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# Combination of an Unbiased Amplification Method and a Resequencing Microarray for Detecting and Genotyping Equine Arteritis Virus

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**This study shows that an unbiased amplification method applied to equine arteritis virus RNA significantly improves the sensitivity of the real-time reverse transcription-quantitative PCR (RT-qPCR) recommended by the World Organization for Animal Health. Twelve viral RNAs amplified using this method were hybridized on a high-density resequencing microarray for effective viral characterization.**

Equine arteritis virus (EAV), the causative agent of equine viral arteritis (EVA), is a member of the *Arteriviridae* family. Its genome is made up of a single positive-strand RNA molecule (1, 2). EAV infects *Equidae* and may be transmitted through the respiratory and venereal routes. EAV can persist in the reproductive tract of stallions only (3). Following the primary infection, up to 70% of stallions can be persistently infected and shed the virus in their semen, sometimes for life. These “shedder” stallions spread the virus in the horse population during breeding or when their semen is used for artificial insemination (4). The financial consequences are potentially huge, since shedder stallions lose their value. Moreover, infection may cause abortion in pregnant mares and the death of young foals (5). The World Organization for Animal Health (OIE) prescribes viral isolation (VI) on cell culture to detect EAV for international trade (6). However, a recent study showed that under certain conditions, the real-time reverse transcription-quantitative PCR (RT-qPCR) assay developed by Balasuriya et al. in 2002 (7) is as sensitive as VI for detecting EAV in semen (8). Therefore, RT-qPCR targeting open reading frame 7 (ORF7) of the EAV genome, encoding the viral nucleoprotein, is increasingly used for diagnosis.

The main challenge to EAV surveillance is detecting EAV in the semen of shedder stallions and the organs of aborted fetuses to prevent costly outbreaks. In particular, when the viral load is very low and thus the amount of viral nucleotides targeted is limited (9). The aim of our study was to increase the sensitivity of the OIE-recommended RT-qPCR method (6) by combining it with an unbiased amplification method using the Phi29 polymerase coupled to a high-density resequencing microarray (RMA) to genotype the viruses detected.

Sixty different samples were used in this study. Of the 48 EAV-positive samples, 31 were from semen (Table 1), 12 were from virus isolation cell culture supernatants, and 5 were tissue samples from the lungs, spleen, or liver of 1 aborted foetus, 3 young foals, and 1 adult. Viral RNA was extracted from semen, cell culture supernatant, or 1 g of tissue sample (previously homogenized in 5 ml of cell medium) with the QIAmp viral RNA minikit (Qiagen, Valencia, CA) following the manufacturer's instructions.

In order to evaluate the gain in sensitivity of the newly devel-

oped method, extracted and purified viral RNA was amplified in triplicate using three different protocols (Fig. 1). First, RNA samples were amplified using the one-step RT-qPCR (assay 1) method recommended by the OIE (7). In parallel, the extracted RNA was treated with Turbo DNase (Life Technologies, Inc., Carlsbad, CA, USA) and then retrotranscribed into cDNA using the SuperScript III first-strand synthesis kit (Life Technologies, Inc.) in the presence of random hexamers. These cDNA samples were then either amplified using qPCR (assay 2) with the same program and the same primers and probe sequences as those used in assay 1 or subjected to random unbiased isothermal Phi29 amplification using the QuantiTect whole transcriptome kit (Qiagen, Valencia, CA), as described by Berthet et al. (10), prior to qPCR amplification (assay 3).

Table 1 presents the results obtained with assays 1, 2, and 3. To ensure that the comparisons between the assays were normalized and standardized, the RNA samples used in each of the three assays were from the same extraction process. Interestingly, all positive samples were detected by all three assays, indicating that isothermal amplification with Phi29 polymerase did not change the specificity of the OIE-recommended RT-qPCR (6). Moreover, no PCR signals were detected from the 12 negative samples, indicating that the assay was highly specific for EAV detection. Furthermore, the analytical sensitivity of each assay was evaluated and

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TABLE 1 The 48 positive EAV samples tested with their origin, year of collection, and copy numbers<sup>a</sup>

| Sample no.      | Sample          |                      | Yr of collection | Assay 1, purified RNA                  |                        | Assay 2, cDNA                   |                        | Assay 3, WTA 1/100                   |                        | Ratio of copy no. (assay 3/assay 1) × 100 |
|-----------------|-----------------|----------------------|------------------|--|------------------------|---------------------------------|------------------------|--------------------------------------|------------------------|---|
|                 | type            | Country of origin    |                  | Copy no.                               | SD                     | Copy no.                        | SD                     | Copy no.                             | SD                     |   |
| 1 <sup>b</sup>  | VI <sup>c</sup> | France               | 2008             | 209 × 10 <sup>3</sup>                  | 19 × 10 <sup>3</sup>   | 282 × 10 <sup>4</sup>           | 84 × 10 <sup>4</sup>   | 504 × 10 <sup>6</sup>                | 120 × 10 <sup>6</sup>  | 24 × 10 <sup>4</sup>                      |
| 2               | Semen           | France               | 2008             | 642                                    | 72                     | 3 × 10 <sup>4</sup>             | 1.3 × 10 <sup>4</sup>  | 3 × 10 <sup>6</sup>                  | 0.4 × 10 <sup>6</sup>  | 46 × 10 <sup>4</sup>                      |
| 3 <sup>b</sup>  | Semen           | France               | 2008             | 16 × 10 <sup>3</sup>                   | 4 × 10 <sup>3</sup>    | 21 × 10 <sup>4</sup>            | 7.2 × 10 <sup>4</sup>  | 7.9 × 10 <sup>6</sup>                | 0.3 × 10 <sup>6</sup>  | 4.75 × 10 <sup>4</sup>                    |
| 4 <sup>b</sup>  | Semen           | France               | 2008             | 745                                    | 135                    | 16.3 × 10 <sup>4</sup>          | 2.1 × 10 <sup>4</sup>  | 8.8 × 10 <sup>6</sup>                | 0.8 × 10 <sup>6</sup>  | 118 × 10 <sup>4</sup>                     |
| 5 <sup>b</sup>  | VI              | France               | 2008             | 4.7 × 10 <sup>3</sup>                  | 1.8 × 10 <sup>3</sup>  | 88.2 × 10 <sup>4</sup>          | 26.8 × 10 <sup>4</sup> | 537 × 10 <sup>6</sup>                | 44.5 × 10 <sup>6</sup> | 1140 × 10 <sup>4</sup>                    |
| 6 <sup>b</sup>  | VI              | France               | 2008             | 47.5 × 10 <sup>3</sup>                 | 6.5 × 10 <sup>3</sup>  | 43 × 10 <sup>4</sup>            | 10 × 10 <sup>4</sup>   | 386 × 10 <sup>6</sup>                | 31.6 × 10 <sup>6</sup> | 81.5 × 10 <sup>4</sup>                    |
| 7               | VI              | Netherlands          | 2012             | 377                                    | 205                    | 2,271                           | 334                    | 32 × 10 <sup>6</sup>                 | 7.7 × 10 <sup>6</sup>  | 848 × 10 <sup>4</sup>                     |
| 8               | VI              | Netherlands          | 2012             | 10.8 × 10 <sup>3</sup>                 | 2.7 × 10 <sup>3</sup>  | 43.3 × 10 <sup>4</sup>          | 6.2 × 10 <sup>4</sup>  | 231.5 × 10 <sup>6</sup>              | 24.5 × 10 <sup>6</sup> | 215 × 10 <sup>4</sup>                     |
| 9               | VI              | Netherlands          | 2012             | 62 × 10 <sup>3</sup>                   | 18 × 10 <sup>3</sup>   | 210.8 × 10 <sup>4</sup>         | 45.7 × 10 <sup>4</sup> | 116.5 × 10 <sup>6</sup>              | 12.8 × 10 <sup>6</sup> | 18.7 × 10 <sup>4</sup>                    |
| 10              | VI              | Netherlands          | 2012             | 33.7 × 10 <sup>3</sup>                 | 2.5 × 10 <sup>3</sup>  | 45.9 × 10 <sup>4</sup>          | 14.3 × 10 <sup>4</sup> | 248 × 10 <sup>6</sup>                | 29 × 10 <sup>6</sup>   | 73.5 × 10 <sup>4</sup>                    |
| 11              | VI              | Netherlands          | 2012             | 5 × 10 <sup>3</sup>                    | 1 × 10 <sup>3</sup>    | 5.9 × 10 <sup>4</sup>           | 0.6 × 10 <sup>4</sup>  | 3.5 × 10 <sup>6</sup>                | 0.1 × 10 <sup>6</sup>  | 6.74 × 10 <sup>4</sup>                    |
| 12              | Semen           | Germany              | 2010             | 6.4 × 10 <sup>3</sup>                  | 1 × 10 <sup>3</sup>    | 2.7 × 10 <sup>4</sup>           | 0.35 × 10 <sup>4</sup> | 0.85 × 10 <sup>6</sup>               | 19,218                 | 1.33 × 10 <sup>4</sup>                    |
| 13              | Semen           | Germany              | 2011             | 3 × 10 <sup>3</sup>                    | 2.5 × 10 <sup>3</sup>  | 3,929                           | 617                    | 1.8 × 10 <sup>6</sup>                | 0.25 × 10 <sup>6</sup> | 5.91 × 10 <sup>4</sup>                    |
| 14              | Semen           | Germany              | 2010             | 15.3 × 10 <sup>3</sup>                 | 4.3 × 10 <sup>3</sup>  | 11.7 × 10 <sup>4</sup>          | 1.7 × 10 <sup>4</sup>  | 8.5 × 10 <sup>6</sup>                | 0.4 × 10 <sup>6</sup>  | 5.52 × 10 <sup>4</sup>                    |
| 15              | Semen           | Qatar                | 2011             | 1 × 10 <sup>3</sup>                    | 121                    | 1.26 × 10 <sup>4</sup>          | 0.35 × 10 <sup>4</sup> | 1.8 × 10 <sup>6</sup>                | 0.28 × 10 <sup>6</sup> | 17.1 × 10 <sup>4</sup>                    |
| 16              | Semen           | Germany              | 2010             | 8 × 10 <sup>3</sup>                    | 7.7 × 10 <sup>3</sup>  | 4 × 10 <sup>4</sup>             | 1.3 × 10 <sup>4</sup>  | 2.6 × 10 <sup>6</sup>                | 0.45 × 10 <sup>6</sup> | 3 × 10 <sup>4</sup>                       |
| 17              | Semen           | Netherlands          | 2010             | 820                                    | 390                    | 3.7 × 10 <sup>4</sup>           | 1.2 × 10 <sup>4</sup>  | 0.73 × 10 <sup>6</sup>               | 71,203                 | 8.99 × 10 <sup>4</sup>                    |
| 18              | Semen           | Germany              | 2012             | 33 × 10 <sup>3</sup>                   | 13 × 10 <sup>3</sup>   | 23.3 × 10 <sup>4</sup>          | 0.5 × 10 <sup>4</sup>  | 10.6 × 10 <sup>6</sup>               | 2 × 10 <sup>6</sup>    | 3.21 × 10 <sup>4</sup>                    |
| 19 <sup>b</sup> | Lung            | France               | 2007             | 539 × 10 <sup>3</sup>                  | 159 × 10 <sup>3</sup>  | 141.3 × 10 <sup>4</sup>         | 14.6 × 10 <sup>4</sup> | 87 × 10 <sup>6</sup>                 | 4.2 × 10 <sup>6</sup>  | 1.62 × 10 <sup>4</sup>                    |
| 20              | Liver           | France               | 2007             | 140 × 10 <sup>3</sup>                  | 77 × 10 <sup>3</sup>   | 5.75 × 10 <sup>4</sup>          | 10.5 × 10 <sup>4</sup> | 61.5 × 10 <sup>6</sup>               | 12.9 × 10 <sup>6</sup> | 4.38 × 10 <sup>4</sup>                    |
| 21              | Lung            | France               | 2007             | 113 × 10 <sup>3</sup>                  | 15.7 × 10 <sup>3</sup> | 44.9 × 10 <sup>4</sup>          | 10.3 × 10 <sup>4</sup> | 31 × 10 <sup>6</sup>                 | 2.8 × 10 <sup>6</sup>  | 2.74 × 10 <sup>4</sup>                    |
| 22              | Lung            | France               | 2007             | 84                                     | 26                     | 1,445                           | 221                    | 7.4 × 10 <sup>6</sup>                | 1.2 × 10 <sup>6</sup>  | 873 × 10 <sup>4</sup>                     |
| 23              | Spleen          | France               | 2007             | 942                                    | 150                    | 2,215                           | 612                    | 94,033                               | 10,357                 | 1 × 10 <sup>4</sup>                       |
| 24              | VI              | France               | 2007             | 174                                    | 48                     | 517                             | 133                    | 51,357                               | 11,365                 | 2.96 × 10 <sup>4</sup>                    |
| 25 <sup>b</sup> | Semen           | France               | 2007             | 133 × 10 <sup>3</sup>                  | 7.5 × 10 <sup>3</sup>  | 49.9 × 10 <sup>4</sup>          | 3.3 × 10 <sup>4</sup>  | 46.9 × 10 <sup>6</sup>               | 4 × 10 <sup>6</sup>    | 3.52 × 10 <sup>4</sup>                    |
| 26 <sup>b</sup> | Semen           | France               | 2007             | 80.5 × 10 <sup>3</sup>                 | 11.2 × 10 <sup>3</sup> | 25.9 × 10 <sup>4</sup>          | 2 × 10 <sup>4</sup>    | 73.3 × 10 <sup>6</sup>               | 3.7 × 10 <sup>6</sup>  | 9.07 × 10 <sup>4</sup>                    |
| 27              | Semen           | France               | 2007             | 46.6 × 10 <sup>3</sup>                 | 33 × 10 <sup>3</sup>   | 12.3 × 10 <sup>4</sup>          | 2.5 × 10 <sup>4</sup>  | 13.25 × 10 <sup>6</sup>              | 1 × 10 <sup>6</sup>    | 2.84 × 10 <sup>4</sup>                    |
| 28              | Semen           | France               | 2007             | 9.6 × 10 <sup>3</sup>                  | 2.3 × 10 <sup>3</sup>  | 1.65 × 10 <sup>4</sup>          | 1,694                  | 1.4 × 10 <sup>6</sup>                | 219,702                | 1.47 × 10 <sup>4</sup>                    |
| 29              | Semen           | France               | 2007             | 6.5 × 10 <sup>3</sup>                  | 701                    | 9,615                           | 1,997                  | 4.1 × 10 <sup>6</sup>                | 615,044                | 6.46 × 10 <sup>4</sup>                    |
| 30              | Semen           | France               | 2008             | 11 × 10 <sup>3</sup>                   | 3 × 10 <sup>3</sup>    | 3.3 × 10 <sup>4</sup>           | 0.8 × 10 <sup>4</sup>  | 12.5 × 10 <sup>6</sup>               | 1.8 × 10 <sup>6</sup>  | 11.3 × 10 <sup>4</sup>                    |
| 31              | Semen           | France               | 2008             | 48.5 × 10 <sup>3</sup>                 | 17.7 × 10 <sup>3</sup> | 14.5 × 10 <sup>4</sup>          | 6 × 10 <sup>4</sup>    | 26.8 × 10 <sup>6</sup>               | 2.9 × 10 <sup>6</sup>  | 5.53 × 10 <sup>4</sup>                    |
| 32 <sup>b</sup> | Semen           | France               | 2008             | 32.5 × 10 <sup>3</sup>                 | 6 × 10 <sup>3</sup>    | 4 × 10 <sup>4</sup>             | 5.9 × 10 <sup>4</sup>  | 21.1 × 10 <sup>6</sup>               | 10.6 × 10 <sup>6</sup> | 6.50 × 10 <sup>4</sup>                    |
| 33              | Semen           | France               | 2007             | 46.5 × 10 <sup>3</sup>                 | 2.3 × 10 <sup>3</sup>  | 10.6 × 10 <sup>4</sup>          | 0.9 × 10 <sup>4</sup>  | 5.8 × 10 <sup>6</sup>                | 0.82 × 10 <sup>6</sup> | 1.25 × 10 <sup>4</sup>                    |
| 34              | Semen           | France               | 2007             | 182                                    | 23                     | 461                             | 31                     | 6,770                                | 2,514                  | 3,720                                     |
| 35              | Semen           | France               | 2008             | 11 × 10 <sup>3</sup>                   | 3 × 10 <sup>3</sup>    | 5.5 × 10 <sup>4</sup>           | 2.6 × 10 <sup>4</sup>  | 0.950 × 10 <sup>6</sup>              | 0.5 × 10 <sup>6</sup>  | 8,670                                     |
| 36              | Semen           | France               | 2008             | 15.2 × 10 <sup>3</sup>                 | 1.5 × 10 <sup>3</sup>  | 7.9 × 10 <sup>4</sup>           | 0.45 × 10 <sup>4</sup> | 1.9 × 10 <sup>6</sup>                | 32,714                 | 1.25 × 10 <sup>4</sup>                    |
| 37              | Semen           | France               | 2008             | 21.2 × 10 <sup>3</sup>                 | 778                    | 6 × 10 <sup>4</sup>             | 0.4 × 10 <sup>4</sup>  | 9.3 × 10 <sup>6</sup>                | 0.8 × 10 <sup>6</sup>  | 4.36 × 10 <sup>4</sup>                    |
| 38 <sup>b</sup> | VI              | France               | 2008             | 200 × 10 <sup>3</sup>                  | 11.5 × 10 <sup>3</sup> | 119 × 10 <sup>4</sup>           | 22.5 × 10 <sup>4</sup> | 196 × 10 <sup>6</sup>                | 20.5 × 10 <sup>6</sup> | 9.78 × 10 <sup>4</sup>                    |
| 39              | VI              | France               | 2008             | 155 × 10 <sup>3</sup>                  | 18.2 × 10 <sup>3</sup> | 9.8 × 10 <sup>4</sup>           | 5.4 × 10 <sup>4</sup>  | 107.8 × 10 <sup>6</sup>              | 16.5 × 10 <sup>6</sup> | 6.96 × 10 <sup>4</sup>                    |
| 40              | VI              | Netherlands          | 2012             | 184 × 10 <sup>3</sup>                  | 31 × 10 <sup>3</sup>   | 84 × 10 <sup>4</sup>            | 8.7 × 10 <sup>4</sup>  | 156.5 × 10 <sup>6</sup>              | 5 × 10 <sup>6</sup>    | 8.49 × 10 <sup>4</sup>                    |
| 41              | Semen           | Germany              | 2010             | 15.8 × 10 <sup>3</sup>                 | 1.2 × 10 <sup>3</sup>  | 4 × 10 <sup>4</sup>             | 1 × 10 <sup>4</sup>    | 4.4 × 10 <sup>6</sup>                | 0.4 × 10 <sup>6</sup>  | 2.78 × 10 <sup>4</sup>                    |
| 42              | Semen           | Germany              | 2012             | 61.3 × 10 <sup>3</sup>                 | 36.3 × 10 <sup>3</sup> | 3.75 × 10 <sup>4</sup>          | 1.3 × 10 <sup>4</sup>  | 1.499 × 10 <sup>6</sup>              | 2.12 × 10 <sup>6</sup> | 245 × 10 <sup>4</sup>                     |
| 43 <sup>b</sup> | VI              | France               | 2008             | 184 × 10 <sup>3</sup>                  | 7.6 × 10 <sup>3</sup>  | 51.6 × 10 <sup>4</sup>          | 13.7 × 10 <sup>4</sup> | 916 × 10 <sup>6</sup>                | 161 × 10 <sup>6</sup>  | 49.8 × 10 <sup>4</sup>                    |
| 44              | Semen           | France               | 2008             | 2.3 × 10 <sup>3</sup>                  | 1.8 × 10 <sup>3</sup>  | 3,248                           | 1,056                  | 7,458                                | 521                    | 326                                       |
| 45              | Semen           | France               | 2008             | 3.7 × 10 <sup>3</sup>                  | 1 × 10 <sup>3</sup>    | 5,418                           | 673                    | 0.65 × 10 <sup>6</sup>               | 18,091                 | 1.77 × 10 <sup>4</sup>                    |
| 46              | Semen           | France               | 2008             | 320                                    | 95                     | 586                             | 146                    | 375                                  | 90                     | 117                                       |
| 47              | Semen           | France               | 2008             | 167                                    | 49                     | 1,502                           | 865                    | 5,566                                | 180                    | 3,320                                     |
| 48 <sup>b</sup> | VI              | USA (Bucyrus strain) | 1953             | 57.8 × 10 <sup>3</sup>                 | 11.8 × 10 <sup>3</sup> | 12.5 × 10 <sup>4</sup>          | 3 × 10 <sup>4</sup>    | 9.9 × 10 <sup>6</sup>                | 0.28 × 10 <sup>6</sup> | 1.72 × 10 <sup>4</sup>                    |
| Mean copy no.   |                 |                      |                  | 53.75 × 10 <sup>3</sup> <sup>d,e</sup> | 11.4 × 10 <sup>3</sup> | 31.10 <sup>4</sup> <sup>d</sup> | 6.6 × 10 <sup>4</sup>  | 112.5 × 10 <sup>6</sup> <sup>e</sup> | 55 × 10 <sup>6</sup>   |   |

<sup>a</sup> Copy numbers obtained with purified RNA (assay 1), purified RNA retrotranscribed into cDNA (assay 2), and purified RNA retrotranscribed and then amplified with the Phi29 polymerase (assay 3) are shown. The gains in sensitivity between assays 1 and 3 are also shown (ratio of copy numbers).

<sup>b</sup> Samples tested on the high-density resequencing DNA microarray.

<sup>c</sup> VI, culture fluid from virus isolation performed on RK-13 cells (ATCC CCL-37), as described in reference 6.

<sup>d</sup> Among these two values,  $P < 0.05$  (using Student's *t* test).

<sup>e</sup> Among these two values,  $P < 0.05$  (using Student's *t* test).

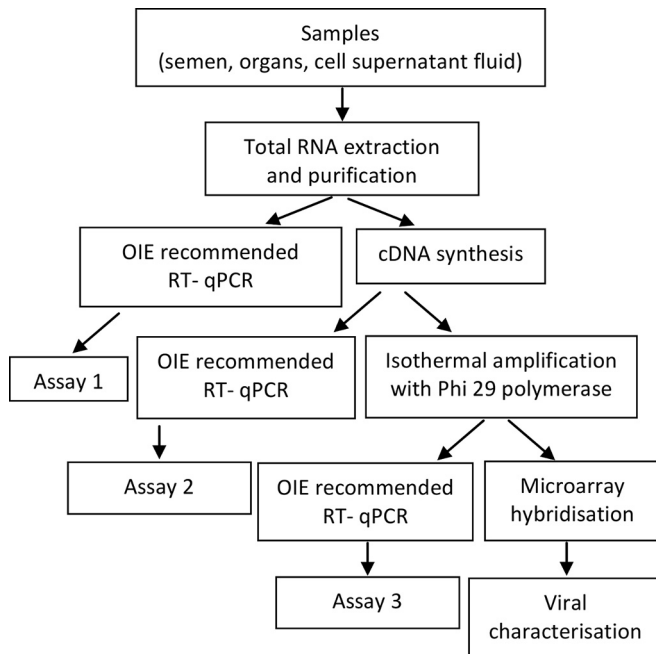


FIG 1 Schematic view of EAV detection by qPCR and RT-qPCR using three different assays (assays 1, 2, and 3).

varied markedly depending on the assay performed. The mean copy numbers of viral genome for the 48 positive samples obtained with assays 1, 2, and 3 were  $53.75 \times 10^3 \pm 11.4 \times 10^3$ ,  $31 \times 10^4 \pm 6.6 \times 10^4$ , and  $112.5 \times 10^6 \pm 55 \times 10^6$ , respectively. Our results show that isothermal amplification with Phi29 polymerase significantly increased the ratio of amplification compared to assay 1. Indeed, the detected amount of DNA with assay 3 was  $10^2$  to  $10^7$  times the amount detected using assay 1 (Table 1). Therefore, compared to the OIE-recommended RT-qPCR, the analytical sensitivity of assay 3 is significantly enhanced (Student's *t* test,  $P < 0.05$ ) (Table 1). These findings were obtained regardless of the sample's origin (culture fluid, raw semen, or homogenized organs).

In addition to enhancing the sensitivity of the current diagnostic method, the second objective of our study was to rapidly genotype the viruses detected. We therefore decided to combine the unbiased amplification of nucleic acids with a high-density resequencing microarray. The two EAV sequences tiled on the microarray cover a region of 267 bp (Table 2) located in the ORF 1 region encoding the nonstructural protein 9 (nsp9) that is involved in RNA synthesis (11). Amplified DNA samples were fragmented and labeled using the GeneChip resequencing assay kit (Affymetrix, Inc., Santa Clara, CA). After overnight hybridization at 45°C, the RMA was washed, stained, and scanned according to the manufacturer's instructions. The raw image file (.DAT) ob-

TABLE 2 EAV partial ORF1 sequences of two 267-nucleotide strains from the *Arteriviridae* family tiled on the resequencing microarray

| Viral strain              | Location along ORF 1 | GenBank Accession no. |
|---------------------------|----------------------|-----------------------|
| CW 96                     | 6579–6845            | AY349167              |
| Bucyrus (EAV ATCC VR-796) | 6575–6841            | DQ846750              |

TABLE 3 Call rate value for each of the 12 strains tested on the RMA<sup>a</sup>

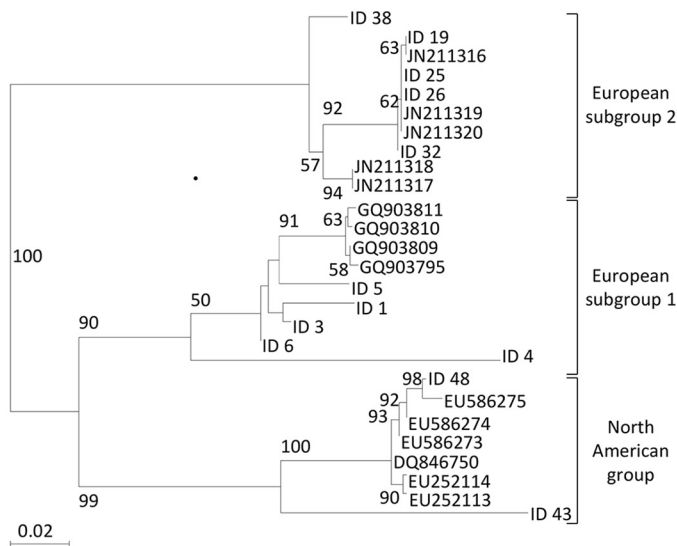
| Sample no. | Call rate value (%) | Phylogenetic group   |
|------------|---------------------|----------------------|
| 1          | 34.69               | European subgroup 1  |
| 3          | 34.69               | European subgroup 1  |
| 4          | 34.69               | European subgroup 1  |
| 5          | 51.43               | European subgroup 1  |
| 6          | 50.61               | European subgroup 1  |
| 19         | 56.33               | European subgroup 2  |
| 25         | 52.65               | European subgroup 2  |
| 26         | 45.97               | European subgroup 2  |
| 32         | 60                  | European subgroup 2  |
| 38         | 71.43               | European subgroup 2  |
| 43         | 73.47               | North American group |
| 48         | 90.2                | North American group |

<sup>a</sup> Call rate value is the ratio of the number of determined bases (A, T, C, or G) to a sequence length of 243 bp.

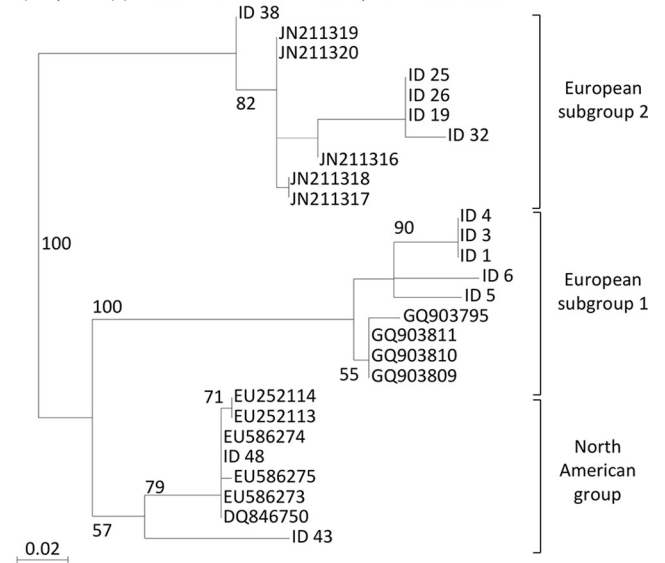
tained after scanning was transformed into a fluorescence intensity file (.CEL) using GeneChip operating software (GCOS) (Affymetrix, Inc.). Bases were called using a derivative of the ABACUS base-calling algorithm (12). Sequences were output in FASTA format, and for each sequence obtained, the call rate value was calculated as the ratio of the number of determined bases (A, T, C, or G) to a sequence length of 243 bp (Table 3). Sequences retrieved from the microarray were used to perform an evolutionary study. The phylogenetic trees were constructed using the maximum-likelihood method, and the tree's statistical robustness was assessed by bootstrap resampling (100 data sets) of the multiple alignments. Phylogenetic reconstructions were performed using SeaView software, version 4 (13). In the literature, EAV isolates have been classified into two distinct groups: the North American group (NA) and the European group (EU) (14). The EU group can be subdivided into two subgroups known as European subgroup 1 (EU-1) and European subgroup 2 (EU-2) (14). This classification was obtained by aligning and comparing either the 3-kb sequences covering the ORF2-to-ORF7 region of the EAV genome, which encodes the structural protein, or a 518-nucleotide portion of ORF5, from positions 11,296 to 11,813, encoding viral glycoprotein 5 (14). A phylogenetic analysis with EAV sequences, retrieved from either GenBank or the 12 samples in our study (samples 1, 3, 4, 5, 6, 19, 25, 26, 32, 38, 43, and 48), was performed according to the internationally recommended rules described above. A phylogenetic tree constructed with the 518-nucleotide sequence obtained following ORF5 sequencing (Fig. 2a) confirmed the classification of known EAV strains. Moreover, samples 1 to 6, 19 to 38, and 43 to 48 were classified as being in the EU-1, EU-2, and NA groups, respectively (Fig. 2b). The products obtained after isothermal amplification from these 12 samples were hybridized on the RMA (Table 1). Surprisingly, the phylogenetic tree obtained with the nsp9 nucleotide sequences retrieved from the microarray separated the strains into the NA and EU groups and divided the EU group into subgroups EU-1 and EU-2 (Fig. 2b). Thus, this result indicates that the 243-nucleotide sequence from the ORF1 region amplified in our study could also be used for genotyping EAV.

This study is the first in the scientific literature to use an unbiased isothermal amplification method using the Phi29 polymerase in combination with a high-density resequencing microarray for diagnosing nidoviruses. Furthermore, amplifying EAV RNA by

a) PhyML In(L)=-2768.3 768 sites GTR 100 replic. 4 rate classes



b) PhyML In(L)=-763.7 243 sites GTR 100 replic. 4 rate classes



**FIG 2** Phylogenetic analysis of the partial ORF5 nucleotide sequences (518 nucleotides in length) (a) and partial ORF1 nucleotide sequences (243 nucleotides in length) (b) of 27 EAV strains. Twelve isolates are specific to this study, and 15 are from previously published EAV strains. Neighbor-joining and maximum-likelihood trees were constructed using SeaView version 4, and equivalent results were obtained. Only the maximum-likelihood trees are shown. The percentages of trees, >50 only, in which the associated taxa clustered together are shown next to the branches. The North American group and European subgroups 1 and 2 are indicated.

whole transcription amplification, which requires an additional time of 10 h, provides much greater sensitivity than the OIE-recommended RT-qPCR method developed in 2002 by Balasuriya et al. (6, 7). Its association with a resequencing microarray has greatly reduced the time taken for genotyping even if it remains more expensive than reference RT-qPCR and Sanger sequencing of PCR amplicons. However, the resequencing microarray is less expensive than next-generation sequencing for EAV genotyping. This method can be recommended for the detection of EAV in semen and aborted fetuses, especially when the viral load is very low. In addition, this study validated the usefulness of the high-density resequencing DNA microarray for the diagnosis of equine viral diseases and the genotyping of RNA viruses such as equine arteritis virus.

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