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ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ESCHERICHIA COLI K99 PILUS ANTIGEN IN FECES

HANY MICHAEL AZIZ

Dept. of Bacteriology, Animal Health Research Institute, Dokki , Giza., Egypt

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SUMMARY

A total of 176 fecal samples were collected from untreated diarrheic cow calves (1 day to 3 weeks old). Each sample was divided equally into three portions. The first portion was subjected to the traditional method and results obtained showed that *E. coli* K99 pilus antigen was isolated from 42 samples in an incidence of 23.86% . The second and third portions were used for Enzyme linked immunosorbent assay (using locally prepared hyper immune serum) and Enzyme immunoassay (EIA) "Pathasure Bovine Enteritis" kit respectively and the results revealed that 46 and 49 samples were positive with an incidence of 26.14% and 27.84% respectively.

The sensitivity and specificity of the traditional method and double antibody sandwich ELISA were 93.7 % , 97.7% and 85.7 % , 94.8% respectively in relation to the enzyme immunoassay (EIA)"Pathasure bovine enteritis kit" the comparative study between the three different methods of diagnosis is discussed.

INTRODUCTION

Diarrhea of newly born calves causes major economic losses directly through both high mortality and the need for expensive treatment and indirectly from poor growth after repeated clinical disease condition and treatment (Thorns et al., 1992).

Enteropathogenic *E. coli* adhesion designated as fimbriae which are species specific, K99 antigen is found on strains from calves. These fimbriae adhere to specific receptors on the enterocytes of the small intestine (Anon, 2002). The K99 antigen is also nominated F5 (Williamson, 2002).

Guinee et al. (1976) concluded that K99 antigen is the useful diagnostic tool for the identification of calf enterotoxigenic *E. coli* strains. Nagy and

Nagy (1982) considered it a good predictor of enteropathogenicity and should be taken as a routine diagnostic procedure.

Piliated E. coli strains always shift to the non piliated phase during culture on regular agar media, thus specific difficult techniques are required to detect K99 antigen (Ørskov et al. , 1975). Therefore the use of specific culture medium is absolutely necessary (Guinee et al., 1976 and Guinee et al., 1977). The probability of identification is increased if more colonies per case have been tested, as some isolates may lose the K99 controlling plasmid during storage (Moon et al.,1976).

Enzyme linked immunosorbent assay (ELISA) technique was performed for several decades in the detection of serum antibodies against different viral diseases. Different types of ELISA a application were used in different sero surveys of animal viral diseases using specific antigen for each causative pathogen. Moreover, ELISA was applied to detect bacterial antibody levels in cows and newborn calves sera and colostrum (Farid et al., 1997 ; Riad, 1997 ; Luginbuhl et al. , 2005 and Varshney et al. ,2007), while its use in direct detection of *E. coli* K99 antigen from fecal samples was not employed in field cases.

Recently, enzyme immunoassay (EIA)) "Pathsure Bovine Enteritis kit" have been introduced and applied internationally to carry out a direct detection in fecal samples the most predominant pathogens causing diarrhea (Rota virus, Corona virus and *E. coli* K99 pilus antigen) in calves. The kit plates were covered by the three specific monoclonal antibodies for the three pathogens thus able to detect either the whole three pathogens or only one of them.

The objective of this study was to spot the prevalence of enteropasthogenic *E. coli* K99 in diarrheic newly born calves and to locally prepare a specific *E. coli* k99 antiserum (polycolonal) to use it in applying the ELISA technique (Double Antibody Sandwich Method) to detect *E. coli* K99 antigen directly in calf fecal samples. Also, to compare between the three diagnostic methods viz: the traditional culture method, enzyme linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA)"Pathasure bovine enteritis kit" in recognition of *E. coli* K99 pilus antigen.

MATERIALS AND METHODS

A total of 176 fecal samples were collected from untreated diarrheic cow calves aging from 1 day to 3 weeks old.

Preparation of fecal samples

Samples were transported to the laboratory as quick as possible; each sample was divided equally into three portions. The first portion was subjected to the traditional culture method. The second portion was vortexed for 2 min. in phosphate buffer saline (1:1) , centrifuged and the superna-

tant was filtered through Millipore filter (0.22 µm) , then treated with Rhozyme 41 (a protease) according to Rigby (1984), used for Enzyme linked immunosorbent assay (ELISA) against the locally prepared *E. coli* K99 antisera,.

The third portion was used to prepare a 10% suspension of the samples should be made in the sample diluents provided. Theirafter was employed for enzyme immunoassay (EIA) "Pathasure Bovine Enteritis" kit.

Preparation of diagnostic hyper immune serum:

Two standard *E. coli* strains ; B41 (O101: K99) and B85 (O9: K99) supplied (WHO Collaborated Center for Reference and Research on *E. coli*, Staten's Serum Institute , Copenhagen , Denmark) were used in the preparation of K99 pilus antiserum according to Kauffman (1966) and Ørskov and Ørskov (1978)

Detection of E. coli K99 pilus antigen in feces:

Traditional method:

The first portion of the fecal samples was separately streaked onto the surface of Minca-Isovitalex agar media and incubated for 24 hr at 37°C. Three colonies from each sample were investigated according to Guinee et al. (1977) .

Determination of *E. coli* K99 antigen was done by the slide agglutination test using K99 hyper immune serum raised in rabbit and was confirmed according to Ørskov and Ørskov (1978) by using the pathogenic *E. coli* immune sera (Denka Seiken Co. Ltd, Japan) .

Enzyme linked immunosorbent assay(Double Antibody Sandwich) for detection of E. coli K99 in feces:

Detection of the *E. coli* K99 antigen in fecal filtrate was carried out according to Voller et al., (1978) and Sobhi (1997).

Coating of the immunoplates was made by 100 ul / well with *E. coli* K99 hyperimmune serum diluted 1: 20 with buffer carbonate and then incubated overnight at 4°C.

Blocking was performed by using 120 ul / well bovine serum albumin (B.S.A). 1: 10 in double distilled water (D.D.W.) and incubated at 37°C for 1hr. in a shaker incubator.

Adding of fecal filtrate: fecal filtrate was diluted 1:50 in B.S.A..100 ul from each samples were dispersed in duplicated wells from each dilution, then incubated at 37°C for 1 hr. in a shaker incubator.

Conjugation: 100 ul / well peroxidase labeled goat anti rabbit conjugate diluted in B.S.A. (1: 3000) was added then incubated at 37°C for 30 min. in a shaker incubator.

Washing: After each one of the previous steps, immunoplates were washed 3 times by washing solution.

Addition of substrate: 100 ul / well ABTS peroxidase substrate were added.

ELISA reader, the plates were read in ELISA

reader at 405 nm and the results were recorded. Positive results are indicated by positive control in the test (well identified *E.coli* K99) and any sample is considered positive if its value reached the volume of positive control or more.

Qualitative enzyme immunoassay(EIA) "Pathasure bovine enteritis kit":

The second portion of the fecal samples were tested directly by the Pathasure bovine enteritis kit for detection of *E. coli* K99 pilus antigen by monoclonal antibodies according to the instructions of the manufacture.

The sensitivity and specificity of the diagnostic methods were calculated according to Thrusfield (1986).

RESULTS

Out of the 528 selected colonies grown on the Minca-Isovitalex agar medium, 42 samples proved serologically as *E. coli* K99 antigen with an incidence of 23.86 %. The correlation between positive cases and the age of new born calf , revealed highest percentage (31.46%) during the first week of age followed by the second week (21.43%), while it was the lowest at the third week (6.45%) (Table 1).

Using both the ELISA and enzyme immunoassay (EIA) "Pathasure bovine enteritis kit" to detect the *E. coli* K99 pilus antigen, the results revealed that 46 and 49 samples were positive with a percentage of 26.14 % and 27.84 % respectively (Table, 2).

The results showed that the sensitivity and specificity of the ELISA was higher than the traditional culture method as shown in Table (3). The sensitivity of the ELISA was 93.7 % and the traditional culture method was 85.7 % when compared the results obtained by the enzyme immunoassay (EIA)"Pathasure bovine enteritis kit" .

Table (1): Prevalence of *E. coli* K99 pilus antigen in diarrheic newly born calves in relation to the age by traditional method

Calves age	No. of samples	Positive cases	
		No	%
1 week	89	28	31.46
2 week	56	12	21.43
3 week	31	2	6.45
Total	176	42	23.86

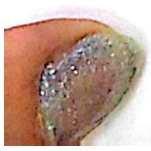

Table (2): Recovery rate of *E. coli* K99 piles antigen in relation to the different diagnostic methods.

Total No. of samples	Positive cases by					
	Traditional method		ELISA		Pathasure kit	
	No.	%	No.	%	No.	%
176	42	23.86	46	26.14	49	27.84

Table (3): The sensitivity and specificity of the traditional method and ELISA in relation to the enzyme immune assay (EIA))"Pathasure bovine enteritis kit".

Method of diagnoses	Sensitivity %	Specificity %
Traditional	85.7%	94.8%
ELISA	93.9%	97.7%

DISCUSSION

Escherichia coli was incriminated to play an important role in diarrhea in new born calves. The confirmation of such incrimination was not proved, for several years, due to the delay of pilus antigen detection as a result of the use of regular ordinary culture media for isolation (Raskova et al.,1976).

Many diagnostic problems appeared during the detection of *E. coli* K99 pili with the traditional culture method as the presence of the A type capsular antigen which masks the development of K99 (Orskov and Orskov , 1977) ; the cultures may lose the K99 controlling plasmid on storage (Moon et al.,1976) and quantitative variation in respect of K99 antigen has also been reported (Isaccson et al., 1977).

Using the traditional culture method, *E. coli* K99 antigen was isolated from cow calves fecal samples in an incidence of 23.86 % (Table 2) , which agrees in the present investigation with the record of Elias (1986); Farid et al., (1992)); Emire et al., (1998) and Solmaz et al., (2000) who recorded an incidence of 33.6%, 24.3%, 32.1% and 26.5% respectively. A lower incidence of 9.1% and 14.9% was recorded by Wray et al.(1993) and Taku et al. (1991) respectively, A higher incidence of 40% and 58% was cited by Valante et al. (1983).

ELISA technique (Double Antibody Sandwich) using the hyper immune serum raised in rabbit showed that it gave better diagnostic results than the traditional culture method. ELISA detects *E. coli* K99 in 46 samples with an incidence of 26.14% and traditional culture method in 42 samples with an incidence of 23.86%. The sensitivity and specificity of traditional culture method and double antibody sandwich Elisa were calculated statistically (Thrusfield, 1986) and it was found to be 93.7 % , 97.7 % and 85.7% , 94.8% respectively in relation to the enzyme immunoassay (EIA)"Pathasure bovine enteritis kit" results (Table 3). This result agreed with Thorns et al. (1992) who mentioned that sensitivity and specificity of ELISA was more than 90% and 93% respectively

Comparison between the traditional culture method, ELISA and enzyme immunoassay (EIA) "Pathasure bovine enteritis kit" was carried out. *Escherichia coli* K99 antigen was recovered from 46 and 49 fecal samples with an incidence of 26.14 % and 27.84% by both ELISA and Pathasure kit methods respectively with corresponding results of 23.86 % using the traditional culture method. It is worthy to denote that all fecal samples which were positive by the culturing method, were also positive by both ELISA and pathasure Kit.

Generally, many diagnostic problems can be overcome by the use of the ELISA. Diagnosis of pathogenic *E. coli* K99 pilus antigen directly in fecal samples with ELISA not only overcomes the problems encountered with the use of the of traditional method but was also found to be highly sensitive, specific and a fast accurate technique, (Holly et al., 1984 ; Schneider et al., 1984 ; Raybould et al., 1987 and Thorns et al., 1992),

Enzyme immunoassay(EIA))"Pathasure bovine enteritis kit" was also considered to be a fast test used in diagnosis of *E. coli* K99 pilus antigen directly in fecal samples (Zschock et al., 1987).

It has been designed and formulated so that it can be performed on the bench with standardized components, and results can be recorded visually. Thus, it is particularly suited for use by practitioners or laboratories where limited technical experts and equipment are available. But unfortunately it

is important to mention that it is not only highly expensive but also not easy to obtain currently and not always available. So we highly recommend the use of the ELISA technique (Double Antibody Sandwich) using locally prepared hyper immune serum (polyclonal).

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