Differentiation of the seven major lyssavirus species by oligonucleotide microarray

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Running title: Differentiation of Lyssaviruses by microarray

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ABSTRACT

An oligonucleotide microarray, LyssaChip, has been developed and verified as a highly specific diagnostic tool for differentiation of the 7 major lyssavirus species. As with conventional typing microarray methods, the LyssaChip relies on sequence differences in the 371-nucleotide region coding of the nucleoprotein. This region was amplified using nested RT-PCR primers binding to the 7 major lyssaviruses. The LyssaChip includes 57 pairs of species-typing and corresponding control oligonucleotide probes (oligoprobes) immobilized on glass slides, and can analyze 12 samples on a single slide within 8 h. Analysis of 111 clinical brain specimens (65 from rabies-suspected animals submitted to the laboratory and 46 of butchered dog brain tissues collected from restaurants) showed that the chip method was 100% sensitive and highly consistent with the “gold standard” fluorescent antibody test (FAT). The chip method could detect rabies virus in highly decayed brain tissues, whereas the FAT did not, and may thereby be more applicable to highly decayed brain tissues than FAT. LyussaChip may provide a convenient and inexpensive alternative for diagnosis and differentiation of rabies and rabies-related diseases.

Keywords: Lyssaviruses; oligonucleotide microarray; species typing.
Rabies is a worldwide zoonotic disease caused by viruses within the genus *lyssavirus*, family *Rhabdoviridae*, with many host species acting as reservoirs for infection (8). There are 7 major species within the genus: classical rabies virus (RABV), Lagos bat virus (LBV), Mokola bat virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus type 1 (EBLV-1), European bat lyssavirus type 2 (EBLV-2), and Australian bat lyssavirus (ABLV). Recently, 4 new members have been approved by the International Committee on Taxonomy of Viruses: Aravan virus (ARAV), Irkut virus (IRKV), Khujand virus (KHUV) and West Caucasian bat virus (WCBV) (18).

Lyssaviruses possess a single-stranded, non-segmented, negative-sense RNA genome of approximately 12kb, which encodes 5 proteins: a nucleoprotein (N), a phosphoprotein (P), the matrix protein (M), a single surface glycoprotein (G) and an RNA-dependent polymerase (L) (29). N gene divergence is commonly used for species-typing of lyssaviruses, since the abundance of N mRNA in infected cells makes it an ideal target for detection and differentiation. Additionally, the N gene has been reported the most conservative of all 5 genes by DNA polymorphism analysis (34).

The illnesses caused by rabies and rabies-related viruses are virtually indistinguishable (28). The FAT – the “gold standard” diagnostic test for rabies – is unable to discriminate between lyssavirus species. Sequence analysis of PCR products has provided the most accurate information, but is not considered the method of choice to determine the presence of double infections (25). Other species-typing methods such as conventional
gel-based PCR assays for detection of LBV and MOKV of lyssaviruses (12), and reverse transcript quantitative real-time PCR (RT-qPCR) for detection of RABV, EBLV-1, EBLV-2 and ABLV, have been reported (13, 31), but there is still no available method for the specific detection of DUVV.

Microarray technology has become a rapid and efficient method in clinical diagnostics (16). Microarray-based detection and genotyping of influenza virus (30), hepatitis B virus (20), foot-and-mouth disease (2), respiratory viruses and food-borne pathogens have been reported (21, 26), and the microarray has additionally been widely used for detecting new pathogens.

Recently, Berthet et al. (2008) have developed a high-density microarray using whole genome amplification (WGA) method for sample preparation (3). Gurrala et al. (2009) have developed a DNA microarray (LP chip) for detection and differentiation of lyssaviruses (15), using random PCR labeling target DNA (32). Recently, Dacheux et al. have reported a resequencing microarray for typing rhabdoviruses (9) with improved whole transcriptome amplification (WTA) (5). De Benedictis et al. (2011) have developed a pyrosequencing method to type lyssavirus species (10). In our study, the LyssaChip has been developed to specifically differentiate the 7 major lyssavirus species based on generic reverse transcription nested PCR (RT-nPCR). It can analyze 12 samples on a single slide within 8 h, thereby showing its potential application for the diagnosis of rabies and rabies-related diseases.
MATERIALS AND METHODS

Viruses and Specimens. Reference strains of the 7 major lyssavirus species were kindly provided by the Animal Health and Veterinary Laboratory Agency, Weybridge, United Kingdom. They were CVS-11 (RABV), RV1 (LBV), RV4 (MOKV), RV131 (DUVV), RV9 (EBLV-1), RV1787 (EBLV-2) and RV634 (ABLV). The four newest members of the lyssavirus genus, ARAV, IRKV, KHUV and WCBV, now considered independent species (18), were not available to us. The LyssaChip analysis of 111 clinical brain tissue specimens submitted by different provinces for laboratory confirmation between 2003 and 2010 was compared with standard FAT (23) and RT-nPCR (19). Of the 111 specimens, 65 were of rabies-suspected animals (dogs, cows, sheep, pigs and raccoon) and the remaining 46 were from restaurants in Hunan Province from dogs slaughtered for consumption (Table 1).

LyssaChip Design and Construction. The twelve-array concept was used to construct the slide chip, named LyssaChip, with each array comprising a maximum of 15 \( \times \) 14 oligoprobes in a 210 \( \times \) 99 \( \mu \)m square (see Fig. 1A). In the chip the clock dial-like layout of specific detection oligoprobes for typing the 7 major lyssaviruses was designed to readily differentiate the species. In addition, positive and negative control oligoprobes (70 nt) were also added to each array to monitor whether the hybridization conditions are correct and specific. The sequence of the positive control was from pGM-T plasmid, and the negative control from Arabidopsis thaliana. To optimize the excitation beam of the
chip reader to 532 nm wavelength a hexachloro fluorescein (HEX) labeled oligomarker (70 nt) with a random sequence was designed and spotted in the center and vertices of each array.

A region within the lyssavirus N gene (371 bp, nucleotides 62-442) was the target for probe detection, which is the RT-nPCR target for detection of pan-lyssaviruses (19). The oligoprobe sequences used for the 7 lyssaviruses were those of Bourhy et al. (2008) for RABV (7), Markoter et al. (2008) for LBV (22), Sabeta et al. (2007) for MOKV (27), Wu et al. (2007) for DUVV (34), Amengual et al. (1997) for EBLV-1, EBLV-2 (1), and Warrilow et al. (2005) for ABLV (33). Specific probes were selected from an extensive alignment comparison of 371 bp N sequences of representative strains of the 7 major taxonomic lyssaviruses retrieved from GenBank in order to cover all published lineages of lyssaviruses, and were then subjected to Blastn homology search on the GenBank website. All selected oligoprobes were of length 65±5 nt with >80% homology and a minimum 25 nt continuously matched region with all targets to be detected and showing the preferential Tm value 70±5°C (32). The 371 bp N gene sequences were aligned by MegAlign of DNASTAR LaserGene software v7.0 (DNASTAR, Wisconsin, USA) and the oligoprobe candidates were designed by Arraydesigner software v4.2 (PREMIER Biosoft, California, USA). Oligoprobes were commercially synthesized (Sangon, Shanghai, China), and contained an aminolink group at the N-terminal. They were diluted to 50 pmol/µl with dilution buffer (CapitalBio, Beijing, China) before use. The Microgrid
II Biorobot system (BioRobotics, Woburn, USA) was used for printing the oligoprobes onto aldehyde-coated glass slides (CapitalBio). The semifinished slides were immediately fixed at 37°C in 70% humidity for 2.5 h, then at room temperature for at least 30 h to allow complete fixation, after which they were washed with 2% SDS (Promega, Madison, USA), 3% NaBH₄ (Sigma-Aldrich, USA) and dried by low-speed centrifugation.

**RNA Extraction, RT-nPCR, and Labeling.** Two sets of pan-lyssavirus RT-nPCR primers were used to amplify the 62-442 nt fragment of the N gene (19), with outer primers N127 and N829 for the first round (nucleotide, 55-899nt), and inner primers NF371 (labeled with biotin-HEX at the 5' end) and NR371 for the second round. Their sequences, along with those of positive control primers (PC) are listed in Table 2.

Samples of brain tissue were prepared as 10% (w/v) suspensions by homogenization in PBS using a TissuLyser II (QIAGEN, Hilden, Germany). Total viral RNAs were extracted using the automatic nucleic acid extraction station epMotion 5075 VAC (Eppendorf, Hamburg, Germany) with QIAamp Virus BioRobot MDX Kit (QIAGEN, Hilden, Germany) as per manufacturer’s instructions. The RNA preparations were used immediately for reverse transcription (RT) with the N127 primer. The RT products were then used to amplify the 371 bp N gene fragment by nested PCR using the above primer sets. PCR was performed at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 40 s. During the second round amplification, the targets were labeled with biotin-HEX.
LyssaChip Hybridization and Analysis. For LyssaChip hybridization, 10µl PCR-labeled detection products were mixed with 2µl PCR-labeled positive control and 10µl hybridization buffer containing 2µl 99% formamide, 2µl 50× Denhardt’s solution (Sigma-Aldrich, USA), 2µl 1× SSC, 2µl 2% SDS (Promega, USA), and 2µl DEPC water (Sangon, Shanghai, China). The mixture was heated at 95°C for 5min to denature dsDNA, followed by chilling immediately on ice for 5 min, then applied to the array and covered with a plastic coverslip (CapitalBio, Beijing, China) to prevent evaporation of the sample during incubation. Hybridization was performed at 43°C for 3.5 h. After hybridization, the slide was washed once for 3 min with 2× SSC containing 2% SDS, followed by a wash for 5 min with 2× SSC and then drying by low-speed centrifugation.

Fluorescent images of the LyssaChip were obtained by scanning the slides with a GenePix 4000A personal scanner (Axon Instruments, California, USA). The fluorescent signals obtained at 532 nm from each spot were analyzed by GenePix Pro 6.0 software (Axon). Median background fluorescence was determined from the region surrounding the hybridization spots and subtracted. Detection signals with a median fluorescence higher than background level ($P < 0.01$) were considered positive.

Data Statistical Analysis. Normalized data from GenePix Pro 6.0 were used for statistical analysis by Prism software v5.02 (GraphPad Software, USA) to conduct Student’s $t$ tests, Kappa test, McNemar Chi-square tests, 95% confidence interval (CI) and produce graphics.
RT-qPCR Differentiation of RABV, EBLV-1 and EBLV-2 (31). LyssaChip results were validated by standard Taqman RT-qPCR, with reaction mixtures as follows: 12µl 10× PCR buffer, 12µl 25 mM MgCl₂, 0.5µl dNTPs (25nM), 0.3µl RNasin (5IU), 0.5µl MMLV (50IU), 0.5µl Ex-Taq polymerase (5IU/µl) (TaKaRa, Dalian, China), 1µl 10% (vol/vol) Triton X-100 (Promega, Madison, USA), 1µl forward primer (20 pmol/µl), 1µl reverse primer (20 pmol/µl), 1µl each of Taqman probes (5µM each), 10µl template RNA and nuclease-free water to a final volume of 50µl. The reactions were carried out in an MX3000P multiplex quantitative PCR system (Stratagene, LaJolla, USA). Reverse transcription and PCR amplification were performed using the following programs: 1 cycle at 42°C for 30 min and 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 20 s.

RESULTS

Oligoprobe Design and LyssaChip Construction. Initially, 78 candidate oligopropbes were designed by Arraydesigner software v4.2 based on multiple alignment of the 371 bp N gene sequences of the 7 major lyssaviruses, then subjected to Blastn search to exclude those with unreasonable GC contents, <80% homology and less than 25 continuously matched nucleotides with target sequences. Fifty oligopropbes that met these parameters were selected and, along with 7 control and oligomarker oligopropbes, were double printed and arrayed on the glass slides as shown in Figure 1A. Finally 6 to 10 specific probes for each virus were selected in order to ensure the detection of all lineages or subtypes within
a species. For instance, a set of 6 probes was selected to specifically detect different RABV lineages including Indian subcontinent, Asian, Africa 2, Arctic-related, Africa 3 and Cosmopolitan (7).

**Detection and Differentiation.** Using the above hybridization conditions the 7 reference lyssaviruses showed hybridization only with their corresponding oligoprobes and no nonspecific hybridization with unrelated oligoprobes (see Fig. 1B). The fluorescence intensities of all expected hybridizations were significantly higher than the background signal ($P <0.01$). These results showed the LyssaChip could specifically detect and differentiate all 7 major species of lyssaviruses.

To test the sensitivity of LyssaChip, 10-fold serial dilutions of plasmids bearing the 371 bp N gene fragment of all 7 viruses were amplified and labeled with HEX by PCR, then hybridized to LyssaChip. The copy number was calculated using the following formula: $[X \, \text{g/µl DNA/ (plasmid length in bp } \times 660)] \times 6.022 \times 10^{23} = Y \, \text{molecules/µl.}$ Starting concentration of the plasmid was $2.46 \times 10^9$ molecules/µl. The detection limit of all species was $2.46 \times 10^5$ molecules/µl, except EBLV-2 which was $2.46 \times 10^4$ molecules/µl (Fig. 2). Results also showed a good linear correlation ($R^2$) between template concentration and fluorescence (graph not shown).

To compare the sensitivity of LyssaChip with those of gel-based RT-nPCR and RT-qPCR, 10-fold serial dilutions of cell-cultured rabies virus, strain SRV9, were tested.
from $10^{6.2}$ to $10^{-0.8}$ TCID$_{50}$/ml. Results showed that the lowest limit of detection of LyssaChip was 0.158 TCID$_{50}$/ml, 10 times more sensitive than RT-nPCR and RT-qPCR.

Cy3 or Cy5 are widely used in the labeling of target sequences by random primer PCR, but the use of biotin HEX in labeling is uncommon. At the beginning of the study Cy3 ddCTP (GE Healthcare, USA) and HEX labeling methods were compared and the result showed that both labeling methods could produce the same fluorescence density ($P>0.05$) (data not shown). However the labeling procedure for Cy3 is more complicated and expensive than HEX, and the latter was therefore used in our sample preparation.

To evaluate the accuracy and specificity of the LyssaChip, 111 laboratory preserved brain specimens (see Table 1) were tested. In the assay of 65 rabies-suspected brain tissues, all were LyssaChip positive, with only 64 and 62 positive by RT-nPCR and FAT respectively. Two specimens were FAT negative and one highly decayed brain tissue was negative by both RT-nPCR and FAT. Among 46 brain tissues of butchered dogs 3 were positive by all three methods. The assay results of the three methods were summarized in Table 3, which showed that the LyssaChip had 100% sensitivity (CI 94.48%-100%) and 93.94% specificity (CI 82.10%-98.63%) as compared with FAT. The LyssaChip had very high consistency with FAT ($\kappa = 0.944$) and RT-nPCR ($\kappa = 0.981$).

**Validation of the LyssaChip in the International Laboratory Test 2010 (ILT-2010) for Rabies.** LyssaChip was tested using ILT-2010 — a set of 12 samples (one each of RABV, EBLV-1, EBLV-2 and ABLV as positive controls, 1 negative
control, and 7 blinded samples) received from the European Union Reference Laboratory for Rabies (EU-RL) at the French Agency for Food, Environmental and Occupational Health Safety (ANSES). The samples were also tested by FAT, RT-nPCR and RT-qPCR at the same time. Results revealed that the 4 methods were 100% consistent, but the LyssaChip was additionally able to differentiate between the species within 8 h, with all 12 samples tested on a single chip slide, while the FAT and RT-nPCR could not differentiate (Table 4).

**ARAV, IRKV, KHUV and WCBV**

Since the 4 new lyssaviruses recently approved by the ICTV were not available to us their detection by LyssaChip could be estimated only by comparison of our probe sequences with the corresponding viral genomic regions. Such comparisons showed that all 50 probes did not have the minimum 25 nt continuously matched sequence essential for hybridization (data not shown). In addition, there were mismatches within the 15 nucleotides at the 3’ end of all probes. Therefore, cross-hybridization of our probes with ARAV, IRKV, KHUV and WCBV seems unlikely.

**DISCUSSION**

For oligonucleotide microarray detection, target cDNA or DNA is usually amplified and labeled by PCR. Sensitivity of such microarray is determined by the target cDNA amplification. Currently, there are two main approaches. In the first, a common primer PCR is used to amplify a genus of pathogens (17), but its ability may be influenced by
mismatched bases between primer and template. Additionally, multiplex PCR is used to amplify pathogens of several different genera (6), but can result in suppression effects on some of the primer pairs, leading to false-negative results (11). In the second, the random primer-directed PCR (15, 32) and Phi29 polymerase-based amplification (3, 5, 9) are used to amplify a wide range of cDNAs. This procedure is widely used in identification of new pathogens, but may yield lower amounts of target DNA copies and may bias amplification, resulting in reduced sensitivity and specificity of a microarray (4). Additionally, random amplification methods are more complex and time-consuming. In the present study we chose the common primer PCR method; i.e., using pan-lyssavirus RT-nPCR to amplify and label the N gene fragment since this method has been previously shown to detect all 7 major lyssavirus species with high sensitivity (19). To avoid primer/target mismatch, 2 sets of primers for RT-nPCR were located within the most conserved region of the N gene of each lyssavirus and the results showed that the LyssaChip rendered the highest sensitivity compared with the conventional methods, indicating the capability to detect low levels of virus particles. Specificity of a species-typing oligonucleotide microarray is determined by the oligoprobes. Excellent probes should accurately identify target cDNAs and show no cross-hybridization with unrelated sequences. Panels of probes were therefore designed for each lyssavirus species, since the existence of lineages or sub-types within a given species makes it impossible to design a common probe to detect all members within a
species. Usually, matching stringency between probe and target is critical for hybridization. It has been reported that, with regard to an oligoprobe of 60-70 nt, any mismatch within 15 nucleotides of the 3’ end could significantly diminish the hybridization and produce false negative results, whereas mismatches of less than 12 nucleotides scattered throughout a sequence at the 5’ end had limited impact on hybridization unless ≥5 of them were continuous mismatches (24). However, Honma et al. (2007) observed that even a single nucleotide mismatch near the 3’ end of a probe significantly reduced the hybridization signal (17), which is more consistent with our observations.

Although blinded sample 5 was determined to be EBLV-1 by LyssaChip, it is interesting to note the difference in the hybridization of EBLV-1-specific probes with the EBLV-1 control sample and blinded sample 5, in which the double-spotted probe (No.33) hybridized with sample 5 but not with the EBLV-1 control (see Fig. 3B). The background information provided by EU-RL revealed that these two samples were different sub-types of EBLV-1, the control sample being EBLV-1-b and the blinded one EBLV-1-a (Table 4). The multiple sequence alignment of all EBLV-1 N gene sequences available from GenBank showed that probe 33 belonged to EBLV-1-a and had 1 mismatched nucleotide with the corresponding sequence of EBLV-1-b at the 13th nt from the 3’ end (Fig. 3A). This emphasizes that when designing specific oligoprobes the possibility of mismatched nucleotides near the 3’ end should be taken into critical consideration. Indeed, this feature
might be used to design probes for differentiating sub-types; e.g., the number 33 probe
could be used for distinguishing between the 2 sub-types of EBLV-1 (Fig. 3).

The LyssaChip was tested in detection of RABV in 111 clinical specimens and showed
comparable specificity with conventional RT-nPCR and FAT. None of the specimens
showed any nonspecific hybridization with the probes of the 6 other major lyssavirus
species. Virus had previously been isolated from 27 of the 68 LyssaChip positive
specimens and the N genes had been sequenced and found to comprise 4 lineages of
RABV (14). This showed that the LyssaChip can detect different genetic variants of
RABV. With respect to sensitivity there were differences between the three methods
(Table 3): for example, a highly decayed brain tissue specimen was FAT and RT-nPCR
negative but LyssaChip positive. This specimen was taken from a clinically rabid dog and
submitted by Chongqing Center for Animal Disease Control and Prevention for
laboratory confirmation. Following its receipt, it was accidentally left in the reception
room for a week at room temperature. By the time it had been retrieved the specimen had
decayed to a very dark grey colour with a strong odious smell. As tested by the three
methods this specimen was positive only by LyssaChip, likely indicating that this is the
most sensitive in terms of detection of decayed specimens. The explanation of this
discrepancy is that the sensitivity of FAT and RT-nPCR could be significantly decreased
since degradation of viral RNA and nucleocapsids could occur with the decay of the
tissue, indicating that the performance of FAT on putrefied specimens is not reliable. In
addition, two more specimens (HuNDN12 and CQWX-2) were also FAT negative, although RT-nPCR and the chip positive (see Table 3). Sample CQWX-2 was from a dog in a village of Wuxi county, Chongqing Municipality, which did not show clinical signs but bitten five villagers without provocation in a single day, and was thereafter killed for rabies diagnosis. Sample HuNDN12 was from a dog which was one of nine culled for safety in a village of Hunan province to prevent further possible rabies transmission since a human rabies case had occurred in the village. Since these two dogs were healthy-looking with no overt clinical signs when they were culled, the difference in detection of virus in their brain tissues might imply that both dogs were in the early incubation stage, at which time the brain tissue viral load might have been too low to be detected by FAT. Calculation of the difference by the McNemar Chi-square test was statistically significant ($P < 0.0001$), thereby indicating that the LyssaChip likely had a higher positive rate. In conclusion, all three methods produced identical results but LyssaChip was more sensitive than FAT and even RT-nPCR in detection of virus in highly decayed specimens. In situation where this might occur, therefore, the LyssaChip could be more useful. Isolates of lyssaviruses other than RABV were not tested since these were not available in China.

The LyssaChip developed in the current study represents a rapid, high-throughput and economical method for the detection and differentiation of the 7 major lyssavirus species. The entire detection procedure takes about 8 h, including 1 h for RNA extraction, 3 h for
RT-nPCR labeling, 3.5 h for hybridization and 30 min for washing and scanning.

Conventional RT-nPCR and sequencing methods are unable to accomplish the same task within such a short time. Additionally, the LyssaChip can assay 12 samples on a single slide, and permits the simultaneous processing of at least 7-8 slides at a reasonable cost (approximately US$ 5 per sample).

Given its high sensitivity, specificity, speed and low cost, the LyssaChip may provide an attractive alternative for clinical laboratories for detection and differentiation of the major lyssaviruses.
ACKNOWLEDGEMENTS

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REFERENCES


Boriskin, Y.S., P.S. Rice, R.A. Stabler, J. Hinds, H. Al-Ghusein, K. Vass, and
P.D. Butcher. 2004. DNA microarrays for virus detection in cases of central nervous

Bourhy, H., J.M. Reynes, E.J. Dunham, L. Dacheux, F. Larrous, V.T. Huong, G.
Xu, J. Yan, M.E. Miranda, and E.C. Holmes. 2008. The origin and


Guigon, P. Dickinson, O. Faye, A.A. Sall, I.G. Old, K. Kong, G.C. Kennedy, J.C.
of broad-spectrum resequencing microarray for genotyping rhabdoviruses. J Virol. 84:
9557-9574.

1932-1938.

PCR: optimization and application in diagnostic virology. Clin Microbiol Rev. 13:
559-570.


TABLE 1. Clinical specimens tested in this study

<table>
<thead>
<tr>
<th>Sample Province</th>
<th>No. of animal brain tissues</th>
<th>Dog</th>
<th>Cow</th>
<th>Sheep</th>
<th>Pig</th>
<th>Raccoon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chongqing</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hunan</td>
<td>57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tianjin</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Shandong</td>
<td>3</td>
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<td>Guangdong</td>
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<td>Shanxi</td>
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<tr>
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<td><strong>Total</strong></td>
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<td><strong>3</strong></td>
<td><strong>2</strong></td>
<td><strong>2</strong></td>
<td><strong>1</strong></td>
<td></td>
</tr>
</tbody>
</table>

Note: <sup>a</sup>: the specimens of cows, sheep, pigs and raccoons were from single rabies outbreaks;

<sup>b</sup>: Of which, 46 were of butchered dogs, with no clinical information available.
## TABLE 2. Primer sets used for LyssaChip sample preparations

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’ to 3’)</th>
<th>Size of products</th>
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<tbody>
<tr>
<td>N127</td>
<td>ATGTAACNCCTCTACAATGG</td>
<td>845 bp</td>
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<tr>
<td>N829</td>
<td>GCCCTGGTTCGACATTCT</td>
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<tr>
<td>NF371</td>
<td>HEX*-ACAATGGAKKCTGACAARATTG</td>
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<td>NR371</td>
<td>CCTGYWGAGCCAGTTVCCYTC</td>
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<tr>
<td>PC forward</td>
<td>HEX-TACCGCGAGACCCACGCTCA</td>
<td>481 bp</td>
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<tr>
<td>PC reverse</td>
<td>GACGCCGGGCAAGAGCAACT</td>
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</table>

TABLE 3. Correlation between detection of lyssaviruses in clinical specimens by LyssaChip, FAT and RT-nPCR.

<table>
<thead>
<tr>
<th>Standard Methods</th>
<th>LyssaChip</th>
<th>Correlation (%)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>FAT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>43</td>
</tr>
<tr>
<td>RT-nPCR</td>
<td>67</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43</td>
</tr>
</tbody>
</table>

<sup>a</sup> FAT was conducted by using FITC-conjugated anti-rabies monoclonal antibody (Fujirebio Diagnostics Inc., Malvern, USA) (23)

<sup>b</sup> these are the highly decayed, BD 12 and CQWX-2 brain tissue specimens (see Discussion).

<sup>c</sup> the highly decayed brain tissue.
<table>
<thead>
<tr>
<th>Specimen</th>
<th>FAT</th>
<th>RT-nPCR</th>
<th>RT-qPCR</th>
<th>LyssaChip</th>
<th>EU-RL diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
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+ positive detection; - negative detection.
Figure Legends:

Fig. 1. The clock dial-like layout of oligoprobe array printed on the glass slide and its hybridization profile of the 7 major lyssavirus species. A: Each microarray has a capacity for 15 × 14 probes. The lyssavirus species-typing oligoprobes (Nos.1-50), positive and negative control probes were spotted in duplicate on each microarray, except for probe 21 spotted in triplicate. The HEX-labeled oligomarker was spotted 8 times: 4 at the center and 4 at the vertices. Probe spot No.1-6: RABV; 7-13: LBV; 14-21: MOKV; 22-27: DUVV; 28-33: EBLV-1; 34-43: EBLV-2; 44-50: ABLV; 51-53: Positive control; 54-56: Negative control; 57: HEX-labeled oligomarker, and each slide contains 12 copies of the clock dial-like microarrays. B: Hybridization of the 7 species of lyssaviruses with LyssaChip. N, negative control; 1, RABV; 2, LBV; 3, MOKV; 4, DUVV; 5, EBLV-1; 6, EBLV-2; 7, ABLV.

Fig. 2. Sensitivity of detection by LyssaChip. The detection of serially-diluted template by the LyssaChip showed high sensitivity in detecting all 7 viruses, and EBLV-2 in particular.

Fig. 3. Sequence analysis of probe 33 (boxed). Twenty two sequences of the entire 1353 bp lyssavirus N gene were obtained from GenBank and aligned by MegAlign software (LaserGene Version 7.0) with Clustal W. The region corresponding to oligoprobe 33
shown from the 3´ end (left) to 5´ end (right). A: The top line is the antisense sequence of probe No. 33 (70 nt). Sequence alignment reveals that EBLV-1 is divided into 2 lineages, EBLV-1-a and EBLV-1-b. Probe 33 belonged to EBLV-1-a, with 98.6% homology with EBLV-1-a and 97.1% with EBLV-1-b. There was a mismatched nucleotide with the EBLV-1-b sequence at position 13 from the 3´ end of the probe. B: LyssaChip detection profile of the EBLV-1 control EBLV-1-b) and blinded sample 5 (EBLV-1-a). Probe 33 is boxed.