunity (Pires Moraes et al., 2000; Zakian et al., 2018).

Calf diarrhoea is easy to diagnose based on clinical signs; however, laboratory diagnosis of etiological factors is the only reliable method for accurate detection of the disease etiology and therapeutic interventions (Cho and Yoon, 2014). Various diagnostic methods are employed to detect enteropathogenic agents (Icen et al., 2013). Diagnosis is done via collecting faeces of diarrhoeic animals by a rectal swab, collecting blood samples, and using laboratory diagnostic tests such as polymerase chain reaction (PCR), Enzyme-linked immunosorbent assay (ELISA), hemagglutination/inhibition assay, and also immunochromatography (Busato et al.,1998; Lanz Uhde et al., 2008; Klein et al., 2009; Cho et al.,2010 and 2012; Icen et al., 2013).

Nowadays, the use of accurate, inexpensive, rapid, simple, and on-farm detection devices is popular in biomedicine, agriculture, and veterinary medicine (Klein et al., 2009; Icen et al., 2013; Zakian et al., 2018). ICG is one of the new technologies with the above-mentioned characteristic, which had recently attracted considerable interest (Koczula and Gallotta, 2016) because of its potential for rapid diagnosis.

In Iran, no comprehensive information is available on the prevalence of infectious causes of neonatal diarrhoea at the national level, though reports are available on individual pathogens responsible for calf diarrhoeai. Most studies in the field of calf diarrhoea in Iran have only focused on the prevalence of diarrhoea causing pathogens (Morshedi et al., 2010; Nazoktabar et al., 2013; Mohebbi et al., 2017; Lotfollahzadeh et al., 2020); however, some of these studies employed non-validated methods to detect diarrhoeic calves. Therefore, the first objective of the present study was to determine whether ICG is an appropriate method with optimal accuracy for the detection of Bovine Coronavirus and Rotavirus in Holstein diarrhoeic calves. The second objective of the current research was to compare the performance of the AgELISA and ICG methods for detecting calf diarrhoea in comparison to the diagnostic golden standard method (RT-PCR).

## MATERIAL AND METHODS

### Calf enrollment and Sampling

Ninety diarrhoeic Holstein calves (45 male and 45 female) were selected from November to December 2018, in five industrial herds of suburbs Tehran, Iran. Clinical examination was performed to assess the health status of calves and faecal samples were collected. Calves that had received antibiotics and corticosteroids, or that suffered from other diseases including pneumonia, omphalitis, or polyarthritis, and calves with detectable clinical congenital abnormalities were excluded from the study. Calves in these farms were administered 2 L colostrum within 6 hours of birth and 2 L of colostrum 12 hours later; after the first day, calves were fed 2 L of milk twice daily.

Sample of faeces was collected by the sterile gloves directly from the rectal mucosa from calves fulfilling the inclusion criteria before they were treated, between 1 to 21 days of age. The identification number, age, breed, and sex were recorded on a standardized form.

**Immunochromatographic test**

Firstly, all the faecal samples were tested on-farm for the presence of RV and BCV by ICG RAINBOW calf scours (BIO K 288, BIO-X Diagnostics, Rochefort, Belgium) kit following the manufacturer's instructions. Also, the part of faecal sample was immediately submitted to the laboratory in sterile bottles cooled on ice packs. Faecal sample of calves was added to the sample tube containing diluent buffer.When faeces were solid, the excess amount was removed using a spatula. To achieve a homogeneous stool suspension, dilution buffer was mixed three times. Subsequently, the sample tube was taped on a hard surface so that all the liquid was collected at the bottom of the tube. The sample tube was inserted into the strip tube and the top of the strip tube was screwed. Thereafter, the device was placed vertically on a flat surface and the results were read after 10 min. Interpretation of the ICG kit was performed following the manufacturer's instructions. The presence of the control band showed a valid test result. The presence of an additional band with a special color indicated a positive reaction to a specific pathogen. Results were recorded as RV and BCV positive or negative, as this kit provides only qualitative results. Samples infected with both RV and BCV viruses were considered Co-infected.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Faecal samples were tested by an antigen ELISA method (BIO K 348, Multiscreen AgELISA/ sandwich, Bio-X Diagnostics, Rochefort, Belgium) for the presence of RV and BCV according to the manufacturer's instructions (Bio-X Diagnostics Rochefort, Belgium).

**RT-PCR ASSAY**

Total RNA was extracted from faecal suspensions using VETEK viral DNA/RNA extraction kit (Intron Biotechnology, Seongnam, South Korea). 200 μL of faeces samples were suspended in 800 μL PBS, then 300 μL of faeces suspension was added to 500 μL lysis buffer. 700 μL of loading buffer was added to the solution at room temperature after 10 minutes. The composition was applied to a VETEK spin column followed by centrifugation at 15000 ×g for 1 minute. Loaded RNA was washed twice using washing solutions before the election. Afterward, the complementary DNA (cDNA) was accomplished by Maxime RT premix kit (Intron Biotechnology, Seongnam, South Korea). The RT-PCR was performed as follows: in a tube, 3 μL of cDNA sample was added to 2 μL of the Reverse and Forward primers and 5.5 μL of nuclease-free water, and eventually, 12.5 μL master mix was added to the solution. Subsequently, the solution was preheated for 5 min at 94 °C, 40 cycles, including 45 seconds at 94 °C, 45 seconds at 52 °C, at 72 °C for 1 min and, a final incubation step at 72 °C for 10 min was applied. The sequences (5'-3') of primers used for detection of BCV were GCCGATCAGTCCGACCAATC and AGAATGTCAGCCGGGGTAT (Tsunemitsu et al., 1999) and for RV were AAGTAGCTGGATTTGATTATTC and GACTCACAAACTGCAGATTCAA (Schwarz, 2002). The amplicons were analyzed in a 1.5% agarose gel and visualized after ethidium bromide staining. PCR products of 407 and 433 bp were detected for BCV and RV, respectively. Samples infected with both RV and BCV viruses were considered Co-infected.

**Statistical analyses and method comparison**

Statistical analyses were performed using statistical software programs (Analyze-it and MedCalc) and significant levels were set at the <0.05. The minimum sample size in the method comparison investigations should be 40 samples and we have chosen population size using the same method that was detailed for sample size selection, as previously reported by Jensen and Kjelgaard-Hansen (2006).

The performance of each diagnostic technique was evaluated according to the results of the RT-PCR test as the diagnostic 'gold' standard (DGS). Individual tests were compared with this diagnostic'gold' standard and among each other by exploring the differences between the respective proportions. The area under the curve (AUC) values for the 3 methods were compared to gold diagnostic standards using a non-parametric approach. An AUC value = 1 indicates a perfect test (Se = Sp = 1); >0.9 typically indicates a highly accurate test, whereas AUC values of 0.7-0.9 indicate moderate accuracy, 0.5-0.7 low accuracy, and 0.5 represents a chance result (Grimes and Schulz, 2005). The sensitivity (Se), specificity (Sp), and likelihood ratio positive (LR+), positive predictive value (PPV), and negative predictive value (NPV) of the RV, BCV, and RV+BCV were calculated using the standard formula. A LR+ >10 indicates that a positive test is good at ruling in diagnosis (Landis and Koch, 1997).

To express a statistically defensible measure for evaluating the performance of a new analytical test as

compared to that of a combined gold standard method, the concordance correlation coefficient was calculated. This method (McBride, 2003; McBride, 2005) has emerged as the best measure of agreement for two methods of measuring. Concordance correlation value was interpreted based on McBride (2005) as poor (<0.90), moderate (0.90-0.95), substantial (0.95-0.99), and almost perfect (>0.99). In addition, the precision and bias of each method were evaluated in comparison to DGS (RT-PCR). Standard techniques for detecting the disease status of study patient determines the accuracy of diagnostic tests.

Associations between prevalence or infection intensity classes and sex or age were investigated using Pearson's $\chi^2$ statistics and p-value descriptive data were generated for all variables. Agreement between three methods ( AgELISA and ICG) for RV and BCV diagnosis was evaluated using the κ statistic (Thrusfield, 1995). Inter-rater agreement (Cohen's Kappa coefficient, κ) was also calculated as a measure of the degree of agreement between each method and the gold standard; values for $\kappa < 0.2$ indicate poor agreement, whereas $0.2 < \kappa \leq 0.4$ indicates fair agreement, $0.4 < \kappa \leq 0.6$ indicates moderate agreement, $0.6 < \kappa \leq 0.8$ reflects substantial agreement, and $\kappa > 0.8$ indicates almost perfect agreement (Bablok and Passing, 1985).

The first set of analyses examined the prevalence of each infective agent based on individual and RT-PCR methods in the studied population, also the correlation between sex and age groups was evaluated. Secondly, test characteristics and the chance of disease detection for each diagnostic method based on infective agents were compared as can be seen from the data in Table 1 and Figure 1.

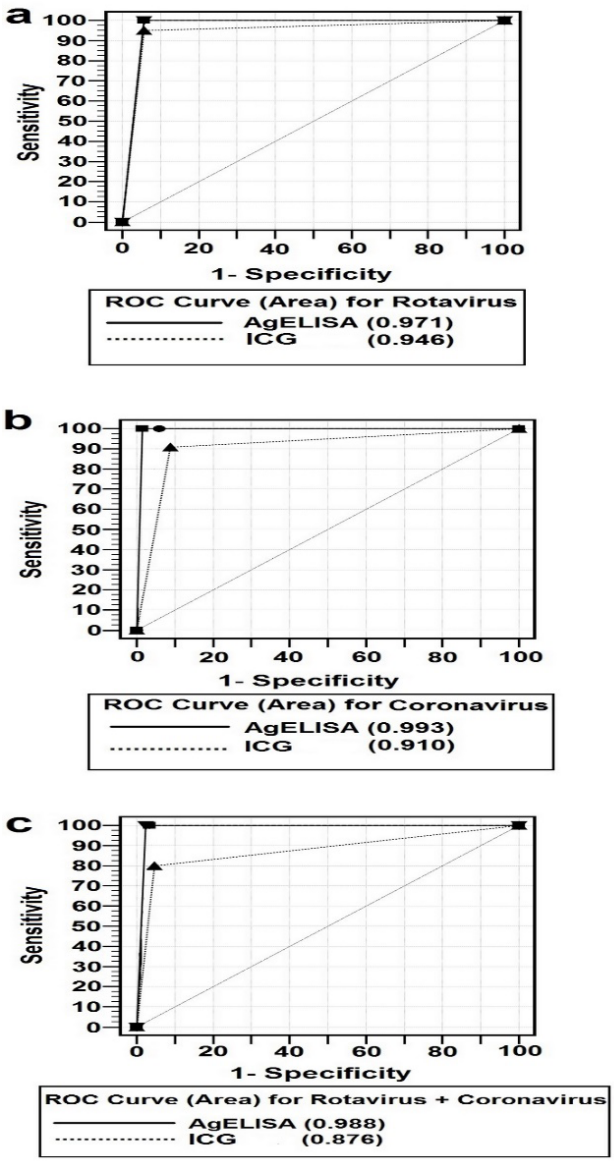


**Figure 1.** Receiver operating characteristic (ROC) curves of the 2 methods for diagnosing bovine coronavirus (BCV), rotavirus (RV), and co-infection of BCV+RV in 90 Holstein calves. The area under the ROC curve (AUC) and 95% confidence interval (CI) are presented for each method (infection was evaluated using AgELISA and another method performed using ICG) in comparison to the combined 'gold' standard (RT-PCR) method. The highest AUC for diagnosing RV (a) for each method was AgELISA=0.971 and ICG =0.946; for diagnosing BCV (b) was equal to: AgELISA= 0.993, and ICG= 0.910; for diagnosingco-infection of BCV+RV (c) for each method was:AgELISA= 0.988 and ICG= 0.876

## RESULTS

### Viral enteropathogens prevalence

The present study evaluated the diagnostic performance of the ICG and AgELISA tests by using RT-PCR and each diagnostic procedure to distinguish reliability, concordance, and bias. The prevalence of viral infection, as determined by each method or diagnostic 'gold' standard method, is shown in Table 2.

The prevalence of RV by using RT-PCR, AgELISA, and ICG methods was 8.89%, 26.7%, and 25.6%, respectively. The prevalence of infection in female calves was higher than in males, but there was no significant correlation between sex and the infection rate (P=0.79). Moreover, the infection rate in age groups 8-14 days was higher than other age groups, nevertheless, a significant correlation between the prevalence of infection with age groups (P=0.28) was not found.

BCV infection rate by using RT-PCR, AgELISA, and ICG methods were equal to14.44%, 28.9%, and

**Table 1.** Sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), likelihood ratio (LR), odd ratio (OR), and area under the curve (AUC) of immunochromatography (ICG) and AgELISA methods in comparison to diagnostic 'gold' standard (RT-PCR) method to diagnose of rotavirus (RV), bovine coronavirus (BCV) and RV+BCV co-infection among diarrhoeic calves in industrial dairy farms, Iran (n=90)

| Test Parameter | Rotavirus | | Bovine coronavirus | | Rotavirus + Bovine coronavirus | |
|---|---|---|---|---|---|---|
| | ICG vs RT-PCR | AgELISA faeces vs RT-PCR | ICG vs RT-PCR | AgELISA faeces vs RT-PCR | ICG vs RT-PCR | AgELISA faeces vs RT-PCR |
| Sensitivity (%) | 95.00 | 100 | 90.91 | 100 | 80.00 | 100 |
| (95% CI) | (75.1-99.9) | (83.2-100) | (71-98.9) | (84.6-100) | (28.4-99.5) | (47.8-100) |
| Specificity (%) | 94.3 | 94.3 | 91.18 | 98.53 | 95.29 | 97.65 |
| (95% CI) | (86-98.4) | (86-98.4) | (81.8-96.7) | (92.1-100) | (88.4-98.7) | (91.8-99.7) |
| NPV (%) | 98.5 | 100 | 96.9 | 100 | 98.8 | 100 |
| (95% CI) | (92-100) | (94.6-100) | (89-99.6) | (94.6-100) | (93.4-100) | (95.7-100) |
| PPV (%) | 82.6 | 83.3 | 76.9 | 95.7 | 50 | 71.4 |
| (95% CI) | (61.2-95) | (62.6-95.3) | (56.4-91) | (78.1-99.9) | (15.7-84.3) | (29-96.3) |
| +LR | 16.62 | 17.5 | 10.30 | 68 | 17 | 42.5 |
| (95% CI) | (6.4-43.3) | (6.8-45.3) | (4.7-22.4) | (9.7-475.8) | (5.9-48.7) | (10.8-167.2) |
| Odds ratio | 313.5 | +∞ | 103.33 | +∞ | 81.00 | +∞ |
| AUC | 0.946 | 0.971 | 0.91 | 0.993 | 0.876 | 0.988 |
| (95% CI) | (0.878-0.983) | (0.913-0.995) | (0.831-0.96) | (0.978-1.00) | (0.79-0.936 | (0.939-100) |

28.9%. We found no statistically significant association between sex and infection rate (P=0.8). The results indicated that the highest incidence was in the second week of birth (36.38%) and the lowest incidence was observed in the first and third weeks of birth (31.81%). However, the Chi-square test did not show any significant differences between infection rate and age groups (P=0.07).

The Co-infection rate of RV and BCV in the current study was 2.22%, 8.90%, and 8.90% by using RT-PCR, AgELISA, and ICG methods, respectively. Results did not show any significant correlation between sex and the infection rate (P=0.78). Results showed that the co-infection rate in 1-7 days was 3 (60%), 8-14 days was 2 (40%), but in 15-21 days age group no positive reaction was observed, and the statistical test showed that there is no significant correlation between age groups and co-infection rate (P=0.26).

## METHOD COMPARISON

### Rotavirus (RV)

Evaluation of diagnostic criteria for detection of RV via each method versus RT-PCR method is represented in Table 1. Sensitivity, specificity, NPV, and PPV were equal to 100 (95% CI: 83.2-100), 94.3 (95% CI: 86-98.4), 100 (95% CI: 94.6-100) and 83.3 (95% CI: 62.6-95.3) for AgELISA method. However, for ICG method the above-mentioned values were 95 (95% CI: 75.1-99.9), 94.3 (95% CI: 86-98.4), 98.5 (95% CI: 92.0-100) and 82.6 (95% CI: 61.2-95.0), respectively (Table 1). The highest diagnostic characteristics and AUC values for AgELISA method were 0.971 (95% CI: 0.913-0.995) and the lowest was for the ICG method with 0.946 (95% CI: 0.878-0.983; Fig. 1A). The differences between AgELISA (P=0.12), and ICG methods (P=0.37) in comparison to RT-PCR method were not statistically significant.

In examining the accuracy and concordance of each of the diagnostic methods for detecting infection with RV against RT-PCR, the values of linear kappa, concordance correlation, and bias correction were equal to 0.88 (95% CI: 0.76-0.99), 0.88 (95% CI: 0.82-0.91) and 0.992 for AgELISA method. For ICG method the values of linear kappa, concordance correlation and bias correction were equal to 0.85 (95% CI: 0.72-0.98), 0.84 (95% CI: 0.77-0.89) and 0.995, respectively (Table 2). The perfect agreement was obtained between RT-PCR method and AgELISA ($\kappa$= 0.88), and ICG ($\kappa$= 0.85).

### Bovine coronavirus (BCV)

Table 1 and Fig. 1 show the performance of individual diagnostic techniques, including a comparison with RT-PCR obtained from the pooled results of AgELISA and ICG for BCV. The results of statistical analysis showed sensitivity, specificity, NPV and PPV of AgELISA method was 100 (95% CI: 84.6-100), 98.53 (95% CI: 92.1-100), 100 (95% CI: 94.6-100),

and 95.7 (95% CI: 78.1-99.9), also for ICG method values was 90.91 (95% CI: 71.0-98.9), 91.18 (95% CI: 81.8-96.7), 96.9 (95% CI: 89.0-99.6) and 76.9 (95% CI: 56.4-91.0), respectively. The highest diagnostic characteristics and AUC belonged to AgELISA method with 0.993 (95% CI: 0.978-1.00) and the lowest was belong to the ICG method with 0.91 (95% CI: 0.831-0.96; Fig. 1B). The differences between AgELISA (P=1.00), and ICG methods (P=0.29) in comparison to RT-PCR were not statistically significant (Table 1).

The values of linear kappa, concordance correlation and bias correction accuracy for AgELISA method was equal to 0.97 (95% CI: 0.91-1.00), 0.97 (95% CI: 0.95-0.98) and 0.999, and for ICG method was 0.77 (95% CI: 0.62-0.92), 0.77 (95% CI: 0.67-0.84) and 0.994, respectively (Table 2). The perfect agreement was obtained between RT-PCR method and AgELISA (κ= 0.97), and a substantial agreement was observed between RT-PCR and ICG (κ= 0.77).

**Rotavirus+Coronavirus**

Results indicated that the sensitivity, specificity, NPV and PPV of AgELISA method was 100 (95% CI: 47.8-100), 97.65 (95% CI: 91.8-99.7), 100 (95% CI: 97.5-100), and 71.4 (95% CI: 29.0-96.3) and for ICG method was 80.00 (95% CI: 28.4-99.5), 95.29 (95% CI: 88.4-98.7), 98.8 (95% CI: 93.4-100), and 50.00 (95% CI: 15.7-84.3), respectively (Table 1). The results obtained from the statistical analysis of ROC curve are presented that the highest and lowest AUC are belong to AgELISA and ICG methods with 0.988 (95% CI: 0.939-100) and 0.876 (95% CI: 0.790-0.939), respectively (Fig 1C). The differences of AgELISA (P=0.5) and ICG methods (P=0.37) in comparison to RT-PCR method were not statistically significant (Table 1).

The values of linear kappa, concordance correlation and bias correction accuracy of AgELISA method was equal to 0.88 (95% CI: 0.58-1.00), 0.82 (95% CI: 0.74-0.87) and 0.984, and for ICG method was 0.59 (95% CI: 0.26-0.91), 0.58 (95% CI: 0.44-0.70) and 0.968, respectively (Table 2). The perfect agreement was obtained between RT-PCR and AgELISA (κ= 0.88), and a moderate agreement was observed with ICG method (κ= 0.59).

## DISCUSSION

One of the most prevalent concerns of the dairy industry is the mortality of newborn calves. (Hansa et al., 2012). With the increase in mortality rate in young calves in the first weeks of life, heavy and irrepara-

**Table 2.** Concordance of results between AgELISA and ICG against diagnostic 'gold' standard (RT-PCR) method by using linear kappa factor, reliability of kappa statistics, bias correction rate and Pearson correlation for the diagnosis of rotavirus (RV), bovine coronavirus (BCV) and RV+BCV co-infection among diarrhoeic calves in industrial dairy farms, Iran (n=90)

| Parameter Test | Prevalence (%) | Reciprocal tests results | | | | Linear Kappa (95% CI) | Reliability of average of Kappa (95% CI) | Concordance Correlation (95% CI) | Bias Correction Accuracy (%) |
|---|---|---|---|---|---|---|---|---|---|
| | | +/+ | +/- | -/+ | -/- | | | | |
| *Bovine coronavirus* | | | | | | | | | |
| AgELISA faeces vs RT-PCR | 23/90 (25.6%) | 22/90 | 1/90 | 0/90 | 67/90 | 0.97 (0.91-1.00) | 0.92 (0.88-0.94) | 0.97 (0.95-0.98) | 0.999 |
| ICG vs RT-PCR | 26/90 (28.9%) | 20/90 | 6/90 | 2/90 | 62/90 | 0.77 (0.62-0.92) | | 0.77 (0.67-0.84) | 0.994 |
| Rotavirus | | | | | | | | | |
| AgELISA faeces vs RT-PCR | 24/90 (26.7%) | 20/90 | 4/90 | 0/90 | 66/90 | 0.88 (0.76-0.99) | 0.92 (0.89-0.95) | 0.88 (0.82-0.91) | 0.992 |
| ICG vs RT-PCR | 23/90 (25.6%) | 19/90 | 4/90 | 1/90 | 66/90 | 0.85 (0.72-0.98) | | 0.84 (0.77-0.89) | 0.995 |
| Rotavirus + Bovine coronavirus | | | | | | | | | |
| AgELISA faeces vs RT-PCR | 7/90 (8.89%) | 5/90 | 2/90 | 0/90 | 83/90 | 0.88 (0.58-1.00 | 0.83 (0.76-0.88) | 0.82 (0.74-0.87) | 0.984 |
| ICG vs RT-PCR | 8/90 (7.78%) | 4/90 | 4/90 | 1/90 | 81/90 | 0.59 (0.26-0.91) | | 0.58 (0.44-0.70) | 0.968 |

ble financial losses are inflicted on livestock farming. Different studies showed that neonatal diseases resulting in calf mortality reduce farm net profit by 38% (Khan and Khan, 1991). Diarrhoea is one of the important problems of dairy farms in Iran (Morshedi et al., 2010; Nazoktabar et al., 2013; Mohebbi et al., 2017), but there are insufficient studies about the causes of diarrhoea in Iran (Lotfollahzadeh et al., 2020). Previous studies have demonstrated that in the colder months of the year, calves are under the greatest risk of diarrhoea (Scott et al., 2004; Uhde et al., 2008), therefore, sampling in the present study was carried out in November and December. As mentioned in the literature review, BCV and RV are the most common viruses involved in neonatal calf diarrhoea (Dash et al., 2012), which led to great economical losses. As a result, using high-precision clinical on-farm methods in the early diagnosis of the cause of diarrhoea plays a very important role in its therapeutic regimes, management protocols, and control procedures. Hence, this study aimed to evaluate the prevalence of BCV and Rotavirus by using ICG and AgELISA and also to examine the performance of these techniques in comparison to RT-PCR.

The prevalence rate of RV in the present study was in agreement with the study conducted by Suresh et al., (2012) and in contrast with the investigation of Nazoktabar et al., (2013). This rather contradictory result may be due to that in the study of Nazoktabar et al. (2013) only faeces samples of calves were examined. Another possible explanation for this is that the age range of calves in their study has been wider than our study (up to 8 weeks vs up to 3 weeks). From point of the prevalence of BCV, the results of this study were different from Dash et al., 2012, Suresh et al., 2012, Kumar et al., 2013 and Mohebbi et al., (2017), but they are broadly consistent with earlier studies conducted by Simenov et al., (1981) and Snodgrass et al., (1986). Additionally, the co-infection prevalence rate of RV and BCV in the current study was 5.56% out of 90 diarrhoeic stool samples processed. These results differ from some published studies (Oliveria Filho et al., 2007; Fernandes Barry et al., 2009), they found a higher co-infection rate of 18.8 and 15.9%, respectively. A possible explanation for this might be that in other studies different breeds (Nelore) in various geographical locations (Brazil) were investigated (Oliveria Filho et al., 2007). In previous studies, mixed infections have been reported in diarrhoeic calves (Cho et al., 2010) and can increase the risk of clinical signs. Diagnosis of co-infections is not surprising due to the presence of faecal-oral route of transmission for enteropathogens. The results of the present study demonstrated that infection, with both viruses, is possible in calves.

A few diagnostic methods are available to detect BCV and RV in stool and blood samples, most of which require laboratory facilities and are time-consuming (Cho et al., 2012; Uhde et al., 2008; Klein et al., 2009; Icen et al., 2013). Rapid assays (e.g., ICG) are able to quickly examine several samples and treatment interventions can be performed immediately and they do not require specialized laboratory equipment (Klein et al., 2009). It is interesting to note that among all employed methods in the present study, ICG had acceptable results due to the less time-consuming origin and is suggested to be used for detection of RV and BCV infection on-farm, as has been shown in previous studies (Izzo et al., 2012), however we believe that ICG positive samples should be tested by AgELISA to determine false positive or negative results.

For the investigation of the rapid assays for RV and BCV, combined ELISA results were considered to be the gold standard because of their high sensitivity and specificity. Among the evaluated pathogens, the ICG revealed different sensitivities and specificities for detecting RV, BCV, and mixed infection. In the study conducted by Cho et al., (2012), which evaluated the diagnostic performance of Enterichek (ICG based method, Bovine Enterichek Biovet Inc., Saint-Hyacinthe, Quebec, Canada) in comparison with a multiplex real-time PCR, it has been shown that diagnostic sensitivity was 42.3% and 100%, and the diagnostic specificity was 60% and 51.4% for RV and BCV. In another research, Klein et al., (2009) assess commercial rapid Kit (FASTest BCV and ROTA Strips, MEGACOR Diagnostik GmbH) for detecting BCV and RV in comparison to real-time PCR with sensitivity and specificity of 71.5% and 95.3% for RV, 60%, and 96% for BCV. Rapid ICG tests (FASTest, MegaCor Diagnostik GmbH) for RV were investigated by Luginbühl et al., (2005) in faecal samples of calves. The Se for detection of RV was low (57%) compared to an AgELISA assay. Compared to real-time PCR as the gold standard, the sensitivity and specificity of the ICG test were high for BCV (90.91% and 91.18%, respectively), RV (94.3% and 95%, respectively), and co-infection (80% and 92.29%) and these results differ from other studies. The agreement quotient (kappa) for RV, BCV, and co-infection with ICG method

in the current study were equal to 0.85, 82.6%, and 98.5% respectively, but in Cho et al., (2012) and Klein et al., (2009) the kappa statics of RV and BCV were 0.6 and 0.95 and 0.91 and 0.6, respectively. A higher NPV and PPV of ICG test for detection of RV (98.5% and 82.6%) and BCV (96.9% and 76.9%) was found in the current study, which was better in comparison to the results obtained by Klein et al (2009) for RV (94% and 76%) and BCV (79% and 91%). The findings of this study are consistent with previous observational studies, which showed appropriate agreement between ICG test and DGS (e.g. RT-PCR) (Luginbühl et al. 2005; Klein et al., 2009; Cho et al., 2012). The best agreement and the highest Se and Sp were obtained between RT-PCR and AgELISA ($k$ = 0.97; Se = 100; Sp = 98.53). The ICG test was less accurate than AgELISA method for identifying RV and BCV in this study. A good correlation between the diagnostic tests was observed (RT-PCR, AgELISA, and ICG). Our findings indicate that sensitivity was higher than 80% and specificity was higher than 91% for all three commercial kits. This is consistent with Van Maanen et al. (2008). Diagnostic Se and Sp and kappa values of RV (ICG based, BIO K 288) are 100%, 95.2%, and 0.96 and BCV (ICG based, BIO K 288) stated by the manufacturer are 89%, 98%, and 0.88, while the obtained concordance of results between ICG kit and ELISA method of using kappa factor for RV and BCV was 0.85 and 0.77. A possible explanation for these results from the point of concordance may be the lack of adequate sampling and not taking faecal samples from healthy calves. A comparison of the findings with those of other studies confirms these rapid assays can be employed easily and reliably in the field to determine the infection status of diarrhoeic calves with RV, BCV, and mixed infection.

Our results suggest that AgELISA, and ICG would be useful for detecting RV and BCV antigens and antibodies in calves' serum and faeces samples.

The generalizability of these results is subject to certain limitations. For instance, these results need to be approved in other farm trial studies with higher population sizes, and other calf diarrhoea pathogens (e.g *C. parvum* and *E. coli*) are prevalent. Although the study has successfully demonstrated that viral enteropathogens are detectable in diarrhoeic calves by using ICG, it has certain limitations in terms of enteropathogens detection in healthy calves without clinical diarrhoea and the comparisons were not performed in clinically healthy calves. The current study has only examined five industrial dairy farms which this issue was addressed in the material and method section. As a result, the samples were nationally representative of the small and confined population, caution must be applied, as the findings might not be applicable to all dairy farms of Iran from point of RV and BCV prevalence.

Our findings express that AgELISA method is more accurate than ICG, but the ICG assay can help improve the speed of diagnosis RV and BCV infections, however new scientific strategies for promoting accuracy and transparency of ICG-based technique in early diagnosis of the cause of diarrhoea should be used. The ICG may also require improvement in terms of cost, as well as a further simplification in terms of equipment if it is to be used for large-scale community diagnosis. We suggest that positive ICG samples should be tested by AgELISA or RT-PCR techniques to avoid false results in farm animals.

## CONFLICT OF INTEREST

The authors whose names are listed certify that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

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