Response to assessment of the relevance of new scientific information (Santos-Vigil et al., 2018) in relation to the risk assessment of genetically modified crops with Cry1Ac by European Food Safety Authority (EFSA)[1]

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Abstract

Following the publication of the EFSA doi:10.2903/sp.efsa.2019.EN-1504 which assessed our scientific publication by Santos-Vigil et al. (2018), we are responding to the points that were criticized and marked as shortcomings in our publication. In general, we disagree with the non-objective criticism made of our work. We have opted for a point by point response format throughout the original text; our answers are in cursive lettering.

Point by point response:

1. Abstract

The outstanding question was whether or not the new scientific information contains elements that could lead the EFSA GMO Panel to reconsider the outcome of its previous risk assessments on genetically modified crops expressing Cry1Ac protein. Santos-Vigil et al. (2018) investigated the allergenic potential and immunological effects of the Cry1Ac protein and compared it with ovalbumin after intragastric administration to BALB/c mice, using a specific model of food-allergy.

Shortcomings in the study design and data interpretation limit the possibility to attribute findings to the intrinsic properties of the Cry1Ac protein. The publication by Santos-Vigil et al. (2018) does not bring new elements that would lead the EFSA GMO Panel to reconsider the outcome of its previous scientific opinions on genetically modified crops with Cry1Ac. Therefore, EFSA considers that the previous risk assessment conclusions on GM crops with Cry1Ac remain valid and applicable. © European Food Safety Authority, 2018

We are aware that more studies are required to determine the potential immunological effects derived from the consumption of GM plants containing Cry1Ac, and we are also aware that the dose of Cry1Ac used in our study was higher than the one reported to be expressed in most GM plants. However, we disagree with the point of view regarding the shortcomings in our study design and data interpretation.

1 Dumont et al., 2018

2. Introduction

No comments

3. Assessment

EFSA assessment described in this Technical Report is structured as follows: 3.1) Summary of previous EFSA opinions of GM plants with Cry1Ac and other relevant information; 3.2) Summary of the publication by Santos-Vigil et al. (2018); and 3.3) Relevance of the publication by Santos-Vigil et al. (2018) for the risk assessment of GM plants with Cry1Ac.

3.1 Summary of previous EFSA opinions of GM plants with Cry1Ac and other relevant information

We concur with the evaluation of the information in the summary regarding no indications of safety concerns for allergenicity of Cry1Ac protein. It is true that evidence supporting the immunogenicity and adjuvanticity of Cry1Ac was attained after applying 3 doses of 50 μg of protein per mouse by different immunization routes, which is a concentration that could not easily be achieved by exposition or consumption of GMO because the levels of Cry1Ac proteins expressed in the GM plants are referred to be low. However, there are variations in the expression levels in the distinct plants and tissues. Moreover, there are stacked events on the market that already produce a much higher overall concentration of Bt toxins than plants producing just one Bt toxin.

We also concur that despite the evidence in relation to rats fed with maize MON810 (expressing Cry1Ab) for 90 days showing no evidence of immunotoxicological effects, and while the study of livestock fed GM products found no evidence of allergic reaction or immunotoxicological effects, it is necessary to develop validated and standardised animal models for the assessment of allergenicity in farm animals. We consider there to be very relevant deficits in current risk assessment, such as the necessity to agree on models to investigate immune reactions and lack of adequate dose-response studies with Bt toxins in respect to their potential immunogenic potentials.

3.2 Summary of the publication by Santos-Vigil et al. (2018)

The objective of the study was to investigate the allergenic potential and other immunological effects of Cry1Ac protein and to compare it with ovalbumin (OVA) after intragastric administration to BALB/c mice, using a specific model of food-allergy that employs cholera toxin as adjuvant to break orally induced tolerance (protocol adapted from Perrier et al., 2010). Several groups of mice were assigned to different treatments as follows: i) vehicle phosphate-buffered saline (PBS); ii) 50 μg or 5 mg of OVA; iii) 50 μg or 5 mg of OVA plus 50 μg of cholera toxin (CT); iv) 50 μg of Cry1Ac alone and v) 50 μg or 5 mg of OVA plus 50 μg of Cry1Ac. Based on a defined protocol of sensitisation, mice were intragastrically administered, once a week for seven weeks, the test substances as described above. On day 49, mice were challenged with a given dose of the corresponding protein OVA or Cry1Ac, administered intragastrically or intravenously and, then, a range of clinical signs and systemic and intestinal immunological parameters were investigated.

It is important to be precise that the allergenic potential intragastric administration of purified Cry1Ac was compared not only with OVA (which was used in our tolerized group)

it was also compared with an allergy positive group using a murine model of food-allergy to ovalbumin (OVA) in which animals are sensitized with the adjuvant Cholera toxin (CT).

The authors reported that groups immunised with Cry1Ac, OVA/Cry1Ac, or OVA/CT developed moderate allergic reactions with a significant systemic IgE response, increased frequencies of intestinal granulocytes and IgE+ lymphocytes in lymphoid tissues (Peyer's patches, splenic and mesenteric lymph-node). At histopathology examination in these groups, inflammation was described in the small and large intestine, mainly consisting of lymphoplasmacytic enteritis and goblet cell hyperplasia. This was associated with an increased number of eosinophils in the lamina propria of the small intestine, or with increased granulocytes in the large intestine. In addition, mice from these three groups were reported to show colon lymphoid hyperplasia. Finally, based on a significant drop in rectal temperature recorded after intravenous challenge, Cry1Ac was also described as being able to induce anaphylaxis.

3.3 Relevance of the publication by Santos-Vigil et al. (2018) for the risk assessment of GM plants with Cry1Ac

Santos-Vigil et al. (2018) assessed the allergenic potential of Cry1Ac protein following an intragastric administration of the protein in a mouse model of food allergy. According to the authors, given that some of their previous studies showed that Cry1Ac is immunogenic and able to induce macrophage activation (Moreno-Fierros et al., 2013; Torres-Martinez et al., 2016), there was the need to further study the in vivo potential of immune and allergic responses to Cry1Ac.To this end, the main objective of the study, as claimed by Santos-Vigil et al. (2018), was to compare the allergenic potential and immunological effects of purified Cry1Ac with that of OVA after intragastric administration to mice.

Our main objective was to determine the allergenic potential and immunological effects of purified Cry1Ac toxin after intra-gastric administration to mice. So the main objective was not just to compare the effect with that of OVA. The group of OVA alone was used as a negative control and the group co-administered with CT was used as allergy positive group.

However, unclarities with regard to the question addressed in the study, shortcomings in the study design and data interpretation limit the possibility to unambiguously attribute findings, raising (or not) safety concerns, to the intrinsic properties of Cry1Ac protein and to draw relevant conclusions for the risk assessment. The main shortcomings identified in the Santos-Vigil et al. (2018) publication are the following:
We believe there are no unclarities with regard to the question addressed, nor in regard to shortcomings in the study design and data interpretation. Based on our results indicating moderate allergenic potential of intragastric administration of Cry1Ac toxin, we are not claiming safety concerns in relation to the consumption of GMO containing this protein, we are just suggesting that risk assessment of GMO products should be performed using adequate food allergy models.

i) the risk assessment relevance of the question posed and hypotheses made are unclear. Even though the authors claim relevance of their findings for the risk assessment of GM plants, they also argue that the doses used in their animal model were very high as compared to doses reached by consuming GM plants expressing Cry proteins assessed so far by the EFSA GMO Panel (Table 1). The authors indicate that additional studies comparing the effects of different levels of Cry1A (Cry1Ab and Cry1Ac) proteins under similar conditions are required to better understand immunogenic/allergenic responses;

Perhaps the risk assessment relevance of the question or hypothesis was not clear enough and we think it is important to evaluate the allergenic potential of Cry1Ac after intragastric administration using an adequate food allergy model. The justification was based on the evidence indicating the immunogenicity and capacity to activate macrophages via MAPKs pathways (Torres Martínez et al. 2016)

It is true that we argue the dose used in the mouse model is very high compared to the doses reached by consuming GM plants expressing Cry proteins assessed so far by the EFSA GMO, but we used a dose we proved was able to induce immune responses via the intragastric route.

ii) the test item used was not characterised in detail and the purity of Cry1Ac protein was not determined. Furthermore, although endotoxin levels were measured for both Cry1Ac and OVA, values are available in endotoxin units per millilitre for Cry1Ac and in endotoxin units per milligram for OVA (according to the manufacturer's information), making it unclear how they compare. Finally, there is no information on the formulation used in the study;

We disagree with this unsuitable pronouncement. Our research group has been working with this Cry1Ac protein for more than 20 years and in our laboratory the purification of the protein (protoxin and toxin) is routinely performed with well-standardized protocols; the integrity and purity is verified with strict quality controls. We do not understand what precisely the points of confusion are, but we will specify some points. The concentration of purified Cry1Ac protein is first quantified by Bradford and the purity is verified by SDS-PAGE 10%. The purified protein is usually obtained at a concentration of 5-7 mg/ml, when the endotoxin levels are determined at this step they are always found below 0.1 EU/ mL with the E-toxate kit (Sigma Chemical Co., St. Louis, USA), so to remove endotoxin remnants, the protein is treated with Affi-Prep polymyxin resin (Bio-Rad, Hercules CA, USA). Finally, the protein is filtered with 0.22 m filters. Regarding OVA (Worthington, Lakewood, USA) the lyophilized protein was solubilized at a concentration of 50 mg/ml, in this stock solution, the endotoxin levels were below 0.05 EU/ mL and this was also treated with polymyxin coupled resin, after this step the protein content was quantified again, concentrated if required and filtered with 0.22 m filters. It is worth mentioning that the endotoxin binding of this affinity resin is 2-4 mg/ml, so after adsorption through polymyxin resin the endotoxin contents in both Cry1Ac and OVA were found below 0.01 EU/ mL.

iii) authors claim to have followed a specific animal model of OVA-mediated allergy to establish food allergy to OVA (Perrier et al., 2010). However, the implications of the deviations added in the sensitisation protocol, the number and age of animals used in the experiment are unclear: the OVA dose used as positive control was suboptimal - 5mg instead of 20mg; the number of animals used per group were 4-5 instead of 10; and animals 6-8 weeks old were used, instead of 3-4 week old;

First, we clearly stated that the administration scheme was adapted and not that it was identical to the food allergy model described by Perrier et al. It is true that there are changes in the age of the mice and OVA dose used between both studies, but the important point is that in our positive group OVA-CT we were also able to induce allergy related effects while in the OVA alone group the mice were tolerized. Moreover, it is worth mentioning that we tested two doses of OVA in these positive and negative groups and with both doses were detected allergy related parameters and tolerization as well, respectively in the OVA-CT and OVA alone groups. As mentioned in the manuscript, the low OVA dose (50 μg) was chosen to make it comparable with the dose tested of Cry1Ac toxin.
As to whether or not the OVA doses tested were suboptimal, it is possible that higher allergenic effects could have been achieved in the positive control OVA-CT group if we had tested a 20 mg OVA dose, but as mentioned above our positive groups are valid.
Moreover, it is important to note that we analysed additional allergy-related parameters, such as the increased frequencies of intestinal granulocytes, IgE+ eosinophils and IgE+ lymphocytes, as well as the presence of colonic lymphoid hyperplasia that were not analysed in the study of Perrier et al. Regarding the number of animals used, we did not use a lower number of animals. It is stated in materials and methods that for the majority of the results shown (Figs. 1–5) two to three independent experiments were performed with 4–5 individuals per group, the number (n) of mice used per group is indicated in each figure legend.

iv) allergic symptoms and specific antibody signal against Cry1Ac and OVA were shown in the groups treated with PBS and OVA only, which were the negative controls. Anti-OVA and anti-Cry1Ac antibodies were measured in sera and (small and large) intestine lavages by indirect enzyme-linked immunosorbent assay (ELISA). It is unclear why the absorbance is shown for one dilution only and why the serial dilutions of the samples and standard curves are not shown; additional proper negative and positive control proteins were not included in the experiment. This is considered necessary to ensure that any effect observed for Cry1Ac protein is indeed specific to this protein only; one-way ANOVA was applied for multiple comparisons between different groups. However, the doses tested in the experiments were different for the different groups. The use of different doses makes it difficult to statistically compare the experimental groups;

The symptoms registered in negative control groups were the scratching around nose, ears and head, which is a common behavior in mice. In contrast, in the allergy positive group and in the Cry1Ac groups additional symptoms were registered, such as scratching more than 10 times, puffiness around the eyes and mouth, diarrhea, pilar erecti for 15 min. While noting the commentary of specific antibody signal against Cry1Ac and OVA in our negative PBS and OVA groups, we disagree because we did not find that. Serial dilutions were probed to estimate the antibody titres, however, when the values recorded for the dilutions are low (around 0.1) the titers cannot be estimated, so in these cases we show instead the value for one dilution within the linear part of the plot for all the groups. Our assays included the appropriate positive and negative controls. We consider the statistical analysis was adequate and its validity is not limited by the different doses tested. Besides this, when Cry1Ac toxin and OVA were compared at similar doses 50 μg their differences in immunologic effects are clear.

v) the authors claim that Cry1Ac, when administered by intragastric route, has a slight adjuvant effect to OVA by eliciting IgG1 and IgA antibody responses in serum (described in the supplementary information), however it is not clear how they reached this conclusion on the basis of the evidence provided;

The slight adjuvanticity is based on the slight, statistically significant increase in anti-OVA IgG1 and IgA antibody responses recorded in the OVA-Cry1Ac group with respect to the OVA alone group.

vi) the authors report that mice sensitised intragastrically with Cry1Ac had a significant temperature drop after intravenous challenge indicating anaphylaxis potential. However, for reasons explained above in paragraphs iii, iv and v, this cannot be attributed unambiguously to Cry1Ac only.

This commentary is unfounded and a lot less can be based on the reasons mentioned in paragraphs iii, iv and v because to perform this experiment we tested several doses of Cry1Ac alone, mice were sensitized via the intragastric route with Cry1Ac and were challenged with Cry1Ac, so we do not understand why the effects should not be attributed to Cry1Ac.

There are other unclarities linked to the protocol used and interpretation of the results. Briefly, the number of experiments (and respective replications) used to collect the results shown in Figures 1 to 5 is unclear. The authors state that the dose selected for Cry1Ac was based on a dose-dependent study with 10, 50, 100 and 200 µg using a different immunisation protocol, but the supporting data are not shown. In addition, the authors fail to explain why the response to OVA (50 μg) dose was larger than the one observed with the higher OVA dose (5 mg) when OVA was co-administered with Cry1Ac. Sera and intestinal lavages were used to measure the cytokine profile. However, for a more meaningful outcome, a kinetics experiment analysing the cytokine profile over time and not at one time point only would have been more informative. Regarding the reported increased colon lymphoid hyperplasia, it is important to highlight that lymphoid aggregates are a common feature of the colonic mucosa in normal rodents (Greaves, 2011). It is, therefore, important to properly sample the intestine, dismissing the

possibility to misinterpret normal anatomical features as pathological findings. Moreover, it is reported that lymphoid hyperplasia observed in animals at the end of the experiment were noted also in animals after 1-month recovery period, however no data is shown. Finally, intestinal segments 0.5–1 cm in length from main areas of the small and large intestine were used to count granulocytes from 20 independent cross-sections per group. The interpretation of these data is unclear because insufficient details are provided on the sampling and the outcome of the comparison is highly dependent upon the location of the sections taken.

To summarise, a comparison of two proteins (OVA and Cry1Ac) administered at different doses without appropriate negative control(s) has limited relevance in a risk assessment frame. It remains unclear if the findings by Santos-Vigil et al. (2018) are linked to Cry1Ac protein only or if other proteins, e.g. strong/weak/”virtually-non” allergens, would behave similarly under the conditions tested.

The authors also deliberate about the contrasting evidence available from studies performed with Cry1Ac vs those performed with Cry1Ab. The reasons underlying this contrasting evidence still remain to be deciphered. Several explanations have been hypothesised: differences in amino acid sequences between proteins (identity between proteins can be higher than 90%), in doses, in routes of administration, in animal models, in experimental protocols and matrices used. Still it remains to be understood if there is a dose-response relationship for the potential adverse effects reported, what test item should be investigated and what in vivo and/or in vitro models should be used.

To this end, studies appropriately designed to provide reliable answers when testing for adjuvant and allergenic potential and more broadly on the effects on the immune system of Cry and other novel proteins are desirable, an aspect also highlighted by the authors. EFSA is moving forward the field of allergenicity assessment and has been proactive in considering new developments in the area (EFSA GMO Panel, 2017). Other EU projects will also contribute to improve the approaches used for the safety assessment (e.g. www.imparas.eu). Future studies on Cry proteins should consider limitations of the currently used models and should be performed using relevant routes of administration at appropriate doses, with appropriate positive and negative control proteins, taking into account possible effects of processing and matrices. As for all risk assessment fields, also for allergenicity absolute safety cannot be guaranteed, therefore a strategy ranking the allergenic potential of known proteins has been suggested as a way forward (FAO/WHO, 2001; Remington et al., 2018; EFSA GMO Panel, 2017). This might then serve as benchmark for the assessment of any novel protein.

The number of mice is indicated in the figure legends. In materials and methods we explained in detail the dose-dependent study just used to select the dose of Cry1Ac; a dose able to induce immune responses following intragastric immunization. The possible explanation of the distinct effects of Cry1Ac obtained with the lower dose of OVA are discussed. It is true that a kinetics experiment analyzing the cytokine profile would be more informative and we will take into account the suggestion that it has not been reported in food allergy models.

Regarding the recorded lymphoid hyperplasia, we know well that the presence of isolated lymphoid follicles is common in the colonic mucosa in normal rodents, but these can be distinguished from the lymphoid hyperplasia detected in the allergy positive group and in the Cry1Ac administered groups. Moreover, the histopathological analysis was performed by a specialized histopathological service. The size of the hyperplasia shown in the images correspond to a mean size representative images but in some mice the hyperplasia recorded was wider. In this response letter, we have attached representative images of colonic lymphoid hyperplasia recorded in mice that were sacrificed after 1-month of recovery period. Regarding the collection of intestinal segments, these were collected from similar regions from all experimental groups.

To summarise, we consider our study to be well-controlled with the appropriate controls, while regarding its relevance in a risk assessment frame, and it should be taken into account for a more complete evaluation of the potential allergenicity of other Cry proteins, because the use of adequate food allergy models and the evaluation of intestinal parameters is required.

We consider our findings were attributable to the Cry1Ac toxin because the purity of the protein tested in this study was adequate. Regarding the possibility that other proteins would behave similarly to Cry1Ac under the conditions tested in present study, we do not know. But it will be interesting to test the effect of co-administration of Cry1Ac with a strong allergenic protein.

*Regarding the discussion of the possible explanations underlying the contrasting evidence between the immunological effects of Cry1Ac and Cry1Ab we have argued, we agree these still remain to be deciphered with more studies. We agree there is a need for additional dose-response studies, in relation of the adverse effects reported attained in our reported study after intragastric application of Cry1Ac at 50 μg doses. Hence, it is true that we highlighted the need for additional studies appropriately designed to provide reliable answers when testing for adjuvant and allergenic potential, and more broadly on the effects on the immune system of Cry and other novel proteins. It is good to know that EFSA is moving forward in the field of allergenicity assessment using relevant routes of administration and improving the assessment. It is important to note that our research group has been interested in studying the immunological properties of Cry1Ac proteins because we have found the protoxin exhibits important protective adjuvant properties, so we do not have any conflicts of interest. We are aware that Cry proteins provide a highly effective means of insect control with good biosafety records (Rubio-Infante and Moreno-Fierros 2016), so its use has been expanded worldwide. We believe our publication (*Santos-Vigil et al 2018) has *contributed to the knowledge of the immunological effects of Cry1Ac toxin and the new information provided should not be negatively judged or disqualified just because it has been considered relevant for the risk assessment of GM plants.*

4. **Conclusions**

In a recent study, Santos Vigil et al. (2018) report that Cry1Ac is moderately allergenic, able to provoke intestinal lymphoid hyperplasia, and even to trigger anaphylaxis in a specific animal model under the experimental conditions tested. Owing to unclarities in the hypotheses tested and shortcomings in the study design, the publication by Santos-Vigil et al. (2018) does not bring new elements that would lead the EFSA GMO Panel to reconsider the outcome of its previous opinions on genetically modified crops with Cry1Ac (Table 1).

It is noted that the EFSA GMO Panel has discussed extensively the potential allergenic and adjuvant capacity of some Cry proteins considering all available information, including literature on the topic (e.g. Vazquez-Padron et al., 1999; Moreno-Fierros et al., 2003; Rojas-Hernandez et al., 2004; Guimaraes et al., 2008; Reiner et al., 2014; Andreassen et al., 2015a,b, 2016; Torres-Martinez et al., 2016; Santos-Vigil et al., 2018). In particular, the adjuvant capacity of Cry proteins is a matter of current scientific debate, where most of the scientists agree on the limited and contrasting evidence available (Rubio-Infante and Moreno-Fierros 2015; Joshi et al., 2016). Most studies in this context have been performed with Cry1Ab and Cry1Ac, and very little is known about a potential doseresponse relationship of Cry protein activity. However, it has been experimentally shown that no adjuvant effect is detectable when Cry proteins are expressed at the levels observed in the GM plants so far assessed by the EFSA GMO Panel, (e.g. Reiner et al., 2014). EFSA and other risk assessment bodies have previously commented on the topic (EFSA, 2009; VKM, 2012). Consequently, on the basis of available knowledge, EFSA and other risk assessment bodies conclude that there are currently no indications of safety concern regarding Cry proteins in the context of the GM plants assessed.

Nonetheless, EFSA is aware of the relevance of the topic and last year initiated a procurement to collect additional information on adjuvanticity in food for further discussion. The resulting external report will be published in the EFSA website by beginning of 2019.

To conclude, the publication by Santos-Vigil et al. (2018) does not present new elements leading the EFSA GMO Panel to reconsider the outcome of its previous opinions on GM crops with Cry1Ac. Therefore, EFSA considers that the previous risk assessment conclusions on GM crops with Cry1Ac remain valid and applicable.

As mentioned before, the purpose of our scientific publication (Santos-Vigil et al 2018) was to contribute to the knowledge of the immunological properties of Cry1Ac, we did not expect to be involved in a debate around risk assessment of GM plants in which the relevance of our work was questioned, but it is good to know EFSA is aware of the relevance of the adequate assessment of allergenicity of Cry proteins.

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Fig 1. Representative images of colonic lymphoid hyperplasia recorded in mice that were sacrificed after 1-month of recovery period.

The persitence of colonic lymphoid hyperplasia was recorded after 35 days of recovery in BALB/c mice receiveng intragastric sensibilization treatments with OVA-Cry1Ac, Cry1Ac and OVA-CT, intragastric challenges on day 49 and were sacrificed until day 84. The images are representative of colon sections from each treatment group (n =6). Sections were labeled with H&E stain.