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Short Communication

Reliability of Reverse Transcription-polymerase Chain Reaction (RT-PCR)-based Assays for the Detection of Circulating Tumour Cells: a Quality-assurance Initiative of the EORTC Melanoma Cooperative Group

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Reverse transcription-polymerase chain reaction (RT-PCR)-based assays detecting occult neoplastic cells are increasingly being used for the study of tumour dissemination and minimal residual disease. However, different methods are employed by various research groups and the results are heterogenous. We prospectively assessed the results from nine laboratories performing tyrosinase RT-PCR assays for the detection of melanoma cells on a series of blind samples. After complete analysis, the results were compared for sensitivity and specificity. All laboratories reported correct results for cDNA standards. Five laboratories attained acceptable specificity and a sensitivity detecting 10 cells in 10 ml of whole blood. Four laboratories had unacceptable specificity and/or sensitivity. This blind study highlights the difficulty of RT-PCR data interpretation and the need for quality assurance between laboratories. Measures to increase the reliability of RT-PCR assays are proposed, which have to be prospectively evaluated in future studies. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

In 1991, a paper by Smith and colleagues [1] described a polymerase chain reaction (PCR) assay for the detection of circulating melanoma cells by the amplification of tyrosinase mRNA, which is exclusively expressed in melanocytes and melanoma cells. This paper was the first of a series describing reverse transcription PCR (RT-PCR)-based assays utilising the amplification of tissue-specific genes for the detection of occult tumour cells for a variety of histologies.

The frequency of tumour cell detection reported by different laboratories varies considerably. This is most obvious in stage IV melanoma patients, where the reported percentage of patients with evidence of tumour cells in blood ranges between 23 and 100% [2–10]. Different methods for sample processing, extraction of RNA, synthesis of cDNA and PCR amplification have been employed in these studies and may account for this discrepancy. The urgent need for quality assurance initiatives for diagnostic PCR has been emphasised by a series of EUROHEP quality control studies on viral nucleic acid-based amplification assays, where only 16–23% of the participating laboratories had reported faultless results [11].

After a 2-day meeting of the EORTC Melanoma Group, where participants of several European laboratories discussed experiences and methodological problems with RT-PCR-based detection of circulating melanoma cells [12], it was decided to perform a collaborative quality control study. This

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study was designed to: (1) assess the reliability of the methods in use; and (2) determine the most common causes of disparities.

MATERIALS AND METHODS

The test panel consisted of three series of samples. For each series, 300 ml of blood was obtained from 2 healthy volunteers and pooled. The resulting 600 ml of whole blood was further processed.

Series 1 (not blind) consisted of cDNA standards. Mononuclear cells were purified from 600 ml of pooled blood by Ficoll hypaque density gradient separation. After RNA extraction and cDNA synthesis, cDNA samples representing the amount obtained from peripheral blood mononuclear cells (PBMC) of 10 ml of blood were mixed with 10-fold dilutions of cDNA from the melanoma cell line SK Mel28 [13], representing a range of $0–10^5$ melanoma cells per sample. These samples were to be amplified as a sensitivity control in the same PCR as the blind samples of the second series.

Series 2 consisted of seven coded 10 ml whole blood samples prepared from 600 ml of pooled blood from 2 further volunteers, to which was added guanidinium isothiocyanate RNA extraction buffer [14] spiked with $0–10^4$ SK Mel28 melanoma cells.

Series 3 consisted of seven coded samples of BPMC prepared by Ficoll hypaque density gradient centrifugation from 600 ml of pooled blood obtained from a third pair of healthy volunteers. Samples of PBMC representing 10 ml of peripheral blood were mixed with RNA extraction buffer containing the same number of melanoma cells as in the whole blood samples of series 2.

The test panel had been prepared in the tumour immunology laboratory at the University of Heidelberg and was distributed by overnight courier on dry ice to the participating laboratories. RNA extraction, purification and cDNA synthesis was performed according to each laboratory's in-house protocol. Most laboratories used the HTYR 1/2 and/or HTYR 3/4 primers [1], one laboratory used the VB17 and VB18 primers [15]. Various PCR protocols were employed, as summarised in Table 1. The results were reported on prepared forms. The evaluation was performed anonymously.

RESULTS

Samples were distributed to 10 European laboratories and results were reported from nine (Table 2). Two laboratories did not participate in the evaluation of series 3. One laboratory performed two different PCR assays (nested PCR and semi-quantitative assessment by combining PCR and Southern blotting) for series 2 and two laboratories performed nested PCR and semi-quantitative analysis for series 3.

Strikingly, all laboratories reported correct results for all samples of the cDNA dilution series (not shown). From whole blood samples (series 2) no laboratory generated faultless results, but four of the nine laboratories (nos 1–3, 5) reported sufficient results, defined as missing only one of the two positive samples with the lowest concentration of melanoma cells. The remaining laboratories reported more false-negative and/or false-positive results. No amplifiable RNA could be extracted in two laboratories (nos 8, 9). Extraction of amplifiable RNA from PBMC (series 3) was successful in all laboratories. Four laboratories (nos 1–3, 5) reported sufficient results, whereas three laboratories had more than one false-negative result (nos 5, 6) or missed a positive sample with 10^2 melanoma cells (no. 9). Two laboratories (nos 3, 6) reported false-positive results, which were, however, not reproducible and judged by the laboratories as probable contamination.

DISCUSSION

The identification of micro-metastatic neoplastic disease has generated considerable interest. The underlying clinical rationale has been that it may be possible to improve clinical outcome by early intervention in patients with micro-dissemination of tumour or preclinical relapse.

For melanoma, most laboratories use the detection of mRNA transcripts of the tyrosinase gene as a marker, since this gene is reported to be exclusively expressed in melanocytes and melanoma cells. Tyrosinase has been detected in a wide range of normal tissues in one study [16], but several reports have consistently stated absence of tyrosinase transcripts in the peripheral blood of healthy volunteers [1–3], 6, 7, 9, 10], certifying that normal melanocytes do not circulate in peripheral blood and blood sampling usually does

Table 1. *Methods employed in different laboratories*

		Reverse transcription		PCR		
Laboratory no.	RNA purification	Priming	RT	Primers*	No. of cycles	Detection
1	Phenol/chloroform (+ CsCl gradient†)	Oligo(dT)	M-MLV	(a) 1/2, 3/4 (b) 1/2	30 + 24 30	EtBr agarose gel Southern blot
2	Phenol/chloroform	Random hex.	AMV	(a) 1/2, 3/4 (b) 1/2	28 + 30 28	EtBr agarose gel Southern blot
3	Phenol/chloroform + DNAse	Oligo(dT)	M-MLV	VB17/18	42	EtBr agarose gel
4	Phenol/chloroform	Oligo(dT)	AMV	1/2, 3/4	30 + 30	EtBr agarose gel
5	Phenol/chloroform	Random hex.	SuperScript	1/2, 3/4	35 + 35	EtBr agarose gel
6	Phenol/chloroform	Random hex.	AMV	1/2, 3/4	30 + 30	EtBr agarose gel
7	Phenol/chloroform + poly A^+ selection	Random hex.	M-MLV	3/4	50	EtBr agarose gel
8	Phenol/chloroform + CsCl gradient	Oligo(dT)	AMV	1/2, 3/4	30 + 30	EtBr agarose gel
9	Phenol/chloroform	Random hex.	M-MLV	1/2, 3/4	35 + 35	EtBr agarose gel

*See [1, 15] for primer sequences. †For series 2 only. RT, reverse transcription; EtBr, ethidium bromide; AMV, avian myeloblastosis virus; M-MLV, Moloney murine leukaemia virus; PCR, polymerase chain reaction.

Table 2. Results from participating laboratories

	No. of SK MEL 28 cells per 10 ml of blood							No. of false	
Laboratory no.	10^4	10^2	10^2	10	10	0	0	Positives	Negative
Series 2 (whole blood)									
1(a)	+	+	+	+	–	–	–	0	1
1(b)	+	+	+	+	–	–	–	0	1
2	+	+	+	+	–		–	0	1
3	+	+	+	+	–		–	0	1
4	+	+	–	–	–	–	+	1	3
5	+	+	+	+	–	–	(+)	(1)	1
6	+	+	+	–	–	+	+	2	2
7	+	+	–	No	–	–	–	0	2
8	No	No	No	No	No	No	No		
9	No	No	No	No	No	No	No		
Series 3 (mononuclear cells)									
1(a)	+	+	+	+	–	–	–	0	1
1(b)	+	+	+	+	+	–	–	0	0
2(a)	+	+	+	+	–	–	–	0	1
2(b)	+	+	+	+	–	–	–	0	1
3	+	+	+	+	+	(+)	–	(1)	0
4	+	–	–	–	–	–	–	0	4
5	+	+	No	+	+	–	–	0	0
6	+	+	–	+	–	–	(+)	(1)	2
7	Not done								
8	Not done								
9	+	–	+	+	+	–	–	0	1

+, positive; –, negative. No, no amplifiable RNA. Results in parentheses, positive results not reproducible, suspected contamination.

not introduce skin melanocytes (and hence contamination) into blood samples. Thus, the presence of tyrosinase transcripts in peripheral blood samples may indicate the presence of melanoma cells. The frequency of tyrosinase mRNA transcript detection in melanoma patients has been correlated with the stage of disease [2] and an association between the intensity of the PCR signal and tumour burden, as well as response to systemic cytokine treatment, has been reported [8]. Most importantly, the detection of tyrosinase transcripts at the time of resection of localised disease has recently been shown to be of prognostic importance [6–10]. The clinical significance of these transcripts in deciding patient management remains to be seen [17] and is currently under investigation in a large randomised interferon trial of the EORTC Melanoma Cooperative Group.

In the future, the results of molecular assays for the detection of occult tumour cells may have consequences for patient management. However, in the current literature there is a wide range of results, consequently these data should be interpreted with great caution. No standard quality control measures have yet been implemented to assure sensitivity and reliability of the assay systems, mostly developed in-house. To assess prospectively whether the results from different laboratories on a panel of samples may be heterogeneous and to determine whether heterogenous results originate from sample processing or from PCR amplification procedures, this quality control study was performed. The result of this initiative is encouraging in that half of the reported results are acceptable, only missing one positive sample with the lowest number of melanoma cells. However, even for these laboratories, it has to be pointed out that the sensitivity of the assays is lower than previously published. Most previous papers state that the tyrosinase assay is able to detect a single tumour cell in 1–10 ml of blood [2–5, 7, 9, 10, 18–22]. In this report, however, approximately 50% of false-negative results occurred at the level of 10 melanoma cells in 10 ml of blood.

Three laboratories reported a sensitivity of less than 1 cell in 1 ml of blood from series 2 and 3, although cDNAs from a dilution of RNA could consistently be analysed with high sensitivity, down to one cell. This observation suggests that the problem of different sensitivities lies with RNA extraction and processing, and not with the PCR assay sensitivity itself. Two further laboratories were unable to extract amplifiable RNA from whole blood samples. Transcribed mRNA is very labile, consequently degradation can pose a problem when analysing biological samples that have not been rapidly or correctly processed. This is a particular problem when analysing blood samples, since red blood cells contain high levels of RNAses [23]. Whether RNA should be extracted from whole blood or from leucocyte preparations is still a matter of debate. Working with whole blood may decrease the purity and quality of isolated RNA, as was also evident in this report, but eliminates the risk of losing tumour cells, which is a concern for density gradient separated leucocyte preparations [12]. Thus, sample preparation, RNA extraction and cDNA synthesis, rather than the PCR protocols, may account for most of the heterogeneity in results of RT-PCR assays. Internal and external standards controlling the whole process of sample preparation as well as PCR amplification are currently being developed [12] and could be helpful to ensure the sensitivity on a per sample basis.

False positive results were reported in both test series. The necessity of stringent methods to detect and avoid systematic, as well as sporadic, contamination in laboratories performing diagnostic PCR cannot be overemphasised, especially if the amplification of a certain gene is performed on a routine basis over a prolonged time period, as PCR products accumulate in the environment of the laboratory.

Several technical problems have been defined and have to be overcome to achieve accurate assay results [24], but it can, nevertheless be foreseen that molecular detection of occult neoplastic cells will give better insights into tumour biology and may benefit our patients in the near future. International quality assurance programmes are an appropriate and important instrument to verify the accuracy of these molecular assays.

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