Curr Microbiol (2014) 68:127–131
DOI 10.1007/s00284-013-0448-9

Microarray Chip Based Identification of a Mixed Infection of *Bovine Herpesvirus 1* and *Bovine Viral Diarrhea 2* From Indian Cattle

Barkha Ratta · Brijesh Singh Yadav · Mayank Pokhriyal · Meeta Saxena · Bhaskar Sharma

Received: 17 May 2013 / Accepted: 22 July 2013 / Published online: 12 September 2013

Abstract *Bovine herpesvirus 1* (BHV1) and *bovine viral diarrhea virus 2* (BVD2) are endemic in India although no mixed infection with these viruses has been reported from India. We report first mixed infection of these viruses in cattle during routine screening with a microarray chip. 62 of the 69 probes of BHV1 and 42 of the 57 BVD2 probes in the chip gave positive signals for the virus. The virus infections were subsequently confirmed by RT-PCR. We also discuss the implications of these findings.

Introduction

Indian cattle and buffalo populations (200 million and 75 million, respectively) are the largest in the world, and India is emerging as one of the largest milk-producing countries of the world. The health and productivity of Indian cattle and buffalo populations are dependent on the absence or the low scale presence of many endemic diseases which are prevalent elsewhere, particularly *bovine viral diarrhea* (BVD) and *bovine herpesvirus 1* (BHV1). BVD is a disease which is endemic in developed countries. It is only in this century [15, 17, 18] that the virus has been isolated from Indian livestock, though serological evidence of the existence of this virus was reported in 1980s [32]. Both BVD virus genotypes 1 and 2 have been reported [4, 16–19, 29].

B. Ratta · B. S. Yadav · M. Pokhriyal · M. Saxena · B. Sharma (✉)
Division of Biochemistry, Indian Veterinary Research Institute, Izatnagar 243122, India
e-mail: bhaskar@ivri.res.in

B. S. Yadav
e-mail: brijeshbioinfo@gmail.com

BVDV, a member of the *Pestivirus* genus of the family *Flaviviridae*, has been linked to a number of clinical conditions of cattle, viz., diarrhea, abortions, congenital abnormalities, and birth of persistently infected animals [24]. Acute BVDV infections in postnatal life are mostly inapparent [2]. BVDV generally induces severe immunosuppression in affected animals. The immunosuppression is associated with transient leukopenia, neutrophil dysfunction, and other immunological disturbances [5, 12]. The immunosuppressive effect impairs the ability of the animal to clear virus or bacteria from its blood and tissues [21, 28].

Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis caused by BHV1 was first reported in the US in 1950s and in India in 1976. BHV1 is a member of genus *Varicellovirus*, subfamily *Alphaherpesviriniae* of the family *Herpesviridae*. BHV1 causes several diseases in cattle including rhinotracheitis [20], vaginitis, balanoposthitis, abortion, conjunctivitis, and enteritis [8]. The World Organization for Animal Health [OIE] lists BHV1 as a List B-notifiable disease. OIEs List B diseases are transmissible diseases that are of socioeconomic and/or public health importance and that are significant in international trade. Acute BHV1 infection, regardless of whether there are clinical signs of disease, leads to latent infection. Latently infected cattle show no disease unless the latent infection is reactivated [13]. In India, the virus has been isolated from states of Orissa [23], Karnataka [22], and Gujarat [10]. The serological evidence for the existence of BHV1 in Indian domestic and wild livestock [34] indicates that this disease is widespread in India.

BHV1 and BVD are the two diseases that are difficult to manage and eradicate because of latent infections. Mixed infections with these two viruses have been reported from different countries [1, 9]. The economic cost of mixed infection has not been determined but is apparently high. In

India, the seroprevalence of both BVD and BHV1 in Indian livestock is known. So far, no report of mixed infections of these viruses from Indian livestock has been reported.

We report the first mixed infection of BHV1 and BVD in Indian cattle using a microarray chip that we have developed, and discuss the implications of the findings and also the use and utility of microarray chips for identifying mixed and emerging infections.

Materials and Methods

Clinical Samples

The clinical sample collected from the institute's veterinary polyclinic was obtained from a cow which had aborted fetus (5 months) and was having fever.

Microarray Chip

We have been designing and testing microarray chips for identification of animal viruses. Different versions of chips have been tested and an earlier version of the chip identified *Newcastle disease virus* from sheep [31]. The chip used here for testing the clinical samples contained 4079 unique probes representing 124 different viruses and 3000 random probes. The probes were designed using e-array (https://earray.chem.agilent.com) or array designer (www.premierbiosoft.com) and were analyzed using BLAST program individually before including the same in the chip. The probe design criteria were (1) length of oligonucleotide probes 50–60. (2) The Tm (melting point) of the probes 700 °C ± 5. (3) The GC content of the probes 40–60 %. (4) The maximum poly nucleotide tract allowed was six. (5) 90 % homology with target sequence with at least 20 nucleotide contiguous matches at 3′ end and less than 85 % homology with nontarget sequence. The random probes included in the chip were taken from MDA (microbial detection array) chip [7]. The chip was fabricated commercially (Agilent Technologies, USA) as per our design.

Sample Preparation, Labeling, and Hybridization

Total RNA was extracted using TRIzol® Reagent (Invitrogen) according to manufacturer's protocol. The quality of total RNA was checked by Nanodrop spectrophotometer and bioanalyzer. The total RNA was labeled with Cyanine 3-CTP labeling dye using Quick-Amp labeling Kit one-color (Agilent) according to manufacturer's protocol. The purified Cy3-labeled samples were hybridized on Agilent's Microarray Hybridization Chamber using Agilent Microarray Hybridization Chamber Kit (G2534A) according to manufacturer's instruction.

Whole Transcriptome Amplification

The total RNA was amplified by Quantitect® Whole transcriptome amplification Kit (QIAGEN, cat no. 207043) according to the manufacturer's protocol.

Data Extraction and Analysis

After hybridization, the arrays were scanned with an Agilent scanner. The microarray data from scanned images were extracted with Agilent Feature Extraction software version 9.5.3. The signal intensities, retrieved from raw data text file generated from hybridized image file using feature extraction software, were sorted by genus and species of the virus.

Many methodologies have been employed for identifying a virus from microarray data. These include hybridization intensity based on expected and observed intensity [35] or on log odd ratio [7], number of probes giving positive signal, or even percentage of probes giving positive signal [27]. We had adopted percentage of probes giving positive signals for making virus call. This method of making a virus call is easy and has 50 % as cut-off for making a virus call is stringent others have kept it at about 30 % [27]. We had kept it at 50 % based on our earlier observation that the closely related viruses give high cross reactivity. In the earlier version of the chip, we used to get 60–70 % cross hybridization of probes between BHV1 and BHV5 and CAV1 and CAV2. This has been brought down to about 30–35 % with the chip that was used in this study. The background intensity for identifying positive signal was arrived from intensity of random probes and was kept at 99th percentile of random probe signal intensity [7].

PCR Amplification of BHV-1 and BVDV-2

The primer sequences used for amplification of BVD2 and BHV1 genes are listed in Table 1. The amplification for BHV1 gE gene was carried out in 50 µl reaction volume containing 1× Taq DNA Polymerase buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 µM of dNTPs, 4 % DMSO, 1 µl of amplified transcriptome, 2 U Taq Polymerase (Fermentas), and nuclease-free water to make up volume of 50 µl. After initial denaturation at 95 °C for 4 min, the amplification was carried out for 34 cycles each of 95 °C–30 s, 61 °C–45 s, and 72 °C–1 min with final extension of 5 min at 72 °C. The amplification for BVD2 was carried out in 50 µl reaction volume containing 1× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer, 200 µM dNTPs, 1 µl amplified transcriptome,

2 U of Taq DNA Polymerase (Fermentas), and nuclease-free water to make 50 µl. After initial denaturation at 95 °C for 4 min, the amplification was carried out for 34 cycles each of 95 °C–30 s, 50 °C–45 s, and 72 °C–1 min with final extension of 10 min at 72 °C. For nested PCR of BVD2, the procedure was essentially the same except that the template was replaced by 1 µl of primary PCR amplicon and primers of set B (Table 1). The annealing temperature was kept at 50 °C.

Result

Microarray Detection of BHV1 and BVD2

Of the many samples tested in one sample from cow, 62 of the 69 probes of BHV1 and 42 of the 57 probes of BVD2 gave positive signals (Table 2). A virus was called to be present if more than 50 % probes for that virus gave signal above cut-off. According to this criterion, both the viruses were present in this particular sample.

Next virus for which we obtained some positive signal was BHV5 (13 of the 37 probes gave positive signal). BHV1 and BHV5 (formerly BHV1.3) are closely related with more than 90 % sequence similarity, and there is always some cross hybridization. We normally get this much cross hybridization with BHV5 when RNA from BHV1-infected cell culture lysate is hybridized with the chip (personal communication). We did not get any further significant signal for other viruses in this sample (Fig. 1). The average signal intensity of BHV1 probes was consistently high as compared to BVD2 probes average signal intensity (Table 2) suggesting that this was probably not necessarily true, BHV1 was the major infection and BVD2 a minor infection.

Validation of Microarray Result

Whole transcriptome PCR results (Fig. 2, amplified product 613 bp for BHV-1 and 198 bp and 105 bp for BVD1, and BVD2 genotype, respectively) corroborated the microarray data. The unambiguous result in both the tests and clinical history (aborted fetus and fever) of the animal makes it almost a certainty that there was a mixed infection of BHV1 and BVD2 in the animal from which the sample was drawn.

Discussion

BHV1, BVD, *respiratory syncytial virus* (RSV), *bovine parainfluenza virus 3*, *bovine adenovirus*, and *coronavirus* are the viruses found which are associated with the bovine respiratory disease complex [6]. In India, cattle movement is frequent; cattle are moved across long distances under highly stressed conditions; shipping fever is known to exist; and, in most cases, it is bacteria, which are isolated from these cases [30]; the underlying virus cause of

Table 1 Sequence of the primers used in the study

Primer name	Primer sequence (5′–3′)	Gene name	Reference
1. BVDV_Set A/F	GTA GTC GTC AGT GGT TCG	5′ UTR	[11]
2. BVDV_Set A/R	GCC ATG TAC AGC AGA GAT		
3. BVDV_Set B/F	CGA CAC TCC ATT AGT TGA GG		
4. BVDV_Set B/R	GTC CAT AAC GCC ACG AAT AG		
5. Bovine herpesvirus 1_F	GTG/AAT TCC GTC GTT ACT TCG GAC CGT TTG	gE	Accession no.
6. Bovine herpesvirus 1_R	GCT G/TC GAC CAA GCG GAG GAT GGA CTT GAG		AJ004801.1

Table 2 Percentage of probes giving positive signal

Virus sample	Virus detected	Number of probes giving positive signal/total number of probes	Average signal intensities	Percentage of probes giving positive signal	Virus prediction
Clinical sample (Bovine)	BHV-1	62/69	30927	89.8	P
	Bovine viral diarrhea virus 2	42/57	6291	73.6	P
	BHV-5	13/37	7382	35.1	A
	Dengue virus-1	1/69	135	1.4	A
	Classical swine fevervirus	1/63	65	1.5	A

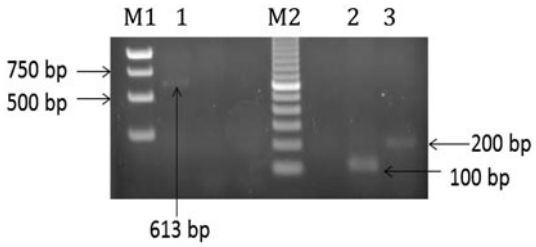

Fig. 1 Agarose gel electrophoresis showing amplification of gE gene of BHV-1 and 5′ UTR region of BVDV from clinical sample. *Lane M1* 1 kb DNA Ladder (Fermentas), *Lane M2* 100 bp DNA Ladder (Invitrogen). *Lane 1* Amplification of 613 bp PCR product of gE gene from WTA–PCR of BHV-1. *Lane 2* Amplification of 100 bp PCR product of 5′ UTR from WTA–PCR of BVDV. *Lane 3* Amplification of 200 bp PCR product of 5′ UTR from WTA–PCR of BVDV

infection remains unstudied. Except for BHV1 and BVD, other viruses involved in bovine respiratory disease complex have not been reported from India though similar viruses from human respiratory cases are well documented [36]. Whether these viruses are really absent from Indian livestock is still an unresolved issue for want of detailed study on their prevalence.

Mixed infection of BVD and other viral diseases have been reported from many places [26, 28], except India. They increase the complexity of the symptoms and economic losses [33]. Among BVD1 and BVD2 genotypes, it is the BVD2 genotype which is more virulent [4]. Among the few clinical samples tested, one sample was positive for BHV1 and BVD2. In India, BVD is still considered as an exotic disease, and handling the virus is confined to a lab with high-security disease though serological evidence [17] indicates otherwise. Large-scale screening of clinical samples may give the actual extent of spread of mixed infections of these viruses. However, based on serological evidences [3, 17, 34], it can be assumed that mixed infection of these viruses could be present on a much larger extent and mostly go unreported due to want of extensive search.

Microarray chips for diagnosis have emerged as highly parallel diagnostic platforms, which that have the ability to detect many known, novel, and emerging pathogenic agents simultaneously. The Virochip developed originally by Palacios is one such chip [25], microbial detection array (MDA) [7] is even more comprehensive chip and has the capability to screen almost all the viruses currently known to exist. Most of microarray chips developed and tested were for human pathogens though they contains probes for animal viruses also. The Virochip was recently used for identifying pathogens in veterinary clinical samples [14, 21]. We had also tested in-house designed chip for testing veterinary clinical samples [35]. Massive multiplexing ability of microarray as compared to other diagnostic techniques is advantageous in identifying multiple infections which may otherwise go unreported. Disadvantage of microarray chip is its high cost which makes its applicability in routine diagnosis highly improbable. Many low-density FDA approved microarrays are now commercially available, e.g., xTag Respiratory Viral Panel (Luminex), Filmarray (Biofire), and TessArray RPM-Flu 3.1 Array (TessArae), ResPlex II v2.0 RVP (Qiagen), they are now used regularly in many clinical microbiology labs. The use of high-density microarrays is limited for want of FDA or other regulatory agencies' approval. Their approval by regulatory agencies required testing against all the pathogens; they are capable of identifying, which is technically not feasible. Current cost of using high-density array is high (200–300 USD). The cost is expected to go down substantially as the methodology gets widely adopted. High-cost microarray can still be used as an adjunct diagnostic method for screening of multiple infections, and in those cases where routine diagnostic methods fail to identify a pathogen from clinical sample.

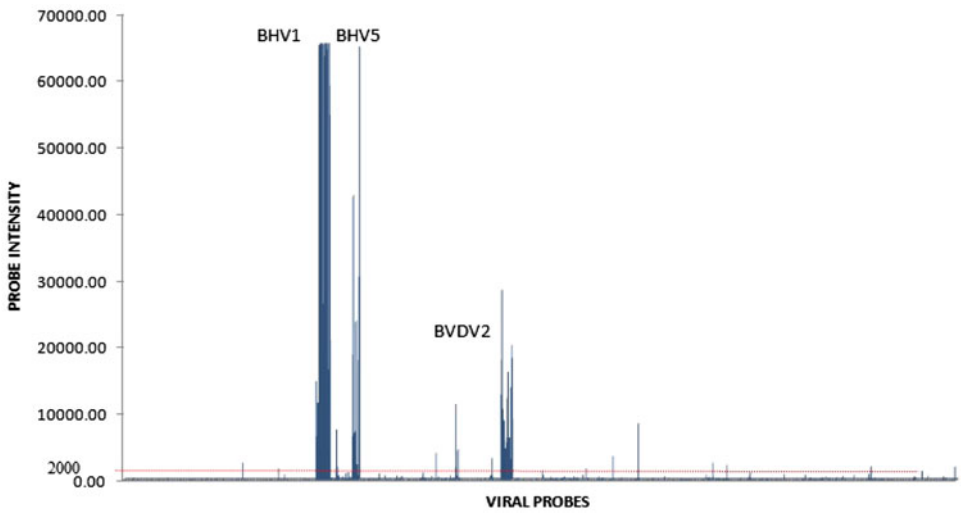

Fig. 2 Pattern of signal intensity observed in microarray chip with clinical sample from cattle

Acknowledgments The authors acknowledge and thank ICAR for funding and Dr. Umesh Dimri for providing clinical samples from institute polyclinic.

References

1. Aly NM, Shehab GG, Abd El-Rahim IHA (2003) Bovine viral diarrhoea, bovine herpesvirus and parainfluenza-3 virus infection in three cattle herds in Egypt in 2000. Rev Sci Tech 22(3):879–892
2. Ames TR (1986) The causative agent of BVD: its epidemiology and pathogenesis. Vet Med 81:848–869
3. Bandyopadhyay S, Chakraborty D, Sarkar T, Pal B, Sasmal D, Biswas TK, Ghosh MK, Sarkar M (2009) A serological survey of antibodies against bovine herpesvirus-1 in yak (*Poephagus grunniens*) in Arunachal Pradesh in India. Rev Sci Tech 28(3): 1045–1050
4. Behera SP, Mishra N, Vilcek S, Rajukumar K, Nema RK, Prakash A, Kalaiyarasu S, Dubey SC (2011) Genetic and antigenic characterization of bovine viral diarrhea virus type 2 isolated from cattle in India. Comp Immunol Microbiol Infect Dis 34:189–196
5. Coria MF, Schmerr MJ, McClurkin AW (1983) Characterization of the major structural proteins of purified bovine viral diarrhea virus. Arch Virol 76:335–339
6. Douglas C, Conlon Jennifer A, Patricia ES (2002) Respiratory viruses and bacteria in cattle. In: Brogden KA, Guthmiller JM (eds) Polymicrobial diseases. ASM Press, Washington DC
7. Gardner SN, Jaing CJ, McLoughlin KS, Slezak TR (2010) A microbial detection array (MDA) for viral and bacterial detection. BMC Genomics 11:668
8. Gibbs EPJ, Rweyemamu MM (1977) Bovine herpesviruses. Part I. Bovine herpesvirus 1. Vet Bull 47:317–343
9. Greig A, Gibson IR, Nettleton PF, Herring JA (1981) Disease outbreak in calves caused by a mixed infection with infectious bovine rhinotracheitis virus and bovine virus diarrhoea virus. Vet Record 108:480
10. Jain L, Kanani AN, Patel TJ, Purohit JH, Jhala MK, Chuahan HC, Chandel BS (2008) Seroprevalence of bovine herpesvirus 1 (BHV-1) in Indian breeding bulls of Gujarat. Buffalo Bull 27:165–169
11. el-Kholy AA, Bolin SR, Ridpath JF, Arab RM, Abou-Zeid AA, Hammam AM, Platt KB (1998) Use of polymerase chain reaction to simultaneously detect and type bovine viral diarrhoea viruses isolated from clinical specimens. Rev Sci Technol 17:733–742
12. Lambot M, Joris E, Douart A, Lyaku J, Letesson JJ, Pastoret PP (1998) Evidence for biotype-specific effects of bovine viral diarrhea virus on biological responses in acutely infected calves. J Gen Virol 79:27–30
13. Lemaire M, Meyer G, Ernst E, Vanherreweghe V, Limbourg B, Pastoret PP, Thiry E (1995) Latent bovineherpesvirus 1 infection in calves protected by colostral immunity. Vet Record 137(3): 70–71
14. Mihindukulasuriya KA, Wu G, Leger J, Nordhausen RW, Wang D (2008) Identification of a novelcoronavirus from a beluga whale by using a panviral microarray. J Virol 82(10):5084–5088
15. Mishra N, Dubey R, Rajukumar K, Tosh C, Tiwari A, Pitale SS, Pradhan HK (2007) Genetic and antigenic characterization of Indian bovine viral diarrhea virus type 2 isolated from Indian goats (*Capra hircus*). Vet Microbiol 124:340–347
16. Mishra N, Pattnaik B, Vilcek S, Patil SS, Jain P, Swamy N, Bhatia S, Pradhan HK (2004) Genetic typing of bovine viral diarrhoea virus isolates from India. Vet Microbiol 104:207–212
17. Mishra N, Dubey R, Galav V, Tosh C, Rajukumar K, Pitale SS, Pradhan HK (2007) Identification of bovine viral diarrhea virus type 1 in Indian buffaloes and their genetic relationship with cattle strains in 5′ UTR. Curr Sci 93(1):97–100
18. Mishra N, Rajukumar K, Tiwari A, Nema RK, Beher SP, Satav JS, Dubey SC (2009) Prevalence of bovine viral diarrhoea virus antibodies among sheep and goats in India. Trop Anim Health Prod 41:1231–1239
19. Mishra N, Rajukumar K, Vilcek S, Tiwari A, Satav JS, Dubey SC (2008) Molecular characterization of bovine viral diarrhea virus type 2 isolate originating from a native Indian sheep (*Ovies aries*). Vet Microbiol 130(1–2):88–98
20. Misra PK, Mishra A (1987) Infectious bovine rhinotracheitis virus infection and infertility in cows, heifers and bulls. Indian J Anim Sci 57(4):267–271
21. Moenning B, Liess V (1995) Pathogenesis of intrauterine infections with bovine viral diarrhea virus. Vet Clin N Am Food Anim Pract 11:477–487
22. Mohan K, Rajasekhar M, Krishnappa G (1994) Isolation of infectious bovine rhinotracheitis virus in Karnataka. Indian vet J 71(2):109–112
23. Nayak BC, Panda SN, Misra DB, Kar BC, Das BC (1982) Note on serological evidence of viral abortion in cattle in Orissa. Indian J Anim Sci 52:102–103
24. Nettleton PF, Entrican G (1995) Ruminant pestiviruses. Br Vet J 151:615–643
25. Palacios G, Quan PL, Jabado OJ et al (2007) Panmicrobial oligonucleotide array for diagnosis of infectious diseases. Emerg Infect Dis 13(1):73–81
26. Potgieter LN, McCracken MD, Hopkins FM, Walker RD (1984) Effect of bovine viral diarrhea virus infection on the distribute on of infectious bovine rhinotracheitis virus in calves. Am J Vet Res 45:687–690
27. Quan PL, Palacios G, Jabado OJ et al (2007) Detection of respiratory viruses and subtype identification of influenza A viruses by GreenChipResp oligonucleotide microarray. J Clin Microbiol 45(8):2359–2364
28. Richer L, Marois P, Lamontagne L (1988) Association of bovine viral diarrhea virus with multiple viral infections in bovine respiratory disease outbreaks. Can Vet J 29:713–717
29. Ridpath JF, Bolin SR (1988) Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. Mol Cell Probes 2:101–106
30. Saxena SP, Hoerlein AB (1966) Studies on shipping fever of cattle. 3. Comparison of *Pasteurella* spp. isolated from shipping fever and other infectious processes. Indian Vet J 43(1):1–8
31. Sharma B, Pokhriyal M, Ratta B, Rai G, Saxena M, Yadav BS, Sen A, Monda B (2012) Isolation of Newcastle disease virus from non-avian host sheep and its implications. Arch Virol 157(8):1565–1567
32. Sood R, Bhatia S, Gounalan S, Patil SS, Pattanaik B (2007) Seroprevalence of bovine viral diarrhoea virus in India: a survey from 1999–2004. Indian J Anim Sci 77:227–229
33. Sudharshana KJ, Suresh KB, Rajasekhar M (1999) Prevalence of bovine viral diarrhoea virus antibodies in India. Rev Sci Tech 18(3):667–671
34. Suresh KB, Sudharshana KJ, Rajasekar M (1999) Seroprevalence of infectious bovine rhinotracheitis in India. Indian Vet J 76(1):5–9
35. Urisman A, Fischer KF, Chiu CY, Kistler AL, Beck S, Wang D, DeRisi JL (2005) E-Predict: a computational strategy for species identification based on observed DNA microarray hybridization patterns. Genome Biol 6(9):R78
36. Yeolekar LR, Damle RG, Kamat AN, Khude MR, Simha V, Pandit AN (2008) Respiratory viruses in acute respiratory tract infection in western India. Indian J Paedtr 75:341–345