Int.J.Curr.Microbiol.App.Sci (2017) *6*(4): 1267-1273

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 4 (2017) pp. 1267-1273**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.604.154

Comparative Evaluation of c-ELISA and i-ELISA for Detection of Bluetongue Virus Antibodies in Sheep

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Keywords

Sheep, Bluetongue, c-ELISA, i-ELISA, Seroprevalance.

Article Info

Accepted:
12 March 2017
Available Online:
10 April 2017

A B S T R A C T

Bluetongue (BT) is an infectious, non-contagious disease of domestic and wild ruminants. Bluetongue virus (BTV) causes severe disease in sheep, which is transmitted by insect vector belonging to *Culicoides* spp. It is particularly a viral disease of sheep, occasionally affecting cattle, buffaloes, goats, camels and other wild ruminants. In present study, out of 382 sheep serum samples, 150 (39.26%) and 130 (34.03%) were positive respectively by c-ELISA and i-ELISA. On comparing relative sensitivity and specificity of i-ELISA to c-ELISA was 86.67 and 100.00 per cent, respectively. Overall agreement between both the tests was 94.76 per cent.

Introduction

Bluetongue (BT) is an infectious, non-contagious, arthropod-borne viral disease, principally of sheep but many domestic and wild animals are also affected by this disease (Akita *et al.*, 1994). The severity of BT in sheep varies depending on the virus, the breed of sheep and environmental stress. Infection in cattle and goats is usually sub-clinical. Etiological agent of the disease belongs to the genus *Orbivirus* in the family *Reoviridae.* Twenty six serotypes of BTV have so far been recognized worldwide and many more may be prevalent in regions where no survey has been made so far (Parsad *et al.*, 2000). BTV infection occurs in most of the tropical, semitropical and temperate region of the world in parallel with the distribution of its vectors. The regions which are considered endemic areas of bluetongue virus are inhabited by 2/3 of sheep and cattle population around the globe between 40^0 North and 35^0 South (Ozawa, 1985). The disease is characterized by high fever, excessive salivation, swollen lips and tongue, petechial hemorrhage, congestion and small ulcer in the mucous membrane of mouth and conjunctiva, coronitis and reproductive disorders leading to abortion or congenital deformities. The disease is a cause for serious concern to the livestock industry.

Studies on Bluetongue in Gujarat is concerned various workers have reported the existence of Bluetongue in Gujarat based on detection of group specific BTV antibodies, Serotypes specific antibodies, antigen detection by AGID, RT-PCR based detection and isolation of BTV (Chandel *et al.*, 2001; Chauhan *et al.*, 2005 and Agrawal, 2009). However, detection of bluetongue virus antibodies from sheep by c-ELISA and i-ELISA and comparative evolution of both test.

Materials and Methods

During the present investigation a total of 382 Sera were collected from sheep from various places of Gujarat state.

c-ELISA

BTV c-ELISA kits used for the present study were made available by courtesy of Dr. M. M. Jochim, President, Veterinary Diagnostic Technology Incorporation, USA. The test was performed as per the protocol of Afshar *et al.*, (1987) as follows

1. The positive, weak positive and negative control sera and all the serum samples to be tested were diluted by adding 50µl of each sample to 200µl of diluting buffer.
2. The antigen coated Divida strip wells were washed twice with 1X washing buffer and dried by tapping the strips on a tissue paper.
3. Two wells meant for diluent control were filled with 100µl of 1X diluting buffer and covered with a tape to serve as diluent control only.
4. 50µl of 1: 5 diluted positive, weak positive and negative sera and all the test sera were run in duplicate.
5. A dilution of BTV antigen specific monoclonal antibody was prepared by adding 25µl of monoclonal antibody to 2.5 ml of diluting buffer and 50µl of this diluted monoclonal antibody was added to all the wells except the diluent control wells.
6. The reagents in each well were mixed gently by tapping the edge of the Divida strips and kept for 2 hours at room temperature.
7. Meanwhile, a dilution of peroxidase conjugated goat anti-mouse immunoglobulin was prepared by adding 25µl of conjugate to 7.5 ml of 1X diluting buffer.
8. Tape was removed from the diluent control wells and all the wells were washed three times with 1X washing buffer and dried by trapping the strips on a tissue paper.
9. 100µl of the diluted conjugate was added to each well.
10. The plate was kept at room temperature for 1 hour.
11. Approximately 10 minutes before the hour ended, the substrate-OPD solution was prepared by adding 1 buffer- substrate capsule to 100ml of deionized water mixed it and 5 ml of this substrate solution was used to dissolve 1 OPD tablet.
12. After washing the wells five times with washing buffer, 100µl of the substrate was added to all the wells and held for 10 minutes in dark chamber for development of colour.
13. After 10 minutes, 50 µl of the stop reagent ($3NH_2SO_4$) was quickly added to all the wells.
14. Finally, the optical density of each well was recorded in an ELISA microplate reader with a 490 nm wave length filter.

To calculate the result, the average OD value was determined for all the negative, weak positive and strong positive duplicate wells. The average OD value of the diluent wells represented the back ground OD and was subtracted from the average OD values of the control and test sera to yield their adjusted

OD values. Then positive/negative ratio were calculated by dividing the adjusted OD values of the positive control and the test sera by adjusted OD values of the negative control serum. These ratios were multiplied by 100 to express them as a percentage of the negative control and this was subtracted from 100 to calculate the percentage inhibition (PI). The samples were considered to be positive for BTV antibody if the PI value was ≥ 50 per cent. The samples with PIs less than 50 per cent were considered negative.

Indirect ELISA

BTV i-ELISA kits developed at Bluetongue virus laboratory, IVRI, Mukteswar were also used for the detection of BTV antibodies in sheep sera. The test was performed as per the standard protocol (Biswas, 2005) for the detection of BTV antibodies in sheep sera.

1. Fifty µl of recombinant VP7 antigen was coated on to 96-well ELISA plate (Maxi Sorp, Nunc, UK) and incubated at 37°C for 1h on a plate shaker. No antigen was added in four wells, which were kept as antigen blank control, in which only PBS was added.
2. Plate was washed three times with washing buffer.
3. After washing 100 µl of blocking buffer was added and the plate was incubated at 37°C on a plate shaker for 1h, and then washed thrice as described above.
4. Fifty µl of positive serum control, negative serum control and test sera (1:10 dilution) were added to respective wells and were incubated at 37°C for 1h on a plate shaker.
5. Following incubation, washing was done as described above.
6. Dilute the donkey anti-sheep HRPO-conjugate (Sigma) 1:6000 in blocking buffer and added (50µl) to all the wells, and the plate was incubated at 37°C on plate shaker for 1h, following which washing was done as describe above.
7. Fifty µl of freshly prepared chromogen-substrate was added to all the wells and incubated at 37°C for 10 to 15 min. Reaction was stopped by adding 50µl of 1M H_2SO_4.
8. Absorbance was measured at 492 nm in an ELISA reader (Sunrise, Tecan, Austria).
9. Test samples showing double or more than double OD492 of the mean negative control value are considered as positive.

Comparative evaluation of C-Elisa and I-Elisa

Performance of the c-ELISA and i-ELISA for the detection of BTV group specific antibodies in sheep was compared. A total of 382 sera were tested from sheep by c-ELISA and i-ELISA. Cross tabulation of c-ELISA and i-ELISA considering c-ELISA as reference test was recorded as per method described by Martin (1977) to determine relative sensitivity and specificity of i-ELISA by following formula as given below.

$$\text{Sensitivity (\%)} = \frac{\text{(i-ELISA and c-ELISA positives)}}{\text{c-ELISA positives}} \text{ X } 100$$

$$\text{Specificity (\%)} = \frac{\text{i-ELISA and c-ELISA negatives}}{\text{c-ELISA negatives}} \text{ X } 100$$

$$\text{Overall agreement (\%)} = \frac{\text{i-ELISA and c-ELISA positives + i-ELISA and c-ELISA negatives}}{\text{c-ELISA positives + c-ELISA negatives}} \text{X } 100$$

Results and Discussion

In the present study, performance of the c-ELISA and i-ELISA for the detection of BTV group specific antibodies in sheep was compared. A total of 382 sera were tested from sheep by c-ELISA and i-ELISA. Of which 150 (39.26%) and 130 (34.03%) samples were found to be positive respectively (Figs. 1 and 2). The results of region wise, district wise, breed wise, sex wise, age wise and animal husbandry practices wise results are given in table 1. Earlier studies carried out by Chauhan *et al.*, (2004b) and Agrawal (2009) recorded 36.11 per cent and 42.28 per cent seroprevalence by c-ELISA respectively in sheep from Gujarat. More or less similar rate of seroprevalence have been reported by Sonawane *et al.*, (2008) in Rajasthan (36.02%).

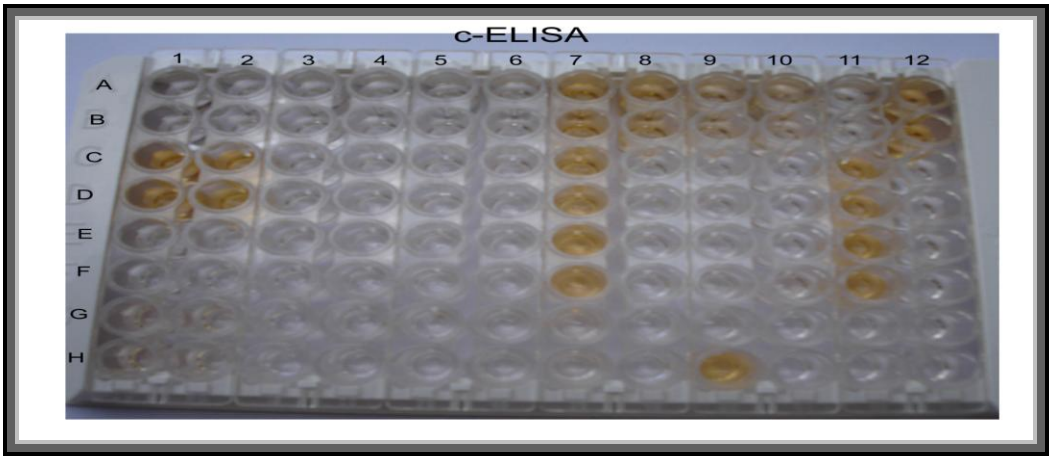
Table.1 Comparison of c-ELISA and i-ELISA for detection of BTV antibodies in sheep sera

Attributes	**No. of serum sample tested**	c-ELISA		i-ELISA	
		No. of sample found positive	Percent age (%)	No. of sample found positive	Percent age (%)
Regions					
North Gujarat	257	100	38.91	88	34.24
Kachchh	76	30	39.47	24	31.57
Saurashtra	49	20	40.81	18	36.73
Total:-	**382**	**150**	39.26	**130**	34.03
Districts					
Banaskantha	166	56	33.73	53	31.92
Mehsana	10	4	40.00	3	30.00
Patan	56	29	51.78	24	42.85
Sabarkantha	25	11	44.00	9	36.00
Kachchh	76	30	39.47	24	31.57
Rajkot	49	20	40.81	18	36.73
Total:-	**382**	**150**	39.26	**130**	34.03
Breed					
Patanwadi	125	61	48.80	53	42.40
Marwari	125	42	33.60	38	30.40
Magara	56	16	28.57	13	23.21
Chokhla	20	7	35.00	6	30.00
Cross Bred (P x R)	56	24	42.85	20	35.71
Total:-	**382**	**150**	39.26	**130**	34.03
Sex					
Female	310	120	38.70	105	33.87
Male	72	30	41.66	25	34.72
Total:-	382	150	39.25	130	34.03
Age					
≤ 1 Year	37	14	37.83	12	32.43
1-2 year	68	29	42.64	24	35.29
2-3 year	112	37	33.03	31	27.67
> 3 year	165	70	42.42	63	38.18
Total:-	**382**	**150**	**39.25**	**130**	**34.03**
Animal Husbandry Practices					
Organized farms	272	108	39.70	91	33.45
Panjarapole	46	19	41.30	16	34.78
Migratory flock	64	23	35.93	23	35.93
Total:-	**382**	**150**	39.26	**130**	34.03

Table.2 Comparative cross tabulation of c-ELISA and i-ELISA

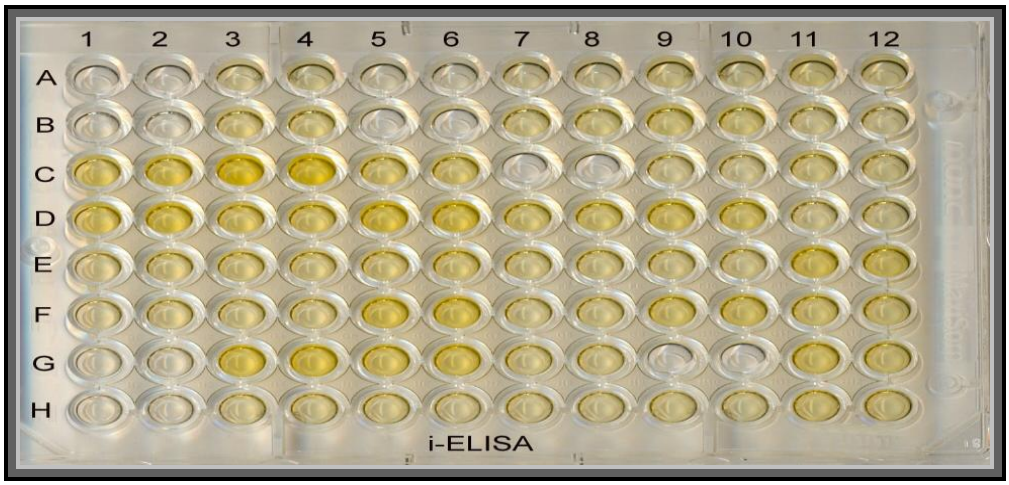
Test		i-ELISA	
		Positive	Negative
c-ELISA	Positive	130	20
	Negative	00	232
Sensitivity (%)		86.67	
Specificity (%)		100.00	
Overall agreement (%)		94.76	

Fig.1 Microtitre ELISA Plate showing results of c-ELISA



Wells A1, A2, B1, B2: Dilution control, Wells C1, C2, D1, D2: Negative control
Wells E1, E2, F1, F2: Weak positive control, Wells G1, G2, H1, H2: Positive control
Rest of the Wells from A3-H12: test serum samples
Wells A3, A4, B3, B4 etc.:- Positive samples, Wells A7, A8, B7, C7 etc.:-Negative samples

Fig.2 Microtitre ELISA Plate showing results of i-ELISA



Wells A1, A2, B1, B2: Conjugate control, Wells C1, C2, D1, D2: Positive control
Wells E1, E2, F1, F2: Negative control, Wells G1, G2, H1, H2: Antigen blank
Wells C3, C4, G3, G4 etc.:-Positive samples, Wells A5, A6, C7, C8 etc.:-Negative samples

However, in contrast to the present findings, lower rate of seroprevalence, 24.66 per cent in Gujarat (Chandel *et al.,* 2004), 21.40 per cent in Kazakhstan (Lundervold *et al.,* 2003) and 6.64 per cent in Punjab (Sodhi *et al*., 1981), whereas higher rate of seroprevalence 76.44 per cent in Iran (Hasanpour *et al.*, 2008), 73.80 per cent in Tamilnadu (Balamurugan *et al.,* 2008), 63.16 per cent in Gujarat (Hinsu *et al.,* 2000) have been recorded.

In present study c-ELISA proved to be the most sensitive in detecting BTV group specific antibodies than i-ELISA as it detected 150 out of 382 samples positive as against 130 samples detected positive in i-ELISA. This corroborates the findings of Das *et al.,* (1997) and Afshar *et al.,* (1989), who detected more number of samples to be positive in c-ELISA compared to i-ELISA. In contrast to the present findings Bhanuprakash *et al.,* (2008) reported 53.50 per cent and 56.80 per cent seroprevalence by c-ELISA and i-ELISA respectively. Considering c-ELISA as the reference test, overall, sensitivity and specificity of i-ELISA were 86.67 per cent and 100.00 per cent, respectively relative to c-ELISA, while overall agreement between both the tests was 94.76 per cent (Table 2). These results are in accordance with the observations reported by Das *et al.,* (1997) and Afshar *et al.,* (1989).

Acknowledgements

The authors are thankful to Dr. Mondal and Dr. Sanchay Bishwas Scientist, Tissue culture lab, division of Virology, Mukteswar and Director of CADRAD, IVRI, Izatnagar for their kind help and cooperation.

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How to cite this article:

Patel, S.S., H.C. Chauhan, B.S. Chandel, K.B. Patel, M.D. Shrimali, A.C. Patel, Manish Rajgor, J.K. Kala, M.G. Patel, M.A. Patel and Patel, B.K. 2017. Comparative Evaluation of c-ELISA and i-ELISA for Detection of Bluetongue Virus Antibodies in Sheep. *Int.J.Curr.Microbiol.App.Sci.* 6(4): 1267-1273. doi: https://doi.org/10.20546/ijcmas.2017.604.154