Detection of *bcr-abl* transcript in chronic myelogenous leukemia patients by reverse-transcription-polymerase chain reaction and capillary electrophoresis

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ABSTRACT

Background and Objective. Capillary electrophoresis (CE) has become an attractive alternative to SLAB gel analysis for direct and accurate detection of amplified product,[1-5] and a few cycles of polymerase chain reactions (PCRs) could be sufficient for both quantitative and qualitative analysis. We try to assess: 1) whether CE could be a practical, non-isotopic method for direct detection of the presence of amplified *bcr-abl* obtained by a reverse transcription (RT)-PCR (qualitative analysis) and 2) whether it is possible to quantify PCR products using a competitive RT-PCR measuring peak areas of CE electropherograms (quantitative analysis).

Design and Methods. The two types of *bcr-abl* chronic myelogenous leukemia (CML) associated transcript products were generated by RT-PCR (qualitative analysis) from 1 μg of total RNA extracted from bone marrow samples of 34 CML patients at diagnosis (median age 47.5; range 18-65; median Sokal's score 0.9; range 0.53-2.78). The PCR products were analyzed by SLAB-gel electrophoresis (SGE) on 2% agarose gels and by CE (128 runs; median 3.3 times for each sample). Furthermore, we assessed the amount of PCR product (quantitative analysis) by a competitive RT-PCR approach and by CE (*bcr-abl* transcripts were expressed as transcript per μg of total RNA examined).

Results. CE separation of PCR products obtained by qualitative RT-PCR showed baseline resolution for the two peaks corresponding to the two types of *bcr-abl* junctions: the *b2-a2* type (343 base pairs, 10 patients) was revealed at 9.33 min [standard deviation (SD) = 0.1] and the *b3-a2* type (418 base pair, 24 patients) at 10.03 min (SD = 0.25). By quantitative analysis we found that there is great interpatient variability in *bcr-abl* expression at diagnosis: the median value of the amount of *bcr-abl* transcript was 78,000 *bcr-abl* transcript/μg total RNA ranging from 17,300 to 750,000. The amount of *bcr-abl* transcript at diagnosis was related to the number of blast cells (mean value 128,859 vs. 331,722 in patients with 0% blast cells and >1% blast cells, respectively; p = 0.004) and Sokal's score (mean value 156,865 vs. 408,800 in patients with Sokal's score <0.8 and >1.2, respectively; p = 0.003).

Interpretation and Conclusions. Our results confirm that CE analysis offers greater resolution and enhanced sensitivity for detection and quantification of *bcr-abl* PCR product in the study of this leukemia. Qualitative analysis by CE of *bcr-abl* product provides a rapid technique (less than 20 min) for the analysis of subnanogram amounts of DNA fragments. CE run times are short, the capillary can be re-used and full automation may be feasible with data acquisition by a computer-controlled step. Competitive/quantitative analysis of *bcr-abl* as analyzed by CE allowed fewer reactions and more precise quantification.
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The molecular definition of chromosomal translocations associated with hematopoietic tumors has provided important tools for the diagnosis and monitoring of patients' response to therapy. A consistent translocation t(9;22) is present in the cells of the majority of chronic myelogenous leukemia (CML) patients.[6,7] Two genes are involved in the translocation, which gives rise to a marker chromosome 22 called Philadelphia or "Ph1": they are *abl* on chromosome 9 and *bcr* on chromosome 22.[7-9] The chimeric transcript *bcr-abl*, generated as the consequence of the reciprocal translocation between the *bcr* and *abl* loci,[8] gives rise to a fusion-protein with neoplastic activity.[9,10] In the vast majority of CML patients (99%) two types of *bcr-abl* junction have been detected by a reverse transcription (RT) polymerase chain reaction (PCR) assay, called *b2-a2* and *b3-a2* junctions respectively, due to the inclusion of 75 base pair (bp) from *bcr* exon 14 (or b3 of a major-breakpoint cluster region, M-BCR).[7,8,11] Several studies have reported on RT-PCR's ability to detect the *bcr-abl* transcript for either the diagnosis or monitoring of the Ph1 clone.[10-17]

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Qualitative assessment of the RT-PCR product was done by agarose SLAB gel or by polyacrylamide gel electrophoresis (PAGE) separation and resolution of the different size of PCR products (75 bp difference). More recently, quantitative detection of *bcr-abl* amplified transcript was obtained by a competitive PCR approach.[18,19]

Although a linear relationship has been observed between the input template and amplification product within the exponential range of amplification,[17-19] this depends on the amount of the starting material (the more abundant the material, the shorter the range), and is influenced by differences in many factors that affect the final result, such as tube-to-tube variation, nature of amplified sequences and the primers' sequences. A competitive PCR could overcome these problems.[18,20] The most reliable approaches to quantitative PCR are those based on the co-amplification of reference templates, which have in common with the target the same sequences for the same primer sites and almost all the same amplified sequences (competitive PCR). A typical competitive PCR experiment was performed by the addition of increasing amounts of known competitors[15] to a fixed amount of the DNA to be quantified, with the ratio between the final amplification products for the two species being evaluated for each point.[15] From this ratio, the amount of the unknown DNA was evaluated. For fine determination by *visual comparison* of the amount of amplified product, we need to find the point at which the amount of *bcr-abl* transcript is equivalent to the amount of competitor. Usually, several reactions have to be performed for each sample because with a single round of amplifications one is unlikely to find the point of equivalence: so further rounds of amplifications with further dilution of competitor within the range previously identified are generally necessary. In any case, the amount of *bcr-abl* has to be expressed as a *ratio* (discontinuous variable) and not as a number (continuous variable).

We felt that capillary electrophoresis (CE), with its higher sensitivity and precise quantification of small amounts of amplified DNA could be valuable for fine quantification of *bcr-abl* transcript in competitive RT-PCR. We studied the feasibility of using CE to detect and quantify *bcr-abl* transcript in 34 CML patients. The purpose of this study was to evaluate CE of bone marrow samples from CML patients at diagnosis for the qualitative separation of *bcr-abl* PCR product formed with a competitive assay during the reaction.

Materials and Methods

Patients, samples and RNA extraction

After informed consent, 34 CML patients were studied at diagnosis. Clinical characteristics of the patients are reported in Table 1. In all cases, the bone marrow samples were examined by RNA extraction as previously described.[14] The amount of extracted RNA was determined by the optical density at 260 nm and its integrity was checked by loading 1 μg on 2% agarose (SLAB) gel. In most cases, 10 to 40 μg of RNA were recovered from 2 to 5 mL of bone marrow.

Markers

The molecular weight marker VI from Boehringer-Mannheim (Milan, Italy) was used (DNA concentration 250 μg/mL in liquid solution of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). For quantitative analysis the two markers M-200 and M-400 (concentration of 10 μgr/μL from Bioventures Inc. (Milan, Italy) of 200 bp and 400 bp were employed, respectively.

Qualitative RT-PCR

RT and PCR amplification were performed as described[14] with few modifications. cDNAs were prepared from 1 μg RNA templates in 50 μL reaction mixtures containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$ of the AZ antisense abl specific primer (20 pmol), 200 μM each of the dATP dCTP, dGTP, dTTP (Perkin-Elmer, Milan, Italy), 10 U placental ribonuclease inhibitor (Boehringer Mannheim, Italy), 200 U Molony Murine leukemia virus (M-MLV) RT (Promega, Milan, Italy), 200 U Molony Murine leukemia virus (M-MLV) RT (Promega, Milan, Italy) (Figure 1; Table 2). The RT reaction was carried out by incubation at 37°C for 1 h. The PCR amplification was performed using 10 μL of the RT product, plus 100 μL final volume with the 2.5 U DNA polymerase from Thermus Acquaticus (Taq polymerase, Perkin-Elmer, Milan, Italy) and AZ-EA122 primer (Figure 1; Table 2) (20 pmol each) and 1 × PCR buffer (Perkin-Elmer, Milan, Italy). The amplification cycles (denaturation: 96°C, 30 s; annealing: 60°C, 30 s; extension: 72°C, 30 min) were performed in a thermal cycler (480 Perkin-Elmer, Italy). A typical program of 30 cycles was completed in 4.5 h. A 343 bp (*b2-a2*) or 418 bp (*b3-a2*) junction product was obtained, as expected.

SGE of PCR amplified products

Samples (10 μL) of the PCR reaction mixture were loaded onto 2% agarose gels (9×7 cm) (BioRad, Milan, Italy) containing ethidium bromide.[14] After electrophoresis (53 mA and 127 V) for 55 min at room temperature, fluorescent DNA bands were visualized on a transilluminator and photographed.[14,21,22]

CE of PCR amplified products

CE was performed on a P/ACE 2100 (Beckman, Milan, Italy) in the reverse-polarity mode (negative potential at the injection end of the capillary). A capillary (100 μm ID, 27 cm total length, 20 cm to the detector, Beckman), coated to reduce electro-osmotic flow, was filled with a sieving polymer buffer system containing hydroxypropyl methylcellulose polymer in a Trisborate-EDTA (TBE) buffer. This buffer was

Table 1. Clinical characteristics of the patients.

ID	Age	Sex	Wbc	Plts	Hb	Blasts	% Ph	Sokal score	Status	Bcr-Abl transcript
1	40	M	57.4	934	13.3	3	100	1.01	Dead FA/MB	361,000
2	64	M	23.5	358	13.6	0	100	0.77	Alive 1st chronic phase	17,300
3	54	M	145	267	13.2	2	100	0.89	Alive 1st chronic phase	750,000
4	32	M	234	315	8.9	2	100	0.92	Alive 1st chronic phase	32,000
5	58	M	17.6	1192	16.6	0	67	1.18	Alive 1st chronic phase	78,000
6	60	M	36.9	191	14.4	0	100	0.71	Alive 2nd chronic phase	36,000
7	18	M	187	93	11.7	0	100	0.58	Alive 1st chronic phase	39,650
8	45	M	177	397	11.4	1	100	1.14	Dead CR post BMT	600,000
9	35	M	87.8	193	14.1	0	100	0.59	Alive 1st chronic phase	27,000
10	65	M	16.3	354	14.9	0	100	0.78	Alive 1st chronic phase	250,000
11	48	F	224	424	11.2	5	100	1.16	Alive 2nd chronic phase	250,000
12	58	M	135	375	9.8	2	100	1.87	Dead FA/MB	650,000
13	41	M	182	582	10.6	1	100	0.70	Alive CR post BMT	350,000
14	54	M	114	197	14	1	100	0.72	Alive 1st chronic phase	23,000
15	48	M	23.7	265	12.9	0	100	0.62	Alive CR post BMT	650,000
16	46	M	68.8	642	15.2	0	100	0.70	Alive 1st chronic phase	36,400
17	57	M	60.8	214	10.6	5	100	1.21	Alive 1st chronic phase	270,000
18	37	F	7.5	104	11.2	1	100	0.59	Alive 1st chronic phase	650,000
19	62	M	300	397	12.7	9	100	2.78	Alive 1st chronic phase	350,000
20	33	M	45	335	16.5	0	100	0.53	Alive 1st chronic phase	23,000
21	31	M	19.3	379	14.2	0	86	0.53	Alive 1st chronic phase	28,000
22	48	M	14.8	247	16.4	0	100	0.62	Alive 1st chronic phase	23,500
23	36	F	186	453	12	1.6	100	0.92	Alive 1st chronic phase	68,000
24	33	M	295.2	405	12.6	10	100	1.80	Alive 1st chronic phase	44,000
25	61	M	26.7	305	15.3	0	100	0.73	Alive 1st chronic phase	230,000
26	46	F	95.2	1298	10	0	100	1.13	Alive CR post BMT	145,000
27	50	M	33.4	999	12.7	1	100	1.00	Alive 1st chronic phase	45,000
28	35	M	218.7	405	11.5	12	100	2.24	Alive 1st chronic phase	350,000
29	65	F	90.3	304	9.7	0	75	0.91	Alive 1st chronic phase	40,300
30	51	F	190.6	453	10.5	1	97	1.07	Dead FA/MB	450,000
31	45	M	36	204	12.5	0	100	0.60	Alive 1st chronic phase	43,000
32	51	F	197	350	10.2	5	100	1.22	Alive FA/MB	650,000
33	32	M	143	389	12.7	4	100	0.94	Dead FA/MB post B	78,000
34	47	F	26.3	516	11.5	0	100	0.71	Alive 1st chronic phase	67,000

ID = identification number; M = male, F = female; Wbc = white blood cells; Plts = platelets; Hb = hemoglobin; Blasts = number of blast cells found; % Ph = % of Ph1 positive cells found; bcr-abl transcript = amount of bcr-abl transcript found.

replaced after each separation. PCR samples were injected hydrodynamically (40 sec at 3.45 kPa) and electrokinetically (60 sec at 40 V/cm). In some analyses, the PCR sample and the molecular weight marker (Molecular Weight Marker VI from Boehringer Mannheim) were injected sequentially and allowed to co-migrate in the capillary. The standard digest was not desalted, but diluted to 20 µg/mL in HPLC-grade water prior to injection.

Temperature of the capillary cartridge was set at 25°C and separation of the PCR products was accomplished at a field of 200 V/cm, but other temperatures were also tested (20°C, 30°C, 37°C, 40°C). Detection was on-line, using the P/ACE standard UV detector at 254 nm. Data was collected at 5 Hz and analyzed using System Gold Software, version 7.12 (Beckman). Each component of the PCR mixture was injected separately to verify elution times.

Construction of competitor template

Two different competitors were generated and used. Two plasmids, TA210b$_3$ and TA210b$_2$ were generated by cloning of b3-a2 and b2-a2 amplified fragments, respectively. Briefly, cDNAs were generated by RT of total RNAs of two patients with CML Ph+ *bcr-abl* with b3-a2 and b2-a2 types of junction, respectively, as previously described.[15] The abl sequence specific primer used for RT was EA130 (Table 2). Initial PCR products for b3-a2 were generated separately (1st PCR reaction) by the use of primers AZ and EA12, respectively. Second PCR products were generated from 0.1 µL of both 1st PCR reactions by a nested PCR (2nd PCR reaction) with primers EA500 and EA122, respectively and agarose gel extra band purified by the phenol-chloroform method. The PCR products of the 2nd PCR reaction were then cloned in a TA-cloning vector by TA-cloning kit (Invitrogen, Milan, Italy) to generate TA210b$_3$ and TA210b$_2$ plas-
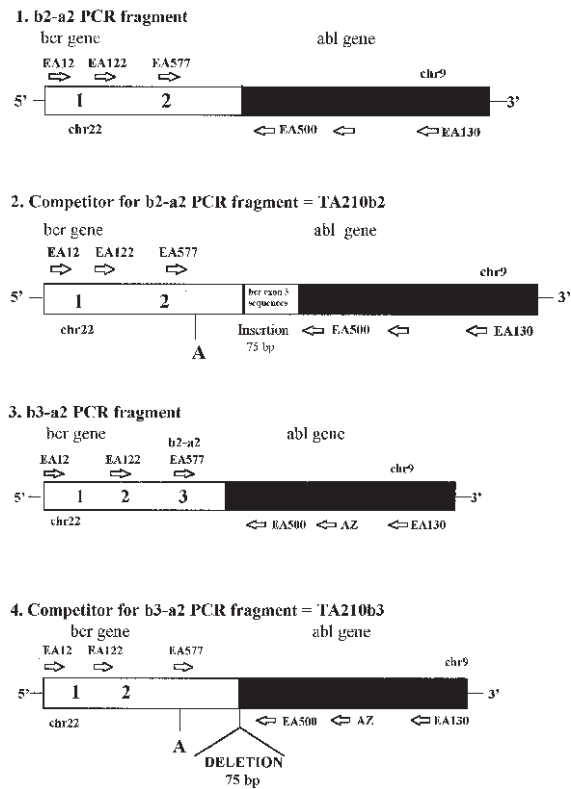
Figure 1. Schematic representation of RT-PCR product of b2-a2 (1) and b3-a2 (3) type of junction and their competitors, respectively.
Black boxes = abl second and third exons; white boxes = bcr exons of M-BCR involved in the translocation fusion cDNA point. Arrows and names = primers used for RT-PCR and nested PCR. EA12, EA122, EA577 are bcr sequence specific primers; EA130, AZ, EA500 are abl sequence specific primers. The bcr-abl specific competitors (2 and 4) were made as described in *Materials and Methods.*

mids. The specificity and sequences were confirmed by further amplification of plasmids with sequence specific primers (EA500, EA122 and EA577) by direct sequences analysis performed as described.[16] Primers for sequencing (seq) are given in Table 2. RT-PCR reaction and cloning procedures were performed as described[15,21-23] and following manufacturers instructions, respectively. The type of junction was confirmed by Southern blot analysis of amplified fragment and hybridization with b3-a2 and b2-a2 probes respectively, as reported.[15,17,24] Probes' sequences are given in Table 1.

TA210b$_3$, TA210b$_2$ were 5.25 kb of size and therefore 1 ng was equivalent to 1.8×10^8 molecules. After linearization of these plasmids with EcoRV restriction digestion, serial dilutions were prepared for each in 1 mmol/L Tris, pH 8.0, 0.1 mmol/L EDTA. Dilutions were made in the range from 10^9 to 10 molecules per 1 μL with steps at every order of magnitude on a logarithmic scale, as previously reported.[19,20]

Table 2. The names of the primers and the probes used, their sequences and references for them.

Name of primer and probe used	Sequence	Ref.
AZ	5' - CCATTTTTGGTTTGGGCTTCACACCATTCC - 3'	9
EA12	5' - GAGCGTGCAGAGTGGAGGGAGAACATCCGG - 3'	9
EA122 (seq)	5' - GTTTCAGAAGCTTCTCCCTG - 3'	9
EA500 (seq)	5' - TGTGATTATAGCCTAAGACCCGGAG - 3'	9
probe b3-a2	5' - GCTGAAGGGCTTTTGAACTCTGCTTA - 3'	9
probe b2-a2	5' - GCTGAAGGGCTTCTTCCTTATTGATG - 3'	9
EA130	5' - ATTGATCCCGCTGCTCAGCAGATACTC - 3'	9
EA577	5' - TCCACTGGCCACAAAATCATACAGT - 3'	9
Abl Ia	5' - CCTCTCGCTGGACCCAGTGA - 3'	15

Competitive PCR

Competitive PCR was performed as previously described.[20] After determination of *bcr-abl* type of junction by qualitative RT-PCR, serial dilutions with a number of TA210b$_3$, TA210b$_2$ competitor molecules were amplified with a fixed amount of cDNA from the patient's samples. RT-PCR conditions were the same as those of the RT-PCR procedures described above.[15,16,20] For competitive assay the same cDNA generated using the AZ primer was amplified with primers EA500 and EA122 (Figure 1; Table 2) under the same PCR conditions. This set of primers yielded PCR products of 313 bp and 388 bp, for b2-a2 and b3-a2, respectively. Rigorous precautions were taken to avoid contamination of PCRs.[15,16] In all the samples, normal *abl* gene was amplified using Abl Ia and AZ primers.[17,24] To ensure reproducible sensitivity of the assay, each run included a dilute positive RNA control (CT): control cDNA was synthesized in 50 mL volume RT reaction from 20 μg of RNA extracted from K562 or BV173 cell lines. Ten μL of this cDNA was used for PCR. All results were discarded for any run if the CT did not show an easily discernible b3-a2 or b2-a2 band. The PCR product was desalted with a Centricon Microconcentrator 30 (Amicon, Italy) to a final volume of 10 μL. The concentrated PCR product was serially diluted to construct a calibration curve. CE of competitive PCR was performed as described above.

Statistical analysis and graphical elaboration of data

The statistical analysis and the graphical elaboration of data were carried out by the computer analysis program MATLAB, kindly provided by Dr. Luigi Montefusco, Faculty of Engineering, University of Florence, Italy.

Results

Qualitative analysis on separation of RT-PCR reaction products from CML patients by SGE gel and CE.
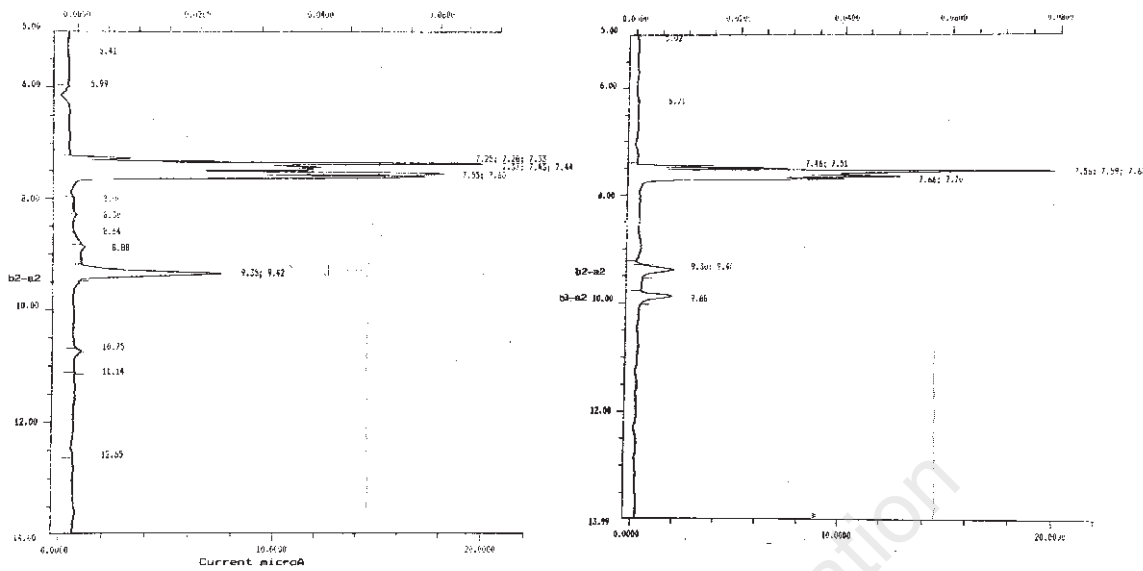
Figure 2.
Left side: Capillary electrophoresis analysis of PCR products by pressure injection.
Capillary electrophoretic separation by pressure injection of b2-a2 PCR product obtained with AZ-EA122 primers.
The peak of the b2-a2 product was revealed at 9.35 minutes. Several peaks corresponding to primers and primers' dimers were detected at ~7.25 minutes. Times are reported on the x axis and UV absorbance at 254 nm on the y axis.
Right side: Capillary electrophoresis analysis of b2-a2 and b3-a2 PCR products.
As in Figures 2 and 3, an electropherogram shows the separation of b2-a2 (revealed at 9.30 min.) plus b3-a2 (revealed at 9.88 min.) PCR co-injected products.
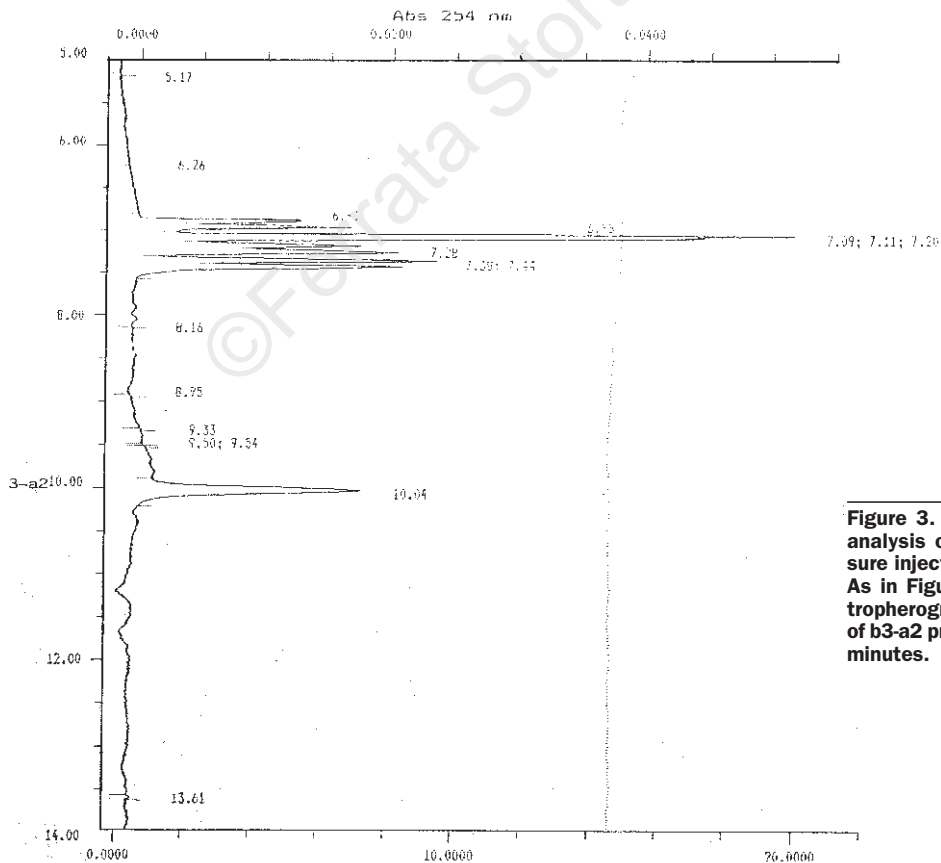
Figure 3. Capillary electrophoresis analysis of PCR products by pressure injection.
As in Figure 2 (left side), an electropherogram shows the separation of b3-a2 products, revealed at 10.04 minutes.

In 34 separate reactions, primer pairs AZ and EA122 were used to generate RT-PCR products from *bcr-abl* transcript of 34 total RNAs RT samples from 34 CML patients, collected at diagnosis. When the PCR products were analyzed by agarose gel electrophoresis, a band at 418 bp, representing the product from the b3-a2 type of junction, and a band at 343 bp, representing the product from the b2-a2 type of junction, were visible.

After amplification, all 34 samples gave rise to an amplified product: 10 were b2-a2 and 24 were b3-a2. Samples of these same PCR products were examined by pressure injection (PI) using CE (128 runs; 30 for b2-a2; 98 for b3-a2 type of junction; median 3.3 times for sample) and the results of representative electropherograms are shown in Figures 2 (left side) and 3. The electropherograms of the reaction product from b2-a2 (Figure 2, left side) and the product from b3-a2 (Figure 3) showed a single main peak, representing 343 bp and 418 bp products, respectively. As a test of the separation capacity of the CE system, a mixture of these two PCR products (Figure 2, right side) and each of them with molecular size markers and primer (data not shown) were prepared, and a sample of each mixture was PI-method injected for analysis. Two peaks, identifiable by comparison with the peaks in Figures 2 (left side) and 3 as the 343 bp and 418 bp products, were observed and clearly separable by this procedure. While the results shown in Figures 2 to 3 were obtained by pressure injection of the sample, a similar separation was achieved using electrokinetic injection (data not shown). After desalting and removing the unincorporated primers of the sample, a ten fold enhancement of sensitivity was obtained using the electrokinetic injection (EI) method (data not shown). When our RT-PCR product was analyzed by CE several clearly resolved peaks were visible: the 418 bp b3-a2 peak (343 bp b2-a2 products) and the primer peak. In this case the main peak of interest was identified using the relative migration times of the primer peak as a reference (Figures 2 and 3). Alternatively, if the sample was analyzed together with the MWM VI, the 394 bp fragment was used as a reference. We found that the median retention time for b2-a2 amplified with AZ-EA122 was 9.33 min (range 8.99-9.40; SD = 0.10) and for b3-a2 was 10.03 min (range 9.58-10.70; SD = 0.25). Comparable results were obtained with products amplified with EA500-EA122 primers (data not shown). There was some false positive or overlapping of the two different types of *bcr-abl* junctions. Better results can be obtained by statistical elaboration, linear regression of median values of peaks of primers and peaks of amplified products, along with easy detection of the type of junction (data not shown).

Quantitative analysis of bcr-abl product using competitive PCR

In an electropherogram, the area under a peak for a particular DNA fragment can be correlated to the quantity of that fragment. To quantify the amount of RT-PCR product in the reaction performed with an unknown amount of template, a competitive analysis was performed. We used $TA210b_2$ or $TA210b_3$ as a competitor in a PCR titration assay, amplified with EA500-EA122 primers as described.[19,20] The PCR products from each reaction were analyzed by SGE and the results were detected by visual comparison between samples and competitor (Figures 4 and 5). In the presence of the same amount of sample, the areas under the sample's peaks increased as the areas under the competitor's peaks decreased (i.e. there was an inverse relationship with the amount of competitor used). The point of equivalence between competitor and sample that had been detected by visual comparison corresponded to the point of exact equivalence between the two areas on CE analysis, thus confirming the validity of the CE method. Usually, only few (four or five) competitive reactions have to be analyzed by CE to determine the *bcr-abl* amount. We applied this method to the CML samples at diagnosis and we found that the median value of the amount of *bcr-abl* transcript at diagnosis time was 78,000 *bcr-abl* transcript/μg total RNA ranging from 17,300 to 750,000. The amount of *bcr-abl* transcript at diagnosis was related to the number of blast cells (median value 128,859 vs. 331,722 in pts with 0% blast cells vs. >1% blast cells, respectively; p = 0.004). The amount of *bcr-abl* transcript at diagnosis was related to Sokal's score: median value

Figure 4. Quantification of *bcr-abl* transcript in a sample from a patient with chronic myelogenous leukemia by competitive PCR.
Different numbers of competitor molecules (s) from 4×10^9 molecules (in lane 1), to 4×10^2 molecules (in lane 8), respectively, were added to a fixed amount of cDNA (c) sample. MWM = molecular weight markers of different size fragments; lane 9 = negative control of RT-PCR (R); c = sample from a patient with CML; s = competitor bands. After amplification, bands were quantified by visual comparison as described.[18] The amount of *bcr-abl* transcript was calculated after correcting for the size difference between the competitor and sample band, as described.[20]
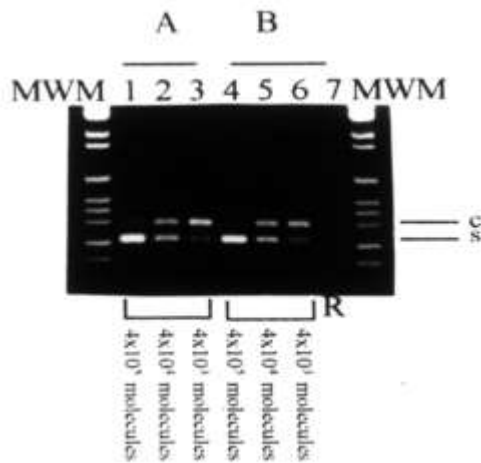
Figure 5. Quantification of *bcr-abl* transcript in two samples from two patients with CML by visual comparison and competitive PCR.
The picture shows the separation on SLAB gel (agarose 2%) of RT-PCR product. The different fragments of molecular weight marker (MWM) are given on the left and right side. A and B = different samples from CML patients A and B, respectively; s = competitor *b2-a2* bands (three aliquots of the competitor molecules and 4×10^3 molecules of *bcr-abl* competitor), c = sample bands from patients with CML; A (lanes 1 to 3) and B (lanes 4 to 6) = 4×10^5 molecules; 4×10^4 molecules, lane 7 = negative control of RT-PCR reaction (R) The equivalence between *s* and *c* is evident in lane 2 for patient A and in lane 5 for patient B.

was 156,865 vs. 408,800 in patients with Sokal's score <0.8 vs. >1.2, respectively; p = 0.003.

Discussion

Automatic detection and quantification of RT-PCR leukemia specific transcript constitutes a useful method for screening and analysis of different types of hematological neoplasms. Unfortunately, these methods are difficult to perform and remain confined to high specialized research laboratories.[25] CE has been suggested as a useful method for accurate detection and on line quantification of amplified product.

The purpose of this study was to evaluate CE for the qualitative separation of *bcr-abl* PCR reaction products and to quantify the amount of *bcr-abl* PCR product formed with a competitive assay during the reaction of samples taken from CML patients at diagnosis. To evaluate CE, RT-PCR products were formed in reactions with *bcr-abl* RNA and the products were analyzed by both SGE and CE.

From a qualitative point of view, our results indicate that the hydroxypropyl-methylcellulose used as a sieving medium for separation was able to resolve the reaction products ranging from 390 to 465 bp. Of additional interest was the separation of the 394 bp different size product of MWM from the 465 bp

b3-a2 product (data not shown). This separation allowed the use of these as migration time standards for qualitative and quantitative analysis. The results of the study also showed that CE could be performed without sample preparation (desalting was not found to be necessary) in qualitative assays if samples could be injected hydrodynamically (PI). CE provides a rapid technique (less than 20 min) for the qualitative analysis of subnanogram amounts of DNA fragments generated by the RT-PCR of the *bcr-abl* transcript. In contrast to SGE, CE run times are short, the capillary can be re-used and full automation may be feasible. Furthermore, data acquisition and analysis are computer-controlled, which reduces error especially when large numbers of PCR products need to be analyzed.

Determination of the specificity of PCR products is usually based on Southern blotting and hybridization of the amplified DNA to sequence-specific radioactive oligonucleotide probes comprised between the PCR primers, or on specific restriction enzymes analysis. The recent introduction of CE for separation of amplified products according to their size enabled us to employ a one-step, non-radioactive protocol to demonstrate the specificity of amplification of *bcr-abl* sequences by PCR.

PCR is one of the most efficient techniques for measuring the expression of neoplastic fusion genes, but easy as it is for qualitative analysis, it is a complicated method for quantification purposes. While the problems associated with developing a quantitative-competitive PCR procedure have been discussed by others (reviewed in ref. #18) and above, the focus of this study with the quantitative PCR experiments was to deal with problems associated with the use of CE for separation, namely a variation in migration time. As the peak area of the fragment is related to its residence time in the detector, slower migrating (large) fragments will remain in the detector window longer than faster migrating (small) fragments. Thus, the peak area may not be perfectly representative of the quantity of DNA passing through the detector. In addition, as DNA fragments from restriction digests, the PCRs are typically contained in a high-salt matrix, and their mobility will vary depending on sample salt concentration. The corrected peak area for a DNA fragment can be compared to a standard curve or to the peak area of a *competitor* to determine absolute amount. The linearity of the standard curve suggests that internal migration time standards, either PCR components such as primer or known-size DNA, are necessary to quantify PCR products accurately. Using this approach we found that there is a great variability in *bcr-abl* expression at diagnosis from patient to patient. How can we explain this difference? Could it be of clinical significance?[26, 27]

Leibowitz *et al*.[28,29] reported the presence of suppressive transcriptional *bcr* promoter factors in the M-BCR region. Three out of four of these could be disrupted by the translocation which gives rise to a

b2-a2 transcript, while only one may be translocated and involved in the b3-a2 generating breakpoint. In the first case (b2-a2), with the absence or silencing of three of the *bcr*-promoter transcriptional factors, the *bcr* promoter may be regulated in a different manner and the transcription of normal bcr and chimeric *bcr-abl* neoplastic transcripts may increase. The increased level of expression of *bcr-abl* may be detected as an increased peak of median area by CE analysis. Several authors have tried to find clinical correlations between types of chimeric transcript and clinical outcome without reaching any firm conclusion. We reported the higher level of WBC count and lacticodehydrogenesis in serum levels in b2-a2 with respect to the b3-a2 transcript carrying patients.[30] These data may all be explained by an increased level of transcription of *bcr-abl* b2-a2 type versus b3-a2 type, and myeloproliferative activity secondary to increased tyrosine-kinase activity.

In conclusion, we have demonstrated the excellent separation efficiency for *bcr-abl* PCR products, and their easy quantification by CE analysis. The enhanced sensitivity of CE leads us to recommend it as an accurate and attractive technique for quantification of *bcr-abl* transcript in the management[25,27,30] of CML patients.

References

1. Gelfi C, Leoncini F, Righetti PG, et al. Separation and quantitation of reverse transcriptase polymerase chain reaction fragments of basic fibroblast growth factor by capillary electrophoresis in polymer networks. Electrophoresis 1995; 16:780-3.
2. Kuypers AW, Meijerink JP, Smetsers TF, Linssen PC, Mensink EJ. Quantitative analysis of DNA aberrations amplified by competitive polymerase chain reaction using capillary electrophoresis. J Chromatogr B Biomed Sci Appl 1994; 660:271-7.
3. Karger BL, Chu YH, Foret F. Capillary electrophoresis of proteins and nucleic acids. Annu Rev Biophys Biomol Struct 1995; 24:579-610.
4. Felmlee TA, Oda RP, Persing DA, Landers JP. Capillary electrophoresis of DNA potential utility for clinical diagnoses. J Chromatogr A 1995; 717:127-37.
5. Nathakarnkitkool S, Oefner PJ, Bartsch G, Chin MA, Bonn GK. High-resolution capillary electrophoretic analysis of DNA in free solution. Electrophoresis 1992; 13:18-31.
6. Guo JQ, Lian JY, Xian YM, et al. Bcr-abl protein expression in peripheral blood cells of chronic myelogenous leukemia patients undergoing therapy. Blood 1994; 83:3629-37.
7. Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973; 24:290-5.
8. Lifshitz B, Fainstein E, Marcelle C, et al. Bcr genes and transcripts. Oncogene 1988; 2:113-7.
9. Shtivelman E, Gale RP, Dreazen O, et al. Bcr-abl RNA in patients with chronic myelogenous leukemia. Blood 1987; 69:971-5.
10. Melo JV, Gordon DE, Cross NC, Goldman JM. The abl-bcr fusion gene is expressed in chronic myeloid leukemia. Blood 1993; 81:158-65.
11. Hughes TP, Morgan GJ, Martiat P, Goldman JM. Detection of residual leukemia after bone marrow transplant for chronic myeloid leukemia: role of polymerase chain reaction in predicting relapse. Blood 1991; 77:874-8.
12. Morgan GJ, Hughes T, Janssen JW, et al. Polymerase chain reaction for detection of residual leukaemia. Lancet 1989; 1:928-9.
13. Sawyers CL, Timson L, Kawasaki ES, Clark SS, Witte ON, Champlin R. Molecular relapse in chronic myelogenous leukemia patients after bone marrow transplantation detected by the polymerase chain reaction. Proc Natl Acad Sci USA 1990; 87:563-7.
14. Goldman JM, Gale RP, Horowitz MM, et al. Bone marrow transplantation for chronic myeloid leukemia in chronic phase. Increased risk for relapse associated with T-cell depletion. Ann Intern Med 1988; 108:806-14.
15. Guerrasio A, Martinelli G, Saglio G, et al. Minimal residual disease status in transplanted chronic myelogenous leukemia patients: low incidence of polymerase chain reaction positive cases among 48 long disease-free subjects who received unmanipulated allogeneic bone marrow transplants. Leukemia 1992; 6:507-12.
16. Martinelli G, Zaccaria A, Farabegoli P, et al. Structural organization of bcr-abl gene in chronic phase and blast transformation in chronic myeloid leukemia patients. Leuk Lymphoma 1993; 11:51-6.
17. Frassoni F, Martinelli G, Saglio G, et al. Bcr/abl chimeric transcript in patients in remission after marrow transplantation for chronic myeloid leukemia: higher frequency of detection and slower clearance in patients grafted in advanced disease as compared to patients grafted in chronic phase. Bone Marrow Transplant 1995; 16:595-601.
18. Cross NC. Quantitative PCR techniques and applications. Br J Haematol 1995; 89:693-7.
19. Thompson JD, Brodsky I, Yunis JJ. Molecular quantification of residual disease in chronic myelogenous leukemia after bone marrow transplantation. Blood 1992; 79:1629-35.
20. Cross NC, Feng L, Chase A, Bungey J, Hughes TP,

Goldman JM. Competitive polymerase chain reaction to estimate the number of bcr-abl transcripts in chronic myeloid leukemia patients after bone marrow transplantation. Blood 1993; 82:1929-36.

21. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989.

22. Testoni N, Martinelli G, Farabegoli P, et al. A new method of "in-cell reverse transcriptase-polymerase chain reaction" for the detection of bcr/abl transcript in chronic myeloid leukemia patients. Blood 1996; 87:3822-7.

23. Zaccaria A, Martinelli G, Buzzi M, Farabegoli P. Will molecular biology contribute to refine prognosis and to select treatment? Leuk Lymphoma 1993; 11:81-9.

24. Martinelli G, Gasparini P, Ambrosetti A, Battista R, Perona G, Pignatti PF. Molecular genetic analysis of bcr breakpoint in Philadelphia chromosome rearrangements and correlation with prognosis and age of chronic myelogenous leukaemia patients. In: Bartsocas CS, Loukopoulos D, eds. Genetics of hematological disorders. Washington: Hemisphere Publishing Corp.; 1990. p. 185.

25. Santini V, Zoccolante A, Bosi A, et al. Detection of bcr/abl transcripts by RT-PCR and their colorimetric evaluation in chronic myeloid leukemia patients receiving allogeneic bone marrow transplantation. Haematologica 1996; 81:201-7.

26. Anonymous. Treatment of the individual patient with chronic myelogenous leukemia. Haematologica 1997; 82:385-6.

27. Carella AM, Frassoni F, Melo J, et al. New insights in biology and current therapeutic options for patients with chronic myelogenous leukemia. Haematologica 1997; 82:478-95.

28. Stewart MJ, Cox G, Reifel-Miller A, Kim SY, Westbrook CA, Leibowitz DS. A novel transcriptional suppressor located within a downstream intron of the BCR gene. J Biol Chem 1994; 269:10820-9.

29. Leibowitz D, Young K, Cox G. Sequence-specific DNA-binding proteins within the Mbcr on the Ph1 chromosome. Genes Chromosomes Cancer 1991; 3:308-12.

30. The Italian Cooperative Study Group on Chronic Myeloid Leukemia. Chronic myeloid leukemia, bcr-abl transcript, response to α-interferon and survival. Leukemia 1995; 9:1648-52.