Report on the Verification of the Performance of a Method for the Detection of "Bt 63" Rice Using Real-Time PCR

9 April 2008

Joint Research Centre
Institute for Health and Consumer Protection
Biotechnology & GMOs Unit

Executive Summary

In September 2006 rice products originating from China and contaminated with the unauthorised genetically modified rice "Bt 63" were identified in the United Kingdom, France and Germany and notified to the Rapid Alert System for Food and Feed (RASFF).

The European Commission established contacts with the Chinese Authorities requesting information on the unauthorised GM line, its genetic structure, the provision of suitable control samples and an event-specific detection method.

The international scientific community undertook efforts to characterise the molecular structure of the insertion region in GM-rice "Bt 63" and developed accordingly detection strategies[1-3].

In October 2006 the Joint Research Centre of the European Commission, namely the Community Reference Laboratory for GM Food and Feed (CRL-GMFF), received samples possibly containing Bt rice as tested by GeneScan with a "Bt 63" construct-specific method. The CRL-GMFF, as a result of the analyses conducted, suggested to the European Network of GMO Laboratories (ENGL) and to the EU enforcement laboratories the use of the detection method developed by Mäde *et al.*[2] targeting the junction region between the *cry1A(b)/cry1A(c)* fusion protein and the *nos* terminator.

In order to support official control activities within the EU, the CRL-GMFF provided, in October 2006 and in December 2006, control samples to enforcement laboratories; the CRL-GMFF continued to assist the laboratories in their needs for control samples and detection issues.

On 09/04/2008 the European Commission issued a Decision for mandatory testing of the food and feed products listed in the Annex to the same Decision, originating in or consigned from China (Commission Decision No 2008/289/EC of 3 April 2008 on emergency measures regarding the unauthorised genetically modified organism 'Bt 63' in rice products)

On 28 February 2008 the CRL-GMFF received a pooled sample of "Bt 63" DNA extracted from single plants previously tested for "Bt 63" presence. This DNA sample was used to assess the specificity and the sensitivity of the construct-specific detection method developed by Mäde *et al*.

The present verification report confirms that the contruct-specific method developed by Mäde *et al* specifically detects "Bt 63" rice.

The limit of detection (LOD) of the method as experimentally established is at least 5 copies of haploid rice "Bt 63" genome.

Content

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1. Introduction

In September 2006 rice products originating from China and contaminated with the unauthorised genetically modified rice "Bt 63" were discovered in the United Kingdom, France and Germany and notified to the Rapid Alert System for Food and Feed (RASFF).

The EC established contacts with the Chinese Authorities requesting information on the GM event, its genetic structure, the provision of suitable control samples and an event-specific detection method.

The analyses conducted on "Bt 63" rice suggested that the unauthorised rice might consist of Bt rice as GM lines Shanyou 63 and Jinyou 63, referred to as "Bt 63" rice. According to Tu *et al.* [(1)], the inserted region of the construct pFHBT1 in "GM Shanyou 63" contains the *act1* promoter and the *Bt cryIA(b)* and *cryIA(c)* fusion genes, followed by the *nos* terminator.

In October 2006 the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF), received from GeneScan samples possibly containing Bt rice as tested by GeneScan with a "Bt 63" construct-specific method. The JRC, as a result of the analyses conducted, suggested to the European Network of GMO Laboratories (ENGL) and the EU enforcement laboratories the use of the detection method developed by Mäde *et al*. [(2)]

This real-time PCR based detection method is construct-specific and it targets the transition region between the Bt *cryIA(b)* and *cryIA(c)* fusion gene and the nopaline synthase (*nos*) terminator.

In order to support official control activities within the EU, the CRL-GMFF provided, in October 2006 and in December 2006, control samples to enforcement laboratories; the CRL-GMFF continued to assist the laboratories in their needs for control samples and detection issues.

On 09/04/2008 The European Commission issued the Decision No 2008/289/EC for mandatory testing of the food and feed products listed in the Annex to the same Decision, originating in or consigned from China.

On 28 February 2008 the CRL-GMFF received from the Chinese Auhtorities a pooled sample of "Bt 63" consisiting of genomic DNA extracted from single plants previously tested for "Bt 63" presence. This DNA sample was used to assess the specificity and the sensitivity of the construct-specific detection method developed by Mäde *et al*. The results of the tests are presented in this report.

2. Experimental design, materials and methods

2.1. DNA extraction

Genomic DNA of "Bt 63" was provided by Prof. D. Zhang (Shanghai Jiaotong University, P.R. China). Bt rice hybrid seeds were sowed in greenhouse, and individual rice plants tested by PCR method. Leaves and stems of the positive Bt rice plants were collected and DNA was extracted using the Plant Genomic DNA Extraction Kit (Shanghai Generay Biotech Co., Ltd, China).

Conventional rice seeds were used to extract DNA at CRL-GMFF according to the validated method for DNA extraction from rice grains/seeds[3].

2.2. DNA concentration and integrity

The concentration of the DNA extracts was determined by fluorescence detection, after extensive homogenization, using the PicoGreen dsDNA Quantitation Kit (Molecular Probes). Suitable dilutions of each DNA extract were prepared in 10 replicates and mixed with the PicoGreen reagent.

DNA concentration was determined on the basis of a five-point standard curve ranging from 0 ng/mL to 500 ng/mL using a Modulus (Turner Biosystems) or a VersaFluor ™Fluorometer (Bio-Rad) as fluorescence detector.

DNA integrity was verified through agarose-gel electrophoresis.

2.3 Linearity/inhibition tests

Linearity and inhibition tests were performed as follows: rice DNA was diluted to a level corresponding to the DNA concentrations intended to be used in the subsequent real-time PCR method. From this sample, named "undiluted" (containing 100 ng DNA for Rice "Bt 63" and 200 ng DNA for non-GM rice sample), a dilution series was prepared. To assess the presence of inhibitors, the Ct values of the diluted samples were plotted against the logarithm of the dilution factor, and the Ct value for the undiluted sample was extrapolated from the equation calculated by linear regression. Subsequently the extrapolated Ct for the sample was compared with the measured Ct.

Linearity tests were conducted on the "Bt 63" rice' and on non-GM rice samples with the rice-specific reference system developed by Mäde *et al.* and based on the *gos9* gene. Criteria for DNA quality acceptance were based on ENGL minimum acceptance criteria: slope between -3.1 and -3.6 and linearity above 0.98[4]. In addition, the ΔCt between extrapolated and measured Ct on the least diluted sample should be less than 0.5.

2.4. Specificity

2.4.1. Bioinformatics analysis

Bioinformatic analysis was conducted by homology search using BLASTN 2.2.16[5], with the sequences of the primers T51F-T51R and with the amplicon of the Mäde method against *i*) the GMO database maintained at the JRC (CCSIS), *ii*) the non-redundant Genebank database and *iii*) the database of patented sequences.

2.4.2. Experimental testing of specificity

The "Bt 63" construct-specific method developed by Mäde *et al*. was tested against 100 ng genomic DNA from a selection of GMOs (Tables 2, 3 and 4 for details).

2.5. Limit of Detection

The sensitivity of the method was assessed through the determination of the limit of detection (LOD). A "Bt 63" DNA sample was diluted to 10 haploid genome copies/μL. Subsequently, a serial dilution was prepared from the first dilution sample to achieve theoretical concentrations of 5, 2, 1, 0.2 and 0.02 copies/μL, respectively. Five μL of each sample of the dilution series were loaded per reaction so to assess the LOD at 50, 25, 10, 5, 1 and 0.1 haploid genome copies. Twenty-one replicates were assessed with the Mäde's method for each dilution.

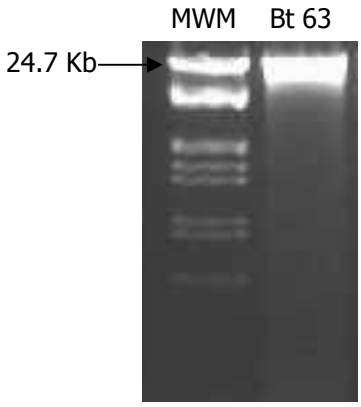
In addition, the plasmid pENGL-00-EM02/06-01M containing the target sequence of the Mäde method cloned in pUC18 was built as a plasmid control sample (PCS). The plasmid concentration was estimated with fluorescence determination as previously described; based on the sequence composition of the PCS and on its molecular weight, a serial dilution was built mirroring the one described for "Bt 63" DNA. The LOD of the detection method developed by Mäde *et al.* was estimated on the pure plasmid serial dilution (15 replicates per concentration level) and on samples non-GM rice DNA (at 100 ng per reaction) spiked with the plasmid (21 replicates per dilution).

3. Results

3.1. DNA integrity

After DNA extraction, the "Bt 63" sample was analysed by gel electrophoresis (70 V for 1 h) on a 1% agarose gel. The results of this assessment for DNA integrity are shown in Figure 1.

Fig. 1. Gel electrophoresis of rice Bt 63 DNA sample



MWM= Molecular Weight Marker, Lamba DNA/*EcoR*I+*Hind*III

As shown in Fig. 1, the rice "Bt 63" DNA was mostly intact and appeared as a high molecular weight sample. It was therefore considered suitable for subsequent analysis.

3.2. Linearity and inhibition tests

The DNA quality has been demonstrated by the results of linearity and inhibition tests shown in Table 1.

Table 1. Linearity and inhibition test results with the species-specific reference system (*gos9*)

Sample	PCR system	DNA dilution factor					Linearity (R^2)	Slope	ΔCt
		1:1	1:4	1:16	1.64	1:256			
		Ct value (mean of 3 wells)							
Rice Bt63	*gos9*	20.0	21.8	23.8	26.0	28.0	0.99	-3.4	0.27
Conventional rice	*gos9*	19.7	21.7	23.7	25.8	28.1	0.99	-3.5	0.24

For both DNA samples the correlation coefficient (R^2) was above 0.99. Also the slopes were within the acceptance criterion. The test for inhibition, performed amplifying the extracts with *gos9* gene, provided a ΔCt value of 0.27 and 0.24 for the "Bt 63" and the conventional rice DNA extracts, respectively.

Altogether these data suggest a suitable quality of the DNA extracted for subsequent applications of real-time PCR.

3.3. Specificity

3.3.1. Bioinformatics analysis

The molecular structure of the insertion region of "Bt 63", and particularly of the *cry1A(b)-cr1A(c)-to-nos* terminator transition, has been published[1,2]. Bioinformatics analysis performed through the Central Core Sequence Information System (CCSIS) maintained in the Biotechnology and GMOs Unit of the JRC-IHCP aimed at assessing whether cross-reactivity could be predicted for the Mäde's method based on the sequence of the T51F and T51R primers and the resulting amplicon.

Homology searches with the sequences of the primer pairs T51F and T51R against the non-redundant Genbank "nt" database found no hits, while when blasting the amplicon sequence (83 bp) against the same database, almost complete match of the region including the T51 forward primer and the T51 probe (from 1 to 60 bp) was evident with targets of the *cry1A(c)* gene. The T51 forward primer confirmed its matches with *cry1A(c)* in the patent database whilst a portion of the T51R primer matched with a different set of patented entries, particularly with short not-annotated regions that could likely represent spacer oligonucleotides.

Blast searches of the T51F-T51R primers against the JRC_GMO database did produce hits with sequences of GMOs. However, most hits involved matching of only one part of the sequence of the two primers. Partial sequences of both T51F and T51R were predicted to anneal to cotton line

MON531 with minimum distance between annealing sites of more than 1700 nucleotides and to carnation Moonlite® target where the distance between annealing sites was at least 5500 nucleotides.

3.3.2. Experimental testing of specificity

Further to the bioinformatics analysis and considering the T51F primer match on the 3' end of the gene econding for CRY1A(c) protein, the specificity of the Mäde detection method was experimentally tested against DNAs of GMOs harboring the *cry1A(c)* gene, as well as on a selection of GMOs, some harboring the termination sequences from the 3' end of the *nos* gene.

Tables 2, 3 and 4 show the GMOs tested with the construct-specific method for detection of rice Bt 63, the presence of *Cry* genes and/or *nos*-terminator in the GMOs and the results of specificity testing.

Table 2. Specificity results for GMOs individually tested with the "Bt 63" Mäde detection method. Ct number is the average of three replicates.

Event Name	***cry* or *3'-nos* genetic elements***	**Bt 63 method (Ct number)**	**Taxon-specific reference system (Ct number / ref system)**
MON15985	*cry1A(c)* *cry2a(b)* *3'-nos*	n.d.	24.3/*adhC* [9]
MON531	*cry1A(c)* *3'-nos*	n.d.	24.1/*adhC* [9]
MON15985xMON1445	*cry1A(c)* *cry2a(b)* *3'-nos*	n.d.	24.1/*adhC* [9]
MON531xMON1445	*cry1A(c)* *3'-nos*	n.d.	24.6/*adhC* [9]
Bt11#	*cry1A(b)* *3'-nos*	n.d.	24.1/*adh* [10]
Positive control 1**	-	29.4	n.d./*gos9*
Positive control 2***	gDNA rice Bt 63	33.9	18.8/*gos9*
No template control	-	n.d.	n.d./*gos9*

n.d. = not determined

Bt11 at 2% GM

* Source: Agbios database [4];

** Positive control 1 = plasmid pGSE28 1000 copies per reaction, GeneScan Lot 060908 "Bt 63"

*** Positive control 2 = FR0633745, rice Bt63 at unknown GM concentration, kindly provided by GeneScan GmbH

Table 3. Specificity results for GMOs individually tested with the "Bt 63" Mäde detection method. Ct number is the average of three replicates.

Event Name	*cry* or *3'-nos* genetic elements*	Bt 63 method (Ct number)	Taxon-specific reference system (Ct number / ref system)
3006-210-23x281-24-236	*cry1A(c)* *cry1F*	n.d.	25.7/*sah7* [7]
Bt176	*cry1A(b)*	n.d.	23.0/*adh* [10]
GA21	*3'-nos*	n.d.	22.9 /*adh* [10]
T25	*None*	n.d.	23.1/*adh* [10]
MON863	*cry3Bb1* *3'-nos*	n.d.	23.0/*adh* [10]
NK603	*3'-nos*	n.d.	22.9/*adh* [10]
MON810	*cry1A(b)*	n.d.	23.2/*adh* [10]
LLRice62	none	n.d.	20.1/*pld* [8]
Positive control**	-	28.16	-
No template control	-	n.d.	*gos9*

n.d. = not determined

* Source: Agbios database [4];

** Positive control = plasmid pGSE28 1000 copies per reaction, GeneScan Lot 060908 Bt63

Table 4. Specificity results for GMOs individually tested with the "Bt 63" Mäde detection method. Ct number is the average of three replicates.

Event Name	*cry* or *3'-nos* genetic elements*	Bt63 method (Ct number)	Taxon-specific reference system (Ct number / ref system)
TC1507	*cry1F*	n.d.	23.6/*adh* [10]
59122	*cry34Ab1* *cry35Ab1*	n.d.	23.8/*adh* [10]
MON89034	*cry1A.105* *cry2Ab2* *3'-nos*	n.d.	24.7/*adh* [10]
MIR604	*nos-t*	n.d.	23.6/*adh* [10]
MON88017	*cry3Bb1* *3'-nos*	n.d.	23.8/*adh* [10]
LY038	*3'-nos*	n.d.	23.6/*adh* [10]
3272	*3'-nos*	n.d.	23.9/*adh* [10]
Rf1	*3'-nos*	n.d.	23.2/*cru* [11]
Rf2	*3'-nos*	n.d.	23.6/*cru* [11]
Rf3	*3'-nos*	n.d.	23.2/*cru* [11]
Ms1	*3'-nos*	n.d.	22.7/*cru* [11]
Ms8	*3'-nos*	n.d.	22.4/*cru* [11]
Positive control**	-	21.19	22.6/*gos9*
No template control	-	n.d.	n.d./*gos9*

n.d. = not determined

* Source: Agbios database and United States Regulatory Agencies Unified Biotechnology Website. [4-6]

** Positive control = 100% Bt63 rice gDNA provided by Chinese Authorities

Altogether these data indicate that, under the conditions described, the detection method developed by Mäde *et al.* to detect rice "Bt 63" does not react with the tested GMOs tested.

3.4. Limit of detection (LOD)

The sensitivity of the Mäde detection method was evaluated through the determination of the limit of detection tested on rice "Bt 63" genomic DNA and based on haploid genome copy numbers as described in the section Experimental Design. Table 5 reports the results of this experiment

According to the definition of the European Network of GMO Laboratories (ENGL), the LOD is the lowest amount or concentration of an analyte in a sample which can be reliably detected but not necessarily quantified, as demonstrated by single laboratory validation. Methods should detect the presence of the analyte at least 95% of the times at the LOD, ensuring ≤5% false negative results[4].

Table 5. Result of LOD of the Mäde's method on rice "Bt 63" genomic DNA

Samples	Bt 63 number of copies	Mean Ct	No Positive/total replicates
100% Bt 63 rice gDNA diluted in water	50	35.1	21/21
	25	36.3	21/21
	10	37.8	21/21
	5	**39.0**	**20/21**
	1	40.8	12/21
	0.1	42.5	2/21
Bt63 positive control	n.d.	33.4	6/6
No Template control	n.d.	n.d.	0/6

n.d. = not determined

Therefore, having regard to the above definition and to the results presented in Table 5, the LOD of the Mäde's construct-specific method for detection of rice "Bt 63" is at least 5 copies. This is in good agreement with the published LOD of 7 copies[2].

In addition to the results presented above and with the intention to assist National Reference Laboratories with the provision of an appropriate positive control samples in suitable amounts, the CRL-GMFF produced a plasmid, (pENGL-00-EM02/06-01M) containing the 83 bp amplicon produced by amplification according to the Mäde detection method.

Further to quality checks, the LOD of the Mäde method was experimentally determined for the pure plasmid (Table 6) serially diluted in water (50 to 0.1 copies) and for the same plasmid spiked in non-GM rice genomic DNA at 100 ng/reaction (Table 7).

As shown in Tables 6 and 7, the Mäde detection method has an LOD of 5 copies both on the pure plasmid containing the target 83 bp amplicon (pENGL-00-EM02/06-01M), and on the plasmid spiked in 100 ng of non-GM rice genomic DNA.

Table 6. Result of LOD of the Mäde method on pure plasmid dilution series

Number of plasmid copies	Mean Ct	St Dev	No Positive/Total replicates
50	34.4	0.3	15/15
25	35.7	0.4	15/15
10	37.5	0.5	15/15
5	**38.7**	**0.9**	**15/15**
1	40.9	1.4	11/15
0.1	43.1	0.5	2/15
PC	33.5	0.3	3/3
NTC	n.d.	n.d.	0/3

n.d. = not determined

Table 7. Result of LOD of the Mäde method on plasmid dilution series spiked in non-GM rice DNA

Number of plasmid copies	Mean Ct	St Dev	No Positive/Total replicates
50	34.3	0.3	21/21
25	35.7	0.3	21/21
10	37.3	0.5	21/21
5	**37.8**	**0.6**	**21/21**
1 #	40.5	0.9	16/21
0.1 #	40.2	1.2	2/21
PC	32.8	0.1	3/3
NTC	n.d.	n.d.	0/3

n.d. = not determined

Samples at 1 and 0.1 plasmid copies were run on a separate plate with PC and NTC

4. Conclusions

The method proposed by Mäde *et al.* targets a DNA sequence spanning between the *cry1A(b)/cry1A(c)* fusion gene and a DNA region linking the fusion gene to the *nos* terminator, as such it has to be considered a construct-specific detection method. Further to bioinformatics analysis run against the major sequence databases, no cross-reactivity with other plant genomes or GMOs can be predicted at this point in time.

Experimental testing of specificity on GMOs currently approved in the EU or in the process of authorisation confirmed the specificity of the method for rice "Bt 63".

As a result of the scientific assessment conducted on the published methods for detection of "Bt 63" rice and as a result of the experimental testing herewith reported, the CRL-GMFF concludes that the detection method published by Mäde *et al*. can specifically detect the presence of "Bt 63" event in rice seeds and grains.

The limit of detection experimentally determined is at least 5 haploid genome copies on rice "Bt 63" genomic DNA, confirmed on plasmid control sample either pure or spiked in non-GM rice DNA.

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