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Reliability of quantitative reverse-transcriptase-PCR-based detection of tumour cells in the blood between different laboratories using a standardised protocol

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Abstract

Differences in methods of reverse-transcriptase (RT)-polymerase chain reaction (PCR)-based detection of tumour cells in the blood gives rise to conflicting results, and standardisation is urgently needed. This pilot study aimed to assess the variation of RT-PCR-based detection of tumour cells in blood between four different laboratories using a commercially available kit with a standardised protocol. This kit allows comparison of results from different laboratories and facilitates the investigation of the influence of pre-analytical parameters. All laboratories analysed identical sets of blood samples spiked with tumour cells in a concentration range of 1–100 tumour cells/ml. To study at which level variation was introduced, three kinds of sample sets were generated in which (i) tumour cell RNA was spiked in the RNA of mononuclear cells (MNC), (ii) tumour cells were spiked in isolated MNC, and (iii) tumour cells were spiked in blood. Real-time quantitative RT-PCR was used to detect and quantify cytokeratin 20 (CK20) expression, which is indicative for the presence of epithelial tumour cells. All laboratories were able to detect CK20 expression in all spiked-RNA samples with limited variation in expression levels between laboratories. There was a positive correlation between the amount of spiked tumour cell RNA and CK20 expression level. RT-PCR analysis of spiked-MNC samples resulted in more variation in the CK20 expression levels between laboratories, however again all spiked samples were reported to be positive by all of the laboratories. The evaluation of spiked-blood samples gave rise to considerable quantitative and qualitative variation between the laboratories. Our results underline the importance and need for standardisation and extended quality control studies in the field of pre-analytics.

Keywords: Cytokeratin 20; Disseminated tumour cells; Interlab comparison; Pre-analytics; Quality control

1. Introduction

Staging of colorectal cancer patients may be improved by detection of disseminated tumour cells in the blood using reverse-transcriptase (RT)-polymerase chain reaction (PCR) [1–3]. Due to a lack of tumour specific markers, other tissue-specific markers are commonly used. These markers are thought to be solely expressed in epithelial cells, including tumour cells derived from epithelial tissues without expression in haematopoietic cells. Cytokeratin 20 (CK20) is an extensively studied tissue-specific marker. Correlation between the presence of CK20 expression in the blood as detected by RT-PCR and worse clinical outcome was demonstrated [1,2]. Nevertheless, the sensitivity to detect CK20-positive patients varies [4–8], and the specificity of CK20 RT-PCR is under debate, because CK20 expression in

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the blood of non-cancerous patients or healthy donors has been reported [2,9–11].

This variation in reported sensitivity and specificity needs to be minimised, before this method can be considered for diagnostic clinical use. Several papers have shown that differences regarding blood sample processing [12], RNA isolation [13], cDNA synthesis [13,14], and PCR amplification [15,16] lead to heterogeneous results even if evaluated in one laboratory. A quality control study of the European Organisation for Research and Treatment of Cancer (EORTC) melanoma group on the detection of disseminated tumour cells, which is the only comparative study among different laboratories, demonstrates that variations are mainly introduced in the course from blood sample processing to cDNA synthesis [17]. Despite the fact that these results clearly stress the need for standardisation of pre-analytical methods across laboratories, few such efforts have been made until now. A prerequisite for the study of pre-analytical aspects is utilisation of a standardised analytical procedure. The introduction of the Roche LightCycler-CK20 Quantification Kit allows for the first time a standardised comparison of results from different laboratories and, consequently, makes it possible to investigate the influence of pre-analytical parameters. Moreover, because this kit is based on real-time quantitative RT-PCR-based detection, it is a precise tool to compare results between laboratories, revealing both qualitative and quantitative differences. Additionally, application of a real-time quantitative RT-PCR might improve the specificity of the assay eventually because it allows distinction between expression due to tumour cells and a low background level of expression [8,13,18].

In our pilot study, this commercially available kit was used to assess the inter-assay variation of RT-PCR-based detection of tumour cells in the blood between different laboratories that all used the same standardised protocol. By this approach, the influence of pre-analytics like blood-sample processing, RNA isolation, and cDNA synthesis was evaluated. Blood samples of healthy donors spiked with various numbers of cultured colon tumour cells were examined. To study at which level the variation was introduced, three kinds of sample sets were generated in which (i) tumour cell RNA was spiked in RNA of mononuclear cells (MNC), (ii) tumour cells were spiked in isolated MNC, and (iii) tumour cells were spiked in blood. The LightCycler Kit was used to detect and quantify CK20 expression. Our results are the first to show that very similar results for samples analysed by different laboratories were obtained when a standardised protocol for RNA isolation and RT-PCR was used. Most variation was observed in the analysis of the spiked-blood samples. The results of our pilot study highlight the need for extended quality-control studies in the field of pre-analytical procedures.

2. Materials and methods

2.1. Sample processing

Samples were prepared by two of the four laboratories. In the first laboratory, 10 ml of blood was collected from a healthy volunteer and pretested for CK20 expression according to the protocol as described below. No CK20 expression was detected in this pretested sample, and 300 ml blood was subsequently collected into a heparin-containing bag. This blood sample was divided into three aliquots of 100 ml. The first aliquot of 100 ml blood was used for the preparation of a set of samples in which RNA from the blood is spiked with RNA from cultured HT29 colon tumour cells. Therefore, MNC were isolated from 100 ml blood by density-gradient centrifugation through Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). MNC were washed twice with phosphate-buffered saline (PBS), snap frozen in liquid nitrogen, and stored at −80 °C until use. For isolation of RNA from MNC and HT29 cells, the High Pure RNA isolation kit (Roche Diagnostics GmbH, Penzberg, Germany) was used according to the manufacturer's instruction. The quantity of RNA was measured by spectrophotometry at 260 nm. Blood RNA was divided into 20 aliquots representing 5 ml blood each. HT29 RNA was added to 16 aliquots in quantities equivalent to 1, 5, 10 and 100 HT29 cells/ml (four samples each). Four aliquots were not spiked with HT29 RNA. Thus, four identical series consisting of five samples each were generated.

The second aliquot of 100 ml blood was used for the preparation of a set of HT29-cell spiked MNC samples. Therefore, 100 ml blood was divided into 20 aliquots of 5 ml. MNC were isolated by density-gradient centrifugation through Ficoll-Hypaque and washed twice with PBS. HT29 tumour cells were added to 16 MNC samples in quantities equivalent to 1, 5, and 10 cells/ml by micromanipulation and 100 cells/ml by serial dilution resulting in four identical sets of spiked-MNC samples. Four MNC samples were not spiked with HT29 tumour cells. Cell pellets were snap frozen and stored at −80 °C.

The third aliquot of 100 ml blood was used for the preparation of a set of HT29-cell spiked-blood samples (blood-A). Again, 100 ml blood was divided in 20 aliquots of 5 ml. HT29 cells were added to 16 aliquots of blood in quantities as described for the second series. Four blood samples were left unspiked. Thereafter, MNC were isolated and washed. The cell pellets were snap frozen and stored at −80 °C. Four identical sets consisting of five samples each were generated.

A second laboratory collected 100 ml heparin blood of another healthy volunteer who showed no CK20 expression in a previously pretested 10 ml blood sample. This aliquot of 100 ml blood was used to prepare

spiked-blood samples (blood-B) in the same manner as the third set of samples of the first laboratory (blood-A). Both laboratories used the same batch of HT29 cells to spike the blood samples.

All samples were coded for blinded analysis and distributed on dry ice by courier to the other participating laboratories. Each laboratory individually isolated RNA from the distributed samples using the High Pure RNA isolation kit according to the manufacturer's instruction, except from the first series of samples distributed by laboratory 1 that already consisted of RNA samples. RNA quantity of individually isolated samples was measured by spectrophotometry at 260 nm at the different laboratories separately.

2.2. Quantitative RT-PCR

The LightCycler-CK20 Quantification Kit (Roche Diagnostics GmbH, Penzberg, Germany), the LightCycler Instrument (Roche Diagnostics GmbH, Penzberg, Germany), and the Relative Quantification Software 1.0 (Roche Diagnostics GmbH, Penzberg, Germany) were used for quantitative RT-PCR according to the manufacturer's instruction with minor modifications. In short, cDNA was synthesised from 1.5 μg RNA isolated from the spiked-MNC and spiked-blood samples and from 20 μl RNA from the spiked-RNA samples (representing 1.5 μg RNA) in a total volume of 40 μl. Reverse transcription was performed in duplicate for the spiked-RNA samples and in single for all other samples. In each PCR, 5 μl cDNA was analysed. Each LightCycler run consisted of triplicate analysis of the 0, 1, 5, 10 cell/ml samples and single analysis of the 100 cell/ml sample, the calibrator sample (supplied with the kit and consists of RNA from HT29 cells), the RT H_2O-negative control sample and the PCR H_2O-negative control sample. Per sample both the target gene *CK20* and the reference gene porphobilinogen deaminase (*PBGD*) were analysed in separate reactions. Per LightCycler run, one sample set was analysed.

Amplification is monitored by fluorescence that is emitted during fluorescence resonance energy transfer between two product-specific fluorophore-labelled probes when both these probes hybridise to the amplicon. The emitted fluorescence is measured in real-time by the LightCycler Instrument. The crossing point represents the cycle number at the beginning of the exponential phase in fluorescence increase and is indicative for the initial concentration of the target sequence in the sample. Crossing points were calculated and imported in the Relative Quantification Software. A normalised ratio of the CK20 crossing point to the PBGD crossing point in a sample relative to the CK20:PBGD ratio in the calibrator sample was calculated automatically for each test sample. The calibrator ratio is always set to 1 000 000 according to the manufacturer's instructions. Therefore, samples with a normalised ratio of 10 000 have a CK20:PBGD ratio 100 times smaller than the calibrator. Measurement of PBGD is used as a control for RNA integrity and loading. The calibrator provides an inter-run normaliser. Samples were loaded into the Relative Quantification Software with the single loading scheme according to the manual of the kit, and the mean normalised ratio of a sample was calculated manually from the CK20-positive tests. A broad description of the relative quantification using this approach has recently been published in Ref. [19].

3. Results

3.1. RNA quality

PBGD crossing points are indicative of RNA quality. Table 1 shows the median and range for PBGD crossing points in the different sample sets. Analysis of identical RNA samples resulted in small differences between laboratories regarding the median crossing points. Each laboratory detected PBGD expression within a range of one cycle except for laboratory 2 that showed a broader range of 1.97–3.38 cycles. PBGD crossing points were comparable in the first and second RT reactions of the RNA samples. PBGD RT-PCR of RNA isolated at the different laboratories for the 10 MNC samples (five samples of the MNC and five samples of the blood-A experiment) distributed by laboratory 1 resulted in quite comparable median crossing points (26.62–27.94 cycles) for laboratories 1, 3, and 4. These crossing points were only slightly increased compared with the median PBGD crossing points of the distributed RNA samples (24.91–26.75). However, laboratory 2 showed a relatively high median PBGD crossing point of 29.06 cycles in the blood-A experiment. Median PBGD crossing points in blood-B samples (28.84–32.45) are somewhat increased compared with PBGD crossing points in the blood-A samples (27.04–29.06).

3.2. Detection of CK20 expression in spiked samples

Not all of the analyses resulted in valid data, and missing values are specified in Table 2. Quantitative data were lost due to computer failure and breakage of capillaries in the second RT of the spiked-RNA samples analysed by laboratory 1 and in the spiked-MNC samples analysed by laboratory 2, respectively. CK20 expression was detected in H_2O control samples of one run analysed by laboratory 1, of two runs analysed by laboratory 2, and of two runs analysed by laboratory 3. Despite this contamination, the quantitative results of two of these runs (two runs analysed by laboratory 3) were in range of the results generated by other

laboratories. Only in one run (analysed by laboratory 1), all other samples were clearly contaminated by CK20 products as CK20 expression was detected in all samples after approximately the same number of cycles (range 0.6 cycles). However, the data resulting from all five runs showing CK20 contamination in H_2O control samples were not considered for further analyses. Because of the limited material available, only laboratory 1 repeated the run. In six samples of laboratory 2 and in one sample of laboratory 3, no PBGD PCR product was detected. Therefore, no normalised ratio could be calculated in those samples.

An overview of all valid results is given in Table 2. All laboratories detected CK20 expression in all HT29-RNA spiked-RNA samples without failure. Normalised ratios were comparable between the different laboratories and all laboratories showed a clear positive correlation between amounts of spiked HT29 RNA and the normalised ratio (Fig. 1a).

In the HT29-cell spiked-MNC samples, again all laboratories detected CK20 expression in all spiked samples without failure (Table 2). Variation in normalised ratios between laboratories was somewhat increased compared with the results of the spiked-RNA samples. Two of three laboratories reported a positive correlation between the number of spiked tumour cells and the normalised ratio.

In the two sets of HT29-cell spiked-blood samples generated by laboratory 1 (blood-A) and 2 (blood-B), only the 100 HT29 cells/ml blood samples were reported CK20-positive without failure by all laboratories (Table 2). In total, CK20 expression was detected in 16 out of 20 samples spiked with 1–10 HT29 cells/ml blood. In 10 out of these 16 positive samples, CK20 expression was detected inconsistently. In four HT29-cell spiked-blood samples, no CK20 expression was detected, and the sensitivity of the experiment was limited to 5 cells/ml for laboratory 2 in the blood-A samples and to 5 and 10 cells/ml for laboratories 1 and 4 in the spiked-blood-B samples, respectively. The detection frequency in the blood-A samples compared with the blood-B samples was 69% (24/35) compared with 40% (12/30), respectively. The variation in normalised ratios between the laboratories was considerable, and in the blood-A samples, only laboratories 1 and 2 showed increasing normalised ratios in samples with increasing numbers of HT29-cells spiked (Table 2 and Fig. 1b). However, the linearity between the number of spiked tumour cells and normalised ratio was not evident. Of all unspiked samples tested, CK20 expression was detected three times in the MNC sample analysed by laboratory 1 and once in the blood-A sample analysed by laboratory 4. Both samples were generated by laboratory 1.

Fig. 2 shows the results of all spiked-RNA,-MNC, and-blood samples all produced by laboratory 1. This figure clearly demonstrates that variation between laboratories was increased and normalised ratios were decreased, when HT29 cells were spiked at an earlier stage of sample processing.

4. Discussion

Conflicting results regarding the sensitivity, specificity and clinical relevance of RT-PCR-based detection of disseminated tumour cells in blood of patients with colorectal cancer are frequently encountered in the literature. This is not only common for CK20 [1,8], which is the RT-PCR marker of interest in our study, but also for other frequently used RT-PCR markers of colorectal cancer cell dissemination like carcinoembryonic antigen [20,21] and guanylyl cyclase C [22,23]. Large heterogeneity in the methods may underlie these conflicting results and makes different studies difficult to compare [12–16]. There is therefore an urgent need for standardisation. However, until now, comparative studies between laboratories are scarce [17].

Table 1
PBGD crossing points specified by laboratory and experiment

	Lab 1		Lab 2		Lab 3		Lab 4	
	Median PBGD CP	Range	Median PBGD CP	Range	Median PBGD CP	Range	Median PBGD CP	Range
RNA 1st RT	26.75	26.31–26.89	27.41[a]	26.06–28.03	26.23	25.64–26.63	24.91	24.61–25.54
RNA 2nd RT	[b]		28.12[c]	26.43–29.81	26.20	25.69–26.51	25.29	25.05–25.52
MNC	27.58	27.06–27.89	27.43	25.93–31.80	26.62	26.20–27.12	27.29	26.06–29.08
Blood-A[d]	27.94	27.72–28.43	29.06	28.32–31.57	27.04[a]	26.21–27.92	27.47	26.53–28.00
Blood-B[e]	32.45	31.68–38.12	29.17	28.75–30.16	28.84	27.57–31.87	31.71	29.79–35.20

CP, crossing point; Lab, laboratory; MNC, mononuclear cells; RT, reverse transcriptase.
[a] Only four of five samples PBGD-positive.
[b] Quantitative data lost due to computer failure.
[c] Only two of five samples PBGD-positive.
[d] Blood samples generated by laboratory 1.
[e] Blood samples generated by laboratory 2.

Table 2
RT-PCR results specified by laboratory and experiment

	Lab 1			Lab 2			Lab 3			Lab 4		
	No. valid determinations	No. CK20+ PCRs	Mean norm. ratio	No. valid determinations	No. CK20+ PCRs	Mean norm. ratio	No. valid determinations	No. CK20+ PCRs	Mean norm. ratio	No. valid determinations	No. CK20+ PCRs	Mean norm. ratio
RNA 1st RT												
0	3	0	0	1[a]	0	0	0[b]			3	0	0
1	3	3	255	1[a]	1	68	0[b]			3	3	275
5	3	3	2002	1[a]	1	1364	0[b]			3	3	1730
10	3	3	3980	0[a,c]			0[b]			3	3	3379
100	1	1	23 570	1[a]	1	44 618	0[b]			1	1	30 468
RNA 2nd RT												
0	0[d]			0[c]			0[b]			3	0	0
1	0[d]			0[c]			0[b]			3	3	290
5	0[d]			2[c]	2	2175	0[b]			3	3	1504
10	0[d]			3	3	4606	0[b]			3	3	3184
100	0[d]			0[c]			0[b]			1	1	37 051
MNC												
0	3	3	342	0[e,b]			3	0	0	3	0	0
1	3	3	836	0[e,b]			3	3	1176	3	3	110
5	3	3	1708	0[e,b]			3	3	693	3	3	475
10	3	3	2491	0[e,b]			3	3	2125	3	3	884
100	1	1	26 362	0[e,b]			1	1	16 659	1	1	4523
Blood-A[f]												
0	3	0	0	3	0	0	3	0	0	3	1	132
1	3	1	549	3	0	0	3	1	145	3	2	192
5	3	1	641	3	3	318	0[c]			3	3	391
10	3	3	3488	1[c]	1	1073	3	3	175	3	2	160
100	1	1	9397	1	1	6763	1	1	11 793	1	1	5925
Blood-B[g]												
0	3[h]	0	0	0[b]			3	0	0	3	0	0
1	3[h]	0	0	0[b]			3	3	955	3	0	0
5	3[h]	1	7568	0[b]			3	1	705	3	0	0
10	3[h]	1	781 124	0[b]			3	1	2209	3	2	25 024
100	1[h]	1	62 652	0[b]			1	1	33 210	1	1	122 516

Lab, laboratory; norm., normalised; MNC, mononuclear cells; RT, reverse transcriptase; PCR, polymerase chain reaction.

[a] Only one determination was performed per sample.

[b] CK20 PCR product detected in one or both of the H_2O samples controlling for contamination.

[c] No PCR product detected in (part of) the PBGD PCRs.

[d] Quantitative data lost due to computer failure.

[e] Second analysis of the samples; in the first analysis, capillaries of the calibrator were broken and no normalised ratios could be computed.

[f] Blood samples were generated by laboratory 1.

[g] Blood samples were generated by laboratory 2.

[h] Second analysis of the samples; in the first analysis, PCR product detected in some of the H_2O samples controlling for contamination.

Our study is the first to assess the influence of pre-analytics on the variation of RT-PCR-based detection of tumour cells in the blood between laboratories all using the same standardised protocol. We have used a commercially available kit for RNA isolation and a commercially available kit for quantitative RT-PCR on the LightCycler Instrument designed to detect disseminated tumour cells in blood and bone marrow samples by measuring CK20 expression. Our results indicate that when a standardised protocol for RNA isolation and RT-PCR is used, reasonable good results in samples analysed by different laboratories can be obtained with respect to two points. First, in the two sets of samples in which tumour cell RNA was spiked in RNA of blood and in which tumour cells were spiked in MNC, valid data were obtained by three laboratories for both sets, and in all these runs, CK20 expression was detected at concentrations as low as one tumour cell per ml of blood. Second, in these runs, increasing concentrations of spiked tumour cells generally resulted in

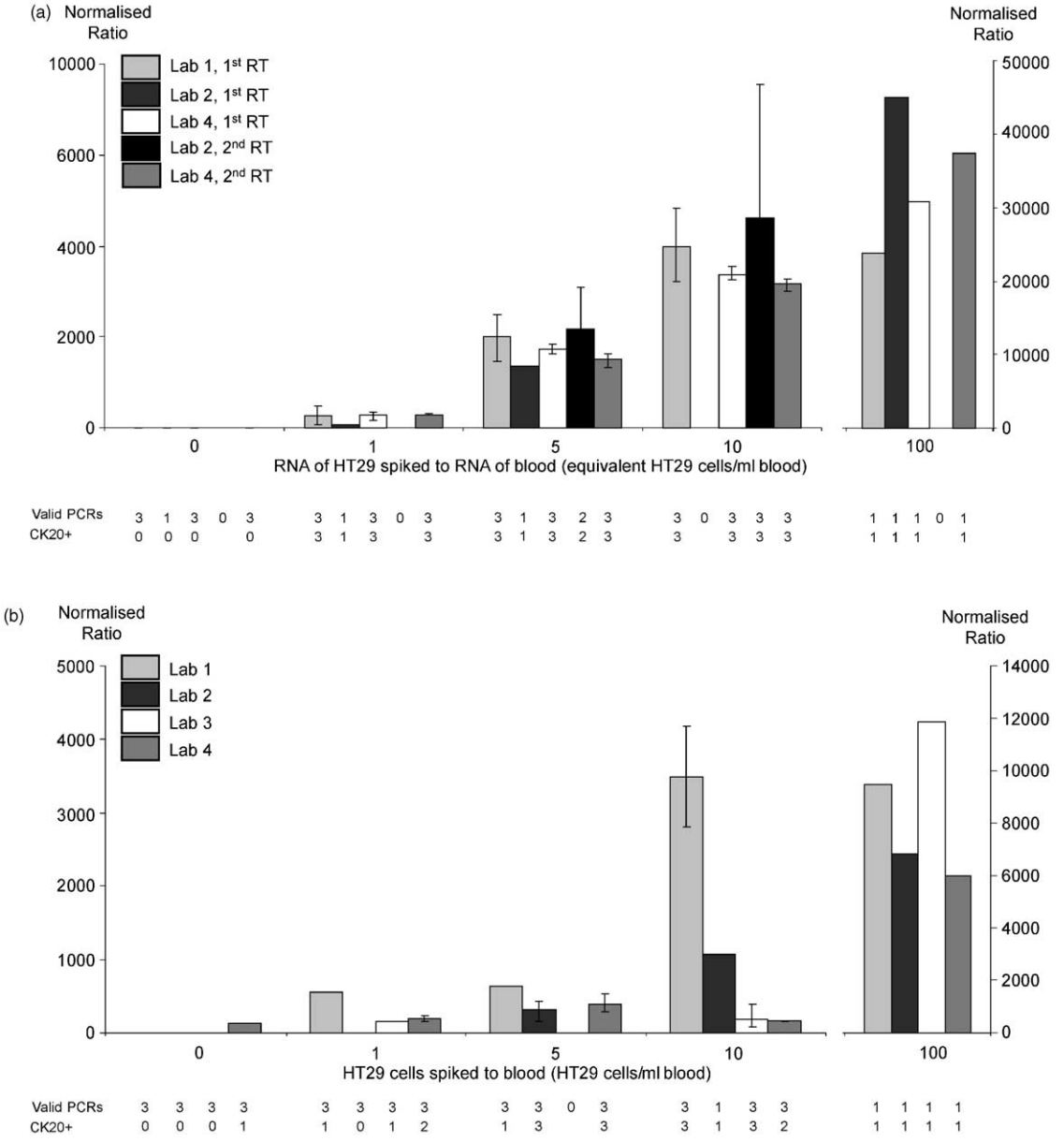

Fig. 1. Comparison of results between laboratories on quantification of CK20 expression in a set of samples in which (a) various amounts of HT29 tumour cell RNA was spiked to RNA of blood and (b) various numbers of HT29 tumour cells were spiked into the blood by laboratory 1 (blood-A). Of the concentrations 0, 1, 5, and 10 HT29 tumour cells/ml blood, three PCR tests were performed per sample, and of the concentration of 100 HT29 tumour cells/ml blood, one PCR test was performed per sample. Mean normalised ratio of positive tests was calculated in a sample and is displayed by a bar. The range of the normalised ratios of the individual tests is indicated by error bar. Note the different scaling of the *y*-axis for the 100 cell/ml samples and the 0–10 cell/ml samples. Number of valid PCR tests and number of CK20-positive (CK20 +) PCR tests are given below the chart.

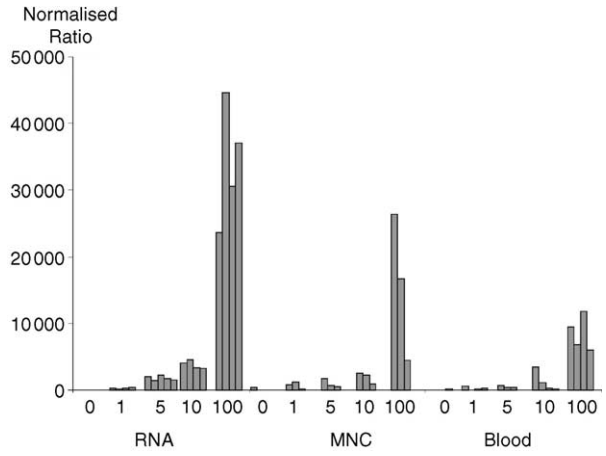

Fig. 2. Quantification of CK20 expression in three sets of blood samples spiked with tumour cells at three different points of sample processing. The results in the samples produced by laboratory 1 are shown. RNA: HT29 tumour cell RNA was spiked to RNA of blood in concentrations equivalent to 0, 1, 5, 10, and 100 tumour cells/ml blood. MNC: HT29 tumour cells were spiked to MNC in concentrations of 0, 1, 5, 10, 100 tumour cells/ml blood. Blood: HT29 tumour cells were spiked to blood in concentrations of 0, 1, 5, 10, 100 tumour cells/ml. For each sample in the concentration range of 0–10 cells/ml, three PCR tests were performed and the mean normalized ratio of positive tests is shown per sample; for the 100 cells/ml sample, one PCR test was performed and the normalised ratio is shown. No distinction was made between the four different laboratories that analysed the samples.

higher CK20 expression levels as detected by the different laboratories. RNA isolation introduced limited extra variation in CK20 expression levels of comparable samples analysed by different laboratories. However, this variation may also be partly due to variations in tumour cell spiking and in MNC harvest.

Despite the uniformity of all spiked-RNA samples distributed, PBGD expression was not detected in all samples. Further analysis of the calibrator samples' fluorescence curves, which show the real-time formation of PCR product, suggests that this is most probably due to a sub-optimal run of the PBGD PCR. This stresses the importance of real-time fluorescence-curve analysis additional to the assessment of the final quantitative results.

Due to CK20 expression in granulocytes [10], these cells need to be depleted from blood samples when CK20 RT-PCR is used to study disseminated tumour cells in blood. Ficoll density gradient separation is the most frequently used method to accomplish this. To evaluate its effect on results between laboratories, two laboratories generated one set of samples each in which tumour cells were spiked directly into blood after collection and before sample processing. Analysis of these samples gave rise to larger variations between laboratories and a reduced sensitivity of the analysis. The lowest concentration of one tumour cell per ml blood could still be detected in four out of seven runs. However, the low tumour cell concentrations were inconsistently CK20-positive. This suggests that target transcripts are present in very low concentrations and amplification is a matter of chance resulting in stochastic effects as described in several previously published studies [24–26]. In the results of the spiked-blood samples, there was no clear relationship between the number of spiked tumour cells and the normalised ratio. However, in three of seven runs, the detected CK20 expression levels in the samples with 100 tumour cells per ml were elevated compared with the samples with 10 tumour cells per ml, and the expression levels in the samples with 10 tumour cells per ml are elevated compared with the samples with one and five tumour cells per ml.

The detection frequency in the spiked-blood samples generated by laboratory 1 (blood-A) compared with the spiked-blood samples generated by laboratory 2 (blood-B) was 69% (24/35) compared with 40% (12/30), respectively. This suggests that sample preparation at the two laboratories may have affected the assay sensitivity. However, the compared number of samples is too limited to evaluate the suggested small difference between the laboratories accurately. More obvious is the difference in RNA quality between the two sample sets as determined by PBGD expression levels. In the blood-B sample set, three out of four laboratories report higher median crossing points for PBGD with a broader range compared with the blood-A sample set. As a low copy number house-keeping gene, PBGD is a good indicator for RNA quality, and low expression of PBGD as detected by high crossing points may give rise to false-negative results [13]. Therefore, reduced RNA quality, which was confirmed by agarose gel electrophoresis of a random set of samples (data not shown), might have brought about the lower detection frequency in spiked samples generated by laboratory 2 compared with laboratory 1. Further research is needed to define a maximum crossing point for PBGD that ensures minimal risk of false-negative results due to inferior RNA quality.

The results on background expression of CK20 in MNC isolated from blood samples of healthy donors and non-cancer patients are conflicting. Studies showing both an absence [5,27] and presence [9,11,23] have been published. Background expression of CK20 in MNC may be due to illegitimate expression [28], presence of a residual limited number of granulocytes [10,16], presence of non-malignant epithelial cells [2], and expression in CD34+ blood cells [23]. The discrepancies between studies may be explained by differences in the sensitivity of the method as a reduced sensitivity results in an improved specificity [16,29]. Despite the negative CK20 results in the pretested blood samples of healthy donors in this study, CK20 expression was detected in two out of 14 unspiked samples. These inconsistent

positive results in unspiked healthy donor blood samples that had been shown to be CK20-negative in previous testing might be caused by very low concentrations of CK20 transcripts resulting in stochastic amplification [24]. This is supported by the low levels of CK20 expression detected in these unspiked samples, which were lower compared with the CK20 expression levels detected in the samples spiked with five tumour cells per ml. Evaluation of differences between the background level of CK20 expression and level of CK20 expression due to tumour cells was beyond the purpose of this study and has to be examined in further studies.

An advantage of a quantitative real-time RT-PCR is the fact that no post-PCR handlings are needed. It has been suggested that this saving of time will reduce the risk of contamination. Surprisingly, contamination was encountered in a relatively high number of runs in this study. Even though gel analysis of the PCR products was not part of the study, laboratories 1 and 3 analysed some PCR products on agarose gels (data not shown). The contamination detected in the two runs of laboratory 3 are most probably due to these post-PCR gel analyses and endorses the suggestion that post-PCR handlings increase the risk of contamination. The reason for the contamination in the runs of laboratories 1 and 2 is not obvious. Furthermore, it is interesting to note that the number of missing data due to various reasons including contamination in this collaborative study is higher than generally reported by individual laboratories.

The idea that the more sample handlings are performed, the more tumour cells are lost seems evident. This thought is nicely illustrated by our results showing gradually reduced CK20 expression levels when tumour cells were spiked to blood samples at an earlier stage in the sample processing. This stresses the necessity for standardised pre-analytical and analytical methods across laboratories during the whole course from blood sample collection to the final PCR result, because any method will result in a loss of signal and the total loss will depend on the number and type of pre-analytical and analytical steps. Our results emphasise that even if standardised protocols are used for all of the steps, the total variation in results increases with the number of pre-analytical steps. This might be due to slight differences in laboratory equipment, working places, or individual laboratory habits. However, the variation in our results highlights the impact of stochastic effects on the highly sensitive RT-PCR method.

In conclusion, quantitative PCR has been shown to be a powerful tool to evaluate both qualitative and quantitative differences in the detection of disseminated tumour cells between laboratories. Use of standardised methods for RNA isolation and quantitative RT-PCR as performed in this study results in fairly comparable data between different laboratories showing CK20 detection without failure in spiked samples even at low frequencies of tumour cells and increasing CK20 quantitative signals with increasing numbers of spiked tumour cells. More variation is introduced by the blood sample preparation method. The present study, which is the first concerted initiative to standardise RT-PCR methods for tumour cell detection across laboratories, indicates that comparable results between laboratories may be possible, but a high level of standardisation is required, especially in the field of pre-analytics. The results of this pilot study stress the need for further comparative collaborative investigations of the pre-analytical procedures currently used. After pre-analytical procedures are further optimised in a standardised manner, the results from this pilot study can be used to define criteria for an acceptable level of inconsistency between laboratories, and, consequently, these criteria can be used to compare results between different laboratories in a more extended study.

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