

# The Identification of Pompe Disease Mutations in Archival Tissues and Development of a Rapid Molecular-based Test

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## تحديد الطفرات المسببة لمرض بومبي في عينات أنسجة محفوظة وتطوير الفحص الجزيئي

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**المخلص:** الهدف: مرض بومبي (مرض تخزين الغليكوجين من النوع الثاني) هو مرض صبغي جسدي متنحي للاختزان في الجسيمات الحالة ينتج عن نقص حمض ألفا غلوكوزيداز. الاستخدام المبكر للعلاج بالاستعاضة الأنزيمية يمكن ان يفيد الاطفال المصابين بهذا المرض ولكن يعوق التشخيص ندرة المرض وتغايرية المظاهر السريرية. في هذه الدراسة، تم استخراج الحمض النووي من الأنسجة الأرشيفية (FFPET) لتحديد الطفرات المسببة لمرض بومبي في عمان وعمل اختبار جزيئي. الطريقة: تم تصميم المشرعات الإلكترونية لمضاعفة مقاطع قصيرة (193-454 قاعدة مزدوجة) من المحورات (2-20) للبحث عن الطفرات باستخدام التسلسل المباشر. النتائج: تم التعرف على طفرتين من الطفرات المتسببة للمرض الوخيم في عينتين. الأولى برمز طفرة  $c.2560C>T$  (p.Arg854X)، والثانية وجدت عند موقع المتقبل المصفر  $c.1327-2A>G$ . تم تصميم الاختبار الجزيئي (PCR-RFLP) للكشف عن الطفرات التي تم تحديدها. الخلاصة: يمكن لهذه الاختبارات تسهيل التشخيص قبل الولادة والمساعدة في تحديد الأفراد الذين يحملون الطفرات في الأسر التي تم تحديد الطفرات فيها.

**مفتاح كلمات:** مرض بومبي؛ غلوكان 4،1 ألفا غلوكوزيداز؛ نسيج؛ الطفرات؛ طرق التنميط الجيني؛ عمان.

**ABSTRACT: Objectives:** Pompe disease (glycogen storage disease type II) is a rare autosomal recessive lysosomal storage disease that is caused by acid alpha-glucosidase deficiency. Early enzyme replacement therapy can benefit infants with the disease but the diagnosis is complicated by the rarity of the disease and the heterogeneity of the clinical manifestations. In this study, DNA extracted from archival postmortem formalin-fixed paraffin-embedded tissues was used to identify Pompe disease mutations in Oman and develop a rapid molecular-based test. **Methods:** Intronic primers were designed to amplify short fragments (193–454 base pairs [bp]) from coding exons (2–20) and screen for mutations using direct sequencing (DS). **Results:** Two mutations known to cause severe disease were identified in two samples. One was a coding mutation,  $c.2560C>T$  (p.Arg854X), and the second was found at a splice acceptor site,  $c.1327-2A>G$ . Polymerase chain reaction- and restriction fragment length polymorphism-based tests were designed for the rapid genotyping of the identified mutations. **Conclusion:** These tests can facilitate prenatal diagnosis and help in identifying carriers in families with the identified mutations.

**Keywords:** Pompe Disease; Glucan 1,4-alpha-Glucosidase; Tissue; Mutations; Genotyping Techniques; Oman.

### ADVANCES IN KNOWLEDGE

- This study identified two genetic mutations causing Pompe disease in two Omani patients.

### APPLICATION TO PATIENT CARE

- The molecular test designed in this study can facilitate prenatal diagnosis and the screening of at-risk infants.

POMPE DISEASE IS A RARE AUTOSOMAL recessive disease, classified as a lysosomal storage disorder. It is caused by an acid alpha-glucosidase (GAA EC. 3.2.1.20) deficiency, which results in the accumulation of glycogen within the lysosomes and the cytoplasm of the

cardiac, skeletal and smooth muscle cells. The clinical spectrum of the disease varies in terms of the age of onset, the disease progression rate and the extent of organ involvement.<sup>1,2</sup>

Because of the continuous clinical spectrum of Pompe disease, Gungor and Reuser proposed three

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subtypes: classic infantile, childhood and adult.<sup>3</sup> The first is characterised by an onset of symptoms within the first year of life, which is always associated with hypertrophic cardiomyopathy and a total lack of acid  $\alpha$ -glucosidase activity; the second subtype covers patients with an onset of symptoms between birth and adolescence, but without persistent and/or progressive cardiac hypertrophy, and the third subtype covers patients with an onset of symptoms between adolescence and late adulthood.<sup>3,4</sup>

Early diagnosis and enzyme-replacement therapy can benefit infants with Pompe disease.<sup>5</sup> To establish a diagnosis of Pompe disease, both clinical evaluation and diagnostic tests are required.<sup>1</sup> Rapid blood-based activity tests were developed for pre-symptomatic diagnoses at birth and for at-risk individuals, to allow optimal conditions for enzyme replacement therapy;<sup>6</sup> however, a second confirmatory test is recommended to support the diagnoses. DNA-based testing is an approach that can rapidly confirm the diagnosis and identify the nature of the mutations (genotypes) that alter the level of residual enzyme activity and are responsible for the clinical phenotype heterogeneity.<sup>7,8</sup>

The *GAA* gene is about 28 Kilo-base pairs (Kb) long, contains 20 exons and maps to human chromosome 17q25.2–q25.3. The first exon is non-coding and the complementary DNA (cDNA) encodes a protein of 952 amino acids.<sup>2</sup> Nearly 250 mutations have been identified as causing Pompe disease, and they are rated by severity and divided into different classes.<sup>9</sup> Some of these mutations are common in certain populations.<sup>2</sup>

The identification of mutations in a population overcomes the problem of a lengthy, time-consuming and expensive search for a mutation approach—due to the size of the gene—by providing a limited number of mutations to be tested. This in turn facilitates diagnoses and aids in the counselling of patients and families with the disease. In this study, we utilised archived formalin-fixed paraffin embedded (FFPE) tissues in pathology laboratories to identify retrospectively *GAA* mutations causing Pompe disease (glycogen storage disease type II) in Oman, which enabled the design of a rapid molecular test for families with the identified mutations.

## Methods

Archived muscle tissues were collected in 2000 and 2002 from two infants of Arab origin (a 3-month-old male and a 4-month-old female, respectively) with clinical presentations consistent with infantile onset Pompe disease. Both infants had presented with bronchitis, hypotonia and hypertrophic cardiomyopathy. Their creatine kinase (CK) levels were high at 571 u/L and 410 u/L, respectively (range 0–6 u/L), and lactate dehydrogenase (LDH) levels were high at 2,415 u/L and 626 u/L, respectively (range 91–180 u/L). The electromyograms (EMG) showed mixed myopathic and neuropathic pictures.

For the DNA extraction, 10 sections of 7 micrometres ( $\mu$ m) were collected from archived muscle biopsies for each infant and kept in 2 ml microcentrifuge tubes. Normal archived muscle tissue sections were used as a control. DNA extraction was performed using a modified phenol-chloroform extraction method.<sup>10</sup> The sections were deparaffinised twice in 1 ml of pre-heated xylene (for 5 mins each) and then re-hydrated with 1 ml of 99% ethanol and 95% ethanol (twice, for 3 mins). For digestion, the tissues were incubated overnight at 60 °C with 300  $\mu$ l of lysis buffer solution containing 100  $\mu$ l of 5 mg/ml proteinase K (the proteinase K was inactivated at 95 °C for 15 mins). To purify the DNA, the samples were extracted twice with 300  $\mu$ l of phenol and once with 350  $\mu$ l of chloroform-isoamyl alcohol (at a ratio of 24:1). Finally, the DNA was precipitated with one ml of 100% ethanol and one U/L of glycogen (20 mg/ml). The mix was incubated at -20 °C for one hr and then centrifuged at 4 °C for 15 mins at 13,000 rpm. After washing the pellet with 1 ml of 70% ethanol, the extracted DNA was left to air-dry and was then resuspended in 50  $\mu$ l of double-distilled water (ddH<sub>2</sub>O). The integrity of the DNA was checked using 1% agarose gel electrophoresis and the quality of the DNA was analysed by polymerase chain reaction (PCR) for different primer sets that amplify different fragment sizes (250–500 bp).

For the PCR and sequencing process, 17 sets of intronic primers flanking exons were designed to amplify the coding regions [Table 1]. Exon 2 is a large exon and was therefore amplified with two overlapping fragments. The primers were designed to amplify short fragments (193–454 bp) suitable for degraded DNA.<sup>11</sup> PCR was performed using a 25  $\mu$ l

**Table 1:** Primer sequences, fragment sizes and annealing temperatures

Exon primers	Sequence (5' to 3')	Fragment size (bp)	Annealing temp. °C
2 A F	ctgagccccctttcttc	365	61
2 A R	cactgttctctgggtgatgg	365	61
2 B F	ccatcaccaggaacagtg	330	61
2 B R	gtgaggctcgtgggtgtc	330	61
3 F	ggtagctgtggggaacat	210	59
3 R	ctggcacagagcccagaa	210	59
4 + 5 F	gggtctctcaggctcgtgt	454	59
4 + 5 R	aggcagcacggaggagac	454	59
6 F	tgaagaatctgtccccaac	342	61
6 R	ggtcatgttctccaccact	342	61
7 + 8 F	ctcatgaagtcggcgttgg	400	61.5
7 + 8 R	ccttcccaaagaccacagt	400	61.5
9 F	cccgcctcatcctcact	252	63
9R	gctggaggcctctgtttct	252	63
10 + 11 F	cactgcagcctctcgttgc	396	58
10 + 11 R	gctaagtctcccaggccaga	396	58
12 F	gaggaagctcctggaacc	202	59
12 R	acaggctgtgaggcagag	202	59
13 F	ctctgcctcatcccagaag	287	63
13 R	cccggcttactctgctg	287	63
14 F	cctgaggaccagcctgact	248	59
14 R	attcccagggagagcttgg	248	59
15 F	agcaccacaagtgttctt	209	61.5
15 R	gcccctgcttaggtcact	209	61.5
16 F	gtatgcctgtgtcccac	193	59
16 R	ggcttaggtgtcccagact	193	59
17 F	cccagaatctcaagcaac	232	61.5
17 R	ctctgcagtgctgtccac	232	61.5
18 F	cacgtgtccttcccttcc	250	61.5
18 R	ccctcacccttctcaacc	250	61.5
19 F	ctgtctgtgacacctccac	239	61.5
19 R	cgatccctgtgccactct	239	61.5
20 F	tgtccattgtgctctctc	200	61.5
20 R	ctccaggtgacacatgcaac	200	61.5

bp = base pairs; temp. = temperature.

reaction volume containing the PCR buffer, 5 µl of genomic DNA, 1.5 millimolars (mM) of magnesium chloride (MgCl<sub>2</sub>), 0.5 unified atomic mass units (U) of Taq DNA polymerase (Promega Corporation, Madison, Wisconsin, USA), 200 micromolars (µM) of deoxyribonucleotide triphosphates (dNTP) and 0.5 µM of each primer.

The PCR was performed under the following conditions: 95 °C for 5 mins followed by 35 cycles of denaturation at 95 °C for 30 secs, annealing at 58–

63 °C for 30 secs and then extension at 72 °C for 30 secs. A final extension step was carried out at 72 °C for 5 mins. The DNA isolated from the peripheral blood was used as positive controls and ddH<sub>2</sub>O was used as a negative control. The product was checked for specificity and yield using 2% agarose gel stained with ethidium bromide.

The direct sequencing (DS) of the PCR products was performed by a commercial company (Macrogen, Inc., Seoul, Korea). Chromatograms were edited and analysed using the software BioEdit, Version 6.0.7 (Ibis Biosciences, Carlsbad, California, USA).

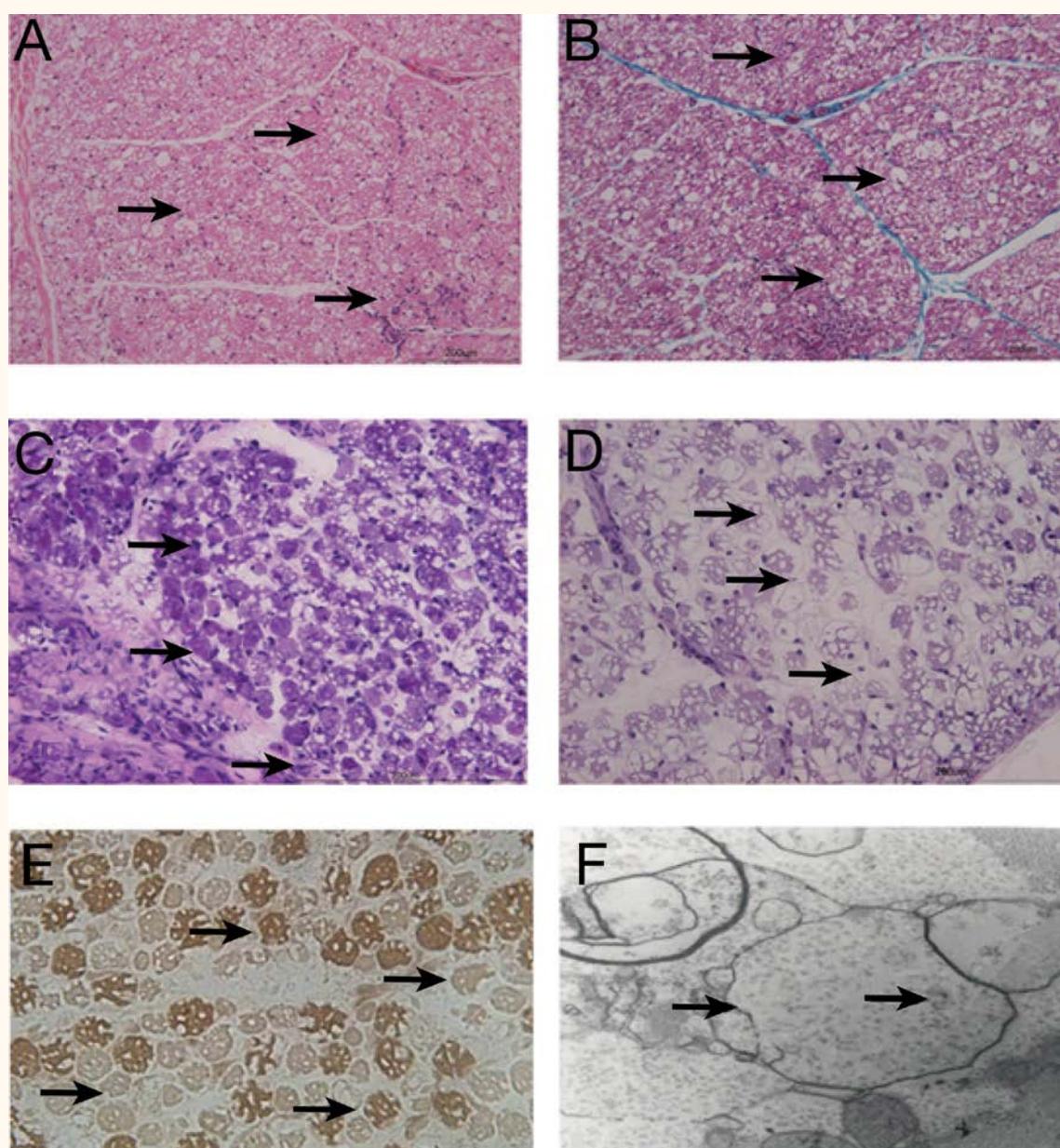
Regarding the restriction fragment polymorphism (RFLP), the mutation at codon 854 in exon 18, c.2560C>T, correlated with a *Bsu36I* restriction site in the T allele. For the restriction fragment length polymorphism (RFLP) reaction, 7 µl of the exon 18 PCR product was digested for 4 hrs with 10 U of the *Bsu36I* gene (New England BioLabs, (UK) Ltd., Hitchin, Herts, UK ) at 37 °C. After the inactivation of the enzyme at 80 °C for 30 mins, the restriction fragments were separated by electrophoresis using a 2.5% agarose gel. The 250 bp PCR product was cleaved into two fragments of 151 and 99 bp in the T allele and uncleaved in the C allele.

The intron 8 splicing site mutation, c.1327-2A>G, correlated with a *SmaI* restriction site in the G allele. For the RFLP reaction, 7 µl of exon 7 and 8 of the PCR product was digested for 4 hrs with 10 U of the *SmaI* (New England BioLabs) at 37 °C. After the inactivation of the enzyme at 80 °C for 30 mins, the restriction fragments were separated by electrophoresis using a 2.5% agarose gel. The 252 bp PCR product was cleaved into two fragments of 162 and 90 bp in the G allele and uncleaved in the A allele.

The project was approved by the Ethics Research Committee of the College of Medicine & Health Sciences at Sultan Qaboos University in Muscat, Oman.

## Results

The examination of the muscle biopsies by light microscopy and electron microscopy for both patients showed vacuolar myopathy with storage of glycogen consistent with Pompe disease [Figures 1 and 2].



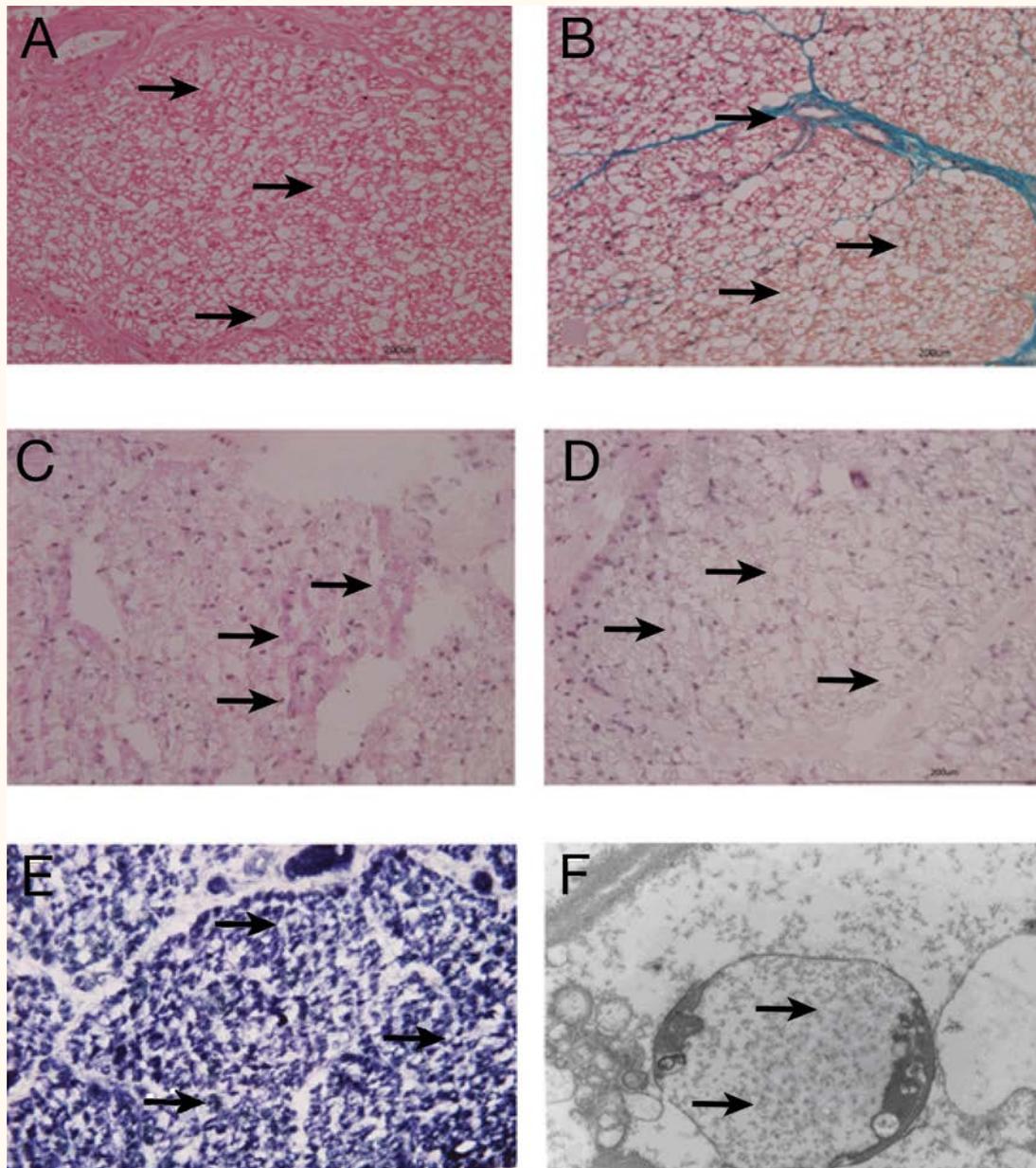
**Figure 1 A to F.** Micrographs of infant 1. **A & B:** Light micrographs showing vacuolated fibres (arrows) stained with haematoxylin and eosin and Masson's trichrome. **C:** Light micrograph showing the rich glycogen accumulation (arrows) with Periodic acid-Schiff (PAS) stain. **D:** Light micrograph showing the digested glycogen with a diastase (arrow) stained with PAS diastase. **E:** Light micrograph showing type I (arrows) and type II (arrows) fibres stained with adenosine triphosphatase (pH = 9.5). **F:** Electron micrograph showing lysosomal vacuoles containing small aggregates of glycogen (arrows).

Scale is 200  $\mu\text{m}$  for A–E and 500 nm for F.

DNA was successfully extracted from the two archived tissues from the patients with Pompe disease using a modified phenol-chloroform extraction method. The checking of the integrity and quality of the DNA by agarose gel electrophoresis and PCR, respectively, indicated a good DNA quality for PCR and post-PCR methods. Two mutations were identified in the Pompe disease samples. Infant 1 (female) was homozygous for mutation

c.2560C>T (p.Arg854X) [Figure 3] and infant 2 (male) was homozygous for mutation c.1327-2A>G [Figure 4]. Neither of the two identified mutations were found in the DNA control sample, nor was the sequence retrieved from the GenBank sequence database (NW\_926918),<sup>12</sup> but both were found in the Pompe Center database.<sup>13</sup>

The PCR and RFLP tests for both mutations were designed to be used as fast confirmatory tests



**Figure 2 A to F.** Micrographs of infant 2. **A & B:** Light micrographs showing the funicular architecture of the tissue; there are many vacuoles of variable sizes within the fibres (arrows) stained with haematoxylin and eosin and Masson's trichrome. **C:** Light micrograph showing the glycogen accumulation (arrows) with Periodic acid-Schiff (PAS) stain. **D:** Light micrograph showing the digested glycogen with a diastase (arrows) stained with PAS diastase. **E:** Light micrograph showing type I and II fibres (arrows) stained with nicotinamide adenine dinucleotide-tetrazolium reductase. **F:** Electron micrograph showing lysosomal vacuoles containing aggregated granules of glycogen (arrows).

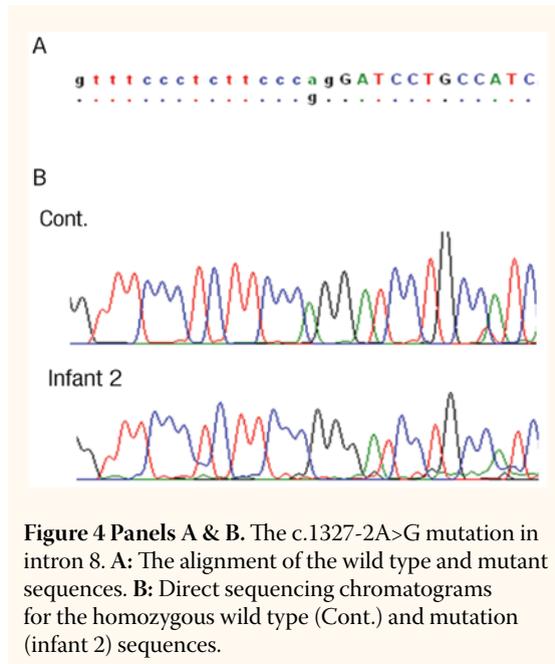
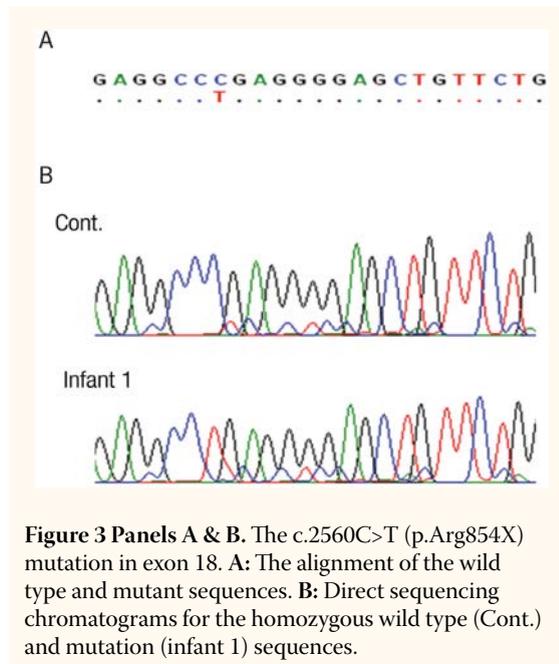
Scale is 200  $\mu\text{m}$  for A–E and 500 nm for F.

for diagnosis and screening. Typical genotyping results are shown in Figures 5 and 6.

## Discussion

Muscle biopsies have always been considered the standard method for diagnosing Pompe disease.<sup>4</sup> However, biopsies are invasive methods requiring anaesthesia, which is not recommended for infants

with Pompe disease. In 2006, enzyme replacement therapy became available and, to ensure the patient's optimal benefit, new rapid and less invasive diagnostic tests were developed.<sup>4</sup> Testing *GAA* in dried blood is one of the methods developed and it is considered to be reliable and suitable for newborn screening, as it only requires a drop of blood to be collected, either from the heel or using the finger stick method.<sup>6</sup> However, the Pompe disease



diagnostic working group emphasised the need for a second confirmatory test to support the clinical and/or biochemical diagnosis due to the absence of developed quality assurance measures.<sup>4</sup>

The detection of Pompe disease-causing mutation(s) is considered a definitive and rapid confirmatory test. It can be utilised in screening at-risk infants and for the identification of carriers, as well as in prenatal diagnosis and the genetic counselling of families with a history of Pompe disease.

In this retrospective study, the pathology archival formalin-fixed paraffin-embedded (FFPE) tissues provided a source of good quality DNA to investigate the molecular bases of Pompe disease in two Omani infants.

Optimisation of the DNA extraction method was very important and the primers for PCR were designed to amplify short fragments (less than 450 bp). The analysis of exons 2–20 of the *GAA* gene using direct sequencing identified two mutations. In infant 1, a c.2560C>T (p.Arg854X) mutation in a CpG dinucleotide, which is susceptible to recurrent mutations, was identified. It is a transition, nonsense mutation at the C-terminal end that is known to be associated with infantile onset Pompe disease.<sup>8</sup> Although found among Pakistani, Mexican-American and French ethnicities, the mutation has been observed in up to 60% of patients of African descent with a common haplotype.<sup>2</sup>

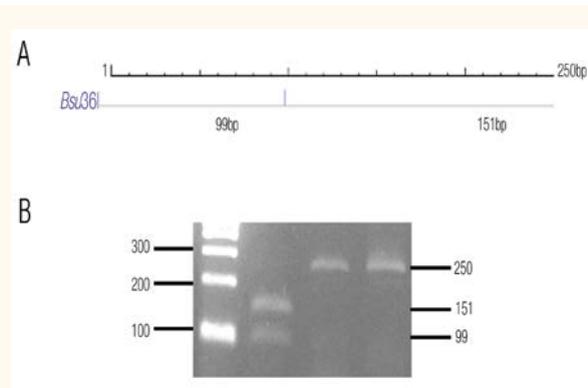
In infant 2, a c.1327-2A>G mutation modifying the splice acceptor site of exon 9 was identified. This mutation is associated with very severe effects, and has been reported in Pompe disease cases from UK, Iran and in patients of Arab origin.<sup>14</sup>

The presence of both mutations as a homozygous genotype is an indicator of parental consanguinity, which has been reported in more Omani patients with inborn errors of metabolism (81%) compared to the general population (33%).<sup>15</sup> Furthermore, consanguineous marriage in Oman (52%) has been found to be dominated by first-cousin marriages (75%).<sup>16</sup>

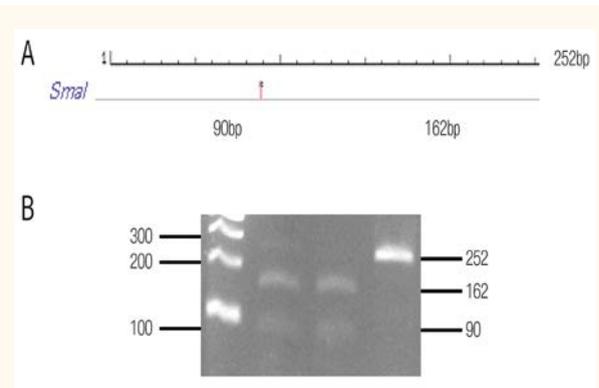
## Conclusion

This study provided valuable information regarding Pompe disease-causing mutations in the *GAA* gene and has enabled the development of specific molecular tests for Omani families. An RFLP test was developed to identify each mutation. The timescale for carrying out the PCR and RFLP test on a routine basis is about 3–8 hrs and is therefore fast compared to other available methods.

To the best of our knowledge, this is the first retrospective study to utilise FFPE tissues in Oman to identify the mutations causing Pompe disease. We were successful in identifying two Pompe disease-causing mutations and developing a rapid specific genetic test that can be used for prenatal



**Figure 5 Panels A & B.** The assay design (A) and results (B) of the polymerase chain reaction restriction fragment length polymorphism analysis for the c.2560C>T (p.Arg854X) mutation in exon 18. A: First, a 250 bp fragment of the GAA gene covering the mutation was amplified by polymerase chain reaction (PCR); next, the PCR product was digested with the Bsu36I restriction enzyme, which recognizes only the T allele, and generated two fragments, 151 bp and 99 bp. B: The electrophoretic separation of the Bsu36I enzymatic restriction products using 2.5% (weight/volume percentage) agarose gel. Lanes: 1 = DNA ladder; 2 = TT homozygote (mutant type) digested product; 3 = CC homozygote (wild type) undigested fragment (250 bp), and 4 = CC homozygote (wild type) undigested fragment (250 bp).



**Figure 6 Panels A & B.** The assay design (A) and results (B) of the polymerase chain reaction restriction fragment length polymorphism analysis for the c.1327-2A>G mutation in intron 8. A: First, a 252 bp fragment of the GAA gene covering the mutation was amplified by polymerase chain reaction (PCR); next, the PCR product was digested with the SmaI restriction enzyme which recognizes only the G allele, and generated two fragments, 162 bp and 90 bp. B: The electrophoretic separation of the SmaI enzymatic restriction products using 2.5% (weight/volume percentage) agarose gel. Lanes: 1 = DNA ladder; 2 = GG homozygote (mutant type) digested product; 3 = GG homozygote (mutant type) digested product, and 4 = AA homozygote (wild type) undigested fragment (252 bp).

diagnosis and carrier screening in Omani families with the identified mutations.

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