Comprehensive Detection and Identification of Seven Animal Coronaviruses and Human Respiratory Coronavirus 229E with a Microarray Hybridization Assay

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Coronavirus \cdot Microarray hybridization \cdot Coronavirus detection

Abstract
Based on microarray hybridization, a diagnostic test for coronavirus infection was developed using eight coronavirus strains: canine coronavirus (CCoV), feline infectious peritonitis virus (FIPV), feline coronavirus (FCoV), bovine coronavirus (BCoV), porcine respiratory coronavirus (PRCoV), turkey enteritis coronavirus (TCoV), transmissible gastroenteritis virus (TGEV), and human respiratory coronavirus (HRCoV). Up to 104 cDNA clones of eight viruses were obtained by reverse transcription PCR with different pairs of primers designed for each virus and a pair of universal primers designed for the RNA polymerase gene of coronavirus. Total RNAs extracted from virus were reverse transcribed, followed by multi-PCR amplification and labeled with Cy3-dCTP. All labeled cDNAs and prepared gene chips were subjected to specific hybridization. The results showed that extensive cross-reaction existed between CCoV, FCoV, FIPV, TGEV and PRCoV, while there was no cross-reaction between BCoV, TCoV and HRCoV. The ultimate specific gene chip was developed with DNA fragments reamplified from the chosen recombinant plasmids without cross-reaction between different coronaviruses.

The hybridization results showed that this gene chip could specifically identify and distinguish the eight coronaviruses and the sensitivity of the chip may be 1,000 \times more sensitive than PCR, indicating that it can be used for the diagnosis of eight coronavirus infections at the same time.

Introduction
Coronaviruses infect a number of different vertebrate species and cause economically important diseases in people, pets, livestock and poultry [1]. Especially with the explosion of SARS in 2003 in several countries, coronavirus research has been becoming more ravishing [2, 3]. These coronaviruses are classified into three groups. One tightly clustered subgroup based on phylogenetic analysis and antigenic cross-reactivity contains canine coronavirus (CCoV), transmissible gastroenteritis virus (TGEV) and human respiratory coronavirus (HRCoV). In fact, all of these viruses are antigenically so similar that they may be regarded as ‘host range mutants’ rather than as separate species [6–9]. Coronaviruses are positive-stranded RNA viruses with exceptionally large genome sizes (up to 31 kb). Reverse transcription-PCR (RT-PCR) is widely used for virus identification [10–12]. However, PCR cannot...
distinguish several viruses at the same time from one sample and a positive amplification can be verified only by subsequent assays to elaborate sequence information. By overcoming this limitation, microarrays and biosensors have become valuable tools for viral discovery, detection, and genotyping [13–16]. Microarrays that contain several thousand different DNA sequences (probes) can theoretically identify several thousand different organisms at one time and exhibit more sensitivity and specificity than those tests which employ a single target sequence. However, the high conservation of the coronaviruses represents a design challenge and a main hindrance for the identification. It is only when coronavirus microarrays can provide information for a wide range of viral strains and have no cross-reaction between different coronaviruses that they are then usable and practical for coronavirus detection.

For this purpose, one coronavirus gene chip was developed by using 104 cDNA clones obtained by RT-PCR technique with different primer pairs (4–15 pairs) designed for TCoV, CCoV, FCoV, FIPV, TGEV, PRCoV, BCoV and HCoV, and a pair of universal primers designed for the RNA polymerase gene of coronavirus. Through two times specific hybridization, cross-reaction clones were deserted and DNA fragments reamplified from recombinant plasmids without cross-reaction between different coronaviruses were chosen to produce the ultimate specific gene chip.

**Materials and Methods**

**Virus and Cells**

Canine coronavirus (TN449 strain, ATCC No. VR-2068), feline infectious peritonitis virus (WSU 79-1146 strain, ATCC No. VR-2128) and feline coronavirus WSU79-1683 strain, ATCC No. VR-989) were propagated in a monolayer of A72 cells. Porcine respiratory coronavirus (AR310 strain, ATCC No. VR-2384) and transmissible gastroenteritis virus (Purdue strain, kept in our laboratory) were propagated in a monolayer of ST cells. Bovine coronavirus (unknown strain, ATCC No. VR-874) was propagated in a monolayer of MDBK cells. Human coronavirus (229E strain, ATCC No. VR-740) was propagated in a monolayer of MRC-5 cells. Turkey enteritis coronavirus (Minnesota strain, ATCC No. VR-911) was an original stock virus. Above eight viruses and cells were all initially obtained from American Type Culture Collection (ATCC).

**Primers**

Primers for RT-PCR were selected using Primer Designer (versions 1.01 and 2.01, Scientific and Educational Software, Durham, N.C., USA). Different (4–15) pairs of primers designed for each virus and a pair of universal primers designed for the RNA polymerase gene of coronavirus were used to amplify TGEV, PRCoV, CCoV, FCoV, FIPV, BCoV, TCoV and HCoV. Sequence analysis and alignments were done with GeneWorks version 2.5.1 (Intelligenetics, Mountain View, Calif., USA). The names and sequences of primers for all viruses used have been listed in table 1.

**Construction of cDNA Clones of Eight Viruses**

All 104 clones were produced by our laboratory. Escherichia coli TGI was offered by Shanghai Veterinary Institute of CAAS and pGEM-T-Easy vector bought from Promega Co. Viral sequence data were obtained from the GenBank database. A pair of universal primers designed for the RNA polymerase gene of coronavirus and different (4–15) pairs of primers designed for each virus were used. Primers were selected to be exclusive to a given virus, as judged by pairwise BLASTN search. The total RNAs were extracted with a QIAamp Viral RNA Mini Kit (Qiagen, Germany) from TCoV stock virus and CCoV, FCoV, FIPV, TGEV, PRCoV, BCoV and HRCoV purified by sucrose density gradient centrifugation were reverse transcribed (M-MLV Rtase cDNA synthesis kit, Takara Biotechnology (Dalian) Co. Ltd, China) and PCR-amplified with the above primers. The PCR products were purified and then linked with pGEM-T-Easy vector and transfected into E. coli TGI [17].

**Amplification and Recovery of Probe Fragments**

The PCR system was used to amplify probe fragments. The reactions were carried out in a final volume of 100 µl containing 2 µl of each primer (10 pmol), 10× buffer 10 µl, dNTP 8 µl, Taq plus 2 µl, dH2O 71 µl and 5 µl plasmid template prepared by boiling lysis from different positive clones store at −70°C. The amplification reaction was carried out in a DNA Thermal Cycler (PerkinElmer Cetus, USA) for 30 cycles after 94°C for 5 min with denaturation at 94°C for 45 s, annealing at 52°C for 45 s and polymerization at 72°C for 60 s. A final extension at 72°C for 10 min was carried out before holding the samples at 4°C. The amplification reaction of universal primers designed for the RNA polymerase gene was 5 cycles after 94°C for 5 min with denaturation at 94°C for 45 s, annealing at 40°C for 45 s and polymerization at 72°C for 60 s, then 30 cycles with denaturation at 94°C for 45 s, annealing at 50°C for 60 s, polymerization at 72°C for 60 s and a final extension at 72°C for 10 min. The amplified product was recovered using PCR Kleen™ spin columns (Bio-Rad Laboratories, Inc., USA). The PCR product was cleaned with a Qiagen QIAquick PCR purification kit. These fragments were designed to have similar annealing stabilities.

**Preparation of Probes and Spotting on Chips**

The concentrations of the above probe fragments were determined by OD260 and OD280 then dried and suspended in 300 ng/µl with 50% DMSO. Spotting was completed using a Bio-Rad printer on slides coated with amino saline. The slides were UV cross-linked at 60 mJ for 25 min and baked for 2 h at 80°C, bathed for 5 min, immediately put into cold absolute ethanol or a refrigerated for 2 min, centrifuged 8 min at 1,000 rpm, dried, a prehybridization solution was added on the slide, prehybridized at 42°C for 1 h, then rinsed twice with distilled water, centrifuged at 1,000 rpm, and stored in a dry dust-free environment. The same virus probes were a designed neighborhood and some QC (quality control, 10 µl HEX), BC (blank control, 50% DMSO), NC (negative control, 127 (SARS)) and EC (PCR product of HLA) were arrayed (see table 2).
**Table 1. Names and sequence of primers for all viruses**

| Primer sets | Sequence of primer | Primer sets | Sequence of primer |
|-------------|--------------------|-------------|--------------------|
| FCoV1       | Fw primer 5'-TGATTGTGCTGTAACCTTG-3' | FCoV2       | Fw primer 5'-TAACACACCTCACCAAGACCA-3' |
|             | Rw primer 5'-CTGGTGAGGTGTTAGTGATG-3' |             | Rw primer 5'-GAGGAAAGAACATATTGTGGGCG-3' |
| FCoV3       | Fw primer 5'-GGAGGTTCATACCTCGATTG-3' | FCoV4       | Fw primer 5'-TGCTATTAGTAACTGGGGCC-3' |
|             | Rw primer 5'-AGTACATACACACTCGGCTG-3' |             | Rw primer 5'-ATACACGCTACTCCACATGC-3' |
| FCoV5       | Fw primer 5'-CATTACACTCAAATGCGACG-3' | FCoV6       | Fw primer 5'-ATGGGTGTAATACCTGTCTG-3' |
|             | Rw primer 5'-ATACACCAACACACTCGTAC-3' |             | Rw primer 5'-CCCTGGAATCTAGTGGTCG-3' |
| FCoV7       | Fw primer 5'-ACACACACCTGACATCGACG-3' | FCoV8       | Fw primer 5'-CTCTCTTCAGGATGTTACAC-3' |
|             | Rw primer 5'-CTCACGAAACACACTGACG-3' |             | Rw primer 5'-CTCTGCTTGCATTAGTGC-3' |
| FCoV9       | Fw primer 5'-GGTTAGGGCTAGTACGACCGAC-3' | FCoV10      | Fw primer 5'-GACTGAGCTCAGGATGTAAC-3' |
|             | Rw primer 5'-TGCAAAACACACTGACGAC-3' |             | Rw primer 5'-CTCTCAGCAAAACACTGAC-3' |
| FCoV11      | Fw primer 5'-GGATACATACACACTCGGACG-3' | FCoV12      | Fw primer 5'-TTAGTGTCAGGATGCCGC-3' |
|             | Rw primer 5'-GAGGAAAGAACATATTGTGGGCG-3' |             | Rw primer 5'-TAACACACCTCACCAAGACCA-3' |
| FCoV13      | Fw primer 5'-CTCAACAGGACCAGATGCACG-3' | FCoV14      | Fw primer 5'-AACACGCTACTCCACATGC-3' |
|             | Rw primer 5'-AGACACCGCTACTCCACATGC-3' |             | Rw primer 5'-GACCGCTACACACTCGGAC-3' |
| FCoV15      | Fw primer 5'-GGTAGGCTTCGACCAGACG-3' | TGEV1       | Fw primer 5'-GATGGGTGTAATACCTGTCTG-3' |
|             | Rw primer 5'-CTGCGGATCTGCGATCGACG-3' |             | Rw primer 5'-CCCTGGAATCTAGTGGTCG-3' |
| TGEV2       | Fw primer 5'-ATGGGGTACGTTACGACG-3' | TGEV3       | Fw primer 5'-TACCTGAGGCATGACAGACG-3' |
|             | Rw primer 5'-CCACCTGATGACATCGACG-3' |             | Rw primer 5'-ACCTGACATGACAGACG-3' |
| TGEV4       | Fw primer 5'-AGTACCTGACAGACG-3' | TGEV5       | Fw primer 5'-ACACACACCTGACATCGACG-3' |
|             | Rw primer 5'-GACACGCTACTCCACATGC-3' |             | Rw primer 5'-GACACGCTACTCCACATGC-3' |
| TGEV6       | Fw primer 5'-GCTGGCTGTCATGACCTGACG-3' | TGEV7       | Fw primer 5'-GACACGCTACTCCACATGC-3' |
|             | Rw primer 5'-GCCACAGGTAAACACACTGACG-3' |             | Rw primer 5'-GACACGCTACTCCACATGC-3' |
| TGEV8       | Fw primer 5'-TTCCTCCGAGGAGGATAGG-3' | TGEV9       | Fw primer 5'-GACACGCTACTCCACATGC-3' |
|             | Rw primer 5'-AGGATGATGAGGATAGG-3' |             | Rw primer 5'-TAAGGCCATGACATCGACG-3' |
| TGEV10      | Fw primer 5'-TTTCACAGGAGGCCGGATG-3' | BCoV1       | Fw primer 5'-CCCCCGATCAGTTATTGTTC-3' |
|             | Rw primer 5'-AGGATGATGAGGATAGG-3' |             | Rw primer 5'-TGCTGTACCTAGCAGACG-3' |
| BCoV2       | Fw primer 5'-GGGCTTTCGATTTAGAGG-3' | BCoV3       | Fw primer 5'-GGGTGTAATACCTGTCTG-3' |
|             | Rw primer 5'-CTGCTGATTTAGAGG-3' |             | Rw primer 5'-ATACGCTACTCCACATGC-3' |
| BCoV4       | Fw primer 5'-TTGATGCAGTAACCTTTAC-3' | BCoV5       | Fw primer 5'-GATGGGTGTAATACCTGTCTG-3' |
|             | Rw primer 5'-CATGACATGAGGATAGG-3' |             | Rw primer 5'-CARAAATACCTGTCTG-3' |
| BCoV6       | Fw primer 5'-TTTCTGCTGATTTAGAGG-3' | BCoV7       | Fw primer 5'-GACACGCTACTCCACATGC-3' |
|             | Rw primer 5'-AGGATGATGAGGATAGG-3' |             | Rw primer 5'-TTAAGGCCATGACATCGACG-3' |
| BCoV8       | Fw primer 5'-ACCGAGATGAGGATAGG-3' | BCoV9       | Fw primer 5'-TAACGCTACTCCACATGC-3' |
|             | Rw primer 5'-GACACGCTACTCCACATGC-3' |             | Rw primer 5'-TTAAGGCCATGACATCGACG-3' |
| BCoV10      | Fw primer 5'-ATGTAAGTCTGCTGATAGG-3' | BCoV11      | Fw primer 5'-GATGGGTGTAATACCTGTCTG-3' |
|             | Rw primer 5'-ACTGCACTGAGGATAGG-3' |             | Rw primer 5'-CARAAATACCTGTCTG-3' |
| BCoV12      | Fw primer 5'-GACGTGCTGCTGATAGG-3' | BCoV13      | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| BCoV14      | Fw primer 5'-GCCCTTCTGTTAGG-3' | BCoV15      | Fw primer 5'-AATAATAGGTGTTGACAG-3' |
|             | Rw primer 5'-TACTGATAGGTGTTGACAG-3' |             | Rw primer 5'-TTAAGGCCATGACATCGACG-3' |
| CCoV1       | Fw primer 5'-ACGTGCTGCTGCTGATAGG-3' | CCoV2       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| CCoV3       | Fw primer 5'-GACGTGCTGCTGCTGATAGG-3' | CCoV4       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| CCoV5       | Fw primer 5'-GACGTGCTGCTGCTGATAGG-3' | CCoV6       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| CCoV7       | Fw primer 5'-GACGTGCTGCTGCTGATAGG-3' | FIPV1       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| FIPV2       | Fw primer 5'-GACGTGCTGCTGCTGATAGG-3' | FIPV3       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| FIPV4       | Fw primer 5'-GACGTGCTGCTGCTGATAGG-3' | FIPV5       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
| FIPV6       | Fw primer 5'-GACGTGCTGCTGCTGATAGG-3' | FIPV7       | Fw primer 5'-GACGTGCTGCTGATAGG-3' |
|             | Rw primer 5'-GACGTGCTGCTGCTGATAGG-3' |             | Rw primer 5'-GACGTGCTGCTGATAGG-3' |
Table 1 (continued)

| Primer sets | Sequence of primer | Primer sets | Sequence of primer |
|-------------|-------------------|-------------|-------------------|
| TCoV1 Fw primer 5'-AGATTAGTTGTTGCTGTGGCC-3' | R CoV1 Fw primer 5'-TCAGACCATGTCGTTCTGGG-3' | FIPV9 | Fw primer 5'-TGACAGGAGTTTCTCAACCC-3' |
| FIPV10 Fw primer 5'-ACATTACAACACACAGAGTC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | FIPV11 | Fw primer 5'-GGTTGAGATGATAAGGAGGC-3' |
| PRCoV1 Fw primer 5'-GTGTTTTGTGGTCGAAATAATGCGC-3' | R CoV1 Fw primer 5'-TGACAGGAGTTTCTCAACCC-3' | PRCoV2 | Fw primer 5'-CCGTGGATGATGTGTTATGATGAGC-3' |
| PRCoV3 Fw primer 5'-GCATCTACACAAACAGACCC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | PRCoV4 | Fw primer 5'-GATTCCGACTACATGTCATGCTGC-3' |
| TCoV1 Fw primer 5'-CAGATGCTAGTTGCTATGGG-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | TCoV2 | Fw primer 5'-ACTCCCTTAAACAGCTTGCTTG-3' |
| TCoV3 Fw primer 5'-CCGCTCGAGTGATGATTGCC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | TCoV4 | Fw primer 5'-ACCACCCATACATGTCATGCTGC-3' |
| TCoV5 Fw primer 5'-GTAGTAGGCGAGCGATTTTG-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | TCoV6 | Fw primer 5'-GAAAAGATGATGATTGCC-3' |
| TCoV7 Fw primer 5'-TTCTCTTGAGGAGGTTGCTGG-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | TCoV8 | Fw primer 5'-ATCGCTATCTGCTGTTATGCTGC-3' |
| TCoV9 Fw primer 5'-AGACGAGGATGATGATTGCC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV1 | Fw primer 5'-CGGCTCGAGGTGATGATTGCC-3' |
| HRCoV2 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV3 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| HRCoV4 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV5 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| HRCoV6 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV7 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| HRCoV8 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV9 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| HRCoV10 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV11 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| HRCoV12 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV13 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| HRCoV14 Fw primer 5'-CCGCTCGAGCTGCTGTTATGCTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV15 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |
| UP FW primer 5'-ACTCATTATGCAGCATTATATTGC-3' | R CoV1 Fw primer 5'-TTCTCTTAAACAGCTTGCTTC-3' | HRCoV16 | Fw primer 5'-CGGCTCGAGCTGCTGTTATGCTGC-3' |

Table 2. Array arrangement of primary coronaviruses chip

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | QC | QC | BCoV1 | BCoV1 | UP1 | UP1 | QC | QC | QC | QC | FCoV1 | FCoV1 | HCoV1 | HCoV1 | QC |
| B | NC1 | NC1 | BCoV2 | BCoV2 | UP2 | UP2 | FIPV1 | FIPV1 | PRCoV1 | PRCoV1 | FCoV2 | FCoV2 | HCoV2 | HCoV2 | NC2 |
| C | TGEV1 | TGEV1 | BCoV3 | BCoV3 | UP3 | UP3 | FIPV2 | FIPV2 | PRCoV2 | PRCoV2 | FCoV3 | FCoV3 | HCoV3 | HCoV3 | BC |
| D | TGEV2 | TGEV2 | BCoV4 | BCoV4 | UP4 | UP4 | FIPV3 | FIPV3 | PRCoV3 | PRCoV3 | FCoV4 | FCoV4 | HCoV4 | HCoV4 | TCoV1 |
| E | TGEV3 | TGEV3 | BCoV5 | BCoV5 | UP5 | UP5 | FIPV4 | FIPV4 | PRCoV4 | PRCoV4 | FCoV5 | FCoV5 | HCoV5 | HCoV5 | TCoV2 |
| F | TGEV4 | TGEV4 | BCoV6 | BCoV6 | UP6 | UP6 | FIPV5 | FIPV5 | CCoV1 | CCoV1 | FCoV6 | FCoV6 | HCoV6 | HCoV6 | TCoV3 |
| G | TGEV5 | TGEV5 | BCoV7 | BCoV7 | UP7 | UP7 | FIPV6 | FIPV6 | CCoV2 | CCoV2 | FCoV7 | FCoV7 | HCoV7 | HCoV7 | TCoV4 |
| H | EC | EC | BCoV8 | BCoV8 | UP8 | UP8 | CCoV3 | CCoV3 | FCoV8 | FCoV8 | HCoV8 | HCoV8 | EC | EC |
| I | TGEV6 | TGEV6 | BCoV9 | BCoV9 | UP9 | UP9 | FIPV7 | FIPV7 | CCoV4 | CCoV4 | FCoV9 | FCoV9 | HCoV9 | HCoV9 | TCoV5 |
| J | TGEV7 | TGEV7 | BCoV10 | BCoV10 | UP10 | UP10 | FIPV8 | FIPV8 | CCoV5 | CCoV5 | FCoV10 | FCoV10 | HCoV10 | HCoV10 | TCoV6 |
| K | TGEV8 | TGEV8 | BCoV11 | BCoV11 | UP11 | UP11 | FIPV9 | FIPV9 | CCoV6 | CCoV6 | FCoV11 | FCoV11 | HCoV11 | HCoV11 | TCoV7 |
| L | TGEV9 | TGEV9 | BCoV12 | BCoV12 | UP12 | UP12 | FIPV10 | FIPV10 | CCoV7 | CCoV7 | FCoV12 | FCoV12 | HCoV12 | HCoV12 | TCoV8 |
| M | TGEV10 | TGEV10 | BCoV13 | BCoV13 | UP13 | UP13 | FIPV11 | FIPV11 | CCoV13 | CCoV13 | FCoV13 | FCoV13 | HCoV13 | HCoV13 | TCoV9 |
| N | NC3 | NC3 | BCoV14 | BCoV14 | UP14 | UP14 | FIPV15 | FIPV15 | CCoV15 | CCoV15 | FCoV15 | FCoV15 | HCoV15 | HCoV15 | NC4 |
| O | QC | QC | BCoV15 | BCoV15 | UP15 | UP15 | QC | QC | QC | QC | FCoV15 | FCoV15 | HCoV15 | HCoV15 | QC |
Samples Processing and Hybridizing

Viral RNAs were extracted from the cryolysate of cell cultures infected with the examined coronavirus strains using a QIAamp Viral RNA Mini Kit (Qiagen, Germany) and reverse transcribed with M-MLV Rtase cDNA synthesis kit (Takara Biotechnology (Dalian) Co. Ltd., China), with oligo-dT as primer, then each cDNA was multi-PCR amplified with different primer sets (see table 3; each virus cDNA with different primer sets, such as CCoV with CCoV1, CCoV2, CCoV3, CCoV4, CCoV5 primer mixture, and CCoV6, CCoV7 primer mixture to amplify separately) and labeled with Cy3-dCTP (PerkinElmer). The labeled fluorescent DNAs were denatured at 96° for 5 min, then placed on a slide and put into a hybridization chamber oven at 42° for 2–3 h. Different multi-PCR samples with Cy3-dCTP were hybridized with the chip. After washing, the slides were scanned on a GenePix 4000B array scanner (Packard Biochip Technologies, Mass., USA) at 10 μm resolution and analyzed with GenePix Pro analysis software.

Sensitivity and Application of the Coronavirus Chip

The 10-fold serial BCoV cDNA dilution was detected by multi-PCR and the coronavirus gene chip using the same primer set. The results were shown by electrophoresis in a 1% agarose gel for 1 h at 90 V, visualized by ethidium bromide and a slide reader. Muscle, heart, liver, spleen, lung and kidney were gathered from the pigs infected with live TGEV, PRCoV, canines infected with live CCoV, felines infected with live FCoV, FIPV, and cattle infected with live BCoV, which were detected with RT-PCR, cell culture and microassay, while 10 canine hearts (7 positive and 3 negative to CCoV analyzed by PCR), 5 feline lungs (3 positive and 2 negative to FIPV analyzed by PCR) and 6 pig lungs (5 positive and 1 negative to PRCoV analyzed by PCR) were collected from clinical animals and detected with microassay [12, 13, 15].

Results

Hybridizing to Different Multi-PCR Samples with the Primary Coronavirus Chip

Different multi-PCR samples with Cy3-dCTP were hybridized with the chip. The slides were scanned by a ScanArray 4000c laser scanning system (Packard Biochip Technologies) with a laser for Cy3 dye (λex543 nm/λem570 nm) at 10 μm resolution. Different intense signals appeared. From the hybridization, extensive cross-reactions between CCoV, FCoV, FIPV, TGEV and PRCoV were found, while there were none between BCoV, TCoV and HRCoV. The results are shown in table 3 and figure 1a–c as samples.

Table 3. Hybridization and amplification of multi-PCR of different coronavirus

| Group | Primer sets | Cross-reaction gene clones of other viruses |
|-------|-------------|--------------------------------------------|
| 1     | BCoV1, BCoV2, BCoV3, BCoV4, BCoV5 | NO |
| 2     | BCoV6, BCoV7, BCoV8, BCoV9, BCoV10 | NO |
| 3     | BCoV11, BCoV12, BCoV13, BCoV14, BCoV15 | NO |
| 4     | TCoV1, TCoV2, TCoV3, TCoV4, TCoV5 | NO |
| 5     | TCoV6, TCoV7, TCoV8, TCoV9 | NO |
| 6     | CCoV1, CCoV2, CCoV3, CCoV4, CCoV5 | TGEV3, TGEV4, TGEV5, TGEV6, TGEV8, FIPV9, FCoV2, FCoV4, FCoV9, FCoV10, FCoV11 |
| 7     | CCoV6, CCoV7 | FIPV3, FIPV4, TGEV4, TGEV5, TGEV7, TGEV8, TGEV9, TGEV10, FCoV2, FCoV3, FCoV9 |
| 8     | FIPV1, FIPV2, FIPV3, FIPV4, FIPV5 | CCoV1, CCoV2, TGEV7, TGEV8, FCoV7, FCoV9, FCoV10, FCoV11, FCoV12 |
| 9     | FIPV6, FIPV7, FIPV8, FIPV9, FIPV10, FIPV11 | FCoV12 |
| 10    | FCoV1, FCoV2, FCoV3, FCoV4, FCoV5 | CCoV6 |
| 11    | FCoV6, FCoV7, FCoV8, FCoV9, FCoV10 | TGEV7, TGEV10, FIPV1, FIPV3, FIPV4 |
| 12    | FCoV11, FCoV12, FCoV13, FCoV14, FCoV15 | TGEV6, TGEV8, TGEV9, TGEV10, CCoV1, CCoV2, CCoV7, FIPV4, FIPV5, FIPV6, FIPV7, FIPV8 |
| 13    | TGEV1, TGEV2, TGEV3, TGEV4, TGEV5 | CCoV3, CCoV4, FIPV3, FIPV4 |
| 14    | TGEV6, TGEV7, TGEV8, TGEV9, TGEV10 | FIPV4, FCoV1, CCoV2, CCoV7, FCoV10 |
| 15    | HRCoV1, HRCoV6, HRCoV8, HRCoV13, HRCoV15 | UP2, UP7 |
| 16    | HRCoV2, HRCoV3, HRCoV4, HRCoV5, HRCoV14 | NO |
| 17    | HRCoV7, HRCoV9, HRCoV10, HRCoV11, HRCoV12 | NO |
| 18    | PRCoV1, PRCoV2, PRCoV3, PRCoV4 | TGEV9, CCoV7 |

NO = No cross-reaction was observed with gene clones from other viruses.
Fig. 1. Detection of gene chip by (a) BCoV multi-PCR product (table 3, group 1), (b) FIPV multi-PCR product (table 3, group 8), and (c) BCoV multi-PCR product (table 3, group 15).

Fig. 2. Hybridization of coronavirus gene chip with (a) FIPV, (b) BCoV, (c) HRCoV, (d) TCoV, (e) PRCoV, (f) CCoV, (g) FCoV, and (h) TGEV multi-PCR probes.

Fig. 3. Hybridization of coronavirus gene chip with universal primer PCR probes.
Design and Verification of Ultimate Coronavirus Chip

The ultimate specific gene chip was developed with the DNA fragments reamplified from the chosen recombinant plasmids without cross-reaction between different coronaviruses, as shown in Table 4. Different multi-PCR samples with Cy3-dCTP using specific primer sets were hybridized with the chip and scanned. Intense signals to the positive and control point and no signal to the negative and blank point were shown. Figure 2a–h shows obvious hybridization in the ultimate gene chip, with which several coronaviruses could be distinguished easily because of no cross-reaction between different coronaviruses.

Hybridization of Coronavirus Gene Chip with Universal Primer PCR Probes

The mixed cDNA of eight coronaviruses was PCR-amplified using a specific universal primer and labeled with Cy3-dCTP, then the PCR product was hybridized with the chip and scanned. The intense signal to the positive and control point and no signal to the negative and blank point were shown. Figure 2a–h shows obvious hybridization in the ultimate gene chip, with which several coronaviruses could be distinguished easily because of no cross-reaction between different coronaviruses.

Sensitivity and Application of the Coronavirus Chip

The 10-fold serial BCoV cDNA dilution was detected by multi-PCR and the coronavirus chip using the same primer sets of BCoV2, BCoV7, BCoV9, BCoV12, BCoV13 and BCoV14. The results showed that $10^{-2}$ diluted cDNA can be detected by multi-PCR and $10^{-3}$ diluted cDNA detected by the chip (shown in fig. 4a–h), indicating that the chip may be $1,000\times$ more sensitive than PCR.

Table 5 shows that the sensitivity of cell culture detection was lower, while the microassay was higher and had comparable sensitivities with PCR. At the same time, the results obtained from field samples with microassay detection showed good concordance with PCR methods.

Discussion

Microarrays and biosensors have become valuable tools for viral discovery, detection, and genotyping, which have been commonly used in gene expressions, diagnosis of disease, discovery of new genes and drug screening [18–21]. Here we have constructed a coronavirus-specific DNA microarray. The assay was designed to be broadly reactive with the genome of many coronavirus species. It is demonstrated that it can detect eight coronaviruses including the HRCoV-229E which are well-recognized human pathogens.

In order to make a coronavirus microarray useful, it must provide information for a wide range of viral strains and not have a cross-reaction between different coronaviruses. We constructed 104 cDNA clones of eight viruses including different genes by using different (4–15) pairs of primers designed for TCoV, CCoV, FCoV, FIPV, TGEV, PRCoV, BCoV and HRCoV, and a pair of universal primers designed for the RNA polymerase gene of coronavirus. Probes were compared to the database to ensure each probe was unique to the respective virus. The probe fragment was then obtained through plasmid PCR using

| QC | QC | BC | BC | BCoV6 | BCoV6 | QC | QC | HRCov3 | HRCov3 | TCoV1 | TCoV1 | QC | QC |
|----|----|----|----|-------|-------|----|----|--------|--------|-------|-------|----|----|
| NC1 | NC1 | FIPV7 | FIPV7 | BCoV7 | BCoV7 | QC | QC | HRCov4 | HRCov4 | TCoV2 | TCoV2 | NC2 | NC2 |
| FIPV2 | FIPV2 | FIPV8 | FIPV8 | BCoV8 | BCoV8 | QC | QC | HRCov5 | HRCov5 | TCoV3 | TCoV3 | BCoV6 | BCoV6 |
| TGEV3 | TGEV3 | FIPV9 | FIPV9 | BCoV9 | BCoV9 | QC | QC | HRCov12 | HRCov12 | TCoV5 | TCoV5 | TCoV7 | TCoV7 |
| TGEV4 | TGEV4 | TGEV5 | TGEV5 | BCoV10 | BCoV10 | QC | QC | HRCov13 | HRCov13 | HRCoV13 | HRCoV13 | TCoV9 | TCoV9 |
| EC | EC | TGEV6 | TGEV6 | BC | BC | QC | QC | HRCov8 | HRCov8 | UP1 | UP1 | UP10 | UP10 |
| PRCoV1 | PRCoV1 | BCoV2 | BCoV2 | CCoV1 | CCoV1 | QC | QC | FCoV6 | FCoV6 | UP3 | UP3 | UP12 | UP12 |
| PRCoV2 | PRCoV2 | BCoV3 | BCoV3 | CCoV2 | CCoV2 | QC | QC | FCoV7 | FCoV7 | UP6 | UP6 | UP14 | UP14 |
| PRCoV3 | PRCoV3 | BCoV12 | BCoV12 | CCoV5 | CCoV5 | QC | QC | FCoV8 | FCoV8 | UP8 | UP8 | NC4 | NC4 |
| NC3 | NC3 | BCoV13 | BCoV13 | CCoV7 | CCoV7 | QC | QC | FCoV9 | FCoV9 | BC | BC | QC | QC |

QC = Quality control, 10 μM HEX; BC = blank control, 50% DMSO; NC = negative control, 127 (SARS); EC = PCR product of HLA.
Table 5. Results showing that the sensitivity of cell culture detection was lower, while the microassay was higher and had comparable sensitivities with PCR. At the same time, the results obtained from field samples with microassay detection showed good concordance with PCR methods.

| Virus infected | Animal n | Tissues | RT-PCR | Chip | Cell culture |
|----------------|----------|---------|--------|------|--------------|
| CCoV canine (2) | muscle | – | – | – | |
|                | heart   | + | + | – | |
|                | liver   | – | – | + | |
|                | spleen  | – | – | – | |
|                | lung    | – | + | – | |
|                | kidney  | – | – | – | |
| canine (1) muscle | – | – | – | |
|                | heart   | + | + | – | |
|                | liver   | – | – | – | |
|                | spleen  | – | + | – | |
|                | lung    | – | – | – | |
|                | kidney  | – | – | – | |
| canine (2) muscle | – | – | – | |
|                | heart   | – | – | – | |
|                | liver   | – | + | – | |
|                | spleen  | – | – | – | |
|                | lung    | – | – | – | |
|                | kidney  | – | – | – | |
| BCoV cattle (2) muscle | + | + | – | |
|                | heart   | – | – | – | |
|                | liver   | – | + | – | |
|                | spleen  | – | – | – | |
|                | lung    | – | – | – | |
|                | kidney  | – | – | – | |
| cattle (2) muscle | – | – | – | |
|                | heart   | – | – | – | |
|                | liver   | – | – | – | |
|                | spleen  | – | – | – | |
|                | lung    | – | – | – | |
|                | kidney  | – | – | – | |
| FIPV feline (3) muscle | – | + | – | |
|                | heart   | – | – | – | |
|                | liver   | – | + | – | |
|                | spleen  | – | – | – | |
|                | lung    | + | + | – | |
|                | kidney  | + | + | – | |
| feline (2) muscle | + | + | – | |
|                | heart   | + | + | + | |
|                | liver   | – | + | – | |
|                | spleen  | – | – | – | |
|                | lung    | + | + | – | |
|                | kidney  | – | + | – | |
| TGEV pig (3) muscle | – | – | – | |
|                | heart   | – | + | – | |
|                | liver   | – | – | – | |
|                | spleen  | – | – | – | |
|                | lung    | – | – | – | |
|                | kidney  | – | – | – | |
| pig (3) muscle | – | – | – | |
|                | heart   | – | – | – | |
|                | liver   | – | – | – | |
|                | spleen  | – | – | – | |
|                | lung    | – | – | – | |
|                | kidney  | – | – | – | |
| PRCoV pig (3) muscle | – | – | – | |
|                | heart   | – | – | – | |
|                | liver   | – | + | – | |
|                | spleen  | – | – | – | |
|                | lung    | + | + | – | |
|                | kidney  | – | – | – | |
| pig (2) muscle | – | – | – | |
|                | heart   | – | – | – | |
|                | liver   | – | – | – | |
|                | spleen  | – | – | – | |
|                | lung    | – | + | – | |
|                | kidney  | – | – | – | |

Detection of different tissues with RT-PCR, the chip and cell culture.
the same primers as clone construction, precipitated with ethanol and suspended in 300 ng/μl with 50% DMSO for spotting purposes. Viral RNAs were extracted from the cryolysate of cell cultures infected with the examined coronavirus strains, labeled with Cy3 fluorescent dyes during PCR with different primer pair sets, and hybridized to the primary gene chip. Extensive cross-reactions between CCoV, FCoV, FIPV, TGEV and PRCoV were found and these clones may exist in a similar site in genes. Through the screening of hybridization, the ultimate specific gene chip was developed with the DNA fragments reamplified from the chosen recombinant plasmids without cross-reaction between different coronaviruses.

In some studies, the sample cDNA was labeled during reverse transcription and then hybridized [18, 22]. In this study we tried to label the cDNAs of BCoV directly with Cy3-dCTP during reverse transcription and subjected them to specific hybridization, however no positive signal appeared. We think the amount of total vRNAs may be so low that the amount of cDNA labeled cannot meet the chip limit required. The amplification of sample cDNAs with PCR is therefore necessary to obtain satisfactory hybridization results. Secondly, the quality and concentration of probes is another important factor related with the signal intensity [23–25]. We obtained the probes by PCR and then purified and concentrated them to make certain that the OD260 was >0.1, the OD260/OD280 was >1.4, and the concentration of probes reached 50 μg/ml.

In summary, a new DNA microarray technology is described exhibiting a useful diagnostic method for comprehensive detection of eight coronaviruses – it had a good correlation with PCR and is 1,000× more sensitive than PCR. It is expected to remain effective with possible mutants and to be of value when other new coronavirus-emes emerge. Because of the same PCR amplification and further Cy3 electrochemistry magnification, the chip method has a more than 1,000× sensitivity than PCR. As we just compare the sensitivity of BCoV detection using the gene chip and PCR methods with the same primer sets, in future we will compare other coronaviruses and different primer sets with these two methods in order to confirm the high sensitivity of DNA microarray technology.

**Fig. 4.** a Detection of 10⁻⁰, 10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ BCoV cDNA by multi-PCR. b–h Hybridization of coronavirus gene chip with (b) 10⁻⁰ BCoV, (c) 10⁻¹ BCoV, (d) 10⁻² BCoV, (e) 10⁻³ BCoV, (f) 10⁻⁴ BCoV, (g) 10⁻⁵ BCoV, and (h) 10⁻⁶ BCoV cDNA multi-PCR probes.
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