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1 **Controversial results of the genetic analysis of a canine distemper vaccine strain**

2
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12 13 **Abstract**

14 Canine distemper (CD) is a highly contagious, often fatal, multisystemic viral disease of
15 receptive carnivores. The presence of a *PsiI* cleavage site on a specific location of the
16 hemagglutinin (H) gene was found to be a hallmark of vaccine strains, thus, a previously
17 published restriction fragment length polymorphism (RFLP) test using *PsiI* theoretically
18 allows the distinction between all currently used vaccine strains and virulent field strains. The
19 RFLP test was carried out on all brands of CD vaccines available in Hungary. The present
20 work describes the extensive sequencing and phylogenetic study of the strain present in
21 Vanguard (Pfizer Animal Health) vaccines, which following the *PsiI* based RFLP test reacted
22 as a wild-type strain. Based on the product description provided by the manufacturer, all
23 batches should have contained a virus strain (Snyder Hill) belonging to the group of vaccine
24 strains (America-1). Extensive genetic analysis involving the full nucleic acid sequence of
25 four other genes (N, M, P and F) of the CDV genome revealed that the incriminated virus

1 strain showed a higher level of genetic identity to wild-type strains from the America-2 group
2 than to any of the strains belonging to America-1 group, therefore the vaccine does not
3 contain the virus strain stated by the manufacturer in its product description and has not been
4 containing it since at least 1992.

5

6 **Keywords:** canine distemper, vaccine strain, phylogenetic analysis

7

8 **Running title:** Canine distemper vaccine strain ambiguity

9

10 **1. Introduction**

11 Canine distemper (CD) is a highly contagious viral disease that has a wide range of receptive
12 carnivore animal families, such as the *Canidae*, *Mustelidae*, *Procyonidae*, *Felidae* and many
13 others. It is caused by the canine distemper virus (CDV) which belongs to the *Morbillivirus*
14 genus of the *Paramyxoviridae* virus family (Appel, 1987; Kabakci et al., 2004; Lednicky et
15 al., 2004). Although live attenuated vaccines have been used for many years to control
16 distemper (Chappuis, 1997; Barrett, 1996; Patel & Heldens, 2009), CDV continues to cause
17 outbreaks in receptive domestic carnivores all around the world, in particular young dogs in
18 breeding units and shelter facilities which most often suffer fatal disease (Appel, 1987;
19 Blixenkrone-Møller et al., 1993; Barrett, 1996; Gemma et al., 1996; Demeter et al., 2006;
20 Martella et al., 2008). Previous genetic and phylogenetic studies have revealed that the virus
21 strains used in the currently applied vaccines form a distinct group (America-1) from the large
22 group of wild-type strains that were reported worldwide in the previous decades (Martella et
23 al., 2007; Demeter, 2009). The most frequently used strains in the manufacturing process of
24 CDV vaccines are the Onderstepoort, Snyder Hill, Rockborn, Lederle, and Convac. The
25 Onderstepoort strain, used worldwide as an attenuated live vaccine, dates back to a disease

1 outbreak among North American rancher foxes in the 1930's (Haig, 1956; Martella et al.,
2 2007). The Snyder Hill strain was isolated in Ithaca, USA in the 1950's from the brain of a
3 dog. The virus was then passaged *in vivo* in dogs before being adapted to cell growth in NL-
4 DKC cells (Brown et al., 1972; Martella et al., 2007). The Rockborn strain is
5 undistinguishable from the Snyder Hill strain (Greene & Appel, 2006), while all the other
6 vaccine strains (Lederle, Convac etc.) also belong to the America-1 group. Nowadays it is not
7 clear whether these strains are still circulating in the field, since they have not been detected
8 over the last five decades (Martella et al., 2007).

9 In recent years the number of vaccinated dogs that got infected with CDV and
10 developed characteristic clinical signs of CD has increased (Appel, 1987; Blixenkron-Møller
11 et al., 1993; Kai et al., 1993; Shin et al., 1995; Gemma et al., 1996; Lan et al., 2006). The
12 molecular analysis of the isolates from the vaccinated yet infected canines demonstrated that
13 the causative agent in these cases was not closely related to any of the currently used vaccine
14 strains (Pardo et al., 2005; Lan et al., 2006; Calderon et al., 2007; Martella et al., 2007). On
15 the other hand, cases when vaccinated animals shed the vaccine strain for a limited period of
16 time were also described (Greene & Appel, 2006). Considering the fact that in case of
17 recently vaccinated animals the virus strain present in the vaccine can interfere with
18 polymerase chain reaction (PCR) based diagnostic tests, leading to diagnostically false
19 positive results, in order to rule out the mentioned misleading factors, a PCR coupled with
20 restriction fragment length polymorphism (RFLP) assay using a *PsiI* cleavage site of the
21 hemagglutinin (H) gene of the viral genome was designed (Demeter et al., 2007). According
22 to the nucleotide sequences deposited in the publicly accessible GenBank, the presence of
23 *PsiI* cleavage site is characteristic only for vaccine strains, thus, *PsiI* RFLP was expected to
24 allow a reliable differentiation of all CDV vaccine strains from the wild-type ones. During the
25 examination of CDV vaccines marketed in Hungary by *PsiI* RFLP, the vaccine strain present

1 in a Vanguard vaccine (Pfizer Animal Health, USA) exhibited wild-type genetic feature
2 remaining undigested by *PsiI*. This study presents the results of the extensive sequencing and
3 phylogenetic investigation of batches of this vaccine brand purchased in different countries
4 (Hungary, Malta, Israel, and USA), as well as batches dating back to 1992 and 1994.

5

6 **2. Materials and methods**

7 *2.1. Vaccine batches*

8 Products of four different manufacturers were purchased and tested during this study. A total
9 number of ten vaccines were investigated, of which six were of the same brand (vaccines
10 number 1 to 6), but purchased in different countries (Hungary, Israel, Malta and United States
11 of America) or were from batches dated back to 1992, 1994 and 2006 (Table 1). All modified
12 live CD vaccines were stored at the recommended temperature of 4 °C.

13

14 *2.2. RNA purification*

15 Vaccines were prepared according to the protocol provided by each manufacturer. Following
16 dilution of the lyophilized component with the solution recommended and supplied together
17 with the vaccine, viral RNA was isolated from each vaccine using QIAamp viral RNA Mini
18 Kit (Qiagen, Germany) according to the manufacturer's instructions. After purification, the
19 RNA samples were stored at -80 °C until the following steps of the investigations.

20

21 *2.3. Primers*

22 The primers used in the present study were designed using the Primer Designer 4 software
23 (Scientific & Educational Software, USA). Sequences of different vaccine and wild-type
24 CDV strains were retrieved from GenBank and were aligned to ensure the attaching of the

1 newly designed primers to the vaccine strains and wild-type strains as well. Primers were
2 synthesized by BIOMI Kft. (Gödöllő, Hungary).

3

4 *2.4. RT-PCR assays, RFLP and gel electrophoresis*

5 Reverse transcription and amplifications were performed in one-step RT-PCR method using
6 the QIAGEN OneStep RT-PCR Kit (Qiagen, Germany), according to the manufacturer's
7 instructions. Reverse transcription was carried out at 50 °C for 30 min. Following an initial
8 denaturation at 95 °C for 15 min, the reaction mixture was subjected to 40 cycles of heat
9 denaturation at 94 °C for 45 sec, the corresponding annealing temperature for each primer
10 pair for 1 min, and DNA extension at 72 °C for 2 min, followed by a final extension of 10 min
11 at 72 °C. The reactions were performed in a SPRT001 PCR Sprint Thermal Cycler (Hybaid
12 Ltd, UK).

13 Following RT-PCR 7.5µl of amplicons was electrophoresed at 80 V for 80 min in a
14 1.2% Tris acetate-EDTA-agarose gel (Merck, Germany), stained with ethidium bromide. The
15 bands were visualized by UV transillumination at 312 nm. Product sizes were determined
16 with reference to 100 bp and 1 kb molecular weight ladders (Fermentas, Lithuania). The *PsiI*
17 (SibEnzyme, Russia) based RFLP was carried out according to the previously described
18 protocol (Demeter et al., 2007).

19

20 *2.5. Sequence analysis and phylogeny*

21 DNA was extracted from the gel with the QiaQuick Gel Extraction Kit (Qiagen, Germany).
22 Fluorescence-based direct sequencings were performed in both directions on the amplicons by
23 BIOMI Kft. (Gödöllő, Hungary) using an ABI 3100 genetic analyzer (Applied Biosystems,
24 USA). Nucleotide sequences were identified by BLAST search against gene bank databases
25 (<http://www.ncbi.nlm.nih.gov/BLAST/>). The complete sequences of each gene were compiled

1 and aligned to the complete genome sequence of CDV using the Align Plus 4 software
2 (Scientific & Educational Software, USA). The nucleotide sequences of the Vanguard vaccine
3 strain were deposited in the GenBank under the following accession numbers: EF095750 (H
4 gene), EU072198 (F gene), EU072199 (M gene), EU072200 (N gene) and EU072201 (P
5 gene). Phylogenetic trees of the nucleic acid and putative amino acid (aa) sequences were
6 established by using sequence data of the analyzed vaccine strains and the ones retrieved from
7 the GenBank (most of the available complete sequences of the analyzed genes of vaccine and
8 wild-type strains as well). In case of the Vanguard vaccine, each gene of the CDV genome
9 was analyzed, except the highly conserved large polymerase (L) gene. Phylogenetic analyses
10 were performed with the help of the ClustalX program and the robustness of the groupings in
11 the neighbor-joining analysis was assessed with 1000 bootstrap resampling. Results of the
12 phylogenetic analysis were verified using Bayesian and maximum likelihood (ML)
13 approaches (Ronquist, F. & Huelsenbeck, 2003) with the MrBayes 3.1.2 program. In order to
14 identify any recombination the recombination detection program (RDP:
15 <http://darwin.uvigo.es/rdp/rdp.html>) was used (Martin & Rybicki, 2000).

16

17 **3. Results**

18 *3.1. RT-PCRs and RFLP analysis*

19 The RT-PCRs resulted amplicons of the expected size (Table 1) in case of every pair of
20 primers used. The RFLP resulted two clearly differentiable bands in case of the currently used
21 vaccine strains at the predicted heights of 294 bp and 816 bp, while the amplicons obtained
22 from field viruses and vaccines 1 to 6 (Table 1) remained undigested (1110 bp).

23 Different batches of Vanguard vaccines (Pfizer Animal Health, USA) dating back to
24 1992, 1994 and 2006 were also involved in the study. Furthermore, batches of the same brand
25 were purchased in different countries as well, such as Hungary, Israel, Malta and the United

1 States of America (Table 1). The RFLP test unequivocally resulted in undigested amplicons in
2 case of all batches from different time and geographical origin.

3

4 *3.2. Genetic analyses*

5 To identify the strain present in the Vanguard vaccines, primers were designed to cover more
6 than half of the viral genome. The nucleotide sequences of all CDV genes, except the highly
7 conserved L gene were determined. The genetic analyses of all investigated genes of the
8 Vanguard vaccines (Table 1) have revealed that the virus strain present in all of them is more
9 closely related to different wild-type strains from the America-2 group, than to the one stated
10 by the manufacturer (Snyder Hill strain) or any of the viruses from the group of vaccine
11 strains (Table 2). As a next step the full nucleotide sequences of the highly variable H gene of
12 all vaccine brands currently used in Hungary were included in the study (Table 1). Since the
13 obtained nucleotide sequences of vaccines 1 to 6 turned out to be 100% similar in case of all
14 batches, the subsequent investigations performed on other domains of the virus genome were
15 carried out using only vaccine number 6. According to the investigation of the complete
16 sequence of the H gene, the investigated virus strain was not positioned anywhere close to the
17 Snyder Hill strain, but it showed a significantly higher similarity to a lesser panda isolate
18 (99.45%) and a wild-type strain (98.90%) isolated from a naturally infected dog (strain 25259,
19 accession number: AY964114) in North America (Figure 1). As expected following the initial
20 RFLP, the virus strains present in vaccines 7-10 were positioned in the group of vaccine
21 strains (America-1). The findings of the neighbour-joining method-based phylogenetic
22 analysis were confirmed by the results of the Bayesian and ML methods as well: the
23 Vanguard vaccine strain showed a considerably higher level of identity with wild-type viruses
24 from the America-2 group (data not shown). RDP could not identify any recombination in
25 case of any of the analyzed genes of the Vanguard vaccine strain (data not shown).

1 The genetic analyses of the full nucleic acid sequences of the F, N, M and P genes
2 only confirmed the initial observations of the H gene analysis: in each case the virus strain
3 present in the Vanguard vaccine was not positioned in the group of vaccine strains (America-
4 1). Unfortunately the nucleic acid sequence of the Snyder Hill strain is not publicly available
5 for these genomic segments, except for a partial, 335 bps long F gene sequence: AY288312
6 and a 390 bps long partial P gene segment: AY286481. Even though it cannot be considered as
7 reliable as complete gene sequence analyses, the genetic analysis of such small segments
8 yielded results identical with the initial investigations performed on the H gene.

10 **4. Discussion**

11 In the present study, we have applied a previously described *PsiI*-based RFLP test (Demeter
12 et al., 2007) that theoretically allows the differentiation of all wild-type strains with known
13 nucleotide sequence from all vaccine CDV strains. The differentiating RFLP analysis carried
14 out on CD vaccine brands currently used in Hungary revealed that the CDV strain present in
15 the Vanguard (Pfizer Animal Health, USA) vaccine reacted as a wild-type strain. According
16 to the statement of the manufacturer, the European Vanguard products used to immunize dogs
17 against CD contain the Snyder Hill strain. Based on the nucleotide sequence available in the
18 GenBank (complete sequence of the H gene of the Snyder Hill strain: accession number:
19 AF259552), the amplicons of the virus strain present in the Vanguard vaccine should have
20 been digested by the *PsiI* enzyme, but they remained undigested, hence reacted as a wild-type
21 virus. In order to elucidate the dilemma, an extensive genetic and phylogenetic analysis of the
22 incriminated virus strain was conducted. Primer pairs that enabled the amplification of
23 complete genes of the CDV genome were designed and the nucleotide sequences of all CDV
24 genes (H, N, M, P and F), except the highly conserved L gene were determined. For
25 phylogenetic purposes the H gene was chosen, because of its vital role in the infectious

1 process (Murphy et al., 1999) this gene is considered as one of the most variable one in the
2 CDV genome (Haas et al., 1997; Harder & Osterhaus, 1997; Iwastuki et al., 1997;
3 Mochizuki et al., 1999; Uema et al., 2005; Martella et al., 2006, McCarthy et al., 2007),
4 therefore it is profoundly suitable for phylogenetic analysis. H gene analysis further
5 emphasized the previous findings: the investigated virus strain was not positioned anywhere
6 close to the Snyder Hill strain, but turned out to be most closely related to a wild-type virus
7 strain isolated from a lesser panda (AF178039). Similar observations were reported by
8 another research group (Pardo et al., 2005), but the investigated segment was too small to
9 obtain relevant results (979 bases). Based on the findings of this research group the virus
10 strain present in the Vanguard vaccine was most closely related to the A75/17 wild-type strain
11 isolated from a dog, but the investigation of the complete sequence of the H gene has revealed
12 that it is more closely related to the lesser panda isolate (99.45 %) and a wild-type strain
13 (98.90 %) isolated in North America from a naturally infected dog (strain 25259, accession
14 number: AY964114) by the same research group (Pardo et al., 2005). The analysis of other
15 genes of CDV has only confirmed the findings of the H gene analysis.

16 In order to extend our investigations in time and geographical respect, Vanguard
17 vaccine vials produced in different time (1992 and 1994) and different geographical origin
18 (Malta, Israel, and USA) were purchased and the same RFLP test was applied to determine
19 whether these vaccines contain the same virus strain as the vaccine purchased in Hungary in
20 2006. The result of the RFLP test and the complete sequencing of the H gene of the virus
21 strains present in all these Vanguard vaccine batches revealed that all of them contain exactly
22 the same virus strain, showing not even one nucleotide difference on this relatively large and
23 highly variable gene. Due to these findings, the vaccine batches dated back to 1992 and 1994
24 and the ones purchased in other countries were not investigated on other segments of the
25 CDV genome, since those results most probably would have only confirmed the previous

1 observations. On the other hand, these findings led to the conclusion that the Vanguard
2 vaccines have been containing the analyzed virus strain since at least 1992.

3 There could be several possible explanations for the findings of the present study, such
4 as (1) the incorrect labelling of the Snyder Hill strain deposited in the GenBank (AF259552) –
5 contradicted by its positioning among the group of vaccine strains in case of the phylogenetic
6 trees constructed based on the nucleic acid sequences of the full length of the H gene (Figure
7 1) and partial segment of the F gene (data not shown); (2) a possible recombination of the
8 seed virus with a wild-type strain – this theory is eliminated by the simultaneous RDP-based
9 analysis of several different genes; (3) due to a taxonomical and technical error (i.e. erroneous
10 laboratory registries) the strain was incorrectly labelled when the vaccine was registered and
11 finally (4) contamination of the seed virus stock used in the vaccine production, as also
12 suggested by Pardo et al. (2005). At this point the last two explanations cannot be
13 demonstrated neither eliminated. The significance of these findings is inconclusive without
14 further studies, as phylogeny can not reveal whether or not a virus is virulent, and there may
15 be little genetic distance between virus passages with dramatic changes in their phenotypes,
16 including virulence. At this point it can only be declared that the vaccine does not contain the
17 virus strain erroneously stated by the manufacturer in its product description and has not been
18 containing it since at least as 1992. Surely the vaccine strain underwent all the required tests
19 before being licensed and proved to be safe to be used in animals, and no claims of inefficacy
20 of the Vanguard vaccines have been reported. There are only a few reports of animals
21 vaccinated with Vanguard vaccines that still developed clinical disease (McInnes et al., 1992;
22 Pardo et al., 2005), but the exact cause of the lack of protection and the exact role of the
23 vaccine strain in the infection was not elucidated, therefore no relevant conclusions can be
24 drawn regarding the efficacy of the Vanguard vaccines, as there are numerous aspects that can
25 interfere with the successful immunization of an animal (Povey, 1986; Greene & Appel,

1 2006). As no documented concerns regarding the safety and efficacy of the Vanguard strains
2 have been raised since at least 1992, it is possible that this recently and apparently
3 successfully attenuated wild-type strain could provide, at least theoretically, a better
4 protection against the currently circulating wild-type CDV strains.

5 On the other hand, these findings suggest that the previously described *PsiI*-based
6 identification of America-1 (vaccine) strains could lead to diagnostically false results in case
7 of animals recently vaccinated with any Vanguard vaccine, or in case of animals with
8 uncertain vaccination history that could have been vaccinated with a Vanguard vaccine.

9

10 **5. Acknowledgements**

11 The authors would like to thank Pfizer Animal Health division of Pfizer Inc. for the support
12 and assistance during the investigations described in the present study.

13

14 **6. Follow-up**

15 Following the notification of the research group, the manufacturer has performed the
16 sequencing of the vaccine master-seed strain used globally for the production of the vaccine.

17 The work has resulted in a regulatory review of, and a change in, the strain designation in the
18 product literature globally in early 2009. This work demonstrated that the distemper strain
19 used in vaccine production was misidentified back in 1956 when it was first acquired.

20 Documentary evidence of these findings was supplied to the European regulatory agencies,
21 and they have accepted the evidence of the misclassification, and the integrity of the
22 registration study data and subsequent production batches. The strain is now identified in the
23 product descriptions as N-CDV.

24

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1 **Figure 1:** Phylogenetic tree constructed based on a total number of 165 complete nucleotide
2 sequences (1824 bp) of the H gene. In case of each strain the accession number along with the
3 species and country of origin is presented (when applicable).
4 * result of a recombination between an “Asia-1” and an “European wildlife” strain (Han et al.,
5 2008)

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Table 1: Description of the vaccines used in the present study

	Vaccine brand	Manufacturer	Batch number	Year of production	Country of origin
1	Vanguard	Norden Laboratories	NA ^a	1992	Hungary
2	Vanguard	Smith Kline	NA ^a	1994	Hungary
3	Vanguard Plus 5	Pfizer Animal Health	L53665	2006	Hungary
4	Vanguard Plus 5	Pfizer Animal Health	A602088C	2006	Israel
5	Vanguard Plus 7	Pfizer Animal Health	L60065	2006	Malta
6	Vanguard Plus 5	Pfizer Animal Health	A602620B	2006	USA
7	Canigen DH(A2)PPi	Ceva Sante Animale	12TU	2006	Hungary
8	Eurican	Merial	L246779	2006	Hungary
9	Nobivac DHP	Intervet	A030C01	2006	Hungary
10	Canvac 8	Dyntec	040407	2007	Hungary

^a not available

Table 2: The level of identity between the nucleotide and amino acid sequence of the H gene and H protein of the strain present in the Vanguard vaccines and those of the viruses from the group of vaccine strains (shaded) and of the wild-type strains, which showed the highest level of identity and the Snyder Hill strain (bolded). The values in italic represent the level of identity at the amino acid level.

	Vanguard	Panda	Dog	Snyder Hill	Convac	Canigen	Onder.	Eurican	Japan
Vanguard	-	98.84	98.35	91.77	<i>90.95</i>	<i>90.95</i>	<i>91.44</i>	<i>90.13</i>	<i>91.44</i>
Panda	99.45	-	<i>97.86</i>	<i>91.61</i>	<i>91.44</i>	<i>90.78</i>	<i>91.28</i>	<i>89.96</i>	<i>91.28</i>
Dog	98.90	<i>98.57</i>	-	<i>90.78</i>	<i>89.96</i>	<i>89.96</i>	<i>90.46</i>	<i>89.14</i>	<i>90.46</i>
Snyder Hill	92.92	<i>92.70</i>	<i>92.32</i>	-	<i>95.39</i>	<i>95.23</i>	<i>95.72</i>	<i>93.91</i>	<i>95.72</i>
Convac	<i>93.09</i>	<i>93.31</i>	<i>92.48</i>	<i>97.09</i>	-	<i>96.87</i>	<i>97.53</i>	<i>95.55</i>	<i>97.36</i>
Canigen	<i>93.03</i>	<i>92.81</i>	<i>92.43</i>	<i>96.87</i>	<i>98.46</i>	-	<i>99.01</i>	<i>98.19</i>	<i>99.17</i>
Onder.	<i>93.25</i>	<i>93.03</i>	<i>92.65</i>	<i>97.09</i>	<i>98.68</i>	<i>99.39</i>	-	<i>97.69</i>	<i>99.50</i>
Eurican	<i>92.59</i>	<i>92.37</i>	<i>91.99</i>	<i>96.32</i>	<i>97.91</i>	<i>99.23</i>	<i>98.84</i>	-	<i>97.69</i>
Japan	<i>93.25</i>	<i>93.03</i>	<i>92.65</i>	<i>97.09</i>	<i>98.68</i>	<i>99.45</i>	<i>99.61</i>	<i>98.90</i>	-

Vanguard: EF095750; Snyder Hill: AF259552; Convac: Z35493; Canigen: DQ903854; Onder: AF378705, Eurican: AF014953; Japan: AB212966; Panda (Lesser Panda): AF178039; Dog (25259): AY964114