Virological and serological examinations for rotaviruses in diarrhoeic calves

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SUMMARY

In this study, polyacrylamide gel electrophoresis (PAGE) method and a commercial latex agglutination (LA) kit designed primarily to detect human rotavirus were performed for the detection of rotaviruses in faeces samples from 49 diarrhoeic calves. In addition, presence of neutralizing antibodies against rotaviruses in serum samples of the same calves were investigated by Serum neutralization (SN) test. Out of the 49 diarrhoeic calves, 7 (14.2%) were positive by LA, 9 (18.3%) by PAGE. On the other hand, rotaviruses were detected in 11 faecal specimens by PAGE and LA test. In contrast, only 2 out of 11 were confirmed positive by electron microscopy (EM). Neutralizing antibodies for rotavirus were demonstrated in 18 (36.7%) of the 49 sera, titers ranging from 1:5 to 1:15. In conclusion, LA test was found very easy to perform and as sensitive as PAGE for diagnosis of rotavirus infections. Latex agglutination appears to be suitable for routine laboratory work not only in research laboratories, but also for veterinary practitioners. Besides, the results obtained have indicated that pregnant dairy cattle should be vaccinated against rotaviruses for transfering of protective antibodies to newborn by colostrum.

Key words: *Diarrhoea, Calf, Rotavirus, PAGE, LA and SN test*

İshalli buzağılarda rotavirusların virolojik ve serolojik olarak araştırılması

ÖZET

Bu çalışmada, polyacrylamide gel electrophoresis (PAGE) metodu ve latex aglutination (LA) test kiti kullanılarak ishalli buzağıların dışkı örneklerinde rotavirusların varlığının araştırılması amaçlanmıştır. Ayrıca, aynı hayvanların serum örneklerinde rotavirus antikorlarının tespiti için serum nötralizasyon (SN) testi uygulandı. LA testi ile 49 ishalli buzağının 7 (%14.2)' sinde, PAGE tekniği ile 9 (%18.3)' unda rotavirus tespit edildi. Ancak, PAGE ve LA testi ile rotavirus tespit edilen toplam 11 örnekten yalnızca 2'sinde elektron mikroskopi (EM) ile rotavirus partikülleri görülebildi. Serum örneklerinin rotavirus yönünden serolojik kontrolü sonucunda, 49 kan serumundan 18 (%36.7)' inde nötralizan antikorlar saptandı. Sonuç olarak, LA testinin uygulaması kolay ve PAGE metodu kadar duyarlı bir teknik olarak gerek araştırma laboratuvarları gerekse pratisyen veteriner hekimler tarafından rotavirusların tespitinde kullanılabileceği kanısına varıldı. Ayrıca, elde edilen bulgulardan buzağıların kolostral yolla yeterli bağışıklık edinebilmeleri için, gebe sığırların rotaviruslara karşı aşılanmalarının yararlı olacağı düşünülmektedir.

Anahtar kelimeler: *İshal, Buzağı, Rotavirus, PAGE, LA ve SN testi*

INTRODUCTION

Diarrhoea, which frequently occurs in young of all species, has been attributed to various causes such as viruses, bacteria, parasites, nutritional imbalance and environmental factors (4,18). Calf diarrhoea is an important cause of economic loss in both dairy and beef herds. The economic loss is due not only to mortality, but also to treatment costs and poor growth (20). Rotaviruses have emerged as an important etiological agent of gastroenteritis in human and animals (4,18,20). They are a major causal agent of diarrhoea cases, either alone or together with some other enteropathogens. Clinical signs of diarrhoea are of little value in the diagnosis of rotavirus diarrhoea, since many bacterial, parasitic and other viral agents cause similar clinical symptoms. The clinical signs, diagnosis, and epidemiology of disease are also similar in all species (16,20).

Rotaviruses cannot be grown readily in tissue culture; therefore, diagnosis of rotavirus related diseases are based on the detection of virus antigen in the faeces, visualization of virions in electron microscopy (EM) or demonstration of an increase in antibody in the serum samples (7,19,21). Enzyme linked immunosorbent assay (ELISA), polyacrylamide gel electrophoresis (PAGE), latex agglutination (LA) test, revers passive haemagglutination (RPHA) test have been widely used to detect rotaviruses in human and animals (1,5,13,15).

The main purpose of this study was to detect the presence of rotaviruses in faecal samples from diarrhoeic calves using PAGE method and LA kit (RotaScreen, Microgen Bioproduct, England) designed primarily for to detect human rotaviruses. Faecal specimens which were positive with PAGE and LA were also examined by EM. In addition, the microtiter system was applied for the detection and titration of neutralizing antibodies against rotaviruses in serum samples.

MATERIALS AND METHODS

Sample collection: Specimens were collected from diarrhoeic calves reared in the private small capacity farms in

Van province and admitted to the Department of Internal Medicine, the Veterinary Faculty. A total of 49 faecal and serum samples were taken from 49 individual calves aged between 1-8 weeks. For PAGE, LA and EM, no pre-treatment of the faeces were applied, and the specimens were stored at -30 °C. However, all serum samples taken for serum neutralization (SN) test were first heat inactivated at 56 °C for 30 minutes, and then stored at -30 °C until used.

Virus strain: The cytopathogenic rotavirus strain (Northern Ireland 75/447) was used throughout the neutralization experiments.

Cell culture: Cell suspension was prepared from Madin Darby Bovine Kidney (MDBK) continuous cell line. The cell culture medium was removed and PBS-Versen-Tyripsin soltion was added to the cell culture bottle in order to dissociate the cells from bottle surface. The aliquot was centrifuged at 1000 rpm for 10 minutes. The cell pellets obtained from centrifugation were resuspended at $3x10^5$ cell/ml in Eagle's MEM supplemented with 5% fetal calf serum.

Polyacrylamide gel electrophoresis (PAGE): The extraction of viral nucleic acid, its resolution and staining was carried out as described by Herring et al.(9) with minor modifications. Briefly, the faecal specimens were diluted 1:4 w/v in extraction buffer containing 1% sodium dodecyl sulfate, an equal volume of phenol-chloroform (3:2) was added and mixture was vortexed and centrifuged for 20 min at 4000 rpm. The aqueous phase was removed. For electrophoresis, 40 µl of the clear supernatant was mixed with 10 µl of blue marker and loaded onto a continuous polyacrylamide gel (7.5%). Then, the gels were run overnight with a 20 mA current (Hoefer Scientific Instruments, USA). Afterwards, gels were fixed, developed and silver-stained. A positive control sample was always included in each gel to compare segmented viral RNA migration pattern.

Latex agglutination (LA) test: A commercial LA kit (RotaScreen, Microgen Bioproduct, England) was performed according to the manufacturer instructions, which had been designed primarily for the detection of human rotaviruses. RotaScreen reagents include test latex coated with rotavirus antiserum, control latex coated with preimmun serum, positive control, and buffer solution. 1 ml aliquots of 10% (w/v) faecal suspension were made up in RotaScreen buffer. After shaking by a vortex, faecal suspensions were centrifuged at 1000 rpm for 10 minutes. Two drop of the each faecal supernatant were placed on a blackside card slide. A drop of test reagent was added to the first drop and the negative control reagent was added to the other drop. Drops weremixed with a wooden stick. The test slide was tilted manually and the reaction was read by eye after 2 minutes. The test was considered positive for rotavirus if distinct agglutination was observed with test latex but not with control latex. If agglutination was observed in the mixture containing control latex, the test was considered uninterpretable.

Serum neutralization (SN) test: The procedure for the detection and titration of serum neutralizing antibodies for bovine rotavirus was done as described previously by Frey and Liess (6). Two-fold dilutions of each inactivated serum samples for the titration of antibody titres (between 1:5 and 1:40) were prepared in 2 rows in microtiter plates using 0.05 ml loops. Subsequently, 0.05 ml of the test virus ($100TCID_{50}$: $10^{-1.7}$/0.05 ml) was added to each well plate which were then sealed and held 1 hours at 37 °C for virus-antibody reaction. Thereafter, cell suspension ($3x10^5$ cell/ml) was added to each well. In addition, 2 wells for the virus control and 2 wells also for the cell control were used. The plates were sealed with nontoxic transparent adhesive tape and incubated at 37 °C in a CO_2 incubator for 4 days. Plates were examined with an inverted microscope (Olympus, Japan).

Electron microscopy (EM): EM preparation was performed as described by Burgu et al. (2). Ten percent (w/v) of each faecal sample was made in distilled water and centrifuged for 10 min. at 3000 rpm. A carbon coated electron microscopy grid was floated on a drop of supernatant for 45 minutes. Then, negative staining was performed using 1% phosphotungstic acid (pH 7.0), blotted dry and examined in a Carl Zeiss 9 S-2 electron microscope.

RESULTS

The results of the analysis of the 49 feacal specimens were given in Table 1. Out of the 49 diarrhoeic calves, 7 (14.2%) were positive by LA and 9 (18.3%) by PAGE. While 5 samples were positive with both methods, 38 samples were negative. The positive / negative ratio of rotavirus found by LA test (7/42) was similar to the ratio found by PAGE (9/40). There was total agreement between the two methods in 43 (87.7%) out of 49 samples tested. Positive specimens detected by PAGE were classified as typical group A rotavirus according to pattern of 11 segments (Figure 1). Out of 11 diarrhoeic samples detected rotavirus positive by PAGE and LA, in only 2 samples could be seen the typical rotavirus particles by electron microscopy (Figure 2).

Out of 49 serum specimens tested by microneutralization test, 18 (36.7%) were found seropositive in 1:5 and above dilutions. Neutralizing dose 50 (ND_{50}) values of the antibody carriers were between 1:5 - 1:15 (Table 2). Out of diarrhoeic calves detected rotavirus positive by PAGE and LA, only eight calves were found seropositive for rotavirus. On the other hand, 10 diarrhoeic calves detected to be negative by PAGE and LA were found also to be seropositive against rotavirus. Twenty eight calves were negative as both virologically and serologically for rotaviruses (Table 3).

Table 1. Comparison of the PAGE and LA test results in 49 faecal samples.

PAGE	LA test		Total
	+	–	
+	5	4	9
–	2	38	40
Total	7	42	49

Table 2. Distribution of antibody titers and detection of neutralizing antibodies against rotavirus in serum samples.

Positive - ND_{50}	Number of calves
1:5	7
1:7.5	8
1:10	1
1:15	2
1:20	-
1:30	-
1:40	-
Negative	31
Total	49

Table 3. Comparison of the serum neutralization values with the results of PAGE and LA test.

Serial No	Case No	PAGE / LA	ND_{50}
01	CN - 01	+ / –	–
02	CN - 05	– / –	1:7.5
03	CN - 06	– / –	1:7.5
04	CN - 08	+ / +	1:5
05	CN - 09	+ / +	1:5
06	CN - 10	+ / +	1:7.5
07	CN - 11	+ / +	–
08	CN - 12	+ / –	1:7.5
09	CN - 13	+ / –	1:10
10	CN - 15	+ / –	1:15
11	CN - 17	– / +	–
12	CN - 21	– / –	1:7.5
13	CN - 24	– / –	1:5
14	CN - 26	– / –	1:5
15	CN - 32	– / –	1:7.5
16	CN - 34	– / –	1:7.5
17	CN - 35	– / –	1:5
18	CN - 40	– / +	1:15
19	CN - 43	– / –	1:7.5
20	CN - 44	– / –	1:5
21	CN - 47	+ / +	1:5
Total		9 / 7	18

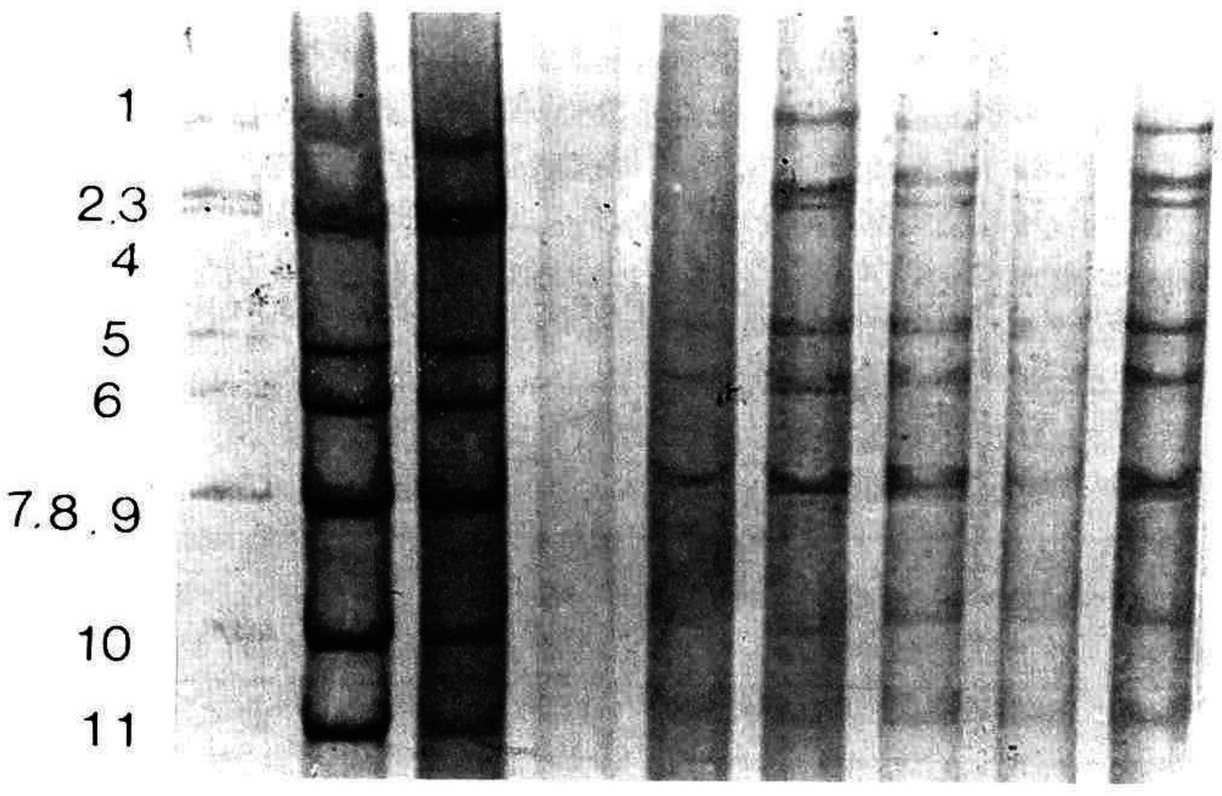

Figure 1. Electrophoretic segment analysis of rotavirus nucleic acids detected in faecal samples (*p: positive control, n: negative faeces, a - g: positive faeces*).

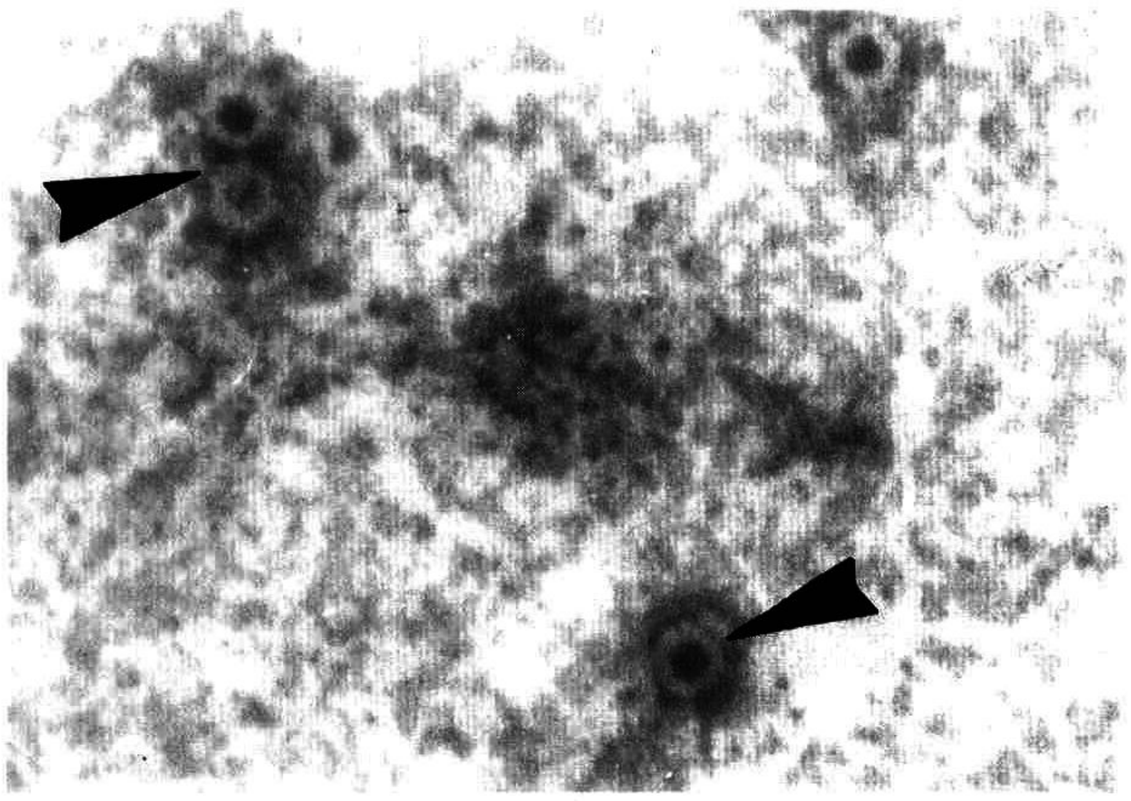

Figure 2. Rotavirus particles detected in faecal sample by electron microscopy (*x 90.000*).

DISCUSSION

Specific, sensitive, economic and practical methods are needed for diagnosis of rotavirus related diseases in veterinary and medicine. Identification of rotaviruses are important because it permits a logical approach to disease control. Although EM have been used as a standard technique for detecting rotaviruses, its routine use for diagnosis is limited beceause of cost, time and equipments (16). The main disadvantage is that a high concentration of viral particles is required. For this reason, specimens for viral diagnosis should be taken at an early stage in clinical disease (7,19). In recent years, alternative methods for the diagnosis of rotaviral diseases, enzyme linked immuno-sorbent assay (ELISA), polyacrylamide gel electrophoresis (PAGE), latex agglutination (LA) test, revers passive haem-agglutination (RPHA) have been used by several workers (3,5,12,21).

Jensen (10) observed a high degree of correlation between LA and ELISA, and found to be a rapid, practical and nonexpensive choice for a laboratory examining a few samples daily by LA because the test requires very little laboratory equipment and has simple procedure. In a research carried out by Burgu et al.(2), three techniques were compared in diagnosis of rotaviruses in faecal samples of diarrhoeic calves. They found that the PAGE and ELISA were more sensitive than the EM. Sukura and Neuvonen (19) found the LA test very easy to perform, more sensitive than the EM method, and rather specific method for detection of rotaviruses. Pai et al. (12) reported that LA test appears suitable for rapid diagnosis of rotavirus related gastroenteritis in small hospitals, emergency wards, or even in the physician's office. Herbst et al. (8) claimed latex agglutination test designed for the detection of human rotavirus suitable for routine laboratory work in research laboratory as well as in veterinary practice. The present study also indicates that PAGE and LA have a similar sensitivity for detection of rotavirus in faecal samples. Since PAGE depends on the identification of a characteristic and distinctive rotavirus electrophoretype, false-positive results are unlikely. However, PAGE method is slow and very laborious compared to LA test. The equipment needed for

PAGE is more sophisticated and experenced staff is needed. On the other hand, the equipment for LA test is simple and can be established in every routine laboratory. The test is easy to perform and does not need special skills.

Kohara et al.(11) suggested that the immunization of cows enhanced the passive immunity levels in calves against bovine rotaviruses. Snodgrass et al.(17) revealed that feeding with immune colostrum of newborn delayed the onset of rotaviral diarrhoea, and reduced its incidence, duration and severity. The serological data obtained from the present study have indicated that calves have not been protected enough by means of antibodies against rotavirus infection. Schwers et al.(14) pointed out that no relationship had been found between the prevalence of bovine rotavirus antibodies and frequency of calf diarrhoea. Similar findings were also observed in the present study.

As a result, LA test was found very easy to perform and as sensitive as the PAGE for diagnosis of rotavirus related infections. Thus, LA test can be suggested to the veterinary practitioners. In addition, pregnant dairy cattle should be vaccinated against rotaviruses for transfering of protective antibodies to newborn via colostrum.

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