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Variability in the levels of PML-RARα fusion transcripts detected by laboratories participating in an external quality control program using several reverse transcription polymerase chain reaction protocols

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Background and Objectives. The detection of *PML-RAR* by reverse transcription (RT) polymerase chain reaction (PCR) in acute promyelocytic leukemia (APL) patients who are in hematologic remission influences therapeutic decision making in several trials. In the light of this, the Spanish group has recently designed an external quality assessment program (EQAP) of RT-PCR detection of *PML-RAR*, which includes a study of sensitivity of the participating laboratories.

Design and Methods. Eighteen laboratories were involved in the program. Ten laboratories followed the method of Biondi *et al.*,[4] 5 employed that of Borrow *et al.*[10] and the 3 remaining used other protocols. The sensitivity was studied in five rounds of quality control. The first two shipments consisted of dilutions of NB4 RNA into non-APL RNA. The third round consisted of serial dilutions of the NB4 cell line into HL60 cells. The fourth and five rounds consisted of plasmid dilutions containing the bcr1 and bcr3 *PML-RAR* isoforms.

Results. The results showed that the distinct methods allow detection of the *PML-RAR* hybrid up to a dilution of 10^{-4}, and exceptionally, up to 10^{-5}. The laboratories following the method of Biondi *et al.* usually detected the 10^{-3} dilution and less frequently the 10^{-4} one, whereas those using other methods usually detected *PML-RAR* transcript in the 10^{-4} dilution, and less commonly in the 10^{-5} dilution. However, each of the PCR methods used by EQAP participating laboratories successfully detected at least 50 copies of *PML-RARα* fusion transcript in plasmid dilution controls.

Interpretation and Conclusions. The results point to heterogeneous sensitivity amongst participating laboratories. This may reflect differences in methodology, although variations in sample quality may also account for discrepant findings.

Key words: *PML-RAR*, RT-PCR, sensitivity, external quality assessment program, acute promyelocytic leukemia

Reverse-transcription polymerase chain reaction (RT-PCR) has been increasingly employed as a rapid and powerful tool for detecting genetic lesions in leukemia. Moreover, it represents the most sensitive method to identify minimal residual disease (MRD) in patients who are in clinical remission after treatment. Compared to RT-PCR assays used to identify other gene rearrangements, the RT-PCR techniques employed for detecting the acute promyelocytic leukemia (APL)-specific *PML-RAR* fusion show inferior sensitivity.[1] In fact, reported *PML-RAR* amplification methods usually reach a 10^{-4} dilution detection threshold, i.e. one or two logs below the sensitivity reported for the *BCR-ABL*[2] and *AML1-ETO*[3] RT-PCR assays used for chronic myeloid leukemia and t(8;21) acute myeloid leukemia, respectively. However, a certain degree of heterogeneity in sensitivity is also found between different APL studies. For example, two of the most widely adopted assays, the techniques originally described by Biondi *et al.*[4] and Chen *et al*.[5] differ by as much as one log in their sensitivity, with reported detection levels of 10^{-4} for the bcr1 transcript using the former method and 10^{-5} for long and short transcripts using the latter.

The sensitivity of the *PML-RAR* RT-PCR assays used for MRD evaluation appears to be extremely important in clinical practice. Using a technique with a 10^{-4} detection threshold, *the Italian Cooperative Group GIMEMA* reported in a prospective monitoring study that virtually all APL patients who converted from PCR-negative to positive during clinical remission underwent hematologic relapse after a median time of 3 months.[6] As a consequence of such findings, patients enrolled in the GIMEMA study who show PCR-positivity for *PML-RAR* (confirmed in two successive marrow samples) are now defined as having *molecular relapse* and given anticipated salvage therapy. By contrast, using a more sensitive assay Tobal *et al.*[7] found that, like patients with other leukemias, some APL patients in long-term remission and presumably cured may nevertheless show PCR-detectable MRD.

In the light of these findings and because prospective monitoring was to be initiated in 1996 for patients enrolled in the Spanish multicenter APL trial, we designed at that time an external quality assessment program (EQAP) for RT-PCR detection of *PML-RAR*. The program originally involved only those Spanish laboratories belonging to institutions participating in the PETHEMA LPA-96 clinical trial, but was subsequently extended to several other European laboratories. The aims of the first part of the study, which involved two rounds of quality control, were to evaluate the concordance of results between participating laboratories for diagnostic evaluation of the fusion gene.[8] The present analysis summarizes the results of a further five rounds of quality control designed to evaluate the sensitivity achieved by the participating laboratories in *PML-RARα* detection.

Design and Methods

The organization of the EQAP program was described in a previous report.[8] It basically consisted of periodic shipments (one every semester) of a set of control samples (between 4 and 7) to all participating laboratories. For each shipment round, one of the laboratories involved in the program was responsible for sample preparation, shipment in dry ice and delivery within 24 h to all other laboratories. The laboratory in charge of sample preparation and shipment varied in each round and was excluded from sample analysis. The results of RT-PCR analyses, to be done blindly on numbered vials, were requested back within 30 days from the date of shipment. As in the preceding rounds, participants were asked to fill in and send back to the Co-ordinating Center (Laboratorio de Biología Molecular, Hospital Universitario La Fe, Valencia, Spain) the forms detailing the following: i) condition of samples on arrival; ii) most relevant technical aspects of the methodology used; iii) amplification results.

At present, the program includes a total of 18 laboratories (Table 1), of which 14 belong to Spanish Institutions and the remaining 4 to Centers in other European countries (Italy, France, UK, and The Netherlands).

Control samples

Sensitivity experiments were performed in five successive EQAP rounds (from the 4th to the 8th) which were carried out during the period October 1998 (4th round)-November 2000 (8th round).

In the 4th and 5th rounds, RNA samples of the APL cell line NB4 were sent. In the 4th shipment the samples consisted of 10-fold serial dilutions (10^{-2},10^{-3}, 10^{-4}) of NB4 RNA into RNA obtained from blood mononuclear cells of patients with chronic myeloid leukemia (CML) (Table 2). Undiluted CML-derived RNA was also used as a negative control in this round. The 5th shipment consisted of undiluted NB4 RNA and 10-fold serial dilutions (10^{-3} through 10^{-5}) of NB4 RNA in RNA derived from the Kasumi cell line. RNA was extracted by the phenol-chloroform procedure as reported by Chomczynski and Sacchi.[9]

The 6th shipment consisted of cellular samples, each one containing a total of 10^7 cells and including two samples at 10^{-2} and one at a 10^{-3} dilution of NB4 into the HL60 cell line. Positive and negative controls consisted of 10^7 undiluted NB4 and HL60 cells, respectively (Table 2).

Table 1. List of participating laboratories.

Person responsible	*Hospital*	*City*
Dr. D Colomer	Clínico de Barcelona	Barcelona
Dr. J Roman	Reina Sofía	Cordoba
Dr. MT Gómez	H Gran Canaria Dr. Negrin	Las Palmas de Gran Canaria
Dr. D Grimwade	University College and Guy's Hospital	London
Dr. E Anguita	Universitario San Carlos	Madrid
Dr. R Bornstein	Doce de Octubre	Madrid
Dr. P Cabello	H Ramón y Cajal	Madrid
Dr. J Benítez	C.to Nac. de Investigaciones Oncológicas	Madrid
Dr. J.A. García-Marco	Puerta de Hierro	Madrid
Dr. JL Vizmanos	Universidad de Navarra	Pamplona
Dr. Ch Chomienne	Hôpital Saint-Louis	Paris
Dr. D Diverio	Universitá degli Studi "La Sapienza"	Rome
Dr. J Jansen	Erasmus Universiteit	Rotterdam
Dr. M Gonzalez	Clínico Universitario	Salamanca
Dr. M Delgado	H Marques de Valdecilla	Santander
Dr JM De Blas	Virgen del Rocio	Sevilla
Dr. I Marugan	Clínico Universitario	Valencia
Dr. E Barragan	Universitario La Fe	Valencia

The 7th shipment consisted of plasmid DNA containing PCR amplification products of the bcr1 and bcr3 *PML-RAR* isoforms (Table 2). These products were obtained using the oligoprimers M2-R5 (for bcr1) and M4-R5 (for bcr3) published by Biondi *et al.*,[4] and cloned in a plasmid vector PCRII-TOPO (TOPO™ TA Cloning®, Invitrogen BV, 970 VT Groningen, The Netherlands). The samples included three 10-fold serial dilutions of the plasmids which contained concentrations of 4,000, 400 and 40 plasmid copies/μL of the bcr1 isoform, and, 2,500, 250, 25 plasmid copies/μL of the bcr3 isoform. A water sample was used in this shipment as the negative control.

The 8th shipment consisted of serial plasmid dilutions containing a full length bcr1 insert into an aqueous solution containing 1 g of salmon sperm DNA at the following concentrations: 0 (reagent blank), 50, 200, 800 and 10,000 plasmid copies/μL (Table 2). In the 7th and 8th shipments, 1mL of control material was used as template in the subsequent PCR reaction.

Reverse transcriptase and PCR protocols

The distinct methods followed between the 4th and 8th rounds of our EQAP are shown in Table 3. Ten laboratories followed the procedure reported by Biondi *et al.*,[4] five laboratories used the protocol published by Borrow *et al.*[10] and each one of the three remaining laboratories followed methods reported by Chen *et al.*[5] or Huang *et al.*,[11] Miller *et al.*[12] and Castaigne *et al.*[13] The majority of laboratories (14/18) amplified *RAR* in parallel as the control gene as previously described,[4,5,10-13] including one which amplified *PML* in addition according to the method of Borrow *et al.*[10] The remaining 4 laboratories which all used the *PML-RARα* assay described by Biondi *et al.* used alternative control gene assays; 3 amplified ABL using primers of Cross *et al.*[2] and one used *AML1* employing primers reported by Satake *et al.*[14]

The main differences between these methods in terms of enzyme type and incubation time for the reverse transcription step are reported in Table 3.

The 7th shipment, which consisted of plasmid-cloned PCR products, was restricted to laboratories with suitable primers to amplify the cloned insert. In this shipment round, the RT protocol was irrelevant since DNA samples were tested. In this EQAP round, three of the participating laboratories followed Biondi's method,[4] one performed Borrow's method[10] modified by the introduction of the P6 primer as reported by Gallagher *et al.*[15] The remaining three laboratories followed the method described by Chen,[5] the BIOMED-1 European Concerted Action[16] and the *real-time* PCR method of Gabert *et al.*[17]

Table 2. Participation and study feasibility.

Round	*Composition*	*Number of samples*	*Number of labs reporting results*	*Validated/ reported results*	*Reasons for exclusion*
4th	NB4 RNA diluted in CML RNA	4	15	52/60	Degraded RNA due to poor shipment conditions (n=4) or delayed analysis (n=4).
5th	NB4 RNA diluted in Kasumi RNA	4	15	56/60	Degraded RNA (n=4)
6th	NB4 cells diluted in HL60 cells	5	13	65/65	
7th	Plasmid containing PCR bcr1 or bcr3 inserts	7	8	49/56	Contamination strongly suspected due to amplification of bcr3 *PML-RAR* in all controls plus water sample
8th	Plasmid containing a bcr1 full length insert	5	16	80/80	

CML= Chronic myeloid leukemia. Reported results: refers to the total number of results returned to the co-ordinating laboratory; Validated results: refers to the number of evaluable results that were included in the final analysis. Reasons for sample exclusion are detailed in the right-hand column.

Table 3. Reverse transcription and PCR methods of participating laboratories.

Methods	Reverse transcriptase		Incubation time	
	AMV	MMLV	<1h (15-50 min)	=1 h (60-90 min)
Biondi *et al.*	2	8	6	4
Borrow *et al.*	4	1	1	4
Huang, Miller, Castaigne	–	3	1	2

The figures within the table relate to the number of laboratories using a particular RT-PCR method.
Abbreviations: AMV, Avian myelomatosis virus reverse transcriptase; MMLV, Moloney murine leukemia virus reverse transcriptase.

Distinct from plasmids sent out in the previous EQAP round, the plasmid sent during the 8th shipment contained a full length bcr1 insert, and all the 16 participating laboratories had primers capable of amplifying the cloned fragment.

Results

Participation and study feasibility

The results of study feasibility are summarized in Table 2. The Co-ordinating Center received data from 15 laboratories in the 4th EQAP. Results from 8/60 samples were excluded, because of delayed delivery and RNA degradation or delayed analysis of the samples. Similarly, results from 4 samples received by 1 laboratory were excluded from the next round in which 60 results were received from 15 laboratories. In the 6th shipment EQAP data were received from 13 laboratories and in the 7th shipment, which consisted of plasmid DNA controls, only 8 laboratories with suitable primer sets participated with data from one laboratory (7 results) excluded because of sample contamination (Table 2). In the 8th shipment, which consisted of plasmid DNA containing a full length bcr1 isoform insert, the results from 16 laboratories were reported.

Sensitivity of PML-RAR detection reported by the laboratories

The results of PML-RAR analysis in RNA samples (4th and 5th EQAP shipments) are given in Table 4. These show that almost all laboratories detected the transcript in the 10^{-2} dilution of the NB4 cell line. Eighteen out of 27 (67%) samples containing the NB4 10^{-3} dilution were reported as *PML-RAR* positive. Positive results were also reported in 5/13 non-APL controls.

With respect to the analysis of cell dilution samples (Table 4, NB4 cells), the results were comparable to those obtained with RNA samples. Hence, *PML-RAR* was detected by all laboratories in the 10^{-2} dilution, whereas some centers (4/13) reported absence of amplification in the 10^{-3} NB4 dilution. None of the laboratories reported positive *PML-RAR* detection in the HL60 cell line negative control. As to the distinction of the *PML-RAR* isoform, in 10/110 results in which *PML-RAR* fusion transcripts were detected the *PML* breakpoint was erroneously reported as bcr3 rather than bcr1. Five different laboratories reported these erroneous results. In four of them the misclassification was restricted to one sample of the 5th shipment. However, for the remaining laboratory the results most likely reflected technical problems since the misclassification affected 5 samples, one from the 4th shipment and 4 from the 5th. Moreover, this laboratory also reported a positive result in the negative control, which was classified as bcr3. These problems were addressed in subsequent EQAP rounds, with the latter laboratory classifying all *PML* breakpoints correctly and reporting no false positives.

Table 4. Results of NB4 RNA and cellular samples.

Methods	RNA NB4 cells (4th and 5th shipments)						NB4 cells (6th shipment)			
	NB4 10^{0}	NB4 10^{-2}	NB4 10^{-3}	NB4 10^{-4}	NB4 10^{-5}	No APL	10^{7} NB4	10^{-2} NB4	10^{-3} NB4	10^{7} HL60
Biondi *et al.*										
PML-RAR positive/total (%) L	7/7 (100)	6/7 (86)	6/13 (46)	5/14 (36)	1/7 (14)	1/7 (14)	6/6 (100)	12/12 (100)	4/6 (67)	0/6 (0)
Borrow *et al.*										
PML-RAR positive/total (%) L	5/5 (100)	4/4 (100)	8/9 (88)	6/9 (66)	3/5 (60)	3/4 (75)	5/5 (100)	10/10 (100)	4/5 (80)	0/5 (0)
Miller, Huang & Castaigne *et al.*										
PML-RAR positive/total (%)	2/2 (100)	2/2 (100)	4/5 (80)	3/4 (75)	0/2 (0)	1/2 (50)	2/2 (100)	4/4 (100)	1/2 (50)	0/2 (0)
Overall results										
PML-RAR positive/total (%)	14/14 (100)	12/13 (92)	18/27 (67)	14/27 (52)	4/14 (29)	5/13 (38)	13/13(100)	26/26 (100)	9/13 (69)	0/13 (0)

The figures within the table relate to the number of results reported.

Sensitivity of the PML-RAR methods

The laboratories that followed Biondi's method[4] reported detection thresholds between 10^{-3} and 10^{-4}. In fact, *PML-RAR* was detected in 6/13 and 5/14 samples for the 10^{-3} and the 10^{-4} RNA dilutions, respectively (Table 4). A false positive result in the non-APL RNA was recorded in 1 of 7 samples for laboratories using this assay. In the EQAP round involving cellular samples (6th), 4/6 laboratories using this method detected the 10^{-3} dilution of NB4 and no false positives were obtained (Table 4).

No difference in *PML-RAR* detection was observed when laboratories using Biondi's method were grouped according to the incubation time of the reverse transcription (RT) step (1h vs.< 1h).

A higher proportion of *PML-RAR* positive results was recorded by laboratories using Borrow's method in the 10^{-4} and 10^{-5} RNA dilutions (6/9 and 3/5, respectively, Table 4). However, a higher proportion of positive results (3/4) in the negative control was also reported by the laboratories using this assay (Table 4). In the analysis of cellular samples 4/5 laboratories detected the 10^{-3} dilution of NB4 (Table 4). For this method most of the participants (4 of 5 laboratories) performed RT with incubation times longer than 1 h.

The results of the 3 remaining laboratories using alternative techniques[11-13] were similar in terms of sensitivity to the ones which followed Borrow's method.[10] Hence, a high proportion of positive results was recorded up to the 10^{-4} dilution (Table 4).

Absolute sensitivity studies with plasmid DNA

As shown in Table 5, all laboratories participating in the 7th round amplified and correctly identified the *PML-RAR* fusion gene in the more concentrated plasmid controls containing 4,000 copies of the bcr1 plasmid/μL and 2,500 copies of the bcr3 plasmid/μL. Four out of 7 laboratories could amplify the bcr1 plasmid at the concentration of 400 copies/μL and 3 of 7 detected the hybrid transcript in the control containing 250 plasmid copies/μL of the bcr3 isoform; however one laboratory erroneously identified the bcr3 fusion gene as bcr1. Only one of seven laboratories reported amplification of the bcr1 plasmid at the concentration of 40 copiesμ/L, but incorrectly identified the fusion gene as bcr3, whereas 2 of 7 laboratories amplified and correctly identified the bcr3 plasmid at the concentration of 25 copies/μL (Table 5).

In the 8th shipment, all the participating laboratories (16/16) detected the *PML-RAR* rearrangement in the control with the lowest bcr1 concentration (50 plasmid copies/μL) (Table 5). As to the negative control, most of the laboratories (14/16) reported it correctly as negative, one reported it as positive and the remaining laboratory as equivocal. No difference could be found when the data reported were classified according to the distinct methods employed by participating laboratories. Whilst the *PML-RAR* fusion gene copy numbers contained within plasmid dilution controls distributed in the 7th and 8th quality control rounds were independently confirmed by *real-time* PCR analysis performed in the co-ordinating laboratory against common plasmid standard curves, the sensitivity reported in the 7th round was worse than in the 8th. This may be accounted for by a variable reduction in *PML-RAR* copy number concentration due to degradation of plasmid DNA or absorptive effects of the sample tubes occurring during transit or storage prior to analysis.

Table 5. Results of the plasmid controls.

Shipment	*Controls*	*Concentration copies/μL*	*PML-RAR+/total (%)*
7th shipment	bcr3 plasmid	2,500	7/7(100)
		250	3/7(43)
		25	2/7(29)
	bcr1 plasmid	4,000	7/7(100)
		400	4/7(57)
		40	1/7(14)
		0	0/7(0)
8th shipment	bcr1 plasmid	10,000	16/16(100)
		800	15/16(94)
		200	16/16(100)
		50	16/16(100)
		0	1/16(6)

Discussion

In this study on the detection of the APL-specific *PML-RAR* hybrid at various dilutions, we observed some important discrepancies which may result from logistic and technical problems. For example, it is possible that a heterogeneous degree of RNA degradation occurred in the various shipments. Moreover, no hybridization step was required to confirm the specificity of amplified products. Besides these caveats, this study confirms the limited sensitivity of the methods commonly used to detect the *PML-RAR* rearrangement. Hence in the analysis of NB4 RNA samples, most laboratories amplified the 10^{-2} dilution, whereas a significant number were not able to detect the rearrangement at a 10^{-3} dilution, and few detected it at 10^{-4}.

The data obtained from the present study suggest that the sensitivity threshold of 10^{-4} originally reported by both Biondi *et al.*[4] and Borrow *et al.*[10] is not

widely reproduced in the setting of a multi-laboratory study, as in the majority of cases a lower sensitivity was obtained, particularly using Biondi's protocol.[4] Although participating laboratories were requested to evaluate sample quality on arrival and successful control gene amplification had to be obtained to validate the results, it is possible that sample shipment to several laboratories might have altered *PML-RAR* stability.

As to the reverse transcriptase phase, the limited number of results did not permit consideration of all the factors that might influence efficiency of this step (primer and RT enzyme used, inclusion of a denaturation step, and incubation time). However, no relevant differences were recorded among the laboratories that performed a prolonged RT incubation (=1h) versus those laboratories which adopted a shorter time (< 1h).

As shown in Table 4, a high incidence of false positivity was found analyzing non-APL RNA samples, which were most frequently reported by laboratories using Borrow's method. The most likely explanation for this was introduction of low levels of contamination by the laboratory preparing the samples. This hypothesis is supported by the fact that in the 6th round, that consisted of cellular samples requiring less laboratory manipulations, no positive result was recorded in the HL60 cell sample (Table 4). Distribution of cellular samples as quality control materials also had the advantage that they permitted evaluation of additional phases of sample processing, including RNA extraction, as well as the RT and PCR steps, which are likely to have an impact on the sensitivity of RT-PCR assays undertaken in a clinical setting. Plasmid dilution standards afford the opportunity to consider the sensitivities of particular PCR primer sets in isolation, without potentially confounding influences generated by inter-laboratory differences in other aspects of the RT-PCR protocol. Our experience with plasmid solutions suggests that they cannot invariably be relied upon to provide stable controls for quality control purposes. Whilst all laboratories successfully detected 50 copies of bcr1 *PML-RAR* transcript in the 8th round, only 1/7 detected 40 copies in the preceding round. This discrepancy in sensitivity is likely to reflect degradation or absorption of plasmid DNAs distributed in the 7th shipment prior to the point of analysis.

In conclusion, our external quality control study on detection of the *PML-RAR* fusion transcript by RT-PCR revealed heterogeneous sensitivities amongst participating laboratories. This is likely to reflect the inherent instability of materials sent for molecular analyses, but may also be accounted for in part by differences between RT and PCR protocols employed by participating laboratories. These issues are important to take into account in the setting of large multicenter studies. It is expected that the quantification of PML-RAR transcripts with specific and highly sensitive quantitative methods developed using real-time PCR equipment[17,18] will, in the near future, offer adequate standardization of sensitivity for optimal comparison of results obtained in clinical trials.

Contributions and Acknowledgments

PB was the principal investigator: he designed the study and was partly responsible for the funding, supervision and critical revision of the manuscript. EB was responsible for the database management. FL contributed to the drafting of the manuscript. DG contributed to critical revision of the manuscript. The remaining co-authors participated in the quality control and in the elaboration and critical revision of the manuscript.

The order of the authorship was made according to the contribution given to the study.

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Potential implications for clinical practice

The *PML-RAR* RT-PCR assay used for minimal residual disease evaluation in APL is critically important for clinical practice, since results are used to guide therapy.[19-22] The standardization of methods and reporting of results of assays used for molecular diagnosis and minimal residual disease monitoring in leukemia will facilitate comparison of outcome between different clinical trials; external quality assessment programs including large numbers of participating laboratories play an essential role in this process and in the provision of reliable results upon which patient management decisions may be based.

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