DMSc PROFICIENCY TESTING PROGRAM FOR α-THALASSEMIA 1 DIAGNOSIS: 13 YEARS EXPERIENCE IN THAILAND

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Abstract. Precise and accurate molecular diagnosis of α-thalassemia 1 allele is one of the key factors for successful prevention and control of Hb Bart's hydrops fetalis (homozygous α-thalassemia 1) in Thailand. Since 2004, the Department of Medical Sciences (DMSc), Ministry of Public Health has established a DMSc proficiency testing program for molecular diagnosis of α-thalassemia 1. The program evaluates clinical laboratory performance based on pre-established criteria in comparison with other member laboratories. Enrollment in the DMSc program expanded from 13 laboratories in 2004 to 44 by the end of 2016. Four blind DNA samples were tested triennially and each laboratory performance at the end of each test cycle were distributed to all participating laboratories. Overall analytical accuracy (99.5%), analytical sensitivity (99.2%) and analytical specificity (99.7%) indicated excellent performance. Only 11/44 participating laboratories in 39 test cycles failed to correctly genotype the samples. Ten of the eleven laboratories resolved the problem by the end of the following test cycle and all within three cycles. The DMSc proficiency testing program will be expanded to cover other molecular genetic tests, such as β-thalassemia, Down syndrome and cancer biomarkers, to ensure the quality of these tests in clinical laboratories throughout Thailand.

Keywords: genetic test, proficiency testing, thalassemia, Thailand.

INTRODUCTION

α-Thalassemia is one of the most common autosomal recessive genetic disorders, characterized by reduction or absence of α-globin chains due to deletion or (less common) mutation of α-globin genes (Winichagoon *et al*, 1992; Harteveld and Higgs, 2010). The phenotype of individuals with deletional type of α-thalassemia is diverse and depends on the number of α-globin alleles that have been deleted or mutated. The most severe form of α-thalassemia is Hb Bart's hydrops fetalis (homozygous α-thalassemia 1 or homozygous α^o-thalassemia), resulting from a deletion of both duplicated α-globin alleles located on chromosome 16. The affected fetus cannot synthesize α-globin chains leading to severe anemia, asphyxia, hydrops fetalis and ultimately stillbirth or neonatal death. Hydropic pregnancies are frequently associated with complications to the mother, and

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most pregnancies in which the affliction is diagnosed are terminated to avoid risk of maternal morbidity (Galanello and Cao, 2011). Reliable, accurate and precise laboratory diagnosis of α-thalassemia 1 carrier is therefore essential to identify couples at risk of conceiving a fetus with Hb Bart's hydrops fetalis and provide such couples with genetic counseling (Traeger-Synodinos *et al*, 2015).

In Thailand, α-thalassemia 1 is usually caused by deletions that remove part or all of the α-globin gene cluster, the most frequent deletion being SEA type (- -^{SEA}) (deletion of approximately 20.5 kb, involving both functional α-globin genes but leaving ζ2-globin gene intact), followed by Thai type (- -^{Thai}) (deletion of approximately 33.45 kb, involving both α-globin genes and ζ2-globin gene (Winichagoon *et al*, 1984; Sangkitporn *et al*, 2003; Siriratmanawong *et al*, 2007). A number of molecular biology techniques have been employed to diagnose α-thalassemia 1, such as gap PCR, direct DNA sequencing, ARMS PCR, quantitative (q)PCR with SYBR Green 1 dye, qPCR with high-resolution melting analysis and droplet digital PCR (Winichagoon *et al*, 1984; Chong *et al*, 2000; Sangkitporn *et al*, 2003; Siriratmanawong *et al*, 2007; Munkongdee *et al*, 2010; Pornprasert *et al*, 2011; Pornprasert *et al*, 2014). The wide range of laboratory techniques employed highlights the importance of standardization and underpins the use of proficiency testing to ensure the accuracy of α-thalassemia 1 diagnosis conducted by clinical laboratories throughout the country.

Proficiency testing is an important component of clinical laboratory quality assurance. It is defined in ISO/IEC 17043 as "evaluation of participant performance against pre-established criteria by means of inter-laboratory comparisons" (ISO/IEC 17043, 2010). Participation in proficiency testing allows laboratories to recognize analytic and interpretative performance, which may indicate internal problems with the systematic nature of the assays, performance characteristics, internal quality control, instrument calibration, assay design, and/or laboratory interpretation (Miller *et al*, 2011; Kalman *et al*, 2013).

Since 2004, the Department of Medical Sciences (DMSc), Ministry of Public Health, Thailand has established a DMSc proficiency testing program for molecular diagnosis of α-thalassemia 1 (SEA and Thai deletion types). The program evaluates participant performance using pre-established criteria through inter-laboratory comparisons. At the beginning, the program followed ISO/IEC Guide 43-1: 1997, and in 2010 the program employed ISO/IEC 17043: 2010 guidelines. Reliability of each participating laboratory is monitored from a Proficiency Testing report, which contains the important indicators in the laboratory's quality assurance. This communication summarizes participating laboratory performances in α-thalassemia 1 diagnosis from 2004 to 2016.

MATERIALS AND METHODS

Organization of DMSc alpha-thalassemia 1 proficiency testing program

The DMSc α-thalassemia 1 proficiency testing program is conducted three times each year. Participation is open to both public and private clinical laboratories. The program is strictly anonymous; laboratories receive a unique ID code from DMSc, which is known only to the laboratory and authorized DMSc personnel.

Preparation of DMSc α-thalassemia 1 test panel

The DMSc α-thalassemia 1 test panel consists of four validated samples of

genomic DNA with at least one α-thalassemia 1 SEA or Thai type-positive DNA sample (the latter included from test cycle 2-2011 onwards). The validity of all DMSc α-thalassemia 1 sample panels were tested by an ISO 15189 certified reference laboratory, using qPCR (DMSc α-thal 1 kit, DMSc, MOPH, Thailand) and subsequently confirmed by direct DNA sequencing. The DMSc α-thalassemia 1 sample panels are shipped in foam boxes with absorbent and dry ice. Fact sheets and report forms are attached with the material, which is sent via an express mail.

Prior to the distribution to participants, an assessment of proper preparation of the sample panels was performed after the samples have been packaged as follows. Ten vials were taken randomly from the packages using an Excel-based random number generator and subsequently tested in triplicate by qPCR (DMSc α-thal 1 kit, DMSc, MOPH, Thailand). For stability assurance, samples were further stored at three different temperatures (-20°C, 4°C and 25°C) and tested at two different periods of time between the moment the laboratories received the test samples and the deadline for sending of results.

Reporting of results and data analysis

Participating laboratories are asked to test samples using their routine protocols and to provide results of genotyping and interpretation within 30 days from receipt of shipment. Laboratory results were evaluated based on concordance of their results with the expected results performed by three reference laboratories, which have been invited based on recommendation from national thalassemia laboratory consultants, including their experience in alpha-thalassemia 1 diagnosis for more than 10 years and accreditation to a recognized international standard ISO 15189. Each sample scored 1 point if reported correctly. Laboratories that scored ≤ 3 (≤ 75% correct) are contacted by e-mail or phone to discuss potential sources of errors. Proficiency testing results are returned to the participating laboratories via express mail within four weeks of receipt.

Normal distributed data are expressed as mean, standard deviation (SD) and coefficient of variation (CV). Qualitative analysis is expressed as positive and negative for α-thalassemia 1 (SEA or Thai deletion type). These binary results were analyzed for accuracy, sensitivity and specificity calculated using the following equations (Fumière *et al*, 2015):

- accuracy (fraction of correct positive and negative results):

$$\frac{(PA+NA) \times 100}{PA+ND+PD+NA}$$

- sensitivity (ability to classifying positive results as positive):

$$\frac{PA \times 100}{PA+ND}$$

- specificity (ability to classifying negative results as negative):

$$\frac{NA \times 100}{PD+NA}$$

where PA is positive agreement, NA negative agreement, PD positive deviation, and ND negative deviation.

Survey of customer satisfaction

A customer survey was carried out to determine customer satisfaction with DMSc α-thalassemia 1 proficiency testing program and to ascertain the needs of participating laboratories. The survey, conducted over a six-week period in 2016, consisted of six questions regarding proficiency testing scheme, sample quality and

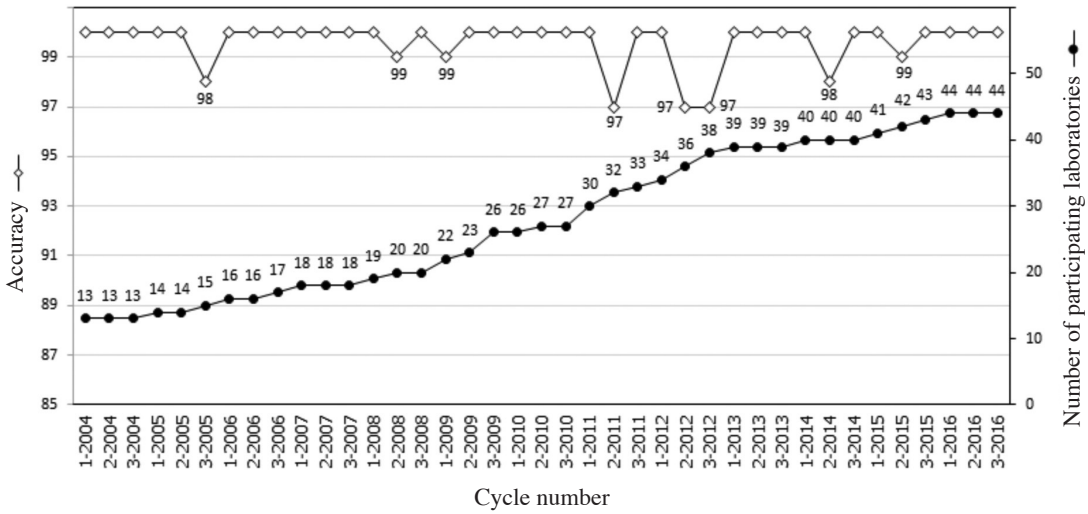

Fig 1–Number of laboratories participating in DMSc α-Thalassemia 1 Proficiency Testing Program, and mean accuracy of each test cycle from 2004 to 2016.

quantity, sample packaging and shipping, proficiency testing frequency, report and communication. Respondents were asked to assign a value to each reply using a five-point Likert scale (Likert, 1932; Vasikaran *et al*, 2016). Score of 1-5 corresponds to poor, unsatisfactory, satisfactory, good and very good, respectively. An option for "not applicable/do not know" was also included.

RESULTS

Since the implementation of the DMSc α-thalassemia 1 proficiency testing program, 39 test cycles have been conducted, with 1,093 panels (4,372 samples) prepared and distributed to participating laboratories. After packaging, 10 vials from the package lot were randomly selected for assurance test. All positive samples were tested positive, the same with negative samples. All samples tested were stable at -20°C, 4°C and 25°C over the period of proficiency testing cycle.

The number of participating laboratories increased from 13 in 2004 to 44 in 2016 (Fig 1). The distribution of participating laboratories in 2016 was as follows: 12 (27%) university hospitals, 24 (55%) Ministry of Public Health hospitals/laboratories and 8 (18%) private laboratories. The most frequently used method was in-house multiplex gap PCR (43%) followed by DMSc α-thal qPCR test kit (39%) and in-house qPCR (18%).

Over a period of 13 years, 96-100% of participating laboratories returned the proficiency testing results after each test cycle, with 75% (33/44) of the laboratories scoring 100% in all test cycles in which they participated. Eleven (25% laboratories had at least one error and only one (2%) laboratory had errors in 2 test cycles. Genotyping error observed in cycle 3-2005, 2-2008, 1-2009, 2-2011, 2-2012, 3-2012, 2-2014, and 2-2015 was 2%, 1%, 1%, 3%, 3%, 3%, 2%, and 1%, respectively (Fig 1). Ten of the eleven laboratories resolved the problem(s) by the end of the following test cycle and all within 3 cycles.

Two education programs were conducted during September-December, 2011 and again during January-April, 2016.

Table 1
Classification of problems identified among unacceptable proficiency testing results.

Problem	Source of problem	Number (%)
Clerical error	Incorrect transcribed proficiency test results to report form.	3 (19%)
	Proficiency test sample mislabeled in the laboratory.	1 (6%)
Methodological problem	Laboratory method unable detect the rare Thai deletion type.	2 (13%)
Equipment problem	Incorrect instrument setting.	2 (13%)
	Equipment malfunction.	1 (6%)
Technical problem caused by personnel error	Staff with inadequate training.	2 (13%)
	Incorrect preparation of reagents.	1 (6%)
	Misinterpretation of test result.	4 (25%)

Table 2
Sensitivity, specificity and accuracy of results of DMSc α-Thalassemia 1 Proficiency Testing Program, 2004-2016.

Number of samples	True positive	False negative	Sensitivity (%)	True negative	False positive	Specificity (%)	Accuracy (%)
4,220	1,856	15	99.2	2,342	7	99.7	99.5

Problems identified when investigating unacceptable proficiency testing results were mainly misinterpretation of test results and clerical errors (Table 1). Overall sensitivity, specificity and accuracy was 99.2%, 99.7% and 99.5%, respectively (Table 2).

Customer satisfaction survey was sent to 44 laboratories participating in cycle 3-2016, with 38 (86%) laboratories responding. Scores were obtained from a five-point Likert scale for 6 attributes. The overall satisfaction scores ranged from 4.3-4.6, with a mean score of 4.4. Respondents indicated the highest level of satisfaction with the proficiency testing frequency, followed by proficiency testing report form, proficiency testing scheme, sample quality and quantity, sample packaging and shipping, and communication.

DISCUSSION

Recent advances in life sciences technologies and increased understanding of the molecular mechanisms underlying health and disease have driven an expansion of the use of molecular diagnostics and have led to an increased role of clinical genetic testing of patients (Kalman *et al*, 2013; Jain, 2009). Currently, more than 1,800 disease-associated genes are identified, and more than 2,000 genetic tests have become available, together with at least 350 biotechnology-based products available in the market (Durmaz *et al*, 2015). The rise in demand for molecular genetic testing requires rigorous quality assurance processes, including proficiency testing or external quality assurance to ensure continued quality of these tests (Ravine and Suthers, 2012).

In Thailand, α- and β-thalassemias are the most common genetic disorders (Fucharoen and Winichagoon, 2011), and accurate and precise molecular diagnosis of carriers of these conditions, together with reliable interpretations, are essential in prevention and control programs of the severe forms of the thalassemias. The DMSc proficiency testing program for molecular diagnosis of α-thalassemia 1 (SEA and Thai deletion types) is the first proficiency testing of a molecular genetic test in Thailand.

The error rate in genetic detection of cystic fibrosis, thrombophilia and hemochromatosis is 1.3-3.8%, 1-3% and 0-8.7%, respectively (Dequeker and Cassiman, 2000; Hertzberg *et al*, 2005; Hertzberg *et al*, 2006; Tosto *et al*, 2009), comparable with the results from this study. The majority (91%) of the problems encountered were resolved by at least the following test cycle once the source(s) of error were identified. It is imperative that all in-house technologies used for molecular diagnosis be validated to ensure that they meet acceptable performance standards and are suitable for the purpose to which they are used (Mattocks *et al*, 2010).

As a result of relatively poor laboratory performance in cycle 2-2011, the education program highlighting analytic and methodological skills were subsequently offered to the participating laboratories. A marked improvement was demonstrated in the next cycle (3-2011) with analytic accuracy of 100%. The education program was conducted again at the beginning of 2016. During the year no genotyping errors were performed by participating laboratories, a gratifying outcome reflecting the success of the scheme and representing an improvement in the quality of α-thalassemia 1 diagnosis in Thailand.

For participating laboratories that found results incompatible with others, corrective actions should be adopted for the improvement of the protocols. Occurrence of unacceptible performance should be used as an opportunity to review procedures and to make the necessary improvements. A careful evaluation, including the receipt of materials and its storage, the evaluation of all steps of the analytical procedure, instrument calibration, adjustments to training procedures, and the fulfilling of report form is important for the identification of critical points and improvement of laboratory performance (Miller *et al*, 2011; Kalman *et al*, 2013).

Although proficiency testing showed satisfactory results, there is still room for improvement. In order to have the greatest value, proficiency testing should evaluate performance in all stages of the testing process, including pre-analysis, analysis, and post-analysis. Proficiency testing samples should be clinical specimens so that they would most closely represent samples actually tested in a clinical laboratory (Kalman *et al*, 2013). In addition, for the analytical phase evaluation, proficiency testing should include a sufficient number of analytes to provide a reasonable estimation of inter-laboratory comparison. However, it is often difficult to obtain samples with rare mutations, such as α-thalassemia 1 Thai deletion type. This limitation could be overcome by spiking a normal sample with PCR-amplified mutant DNA. This procedure has proven suitable for quality control and proficiency testing of cystic fibrosis, Tay Sachs disease, Canavan disease, familial dysautonomia, mucolipidosis IV, Niemann-Pick disease type A, Fanconi anemia type C, Bloom syndrome, Gaucher disease, and glycogen storage disease (Christensen *et al*, 2007; Kalman *et al*, 2009).

For post-analysis phase evaluation,

participants should be required to submit results in their usual clinical reporting format. This allows evaluation of the interpretation of a laboratory's results and other important elements in the laboratory report, such as name of the laboratory, title of analysis, identification number, arrival date of sample, type of specimen, method used, list of mutations tested, analysis sensitivity and specificity, detection rate, description of the genotype, participation in PT/ EQA, reporting date and signature (Tosto *et al*, 2009).

In summary, this study shows that the DMSc proficiency testing program for α-thalassemia 1 (SEA and Thai deletion types) is an important tool for assessing laboratory competence, evaluating laboratory testing process and improving laboratory performance. The results indicate excellent performance of the participating laboratories. In the near future, the DMSc proficiency testing program will be expanded to cover other molecular genetic tests, such as β-thalassemia, Down syndrome and cancer biomarkers, to ensure the quality of these molecular genetic tests in clinical laboratories in Thailand.

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