European Commission

JRC CONFERENCE AND WORKSHOP REPORT

33rd ENGL Meeting

Meeting Report

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Joint Research Centre

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Report

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33rd ENGL Meeting

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1. Welcome of the Chair

The Chair welcomed the participants and introduced herself as the new Head of the Unit Food & Feed Compliance (JRC F.5). She introduced the presentations planned for the meeting.

2. **The European Animal breeders' perspective on** the potential use of new genomic techniques in Animal Breeding programs (A. Granados Chapatte, EFFAB, FABRE TP)

The speaker explained that the European Forum of Farm Animal Breeders (EFFAB) is a recognised association of animal breeders including members from EU and from other countries operating in EU. The association promotes research and innovation on animal genetics in poultry, ruminants, insects, aquatics and pigs and supports research cooperations between private and public sectors. EFFAB is endorsing a responsible, sustainable and balanced breeding (Code EFABAR) as well as a dialogue on sustainable animal breeding and farming knowledge. Instead of focusing only on better production, a susteinable breeding program pursues a balanced and responsible combination of several traits, such as animal health and welfare, maintainance of genetic diversity, better use of resources, resistance to diseases and reduction of ammonia and carbon emmissions. The EFABAR code of good practice includes also a responsible use of technology to meet six pillars. All the members, or at least those involved in breeding programs, must apply this code; they do not use transgenesis but may use genome editing to their scope.

The speaker illustrated potential applications in which the members are exploring these opportunities such as resistance to Porcine Reproductive Respiratory Syndrome (PRRS) and other diseases, hornless cattle, sterility in GM fish (salmon) to avoid crossing to wild animals and preserve biodiversity.

According to the speaker, Increased knoledge of animal genetics and safety data will help drafting a legislation adapted to scientific and technological progress, addressing the product instead of the technology. Under the GMO Directive the risk assessment for GM animals may be long and costly, but the use of NGTs could make animal programs accessible also to small-to-medium enterprises (SMEs). The EFFAB association needs clear, smart legislation on genome editing, a definition of safety data, guidelines for potential applications, a framework of Responsible Research and Innovation (RRI) in which the members could operate and detection methods. Public acceptance is also important and the association promotes a series of initiatives to explain potential usefull applications.

Questions

Participants enquired on traceability of animals modified by NGTs, on transparency of the breeding programs, their effect on sustainability and on the strategies followed by the association to ensure implementation of the EFABAR code.

The speaker explained that the association provides animals and semens to farmers. The semens are traceable but NGT animals are challenging since farmers are themselves breeders. It is also difficult to measure progress on breeding programs since information from different companies or countries is not always available. Improvements on leg strength in chickens can be documented but the association is not good in communicating them. Moreover, sustainability is a broad concept, covering environmental and social aspects. New breeding programs could support sustainability while NGTs could improve specifically animal health and indirectly also sustainability by decreasing mortality or slaughter of animals (reduction of resources).

A representative from Belgium requested wether breeders could agree to provide information on the modified sequences and methods for their traceability. The speaker urged a discussion for defining a solution to the traceability issue.

3. Genome editing in animals: current state, traits of interest, and technology developments (C. Tait-Burkard, Roslin Institute, UK)

Since 2005, the price reduction in genome sequencing has made the identification of the genetic markers associated to the different traits more affordable and their modification by genome editing feasible. Genome editing avoids the risks of inbreeding and loss of productivity during crossbreeding/genetic selection and allows integration of certain traits when crossbreeding is not possible. The speaker illustrated some applications in animals

to reduce antimicrobial use and food waste in the production chain and improve animal welfare mostly by reducing vulnerability to diseases. The speaker provided the following examples:

Resistance to viral infection by the PRRS virus (PRRSV)

A 450 bp deletion in the CD163 receptor blocking the interaction with the virus and the induction of a cell response is under Food Drug Administration (FDA) regulatory submission. This deletion is not present in nature and could be used for detection and traceability of the modified animals.

Environmental resilience/heat stress

A truncation in a Prolactin receptor has been selected in nature primarily in Caribbean and South America slick-haired breeds to reduce heat stress. The modification has been introduced by NGTs in new cattle varieties. In Brazil and USA, beef cattle with this trait is considered low risk and equivalent to natural breeds even if the modification has been introduced by biotechnology.

Animal welfare/dehorning in cattle

Celtic cattle breeds do not have horns. This trait can be introduced by genome editing in other cattle varieties without changing other important traits.

Better production /improving muscle gain

This feature when selected by natural crossbreeding can be associated with birth defects. A more efficient food conversion trait has been genetically introduced in tilapia in Argentina, red sea bream and tiger puffer in Japan. Sterility could be very important to avoid crossing with wild varieties.

AquAdvantage salmon

This fish approved in the USA has an additional ocean pout promoter stimulating expression of growth hormone independently of the season. It is very sustainable because the transgenic fish consumes 10 times less food to reach adult size.

An enviropig expressing *E. coli* phytase has been developed to reduce phosphate emissions but it is not yet approved.

The speaker covered other technological advances to generate genome-edited animals such as cloning, zyogote injection and transfer, use of spermatogonial stem cells and mating to sterile surrogate hosts.

A participant requested whether non-target unintentional effects could introduce unexpected traits. The speaker acknowledged that off-targets effects may happen but comprehensive computational analyses may reduce the risk of targeting other genes. Moreover, off-target mutations located outside the coding regions may not matter or actually increase biodiversity.

4. Metagenomic characterization of multiple genetically modified *Bacillus* contaminations in commercial food enzyme products (J. **D'aes,** Sciensano, BE)

Genetically Modified Microorganisms (GMMs) producing enzymes, vitamins and additives often contain antimicrobial resistance (AMR) genes as selectable markers, which may spread via horizontal gene transfer after ingestion, especially if inserted in a plasmid. The presence of these GMMs in the final products is not authorised in EU. Official Control Laboratories (OCL) have difficulties in performing adequate controls since information on GMM transgenic constructs is confidential and not accessible. A classical GMM detection strategy includes first a qPCR screening for common AMR genes and shuttle vector pUB110 on DNA extracted from in vitro microbial isolates and secondly a qPCR for the identification of the GMM targeting the alpha-amylase or protease enzyme constructs.

The speaker presented a metagenomics approach for confirming/replacing the classical analytical PCR strategy. This is a non-targeted open approach where all genomic DNA extracted directly from the sample matrix is shotgun-sequenced at high depth and then assembled in the different genomes (chromosomes plus plasmids) represented by the sequencing data. The benefits are that no prior knowledge is required, the GMMs do not need to be cultured in vitro and that novel GMM constructs can be eventually discovered. The laboratory used as a case study, common food enzyme products presenting cross-contamination with different GMMs, which had been partially characterised by PCR analysis.

Two platforms were used for sequencing; the Illumina MiSeq system to obtain highly accurate short (250 bp)

sequences and the MinION device from Oxford Nanopore Technologies providing long read sequences (up to 100 kilobases) to complete the assembly of the genomes. The taxonomic profiles of the raw data indicated that the large majority of the reads were belonging to *Bacillus* strains. The laboratory focused therefore on the *Bacillus* species and followed a bioinformatics assembly strategy including pre-processing, filtering and binning procedures for a quality control of the sequencing data.

By using the metagenomics approach, the laboratory was able to assemble transgenic constructs detected by PCR 1st and 2nd line screening, to confirm the presence of two GMM amylase constructs that previously could not be fully characterised because present in not viable cells and to detect three uncultivable *Bacillus* species, of which two were newly identified. The approach required several manual analyses and the novo sequence assemblies. Availability of a database curated with GMM data could allow the application of this strategy to routine analysis and the development of detection methods.

Questions

A representative from the JRC enquired on the use of the approach for the detection of NGT strains. The speaker remarked the difficulty in using the strategy without prior knowledge of the mutation and without sufficient sequencing depth. She further clarified that the approach could not be used for quantitative purposes.

5. NGS strategy to detect a single-point variation in CRISPR/Cas plants (M. A. Fraiture, Sciensano, BE)

The speaker illustrated a targeted high-throughput sequencing approach for detecting a single nucleotide variation (SNV) in a genome-edited plant. The project was funded by the Belgium Federal public service for developing and evaluating approaches of NGTs products detection.

NGT modifications of one or few nucleotides can be very challenging at the analytical and interpretation level. Fraiture *et al*. 2022 were able to specifically detect and quantify a single base pair insertion from a gene edited rice line using ddPCR, a technology that is commonly used by enforcement laboratories. However, prior knowledge is required for the design of primers and probes and is not easily exploitable for multiplexing. Furthermore, the development of each method is a case-by-case process and it is not always possible. Conversely, a targeted high throughput sequencing PCR-based enrichment approach could be more amenable to multiplexing strategies. Prior knowledge and specific design is still required. The approach is sensitive, but potential sequencing errors and bioinformatics bias could hinder the detection of small size modifications. Finally, multiple variation points in the same organism, including off-target sites, could be associated to a species or phenotype for the identification of genome-edited organisms.

The laboratory tested the approach on the same genome-edited rice line of the ddPCR method using rice seed samples containing different percentages (from 100 % to 0.1 %) of the mutated rice line in the parental rice line. Additionally the approach was tested on different mixtures (from 100 % to 0.1 %) of mutant rice lines in rice noodles and crop combinations. The strategy included DNA extraction, PCR enrichment steps with the ddPCR primers, sequencing on Illumina MiSeq platform and data analysis with an in-house pipeline to identify the nucleotide variations and their frequency. The laboratory was able to detect the expected SNV at the 0.1 % level without false positives or negative results and with a good estimation of the frequency even in processed rice samples or crop mixtures. However, no conclusions could be obtained on the origin of the mutation (natural, radiation/chemical or genome editing). The laboratory is planning to investigate other targets in the same organism by high−throughput sequencing to determine if an ambiguous identification of a plant generated by genome editing is possible. As a first step, a high-quality reference database of genome-edited plants is needed to develop methods and unambiguously identify NGT products.

Questions

Participants requested if the approach could have the same sensitivity in detecting base substitutions or complex genomes. The speaker remarked the need for further testing, of high quality sequences for designing primers for the target regions and the difficulty in applying the approach to plants with many repetitive regions.

A JRC representative reminded that off-target effects might be lost in crossbreeding and that a high quality reference database containing all possible sequences of different varieties and genome variations may not be practicable. The speaker acknowledged the difficulty in developing a complete reference database.

6. ENGL Guidance on the selection and use of DNA extraction methods (T. Prins, WUR, NL)

The chair summarised the mandate and the work performed by the members of the WG on DNA extraction. The WG was formally established at the beginning of 2018 with the aim of harmonising and providing guidance for best practices in the selection of DNA extraction methods as well as supplying a dedicated webpage on ENGLnet for sharing information and solutions. The work was organised in eight subtasks. The chair reminded that a compendium on "DNA issues and solutions", based on a workshop organised in 2017, has been uploaded in ENGLnet in February 2020 including a trend analysis for most common DNA extraction problems and related

recommendations. The chair illustrated the eleven chapters of the guidance document providing a literature review of DNA extraction methods, a practical guidance on in-house validation and verification of DNA extraction and considerations for inter-laboratory validation studies. Other chapters cover DNA quantity and quality check in routine analyses and Decision Support Systems (DSS) for the selection of appropriate methods according to the type of sample. Publication of the guidance is foreseen in early 2023.

Members of the group expressed different positions for cases in which the positive actin gene control and the 35S and T-nos targets are not detected by PCR, suggesting a non-sufficient DNA quantity in the sample. A representative from Germany proposed spiking the sample with a low amount of known DNA to prove a successful extraction. A WG member encouraged using the developed web resources to address extraction problems from difficult matrixes and to build expertise from collective experiences. A representative from Belgium suggested having a blacklist of matrixes from which DNA extraction is not possible.

7. Multiplex digital PCR for the detection of soybean events (D. Mäde, DE)

Currently 18 soybean GM lines are authorised in the EU but many do not carry conventional screening elements, therefore event-specific methods need to be included in the screening design for their coverage. Multiplex real-time PCR or ddPCR methods can be used as an alternative option. Since ddPCR platforms have only two channels, the laboratory devised three systems with FAM and HEX labelled probes and optimised the conditions to distinguish the varieties at the fluorescence level. The laboratory tested the performance of duplex ddPCR methods including the event-specific and lectin systems on CRM at 10 %, 1 % and 0.1 % GMO and converted the results from copy/copy into mass/mass using the conversion factors established in the validation studies (all very close to the value of 1). The laboratory then evaluated if the performance of the ddPCR approach was in line with the ENGL MPR document. A sample was considered positive if at least three droplets were positives. According to Poisson statistics, three copies of the target are required to have a probability of 95 % that at least one copy is present. According to the ENGL MPR, the variability between copy number measurements shall not exceed 25%. Poisson probability p=0.95 for reaching a 25% variability could be obtained with at least 55 copies of the target while the minimal copy number for reliable quantification was equal to 42 copies.

Questions

The participants enquired over the concentrations of primers and probes used in the ddPCR reactions, requested if *in silico* analysis were performed to identify cross reactivity and if controls were included to identify non-specific effects. The speaker explained that the system was optimised for distinguishing the varieties at the fluorescence level and therefore, that the conditions were not identical to the real-time PCR methods. He added that *in silico* analysis were not performed and that the approach was designed for qualitative detection, not for quantification. He further remarked that the work was not yet published and that the laboratory was planning to transfer all protocols to the ddPCR platform for economic reasons.

8. CEN and ISO activities (L. Grohmann, BVL, DE)

A representative from Germany described the Committees involved in developing standards on GMO detection in CEN and ISO organisations. He explained that the general aspects are addressed at the technical committee (TC) level (TC 34 on *Food products* in ISO and TC 275 *Food analysis - horizontal methods* in CEN), while the expert work is performed at the working group (WG) level (Sub-Committee (SC) 16 *Horizontal methods for biomarker analysis* in ISO and WG 11 *Genetically modified foodstuffs* in CEN). He illustrated the entire workflow from new work item proposals to technical reports to final publication of an EN and ISO standards.

He informed that CEN/TC 275/WG11 changed back its scope to 'standardisation of methods for detection and/or determination of GM foodstuffs'. WG11 published in 2019 two documents on validation of qualitative real-time PCR methods, part 1 on single-laboratory validation and part 2 on collaborative studies. The first document was revised and published in 2021 to implement in the Annex the source code of the R software tool for automatic LOD95% calculation. The document was submitted as a new work item proposal to ISO/TC 34/SC 16 and approved to become a joint and full ISO/CEN standard. WG11 members recommended the adoption of ISO 22753 on 'general requirements and procedures for evaluating GM content in seeds or grains by a group testing (sub-sampling) strategy and statistical evaluation'. The document is in agreement with the ENGL document 'seed testing' (2015). A decision in April 2022 with 12 national members approved the document as European standard.

The main EN ISO standards for GMO analysis are EN ISO 21569 (qualitative PCR), EN ISO 21570 (quantitative PCR), EN ISO 21571 (Extraction) and EN ISO 24276 (general requirements and definitions). These standards were published in 2005 and amended in 2013. Since then, methods for GMO detection undergo an independent systematic review at ISO level. Under request of some members, ISO/TC 34/SC 16 determined in 2022 a possible roadmap for the revision of these documents. The project on ISO 21569 could start in 2023 while the one on ISO 21570 in 2025.

European countries (33) are participating also to ISO/TC 34/SC 16 covering horizontal methods for biomarker

analysis. New work item proposals concerning GMO detection (2022) in this ISO subcommittee are the followings:
ISO/TS 21569-7 Part 7: *Real-time PCR based methods for the detection of CaMV and Agrobacterium Ti-plasmid derived DNA sequences;* it should be published at the end of the year.
ISO/TS 21569-8 Part 8: *DNA extraction from alfalfa seeds and real-time PCR based event-specific detection methods for GM alfalfa lines J101, J163 and KK179*, vote ongoing.
ISO/TS 21569-9 Part 9: *Construct-specific real-time PCR based screening method for the detection of the P35S-nptII DNA sequences*, vote ongoing.
JWG 12 molecular biomarkers of agricultural fibers:
ISO/NP TS 5354-1 Molecular biomarkers of agricultural fibers - Detection of DNA in textiles derived from Cotton - Part 1: extraction of DNA from cotton and cotton-derived textiles, working draft.
ISO/NP TS 5354-2 Molecular biomarkers of agricultural fibers - Part 2: Overview of target sequences for use in PCR-based detection methods for cotton GM events, working draft

The speaker informed that ISO standards providing terms and definitions such as ISO 5058-1:2021 (en) *Biotechnology - Genome editing - Part 1: Vocabulary* and ISO 16577:2022 (en) *Molecular biomarker analysis - Vocabulary for molecular biomarker analytical methods in agriculture and food production* are freely accessible via ISO Online Browsing Plattform (OSB) https://www.iso.org/obp/ui.

9. GMO Global Conference 2023 (H. Broll, Bfr, DE)

A representative from Germany announced the organisation of an international conference on GMO analysis and new breeding techniques (NBTs) on 14-16 March 2023 in Berlin. The speaker reviewed the recent initiatives of the Commission on the evaluation of the EU legislation on GMOs following the Decision of the EU Court of Justice on NGT products. He recalled the first global conferenced on GMO analysis organised in Como in June 2008. Different organisations, including BVL, BfR, JKI, BMEL and JRC will cooperate on the conduction and planning of the event. He informed that the conference will have a hybrid format (presence and online) with 330 colleagues physically present. He encouraged the participants in registering early to the event. The Scientific Programme Advisory Board is composed of colleagues from different regions of the world. In the draft program, sections will be dedicated to global capacity building and new approaches for detection of NBT applications. The Chair informed that a visual identity and a website have been designed for the conference.

10. AOB

The Chair informed that the 2023 ENGL plenary would be organised in the last week of September or first week of October, possibly in a hybrid format. Information will be provided as soon as the dates will be defined. She thanked the participants for their contributions and closed the meeting.

Annex 1: agenda

33rd ENGL Meeting
30 September 2022

	Time	Topic	
1	8:45	▪ Welcome of the Chair	
2	9:00	▪ The European Animal Breeders' perspective on the potential use of new genomic techniques in Animal Breeding programs (A. Granados Chapatte, EFFAB, FABRE TP)	
3	9:45	▪ Genome editing in animals – Current state, traits of interest, and technology developments (C. Tait-Burkard, Roslin Institute, UK)	
	10:30	*Coffee break*	
4	11:00	▪ Metagenomic characterization of multiple genetically modified *Bacillus* contaminations in commercial food enzyme products (J. D'aes, Sciensano, BE)	
5	11:45	▪ NGS strategy to detect a single-point variation in CRISPR/Cas plants (M.A. Fraiture, Sciensano, BE)	
	12:30	*Lunch break*	
6	13:45	▪ ENGL Guidance on the selection and use of DNA extraction methods (T. Prins, WUR, NL)	
7	14:30	▪ Multiplex digital PCR for the detection of soybean events (D. Made, DE)	
	15:15	*Coffee Break*	
8	15:45	▪ CEN and ISO activities (L. Grohmann, BVL, DE)	
9	16.30	▪ GMO Global Conference 2023 (H. Broll, Bfr, DE)	
10	16:45	▪ AOB	
	17:00	*End of meeting*	

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