European Food Safety Authority

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STATEMENT OF EFSA

Scientific advice on the suitability of data for the assessment of DNA integration into the fish genome of a genetically modified DNA plasmid-based veterinary vaccine[1]

European Food Safety Authority[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

Pancreas disease caused by salmonid alphavirus in farmed Atlantic salmon (*Salmo salar*) leads to high mortality rates post infection and histopathological lesions in several organs. As protection against pancreas disease, Novartis developed a prophylactic DNA plasmid-based vaccine to be administered to salmon as naked plasmid in a single intramuscular injection. In order to assess the legal status of the fish vaccinated with this new vaccine with regard to the legislation on genetically modified organisms, the European Commission suggested that the company carry out a scientific study on the integration/non-integration of the plasmid DNA into the fish genome. Subsequently, the European Commission requested EFSA to give scientific advice on the study design and the conclusions drawn by the company. PCR based analysis of genomic DNA from muscle samples, taken from at or around the injection site 436 days post vaccination, led the company to conclude that integration of plasmid DNA into the fish genome is extremely unlikely. After an assessment of the study, EFSA considers that the study presented by Novartis Animal Health on the integration/non-integration of DNA plasmid-based vaccine into the salmon genomic DNA provides insufficient information on the potential integration of plasmid DNA fragments into the fish genome due to a limited coverage of the plasmid DNA by the detection method provided, the limited number of samples analysed and an insufficient limit of detection and method validation. Therefore, EFSA is of the opinion that the results from the integration/non-integration study submitted by Novartis Animal Health are not sufficient to support the conclusion of non-integration of plasmid DNA into the fish genome drawn by the company.

KEY WORDS

DNA plasmid-based vaccine, genomic DNA, salmon, study design, DNA integration, genetically modified organisms, Directive 2001/18/EC

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[2] Correspondence: GMO@efsa.europa.eu

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SUMMARY

Pancreas disease caused by salmonid alphavirus in farmed Atlantic salmon (*Salmo salar*) leads to high mortality rates post infection and histopathological lesions in several organs. A new DNA plasmid-based vaccine was developed by Novartis to be injected prophylactically in salmon and to confer protection against the pancreas disease caused by this virus.

In order to assess the legal status of fish vaccinated with a new DNA plasmid-based vaccine developed by Novartis with regard to the legislation on genetically modified organisms, the European Commission suggested that the company carry out a scientific study on the integration/non-integration of the plasmid DNA into the fish genome. Novartis Animal Health submitted a study assessing the integration potential of the DNA plasmid-based vaccine in the salmon genome (Report No NAHC/RD/01/02). Genomic DNA isolated from muscle samples collected from at or around the site of injection 436 days after vaccination, were analysed after several rounds of field inversion gel electrophoresis with a plasmid-specific quantitative PCR primer pair and probe. After analysis of the results, Novartis concluded that it would be extremely unlikely for plasmid DNA to be integrated into the fish genome.

On 28 January 2013, EFSA was requested by the European Commission to give scientific advice on the study design and the conclusions drawn by the company. After assessing the data presented in the integration/non-integration study submitted by Novartis Animal Health, EFSA considers that the study presented by Novartis Animal Health on the integration/non-integration of DNA plasmid-based vaccine into the salmon genomic DNA provides insufficient information on the potential integration of plasmid DNA fragments into the fish genome due to a limited coverage of the plasmid DNA by the detection method provided, the limited number of samples analysed and an insufficient limit of detection and method validation. Therefore, EFSA is of the opinion that the results from the integration/non-integration study submitted by Novartis Animal Health are not sufficient to support the conclusion of non-integration of plasmid DNA into the fish genome drawn by the company.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The pharmaceutical company Novartis asked the European Medicines Agency (EMA) Committee on Veterinary Medical Products (CVMP) in June 2010 whether a fish vaccinated with a DNA plasmid-based vaccine falls within the scope of Directive 2001/18/EC or not.

The plasmid used for the vaccination has an origin of replication that allows high copy number replication in bacterial cells. Regarding eukaryotic control elements, Novartis states that the vector contains only promoters, enhancers and terminators. The company says that there are no other known control elements for eukaryotes. Based on these informations, the plasmid DNA could only replicate in the fish if it were integrated into the fish genome.

DG SANCO suggested to Novartis to carry out scientific studies on the integration/non-integration of the DNA plasmid-based vaccine to clarify whether or not there is recombination between the DNA plasmid and the fish DNA, in order to assess the legal status of a fish vaccinated with this plasmid.

Novartis performed an integration/non-integration study and forwarded the outcome of this study to the European Commission.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

EFSA is requested to give scientific advice on whether the design of the integration/non-integration study and the conclusion drawn by the company leading to the interpretation that there is no integration, are scientifically sound.

ASSESSMENT

1. INTRODUCTION

Pancreas disease (PD) caused by salmonid alphavirus in farmed Atlantic salmon (*Salmo salar*) leads to high mortality rates post infection and histopathological lesions in the pancreas, heart and skeletal muscle. While PD is generally diagnosed in growing fish in their second year at sea, infection can occur in all phases of the marine production cycle.

As protection against PD, Novartis developed a prophylactic DNA plasmid-based vaccine to be administered to salmon as naked plasmid in a single intramuscular injection. In order to assess whether fish vaccinated with the DNA plasmid-based vaccine should be considered a genetically modified organism (GMO) as defined by Directive 2001/18, the European Commission suggested that the company carry out a scientific study on the integration/non-integration of the plasmid DNA into the fish genome. Subsequently, the European Commission requested EFSA to give scientific advice on the design of the study and the conclusions drawn by the company leading to the interpretation that there is no integration of plasmid DNA into the salmon genome.

In the following sections the outcome of EFSA's assessment of the study submitted by Novartis Animal Health is presented.

2. OVERVIEW OF THE INTEGRATION STUDY NAHC/RD/01/12 AS PERFORMED BY NOVARTIS ANIMAL HEALTH

2.1. Study design and results

On the initial day of the study, pre-smolt Atlantic salmon of average weight (9.0 ± 1.4 g) were injected intramuscularly with 2× or 10× the dose of plasmid DNA intended to be injected for commercial vaccination. For the integration study, 10 individual epaxial muscles from at and around the site of injection were harvested at 436 days post vaccination (DPV) from salmon injected with 2× the vaccination dose, and total DNA was isolated. For a simultaneously performed biodistribution study, both muscle and non-injection site organs were analysed. Non-injection site organs of 10 fish were sampled at intervals from day 1 to 60 after vaccination and analysed with quantitative polymerase chain reaction (qPCR) for the distribution of plasmid DNA. Only results for the group vaccinated with the 2x dose are included in the report. On day 1, plasmid was detected in gut, spleen, gonads, head kidney and heart. By day 60, all organs had plasmid levels below the detection limit. Muscle samples of 10 fish were collected from day 1 until 331 from both 2x and 10x vaccinated groups. These samples were positive for plasmid DNA and showed a decreasing plasmid level through time (at day 331 less than 1% of the plasmid concentration measured at day 1 post vaccination remained).

To distinguish between high molecular weight (HMW) DNA, such as genomic DNA (gDNA), and smaller DNA, such as plasmid DNA, a field inversion gel electrophoresis (FIGE) technique was used for the integration study. In FIGE, every 180 degree re-orientation of the applied electric field contributes to the separation of small DNA fragments from large DNA fragments in the gel (Carle and Carle, 1992). To eliminate potential plasmid concatamers, all DNA was first digested with a restriction enzyme that digests the plasmid DNA at a single site, but is a rare cutter in genomic fish DNA. After electrophoresis, HMW DNA was visualised and fragments larger than 17 kb were excised, eluted from the gel and analysed for the presence of plasmid DNA sequences with qPCR.

The qPCR assay was performed with primers and probe that bind to the plasmid DNA sequence, giving rise to a PCR product spanning the intersection of vector backbone and insert. The limit of detection (LOD) was validated to 10 plasmid DNA copies per µg of salmon gDNA, while the lower limit of quantification was set at 50 plasmid copies per µg gDNA. All samples were tested and reported to be free of the presence of PCR inhibitors.

HMW DNA samples were analysed individually by qPCR. Samples below the LOD were tested for the presence of the 18S rRNA gene to confirm the initial presence of gDNA. Samples that tested above the LOD were subjected to additional rounds of FIGE to improve the separation of HMW DNA from plasmid DNA. In order to have enough DNA to visualise the HMW DNA on the gels in these additional rounds, DNA of samples with similar qPCR values was pooled after the first and second FIGE separation rounds. With every sample run, salmon gDNA of non-vaccinated fish and no-template controls were added to the plate set-up. For the quantification of plasmid DNA copy numbers, duplicate qPCR runs were performed, and compared with a standard prepared by serial dilution.

Before the first round of FIGE, the average plasmid copy number detected in samples obtained on day 436 from at and around the injection site of the epaxial muscle was about 5.20×10^3 copies/µg HMW DNA, 0.11 % of the day 1 average level. Three sequential FIGE runs were performed, and after every run the qPCR values of each pooled sample decreased. After the third FIGE run, the company reported that none of the samples had qPCR values above the LOD.

From these experiments, the company reported that the risk associated with the integration of up to 9.9 (below LOD) copies of plasmid is 220 times lower than the risk to the genome associated with a spontaneous mutation. As the estimated mutations per gene (i.e. integration events per µg gDNA/genes per µg gDNA) was calculated to be 8.44×10^{-10}, the company claimed that over 84 billion fish should be analysed to find one integration event, and deduced that increasing the number of vaccinated fish tested would not appreciably increase the odds of detecting an integration event.

2.2. Conclusion by the company

Novartis stated that the magnitude of plasmid DNA copy decrease detected in association with gDNA in each successive purification round was consistent with extrachromosomal plasmid DNA co-purifying with salmon gDNA. It was argued that any plasmid-specific signal remaining in samples after FIGE was likely caused by extrachromosomal plasmid contamination that was physically trapped inside the gDNA, resulting in co-migration with HMW DNA on the FIGE gel. Finally, Novartis concluded that the potential for plasmid DNA integration into the fish genome when administered at the intended vaccination dose should be considered extremely unlikely and that plasmid-vaccinated salmon should not be considered as genetically modified organisms.

3. EFSA'S SCIENTIFIC ADVICE ON THE INTEGRATION STUDY NAHC/RD/01/12 FROM NOVARTIS ANIMAL HEALTH

After assessment of the study presented by Novartis Animal Health on the integration/non-integration of DNA plasmid-based vaccine into fish gDNA, EFSA detected several shortcomings in the study design and interpretation of the results.

3.1. Detection method

Since the DNA plasmid used for vaccination is several kilobases long, the use of a single qPCR primer pair is insufficient to properly cover the detection of potential plasmid integration. No evidence was provided to suggest that only the full plasmid or fragments detectable by the chosen qPCR primers would integrate into the fish genome. It cannot be excluded that other fragments of the plasmid, not detectable by the chosen PCR primers, would integrate into the gDNA should such an event occur.

Furthermore, the scientific literature (Ledwith et al., 2001; Manam et al., 2001) reports a detection limit of 1 plasmid copy per µg gDNA. The detection limit of the PCR method used in this report is 10 plasmid copies per µg gDNA. This detection limit would not be sufficient for detection of low-frequency integration events.

Finally, no proper method validation for the qPCR was used. For example, the determination of the LOD has not been repeated sufficiently per DNA level (no level at or below one copy) (e.g. see

reference AFNOR XP V03-044-2008 F) and no explanation was given for the observation of Ct values of around 37 for the zero copy reaction.

3.2. Design of the integration study

First, the collection of samples for the genome integration analysis only at a late stage (436 DPV) in the study raises concerns. At 436 DPV the plasmid copy numbers in muscle tissues were already strongly reduced compared with the DNA plasmid copy number at day 1 post vaccination. Considering that the integration potential is higher in the presence of higher plasmid copy numbers, analysis of integration at an earlier time point would improve the sensitivity of the study. Other studies have usually checked integration events at an earlier stage when plasmid concentrations are higher (Kanellos et al., 1999; Wang et al., 2004; Doukas et al., 2011).

If integration can be found at these earlier stages, but not at later stages, e.g. as a result of the removal of cells by the immune response, this information would still be useful in order to determine integration potential. It would be useful to carry out an integration study at different time points after vaccination.

Secondly, the 10× vaccinated group was not included in the integration study. In the 10× group the plasmid levels would be significantly higher, leading to a more robust range in which integration potential could be tested. In addition, the different tissue types analysed for the integration study are limited. For example, no tissue samples with higher cell division rates, like gonads, spleen and anterior kidney, were tested in order to exclude that this could potentially lead to more frequent integration events in these tissues.

Third, technical difficulties during multiple rounds of DNA purification used to separate HMW DNA from plasmid DNA resulted in pooling of DNA and eventual analysis of only three individual samples. These cannot be considered representative for the total fish population vaccinated.

Finally, the calculation of risk associated with the potential integration of up to 9.9 plasmid copies into the fish gDNA compared with the risk associated with spontaneous mutation in the fish genome does not contribute added value in determining whether or not integration occurred. The claim that 84 billion fish would need to be analysed to find one integration event is not supported by the calculation provided, since the estimated integration frequency refers to genes and not fish.

CONCLUSIONS

The study presented by Novartis Animal Health on the integration/non-integration of DNA plasmid-based vaccine into the salmon gDNA provides insufficient information on the potential integration of plasmid DNA fragments into the fish genome due to a limited coverage of the plasmid DNA by the detection method provided, the limited number of samples analysed and an insufficient limit of detection and method validation. Therefore, EFSA is of the opinion that the results from the integration/non-integration study submitted by Novartis Animal Health are not sufficient to support the conclusion of non-integration of plasmid DNA into the fish genome drawn by the company.

DOCUMENTATION PROVIDED TO EFSA

1. Letter, received 29 January 2013, with supporting document from Novartis Animal Health, to Catherine Geslain-Lanéelle, Executive Director EFSA (ref Ares (2013) 104417), requesting for EFSA's scientific advice on the suitability of data for the assessment of DNA integration into the fish genome of a genetically modified DNA plasmid-based veterinary vaccine and comprising the following supporting document:
    - Final study report (Report No: NAHC/RD/01/12): Assessment of the biodistribution and potential for integration of the plasmid DNA vaccine in Atlantic salmon (*Salmo salar*). August 2012. Submitted by Novartis Animal Health.

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