Studies of the Antigenic relationships between Bluetongue virus serotypes 2, 9 & 15 isolated in Andhra Pradesh, India

Deepthi Balam*[1], Sreenivasulu Daggupati, Himaja Maddireddy[1]

Department of Microbiology,
Veterinary College, Sri Venkateswara Veterinary University, Tirupathi, Andhra Pradesh, India
1. State Department of Animal Husbandry, Andhra Pradesh, India
* Corresponding author email: drdeepthib@gmail.com
Received: 16-04-2011, Accepted: 21-05-2011, Published Online: 05-09-2011
doi: 10.5455/vetworld.2011.444-448

Abstract

The presence of multiple serotypes of the midge-borne bluetongue virus and lack of effective vaccine are the major impediments in controlling bluetongue in sheep. Attempts are being made to develop a vaccine employing the available serotypes to control the disease in the state. Hence, it is essential to identify the antigenic relationships among the serotypes to identify the candidate strains to be incorporated in the preparation of vaccine. To understand the antigenic relationships between Bluetongue virus -2, 9 and 15 serotypes, the viruses were propagated in BHK21 cell lines, purified using PEG precipitation method and purified virus used to raise hyper immune serum in rabbits. Neutralizing antibodies for the BTV serotypes were detected by day 21 PI. Reciprocal cross neutralization test was employed to determine the R% values between BTV-2, 9 and 15 which indicated the extent of antigenic relationships among the serotypes. R% value between BTV-2 and BTV-9 was recorded as 2.8. R% value of 3.53 and 2.8 were observed between BTV-2 & 15 and BTV-9 & 15 respectively. The R% values recorded in the present study revealed a weak antigenic relationship between the BTV serotypes, indicating that the serotypes are highly divergent.
Key words: Bluetongue virus; serotypes; antigenic relationship.

Introduction

Bluetongue (BT) is a non-contagious, arthropod borne viral hemorrhagic disease of ruminants, particularly of sheep and occasionally cattle and some species of deer. It is also associated with abortion storms and high mortalities. The disease, once confined to the exotic sheep *viz* Southdown, Rambouillet, Russian Merino and Corriedale, recently became established in native sheep of South India, causing severe outbreaks in the region. The disease is reported annually from the south Indian states, Andhra Pradesh, Karnataka, Tamil Nadu and Maharashtra and is causing socioeconomic problems among the sheep farmers. (Sreenivasulu et al. 2004). There are now 24 serotypes of BTV in the world. However 25th serotype was also reported from Kenya (Davies *et al.*, 1992). Of these 21 serotypes have been reported in India (Sreenivasulu *et al*., 2004). BTV serotype 2 was isolated from native sheep of Chittor district, Andhra Pradesh in 1999 (Sreenivasulu *et al.*1999).

Subsequently, BTV serotype 9 from Mehaboobnagar district in 2002 and BTV serotype 15 from Nalgonda district in 2003 were also isolated from outbreaks in the state (Bommineni et al.2008). These outbreaks were reported to the animal husbandry department of the state government and not to the OIE since the state government is the controlling authority. There is an urgent need to develop the vaccine to control the disease in the state. Control of BT in an endemic region depends primarily on the active immunization of susceptible animals. This requires the identification of the circulating serotypes and determination of their serological relationships. However information is not available on the antigenic relationships among the viruses isolated from South India, particularly from Andhra Pradesh. The current study was thus aimed at determining the antigenic relationships among BTV serotypes -2, 9 and 15 isolated in Andhra Pradesh, to facilitate the selection of viruses for inclusion in the vaccine formulated for disease control.

Materials and Methods

Bluetongue viruses: BTV-2/TPT, BTV-9/MBN and BTV-15/N12 isolates recovered from bluetongue outbreaks which occurred in Andhra Pradesh, and stored at the department of Microbiology, College of

Veterinary science, Tirupati, Andhra Pradesh, India, were used in this study at 32nd, 4th and 5th passage levels respectively. The viruses were grown in BHK21 clone 13 cell line for further work.

Purification of Bluetongue virus: The method of Hubschle (1981) with slight modification using Polyethylene Glycol (PEG 6000) and sodium chloride at the rate of 7% and 2.3% respectively, was used for virus purification. The presence of virus in the final pellet was determined by RNA extraction and the product was subjected to agarose gel electrophoresis. The migratory pattern of RNA was compared with the standard pattern of Bluetongue virus RNA which was established and is common to all serotypes.

Antisera: Antisera were raised against purified BTV-2, 9 and 15 in 8-month old serologically negative rabbits as described by Huismans and Erasmus, 1981. In brief, protein content of the antigen (purified virus) was quantified by spectrophotometry and the priming dose was administrated after blending 0.5ml of purified BTV (500µg of protein) with an equal quantity of Freund's complete adjuvant. Subsequently, three boosters were administered along with Freund's incomplete adjuvant. The rabbits were bled a week following administration of the last dose of inoculum and the serum was collected. All the sera were heat inactivated at 56^0C for 30 min, sterilized by filtration through a 0.45µm membrane filter and stored at -20^0C until used.

Agarose gel immune diffusion test (AGID): The rabbit sera were tested for the presence of BTV group specific antibodies using respective purified viruses as antigen and the uninfected cell culture as control. AGID detects group specific antigen, NS3 which is common to all the serotypes.

Microtitre Serum Neutralization Test (MSNT): The quantal microtitre assay was performed using decreasing serum-constant virus (beta) method (Parker et al., 1975) in sterile flat-bottomed microtitre tissue culture plates. This method was employed to detect the titre of the hyper immune sera raised against each of the virus serotypes and also to determine cross neutralization titres between the serotypes.

Initially, 1:10 dilutions of the sera were made. The first rows of the microtitre plates were filled with 75µl of diluent, and the second to the last rows contained only 50µl of the same diluent. Each serotype was tested in duplicate, where 25µl was added to the first well of the designated columns, following which serial two fold dilutions from 1/160 to 1/20480 were prepared by transferring 50µl from the wells in the first rows to subsequent wells, and the same quantity was discarded following transfer into the last well. Virus containing 100TCID50 at volumes of 50µl were added to each dilution of the sera. The sera-virus mixtures were incubated for one hour at 37 degrees Celsius and 5% Co2.. BHK21 cell suspension in growth medium (Himedia) at a concentration of 1 x 10^6cells/ml was added to all the wells in 100 µl volumes. Cell controls contained only 100µl of cell suspension in growth medium and virus controls contained 100µl cell suspension and 50µl of diluted virus (100 TCID50). The plates were sealed and incubated at 37 degrees Celsius in a humidified CO2 atmosphere until a complete monolayer developed in the cell controls and complete CPE in the virus controls was observed. The test was often read following 3-5 days of incubation. The titre of the serum was regarded as the highest dilution at which the virus was neutralized. The test was read as positive, in wells with more than 80% of the cell sheet intact, indicating neutralization of the BTV by the antibodies in the hyper immune serum.

Reciprocal cross neutralization test: The protocol employed for conducting cross neutralization tests was essentially similar to Micro Serum Neutralization Test with slight modifications. Each virus serotype under study was neutralized with the hyper immune sera raised against the three serotypes. The homologous serum was titrated from an initial dilution of 1:10, while the heterologous sera were titrated from initial dilutions of 1:4, to detect cross-reactions even at a lower dilutions of the sera. The serological relationships (R) between the BTV serotypes 2, 9 and 15 were calculated using the formula as described by Dopazo et al. (1996).

$$R = \sqrt{r_1 \times r_2} \times 100$$

R - value is defined as the ratio of the heterologous to homologous serum titres. Ratio r1 was calculated by dividing the heterologous titre obtained with serum 2 by the homologous titre obtained with serum 1, and the ratio r2 was determined by dividing the heterologous titre obtained with serum 1 by the homologous titre obtained with serum 2. The R value gives the extent of the antigenic relationship between the two viruses.

Sequence analysis: Multiple sequence alignment of the VP2 gene (sequence length of 3Kilo Base pairs) of BTV-2, 9 and 15 South African reference serotypes (accession numbers AJ585123.1, AJ58130 and AJ585136) was carried out by using sequence data obtained from Genbank using BLAST tool V.2.2.17. and the alignment was edited using Bio edit V.5.09.

Percent homology and divergence between the BTV serotypes 2, 9 and 15 was generated by using Martinez Needleman–Wunsch algorithm of MegAlign software V.4.03.

Results

Bluetongue viruses: BTV-2/TPT, BTV-9/MBN and BTV-15/N12 isolates grown in BHK 21 cell lines yielded infective titres of 4.6, 4.92 and 5.63 TCID 50/ml respectively

Detection of Bluetongue virus group specific antibodies in hyperimmune sera of rabbits: Sharp precipitation lines were observed between the antigen and the antisera wells in agarose gels indicating the presence of BTV group specific antibodies. No precipitation lines were observed with the control wells containing control cell culture

Detection of neutralizing antibodies in the hyperimmune sera by microtitre neutralization tests: The neutralizing antibodies to BTV serotypes 2, 9 and 15 were noticed from day 21 post infection (PI) and continued to increase reaching a maximum by day 45 PI. The neutralizing (SN50) antibody titres against BTV-2, 9 and 15 are shown in Table-1. The serum neutralizing titres induced against BTV-2, 9 and 15 persisted until the end of the experiment, on day 60 PI.

Table-1. SN_{50} titres of rabbits inoculated with the three BTV serotypes.

Antisera against BTV serotype	SN_{50} titres on days post inoculation 0	21	45	60
BTV – 2	0	64	320	320
BTV – 9	0	32	256	160
BTV – 15	0	128	640	640

Reciprocal cross neutralization tests: Table-2 indicates the cross neutralization titres and titre ratios of homologous and heterologous sera . The R-values and R% values are shown in Table-3. R-value for BTV-2 and BTV-15 is 28.3 indicating a relatedness of 3.53%. R-value for BTV-2 and 9 is 35.77 showing a relatedness of 2.8%. Similarly, BTV-9 and 15 also showed a relatedness of 2.8%.

Sequence analysis: The nucleotide and amino acid homologies of the BTV reference serotypes ranged between 47-53% and 29-41% respectively, indicating weak relationships between the serotypes under study.

Discussion

Insect vector transmission, prevalence of multiple serotypes, broad host range and non-availability of a suitable vaccine are playing a major role in establishing and causing regular outbreaks of BT in the Southern region of India. Since there is no control over the prevalence of bluetongue virus serotypes and vector control is a difficult task, the best alternative is to develop a suitable vaccine to control the disease.

Table-2. Cross neutralization titres and titre ratios of BTV serotypes 2, 9 and 15

HIS* against BTV serotype	Cross neutralization titre with BTV serotypes BTV- 2	BTV-9	BTV- 15
BTV – 2	320	8	32
BTV – 9	8	256	16
BTV – 15	8	8	640
	Titre ratio with		
	BTV- 2	**BTV-9**	**BTV- 15**
BTV – 2	1	1/40	1/10
BTV – 9	1/32	1	1/16
BTV – 15	1/80	1/80	1

Isolation and identification of circulating serotypes is very essential for the development of vaccine, and it is in that context that several BTV-2, 9 and 15 strains were isolated from native sheep of Andhra Pradesh. An inactivated vaccine using BTV-2, 9, 15 serotypes was subsequently prepared and it is currently under field evaluation. To refine the vaccine, it is necessary to identify candidate vaccine strains based on the antigenic relationships. However, all the available serotypes cannot be included in the preparation of vaccine due to the interference phenomenon which was also reported among the bluetongue virus serotypes (Roy *et al*., 1990). Hence, the present study was under taken to identify the antigenic relationship between BTV-2, 9 and 15 serotypes present in Andhra Pradesh. which are being used in the BT vaccine in the state.

Table-3. R value and R % values of BTV-2, 9 and 15.

BTV Serotypes	R value	R% value
BTV-2 and 15	28.3	3.53%
BTV-2 and 9	35.77	2.8%
BTV-9 and 15	35.77	2.8%

New Zealand white rabbits were utilized to raise hyper immune serum against BTV-2, 9 and 15 serotypes. Antibody response directed against VP2 polypeptide causes neutralization of BTV *in vivo* and *in vitro* (Huismans *et al*.,1983). Groocock *et al*., (1982) and Chander *et al*., (1990) reported the appearance of neutralizing antibodies in sheep inoculated with BTV- 20 on 2nd and 3rd week PI. Sreenivasulu and Subba Rao (2000) also demonstrated the appearance of neutralizing antibodies by Day 9 PI in sheep and goat inoculated with BTV- 2. All the three serotypes of BTV in the present investigation were immunogenic inducing both neutralizing and

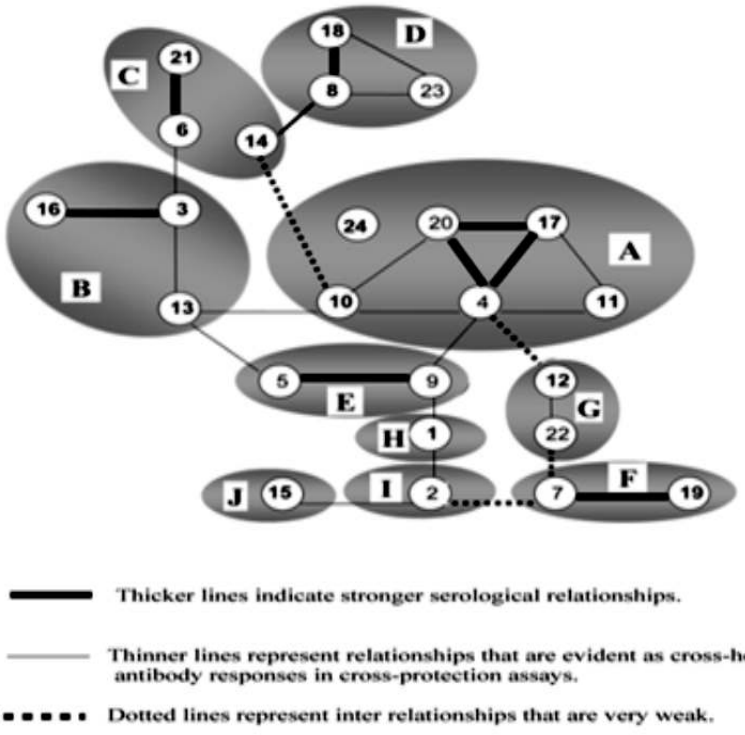

Figure-1. Serological relationships beteween BTV Serotypes (Mann *et.al*. 2007)

precipitating antibodies.

Studying antigenic relationships among the BTV serotypes is important in the selection of candidate strains to be included in vaccine preparations. Dopazo *et al.*, (1996) used reciprocal cross neutralization test to study serological relatedness among aqua reoviruses and found that the test was most suitable in grouping related viruses. R% value of 100 between two viruses indicated that they were serologically identical. The R% values obtained in this study indicate a weak antigenic relationship between the three BTV serotypes. Brooksby (1968) also used the R% values to study the antigenic relationships among various strains of foot and mouth disease virus (FMDV) and proposed the R% value of 70% or more to group the FMDV strains into the same subtype, 32-70% to identify different subtypes, 10-32% for widely different subtypes and less than 10% for different serotypes of FMDV. Hence, R% values of less than 10% are considered to imply that the viruses are antigenically unrelated. The R% values of 2.8, 3.5 and 2.8 obtained for BTV serotypes 2, 9 and 15 is less than 10% and suggest the serotypes are antigenically unrelated to each other.

Erasmus (1990) established the serological relationships between 24 BTV serotypes using plaque reduction assays (Fig.1). Maan *et al*., (2007) correlated these serological findings with the nucleotide and amino acid homologies and grouped the 24 BTV serotypes into 10 nucleotypes (A-J). Serotypes having less than 35% differences in their nucleotide and amino acid sequences were grouped into one nucleotype. BTV serotypes 2, 9 and 15 belonged to three different nucleotypes, I, E and J respectively.

Nucleotide sequence variations in segment-2 of the variable VP2 gene correlate with differences in virus serotypes (Maan *et al*., 2007), thus the gene is utilized for serotyping viruses on a molecular level. Since full length sequences of the indigenous BTV serotypes was not yet published, reference BTV serotypes were used in the study. Though there may be slight variation between the sequences of reference and indigenous serotypes, it is expected that the results will give an idea as to the extent of the relationships between the serotypes.The genomic relationships between the reference BTV serotypes 2, 9 and 15 and the R% values (serological relationship) of the indigenous BTV serotypes 2, 9 and 15 were taken for

consideration. R% values obtained using reciprocal cross neutralization test using BTV-2, 9 and 15 isolated in the native sheep of Andra Pradesh and the genomic analysis of BTV-2, 9 and 15 reference serotypes revealed very weak antigenic relationship and high sequence divergence. It has thus been appropriate to include the three isolated BTV-2, 9 and 15 serotypes in the experimental vaccine to protect sheep against BTV in Andra Pradesh. Further investigations to isolate and characterize other circulating BTV serotypes in the region in the event of outbreaks are however imperative, to identify suitable vaccine candidates.

The R% values recorded in the present study revealed a weak antigenic relationship between the BTV serotypes, indicating that the serotypes are highly divergent.

Acknowledgement

The authors are grateful to Sri Venkateswara Veterinary University, Tirupati, Andra Pradesh for the facilities provided.

References

1. Brooksby JB. (1968). Variants and Immunity: definitions for serological investigations. International symposium on Foot–and-Mouth Disease: Variants and Immunity, Lyon, 1967. Symposia series in Immunological Standardization. 8: 1-10.
2. Bommineni YR, Reddy YN, Rao PP, Sudheer D, Susmitha B, Sarma BJ and Subba Rao MV. (2008). Isolation of bluetongue virus serotypes 2, 9 and 15 from sheep in India. *Veterinary Record*, 163: 545-546.
3. Chander S, Prasad G and Jain NC.(1990). Clinical, hematological and immunological studies on sheep experimentally inoculated with two strains of bluetongue viruses of serotype 1. *Indian Journal of Virology*. 6: 23 – 31.
4. Dopazo CP, Bardin I, Rivas C, Cepeda C and Barja JL.(1996). Antigenic differences among aquareoviruses correlate with previously established genogroups.*Diseases of Aquatic organisms*. 26: 159 – 162.
5. Erasmus BJ (1990) Bluetongue virus. In Virus infections of ruminants, Vol.3 pp. 227 – 237. Edited by Z. Dinter & B. Morein. New York: Elsevier Science Publishers.
6. Groocock CM, Parsonson IM and Campbell CH (1982). Bluetongue virus serotypes 20 and 17 infections in sheep. Comparison of clinical and serological responses. *Veterinary Microbiology* 7: 189 – 196.
7. Hubschle OJB, Lorenz RJ and Mathaka HD. (1981). Enzyme linked immunosorbent assay for detection of bluetongue viral antibodies. *American Journal of Veterinary Research* 42: 61-65.
8. Huismans H and Erasmus B J (1981). Identification of serotype–specific and group–specific antigens of bluetongue virus. *Ondersteeport Journal of Veterinary Research* 48: 51 – 58.
9. Huismans H, Vanderwalt NT, Cloete M and Erasmus BJ. (1983). The Biochemical characterization of bluetongue virus outer capsid polypeptides: In double stranded RNA viruses. Proc. I Inter. Symp. St. Thomas Virgin Islands, R. W. company and DHL Bishop (eds.) Elsevier, New York. 765-772.
10. Maan S, Maan NS, Samuel AR, Rao S, Attoui H and Mertens PPC (2007). Analysis and Phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *Journal of General virology* 88: 621 – 630.
11. Mehrotra ML and Shukla DC (1990). Sero prevalence, diagnosis and differential diagnosis of bluetongue virus disease in India. *Indian Journal of Virology* 1-2: 98 – 103.
12. Parker J, Herniman KAJ, Gibbs EPJ and Sellers RF. (1975). An experimental inactivated vaccine against Bluetongue. *Veterinary Record* 96: 284-287.
13. Roy P, Urakawa T, Van Dijk AA and Erasmus BJ. (1990). Recombinant virus vaccine for Bluetongue Disease in Sheep. *Journal of virology*. 64: 1998 – 2003.
14. Sreenivasulu D, Subba Rao MV, Reddy YN and Gard GP (2004). Overview of bluetongue disease, viruses, vectors surveillance and unique features. The Indian subcontinent and adjacent regions. *Veterinaria Italiana* 40(4): 73-77.
15. Sreenivasulu D and Subba Rao MV. (2000). Clinical, virological and serological responses of sheep and goat inoculated with bluetongue virus type2. *Indian Journal of Animal Sciences*. 70(2): 151-153.

* * * * * * * *