

An Evaluation of Minimal Residual Disease in Childhood Acute Lymphoblastic Leukaemia

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Abstract

Evaluation of minimal residual disease (MRD) in acute lymphoblastic leukaemia (ALL) is important for disease prognostication and early relapse detection. In this study, the rearranged third complementarity-determining-region (CDR-HI) of immunoglobulin heavy chain (IgH) was used as a surrogate tumour marker for MRD evaluation. DNA obtained from marrows at diagnosis was amplified by the polymerase chain reaction (PCR) using a pair of consensus primers. After 2 rounds of DNA amplification and polyacrylamide gel separation, the nucleotide sequences of 87.5% (21/24) consecutive children with B-lineage ALL were obtained by automated sequencing. There were between 1-4 rearrangements per patient. Although the J5 and J6 joining regions were preferentially used, the rearranged sequences were unique for all 25 sequences obtained. Oligoprobes to the DNJ region were constructed and quantitation in 7 patients showed a detection sensitivity of 1 leukaemic cell in 10^4 to 10^5 normal cells compared to 3 in 100 using conventional morphological criteria. Serial bone marrow showed progressive decrease in the quantity of leukaemic cells, and no leukaemic sequences were detected during cessation of therapy in 4/7 patients. One patient with detectable MRD, absconded treatment and eventually relapsed. These results are consistent with the need to eliminate the leukaemic clones below MRD detection levels before the end of therapy at 2 years. In conclusion, this study describes a novel, simplified and sensitive method of MRD detection in childhood leukaemia.

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Introduction

With improved long-term survival for many childhood cancers, therapy has shifted from "cure at all costs" to "cure at minimal cost". For childhood acute lymphoblastic leukaemia (ALL), the most common childhood malignancy, 5-year event-free survival (EFS) has improved tremendously from no survivors* in the 1960s to 70% in the 1990s.²⁻⁴ Our experience in the National University Hospital has also shown comparable improvement, from no survivors before 1973 to 38% 2-year EFS for the 1973-1976 cohort,⁵ and 63% for the 1988-1992 cohort.⁶

Long-term follow-up studies of survivors of childhood ALL, however, have revealed significant morbidities in the earlier protocols. These included secondary malignancies,^{7,8} sterility,⁹ endocrine dysfunction,¹⁰ cardiomyopathy,¹¹ and decreased IQ.^{12,13} There is,

hence, an urgent need to modify therapy to decrease treatment related side-effects and ensure the return of survivors to a normal lifestyle.

On the other hand, salvage therapy remains poor for a significant proportion (up to 30%) of patients who relapse.¹⁴ Our experience has also shown no survival for patients relapsing during therapy.⁶ Attempts to stratify patients into risk groups help tailor the intensity of therapy to the risk of relapse¹⁵ but does not identify all potential relapsers.

Recently, molecular quantitation of residual leukaemia during disease remission promises to better identify those at risk of relapse.¹⁶ This technology provides the capability to detect and quantitate minute amounts of residual leukaemic cells (up to 1 leukaemia cell in 10^5 normal cells) before clinical relapse, and this defines the study of minimal residual disease (MRD).¹⁷

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This method of MRD detection has increased sensitivity of tumour detection by several log-fold compared to conventional bone marrow morphology, which only detects relapse at blast counts of more than 3% (3 blasts in 100 normal cells), or an estimated leukaemic burden of up to 10^{10} cells.¹⁸

The early detection of an increase in the leukaemic cell population before overt clinical relapse is important as intensifying therapy at this stage has been shown to improve survival.¹⁹ The quantitation of MRD also documents objectively the efficacy of different parts of a protocol and provides an unambiguous comparison between protocols which has been hampered by the various complexity of risk assignment.¹⁴

In this study, MRD was assessed in children with B-lineage ALL. Twenty-four consecutive patients were evaluated using molecular methods to identify immunoglobulin heavy chain (IgH) rearrangements of the leukaemic clones. A simplified method of MRD quantitation is described. Our results indicate that this method of detection is extremely sensitive and has the potential of early identification of patients at risk of relapse.

Materials And Methods

Patient Population

Twenty-four consecutive children (<15 years) with B-lineage ALL presenting to the National University Hospital between 1992 and 1995 were entered into the study. The diagnosis of B-lineage ALL was confirmed using the French-American-British morphological criteria and immunophenotyping. Diagnostic bone marrow samples were obtained prior to therapy with the modified Pediatric Oncology Group ALL protocol⁶ or the National University Hospital ALL protocol.⁶ Serial bone marrow samples were obtained at various phases of treatment. Marrow mononuclear cells were isolated using Ficoll/Hypaque density gradient fractionation.

DNA Extraction and Amplification by Polymerase Chain Reaction (PCR)

DNA from the bone marrow mononuclear cells was

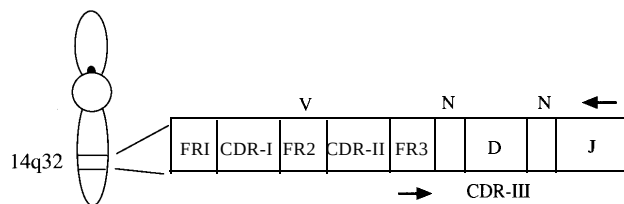


Fig. 1. The IgH gene is located in chromosome 14q32. The CDR-III region consists of the framework 3 (FR-3), diversity (D) and joining (J) segments. The N-regions are the random addition and deletion of nucleotides at the joining junctions of the 3 segments. The locations of the consensus primers used in this study are indicated by the arrows.

extracted using phenol/chloroform extraction,²⁰ precipitated using ethanol, resuspended in water, and stored at -70°C . The PCR primers used were the consensus primers for the Joining (J) region and the variable framework FR-3 region of the heavy chain of immunoglobulin²¹ (Fig. 1):

VH consensus:

5'-CTGTCGACACGGCCGTGTATTACTG-3', and

JH consensus:

5'-AACTGCAGAGGAGACGGTGACC-3'.

Thirty-five amplification cycles consisting of denaturation at 94°C for 2 minutes, annealing of primers at 55°C for 30 seconds, and DNA extension at 72°C for 30 seconds were used in a thermal cycler (Cetus, USA).

Polyacrylamide Gel Electrophoresis (PAGE) of Amplified DNA and DNA Sequencing

Amplified DNA was separated by 8% PAGE and stained with Sybrgreen® (Sybex, USA). The bands between 50-200 bp were excised, as this is the expected size range for the rearranged CDR-III. DNA was eluted by soaking in Tris-HCl 10M/EDTA 1M buffer (pH 8) overnight at 37°C , precipitated and resolubilised in water.

As the amount of eluted DNA was insufficient for DNA sequencing, it was reamplified by a second round PCR and separated using similar conditions as described above. The DNA bands were eluted for automated DNA sequencing (Applied Biosystems Inc, USA). The D and J region sequences were identified by comparing with published sequences.^{22,23}

Constructing the Allele-Specific Oligomers

For 7 patients, oligoprobes (18-25 bp) to the D-N-J region of CDR-III were used as unique surrogate tumour markers for subsequent MRD detection in remission marrows. Sequences with hairpin loops and interprobe annealing were excluded using the computer software Oligo™ (version 3.4). The IgH CDR-III of remission bone marrows of each patient were amplified, transferred on to a nylon membrane by Southern blot, and hybridised with their respective radiolabelled oligoprobes.

Standardisation of MRD Quantitation and Oligonucleotide Hybridisation

In order to quantitate MRD in remission marrow, a standard curve was constructed using serially diluted diagnosis DNA. These dilutions were made with DNA from normal marrow cells to simulate those of remission marrows which would contain a predominance of normal cells. To ensure consistency of PCR amplification, a system of dilution with duplicates and internal checks was devised (Fig. 2). Our initial experiments showed

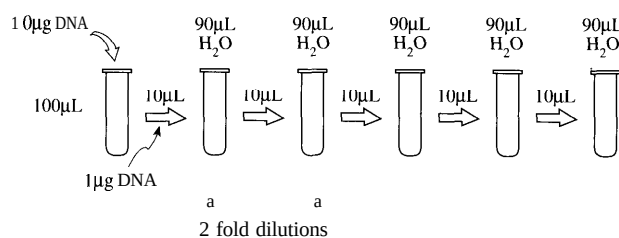


Fig. 2. Schematic representation of the dilution method devised for quantitation of MRD. To reduce the errors of pipetting small volumes of DNA, every dilution involves dilution of 10 µL of the preceding concentration into 90 µL of water. Two-fold dilutions are made for the range where the log-linear region of the sigmoid curve is expected (range between 1 to 100 ng of leukaemic DNA).

that the standard curve was sigmoid with a narrow exponential range at DNA concentration of 1 to 100 ng. DNA in this range was diluted 2-fold compared to 10-fold dilutions outside this range (Fig. 2).

For each MRD quantitation experiment, 30 to 40 PCR reactions, which included duplicates of serially diluted diagnosis DNA and triplicates of remission marrows, were carried out. PCR products of the serially diluted DNA of diagnosis and remission marrows were separated by 1.5% agarose gel electrophoresis and transferred to Hybond-N membranes (Amersham International, England) by Southern blot.²⁴ The oligonucleotide probes were end-labelled with T₄

polynucleotide kinase (Amersham International, England) using [γ -³²P]dCTP. Membranes were prehybridized with buffer containing 3X SSPE (1X SSPE = 0.15 M NaCl, 0.011M NaH₂PO₄, 0.002 M EDTA), 1X Denhart's solution, 0.5% sodium dodecyl sulphate (SDS), 1 mg/mL salmon sperm DNA for 1 hour. This is followed by hybridisation with radiolabelled oligoprobe in the same buffer for 1 hour at 5°C below the calculated T_m of the oligoprobe. The membrane was then washed using 2X SSC (1X SSC = 0.5 M NaCl, 15 mM sodium citrate)/0.1% SDS at 2°C below the calculated T_m of the oligoprobe for 5 to 10 minutes and exposed to X-ray film (Kodak X-omat AR, Kodak) at -70°C overnight. A standard curve was constructed from densitometric readings of autoradiographed bands obtained from known amounts of serially diluted leukaemia DNA.

Results

DNA Amplification and Sequencing of IgH CDR-III

A total of 24 patients with B-lineage ALL were studied (Table I). Successful sequencing of at least one leukaemic rearrangements of the IgH CDR-III region per patient was achieved in 21/24 (87.5%) of patients. For each patient sample, there were between 1-4 bands within the 50-200 bp region of interest, though majority have 1 or 2 bands only (Fig. 3, Table I). Interestingly, we found

TABLE I: SUMMARY OF PATIENT DATA

No	Pt	Age/ mths	TW at diagnosis	Sex	FAB	cALLA	Status	No of bands*	Blasts diagnosis (%)
1	L1	52	3.1.	M	L1	+ve	4-y EFS	1	98%
2	L3	52	47.9	F	L1	+ve	4-y EFS	1	80%
3	L4	57	9.7	F	L2	+ve	4-y EFS	1	78%
4	L6	74	262.8	M	L1	-ve	4-y EFS	4	99%
5	L8	161	48.2	F	L1	+ve	4-y EFS	1	90%
6	L9	98	83.1	F	L2	+ve	3-y EFS	1	100%
7	L10	123	60.9	M	L2	-ve	3-y EFS	1	100%
8	L11	58	75.6	M	L2	+ve	abscond		95%
9	L13	67	115.0	F	L2	+ve	3-y EFS	1	100%
19	L28	21	8.1	F	L2	+ve	3-y EFS	failed	91%
10	L14	20	92.0	M	L1	+ve	3-y EFS	1	83%
11	L15	65	10.1	M	L2	+ve	2-y EFS	2	92%
12	L16	96	53.0	F	L2	+ve	2-y EFS	3	100%
13	L18	149	3.2	M	L1	+ve	abscond	2	100%
14	L19	33	69.2	F	L1	+ve	2-y EFS	1	89%
15	L22	42	3.5	M	L2	+ve	2-y EFS	1	99%
16	L23	52	68.8	F	L2	+ve	on Rx	2	99%
17	L24	41	7.0	F	L1	+ve	on Rx	2	80%
18	L27	84	5.4	M	L1	+ve	on Rx	2	30%
20	L29	146	3.9	F	L1	+ve	on Rx	4	few
21	L37	52	7.7	F	L2	+ve	on Rx	2	93%
22	L38	37	3.7	F	L1	+ve	on Rx	3	46%
23	L39	84	69.2	F	L2	+ve	on Rx	2	93%
24	L40	36	6.3	M	L1	+ve	died	2	43%

*The number of bands detected after PAGE separation of the PCR product

Pt: patient; TW: total white; FAB: French-American-British classification; cALLA: common acute lymphoblastic leukaemia antigen; EFS: event-free survival; PAGE: polyacrylamide gel electrophoresis; PCR: polymerase chain reaction

TABLE II: THE DNJ SEQUENCES FOR THE LEUKAEMIC DNA CDR-III REARRANGEMENTS

Pt	D Region	N Region	J Region
L1	DXP' 1	TTACTATGGTTCGGGGAGTTAT (-6)	<u>cccc</u> (-5) <u>TACTACTACTACGGTATGGACGTCT</u> J6
L3	D23/7	CATTTTGGAGTGGTTATTA (-4)	<u>aggatcatg</u> (-12) <u>ATGCTTTTGATGTCTGGGGCCAAGGG</u> J3
L4	DLR4	GTAGTAGTACCAGCTGCT (-5)	<u>cacgg</u> (-4) <u>TGGTTCGACCCCTGGGGCCAAGGGAA</u> J5
L6b	DLR2	TGTAGTGGTGGTAG (-10)	tctactccgatc (-3) ACTACTACTACTACGGTATGGACCCC J6
L8	DLR4	ATATTGTAGTAGTACCAGCTGCT (-5)	<u>ccccct</u> (-5) <u>TACTACTACTACGGTATGGACGTCT</u> J6
L10	D21/9	GATAGTAGTGGT (-9)	ggg (-5) TACTACTACTACGGTATGGACGTCT J6
	DM2	(-7) CCGGAACCCAC (0)	tccatct (-5) ACTACTACTACTACGGTATGGACG J6
L11	DLR2	GGGTGGTAGCTGCTACTCC (0)	ggtc (-3) TACTACTACTACGGTATGGACGTCT J6
L14	DXP1	GTATTACGATATTTTGACTGGTTAT (-6)	ca (-5) TACTACTACTACGGTATGGACGTCT J6
L15a	DM1	AAC <u>TGGA</u> ACTA (-1)	<u>gattggg</u> (-4) <u>TGGTTCGACCCCTGGGCCAGCAA</u> J5
L15b	DN1	GGGTATAGCAGCAG (-7)	(-14) TACGGGTATGGACCCCT J6
L16	DLR4	TATTGTAGTAGTACCAGCTGCT (-5)	<u>cacgg</u> (-4) TGGTTCGACTCCTGGGGCCAGGGACC J5
L18	DXP1	ATTACGATATTTTGACTGGTTA (-7)	<u>ccct</u> (-4) <u>CTACTACTACTACGGTATGGACGTCT</u> J6
L19	DLR3	ATATTGTGGTGGTGATTGCTATTCC (0)	<u>gctaag</u> (-8) <u>ACTACTACGGTATGGACGTCTGGGG</u> J6
L22	DXP1	ACGATATTTTGACTGGTTAT (-6)	cccaatttt (0) ACTACTTTGACTACGGGGCCAAGGAACCC J4
L23	DLR4	AGTAGTACCAGCTGCT (-5)	ct (-2) ACTGGTTCGACCCCTGGACCAGGA J5
L24	DN1	GCGTATAGCAG (-10)	gttttggg (-2) ACTGGTTCGACCCCTGGGGCCAGGG J5
L27	DN4	GAGTATAGCAG (-7)	gtctcgggg (-2) ACTGGTACTTCGATCTCTGGGGG J2
L28	DLR4	AGTACCAGCTGCT (-5)	ctcatct (-8) TACTACTACGGTATGGACGTCTGGG J6
L29	DLR4	TAGTACCAGCTGCT (-5)	cacctc (-5) TACTACTACTACGGTATGGACGTCT J6
L37a	DXP1	ACGATATTTTGACTGGTTAT (-6)	cccaacttt (0) ACTACTTTGACTACTGGGGCCAGGG J4
L38	DLR4	GTAGTAGTACCAGCTGCT (-5)	cacctctcg (-7) CTACTACTACGGTATGGACGCC J6
L39	DLR4	TAGTAGTACCAGCTGC (-6)	aaacctcaaagtatctccga (-2) ACTGGTTCGACCC J5

Pt: patient; CDR: complementarity-determining-region

Underlined text denotes sequences used for oligoprobe construction.

Figures in parenthesis denote the number of nucleotides deleted.

In some patients, more than one band are sequenced. The DNA probes used for the 7 patients for remission detection are as underlined. Note the preferential utilisation of the J5 and J6 segments in these patients. This, however, did not compromise the diversity or uniqueness of each rearrangement because of the random deletions and addition of nucleotides in the N regions.

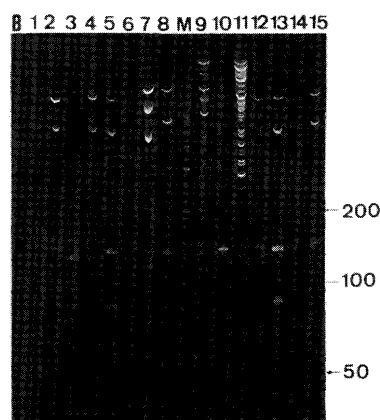


Fig. 3. Photograph of a representative PAGE gel used to separate the PCR products. The band size of interest is between 50-200 bp as indicated. Most samples demonstrate between 1-4 bands of rearrangement (Table I).

preferential usage of the J5 (24%; 6/25) and J6 (56%; 14/25) during recombinations compared to J1-4 (Table II).

Construction of Oligoprobes and Quantitation of MRD

Based on the D-N-J sequences, oligoprobes were constructed for 7 patients (Table II). Hybridisation of

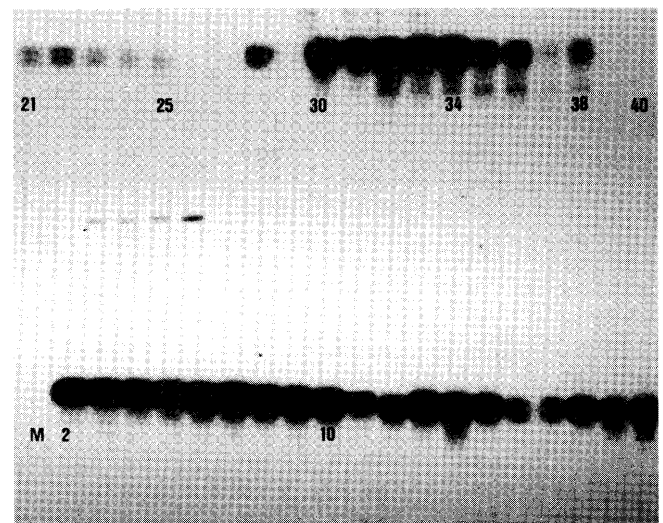


Fig. 4. Autorad of patient L19 showing serial dilution of the marrow from lanes 2-27. Ten-fold dilution of the early remission marrow in lanes 30-33, marrow at 2 months (lanes 34-36) and marrow at 8 months (lane 37). Refer to Figure 6(L19) for interpretation. Lanes 28, 29 and 38 are test concentrations of DNA.

these probes with PCR amplified serial dilutions of diagnosis marrow DNA yielded detection sensitivity of one leukaemic cell in 10^4 to 10^5 normal cells (Fig. 4). This

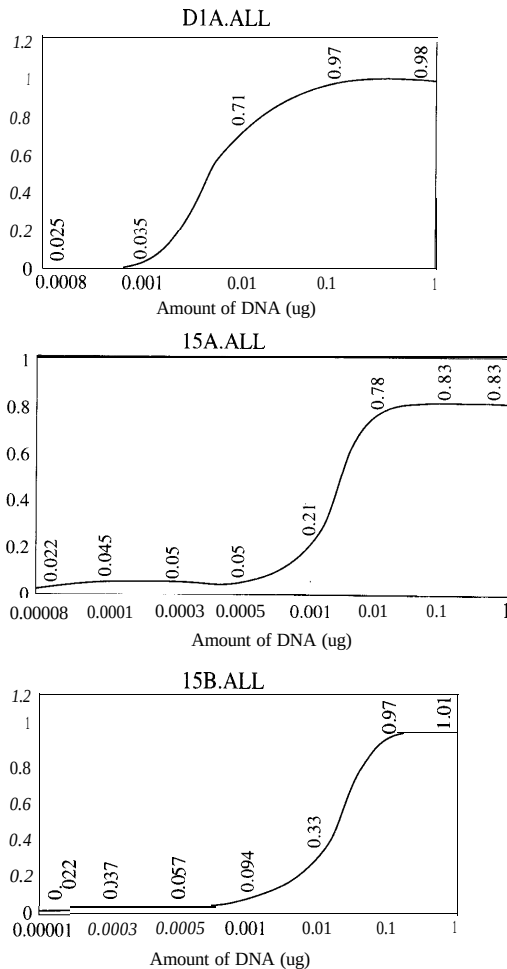


Fig. 5. Three representative sigmoid curves from 2 patients. Note the sigmoid curves follow essentially the same shape for all patients, except they are shifted to the right or left with different probes and patient DNA. The log-linear part of the curve is narrow extending only for two 10-fold dilutions.

is calculated from the assumption that one cell contains 6 pg of DNA.¹⁸ Oligoprobe hybridisation was specific as it did not bind DNA from normal dilution marrows (data not shown).

In order to accurately quantitate the remission marrow, standard curves were constructed for each patient (Fig. 5). The standard curve for each patient was sigmoid with a narrow linear portion extending only over two 10-fold dilutions. Blinded samples of known dilutions of diagnostic marrow were used as controls for MRD quantitation and showed an intrassay variation of $\pm 20\%$. For the 7 patients, quantitation of MRD in remission marrows are summarised in Figure 6. Most patients showed a decrease in leukaemic cells below 8×10^3 by the end of induction therapy. For the 4 patients who have completed therapy, there were no detectable leukaemic cells before cessation of therapy. There was progressive decrease in the quantity of leukaemic cells with therapy.

Discussion

In this study, a simplified method of molecular quantitation of MRD for B-lineage childhood ALL is described. Rearrangement of the CDR-III region of the IgH chain was chosen because of its extreme variability, and the high likelihood (98%) of rearrangements in B-lineage ALL,²⁵ the most common subtype of childhood ALL. These rearrangements, which were unique for each patient, were used as surrogate markers of the leukaemic blasts for the evaluation of subsequent remission marrows.

In contrast to previous reports, which involved the more tedious and laborious method of subcloning am-

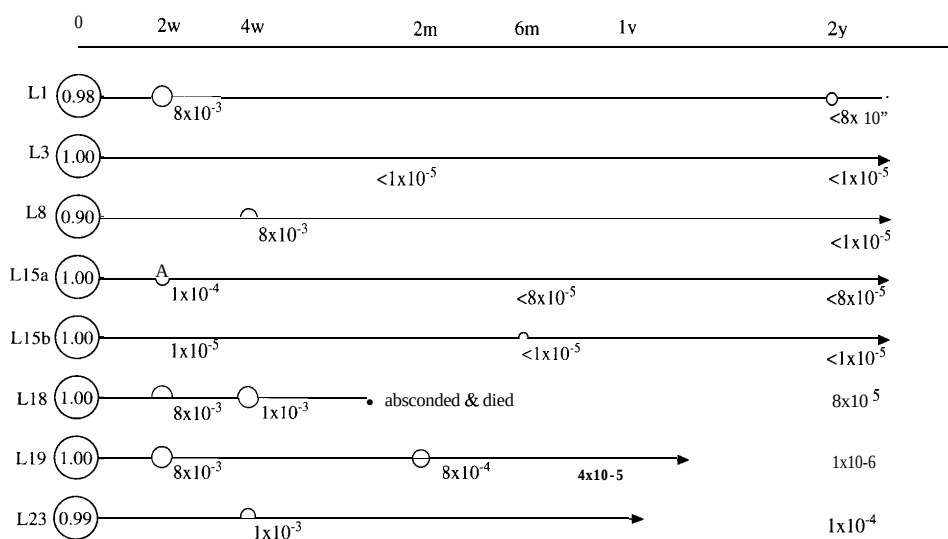


Fig. 6. Quantitation of minimal residual disease in serial marrows of 7 patients. The time is indicated at the top. The amount of leukaemic cells indicated inside or beside the circles. The amount is written as the ratio of 1 leukaemic cell to normal cell. For example 8×10^{-3} implied the method is able to detect 1 leukaemic cell in 8×10^3 normal cells. There is variable sensitivity of detection using different probes, ranging from 8×10^{-3} to 1×10^{-6} .

plified leukaemic CDR-III by ligation into plasmids and transfecting them into *E. coli*,^{19,26-28} the results of this study indicated the feasibility of 2 rounds of PCR amplification and PAGE separation to identify CDR-III rearrangements in 87.5% (21/24) of the patients studied. This compared favourably with similar single pair consensus primer methods which employed plasmid ligation, with success of sequencing in 49% to 80%.^{19,26-28} Our improved results were also likely to be due to availability of leukaemic DNA without resorting to stored slides. As noted by Brisco et al,¹⁹ who utilised DNA extracted from slides, only 49% were successful.

Due to the small size (50-200 bp) of the rearranged CDR-III, manual DNA sequencing was unsuccessful and necessitated automated DNA sequencing. This was due to the inherent inability of manual method to sequence up to 30 bp from the primer. Most published studies also employed automated sequencing as ligation into plasmids require sequencing of the many colonies grown.^{19,26,27} Interestingly, we identified preferential usage of the J5 (6/25) and J6 (14/25) regions by the leukaemic blasts compared to J1-J4 (total 4/25) regions. This is in contradistinction to normal peripheral blood B cells which have preferential usage of J4 (52%), compared to J6 (22%) and J5 (15%).²⁹ This significance is unknown. Despite this less than expected variability, the random additions and deletions of nucleotides in the N region maintained specificity. The absence of identical sequences in the 25 sequences identified in this study (Table II), confirmed the uniqueness of using the CDR-III region as a leukaemic marker.

In the remaining 12.5% (3/24) of patients, whose rearrangements could not be identified, failure of PCR amplification may have been due to extreme variability V region sequences which were not compatible with the V-H consensus primers used. Deane and Norton³⁰ used a family of 7 consensus primers for the V region and a single consensus J primer improved detection of rearrangement to 90%.

To ensure accurate quantitation of the MRD, we designed a relatively simple cross-check method involving 30 to 40 PCR reactions. This was necessary as quantitation using PCR is notoriously problematic³¹ because PCR exponentially amplifies target DNA, such that a small change in amount of target DNA may result in a significant difference. This involved initial serial 10-fold dilutions of the diagnostic DNA to identify the linear component of the sigmoid curve, which was further diluted in 2-fold dilutions. To ensure reproducibility, all the dilutions were made in duplicates. Blinded samples of known concentrations of target DNA were also added to assess the accuracy of quantitation. Remission marrows were amplified in triplicates and diluted 10-fold until 10^{-3} μ g to ensure that the quantitation fell within the linear region of the standard curve which

therefore maximised the accuracy of quantitation. A total of 40 PCR reactions were carried out per patient sample. Using this system, our detection limit varied from 1 leukaemic cells in 10^4 to 10^5 normal cells. This level of sensitivity is comparable with other studies.^{19,26,27}

We were able to demonstrate detection of 8×10^{-3} to 1×10^{-5} leukaemic cells in the early remission marrow in 6/7 patients studied at the time of this report. Clearing of leukaemic blasts below our detection sensitivity was documented in the marrow before stopping therapy in all 4 patients who continued in complete remission. In 1 patient with detectable MRD after the end of induction (L18), who absconded, presented 2 months later with relapse. This is consistent with the need to clear the leukaemic cells below our detection level, prior to likelihood of cure. We have identified a total of 5 patients with B-lineage ALL who have relapsed. Work is currently being carried out on these 5 patients so as to determine the usefulness of this method for the early detection of relapse.

The strengths of this study lie in the simplified method of detection of rearrangement of the CDR-III region without need of the laborious and technically challenging subcloning, and yet not compromising efficacy, and an accurate method of quantitation. We discovered that the serial dilution of the leukaemic DNA for PCR was not linear thus throwing doubt on quantitative studies using only small number of dilutions. The narrow linear range of the standard curve over two 10-fold dilutions made it necessary to have smaller fold dilutions in this range. We hence provide a reliable method of quantitating MRD which are within the reach of the ASEAN and developing countries.

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REFERENCES

1. Poplack D G, Reaman G. Acute lymphoblastic leukemia in childhood. In: Poplack D G, editor. The Leukemias. *Pediatr Clin North Am*

- 1988; 35:903-32.
2. Rivera G K, Pinkle D, Simone J V, Hancock M L, Crist W M. Treatment of acute lymphoblastic leukemia-30 years' experience at St. Jude Children's Research Hospital. *N Engl J Med* 1993; 329:1289-95.
3. Chessells J M, Bailey C, Richards S M, for the Medical Research Council Working Party on Childhood Leukaemia. Intensification of treatment and survival in all children with lymphoblastic leukaemia: Results of the UK Medical Research Council trial UKALL X. *Lancet* 1995; 345:143-8.
4. Ritter J, Creutz U, Reiter A, Riehm H, Schellong G. Childhood leukemia: Cooperative Berlin-Frankfurt-Münster trials in the Federal Republic of Germany. *J Cancer Res Clin Oncol* 1990; 116:100-3.
5. Quah T C, Wong H B, Yip W C L, Vellayapan K, Tay J S H, Chia K B. Treatment and follow up of acute leukaemia in children 1973-82. *J Singapore Pediatr Soc* 1983; 25:63-9.
6. Quah T C, Sun L, Chew F T, Yeoh A, Lee B W. Survival of childhood leukemia in Singapore. *Med Pediatr Oncol* 1996; 26:318-24.
7. Pui C-H, Ribeiro R C, Hancock M L, Rivera G K, Evans W E, Raimondi S C, et al. Acute myeloid leukemia in children treated with epipodophyllotoxins for acute lymphoblastic leukemia. *N Engl J Med* 1991; 325:1682-7.
8. Neglia J P, Meadows A T, Robison L L, Kim T H, Newton W A, et al. Second neoplasms after acute lymphoblastic leukemia in childhood. *N Engl J Med* 1991; 325:1330-6.
9. Leiper A D, Grant D B, Chessells J M. The effect of testicular irradiation on Leydig cell function in pre-pubertal boys with acute lymphoblastic leukaemia. *Arch Dis Childhood* 1983; 58:906-10.
10. Voorhess M L, Brecher M L, Glicksman A. Hypothalamic-pituitary function of children with acute lymphoblastic leukemia after three forms of central nervous system prophylaxis. *Cancer* 1986; 57:1287-91.
11. Lipshultz S E, Colan S D, Gelber R D, Perez-Atayde A R, Sallan S E, Sanders S P. Late cardiac effects of doxorubicin therapy for acute lymphoblastic leukemia in childhood. *N Engl J Med* 1991; 324:808-15.
12. Ochs J, Mulhern R, Fairclough D, Parvey L, Whitake J, Ch'ien L, et al. Comparison of neuropsychologic functioning and clinical indicators of neurotoxicity in long-term survivors of childhood leukemia given cranial irradiation or parenteral methotrexate: a prospective study. *J Clin Oncol* 1991; 9:145-51.
13. Jankovic M, Brouwers P, Valsecchi M G, Veldhuizen A V, Huisman J, Kamphuis R, et al. Association of 1800 cGy cranial irradiation with intellectual function in children with acute lymphoblastic leukaemia. *Lancet* 1994; 344:224-7.
14. Chessells J. Treatment of childhood acute lymphoblastic leukaemia: Present issues and future prospects. *Blood Rev* 1992; 6:193-203.
15. Pui C-H. Medical progress: Childhood leukemias. *N Engl J Med* 1995; 332:1618-30.
16. Carr T, Stevens RF. Minimal residual disease in childhood acute lymphoblastic leukaemia (commentary). *Lancet* 1994; 343:190.
17. Potter M N. The detection of minimal residual disease in acute lymphoblastic leukaemia. *Blood Rev* 1992; 6:68-82.
18. van Bakkum D W. Residual reflections on the detection and treatment of leukemia. In: Lowenberg B, Hagenbeek A, editors. *Minimal Residual Disease in Acute Leukemia*. Boston: Martinus Nijhoff, 1984:385-90.
19. Brisco M J, Condon J, Hughes E, Neoh S-H, Sykes P J, Seshadri R, et al. Outcome prediction in childhood acute lymphoblastic leukaemia by molecular quantitation of residual disease at the end of induction. *Lancet* 1994; 343:196-200.
20. Manak M M. Sample preparation. In: Keller G H, Manak M M, editors. *DNA Probes*. 2nd ed. England: Macmillan Publishers, 1993:27-68.
21. Yamada M, Hudson S, Toumay O, Bittenbender S, Shane S S, Lange B, et al. Detection of minimal disease in hematopoietic malignancies of the B-cell lineage using third-complementarity-determining region (CDR-III)-specific probes. *Proc Natl Acad Sci USA* 1989; 86:5123-7.
22. Ravetch J V, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. *Cell* 1981; 27:583-91.
23. Ichihara Y, Matsuoka H, Kurosawa Y. Organization of human immunoglobulin heavy chain diversity gene loci. *EMBO J* 1988; 7:4141-50.
24. Southern E. Detection of specific sequences of among DNA fragments separated by gel electrophoresis. *J Mol Biol* 1975; 98:503-8.
25. van Dongen J J M, Wolvers-Tettero I L M. Analysis of immunoglobulin and T cell receptor genes. Part II: Possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. *Clin Chim Acta* 1991; 198:93-174.
26. Yamada M, Wasserman R, Lange B, Reichard B A, Womer R B, Rovera G. Minimal residual disease in childhood B-lineage lymphoblastic leukemia. *N Engl J Med* 1990; 323:448-55.
27. Kitchingman G. Residual disease detection in multiple follow-up samples in children with acute lymphoblastic leukemia. *Leukemia* 1994; 8:395-401.
28. Jonsson O G, Kitchens R L, Scott F C, Smith R G. Detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin hypervariable region specific oligonucleotide probes. *Blood* 1990; 76:2072-9.
29. Yamada M, Wasserman R, Reichard B A, Shane S, Caton A J, Rovera G. Preferential utilization of specific immunoglobulin heavy chain diversity and joining segments in adult peripheral blood B lymphocytes. *J Exp Med* 1991; 173:395-407.
30. Deane M, Norton J D. Immunoglobulin heavy chain variable region family usage is independent of tumor cell phenotype in human B lineage leukemias. *Eur J Immunol* 1990; 20:2209-17.
31. Ouspenskaia M V, Johnston DA, Roberts W M, Estrov Z, Zipf T F. Accurate quantitation of residual B-precursor acutelymphoblastic leukemia by limiting dilution and a PCR-based detection system: a description of the method and principles involved. *Leukemia* 1995; 9:321-8.