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Ability to differentiate between cp and ncp BVDV by microarrays: Towards an application in clinical veterinary medicine?

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Abstract

Microarray expression profiling provides a comprehensive portrait of the transcriptional world enabling us to view the organism as a 'system' that is more than the sum of its parts. The vigilance of cells to environmental change, the alacrity of the transcriptional response, the short half-life of cellular mRNA and the genome-scale nature of the investigation collectively explain the power of this method. These same features pose the most significant experimental design and execution issues which, unless surmounted, predictably generate a distorted image of the transcriptome. Conversely, the expression profile of a properly conceived and conducted microarray experiment can be used for hypothesis testing: disclosure of the metabolic and biosynthetic pathways that underlie adaptation of the organism to infectious processes; the identification of co-ordinately regulated genes; the regulatory circuits and signal transduction systems that mediate the adaptive response; and temporal features of developmental programmes. The study of viral pathogenesis by microarray expression profiling poses special challenges and opportunities. Although the technical hurdles are many, obtaining expression profiles of an organism growing in tissue will probably reveal strategies for growth and survival of the virus in the host's cells. Here, we show data obtained using a tailored microarray system based on synthetic polynucleotides derived from human sequences (SIRS-Lab GmbH, Jena, Germany) to study the effect of cytopathogenic (cpe) and non-cytopathogenic (ncp) bovine viral diarrhoea virus (BVDV) infection of bovine macrophages, focusing on intracellular signalling molecules. Of the 575 genes present on the array, more than 70% showed a reaction with the oligonuleotides spotted on the array, and 26 genes were differentially expressed comparing cDNA derived from cpe and ncp infected cells. These data will help to further understand our knowledge regarding BVDV infection, and will especially help to understand differences in cellular responses to cpe and ncp biotypes.

Keywords: Microarray; Innate immunity; TLR; BVDV; Bovine

1. Introduction

The pestiviruses – bovine viral diarrhoea virus (BVDV), classical swine fever virus and border disease virus of sheep, together with the flaviviruses

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and hepatitis C virus – are a closely related group of small enveloped viruses, the *Flaviviridae*, with a single-stranded, positive-sense RNA genome of approximately 12.5 kb. The RNA is translated into a single virus polyprotein that is processed by both viral and host proteases to either 11 or 12 virus polypeptides dependent on the virus biotype. The viruses are generally non-cytopathogenic (ncp), although BVDV associated with the development of mucosal disease in persistently infected (PI) animals is a cytopathogenic (cp) biotype, which produces an additional virus polypeptide, NS3, which represents the C-terminal two-thirds of NS2-3 present in ncp BVDV strains (Donis and Dubovi, 1987).

Several groups have recently attributed the persistent infection to the ability of ncp BVDV to interfere with the induction of interferon (IFN) type I production in macrophages (MΦ) (Baigent et al., 2002, 2004; Charleston et al., 2001; Schweizer and Peterhans, 2001). The lack of IFN induction observed with ncp BVDVs and the failure to induce apoptosis might have advantages for the survival of the virus (Schweizer and Peterhans, 1999, 2001). The induction of apoptosis by cp BVDV appears to be an active process of induction since cells infected with ncp BVDV and subsequently infected with cp BVDV still undergo apoptosis (Zhang et al., 1996); these results indicate that ncp BVDV does not inhibit apoptosis. The mechanism of induction of apoptosis by cp BVDV is controversial, but it has been suggested that the accumulation of viral RNA in cells infected by cp BVDV could activate the double-stranded RNA-activated protein kinase (PKR) and initiate apoptosis (Vassilev and Donis, 2000). However, more recent studies on the cp strain NADL have demonstrated that apoptosis induced by cp BVDV can be mediated through endoplasmic reticulum (ER) stress, but the initiating trigger of the apoptotic pathway is not clear (Jordan et al., 2002).

The observation that ncp BVDV specifically interferes with the induction of IFN type I by inhibiting the transcription factors IFN regulatory transcription factor (IRF) 3 and 7 may be relevant in view of the role an innate immune system plays in the establishment of the adaptive immune response, and understanding these differences may help to identify genes specifically activated by ncp or cp BVDV, which can be used as diagnostic markers. Both IRF3 and IRF7 have been shown to be involved in the signalling cascade activated by a specific group of pattern recognition receptors, the Toll-like receptors (TLR). Whereas most TLR seem to be expressed on the cellular surface, TLRs involved in the generation of the innate immune response to viruses, such as TLR3, TLR7 and TLR8 are present within vesicles in the cytoplasm or even free in the cytoplasm. Activation of these TLRs by dsRNA, which is unavoidably formed during the replication of DNA and RNA viruses, is a key trigger of IFN production in virtually all cells and leads to transcriptional activation of IFNβ gene(s) via NF-κB and other mechanisms (Fig. 1).

Dissection of the activation of the TLR/IFN type I pathway becomes increasingly complex with more and more signalling molecules being described to be involved. Despite the fact that intracellular molecules share a high cross-species homology, current reagents to receptors and adaptor molecules do not seem to cross-react convincingly (Werling and Jungi, unpublished observation). However, these investigations may be helped by the development of bovine-specific microarrays, which are currently already in use (Aho et al., 2003; Coussens et al., 2002, 2003; Yao et al., 2001). Despite the undoubted beneficial use of such large arrays for research, their analysis requires extensive bioinformatics input and is often very time-consuming. We therefore investigated the applicability of utilising a commercially available "tailored" microarray, containing 547 probes for human genes involved in TLR signalling, type I IFN production and NF-κB signalling to dissect differences in cellular activation induced by cells exposed to either cp or ncp BVDV. Identifying genes uniquely responding to either virus exposure could lead to the development of new diagnostic tools.

2. Materials and methods

2.1. Animals and cell culture

Blood was collected from Holstein Friesian cattle. For generation of monocyte-derived macrophages (MΦ), PBMC isolated by an adapted Ficoll-metrizomate procedure were sealed in Teflon bags (10–20 ml, 4×10^6 PBMC ml^{-1}) as described previously (Werling et al., 2004), and cultured for 6–8 days at 37 °C in

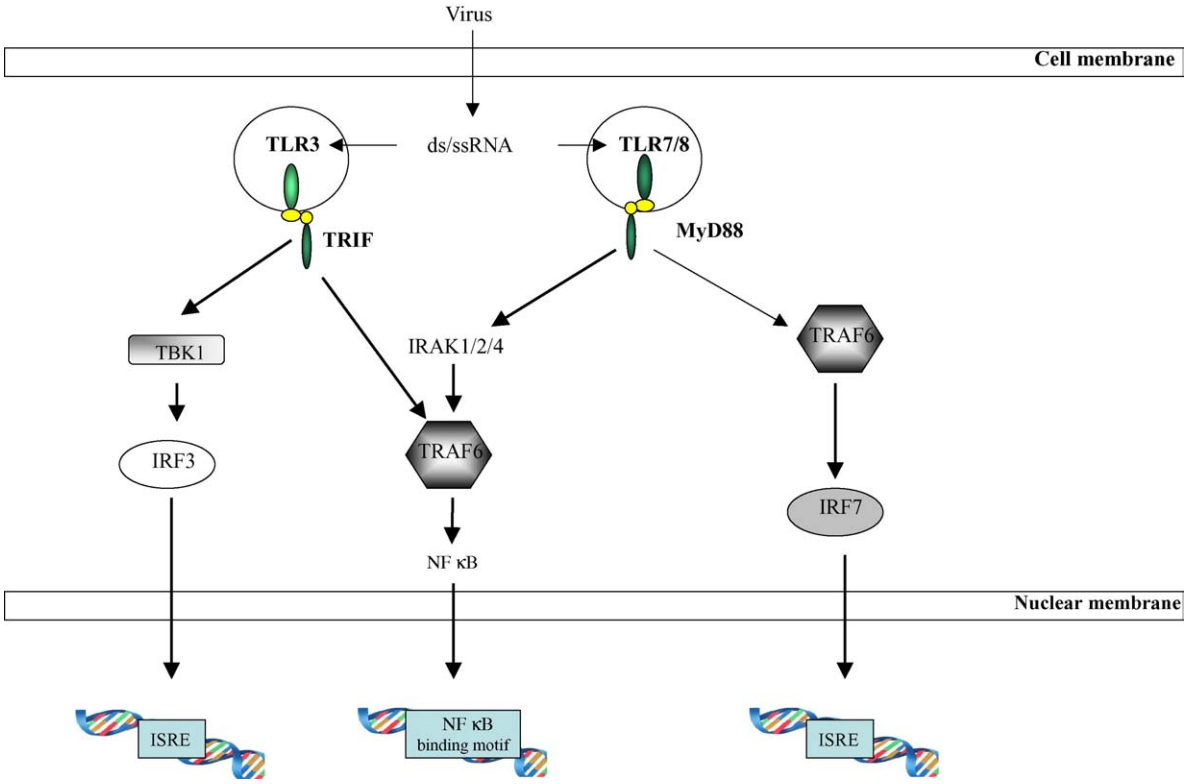

Fig. 1. Activation of IRF3 and IRF7 by double-stranded (ds) and single-stranded (ss) RNA. Replication of viruses within the cells leads to activation of TLR3 and TLR7/8 by either ds or ssRNA. Subsequently, binding of dsRNA to TLR3 stimulates either NF-κB activation via TRAF6, or activation of IRF3 via TBK, leading to the activation of interferon-stimulated response elements (ISRE). In contrast, binding of ssRNA to TLR7/8 can activate either NF-κB-depending gene transcription or, via IRF7, activation of ISRE. IRAK: IL-1-receptor-associated kinase; IRF: IFN regulatory factor; NF-κB: nuclear factor-κB; TIR: Toll/IL-1 receptor; TLR: Toll-like receptor; TRAF: TNF-receptor-associated factor; TRIF: TIR-domain-containing adaptor protein inducing IFNα; TBK1, TANK-binding kinase 1; ISRE: IFN-stimulated response element.

a humidified atmosphere of 5% CO_2. The medium was RPMI 1640 containing 10 mM HEPES (pH 7.4), 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 1% (v/v) nonessential amino acids for MEM (Invitrogen), 0.4% (v/v) vitamin solution for MEM (Invitrogen), 2 μM glutamine (Invitrogen), 40 μg ml^{-1} folic acid, 1 mM sodium pyruvate (Invitrogen), 2.5 μM amphotericin B (Invitrogen) and 15% heat-inactivated FCS (Invitrogen). During this time, monocytes had matured to non-activated MΦ, which optimally responded to LPS by NO generation and TNF production (Jungi et al., 1996). From the cell mixture of variable composition, MΦ were purified by selective adherence to microtitre plate wells for 3 h. After washing, T-cell contamination was estimated to be 1–2%, based on immunocytochemical analysis (unpublished observation), with a viability of > 98%.

2.2. Cultivation of bovine viral diarrhoea virus

Both the cp and ncp PEC 515 isolates of BVDV were grown in primary foetal bovine lung cells (FBLs) cultured in DMEM (Invitrogen) containing 1% antibiotic/antimycotic (Gibco) and 2% FCS (PAA, Austria). Serial dilutions of each virus were cultured on FBLs for five days before the virus titre ($TCID_{50}$) was determined either by microscopic examination for cp effect or the presence of intracellular virus was determined using the immunoperoxidase assay on fixed cells (ncp). In brief, cells were acetone fixed and labelled with an anti-BVDV hyperimmune serum

V182 (Brownlie, personal communication) washed and counter-labelled with an HRP-conjugated anti-bovine secondary antibody (Sigma). The presence of virus was then visualised with AEC colour substrate (Sigma) under a light microscope. In both cases the virus titre was calculated using the formula from Spearman and Karber (Villegas, 1998).

2.3. RNA isolation

MΦ were infected with cp and ncp PEC515 at a MOI of 10:1 for 6 h. Thereafter, cells were washed once with cold PBS, before lysis of the cells. Total RNA was isolated from infected cells after 6 h of incubation using the total RNA extraction kit (Qiagen), followed by DNAseI treatment (Ambion). The RNA was analysed on a 1% agarose denaturing gel to assess quality and potential degradation. Before cDNA synthesis, the total RNA was spiked with *Arabidopsis thaliana* mRNA transcripts (ara1).

2.4. RNA labeling

Seventeen micrograms of total RNA samples isolated from both infected and control cells were converted into first-strand cDNA synthesis mix using SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) and oligo(dT)18 primer, substituting a fraction of dTTP in the newly synthesized strands with aminoallyl-dUTP (AA-dUTP). Conversion of formed RNA/cDNA duplexes into single-stranded cDNA was performed via RNA alkaline hydrolysis. For the first two experiments, cDNA resulting from total RNA obtained from the infected cells was labelled with AlexaFluor 647 monofunctional NHS-ester and cDNA from control cells was labelled with AlexaFluor 555 monofunctional NHS-ester (Invitrogen, Carlsbad, CA) by chemical coupling to incorporated AA-dUTP. The next two experiments represented ‘‘dye-swap’’ hybridizations and, thus, the reversed labelling pattern for above RNA was used.

2.5. DNA microarrays

Combined arrays (Lab-Arraytor® human 60 1–6) fabricated for internal R&D purposes of SIRS-Lab GmbH were used in this study (http://www.sirs-lab.de/cgi-bin/site.pl?scr=0&site=content/en/produkte/produkte). They represent a platform of spotted arrays and consist of spots of single-stranded 56–70 bases long polynucleotide probes interrogating transcription of human genes involved in inflammation, TLR signaling, apoptosis, cell cycle control and related processes. The entire array is composed of three identical separate arrays, each consisting of 705 probe spots distributed over four subarrays. 593 of them carry probes for human genes and 112 represent positive, negative and spiking control elements. These comprehensive controls aided in obtaining a quality control measurement for each experimental step, starting with cDNA generation to post-hybridization washing steps. Thus, the entire array consists of a total of 2115 polynucleotide probe spots. A GAL-file describing the array layout was generated by the OmniGrid software which controls the GeneMachines spotter and is used by the array analysis software GenePixPro 4.0 (Axon Instruments, Foster City, CA).

2.6. Microarray hybridization

Co-hybridization of AlexaFluor 647- and AlexaFluor 555-labelled cDNAs to combined microarrays was performed for 14 h at 42 °C in formamide-based hybridization buffer using the HS 400 Hybridization Station (Tecan Group Ltd., Switzerland). After completion, arrays proceeded through washing and drying steps, and immediate fluorescence scanning on an Axon GenePix4000B scanner (Axon Instruments, Foster City, CA).

2.7. Microarray data pre-processing

Hybridization signal intensities were measured using the GenePix Pro 4.0 software (Axon Instruments). Data pre-processing included spot detection and background subtraction, spot flagging according to defined signal-to-noise threshold values, normalization and transformation of the signals obtained from both laser channels, and averaging of data from the three array replicates and determination of thresholds. For the first two steps, the GenePix Pro 4.0 Analysis Software was used. For the third step, the approach from Huber et al. (2003) was applied including the variance-stabilised transformation. Thresholds of differentially expressed genes were determined from the interval spread of the signal ratios

from the red versus green channel of positive control spots.

2.8. RT-PCR and real-time PCR for bovine TLR

To validate results obtained using this human array, we analysed expression of TLR2 and TLR4 by quantitative PCR. The relative amount of TLR transcribed by MΦ exposed to cp or ncp BVDV was assessed using the TaqMan™ real-time PCR technology as described recently (Werling et al., 2004). Briefly, total RNA was extracted from lysed cells using the RNeasy mini kit (Qiagen) and treated with RNase-free DNase I (Amersham Pharmacia Biotech, Uppsala, Sweden) to remove contaminating genomic DNA. cDNA was synthesized using SuperScript II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA) in a reaction volume of 30 μl on a MWG (Applied Biosystems, Foster City, CA). The primers and TaqMan probes were designed as described (Werling et al., 2004). TaqMan PCR for the 18S ribosomal RNA control (Applied Biosystems) and bovine TLR were run as multiplex PCR in the same well and calculated using the comparative C_T method (User Manual 2, Applied Biosystems). The PCR reactions contained 300 nM of each primer, 200 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems), 1.25 μl of the 18S RNA control, and 2.5 μl of the diluted cDNA sample in a total volume of 25 μl. The samples were placed in 96-well plates and amplified in an automated fluorimeter (ABI Prism 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

3. Results

Despite the fact that the array consisted of polynucleotides derived from human gene sequences, more than 70% of the genes represented on the array hybridized with cDNA derived from bovine MΦ. Subsequent analysis showed that a set of kinases responding to a number of mitogenic, metabolic stimuli and stress (MAP kinases, transcription factor Jun) as well as molecules involved in apoptosis and the innate immune response (TLR family members) were upregulated in cp BVD-infected MΦ compared to ncp infected-MΦ (Table 1). In addition, this set included a range of cytokines, chemokines, their receptors and regulators (Table 1). It is noteworthy that IFNA7 and IFNAR1 were among them. The latter forms one of the two chains of a receptor for IFNα/β. Binding and activation of this receptor stimulates Janus protein kinases (JNK), which in turn phosphorylate several proteins, including STAT1 and STAT2 which were also found to be upregulated. Another plausible result concerned upregulation of IRF3 which is known to form a complex with CREBBP upon serine/threonine phosphorylation followed by translocation of the formed complex to the nucleus and activation of the

Table 1
Microarray analysis of genes differentially expressed in bovine macrophages incubated with either cp or ncp BVDV strain PEC ($n = 2$)

	Gene	Changes in expression
Environmental stress	JUN	2.23
	MAP3K1	1.96
	MAPK11	1.42
	MAPK14	1.31
Apoptosis	BCL2	3.21
	CASP4	2.86
	STAT1-2	2.41
	MAP2K6	1.97
	CASP1	1.89
	BAK1	1.25
Cytokines/Chemokines, their receptors and regulatory factors	CXCL4	6.03
	CCL4	2.42
	IFNA7	2.00
	IRF3	1.86
	IFNAR1	1.59
	IL7	1.47
	IL17BR	1.34
	IL17C	1.33
TLR pathway	TLR4	2.70
	TLR2	2.42
	TLR7	2.28
	TLR3	1.98
	MYD88	1.29
Complement	C6	1.39
	C1QR1	1.27
Cell cycle	CDKN1A	1.28

Expression changes are given as increased expression of cp versus ncp-BVDV infected macrophages.

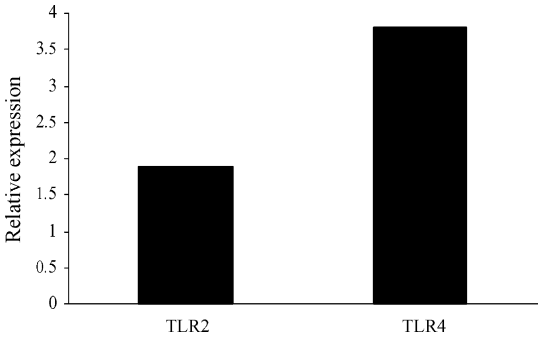

Fig. 2. Presence of mRNA transcripts for TLR2 and TLR4 in bovine macrophages either infected with cp or ncp BVDV. Bovine MΦ were incubated with cp or ncp BVDV strain PEC for 6 h, and presence of mRNA transcripts analysed by quantitative PCR as described. For both, TLR2 and TLR4, cp infected MΦ seem to contain two to four times more transcripts compared to cDNA generated from ncp-infected MΦ. Data are expressed as relative differences, with values obtained for ncp BVDV samples set as 0 ($n = 1$).

transcription of interferons alpha and beta, as well as other interferon-induced genes.

3.1. Verification of microarray data by quantitative PCR

Quantitative PCR was used to verify differential expression of TLR2 and TLR4 observed via microarray analysis. qPCR performed with the same cDNA pair which was used for microarray experiments showed that cDNA derived from cp infected MΦ contained about two and three times more transcripts for TLR2 and TLR4, respectively (Fig. 2).

4. Discussion

The aim of this study was to identify potential gene candidates that would help to identify and discriminate between cells of animals infected with either ncp (possible PI animals) or cp BVDV, which may help in establishing microarrays as a new tool for diagnostics. However, it became evident, that microarrays, even such with limited gene numbers (‘‘tailored’’ arrays) will at the current stage not be suitable as diagnostic tools. Despite the fact that the ‘‘tailored’’ microarray containing polynucleotides derived from human genome sequences used in the present study showed a high degree of cross-reactivity with bovine cDNA (70% of the spotted polynucleotides reacted) and identified some genes upregulated in MΦ infected with cp BVDV, none of these candidates showed such a significant and corroborative differential expression which could be used as a diagnostic marker under the present culture conditions (CXCL4, or platelet factor 4, awaits sequence annotation, primer design and quantitative PCR proof).

Our study identified several genes involved in stimulating the innate immune response that were differentially regulated in cp BVDV-infected but not in ncp BVDV-infected MΦ, such as TLR2 and TLR4. While it is difficult to understand why MΦ exposed to different forms of a virus should differentially regulate expression of TLRs, which are mainly involved in anti-bacterial defence, our approach clearly highlights the ability of microarray technology to differentiate between such infection. To date, no explanation can be given for this observation in our BVDV model. However, it is worth mentioning that HCV core and NS3 protein are known to trigger TLR2-mediated pathways (Dolganiuc et al., 2004). Whereas cellular uptake was TLR2-independent, the HCV core protein co-localised intracellular with TLR2 leading to subsequent activation of IRAK, phosporylation of p38 MAPK, ERK, JNK and subsequently AP-1 and NF-κB. Similarly, mRNA expression of several TLRs, including TLR2, was upregulated during infection of MΦ with influenza A and Sendai virus. This upregulation was reversible by incubating the cells with neutralizing anti-IFN type I antibodies (Miettinen et al., 2001). These results clearly indicate a common IFN/TLR pathway during viral infection, with IRF3 being the potential link between a viral and bacterial TLR activation pathway (Doyle et al., 2002).

Our data suggest that infection of MΦ with cp BVDV lead to differences in the expression of TLR7, JUN and IRF3 compared to MΦ infected with ncp BVDV. Previous work has shown that ncp BVDV infections fail to induce NF-κB activation and although IRF3 is translocated from the cytoplasm to the nucleus, this IRF3 is actively prevented from binding DNA (Baigent et al., 2002). Interestingly, recent work on HCV has suggested that the HCV NS3 may play a role in the inhibition of IRF3 (Foy et al., 2003). Thus, other IRF family members may co-operate with NF-κB to trigger IFNβ induction by cp BVDV (see Fig. 1 for scheme). However, neither cp nor ncp BVDV were

shown to interact with IRF7, a transcription factor involved in TLR7 signalling, or other transcription factors such as ATF2. However, some c-Jun phosphorylation was observed during cp BVDV infections (Baigent et al., 2004). This phosphorylation could be based on ER stress, which has been associated with c-Jun phosphorylation (Harding et al., 2002; Urano et al., 2000), since cp BVDV induces the ER stress responses (Jordan et al., 2002).

As one feature of cp BVDV infection is the induction of apoptosis, differences in the expression of some proteins involved in apoptotic processes in cDNA derived from MΦ infected with cp BVDV were observed (Table 1). Ncp BVDV has been shown to inhibit apoptosis in a very unique manner which seems to be specific for dsRNA, but not to cell death induced by staurosporine or actinomycin D (Schweizer and Peterhans, 2001). A number of reports suggest that triggering and execution of virus-induced apoptosis and of IFN synthesis may have common pathways, with IFNα/α being essential mediators or to potentiate apoptotic cell death in virus-infected cells (Balachandran et al., 2000; Tanaka et al., 1998). The core protein of HCV, a flavivirus closely related to BVDV in its genome structure, has been reported to be immunosuppressive and to inhibit apoptosis, but the results are contradictory (Dumoulin et al., 1999; Large et al., 1999).

Despite the single time-point analysed and the limited verification of microarray data due to unavailable sequence annotations, the results suggest that the use of arrays representing only a limited set of genes involved in a very defined pathway may be of better use than microarrays currently available. This targeted approach allows the user to focus on individual (signalling) pathways and may help to identify potential markers for diagnostic use. Tailored arrays offer the potential to be used as diagnostic tools to discriminate between infections with both a broad range of micro-organisms or with closely related micro-organisms such as cp and ncp viruses.

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