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Experimental animal inoculation and cross neutralization studies of bluetongue virus serotype-4

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Abstract

In this study, plaque purified bluetongue virus serotype-4 (BTV-4) was used with an objective of raising hyper immune serum (HIS) for serum neutralization studies. The plaque purified BTV-4 followed by RT-PCR confirmation was inoculated at a dose of 1ml of $10^{6.46}$ TCID₅₀ (Tissue Culture Infective Dose 50) in Deccani sheep which were confirmed as seronegative for BTV by CELISA, along with a booster dose 15 days post infection (dpi.). Mild clinical signs were observed and the infected animals were found to sero convert to BTV on 7dpi. Sera collected from these animals were then tested for type specific neutralizing antibody titer by serum neutralization test (SNT) in Vero cells. The titre ranged from 1/80 on 7dpi. to 1/640 by 28 dpi. Interestingly, the serum was also found to neutralize BTV-9 and BTV-10 in a one way cross neutralization reaction.

Keywords: BTV-4, hyperimmune serum, vero cells, antibody titer, cross-neutralization.

Introduction

Bluetongue (BT) is an infectious, non contagious, economically important viral disease of sheep and other domestic and wild ruminants caused by bluetongue virus (BTV). BTV is a segmented double-stranded RNA virus of genus *Orbivirus* under family *Reoviridae*. The segmented nature of BTV genome allows reassortment between different serotypes and strains. Presently, worldwide 27 distinct BTV serotypes with the possible addition of two more serotypes are in circulation (Hofman *et al.*, 2008; Maan *et al.*, 2011; Zientara *et al.*, 2014) ^[1, 2, 3]; in India 23 serotypes have been recognized (AINP-BT, 2012; Rao *et al.*, 2014, Krishnajoithi *et al.*, 2016) ^[4, 5, 6].

Virus neutralization test (VNT) is considered as the gold standard for serotyping of BTV which requires plaque purified viruses and serotype specific sera. But some of the Asian viruses differ substantially from the reference viruses (Maan *et al.*, 2012; Rao *et al.*, 2012a,b) ^[7, 8, 9] as a result of which locally isolated serotypes may not be neutralized by reference sera. Therefore, it is essential to raise type-specific sera against different serotypes isolated locally (Rao *et al.*, 2014) ^[5]. Therefore, BTV-4 which was plaque purified and confirmed by RT-PCR was used in this current study to raise hyper immune serum and also to study its neutralization behavior with other available plaque purified BTV serotypes.

Materials and Methods

Animal Inoculation

For antibody production, BTV seronegative 6-12 month-old Deccani sheep were selected through competitive enzyme-linked immune sorbent assay (cELISA) kit protocol (Afshar *et al.*, 1987) ^[10]. For this test, blood samples from each sheep were collected separately in serum vacutainers and kept at 4°C. Serum was separated by centrifuging the blood at 4000rpm, 4°C for 15 minutes and the serum was aliquoted and stored at -20°C. Seronegative sheep as tested by cELISA kit (cELISA, Veterinary Diagnostic Technology, Inc, U.S veterinary License #336) were shifted to insect proof animal house and enough time was given for acclimatization. 1ml of $10^{6.46}$ /ml TCID₅₀ virus was inoculated to sheep by two routes i.e., 0.5ml via intradermal; 0.5ml via subcutaneous routes at shoulder region. For control animal, 1ml of plain MEM was given to compare results. Before inoculation, animals were examined for their general health status i.e., temperature, physical appearance. After that selected site for

infection was shaved and sterilized with 70% ethanol. On 15th day of infection one more injection of same dose was given to both animals. From the day of inoculation animals were examined regularly for typical clinical signs. Serum samples were collected in sterile serum vacutainers (BD Vacutainer® Ref.365078) on 0, 7, 14, 21, 28 days post inoculation (dpi.) to assess seroconversion and to calculate antibody titer.

Virus titre Calculation

TCID50 (Tissue culture infective dose 50) was calculated for available plaque purified types (BTV-1, 2, 4, 9, 12, 16, 21, 23, 24) in 96 well tissue culture plate. For this, Vero cells were seeded at the rate of 1x10⁴ per well in MEM with 10% FBS and incubated in CO₂ incubator at 37°C. At 80-90% confluence, monolayers were infected with 100µl of 10⁻¹ to 10⁻⁸ serially diluted virus in 1% growth medium and incubated in CO₂ incubator. Plates were observed at 12-18 hrs. interval for characteristic CPE under inverted microscope and final readings were taken on 5th day of infection by comparing against cell control and virus control. Virus titer was calculated according to Reed and Munch method (1938) [11].

Serum Neutralization Studies (SNT)

Type specific neutralizing antibody titer of BTV-4

BTV-4 serotype specific neutralizing antibody titers of weekly collected serum samples from virus inoculated animals, were estimated using different dilutions of serum against 100 TCID50 BTV-4 virus i.e, constant-virus-variable-serum method.

Cross neutralization studies

In cross-neutralization studies 1/20 dilution of 21st day hyper immune serum (Parker *et al.*, 1975) [12] was tested against available plaque purified types (BTV- BTV-1, 2, 9, 10, 12, 16, 21, 23, 24). Cross-neutralization studies were carried out according to Sairaju *et al.* (2014) [13] i.e., 100 TCID50/ 50 µl of virus was incubated with 50 µl of 1:20 diluted serum at 37°C for 1 hr. and then inoculated onto confluent monolayers of Vero cells in 96-well tissue culture plates. Along with infected wells cell control, serum control, virus control wells were maintained and incubated with 5% CO₂ at 37°C. Plates were observed under inverted microscope at 12 hr. interval for CPE and the final titer was taken on the fifth day.

Results

Experimental animal infection

Animal with tag no.223 (Animal 1) developed mild pyrexia on evening hours on 6 dpi (103.6⁰ F) which persisted for 4 days and another animal with tag no.233 (Animal 2) developed pyrexia (104.6⁰ F)10-12 hours earlier to animal 1 and persisted for 6dpi (Fig 1) whereas control animal showed normal rectal temperature (102.8⁰F-103.2⁰F). Both exhibited edema of buccal area and lips on 10 dpi (Fig 2.C), nasal discharges on 11 dpi. Hyperemia of buccal mucosa (Fig 2.D) was observed in animal 1 on 17 dpi. and on 16 dpi. in animal 2. Control animal was found to be normal as before inoculation during this entire study (Fig 2.A, 2.B).

Serological findings

Serum samples of control and infected animals collected weekly were subjected to group-specific serologic test, cELISA for confirmation of seroconversion. Both inoculated sheep were found to seroconvert by 7th day while control

remained seronegative. Both the animals were seropositive till the end of the experiment i.e, 28 dpi. Type specific neutralizing antibody titer was observed to increase from 1/80 to 1/640 during 7th day to 28th dpi (Table 1). TCID50 was calculated for available plaque purified types (BTV-1, 2, 9, 12, 16, 21, 23, 24) which were used in serum neutralization tests. Titers for BTV-1, 2, 9, 12, 16, 21, 23, 24 are given in Table 2.

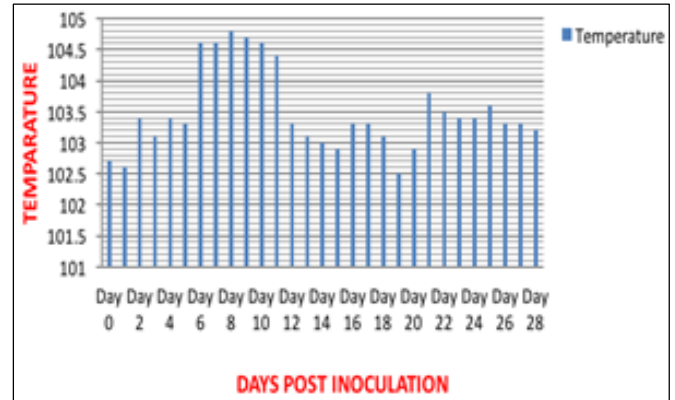


Fig 1: Temperature details of animal with tag no.233. Showing pyrexia between 6-11 dpi.



Fig 2: Clinical signs in deccani sheep infected with BTV 4.

Fig 2.A and 2B are control sheep with no apparent clinical signs and pale pink-normal buccal mucosa. Fig 2.C and 2.D are BTV 4 inoculated sheep exhibiting edema around buccal region and hyperemia in buccal mucosa, respectively.

Table 1: Type specific neutralizing antibody titers of serum collected from experimental animals.

Animal tag no.	Neutralizing antibody titer				
	0 dpi.	7 dpi.	14 dpi.	21 dpi.	28 dpi.
223	0	1/80	1/320	1/640	1/640
233	0	1/160	1/320	1/640	1/640

Table 2: TCID50 titers of BTV serotypes used in the current study.

BTV serotype	TCID50 calculation
BTV-1	$10^{4.375}/100 \mu\text{l}$
BTV-2	$10^{4.167}/100 \mu\text{l}$
BTV-4	$10^{5.46}/100 \mu\text{l}$
BTV-9	$10^{3.833}/100 \mu\text{l}$
BTV-12	$10^{4.5}/100 \mu\text{l}$
BTV-16	$10^{4.682}/100 \mu\text{l}$
BTV-21	$10^{4.375}/100 \mu\text{l}$
BTV-23	$10^{4.5}/100 \mu\text{l}$
BTV-24	$10^{3.833}/100 \mu\text{l}$

Cross neutralization studies

For cross-neutralization studies, serum of 21st day was tested against all available plaque purified serotypes (BTV-1, 2, 9, 10, 12, 16, 21, 23, 24). In these studies, BTV-9 and BTV-10

serotypes were found to be neutralized with BTV-4 serum, thus revealed heterotypic cross neutralization, but didn't show any cross neutralization with other available plaque purified serotypes (Table 3).

Table 3: Cross Neutralization Studies

Plaque purified BTV serotype	SNT result with 21 st day hyper immune serum of BTV-4 serotype	
	Animal tag no.223	Animal tag no.233
BTV-1	CPE +ve	CPE +ve
BTV-2	CPE +ve	CPE +ve
BTV-9	CPE -ve	CPE -ve
BTV-10	CPE -ve	CPE -ve
BTV-12	CPE +ve	CPE +ve
BTV-16	CPE +ve	CPE +ve
BTV-21	CPE +ve	CPE +ve
BTV-23	CPE +ve	CPE +ve
BTV-24	CPE +ve	CPE +ve

CPE negative indicates neutralization of tested BTV serotype with BTV 4 HIS indicating cross neutralization.

Discussion

BTV seronegative sheep injected with plaque purified BTV-4 developed pyrexia on 6 dpi. with 103.5F and 104.6F temperature. Pyrexia was maintained for 4-6 days which was in line with previous observations of Chander *et al.* (1990) [14] and Sreenivasulu and Subba rao (2000) [15]. However, pyrexia was not observed in either animal upon booster dose, which might be due to development of neutralizing antibodies with a titer of 1/320 by 15th day. Parker *et al.* (1975) [12] considered a titer of 1/20 or more as positive for neutralization. Clinically, both animals exhibited mild signs only and this can be attributed to virus attenuation upon being passaged 13 times in Vero cell line. Similar observations were made previously by Chander *et al.* (1990) [14]. It has been known that experimental inoculation of sheep with cell culture adapted BTV results in mild clinical manifestations (Jeggo *et al.*, 1983) [16].

BTV-4 serotype confirmation by RT-PCR was conducted using available primers of BTV types (BTV-1, 2, 4, 9, 10, 12, 16, 21, 23, 24) before and after animal inoculation. But, according to Maan *et al.* (2012) [7] and Susmitha *et al.* (2012) [17], RT-PCR solely was not perfect for typing due to genetic diversity within and between serotypes and topotypes of BTV. Therefore cross neutralization studies were carried out using HIS of BTV-4 against available plaque purified serotypes (BTV-1, 2, 4, 9, 10, 12, 16, 21, 23, 24). But, despite plaque purification, HIS of BTV-4 was found to neutralize BTV-9 and BTV-10 viruses. This cross neutralization behavior is in agreement with previous observations of Zulu and Venter (2014) [18]. Erasmus (1990) [19] also observed some cross or heterotypic antibody response in cross protection assays between BTV-9, 10, 11 each with BTV-4. Heterotypic

cross neutralization of this present study indicates antigenic relationship between BTV-9, BTV-10 with BTV-4. To overcome this ambiguity available HIS of BTV-9, BTV-10 serotypes, was used in another SNT against plaque purified BTV-4 of this study, to find out antigenic similarity, if any. Interestingly, HIS of neither BTV-9 nor BTV-10 could neutralize the BTV-4 indicating that there was only one-way cross neutralization but not two-way.

Conclusion

Plaque purified BTV-4 infected to seronegative sheep elicited mild clinical signs. Further antibody response was noticed from 7 dpi. BTV-4 hyper immune serum was also found to cross neutralize BTV-9 and 10 serotypes. However, this was only one way cross neutralization since hyper immune sera against BTV-9 and BTV-10 could not neutralize plaque purified BTV-4. Therefore, further studies in this aspect are warranted to draw final conclusions.

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References

- Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G, Thuer B. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg. Infect. Dis.* 2008; 14:1855-1861.
- Maan S, Maan NS, Nomikou K, Veronesi E, Bankowska BK, Manjunatha N *et al.* Complete Genome characterization of a novel 26th Bluetongue virus serotype from Kuwait. *PLoS One*, 2011; 6:e26147.
- Zientara S, Sailleau C, Viarouge C, Höper D, Beer M,

- Jenckel M *et al.* Novel bluetongue virus in goats, Corsica, France. *Emerg. Infect. Dis.* 2014; 20:2123-2132.
4. AINP-BT Annual Report, All India Network Project on Bluetongue. Indian Council of Agricultural Research, New Delhi, India, 2012.
 5. Rao PP, Hegde NR, Reddy YN, Krishnajyothi Y, Reddy YV, Susmitha B, *et al.* Epidemiology of Bluetongue in India. *Transboundary and emerging diseases* 2014; 10.1111/tbed. 12258.
 6. Krishnajyothi Y, Maan S, Kandimalla K, Maan NS, Tutika RB, Reddy YV *et al.* Isolation of Bluetongue Virus 24 from India—An Exotic Serotype to Australasia. *Transboundary and emerging diseases.* 2016; 63:360-364.
 7. Maan NS, Maan S, Guimera M, Nomikou K, Morecroft E, Pullinger G *et al.* The genome sequence of a reassortant bluetongue virus serotype 3 from India. *Journal of virology.* 2012; 86:6375-6376.
 8. Rao PP, Reddy YN, Hegde NR. Complete genome sequence of bluetongue virus serotype 9: implications for serotyping. *Journal of virology,* 2012a; 8:8333.
 9. Rao PP, Reddy YV, Meena K, Karunasree N, Susmitha B, Uma M *et al.* Genetic characterization of bluetongue virus serotype 9 isolates from India. *Virus genes.* 2012b; 44:286-294.
 10. Afshar A. Bluetongue: laboratory diagnosis. *Comparative immunology, microbiology and infectious diseases,* 1994; 17:221-242.
 11. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *American journal of epidemiology.* 1938; 27:493-497.
 12. Parker J, Herniman KA, Gibbs EP, Sellers RF. An experimental inactivated vaccine against bluetongue. *The veterinary record.* 1975; 96:284-287.
 13. Sairaju V, Susmitha B, Rao PP, Hegde NR, Meena K, Reddy YN. Type-specific seroprevalence of bluetongue in Andhra Pradesh, India, during 2005–2009. *Indian Journal of Virology.* 2013; 24:394-397.
 14. Chander S, Prasad G, Jain NC. Clinical, haematological and immunological studies on sheep experimentally inoculated with 2 strains of bluetongue virus type 1. *Indian Journal of Virology.* 1990; 6:23-31.
 15. Sreenivasulu D, Subba Rao MV. Clinical, virological and serological response of sheep and goat inoculated with bluetongue virus type 2. *Indian Journal of Animal Sciences.* 2000; 70:151-153.
 16. Jeggo MH, Gumm ID, Taylor WP. Clinical and serological response of sheep to serial challenge with different bluetongue virus types. *Research in veterinary science.* 1983; 34:205-211.
 17. Susmitha B, Sudheer D, Rao PP, Uma M, Prasad G, Minakshi P *et al.* Evidence of bluetongue virus serotype 21 (BTV-21) divergence. *Virus genes,* 2012; 4:466-469.
 18. Zulu GB, Venter EH. Evaluation of cross-protection of bluetongue virus serotype 4 with other serotypes in sheep. *Journal of the South African Veterinary Association,* 2014; 85:01-02.
 19. Erasmus BJ. Bluetongue virus. *Virus infections of ruminants,* 1990; 3:227-237.