Evaluating methods for Avian avulavirus-1 whole genome sequencing

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ABSTRACT

Background: Avian avulavirus-1 (AAV-1, previously Newcastle Disease Virus) is responsible for poultry and wild birds' disease outbreaks. Numerous whole genome sequencing methods were reported for this virus. These methods included cloning, specific primers amplification, shotgun PCR approaches, Sequence Independent Single Primer Amplification and next generation sequencing platform kits.

Methods: Three methods were used to sequence 173 Israeli Avian avulavirus-1 field isolates and one vaccine strain (VH). The sequencing was performed on Proton and Ion Torrent Personal Genome Machine and to a lesser extent, Illumina MiSeq and NextSeq sequencers. Target specific primers (SP) and Sequence Independent Single Primer Amplification (SISPA) products sequenced via the Ion torrent sequencer had a high error rate and truncated genomes. All the next generation sequencing platform sequencing kits generated high sequence accuracy and near-complete genomic size.

Results: A high level of mutations was observed in the intergenic regions between the avian avulavirus-1 genes. Within genes, multiple regions are more mutated than the Fusion region currently used for typing.

Conclusions: Our findings suggest that the whole genome sequencing by the Ion torrent sequencing kit is sufficient. However, when higher fidelity is desired, the Illumina NextSeq and Proton torrent sequencing kits were found to be preferable.

1. Introduction

Newcastle Disease Virus, currently named Avian avulavirus 1 (Amarasinghe et al., 2017) is the causative agent of Newcastle disease (ND). The disease affects over 200 species of birds including wild birds, pigeons and poultry. Newcastle disease outbreaks can have a devastating effect on flocks with mortality approaching 100%. AAvV-1 was first described in 1926 in Newcastle-on-Tyne England (hence the name) and on the island of Java (Alexander, 2001).

AAV-1 is a member of the order Mononegavirales, family Paramyxoviridae, genus Avulavirus (Afonso et al., 2016; Amarasinghe et al., 2017; De Battisti et al., 2013). The virus has an approximately 15.2 kb ssRNA genome with varying lengths (Shi et al., 2011). The viral genome is composed of six genes expressing six proteins: nucleoprotein [NP], matrix, phosphoprotein [P], [M], fusion [F], hemagglutinin-neuraminidase [HN], and RNA polymerase [L]. AAV-1 has only one serotype but has varying degrees of virulence (Gogoi et al., 2015). The F protein is cleaved between amino acid 111–117. The AAV-1 virulence
is correlated with the frequency of basic amino acids in the F protein proteolytic cleavage site (Gogoi et al., 2015). Accordingly, AAvV-1 has five virulence categories or pathotypes. These virulence pathotypes are according to severity, starting with the highly virulent to the non-virulent: Velogenic neurotropic, Velogenic viscerotropic, Mesogenic, Lentogenic and asymptomatic (Ganan et al., 2014).

The AAvV-1 genome was previously sequenced using several methods, including: cloning (De Leeuw and Peeters, 1999), sequencing portions of the viral genome (Barbezange and Jestin, 2003, 2005), or sequencing the full genome using target specific primers (Absalón et al., 2014; Meng et al., 2012; Munir et al., 2011). Prior to whole genome sequencing kits, most publications used target specific primer amplification of the viral genome. This method utilizes AAvV-1-universal primers that can be applied to any AAvV-1 subtype as well as a rapid amplification of cDNA ends (RACE) to obtain the viral genome 5′ and 3′ end sequences (Chenchik et al., 1996; Li et al., 2005; Schaefer, 1995; Shi et al., 2011; Shi and Jarvis, 2006). Pyrosequencing and shotgun PCR methods were also successful in producing a whole genome sequence or parts of the whole genome (Campana et al., 2014; Ma et al., 2017; Djikeng et al., 2008; Karlsson et al., 2013; Van Borm et al., 2013).

In this study we evaluated the performances of three different methods for AAvV-1 whole genome sequencing during AAvV-1 epizootic research. The three methods used were the target specific primer (SP) approach followed by SISPA, a shotgun PCR approach, as well as produced than the Ion Torrent sequencing kits (Jahresara et al., 2016; Karlsson et al., 2013).

2. Materials and methods

2.1. Viral isolation

Five tracheal or cloacal swabs were pooled in a tube containing 2-3 ml PBS solution (HyLabls, Cat. BP 532/100S). The sample was incubated for 1 h, at room temperature. A 250 μl aliquot of the sample was added to a 2 ml Eppendorf containing 1.5 ml of PBS and 250 μl of X10 antibiotics solution for injection (HyLabls, Cat. TT359). The solution was incubated for 1 h at room temperature before injection to 9–11 days old embryonated chicken eggs (ECE's). A total of 200 μl of the prepared solution was injected per egg. Eggs were examined for seven days post injection using an egg lamp. Eggs containing dead embryos were opened and the allantoic fluid was collected and stored at −20 °C.

2.2. Viral RNA purification

AAvV-1 RNA purification was based on previously published methods (Dimitrov et al., 2014; Jahresasara et al., 2016; Hussain et al., 1989). Briefly, a total of 6 ml thawed allantoic fluid were added to 4 ml PBS. The samples were centrifuged in the Beckman Optima XE-90K centrifuge at 9100 g for 30 min at 4 °C to remove tissues and cells. The clear supernatant was transferred into clear ultra centrifuge tubes (Beckman #344058) and was supplemented with 25 ml of PBS to a final volume of 35 ml. The samples were centrifuged in the Beckman Optima XE-90K with the swing rotor (SW 28) at 70,000g for 3 h at 4 °C. Following centrifugation, the supernatant was decanted and the pellet containing the virus was resuspended in 110 μl PBS (final volume 170 ul). The viral suspension was transferred to an Eppendorf tube containing 30 μl of DNaSe-RNase buffer. The DNaSe-RNase buffer was comprised of 10 units of DNaSe and 4.5 units of RNaseA suspended in x1 DNaSe buffer (NEB Cat. M0303L and Sigma Cat. R4875, respectively). The viral suspension was incubated at 37 °C for 2 h to decrease host DNA and RNA. RNA purification was performed using the Trizol LS kit (Thermo Fisher, Cat 10296028) according to the manufacturer's instructions. Viral RNA was stored at −80 °C.

2.3. Sequence specific primer amplification of AAvV-1 RNA

A total of 260 AAvV-1 full genome sequences from NCBI and one Israeli AAvV-1 vaccine (VH) sequence were used (sequence courtesy of Dr. Benet-Noach, Phibro Israel). The sequences were aligned using Clustal Omega v1.1.0 (Sievers et al., 2011). The resulting reverse transcription primers and PCR primers (see Table 1) were designed to amplify 3.5 kb overlapping amplicons of the AAvV-1 genome.

Optimized reverse transcription was performed using Agilent's Accuscript high fidelity Reverse Transcriptase (Cat. 600089) with the following modifications. A total of 100 ng RNA template was supplemented with 2 μl of accuscript buffer (×10), 2 μl of 10 μm reverse transcription primers mix, 0.8 μl of dNTP's 100 mM and DEPC treated DDW to a final volume of 10 μl. Pre-boil was performed at 65 °C for 15 min and the tubes were allowed to cool slowly to room temperature, according to the manufacturer's instructions. The pre-boiled mix was supplemented with 3 μl of DEPC DDW, 2 μl DTT, 3 μl of 20% DMSO solution, 1 μl of Betain 5 M solution and 1 μl of Accuscript reverse transcriptase enzyme. The reverse transcription was performed at 37 °C for 2 h followed by a heat inactivation step at 70 °C for 15 min.

The Q5 high fidelity PCR mix (NEB, Cat. M0493) was optimized to efficiently amplify up to 6 kb of the viral genome. To this end, 2 μl of the reverse transcription reaction were used as template supplemented with 2 μl of 10 μM forward and reverse primers, 2 μl of 10 mM dNTPs, 4 μl of NEB Q5 buffer (×5), 0.4 μl Q5 enzyme, 3 μl of 20% DMSO solution, 3 μl of Betain 5 M solution and 3.6 μl DEPC DDW (20 μl final reaction volume). The PCR reaction was performed in the S1000 thermal cycler (Biorad). The reaction conditions were as follows: 95 °C for 3 min, 35 cycles of 95 °C – 20 s, 35 °C – 30 s, 72 °C – 45 s followed by a final step of 72°C - 10 min. The PCR results were examined in a 1.5% agarose gel using Ethidium bromide prior to sequencing. The PCR reactions were performed using Strategex's PCR and enzymatic reaction purification kit (MBRacpe kit, Statec, Cat. 1,020,220,300) according to the manufacturer's instructions.

2.4. SISPA

The SISPA protocol was performed according to the literature with the modifications listed below (Djikeng et al., 2008). Briefly, 100 ng of

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**Table 1**

| AAvV-1 universal reverse transcription primers (AAvV-1uniRT) and amplification primers (AAvV-1final). |
|---------------------------------------------------------------|
| **Final primers** | **Sequence** | **From** | **To** | **Product** |
|-------------------|--------------|----------|--------|-------------|
| AAvV-1uni RT_1     | gcattgYgaRctgacatctg | 2427     | 2461   |             |
| AAvV-1uni RT_2     | caaatgcYtcctgacatctgacatctg | 4207     | 4236   |             |
| AAvV-1uni RT_3     | cctcgacatctgacatctgacatctg | 5027     | 5053   |             |
| AAvV-1uni RT_4     | acYctgacatctgacatctgacatctg | 7757     | 7776   |             |
| AAvV-1uni RT_5     | gcRgacaacatctgacatctgacatctg | 8409     | 8431   |             |
| AAvV-1uni RT_6     | ggYccaagacatctgacatctgacatctg | 10,546   | 10,570 |             |
| AAvV-1uni RT_7     | gccaagacatctgacatctgacatctg | 12,975   | 12,994 |             |
| AAvV-1uni RT_8     | tatteggYggagntctgacatctgacatctg | 14,223   | 14,243 |             |
| AAvV-1uni IF      | gcRgacaacatctgacatctgacatctg | 656      | 673    | 3281        |
| AAvV-1final IF     | tcYccaagacatctgacatctgacatctg | 3915     | 3937   |             |
| AAvV-1final IF     | agYctgacatctgacatctgacatctg | 3891     | 3947   | 3533        |
| AAvV-1final IF     | ccctcgacatctgacatctgacatctg | 7353     | 7424   |             |
| AAvV-1final IF     | gcYgacatctgacatctgacatctg | 5652     | 5671   | 2851        |
| AAvV-1final IF     | agYgacatctgacatctgacatctg | 8482     | 8503   |             |
| AAvV-1final IF     | cmYgacatctgacatctgacatctg | 8472     | 8500   | 3442        |
| AAvV-1final IF     | tcaatgcYgRaggagntctgacatctgacatctg | 11,890   | 11,914 |             |
| AAvV-1final IF     | aacacYgacatctgacatctgacatctg | 10,905   | 10,924 | 3791        |
| AAvV-1final VR     | ccYgacatctgacatctgacatctg | 14,677   | 14,696 |             |
purified RNA were reverse transcribed using Agilent Accuscript reverse transcriptase using 1 μl of 10 mM FR26RV-N primer (5’ GCCGGAGCT CTGCCAGATATC GNNNNNNN 3’), 2 μl Agilent RT buffer, 0.8 μl of 100 mM dNTPs and DEPC DDW were added to a final volume of 13 μl. The pre-boil was performed at 65 °C for 15 min and the samples were allowed to cool to room temperature. The reverse transcription reaction supplemented contained 2 μl DTT, 3 μl DMSO 20%, 1 μl Betain 5 M and 1 μl Agilent accsscript reverse transcriptase, reaching a final reaction volume of 20 μl. The reaction mix was incubated for 1 h at 37 °C followed by heat inactivation at 75 °C for 15 min. The reverse transcription reaction was supplemented with Klenow fragment reaction mix. The Klenow reaction contained 5 μl NEB buffer 2 (X10), 1 μl FR26RV-N primer, 1 μl (5 units) of Klenow fragment (NEB Cat. M0210S), 1 μl of 10 mM dNTPs and DEPC DDW to a mix volume of 30 μl. The Klenow reaction was incubated at 37 °C for 1 h followed by heat inactivation at 70 °C for 10 min. The reaction was purified using Stratagene’s PCR and enzymatic reaction purification kit, as before, having a final elution volume of 20 μl.

The PCR reaction was performed using the Q5 high fidelity polymerase kit, as listed above, to generate full length templates (see Appendix A and Supplementary Information). Of these, 173 were field isolates (10 wild bird; 31 backyard birds; 132 commercial poultry) and the Israeli AAoV-1 vaccine (VH) were sequenced using different platforms. The viruses were sequenced using three different methods including the specific primers (34), SISPA (57) and 96 samples via NGS sequencing kits (8 IonTorrent, 84 Proton Torrent, 2 Illumina MiSeq and 2 Illumina NextSeq). Several samples were sequenced using multiple methods. Except for the VH, all these methods utilized enriched viral RNA that was purified from the allantoic fluid (Jakhesara et al., 2016). The VH genome was purified from a commercial vaccination tablet.

For the template-specific primers method, AAoV-1 universal primers were designed using the entire Pubmed database AAoV-1 sequences. Eight forward AAoV-1 universal primers were used for the reverse transcription reaction step and five universal primer pairs amplified 3.5 kb overlapping segments of the 15.2 kb viral cDNA. The resulting PCR products were purified prior to library preparation and sequenced via the Ion Torrent PGM platform kits followed by genome assembly. Preliminary tests using the lentogenic VH vaccine RNA as a template generated a contig of 14,000 nt's. The resulting VH contig covered ~94% of the viral genome (680–14,680 nt's). Following this test, 34 AAoV-1 velogenic field isolates were sequenced using this method. However, in all the field isolates the first amplicon (656–3937) was missing, resulting in a partial contig covering 72% of the viral genome (3890 nt–14,680 nt; see Fig. 1). The first amplicon PCR failure presumably occurred due to a poly-C region in the AAoV-1 genome located in a ~100 bp region near 1700 nt (1656–1761). The velogenic AAoV-1 field isolates have a poly-C region containing 3–4 repeats of 5–6 tandem Poly-Cytosine while the VH sequence had a smaller poly-C region containing only 3 repeats of 3–4 tandem repeats (data not shown). This region has a highly consistent drop in viral genome coverage in all the velogenic samples sequenced, regardless of the amplification method or NGS platform used (see Figs. 1 and 2). This is likely due to G-quadruplex (Chambers et al., 2015).

The SISPA method was developed to sequence novel RNA viruses (Froussard, 1992). It has been shown to be efficient and to successfully sequence the AAoV-1 genome as well as other RNA viruses (Allander et al., 2001; Dijkeng et al., 2008; Dijkeng and Spiro, 2009). The standard SISPA primers were examined in silico to verify no match to the AAoV-1 known sequences, thus avoiding technical issues (Karlsson et al., 2013). The SISPA method resulted in a contig coverage of over 99% of the viral genome. Additional 56 AAoV-1 isolates were sequenced using the SISPA method via the Ion Torrent sequencer. This resulted in an average coverage of 98–99% of the viral genomes (see Figs. 1 and 2).

The Ion Torrent, Illumina MiSeq and NextSeq RNA library preparation and sequencing kits were also examined using the VH vaccine and Israeli isolates. Purified VH RNA sequenced using the Ion Torrent sequencing kit resulted in 99.98% of the viral genome with the small exception of 5–7 mismatches compared to the known sequence. These mismatches were found only at the ends of the viral genome (at the sense 5’ end). The VH RNA was successfully sequenced using Illumina
NextSeq reaching an average depth of 80,000 reads/nt, and a perfect 100% match to the known VH sequence. A total of 6 AAvV-1 field isolates were sequenced using the Ion Torrent sequencing kit followed by a second set of 82 isolates sequenced using the ProtonTorrent sequencing kit with a final average cover of > 99% of the viral genome (see Figs. 1, 2).

All three sequencing platforms and their kits (IonTorrent, ProtonTorrent and Illumina) had similar nucleotide profile with few N reads or gaps. The SP and SISPA methods had higher N reads compared to the NGS sequencer kit average (8.9 and 10.3 respectively) (see Fig. 3).

Given the high sequencing accuracy and coverage of the NGS methods, we addressed the mutational load at each site within a host (sample). The high coverage allows us to detect not only the main consensus sequence, but also the quasi-species of the virus genomic population. A high mutation load was seen in SISPA and the specific primers sequences all along the viral genome, suggesting that these observed mutations are mainly sequencing errors. However, in the NGS based methods, mutations are limited to specific regions. The high mutation and deletion rate is observed in all sequencing methods near the 3′ and 5′ ends of the viral genome is possibly due to the folding of the viral genome ends leading to misreading. The mutation hotspots in the viral genome mutations are mainly between the genes, regardless of the method or NGS sequencer used (see Fig. 4a and b). Note that the

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**Fig. 1.** Average coverage of each position in the AAvV-1 viral genome sequences, per method. The viruses were sequenced using three different methods including the specific primers (34), SISPA (57) and 96 samples via NGS sequencing kits (8 IonTorrent, 84 Proton Torrent, 4 Illumina). In all the methods a drop in the coverage near nucleotide 1700 is seen, possibly due to the G-quadruplexes. The Illumina and Proton Torrent have the highest coverage. The lowest coverage was obtained by the specific primers and SISPA methods (sequenced via the Ion Torrent).

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**Fig. 2.** Fraction of covered samples in each position along the sequence per method as measured by the fraction of samples that have at least five reads for a given position (otherwise, the position is assigned an N). The coverage drop at position 1,700 is clearly observed, as well as the coverage drop in the 5’ and 3’ ends of the viral genome. Intragenic regions also have a high error rate.
average mutation rate with a sample in the F gene typically used to classify the virus into sub-types is lower than in most other genes.

To further estimate the variability, we computed the fraction of NS mutations. While, there is no practically no difference between sequencing methods, as expected intergenic regions have significantly higher NS fractions compared with genic regions (Data not shown).

4. Discussion

Brute force NGS sequencing approach (i.e. total DNA or RNA purification and sequencing) has its own advantages (Li et al., 2016). It is mostly used for sequencing a small number of samples at low coverage. However, for sequencing of tens to hundreds of samples it is more cost-effective to remove the host genomic DNA or RNA, as much as possible (Dimitrov et al., 2017; Thomson et al., 2016; Tyler et al., 2016). This can be done using the physical properties of the virion (Jakhesara et al., 2016; Hussain et al., 1989) or by using purification kits post DNA or RNA purifications (Dimitrov et al., 2014, 2017). The latter involves a considerable loss of target genomic material due to the purification steps losses. During this study we opted for the former approach for viral RNA purification (Temmam et al., 2015). This method resulted in 90–95% viral RNA sequences (data not shown). The low host DNA and RNA enabled loading the NGS platforms with a large pool of tagged cDNA samples while still obtaining high coverage. For the Ion Torrent and Proton Torrent up to 12 and 16 samples were loaded per sequencing run, respectively.

Several methods and sequencing platforms were reported to amplify the AAvV-1 genome. The specific primer approach was mostly used for AAvV-1 whole genome sequencing (Absalón et al., 2014; Meng et al., 2012; Munir et al., 2011; Wei et al., 2008). However, the partial sequence obtained, the need for the RACE method to amplify the viral genome ends, the sequence inaccuracies and the amplification termination near position 1700 nt’s of the field isolate genomes all suggest that such a method is not appropriate for whole genome sequencing of AAvV-1 (Chenchik et al., 1996; Li et al., 2005; Liu and Gorovsky, 1994; Schaefer, 1995; Shi and Jarvis, 2006).

The SISPA method was also shown to reliably sequence the AAvV-1 genome (Allander et al., 2001; Chrzastek et al., 2017; Djikeng et al., 2008; Karlsson et al., 2013). The sequencing of other viruses using this method has failed, possibly due to technical issues (Karlsson et al., 2013). In our hands, SISPA successfully sequenced the AAvV-1 genome via the Ion Torrent PGM. Similarly to the specific primers method, it was found to have high error rates resulting in a considerably higher mutation rate compared to the sequencing kits of the NGS platforms. The SISPA was usually unable to sequence 100-200 bp from the 5’ and 3’ ends of the viral genome. This problem could possibly be resolved using a higher coverage, at the cost of running fewer samples per chip.

Our findings concur with the reports of PCR amplification bias in NGS sequencing which were recently reported in several articles (Chrzastek et al., 2017; Dimitrov et al., 2017; Jones et al., 2015; Pinard et al., 2006; Thomson et al., 2016; Tyler et al., 2016; Van Borm et al., 2016). While PCR amplified samples can be used for sequencing low titer samples, the bias involved should be considered and avoided when possible (Aigrain et al., 2016; Chrzastek et al., 2017; Dimitrov et al., 2017; Huggett et al., 2015; Thomson et al., 2016; Tyler et al., 2016; Van Borm et al., 2016). In our hands, the Illumina NextSeq, ProtonTorrent and Ion Torrent sequencing kits generated nearly full viral genome (98–99% for the Ion and proton torrent systems and 100% for the Illumina NextSeq) with few mismatches at the 3’ and 5’ ends. From our experience, it is advisable to opt for a high coverage sequence (a minimum coverage of 5000, preferably 50,000) to cover the 1700 nt position (the poly-C region) as well as the 7000 nt region. We therefore recommend using the Proton Torrent or the Illumina Nextseq sequencing kits for whole genome sequencing of AAvV-1 isolates.

Given the high fidelity of the NGS based sequencing, it is possible to study beyond the main variant. The quasi-species cloud (surrounding each main variant) is highly mutated in the intergenic regions, and in the 3’ and 5’. We have found a mutation rate of 30 mutations/year in the viral genomes examined (data not shown). Such mutations are expected, since their effect on the viral fitness may be limited. Considering the viral reverse transcriptase mutation rate, we expected a higher mutation in the viral genome (Menéndez-Arias, 2002; Menéndez-Arias et al., 2017). The AAvV-1 found mutation rate suggests there is a natural selection process. This process is possibly affecting the expected mutation rate and limiting the regions that can be affected (Fan et al., 2017; Miller et al., 2009).
5. Conclusions

We have evaluated different methods of AAvV-1 full genome sequencing. The main conclusions are a clear recommendation to purify the viral genome prior to sequencing. PCR amplifications of the viral genomes, either using shotgun PCR (SISPA in this case) or via target specific primers have generated poor results compared to NGS platform kits. Sequencing kits of the Illumina NextSeq and Proton torrent PGM have successfully resulted in 99–100% of the viral genome at a high coverage. The high coverage obtained via NGS methods shows a map of mutations in the AAvV-1 genome which are mainly in intergenic regions.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2019.100004.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Tal Saar, Ben Izhak Meirav, Wachtel Chaim and Wiseman Anat and Broun Tzipi have made substantial contribution to the experimental design and acquisition of data. Yechezkel Elinor, Golan Einav, Hadash Ruth, Adi Turjeman, Banet Noach Caroline, Michal Bronstein and Avishai Lublin have all have
made substantial contributions to acquisition of data, methodology and resources.

Berman Elyakum, Raviv Ziv, Pirak Michael, Klement Eyal and Louzoun Yoram have made substantial contributions to analysis and interpretation of data, as well as funding acquisition.

Tal Saar, Wiseman Anat and Louzoun Yoram have been involved in drafting the original manuscript draft as well as reviewing and editing the final draft.

Pirak Michael, Klement Eyal, Raviv Ziv and Louzoun Yoram have been involved in Project administration.

**Appendix A**

Table of AAvV-1 isolates and their origin.

| Sample number | Source | Specific primers | SISPA | Platform sequencing kits |
|---------------|--------|------------------|-------|--------------------------|
|               |        |                  |       | Ion torrent | Proton torrent | MiSeq | NextSeq |
| 1818          | EPB    | v                |       | v           | v            | v      | v       |
| 11278         | EPB    | v                |       | v           | v            | v      | v       |
| 120420        | EPB    | v                |       | v           | v            | v      | v       |
| 173849        | EPB    | v                |       | v           | v            | v      | v       |
| 176674        | EPB    | v                |       | v           | v            | v      | v       |
| 120147        | EPB    | v                |       | v           | v            | v      | v       |
| 120430        | EPB    | v                |       | v           | v            | v      | v       |
| 195810        | EPB    | v                |       | v           | v            | v      | v       |
| Lasota        | Phibro | v                |       | v           | v            | v      | v       |
| 120809        | EPB    | v                |       | v           | v            | v      | v       |
| 124026        | EPB    | v                |       | v           | v            | v      | v       |
| 141353        | EPB    | v                |       | v           | v            | v      | v       |
| 120860        | EPB    | v                |       | v           | v            | v      | v       |
| 120654        | EPB    | v                |       | v           | v            | v      | v       |
| 120423        | EPB    | v                |       | v           | v            | v      | v       |
| 124027        | EPB    | v                |       | v           | v            | v      | v       |
| 123498        | EPB    | v                |       | v           | v            | v      | v       |
| 137699        | EPB    | v                |       | v           | v            | v      | v       |
| 120807        | EPB    | v                |       | v           | v            | v      | v       |
| 137495        | EPB    | v                |       | v           | v            | v      | v       |
| 123526        | EPB    | v                |       | v           | v            | v      | v       |
| 121808        | EPB    | v                |       | v           | v            | v      | v       |
| 104477        | EPB    | v                |       | v           | v            | v      | v       |
| 138692        | EPB    | v                |       | v           | v            | v      | v       |
| 137592        | EPB    | v                |       | v           | v            | v      | v       |
| 122509        | EPB    | v                |       | v           | v            | v      | v       |
| 137569        | EPB    | v                |       | v           | v            | v      | v       |
| 99188         | EPB    | v                |       | v           | v            | v      | v       |
| 114599        | EPB    | v                |       | v           | v            | v      | v       |
| 121118        | EPB    | v                |       | v           | v            | v      | v       |
| 125877        | EPB    | v                |       | v           | v            | v      | v       |
| 118279        | EPB    | v                |       | v           | v            | v      | v       |
| 115038        | EPB    | v                |       | v           | v            | v      | v       |
| 114112        | EPB    | v                |       | v           | v            | v      | v       |
| 141759        | EPB    | v                |       | v           | v            | v      | v       |
| 142551        | EPB    | v                |       | v           | v            | v      | v       |
| 142626        | EPB    | v                |       | v           | v            | v      | v       |
| 142784        | EPB    | v                |       | v           | v            | v      | v       |
| 143114        | EPB    | v                |       | v           | v            | v      | v       |
| 143696        | EPB    | v                |       | v           | v            | v      | v       |
| 144702        | EPB    | v                |       | v           | v            | v      | v       |
| 147004        | EPB    | v                |       | v           | v            | v      | v       |
| 149165        | EPB    | v                |       | v           | v            | v      | v       |
| 152628        | EPB    | v                |       | v           | v            | v      | v       |
| 153812        | EPB    | v                |       | v           | v            | v      | v       |
| 156916        | EPB    | v                |       | v           | v            | v      | v       |
| 159057        | EPB    | v                |       | v           | v            | v      | v       |
| 166631        | EPB    | v                |       | v           | v            | v      | v       |
| 168757        | EPB    | v                |       | v           | v            | v      | v       |
| 169730        | EPB    | v                |       | v           | v            | v      | v       |
| 170338        | EPB    | v                |       | v           | v            | v      | v       |
| 170394        | EPB    | v                |       | v           | v            | v      | v       |
| 175271        | EPB    | v                |       | v           | v            | v      | v       |
| 176851        | EPB    | v                |       | v           | v            | v      | v       |
| 201029        | EPB    | v                |       | v           | v            | v      | v       |
| 203145        | EPB    | v                |       | v           | v            | v      | v       |
| 203308        | EPB    | v                |       | v           | v            | v      | v       |
| 203762        | EPB    | v                |       | v           | v            | v      | v       |
| 205659        | EPB    | v                |       | v           | v            | v      | v       |
| 205770        | EPB    | v                |       | v           | v            | v      | v       |
| 206519        | EPB    | v                |       | v           | v            | v      | v       |
| 207436        | EPB    | v                |       | v           | v            | v      | v       |
| 209108        | EPB    | v                |       | v           | v            | v      | v       |
Note: Specific primers and SISPA samples were sequenced using the IonTorrent. 
Acronyms: EPB - egg and poultry board. KVI - Kimron Veterinary institute.

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