COST-EFFECTIVENESS IN ESTABLISHING HEMOPHILIA CARRIER DETECTION AND PRENATAL DIAGNOSIS SERVICES IN A DEVELOPING COUNTRY WITH LIMITED HEALTH RESOURCES

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Abstract. The cost-effectiveness of carrier detection and prenatal diagnosis for hemophilia at the International Hemophilia Training Center, Bangkok, Thailand was studied. From 1991 to 2002, 209 females from 124 families with hemophilia A and B were included. There were 180 hemophilia A carriers and 29 hemophilia B carriers which could be classified into 78 obligate and 131 possible carriers. The phenotypic analysis for possible carriers involved the determination of levels of factor VIII or IX clotting activity (FVIII:C, FIX:C) and the ratio of FVIII:C and von Willebrand factor antigen. The result revealed that 49 females (37.4%) were diagnosed as carriers, 65 females (49.6%) were normal and 17 females (13%) were undetermined. Additional genotypic analysis was provided to 46 families with 74 females with obligate, proven or undetermined carriers within the reproductive life. The polymorphisms associated with factor VIII and IX genes were used including *Bcl* I for the factor VIII gene and combined use of *Mse* I, *Sal* I, *Nru* I, *Hha* I and *Dde* I for the factor IX gene. The informative rate was 59.4% (44/74). Consequently, 12 prenatal diagnoses for fetus at risk were performed. Sex determination was initially determined and followed by the diagnosis of hemophilia through informative gene tracking and/or the measurement of fetal levels of FVIII:C or FIX:C. The result revealed that 3 male fetuses were affected. The total cost of carrier detection and prenatal diagnosis that the families had to pay in the government hospital was 238,600 Baht (US$ 5,965). It was compared to the estimated cost of minimal replacement therapy using lyophilized cryoprecipitate for the survival time of 30 years in one patient with hemophilia of 1,012,500 Baht (US$ 25,312.5). The cost of prevention was much less than the replacement therapy. In conclusion, it is cost-effective to establish the service for carrier detection and prenatal diagnosis for hemophilia especially in developing countries with limited health resources.

INTRODUCTION

Hemophilia is a serious bleeding disorder found in 1 out of 10,000 in the population in both developed and developing countries (Kasper *et al*, 1992). Most of the hemophiliacs in developing countries are in the pediatric age group, they seldom reach adulthood because of a lack of adequate replacement therapy (Evatt and Robillard, 2000). A previous study in Thailand reported that patients with presumptive diagnosis of hemophilia by history, who did not receive replacement therapy, succumbed to bleeding complications at the mean age of 8 years and 9 months. The median survival times of patients with severe and moderate degrees of hemophilia receiving treatment at the comprehensive hemophilia center in Thailand were 35 and 38 years, respectively (Chuansumrit *et al*, 2000). Still, they are much lower than those in developed countries.

The prevention of new cases of hemophilia is essential in developing countries with limited health resources. Accurate carrier detection analy-

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sis should be established at the comprehensive care level either by phenotypic or genotypic analysis, while prenatal diagnosis should be set-up in the reference center for hemostatic disorders (Isarangkura, 2002).

The present study reports the cost-effectiveness of establishing a service for carrier detection and prenatal diagnosis of hemophilia at the International Hemophilia Training Center, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand.

MATERIALS AND METHODS

Subjects

From 1991 to 2002, 209 females from 124 families with hemophilia were included. There were 180 hemophilia A carriers and 29 hemophilia B carriers who were classified into 78 obligate and 131 possible carriers. Obligate carrier status was defined by pedigree tracking without the need of laboratory confirmation. They included females with hemophiliac fathers, with two hemophiliac sons, and with one hemophiliac son combined with one brother or uncle on the maternal side with hemophilia. Possible carriers included those females with an obligate carrier mother, with one hemophilia son and those with a family history of hemophilia alone. The possible carriers required laboratory confirmation either by phenotypic or genotypic analysis.

Additional genotypic analysis was performed in 46 families with 74 females who were obligate, proven or undetermined carriers within the reproductive life. They included mothers, sisters and maternal aunts of the hemophiliac patients. The prenatal diagnosis was offered to females with obligate and proven carriers who risked having offspring with a severe or moderate degree of hemophilia. The prenatal diagnosis was performed by chrorionic villi sampling at 10-12 weeks of gestation and/or fetal blood sampling at 16-22 weeks of gestation.

Phenotypic analysis of hemophilia carrier

Two blood samples, taken one week apart, were drawn from females at risk, who denied ingestion of oral contraceptives or pregnancy, into 1/10 volume 3.2% sodium citrate by a two-syringe technique. The samples were immediately put on ice and centrifuged at 3,000 rpm for 15 minutes to obtain platelet poor plasma which was stored at -70°C before testing. The levels of factor VIII clotting activity (FVIII:C) and von Willebrand factor antigen (vWF:Ag) were determined in females at risk for being hemophilia A carriers while the levels of factor IX clotting activity (FIX:C) were determined in females at risk for being hemophilia B carriers. The cut-off level of FVIII:C or FIX:C for determining hemophilia A or B carriers was less than 50%. Additionally, the cut-off ratio of FVIII:C to vWF:Ag for determining hemophilia A carriers was 0.6 (Pintadit *et al*, 2000).

The levels of FVIII:C and FIX:C were determined by the one-stage method based on the partial thromboplastin time (Hardistry and Macpherson, 1962; Pitney, 1975) with factor VIII or IX deficient plasma as substrate. The hemophiliac patients, whose FVIII:C or FIX:C was less than 1%, were used as the substrate. vWF:Ag was measured by ELISA technique using rabbit polyclonal anti-vWF antibody and peroxidase conjugated immunoglobulin to vWF obtained from Dako, Denmark. FVIII:C, FIX:C and vWF:Ag were expressed as percent activity in relationship to normal plasma. A 10-donor plasma pool, kept at -70°C was used as the normal plasma.

Genotypic analysis for hemophilia carrier

DNA was extracted from 10 ml of EDTA anticoagulant treated whole blood using proteinase-K digestion followed by phenol/chloroform extraction (Kunkel *et al*, 1977). The polymorphisms associated with the factor VIII and IX genes were used including *Bcl* I for the factor VIII gene and combined use of *Mse* I, *Sal* I, *Nru* I, *Hha* I and *Dde* I for the factor IX gene. Amplification of factor VIII and IX sequences for analysis of polymorphisms (Saiki *et al*, 1988; Kogan and Gitschier, 1990) is briefly described. Using the published primers (Table 1) 250 ng of genomic DNA were amplified in a 50 μl total volume with 200 μM dNTPs (Pharmacia) and 1 unit Taq polymerase (Perkin-Elmer). Following an initial denaturation at 94°C for 5 minutes, thermocycling at 94°C for 1 minute, 55°C-60°C for 1 minute and 72°C for 2 minutes was carried out for a total

of 30 cycles. Following amplification, 15 μl of amplified product was digested with the appropriate restriction enzymes (*Bcl* I, *Mse* I, *Sal* I, *Nru* I, *Hha* I supplied by New England, Biolabs) according to the manufacturer's instruction and then size fractionated by gel electrophoresis on 6-12% native polyacrylamide gel. *Dde* I amplified products did not require restriction enzyme digestion prior to analysis as the polymorphism is due to a 50 bp insertion/deletion. Products were fractionated as described above.

Prenatal diagnosis for hemophilia

Sex determination. The sex determination was initially performed by direct chromosome preparation from chorionic villi sampling or short term culture from lymphocytes (Kangwanpong, 1992) between 1994 and 1996. Starting in 1997, the DNA amplification for the X and/or Y chromosome by using primers, 5′-CTCTGATGGTT GGCCTCAAG-3′ and 5′ ACCTTGCTCAT ATTATACTTGACAAAG-3′ from David Bailey (personal communication) was as follows: 500 ng of genomic DNA was amplified in 50 μl reaction volume consisting of 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin; 200 μM dNTPs (Pharmacia); 150 ng of each primer and 1 unit Taq polymerase (Perkin-Elmer). Following an initial denaturation at 94°C for 7 minutes, thermocycling at 94°C for 1 minute, 61°C for 1 minute and 72°C for 1 minute was carried out for a total of 30 cycles. The approximate product sizes are 500 bp from the X and 350 bp from the Y chromosome.

Genotypic analysis. The transcervical chorionic villi sampling under ultrasound guidance was performed at 10-12 week gestation. The samples were checked microscopically to exclude contamination with maternal decidua before being resuspended in 0.15 mM NaCl, 0.25 mM EDTA, 0.5% sodium dodecyl sulfate. This was followed by proteinase-K digestion and phenol/chloroform extraction. Then, the DNA was precipitated with ethanol and reconstituted in water. Alternatively, a small piece of chorionic villi was washed by TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.9) and boiled in 40 μl water for 15 minutes. Following centrifugation, 3 μl of supernatant was used in DNA amplification (Goodeve *et al*, 1998).

Phenotypic analysis. The trans-abdominal fetal blood sampling from the cord insertion under ultrasound guidance was performed at 16-22 weeks of gestation. The purity of the fetal blood was checked by staining with Amido black. The actual amount of 0.5 ml fetal blood was mixed with 0.2 ml of anticoagulant of 3.2% sodium citrate. The levels of FVIII:C or FIX:C was assayed as described above. Since the ratio of fetal blood and anticoagulant was 2.5:1, the levels of fetal FVIII:C or FIX:C were corrected accordingly to the ratio of whole blood and anticoagulant of 9:1.

Purity of fetal blood. The purity of fetal blood was determined by staining with Amido black (Betke and Sanguansermsri, 1972). A 30-minute completely air-dried blood smear was fixed in 80% ethyl alcohol. The slide was stained for 3 minutes by Amido black dissolved in 80% ethyl alcohol with a pH of 2 by 1 N HCl. Subsequently, the slide was rinsed in tap water for 1 minute and air-dried. Red cells containing HbF were presented as dark blue cells and those containing no HbF were presented as ghost cells.

RESULTS

The phenotypic analysis was performed among 131 females with possible carriers. The number of females at risk for hemophilia A and B carriers was 117 and 14, respectively. The results revealed 49 females (37.4%) were diagnosed as carriers (45 hemophilia A carriers and 4 hemophilia B carriers) and 65 females (49.6%) were diagnosed as normal. However, 17 females (13%) were undetermined as carriers or normal. Additional genotypic analysis was performed among 46 families (41 hemophilia A, 5 hemophilia B) among 74 females at risk (50 possible carriers, 24 obligate carriers) within the reproductive life. The polymorphisms associated with factor VIII and IX genes of restriction fragment length polymorphisms were used for the genotypic analysis.

The combination of genotypic and phenotypic analysis among 50 females with possible carriers is shown in Table 2. The informative rate

Table 1
Primers used for the study of polymorphisms associated with factor VIII and IX genes.

Site	Restriction enzyme		Primers Reference
Intron 18	*Bcl* I	5′ TAAAAGCTTTAAATGGTCTAGGC 3′ 5′ TTCGAATTCTGAAATTATCTTGTTC 3′	Peake *et al* (1993)
5′ flanking region	*Mse* I	5′ GATAGAGAAACTGGAAGTAGACCC 3′ 5′ TTAGGTCTTTCACAGAGTAGATTT 3′	Peake *et al* (1993)
5′ flanking region	*Sal* I	5′ CTCGTTGTGCACATGTACCC 3′ 5′ CAATACCACCCTATCCTTCGTCGA 3′	Toyozumi *et al* (1995)
Intron 1	*Nru* I	5′ ACTGCCCATTCTCTTCACTTGT 3′ 5′ CTGTCAAATCATGTAATCAAAATTTCG 3′	Toyozumi *et al* (1995)
3′ flanking region	*Hha* I	5′ ACAGGCACCTGCCATCACTT 3′ 5′ AGATTTCAAGCTACCAACAT 3′	Peake *et al* (1993)
Intron 1	*Dde* I	5′ GGGACCACTGTGGTATAATGTGG 3′ 5′ CTGGAGGATAGATGTCTCTATCTG 3′	Peake *et al* (1993)

Table 2
Combined phenotypic and genotypic analysis among 50 females with possible carriers.

	Status of females				Total (%)
	Hemophilia A carrier	Hemophilia B carrier	Normal	Undetermined	
Non-informative genotypic analysis	11[a]	1[a]	8[a]	2[a]	22 (44)
Same phenotypic and genotypic analysis	15	1	7	0	23 (46)
Normal phenotypic analysis but diagnosed as carrier by genotypic analysis	3	1	0	0	4 (8)
Undetermined phenotypic analysis but diagnosed as carrier by genotypic analysis	1	0	0	0	1 (2)
Total					50

[a]defined by only phenotypic analysis.

was 56% (28/50) while the remainder was non-informative. The genotypic analysis was helpful in the additional diagnosis of 4 hemophilia A carriers and 1 hemophilia B carrier. The genotypic analysis was also performed among 24 female obligate carriers. The informative rate was 66% (16/24) while the remainder was non-informative. Therefore, the overall informative rate among the studied carriers was 59.4% (44/74).

Twelve prenatal diagnoses for fetus at risk of hemophilia were performed on 9 females as shown in Table 3. The fetal sex was simultaneously determined with the diagnosis of hemophilia by an informative genetic marker. In case of non-informativeness, the measurement of fetal levels of FVIII:C or FIX:C was performed. Moreover, one female (No.3), who possessed a female offspring with a carrier state, requested to have amniocentesis for the exact number of chromosomes to exclude Turner's syndrome of 45, XO. The prenatal diagnosis of a hemophilia carrier status for the female offspring was not always required. Unfortunately, one female (No.5), who refused the determination of hemophilia in her male fetus, was affected by hemophilia. There was no fetus loss induced by the obstetric procedures.

The cost of phenotypic and genotypic analysis for females and family members for determining the carrier status and searching for genetic informative markers is shown in Table 4. Additionally, the cost of prenatal diagnosis was calcu-

Table 3
The prenatal diagnosis of hemophilia during 1994 to 2002.

No.	Date	Type of carrier	Risk to hemophilia	Chorionic villi sampling	Fetal blood sampling	Sex determination	Determination of hemophilia	
							Method	Result
1	30 Sep 1994	Obligate	A	✓	-	46 XX[a]	RFLP *Bcl* I	Carrier
2	26 Apr 1996	Proven	A	-	✓	46 XY[b]	FVIII:C 73%	Normal male
3	22 Apr 1997	Proven	A	✓	-	X	RFLP *Bcl* I	Carrier
4[c]	17 Feb 1998	Proven	A	✓	-	XY	RFLP *Bcl* I	Normal male
5	30 Mar 1998	Proven	A	✓	-	XY	-	Hemophilia[d]
6[c]	18 Jan 2000	Proven	A	✓	-	XY	RFLP *Bcl* I	Hemophilia
7	18 Apr 2000	Proven	A	✓	-	X	-	Female[f]
8	18 Feb 2000	Proven	A	-	-	Female[e]	-	Female[f]
9	31 Aug 2000	Obligate	B	-	✓	XY	RFLP *Hha* I FIX:C 21%	Normal male
10	28 Nov 2000	Obligate	A	-	✓	XY	FVIII: 67%	Normal male
11	20 Nov 2001	Obligate	A	✓	-	X	RFLP *Bcl* I	Carrier
12[c]	3 Dec 2002	Proven	A	✓	-	XY	RFLP *Bcl* I	Hemophilia

[a]Direct chromosome preparation, [b]short term culture of lymphocyte, [c]the same female, [d]the mother refused to have prenatal diagnosis of hemophilia, and unfortunately the male offspring was affected with hemophilia, [e]sex determination by ultrasound, [f]the status of normal or female was not determined postnatally.

Table 4
The cost of phenotypic and genotypic analysis for females at risk and prenatal diagnosis among 12 pregnancies.

	Number	Cost per case		Total cost	
		Baht	US$	Baht	US$
Phenotypic analysis					
Hemophilia A carrier	117	400	10	46,800	1,170
Hemophilia B carrier	14	200	5	2,800	70
Genotypic analysis					
Hemophilia A family	41	3,000	75	123,000	3,075
Hemophilia B family	5	6,000	150	30,000	750
Prenatal diagnosis					
Female at risk	12	3,000	75	36,000	900
Total				238,600	5,965

Table 5
The estimated cost of minimal replacement therapy with lyophilized cryoprecipitate (LC) in one patient with hemophilia A.

Age (years)	Bleeding episode/year	Number of utilized LC[a]/year	Total	
			Baht	US$
1-10	10	40	200,000	5,000
11-15	10	100	200,000	6,250
15-30	5	75	562,500	14,062.5
Total			1,012,500	25,312.5

[a]Each bottle of lyophilized cryoprecipitate (LC) costs 500 Baht (US $ 12.5).

lated. The total cost of carrier detection and prenatal diagnosis that the families had to pay in the government hospitals was US$ 5,965. The estimated cost of minimal replacement therapy on demand of bleeding episodes for the survival time of 30 years in one patient with hemophilia is shown in Table 5. The replacement therapy was based upon the lyophilized cryoprecipitate which costs US$ 12.5 per bottle. It is heat-treated at 60°C for 72 hours, which is produced by the National Blood Center, Thai Red Cross Society. The cost of replacement therapy for only one patient with hemophilia A was 4 times that for carrier detection and prenatal diagnosis. Actually, there were three pregnancies with affected fetuses with hemophilia A among 209 females. Therefore, the cost of carrier detection and prenatal diagnosis was much less than replacement therapy for patients with hemophilia.

DISCUSSION

The effective prevention of hemophilia, especially in developing countries, is through accurate carrier detection and prenatal diagnosis. The facilities of medical personnel, equipment and reagents can be shared with other available services. For example, the prevalence of thalassemia and hemoglobinopathy traits is 40% of the Thai population. The prenatal diagnosis for females at risk of having offspring with thalassemia has already been established in University Hospitals in Thailand. Therefore, these facilities can be shared for the prenatal diagnosis of hemophilia.

The initial step is to identify obligate carriers and to refer possible carriers for laboratory testing. The cost of assaying FVIII:C, FIX:C and vWF:Ag for the phenotypic analysis of carriers can be decreased by using 'in-house' preparation of reagents such as factor VIII deficient plasma, factor IX deficient plasma and normal plasma. Their cost is much less than for commercial reagents. Factor VIII deficient and factor IX deficient plasma can be drawn from hemophiliac patients with severe degree (FVIII:C or FIX:C <1%) and without inhibitor. Normal plasma can be prepared from 10-20 healthy volunteers who forego ingestion of any medication for at least one week before blood drawing. Males and females were included equally. The ELISA for vWF:Ag can be prepared by an 'in house' method. The rabbit polyclonal anti-vWF antibody can be purchased from pharmaceutical companies. The 'in-house' ELISA is less expensive than commercial kits. By the means of modification, the phenotypic analysis for hemophiliac carriers can be established for service at an affordable price for most families.

Phenotypic analysis for determining the carrier status of hemophilia A and B is limited. Two subsequent blood testings are required. Approximately 10-20% of obligate carriers had levels of FVIII:C or FIX:C less than 50% (Pintadit *et al*, 1996; Rurgkhum *et al*, 2002). The addition of vWF:Ag to calculate the ratio of FVIII:C and vWF:Ag markedly improved the discrimination between carrier and non-carrier status. Ten to twenty percent of females still could not be diagnosed as carriers or normal. A more accurate tool of genotypic analysis is required. Genotypic analysis is provided to obligate, proven and undetermined carriers during reproductive life only.

Genotypic analysis using polymorphism analysis is an effective procedure with an accuracy rate of greater than 99% if there is an informative marker. The allelic frequencies of DNA polymorphisms associated with factor VIII and IX genes in the Thai population are lower than those of Caucasians (Goodeve *et al*, 1994; Sasanakul *et al*, 2000). Therefore, the genotypic analysis is limited by non-informativeness. Moreover, an affected individual with hemophilia must be included in the study. The important females (for example, mothers) within the family must be heterozygous (informative) for the particular DNA polymorphism. Then, the informative marker can be used for tracking the carrier status among females at risk and for prenatal diagnosis of hemophilia in that family. DNA polymorphism analysis is of limited use in a family with no history of hemophilia (sporadic case) and carrier status can only be excluded if the female does not possess the same polymorphic site as the hemophiliac patient.

Sex determination of a fetus at risk is the initial step for the prenatal diagnosis of hemophilia since the male fetus is mainly affected. Sex determination has developed from direct chromo-

some preparation requiring 25 mg of chorionic villi sample and the short term culture of lymphocytes requiring 0.5 ml of fetal blood to the DNA amplification technique requiring the minute amount of 5 mg of chorionic villi sample. The presence of the X chromosome by the DNA amplification technique will only be compatible with a female fetus but cannot rule out the status of 45, XO, who could be a female hemophiliac if she receives the maternal hemophilia X chromosome. Although the chance of a female hemophiliac with Turner's syndrome is rare, it is still a possible risk. Therefore, an additional long-term culture of amniocytes from amniocentesis at 16-18 weeks of gestation for determining the exact number of chromosomes is suggested for a female fetus with carrier status.

The present study reports 12 prenatal diagnoses among 9 females. The prenatal diagnosis was accurately performed using the informative markers of *Bcl* I and *Hha* I in eight pregnancies while the factor assay was performed in two pregnancies without informative markers. The diagnosis of hemophilia was not performed in three pregnancies with female fetuses and another with a male fetus. The female at risk with a male fetus refused to have further investigation, preferring to take the 50% chance of a normal son. Unfortunately, the male fetus was affected by hemophilia. This reflects that prenatal diagnosis is not completely accepted among couples at risk since the termination of pregnancy with an affected male fetus is heart-breaking for the parents.

In cases of non-informative genotypic analysis, the measurement of levels of FVIII:C or FIX:C in the fetal blood is an alternative to prenatal diagnosis. The advanced technology of ultrasound allows the pure fetal blood obtained from the cord insertion. An obstetrician skilled in this procedure is essential for success. The risk of fetal loss is 1-2% from fetal blood sampling and 2-3% from chorionic villi sampling (Daffos *et al*, 1983; Hogge *et al*, 1986). In the present study, no fetal loss occurred from the 11 pregnancies who received obstetric procedures of chorionic villi sampling and/or fetal blood sampling.

The establishment of service for carrier detection and prenatal diagnosis for hemophilia in developing countries with limited health resources is cost-effective. It should be adopted by other developing countries where hemophilia treatment is inadequate. The utilized budget for prevention is much less than the budget for replacement therapy. In the present study, the replacement therapy of locally prepared lyophilized cryoprecipitate is estimated from the treatment on demand of bleeding episodes. If the commercial factor concentrates are used, the cost will be tremendously more than those used for prevention.

In conclusion, prevention of new cases with hemophilia should be emphasized among females at risk. The initial step is to identify the obligate carriers and to refer the possible carrier for phenotypic analysis. The additional genotypic analysis is provided to obligate, proven and undetermined carriers within the reproductive life. Then, prenatal diagnosis by either tracking the informative polymorphisms or measuring the levels of FVIII:C or FIX:C should be provided to females at risk. The overall cost of prevention is much less than the replacement therapy for hemophiliac patients. Therefore, the service for carrier detection and prenatal diagnosis should be established in developing countries with limited health resources.

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