and wound, in 2007 (n=20 isolates), 2006 (n=20 isolates), 2005 (n=20 isolates), 2004 (n=20 isolates) and 2003 (n=6 isolates).

All isolates were revived from frozen storage and were identified phenotypically using a combination of conventional identification methods (e.g., oxidase) as well as the API identification scheme (API 20NE; bioMérieux, Les Halles, France). The presence of LES markers (LES-PS21 and LESF9) was determined by molecular methods, as recently described.[3]

Results indicated that none of the equine isolates were LES strains, as defined by Fothergill *et al*.[3] Recently, there have been several reports describing the emergence of the LES of *P. aeruginosa* in CF patients.[1] This LES strain has been reported as the most frequently isolated clone obtained from CF patients in England and Wales.[2] This epidemic strain has also been reported to cause superinfection[4] and is associated with greater morbidity in patients than is the case with other non-LES *P. aeruginosa* strains.[5] In addition, it has been shown to be highly transmissible from a CF patient to non-CF parents,[4] and from a CF patient to a cat,[6] which resulted in increased morbidity for recipients of LES PA in both reports. Hence, it is a priority to define the carriage of these markers in *P. aeruginosa*, both within and outside the CF community.

This study was not able to demonstrate the presence of LES strains in a comprehensive collection of clinically significant *P. aeruginosa* from a non-CF source, suggesting that LES is a CF-related phenomenon. However, further studies are required to determine the carriage of LES markers in non-CF-associated *P. aeruginosa* in other clinical and non-clinical isolates in order to determine the uniqueness of LES within the CF population.

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Molecular characterisation and diagnosis of Hb J-Taichung (129[H7]Ala→Asp) in a Taiwanese family subject

T-H. LIN[*], T-K. ER[#,¥], S-C. LIU[*], I-C. LIN[§], H-L. CHENG[§], C-T PENG[†], H-C. SHIH[*,‡] and J-G. CHANG[#,¥]

[*]Department of Laboratory Medicine and [†]Children's Medical Center, China Medical University Hospital; ‡School of Post-Baccalaureate Chinese Medicine and §Department of Laboratory Biological Science and Technology, China Medical University, Taichung; [#]Department of Laboratory Medicine, Kaohsiung Medical University Hospital, Kaohsiung and [¥]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

Thalassaemia and haemoglobin variants are the most common genetic disorders in Taiwan. The frequency of α-thalassaemia is 3–5% and that of β-thalassaemia is 1–3% in the Taiwanese population. Haemoglobin (Hb) J-Taichung is a rare β-chain variant first describe in a Chinese female by Blackwell *et al*.[1] in 1969, and not found in other ethnic populations.

Hb J-Taichung is a non-pathological (stable) β-chain variant characterised by mutation at codon 129 of the β-globin gene exon 3 that changes alanine (GCC) to aspartic acid (GAC). Several other Hb variants depend on mutations at this region and include Hb Crete (β129 [H7] Ala→Pro) and Hb La Desirade (β129 [H7] Ala→Val). However, these mutations would not create a *Tse*I site.

In the present case study the proband was a 14-year-old Taiwanese girl admitted to the Children's Medical Centre, China Medical University Hospital, Taichung, following a four-month history of pallor. Informed consent to all investigations was obtained.

Peripheral blood samples, anticoagulated with

Table 1. Results of haematological indices and serum iron-related tests.

Parameter	Father	Mother	Brother	Proband
RBC (x10^6/μL)	4.98	4.70	5.82	4.87
Hb (g/dL)	16.1	12.9	15.6	10.0
Hct (%)	47.6	39.8	47.2	32.3
RDW	13.0	14.0	14.5	19.3
MCV (fL)	95.6	84.7	81.1	66.7
MCH (pg)	32.3	27.4	26.8	19.1
MCHC (g/dL)	33.8	32.4	33.1	30.7
HbA (%)	97.3	64.8	65.5	75.0
HbA2 (%)	2.7	3.3	3.3	3.5
HbF (%)	<0.1	<0.1	<0.1	<0.1
HbX (%)	–	31.8	31.1	21.4
Transferrin (mg/dL)	239.6	290.5	232.8	293.2
TIBC (μg/dL)	342.6	415.4	332.9	419.3
Fe (μg/dL)	82	80	143	19
Ferritin (ng/mL)	120.2	135.1	210.6	5.9

Correspondence to: Dr. Jan-Gowth Chang or Dr. Hung-Chang Shih
Emails: jgchang@ms.kmuh.org.tw / phd.shih@gmail.com

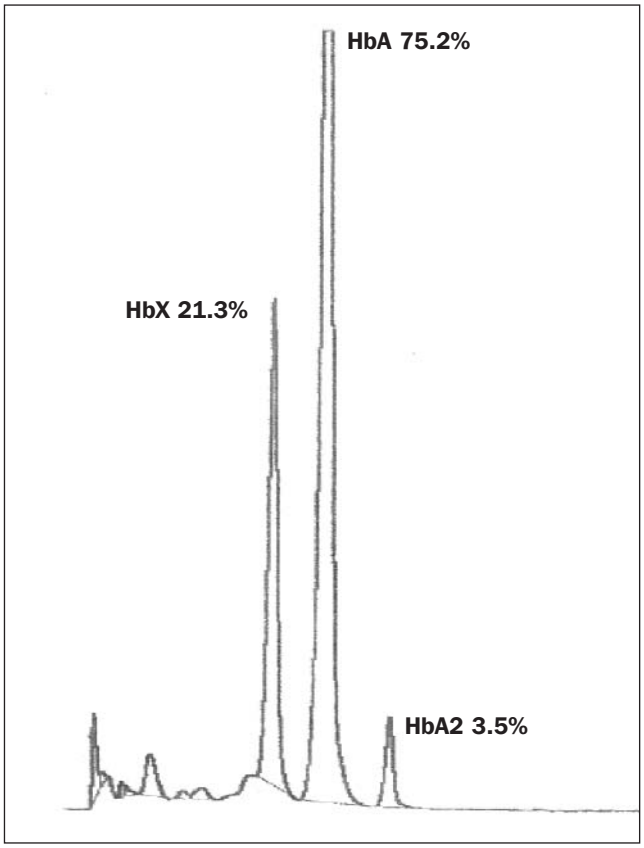

Fig. 1. High-performance liquid chromatogram showing an abnormal peak (HbX).

ethylenediamine tetraacetic acid (EDTA) were obtained from the patient and her family members (Table 1). Mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) values have greatest usefulness in longitudinal quality control and contribute little to the clinical evaluation of anaemia. Haemoglobin concentration and erythrocyte indices were obtained using an automated blood cell counter (Sysmex XE-2100 and SP-1000i; Sysmex, Chuo-Ku, Kobe, Japan).

Haemoglobin quantification and type were examined by automated high-performance liquid chromatography (HPLC) (Primus CLC385; Kansas City, Missouri, USA). In the authors' experience, HbA2 in normal adults, α-thalassemia-1 carriers and β-thalassaemia carriers are 2–3.5% and >3.5%, respectively.

Genomic DNA was prepared from peripheral blood leucocytes using a standard method and eluted 150 μL DNA (illustra GFX Genomic Blood DNA Purification kit, GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). Identification of α-globin and β-globin genes was performed using an allele-specific polymerase chain reaction (PCR) and direct sequencing analysis.

The α-globin genes were amplified with primers P1 (forward primer, 5′ non-coding area: 5′-CTCTTCTGGTCCCCACAGAC-3′) and P2 (reverse primer, 3′ non-coding area: 5′-AGGGGCAAGAAGCATGGCCA-3′) to amplify the entire coding region and two introns. The α2-globin genes were amplified with primers P1 and P3 (reverse primer, 3′ non-coding area: 5′-CAGGAAGGGCCGGTGCAAGGAG-3′) to amplify the entire coding region and two introns.

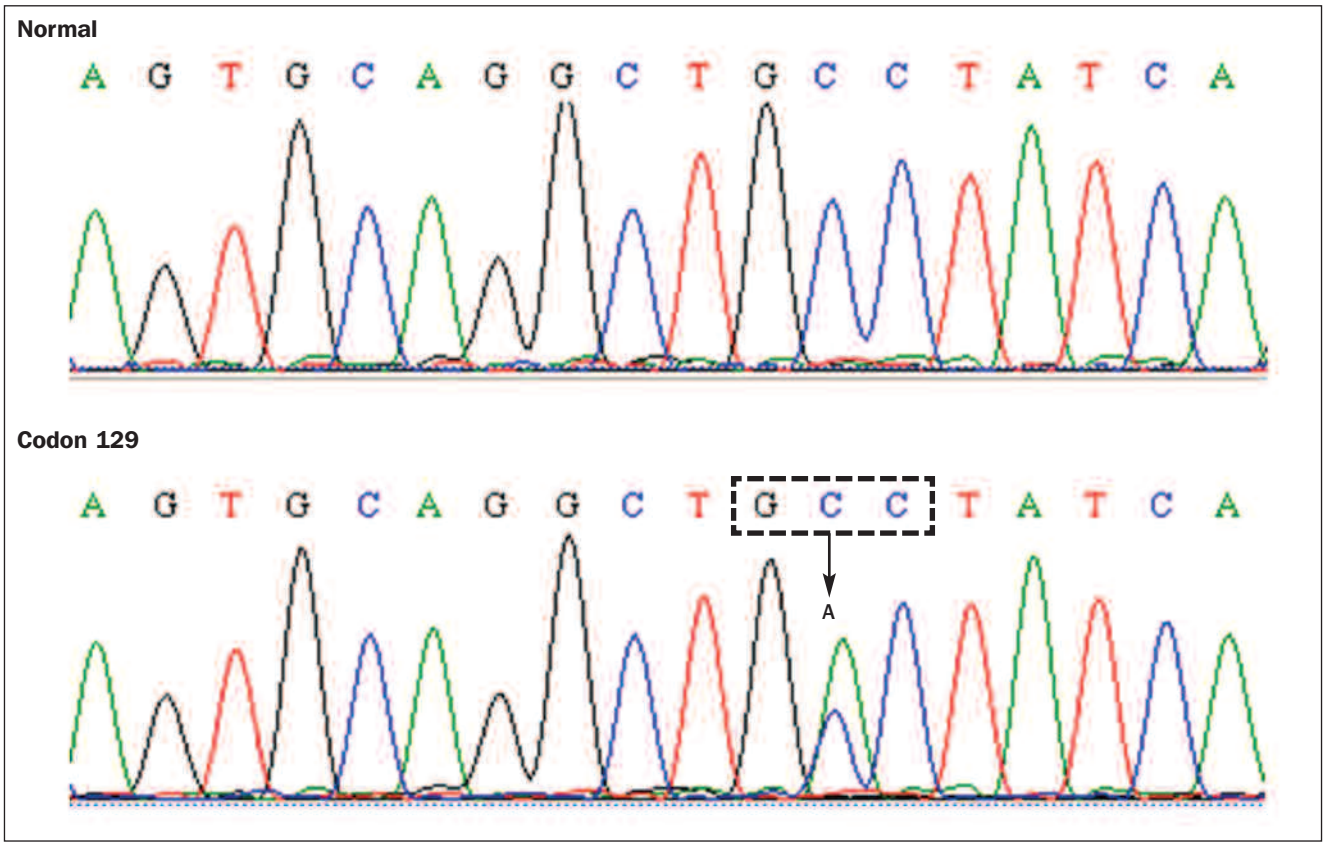

Fig. 2. Results of direct sequencing of the β-globin gene exon 3 region showed an A→C substitution at the second base of codon 129, indicated on the lower portion. The normal sequence is shown on the upper portion.

The β-globin gene was amplified with primers P4 (forward primer, 5′ non-coding area: 5′-GCTTACCAAGCTGTGATTCC-3′) and P5 (reverse primer, 3′ non-coding area: 5′-GGACTTAGGGAACAAAGGAACC-3′) to amplify the entire coding region and two introns.

The PCR amplification was carried out in a 50 µL reaction containing 500 ng genomic DNA, 50 ng each primer (PI +P2 or PI + P3 or P4 + P5), 0.3% DMSO, 50 µmol/L each dNTP, 1xPCR buffer and 2.5 units *Thermus aquaticus* (*Taq*) polymerase (Perkin Elmer, Norwalk, CT, USA). The PCR conditions were 35 cycles of 2 min at 94˚C (denaturation), 2 min at 60˚C (annealing) and 3 min at 72˚C (extension), and a final extension of 5 min at 72˚C, using a Perkin Elmer Cetus PCR thermocycler. The products were subjected to isolation and sequencing analysis as described previously.[2,3]

In addition to primers P1, P2, P3, P4, and P5, the sequencing primers for the α-globin gene wereas follows: P6 (reverse primer, intron 1: 5′-CAGGACGGTTGAGGGTGGCCT-3′), P7 (forward primer, intron 1: 5′-ACCCCACCCCTCACT CGCTT-3′), P8 (reverse primer, intron 2: 5′-TGCGAGGAAGGCGCCATCTC-3′) and P9 (forward primer, intron 2: 5′-GCAGAGGATCACGCGGGTTG-3′. The sequencing primers for the β-globin gene exon 3 region were P10 (reverse primer: 5′-ATGCACTGACCTCCCACATTCCCT-3′) and P11 (forward primer: 5′-TGCTAATCATGTTCATACCT-3′).

The PCR restriction fragment length polymorphism (PCR-RFLP) was developed for specific identification of the target $\beta^{\text{J-Taichung}}$ gene. In order to confirm the specific DNA sequence data, Hb J-Taichung can be caused by a mutation at codon 129 of the β-globin gene exon 3, and therefore selective amplification and restriction digestion of the PCR product was performed using the *Tse*I restriction enzyme (New England Biolabs, UK).

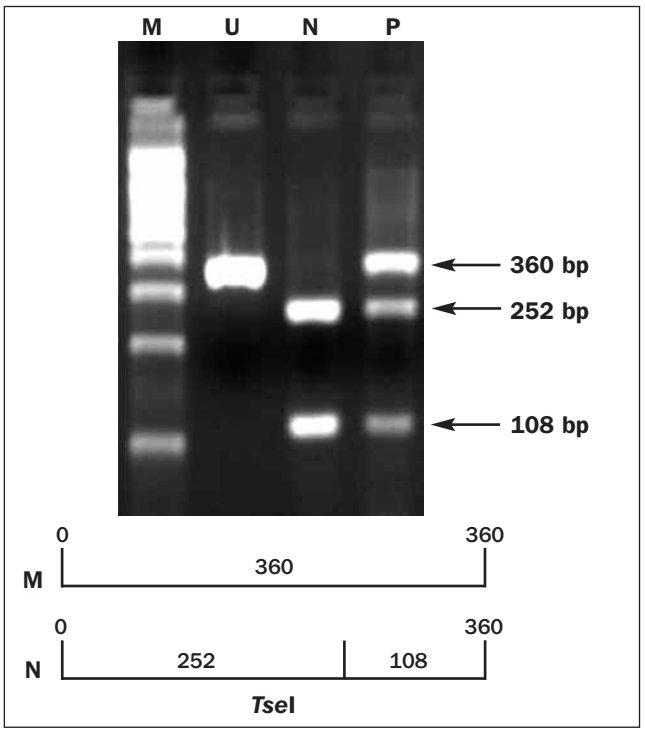

Fig. 3. Results of restriction enzyme *Tse*I digestion of PCR products of the β-globin gene exon 3 region showed that the normal control had 252 and 108 bp digested fragments from the normal allele, and a 360 bp undigested fragment from the mutant allele. The uncut (U) control also had a 360 bp fragment. M: 100 bp marker, U: uncut control, P: proband.

This study used the primer pairs P13+P14 for high-resolution melting (HRM) analysis as previously described.[4] The primers were all of standard molecular biology quality (Protech Technology Enterprise, Taiwan). The 309-bp PCR products were resolved on 2% agarose gel and visualised after staining with ethidium bromide.

The PCR reactions were carried out in duplicate in a final volume of 20 µL with 1x buffer containing *Taq* polymerase, nucleotides, ResoLight dye and 30-ng DNA. The primers and $MgCl_2$ were used at a concentration of 0.25 µmol/L and 2.5 mmol/L, respectively. The HRM assay was conducted using the LightCycler 480 and Gene-Scanning software (version 1.0; Roche Diagnostics).

The PCR program required an SYBR Green I filter (533 nm) and comprised an initial denaturation-activation step at 95˚C for 10 min followed by a 45-cycle program (denaturation at 95˚C for 15 sec, annealing at 52˚C for 15 sec and elongation at 72˚C for 15 sec). The melting program included three steps: denaturisation at 95˚C for 1 min, renaturisation at 40˚C for 1 min and

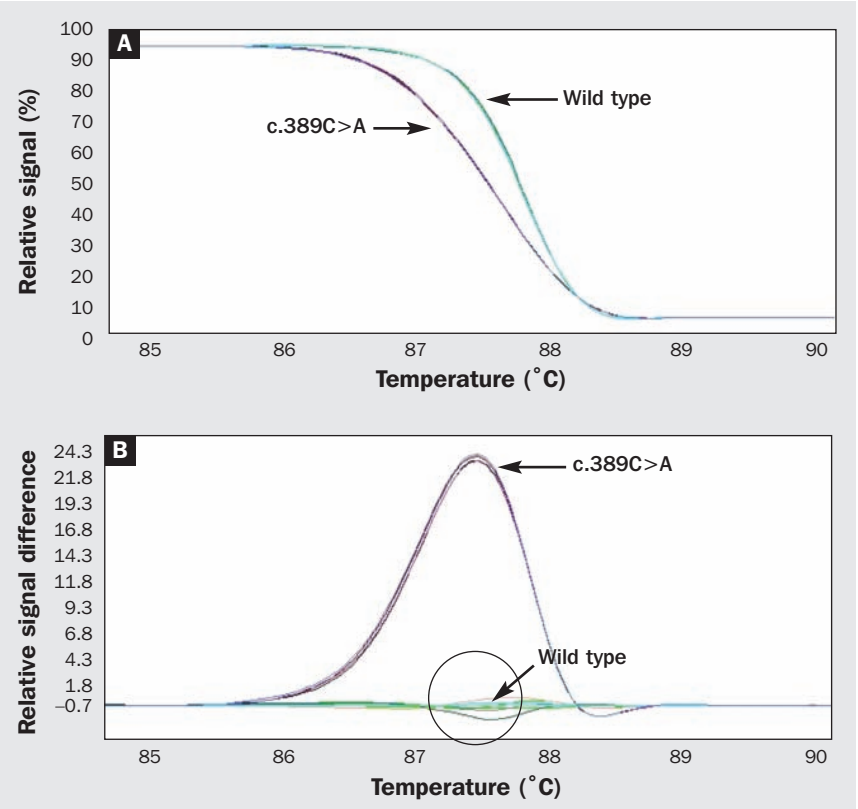

Fig. 4. Screening of the Hb variant c.389C>A. A) Normalised melting curves; B) Difference plots with primer set (P13+P14). c.389C>A can be distinguished easily in the normalised and temperature-shifted difference plot.

subsequent melting that consisted of a continuous fluorescent reading from 60 to 90°C at the rate of 25 acquisitions per degree centigrade.

Haematology and serum iron data from the proband and her family are shown in Table 1. The proband's complete blood count data showed mild hypochromic anisocytosis (Hb: 9.9 g/dL, haematocrit [Hct]: 32.3%, MCV: 62.4 fL, MCH: 19.1 pg, MCHC: 30.7 g/dL). Ferritin was low (5.94 ng/mL) and she had an abnormal haemoglobin electrophoresis pattern (HbA: 75.2%, HbA2: 3.5%, HbX: 21.4%; Fig. 1).

Investigation confirmed that the proband was a β-chain variant with IDA. Her mother and brother had the same abnormal haemoglobin electrophoresis pattern with HbX levels of 31.8% and 31.1%, respectively. The GCC →G*A*C at codon 129 in the β or βH7 position was shown after direct DNA sequencing (Fig. 2). Furthermore, the mutation area was amplified using the same primers (P10+P11), and the PCR products were digested with *Tse*I (Fig. 3). In addition, it was possible to distinguish Hb J-Taichung from wild-type DNA by HRM analysis (Fig. 4).

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