Viral diagnosis in Indian livestock using customized microarray chips

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Abstract:
Viral diagnosis in Indian livestock using customized microarray chips is gaining momentum in recent years. Hence, it is possible to design customized microarray chip for viruses infecting livestock in India. Customized microarray chips identified *Bovine herpes virus-1* (BHV-1), *Canine Adeno Virus-1* (CAV-1), and *Canine Parvo Virus-2* (CPV-2) in clinical samples. Microarray identified specific probes were further confirmed using RT-PCR in all clinical and known samples. Therefore, the application of microarray chips during viral disease outbreaks in Indian livestock is possible where conventional methods are unsuitable. It should be noted that customized application requires a detailed cost efficiency calculation.

Keywords: DNA microarray, viral diagnosis, livestock, RT-PCR (reverse transcriptase polymerase chain reaction).

Background:
Microarray is a nucleotide sequence probe based pathogen identification method with massive multiplex ability [1]. Polymerase chain reaction (PCR) and real time PCR are the two sequence based methods which are increasingly used in diagnosis. One of the problems with PCR based method is limited multiplexing capability though mass tag PCR could overcome this difficulty [2]. The meta-genomics incorporated with microarray and sequencing techniques are helping viral detection and characterization [3]. New generation sequencing is another approach for diagnosis but is currently prohibitive to use and is computationally very expensive.

DNA microarrays fall in between PCR and de novo sequencing in both flexibility and cost [4]. The first broad range virus chip developed was used for identification of SARS-corona virus [2, 5]. First chip for detecting a specific animal virus FMDV (foot and mouth disease virus) was reported by Baxi *et al.* [6]. Subsequently, a chip to detect three animal viruses; FMDV, vesicular stomatitis virus and swine vesicular disease virus was developed [7]. A chip for detection of vesicular or vesicular lesion in livestock has also been reported. This chip contained 412 probes for 21 viruses causing vesicular or vesicular like diseases, 177 probes for arbo viruses and 95 probes for avian viruses [8]. Microarray chip was developed to characterize 20 MRSA (*Methicillin Resistance staphylococcus Aureus*) in livestock's in Switzerland [9].

Microarray chips have not been used as diagnostics of viruses in India. We have been working on developing broad range microarray chip for virus diagnosis in Indian livestock. We have earlier reported the identification of Newcastle disease virus in sheep [15] and mixed infection of bovine viral diarrhea virus subtype 2 and bovine herpes virus 1 [17] in cattle. We have also created an oligo-nucleotide probe catalogue for making customized microarray chip for virus diagnosis [11]. One of the biggest disadvantages of this technique is cost and this is prohibitive in Indian circumstance [10]. Therefore, it is of interest to identify viruses causing infection in Indian livestock using virus specific designed microarray chip to screen known and unknown clinical samples.

Methodology:
Virus samples
Bovine herpes virus -1 (BHV-1) grown in Madin Derby Bovine Kidney (MDBK) cell lines, *Canine Adenovirus-1* (CAV-1) grown

in Madin Derby Canine Kidney (MDCK) cells were used. The clinical samples were randomly collected from the Indian Veterinary Research Institute (IVRI) polyclinic; some samples were collected from Centre for Animal Disease Research and Diagnosis (CADRAD), IVRI, Izatnagar.
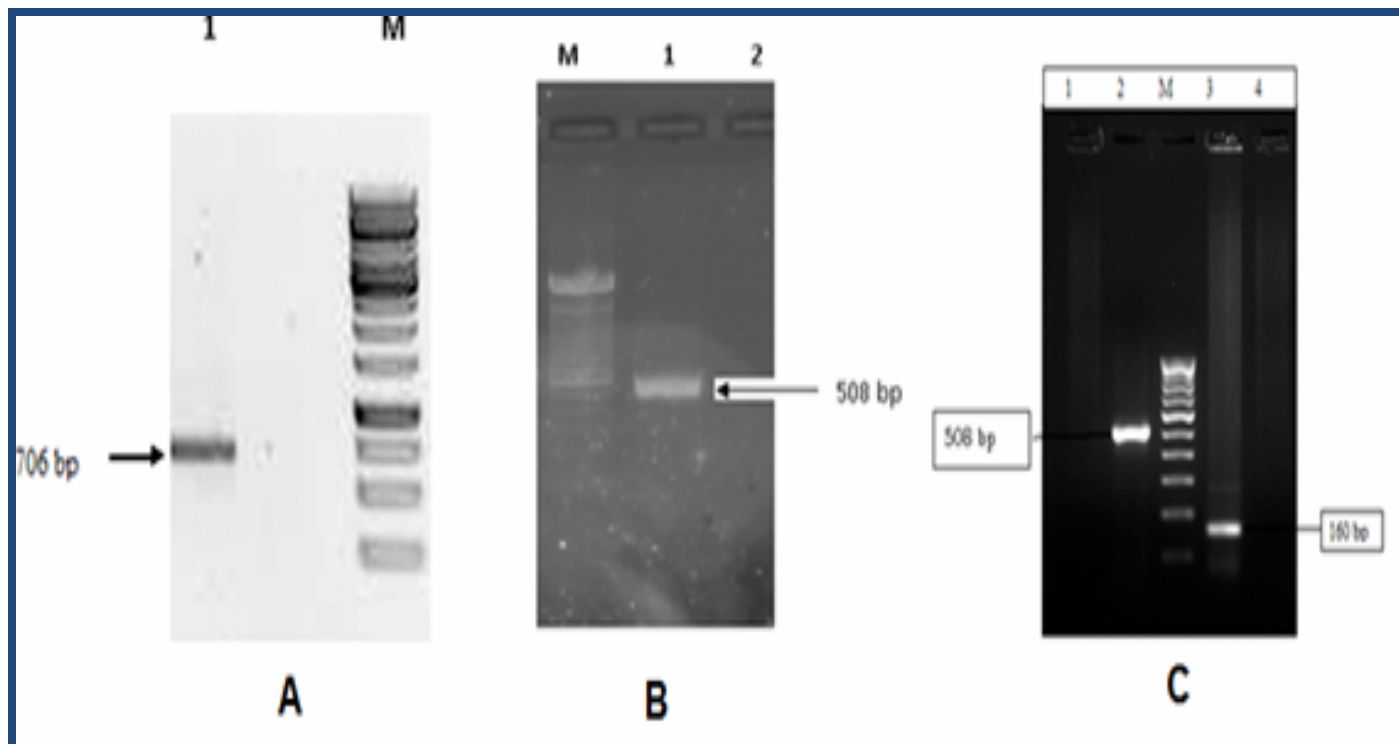
Figure 1: RT-PCR confirmation of positive viruses: **A)** PCR amplification of gE gene of BHV-1 from known BHV1 sample, Lane 1: Amplification of 706 bp PCR product of gE gene, Lane M: 1kb DNA Ladder; **B)** PCR amplification of E3 gene from known CAV-1, Lane M: 100 bp DNA Ladder (Invitrogen), Lane 1: Amplification of 508 bp PCR product of E3 gene from CAV-1, Lane 2: Negative control; **C)** PCR amplification of VP2 gene of CPV-2 from clinical sample 2 and E3 gene of CAV-1 virus from clinical sample 3, Lane M: 100 bp DNA Ladder, Lane 1: Negative control, Lane 2: Amplification of 150 bp WTA-PCR product of VP2 gene from CPV-2, Lane 3: Amplification of 508 bp WTA-PCR product of E3 gene from CAV-1, Lane 4: Negative control.

Microarray probe design
Array designer 4.0 (http://www. premierbiosoft.com) and e-array (https://earray.chem.agilent.com/earray/) probe designing software were used for making both unique and conserved probes. The detailed protocol of designing probe was already published elsewhere **[12].** The microarray chip was custom made using Agilent (Agilent technologies, USA) in 8X15K format.

Sample preparation, labeling, hybridization, scanning and data analysis
The protocol originally developed by Wang *et al* **[2]** was used for sample processing and labeling. This protocol in brief include total RNA extraction with Trizol, conversion to cDNA with a random nonamer anchored primer 5'-GTTTCCCAGTCACGATA-(N9)-3' (Primer A), conversion to ds cDNA using sequenase and PCR amplification of ds cDNA with primer 5'-GTTTCCCAGTCACGATA-3' (Primer B). In the next step PCR is repeated with the same primer but in presence of amino-allayl dNTPs. The product of this reaction is labeled with Cy3 dye in dark and purified. The hybridization was done overnight at 65°C in Agilent hybridization chambers. After washing the chips as per Agilent protocol, the chips were scanned and the signal intensities from scanned images were arranged according to the corresponding genus and species of the virus.

Virus screening with the microarray chip
Presence of a virus in the sample was predicted based on two methods. Average signal intensity was used to predict the presence of a particular virus in one method. Virus was predicted to be present if the signal intensity (1500) was above cut off value. The other prediction parameter used was percentage of probes giving signal above cut off value. Virus was predicted to be present if 40% or more probes of a virus show signal above cut off. This method is more reliable as compared to average signal intensity method.

Result validation
The RNA extracted from the clinical samples which were positive for a virus was amplified by whole transcriptome amplification kit as per manufacture's recommendation (QuantiTect whole transcriptome kit, Qiagen). PCR was done with the primers reported in literature.

Results & Discussion:
The use of microarray chip for virus identification require processing the clinical samples in such a way that sufficient quality and quantity of target nucleic acid is obtained for hybridization. We used the protocol described by Wang *et al* (2003) for RNA processing **[2].** The microarray chip used contained 4079 viruses specific probes for 124 viruses and their subtypes and 3,988 conserved probes for 146 viral genera and

BIOINFORMATION

3000 random probes. A database of microarray probes was also created and made available online for developing custom designed microarray probes **[11].** The length of probes (60 mer) was similar to Palacios *et al.* (2007) **[12]** and Gardner *et al.* (2010) **[13].** The Tm was kept at 70°C ±5. This is because the GC content of virus varies from less than 40% **[14]** in some viruses to more than 60% (herpes viruses). The number of probes for each virus species was minimum 10 or more (average probes per virus is 32). This is larger than the probe number in Green e-chip (average less than 5 probes per virus) **[15, 16].**

The microarray data analysis based on average and percent of probe signal intensity predicted cell culture adapted viruses **Table 1 (see supplementary material).** In one clinical sample (bovine nasal swab), microarray chip screening predicted presence of two viruses [BHV1 and bovine viral diarrhea 2 (BVD2)] **(Table 1)**. This is the first such report of mixed infection of BHV1 and BVD2 from India **[17].** In one sample canine parvovirus 2 was identified and in another canine adeno virus-1 was identified **(Table 1).** The rests of the clinical samples were negative for viruses. All the viruses were subsequently confirmed to be present in the microarray chip by RT-PCR **Figure 1 (A, B & C).**

Many microarray based diagnostic chips have been used for identifying viruses during the last decade. This is an expanding field where many groups are engaged in developing microarray chips. Each group has developed their own strategy **[2, 5, 13, 14, & 18]** and their approaches may be distinguished according to the range of pathogens targeted, the probe design strategy, and the array platform used. The latest and the most comprehensive chip recently reported is called microbial detection array **[13].** We report a comprehensive chip for the identification of animal viruses reported from India after testing the chip with clinical samples from healthy and diseased animals and removing all cross-reacting probes.

Conclusion:
We have designed and tested a microarray chip for the identification of viruses causing diseases in Indian livestock. The chip contains unique and conserved probes for the identification of specific and genera specific viruses. The chip was tested against known viruses and clinical samples including screening of blind clinical samples. The viruses identified by the chip were confirmed subsequently by RT-PCR.

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Supplementary material:

Table 1: Average signal intensity and percent of probes giving positive signal in samples

Virus sample	Virus detected	Average signal intensity	Virus prediction	(%) Positive probes$	Virus prediction
Known BHV-1	BHV-1	14539	P	68/069 = 98.8%	P
Known CAV-1	CAV-1	29337	P	60/090 = 66.6%	P
Clinical sample 1	BHV-1	33927	P	62/069 = 89.8%	P
	BVDV-2	6181	P	42/057 = 73.6%	P
Clinical sample 2	CPV- 2	7432	P	09/010 = 90.0%	P
Clinical sample 3	CAV- 1	7028	P	43/090 = 47.6%	P
Clinical sample 4	BHV- 4	1159	A	32/286 = 11.0%	A
Clinical sample 5	BPV-1	629	A	05/049 = 10.0%	A
Clinical sample 5	BHV- 2	177	A	05/100 = 05.0%	A

(%) Positive probes$ = Number of positive probes/Number of probes

BHV-1 = bovine herpes virus-1; CAV-1 = canine adeno virus-1; CPV-2 = canine parvo virus-2; BVDV-2 = bovine viral diarrhea-2