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Establishment of a National Taiwan Standard and Working Reagent for Human Parvovirus B19 DNA Nucleic Acid Amplification Techniques (NAT)

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ABSTRACT

Mandatory requirements for plasma pools containing a maximum concentration of human parvovirus B19 DNA will be established in Taiwan in the near future. To facilitate the implementation of the policy, a National Standard and working reagent were established for human parvovirus B19 DNA nucleic acid amplification techniques (NAT). The standards are intended to be used for the quality control of B19 NAT assays and as a quantitative reference material for B19 NAT testing. A collaborative study, including 10 laboratories from seven countries, was done in order to establish the National Standard and working reagent for B19 DNA NAT assays by calibration, in International Units (IU), against the WHO International Standard for B19 (99/800). Participants were requested to test the candidate materials and the WHO International Standard by the NAT assay in routine use in the laboratory. The mean B19 DNA content of the standards was determined by each laboratory in three independent assays and the results were analyzed statistically. Overall, a high level of agreement among results was achieved from different laboratories. Consequently, the first National Standard and working reagent for B19 DNA NAT assays with an assigned value of 1.9×10^6 IU/mL and 2.0×10^4 IU/mL, respectively, were established. The results of the stability analysis indicated that both reagents were stable for 4 weeks at 25°C, for 8 weeks at 4°C, for at least 18 months at -20°C or -80°C.

Key words: National Standard, nucleic acid amplification techniques (NAT), human parvovirus B19

INTRODUCTION

Currently, plasma products have outstanding viral safety with respect to enveloped viruses including HIV, HBV, and HCV by regulations such as donor screening, and virus inactivation/removal procedures and following the cGMP requirements during manufacturing. However, human parvovirus B19 (B19), a small (in size 18-26 nm) non-enveloped virus, is difficult to remove by filtration and also resistant to many virus inactivation procedures, such as solvent/detergent (S/D) and dry heat treatment. The presence of B19 DNA in plasma pools as well as plasma products, especially in coagulation factor VIII products, has been reported and poses a potential risk of B19 infection by these plasma products to recipients, especially pregnant women and people with weakened immune system. The prevalence and levels of B19 DNA in coagulation factor VIII products prepared from plasma without B19 DNA screening were high but varied among products from different manufacturing procedures. B19 DNA screening of plasma by nucleic acid amplification techniques (NAT) effectively lowered the B19 DNA level in the final products and in the majority of cases rendered it undetectable and hence potentially reduced the risk of B19 transmission[1-7].

Human parvovirus B19, a member of the *Parvoviridae* family, normally causes an asymptomatic or mild infection in children and a rash and transient symmetrical polyarthralgia in adults[8-9]. However, B19 is a common human pathogen associated with a wide variety of diseases. Besides erythema infectiosum (fifth disease), B19 has been implicated in arthritis, transient aplastic crisis, chronic anemia in immunocompromised patients, hydrops fetalis, and fetal death[10-13].

The European Pharmacopoeia (EP) demands screening of B19 DNA in plasma for further manufacture and has set a limit of less than 10^4 IU/mL of B19 DNA for the final manufacturing pool (monograph Human anti-D immunoglobulin, 0557) and monograph Human plasma (pooled and treated for virus inactivation, 1646). In

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USA, the FDA also proposes the same limit to reduce the viral load of parvovirus B19 in manufacturing pools. To improve the safety of plasma products, a new requirement was announced by the Department of Health in Taiwan on December 19, 2002. The announcement recommended that plasma be screened for B19 DNA by NAT and the cut-off limit for manufacturing plasma pools be less than 10^5 IU/mL. The Blood Directive Act, for the enhancement of the safety and quality of blood derivatives and the assurance of a stable supply quality in order to protect the health of local people, was enacted in 2005. We believe that mandatory requirements for plasma pools containing a defined concentration of B19 DNA, will be established in Taiwan in the near future.

The first World Health Organization (WHO) International Standard for B19 NAT assays was established in 2002 and was essential for introduction of screening of plasma pools for B19 DNA by NAT assays[14]. Since the WHO International Standard for B19 DNA (WHO IS, 99/800) is available only in limited amounts, a European Pharmacopoeia Biological Reference Preparation (BRP) for B19 Virus DNA Testing of Plasma Pools by NAT has been established[15]. To facilitate the implementation of the policy in Taiwan, it was necessary to establish a National Standard and working reagent for human parvovirus B19 DNA NAT assays.

The objectives of this study were to prepare the candidate materials for the National Standards for B19 DNA NAT assays and to calibrate the B19 DNA content of the candidate standards against the WHO International Standard for B19 DNA NAT assays (99/800).

MATERIALS AND METHODS

I. *Preparation of Candidate Materials*

Two materials were prepared for the National Standard candidate and working reagent candidate, samples A and B, respectively. Samples A and B were liquid preparations, and were prepared by diluting a human plasma with high titer of B19 DNA in pooled human cryosupernatant. The B19 DNA positive human plasma had a titer of B19 DNA about 2×10^{11} IU/mL, and was negative for HBV DNA, HCV RNA, HIV RNA, and HAV RNA, and negative for HBsAg, Anti-HCV, Anti-HIV, and Anti-B19. The cryosupernatant was negative for HBV DNA, B19 DNA, HCV RNA, HIV RNA, and HAV RNA, and negative for HBsAg, Anti-HCV, Anti-HIV, and Anti-B19. Samples A and B contained B19 DNA around 10^6 IU/mL and 10^4 IU/mL, respectively.

II. *Design of the International Collaborative Study*

The international collaborative study was aimed to calibrate the titers of parvovirus B19 DNA National Standard and working reagent prepared by the Bureau of Food and Drug Analysis (BFDA) of Taiwan. Sample A was the candidate for BFDA parvovirus B19 DNA National Standard and sample B was the candidate for BFDA parvovirus B19 DNA working reagent.

Including our laboratory, a total of 10 laboratories participated in this study. Participants received 3 vials of each sample and 1 vial of the WHO International Standard for B19 DNA (WHO IS, 99/800), and were requested to perform three independent assays for B19 DNA using the two samples and the WHO IS. Before use, the WHO IS was reconstituted in 0.5 mL water to give a final concentration of 1×10^6 IU/mL. If the reconstituted material was not used immediately, the protocol recommended that laboratories aliquot the material into suitable volumes, which were stored at or below -70°C. For each assay, serial dilutions of the WHO IS were prepared using the appropriate diluent. The recommended diluent for the study was B19 DNA negative human plasma. These serial diluted IS were used to calibrate samples A and B by creating a standard curve. If a commercial kit was used, the IS could be treated as a third unknown sample (sample C) and quantitated in parallel with samples A and B.

The statistical analysis was performed by the maximum likelihood method[14]. Briefly, a single estimate was obtained for each sample in each laboratory and for each assay method, by calculating the mean of repeat data within a single assay. The overall mean and SD were then calculated from the results of all participants, excluding the laboratories with values that lay outside the mean ± 2 SD.

III. *Stability Study on Samples A and B*

Vials of each sample were incubated at 4°C, 25°C, -20°C, and -80°C, and 3 vials were removed at regular intervals for three independent tests. B19 DNA was extracted from 200 μL of samples using the High Pure Viral Nucleic Acid Kit (Roche, Germany). Briefly, the samples were digested in a proteinase K/guanidinium buffer and the nucleic acids were bound to a silica filter, washed with ethanol and eluted in 50 μL of water. Two different assay methods were used for quantitative analysis: a commercial kit (RealArt^{TM} Parvo B19 LC PCR Kit; Qiagen, Germany), and an in-house method. For each method, 5 μL of sample was amplified in the LightCycler Real-time PCR System (Roche Molecular Biochemicals, Germany).

RESULTS

I. *Assay Methods*

Nine of the participants performed the quantitative assays; six laboratories used the LightCycler (Roche), two laboratories used ABI Prism 7000/7700 (Applied Biosystems), and one laboratory used the Cobas S201

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TaqScreen DPX (Roche, in development, not commercially available). One of the participants performed the nested PCR using the Perkin Elmer Thermocycler 9600 (Perkin-Elmer Applied Biosystems). For DNA extraction, the majority of participating laboratories used commercial extraction kits such as the QIAamp series kits (Qiagen), High Pure Viral Nucleic Acid Kit (Roche), and MagNa Pure LC Total Nucleic Acid Isolation kit (Roche). Among them, one laboratory (Lab 10) returned data analyzed from three different assay methods, and the results were reported separately (10A, 10B, 10C). The overall results were therefore based on a maximum of 12 data sets. Six data sets were obtained by in-house assays, and six data sets by commercial assays. The methods used are summarized in Table 1[16-17].

II. *Estimated Value of B19 DNA for Samples A and B*

The estimated values of B19 DNA, relative to the International Standard, for sample A and sample B for each laboratory were listed in Table 2 and Table 3, and showed in Figure 1 (A) and (B) for sample A and sample

Table 2. The estimated values of parvovirus B19 DNA (Log IU/mL)[a] for sample A by 10 laboratories

Lab code	Mean	Minimum	Maximum	CV
1	6.20	6.13	6.26	1.05%
2	6.39	6.26	6.61	3.00%
3	6.25	6.18	6.32	1.17%
4	6.27	6.23	6.35	1.11%
5	6.05	5.59	6.37	6.74%
6	6.41	6.24	6.62	3.06%
7	6.03	6.02	6.04	0.20%
8	6.42	6.35	6.51	1.32%
9	6.61	6.45	6.83	3.01%
10A	6.01	5.91	6.08	1.51%
10B	6.26	6.24	6.27	0.24%
10C	6.32	6.25	6.37	1.04%
Overall	6.27	6.01	6.61	2.86%

[a] The measurement was performed using WHO International Standard for human parvovirus B19 DNA (WHO IS, 99/800) as the standard.

Table 1. An overview of the assays used in the collaborative study

Lab code[a]	Short description of assay method	Region for primer design [b]
1	QIAamp DNA mini kit (Qiagen) RealArt Parvo B19 LC PCR kit (Qiagen) / LightCycler	VP
2	QIAamp DSP Virus kit (Qiagen) RealArt Parvo B19 LC PCR kit (Qiagen) / LightCycler	VP
3	MagNa Pure Total Nucleic Acid Extraction Large Volume (Roche) In-house Assay (TaqMan) / LightCycler	NS
4	MagNa Pure LC Total Nucleic Acid Isolation kit (Roche) Parvovirus B19 Quantification Kit (Roche) / LightCycler	NS
5	QIAamp Viral RNA kit (Qiagen) In-house Assay (TaqMan) / ABI 7700	Unknown
6	Parvo SuperCycle PCR Assay	Unknown
7	Cobas s201 TaqScreen DPX (Parvo/HAV) (in development)	Unknown
8	NucliSens (Organon) In-house Assay (Nested PCR) / Perkin Elmer Thermocycler 9600	VP
9	SMI-TEST EX-R&D (Nippon Genetics) In-house assay (TaqMan) / ABI 7000	NS
10A	High Pure Viral Nucleic Acid Kit (Roche) In-house Assay (Hybridization probe) / LightCycler	VP
10B	High Pure Viral Nucleic Acid Kit (Roche) Parvovirus B19 Quantification Kit (Roche) / LightCycler	NS
10C	High Pure Viral Nucleic Acid Kit (Roche) RealArt Parvo B19 LC PCR kit (Qiagen) / LightCycler	VP

[a] One laboratory (Lab 10) returned data from three different assay methods, and the results are reported separately. The overall results are therefore based on a maximum of 12 data sets.

[b] VP: Viral capsid protein gene.
NS: Nonstructural gene.
Unknown: The participant laboratory/manufacture did not disclose the target region.

Table 3. The estimated values of parvovirus B19 DNA (Log IU/mL)[a] for sample B by 10 laboratories

Lab code	Mean	Minimum	Maximum	CV
1	4.31	4.25	4.37	1.34%
2	4.36	4.27	4.42	1.81%
3	4.24	4.18	4.28	1.34%
4	4.08	4.02	4.15	1.67%
5	4.45	4.34	4.53	2.08%
6	4.25	4.04	4.61	7.46%
7	4.11	4.05	4.18	5.17%
8	4.65	4.38	4.85	1.53%
9	4.56	4.46	4.64	2.06%
10A	4.03	3.84	4.34	6.81%
10B	4.35	4.30	4.39	1.09%
10C	4.34	4.21	4.46	2.83%
Overall	4.31	4.03	4.65	4.33%

[a] The measurement was performed using WHO International Standard for human parvovirus B19 DNA (WHO IS, 99/800) as the standard.

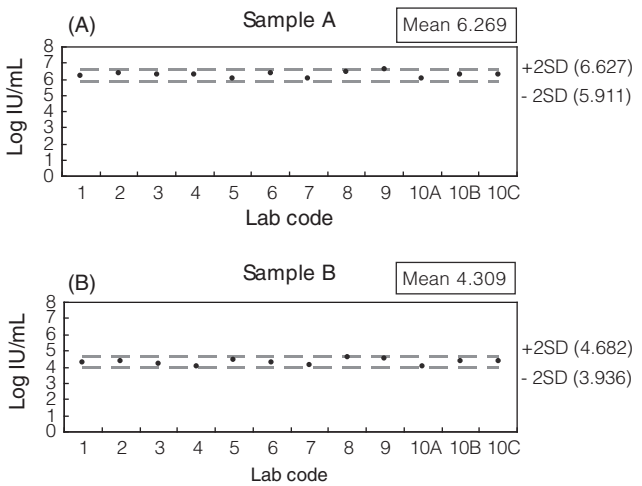

Figure 1. The estimated values of parvovirus B19 DNA (Log IU/mL) for sample A (A) and sample B (B) by 10 laboratories. The dotted lines indicate the mean ± 2SD.

B, respectively. All the data were within a range of mean ± 2SD for the sample A and sample B. Therefore, the overall mean and SD were calculated using the results from all participants. The number of laboratory with various value of B19 DNA estimate is shown in Figure 2 (A) and (B) for samples A and B. Each box represents the estimate from one laboratory and/or assay method, in which the gray color represents a laboratory using the nested PCR assay. All the data were within a range of 1.0 Log for each sample, indicating that all laboratories were in good agreement with the estimates. The overall means

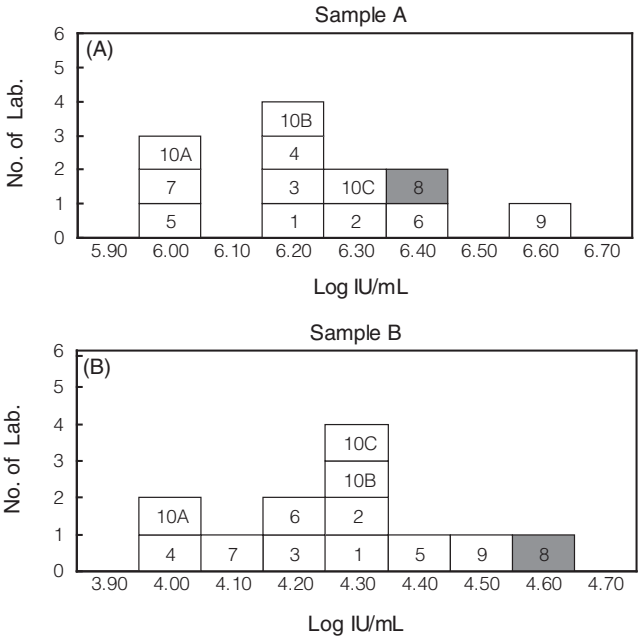

Figure 2. The histogram of estimated values of parvovirus B19 DNA (Log IU/mL) for sample A (A) and sample B (B) by 10 laboratories. The number labeled on the box represented the laboratory code number.

Table 4. Overall mean estimates of parvovirus B19 DNA (Log IU/mL) for samples A and B

Sample	Mean		95% confidence interval (95% CI)	
	Log IU/mL	IU/mL	Log IU/mL	IU/mL
A	6.27	1.9 E+06	6.16~6.38	1.5 E+06~2.4 E+06
B	4.31	2.0 E+04	4.20~4.41	1.6 E+04~2.6 E+04

for sample A and sample B are shown in Table 4, along with the 95% confidence intervals.

III. *Stability Analysis of Samples A and B*

Vials of each sample were stored at 4°C, 25°C, -20°C, and -80°C, and 3 vials were removed for testing at regular intervals, one-week intervals for 25°C, one- or two- week intervals for 4°C, three- or six-month intervals for -20°C, and -80°C. Triplicate samples were assayed at each time-point for each temperature in 3 independent tests. The calculated mean concentration (IU/mL) for each time point/temperature is shown in Figure 3. The results of the stability analysis indicated that samples A and B were stable after storage at 4°C for 8 weeks, 25°C for 4 weeks, -20°C for 18 months, and -80°C for 18 months.

DISCUSSION

In this international collaborative study, a high

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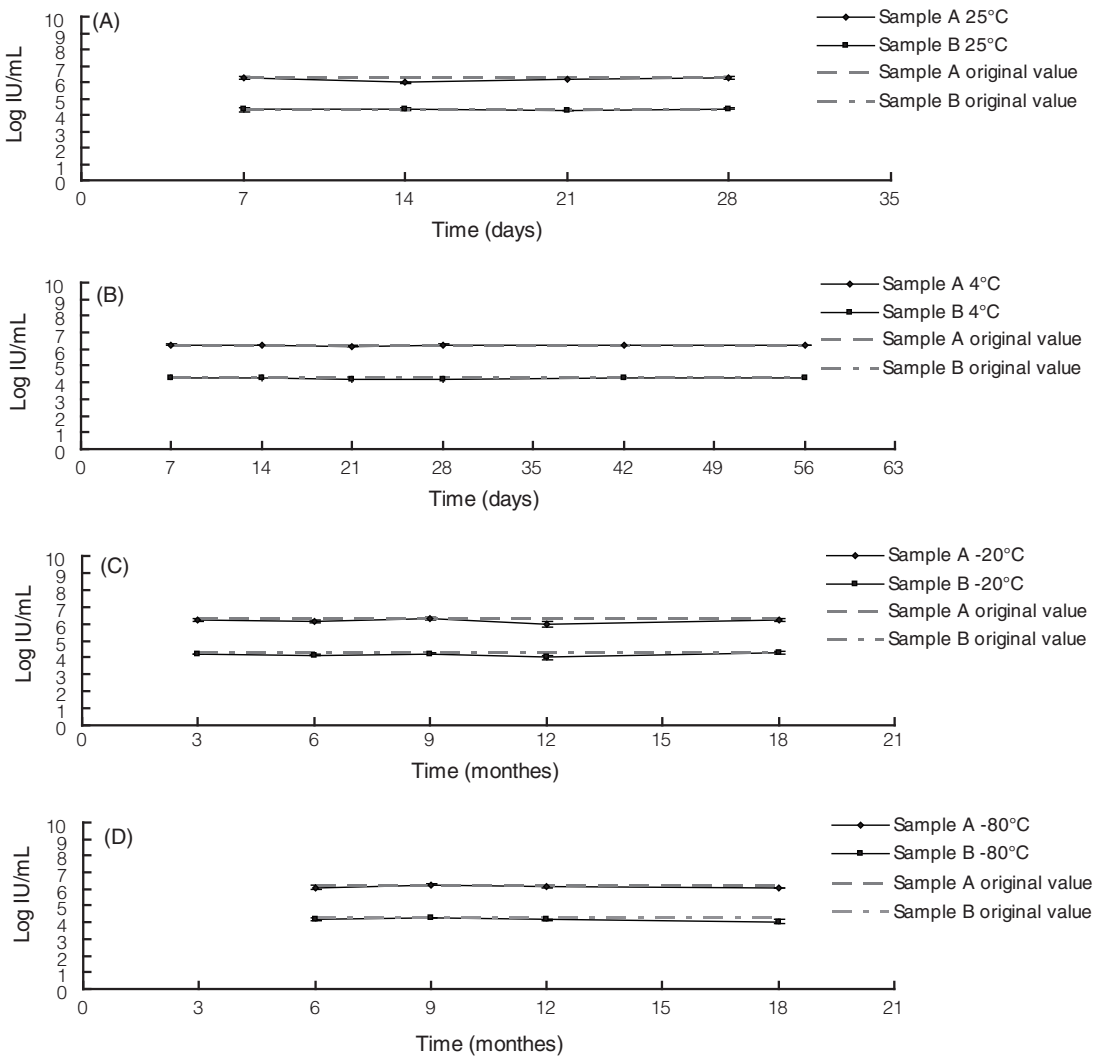

Figure 3. Stability analysis of parvovirus B19 DNA present in samples A and B after storage at different temperatures. (A) 25°C for 4weeks, (B) 4°C for 8weeks, (C) -20°C for 18 months, (D) -80°C for 18 months. The solid line and the dotted line represent the tested values and the original values before storage, respectively.

level of agreement among the results obtained from different laboratories was observed. The first National Taiwan Standard and working reagent for B19 DNA NAT assays with an assigned value of 1.9×10^6 IU/mL and 2.0×10^4 IU/mL, respectively, were established. The results of the stability analysis indicated that the B19 DNA National Standard and working reagent were very stable. They could be used nationally for the quality control of B19 DNA assays. The parvovirus B19 DNA present in this National Standard and working reagent belonged to genotype 1. The genotype 2 parvovirus B19 has been identified in plasma pools that are undergoing batch release in Europe. It was reported that the currently used Roche assay could not detect genotypes 2 and 3, unlike the Artus assay[16]. However, the newly designed Roche assay can detect all the three genotypes (personal communication). Although genotypes 2 and 3 are rare, especially genotype 3, a recent decision by the SoGAT committee (B19 meeting at NIBSC on 2 March, 2007) supported the European Pharmacopoeia view that all assays must detect all the three genotypes. The discussion also focused on the availability of genotypes 2 and 3 materials and the calibration of these materials against the WHO IS (genotype 1). On the other hand, in the study of Ekman A etc., analysis of the P6 promoter showed there is no difference in activity among the three genotypes of B19 virus[19]. Moreover, there is no difference among the different genotypes in their ability to infect permissive cells *in vitro*. Data were also presented to show the serological cross-reactivity between B19 virus genotypes 1 and 2 using recombinant antigens and sera from individuals infected with known genotypes. There is no evidence, so far, that genotypes 2 and 3 behave differently from genotype 1 and they do not have a different clinical outcome from genotype 1[18-19]. Therefore, the first Taiwan National Standard and work-

ing reagent for B19 DNA NAT assays will be used for the quality control of B19 assays before the genotype 2 and 3 standards are available.

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REFERENCES

1. Saldanha, J. and Minor P. 1996. Detection of human parvovirus B19 in plasma pools and plasma products derived from these pools: implications for efficiency and consistency of removal of B19 during manufacture. Br. J. Haemato. 93: 714-719.
2. Willkommen, H., Schmidt, I. and Löwer, J. 1999. Safety issues for plasma derivatives and benefit from NAT testing. Biologicals. 27: 325-331.
3. The European Agency for the Evaluation of Medicinal Products Evaluation of Mediicines for Human Use. 2000. EMEA Workshop on viral safety of plasma-derived medicinal products with particular focus on non-enveloped viruses. CPMP/BWP/BPWG/4080/00.
4. Blümel, J., Schmidt, I., Willkommen, H. and Löwer, J. 2002. Inactivation of parvovirus B19 during pasteurization of human serum albumin. Transfusion 42: 1011-1018.
5. Blümel, J., Schmidt, I., Effenberger, W., Seitz, H., Willkommen, H., Brackmann, H. H., Löwer, J. and Eis-Hübinger, A. M. 2002. Parvovirus B19 transmission by heat treated clotting factor concentrates. Transfusion 42: 1473-1481.
6. Wu, C. G., Mason, B., Jong, J., Erdman, D., McKernan, L., Oakley, M., Soucie, M., Evatt, B. and Yu, M. Y. 2005. Parvovirus B19 transmission by a high-purity factor VIII concentrate. Transfusion 45: 1003-1010.
7. Geng, Y., Wu, C. G., Bhattacharyya, S. P., Tan, D., Guo, Z. P. and Yu, M. Y. 2007. Parvovirus B19 DNA in factor VIII concentrates: effects of manufacturing procedures and B19 screening by nucleic acid testing. Transfusion 47: 883-889.
8. Mcomish, F., Yap, P. L., Jordan, A., Hart, H., Cohen, B. J. and Simmonds, P. 1993. Detection of parvovirus B19 in donated blood: a model system for screening by polymerase chain reaction. J. Clin. Microbiol. 31: 323-328.
9. Anderson, M. J., Jones, S. E., Fisher-Hoch, S. P., Lewis, E., Hall, S. M., Bartlett, C. L. R., Cohen, B. J., Mortimer, P. P. and Pereira, M. S. 1983. Human parvovirus, the cause of erythema infectiosum (fifth disease). Lancet ii: 1378.
10. Brown, T., Anand, A., Ritchie, L. D., Clewly, J. P. and Reid, T. M. S. 1984. Intrauterine parvovirus infection associated with hydrops fetalis. Lancet ii: 1033-1034.
11. Pattison, J. R., Jones, S. E., Hodgson, J., Davis, L. R., White, J. M., Stroud, C. E. and Murtaza, L. 1981. Parvovirus infections and hypoplastic crisis in sickle-cell anemia. Lancet i: 664-665.
12. White, D. G., Woolf, A. D., Mortimer, P. P., Cohen, B. J., Blake, D. R. and Bacon, P. A. 1985. Human parvovirus arthropathy. Lancet i: 419-421.
13. Schmidt, I., Blümel, J., Seitz, H., Willkommen, H. and Löwer, J. 2001. Parvovirus B19 DNA in plasma pools and plasma derivatives. Vox Sang. 81: 228-235.
14. Saldanha, J., Lelie, N., Yu, M. W. and Health, A. 2002. Establishment of the first World Health Organization International Standard for human parvovirus B19 DNA nucleic acid amplification techniques. Vox Sang. 82: 24-31.
15. Nübling, C. M., Daas, A. and Buchheit, K. H. 2003. Collaborative study for establishment of a european pharmacopoeia Biological Reference Preparation (BRP) for B19 virus DNA testing of plasma pools by nucleic acid amplification technique. Pharmeuropa Bio. 2: 27-33.
16. Baylis, S. A., Shah, N. and Minor, P. D. 2004. Evaluation of different assays for the detection of parvovirus B19 DNA in human plasma. J. of Virol. Methods 121: 7-16.
17. Hokynar, K., Norja, P., Laitinen, H., Palomaki, P., Garbarg-Chenon, A., Ranki, A., Hedman, K. and Soderlund-Venermo, M. 2004. Detection and differentiation of human parvovirus variants by commercial quantitative real-time PCR tests. J. Clin. Microbiol. 42: 2013-2019.
18. Blümel, J., Eis-Hübinger, A. M., Stühler, A., Bönsch, C., Gessner, M. and Löwer, J. 2005. Characterization of parvovirus B19 genotype 2 in KU812Ep6 cells. J. Virol. 79: 14197-14206.
19. Ekman, A., Hokynar, K., Kakkola, L., Kantola, K., Hedman, L., Bondén, H., Gessner, M., Aberham, C., Norja, P., Miettinen, S., Hedman, K. and Söderlund-Venermo, M. 2007. Biological and immunological relations among human parvovirus B19 genotypes 1 to 3. J. Virol. 81: 6927-6935.