REVIEW ARTICLE

# Molecular Diagnosis of Haemophilia A in Clinical Practice

Habart D.
*Institute of Hematology and Blood Transfusion, Prague, Czech Republic*

**SUMMARY**

Understanding the pathogenesis of haemophilia A has allowed for detailed diagnosis of the condition at a molecular level. Evaluation of the interaction between factor VIII and von Willebrand factor has been utilised to distinguish haemophilia A from von Willebrand disease. The discovery of a wide spectrum of mutations in the factor VIII gene and their association with different severity of the disease has allowed for the development of a rational strategy for mutation detection in clinical settings. Characterisation of the genetic defects is required for carrier detection and antenatal testing, and this also helps to predict risk of factor VIII inhibitor development. Research is ongoing to establish less invasive prenatal testing and to move the testing to pregravid period.
**Key words:** haemophilia A, von Willebrad factor, factor VIII, factor VIII inhibitor, mutation, inversion, polymorphism, prenatal testing, preimplantation genetic diagnosis.

*Čas. Lék. čes., 2005, 144, pp. 795-800.*

## INTRODUCTION

Haemophilia A is an inherited deficiency of coagulation factor VIII. It is caused by mutations in the factor VIII gene (26 exons, 180 kb, mRNA ~9kb), that is localised on the chromosome Xq28. The plasma level of factor VIII correlates with severity of the bleeding. This enables classification of the patients at an early childhood into three prognostic groups and then to choose a suitable therapeutic approach. Substitution therapy with factor VIII concentrates may be complicated by antibody response directed against the factor VIII (inhibitor). The correlation between the risk of inhibitor development and character of causal mutation allows, to a certain extent, prediction of this serious complication. Plasma level of the factor VIII is influenced by its binding to von Willebrand factor (vWF). Haemophilia A therefore needs to be distinguished from von Willebrand disease, which is associated with abnormal vWF. The relatives of haemophiliacs are also of concern - determination of carriers and haemophilia transmission to the next generations is required. Laboratory tests have been established that facilitate prenatal and postnatal diagnosis and prediction of the course of the disease and its specific complications.

## PREDICTION OF CLINICAL SEVERITY

Plasma activity of factor VIII generally correlates well with clinical severity of the disease. Haemophilia A is classified as severe (FVIII<1%), moderate (1-5%) and mild (5-40%) to estimate future course of the disease.

In patients with severe haemophilia, frequent (several times a month) and spontaneous bleeding especially into weight-carrying joints and muscles should be expected. These patients represent about half of haemophiliacs and usually require long-term and expensive prophylactic administration of the factor VIII concentrates since early childhood. There is also an increased risk of the inhibitor development in these patients. Repeated bleeding with insufficient treatment leads to the development of haemophilic arthropathy and to severe derangement of the musculo-skeletal system. This may require an orthopaedic treatment, which is both demanding and expensive. Determination of carriers in these families has significant practical impact.

In the patients with moderate form of haemophilia, joint or soft tissue bleeding owing to minor injuries should be expected, usually several times a year.

Patients suffering from a mild form of haemophilia bleed mainly during surgical procedures performed without previous haematological preparation.

For clinical classification of haemophilia, determination of the factor VIII activity in plasma (FVIII:C) is sufficient, whether using one-stage (1, 2), two-stage (3) or chromogenic (4, 5) assay. New methods based on thrombin generation (6, 7) seem suitable also for laboratory follow-up of the efficacy of substitution treatment.

## DIFFERENTIATION FROM VON WILLEBRAND DISEASE

Differentiation of haemophilia A from von Willebrand disease is a critical requirement for sound genetic counselling, owing to different mode of inheritance. It is also important for rational treatment of each of the diseases.

The stabilising influence of von Willebrand factor on the factor VIII in plasma is reflected by markedly decreased factor VIII (about 3% of normal) in the patients with complete deficiency of vWF

Address for correspondence:
David Habart, MD., Ph.D.
Institute of Hematology and Blood Transfusion
128 20 Prague 2, U Nemocnice 1
Czech Republic
E-mail: dh@uhkt.cz

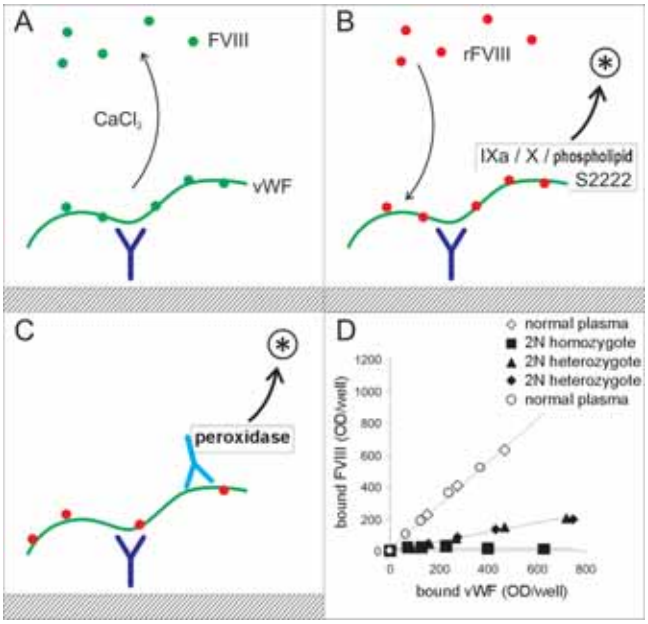


**Fig. 1.** Determination of the factor VIII binding to von Willebrand factor
The principle is explained in Annex 1.
A) Endogenous factor VIII is released from binding to vWF by increase of $Ca^{2+}$ concentration.
B) Recombinant factor VIII (rFVIII) does not contain the admixture of vWF. Unbound rFVIII is washed away. The bound rFVIII is detected by a chromogenic functional test or immunologically.
C) Immunological detection of immobilized vWF is done by using different wavelength.
D) The tested sample is prepared in several dilutions. The FVIII binding to vWF is assessed from the slope of fitted curve.

**Tab. 1.** Prevalence of the factor VIII inhibitor in the patients with haemophilia A with respect of the type of mutation (15)

| Prevalence (%) | Type and location of mutation |
|---|---|
| 68 | deletion of more than one exon |
| 50 | nonsense mutation in the light chain of the factor VIII protein |
| 34 | intron 22 inversion |
| 21 | insertion and deletion outside the continuous segments of several adenines |
| 14 | nonsense mutations in heavy chain of the factor VIII protein |
| 12 | mismatch mutation in light chain of the factor VIII protein |
| 12 | deletion of one exon |
| 6 | insertion and deletion in continuous segments of several adenines |
| 4 | mismatch mutations in heavy chain of the factor VIII protein |
| 2 | splice-site mutations |

(type 3 vWD). Von Willebrand disease is highly heterogeneous disorder. While it is easy to distinguish the type 3 vWD (undetectable level of vWF), more complex approach is required for differentiation the other subtypes. Possible co-existence of both disorders in an individual family should also be borne in mind (8).

The main diagnostic problem is differentiation of mild and moderate forms of haemophilia A from type 2N vWD (Normandy), because both disorders are characterised by low level of factor VIII with normal vWF (9). In patients with type 2N vWD, the structure of both gene and protein of the factor VIII are normal, while mutations in the vWF gene alter the ability of von Willebrand factor protein to bind and stabilise factor VIII (some of these mutations influence also the adhesive function of vWF).

A specific binding assay (vWF: FVIIIB) has been developed to detect diminished binding of normal factor VIII to tested von Willebrand factor (10). The principle and interpretation of the assay are explained in Technical Annex 1 and in the Fig. 1. When the functional assay is unavailable, the presence of specific type 2N mutations in vWF gene is sought (Technical Annex 2). However, negative finding does not completely exclude the type 2N vWD as a consequence of a novel mutation. Functional assay then needs to be performed.

## PREDICTION OF INHIBITOR DEVELOPMENT

In some patients the treatment with factor VIII concentrates induces specific alloantibodies that inhibit the function of factor VIII (inhibitor). The inhibitor significantly impacts on further treatment of the patients. The inhibitor arises mostly during the childhood in the course of the first 25 treatment days (11, 12). The occurrence of inhibitors has been reported in the range of 10–35% of severe haemophiliac patients, depending on whether only clinically significant inhibitor with an impact on further therapy or also transient detection the antibodies is included. Risk factors associated with the inhibitor development have not yet been fully characterised. However, it is evident that they are both inborn and acquired in nature (13). It follows that in patients with increased genetic risk, the external risk factors should be minimised (desmopresin treatment (14), commencement of prophylactic therapy at higher age, limited switches between the factor VIII concentrates).

Estimation of the genetic risk is based on family history and knowledge of causal mutation. The presence of the inhibitor in a relative haemophiliac is considered a risk factor. Extensive studies of a large numbers of patients with the inhibitor have permitted stratification of mutations in the factor VIII gene according to the risk of the inhibitor development, as is indicated in Tab. 1 (15). For instance, the intron 22 inversion – which occurs in almost half of the patients with severe form of haemophilia A – is associated with transient or permanent occurrence of the inhibitor in about one third of the cases. The highest risk is associated with gross deletions (inhibitor is present in about two-thirds of patients), which themselves are infrequent cause of severe haemophilia (16).

## CARRIER DETERMINATION

Haemophilia A is a gonosomal recessive disorder. Males usually suffer from bleeding while females are asymptomatic carriers. The investigation of carriership should be commenced in asymptomatic girls with family history of haemophilia at about 10 years of age. Carrier determination is important for family planning, antenatal testing and for proper conducting the labour (care must be exercised to avoid risk of intracranial bleeding in a haemophiliac). Decreased factor VIII activity as observed in some carriers, is associated with increased risk of bleeding after teeth extraction or surgical procedures, and may lead to hypermenorrhea. A common cause of the decrease is non-random X-chromosome lyonization (17, 18), which leads to preferential utilisation of the damaged factor VIII gene. This can be verified by a laboratory test (Technical Annex 5).

In specific cases it is possible to identify carriers by simple pedigree analysis – "definite" carrier is the daughter of a haemophiliac, the mother of two haemophiliacs and the mother of a haemophiliac with family history of haemophilia traceable in the female line. Indirect methods of molecular genetics utilise polymorphic markers

to trace the affected allele of the factor VIII gene throughout tested family (Technical Annex 4). This approach, however, may demand sampling of extensive number of family members. In addition, the indicated father is not always reliable. There is also a risk of error owing to recombination between the marker and causal mutation (<0.1% and 5% in case of use of intragenic or extragenic polymorphisms). The most reliable approach is direct determination of disease causing mutation. This is technically demanding and can be time-consuming. This approach allows for determination of carriers even among distant family members without sampling other relatives except of the haemophiliac (Technical Annex 3). The formerly used ratio of factor VIII level and vWF (19) serves now for rough orientation only.

## ANTENATAL TESTING

Invasive sampling such as chorionic villus sampling (CVS) or amniocentesis (AMC) carries about 1-2% risk of fatal and non-fatal complications to the foetus. It has to be performed by an experienced team. When indicating antenatal testing, this risk has to be weight against the benefit - a possibility to make an informed decision regarding the abortion of the affected foetus, which is based on the results obtained from the sample (20, 21). As the severity of haemophilia remains stable within an individual family, the partners can base their decision on their own experience with the disease, while they are informed by a clinician about progress in haemophilia treatment. Prenatal testing is generally indicated in families with severe or moderate forms of haemophilia. In families with the mild disease such indication is rare. Preferably, the chorionic villus sampling is performed within the $10^{th}$–$13^{th}$ week of gestation. Indication for amniocentesis (between $15^{th}$–$16^{th}$ week) is warranted for pregnant women over 35 years of age or with another genetic disorder in family. The presence of Y chromosome in the tissue is detected rapidly by a PCR-based method (Technical Annex 6). If the foetus is male, the diagnosis of haemophilia is established either directly by proving the presence of causal mutation or indirectly by showing the presence of the affected allele (Technical Annex 3 and 4). The results are concluded within several days, as far as PCR-based methods can be used. The chromosomes are tested in parallel by cytogenetic methods to exclude presence of unexpected aberrations (results obtained after 2 or 3 weeks).

Occasionally, a carrier of severe haemophilia A is pregnant with a male foetus and at the same time she is homozygous (e. g. uninformative) in all polymorphisms while causal mutation has not yet been determined. In such cases chordocentesis is performed at $16^{th}$-$18^{th}$ week of gestation and antenatal diagnosis is established by factor VIII determination in the umbilical cord blood (22) (Technical Annex 7).

## NOVEL APPROACHES

In female foetus the knowledge of antenatal diagnosis is not needed. It follows that, if the sex of the foetus was known, the invasive examination would not be indicated in about half of the cases. Therefore, less invasive procedures for sex determination are being developed, based on the venous blood sample taken from a pregnant woman at an early stage of gestation. This technique has been successfully used by our centre, so far on an experimental basis (Technical Annex 6) (23).

In principle, the assisted reproduction techniques shifted the decision regarding the fate of the affected embryo to the period before implantation into the mother's uterus (Technical Annex 8) (24). At present in the Czech Republic, selection by the sex can be performed as an ethical makeshift solution. However, timed diagnosis could allow for the implantation of selected unaffected embryos of either sex. When established, this approach could be preferred by haemophilia families to current practice of antenatal testing associated with abortion of already developed though affected foetus.

## TECHNICAL ANNEX

### *1. Determination of binding of factor VIII to von Willebrand factor*

Specific functional assay based on the ELISA principle (see Fig. 1) allows detection of the decreased factor VIII binding to von Willebrand factor (25). The test is based on the patient's plasma and requires 4 days. Von Willebrand factor from patent's plasma is first immobilized on the solid phase and endogenous factor VIII is removed (Fig. 1A). Then the amount of added recombinant factor VIII that is bound to the binding sites on the examined molecule of von Willebrand factor, is determined (Fig. 1B). Control of the immobilisation efficacy of the tested vWF on the solid phase is performed (Fig. 1C). Patients with type 2N von Willebrand disease show decreased or undetectable amount of bound factor VIII (Fig. 1D).

### *2. Detection of selected mutations in the vWF gene*

Mutations characteristic for type 2N von Willebrand disease are usually detected by direct sequencing of exons 18-21 and 24 gene for vWF. These exons code a binding site for factor VIII and contain several polymorphisms.

### *3. Detection of mutations in the factor VIII gene*

Detection of causal mutation is technically demanding and time-consuming due to the considerable size of gene and high variability of genetic defects. First, the mutation is detected in a haemophiliac who represents the given family (index patient). A definite carrier by pedigree analysis can supplemet for the haemophiliac, if he is unavailable *. Choice of a suitable method is guided by severity of the disease and pedigree analysis (Fig. 2). In the Czech Republic, the work has been facilitated by "National registry of patients with inherited coagulation disorders", which is being created through a long-term collaboration of all nine haemophilia cen-

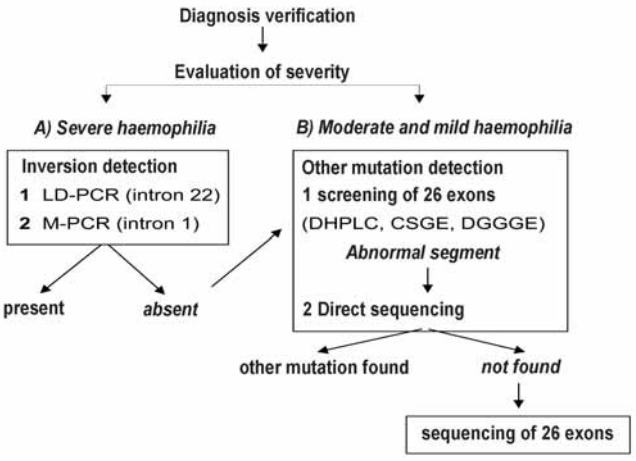


**Fig. 2.** Strategy for mutation detection in the factor VIII gene
It should be based on verified diagnosis and assessed severity of haemophilia A. In the patients with severe form of the diseases, the presence of both inversions is first determined (A). If the inversions are ruled out in severe haemophilia and in all cases of moderate and mild haemophilia, we screen the entire gene for the factor VIII and directly sequence only the abnormal segments (B).

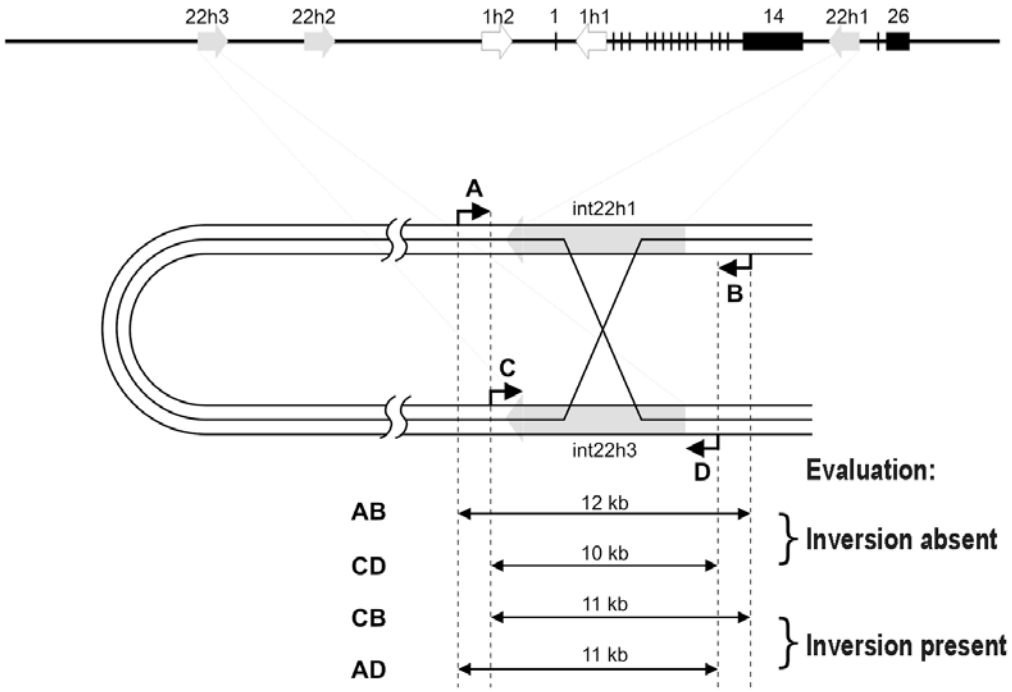


**Fig. 3.** The principle of detection of the intron 22 inversion of the factor VIII gene
Inversions arise as a consequence of homologous recombination of the region h1 within intron 22 (grey arrows) or within the intron 1 (white arrows) with one of the extragenous copies (h2 or h3). The primers A-D (arrows with foot) are designed so that LD-PCR across the homologous regions located inside and outside the factor VIII gene give rise to amplicons of different length. Rearrangement of the homologous regions as a consequence of the inversion leads to novel amplicons of different length. Only the inversion of intron 22 is shown, while the principle of the detection of the intron 1 inversion is similar.

tres in our country and is managed at ÚHKT (26). The registry comprises information regarding causal mutations detected in the index patients.

In almost half of the cases the cause of severe haemophilia A is an inversion of the factor VIII gene. The breakage site is commonly found in the intron 22 (about 45% of severe cases (27)), rarely in the intron 1 (2-5% of severe haemophilic cases), (28, 29)). Recently, it has become possible to detect both the inversions by PCR amplification of genomic DNA across the respective breakage sites (28, 30, 36). In case of the intron 22 inversion, very long and CpG rich segments need to be amplified by means of long-distance PCR (LD-PCR) (Fig. 3). In case of the intron 1 inversion, significantly shorter segments are amplified by multiplex PCR (M-PCR).

Except for the inversions, the haemophilia A causing mutations are usually unique and dispersed all over the entire length of the factor VIII gene. Their detection can be based either on the genomic DNA derived from peripheral leukocytes or on the mRNA ectopically transcribed in these cells (31). Amplification of the entire coding region (26 exons) together with the adjacent non-coding sequences can be performed. The other option is reverse transcription (RT-PCR).

of the factor VIII mRNA (9 kb) into several segments of cDNA. The presence of gross deletion is suspected when amplification failure occurs. The deletion is further characterised by Southern blotting. The amplified segments of DNA or cDNA can be either directly sequenced or screened for presence of a mutation. Direct sequencing of the extensive regions is expensive and demanding; hence many laboratories have preferred a two-step procedure (see Fig. 2). In the first step, the amplified segments are screened for the presence of an abnormal sequence by one of the standard scanning techniques (SSCP, DGGE, SCGE, HPLC, CCM, PTT), the sensitivity of which ranges from 80% to 97% (32). In the second step, the abnormal fragment only is directly sequenced. Characterised mutations are finally compared with the international database (33). At our institute, we utilise the genomic DNA (easier transport) and employ the scanning techniques DGGE (34), CSGE (35) and more recently, HPLC (collaboration with the Institute of Biochemistry and Experimental Oncology of the 1st Medical Faculty, Charles University).

***4. Investigation of polymorphisms in the factor VIII gene***

When utilising the indirect methods, the DNA of both parents of the suspected female carrier and possibly grandparents and other relatives is necessary in addition to that of the propositus and haemophiliac. The combined informativeness of the commonly used polymorphisms in the introns 19 (RFLP-Hind III) or 18 (RFLP-Bcl1) and VNTR-PCR in the introns 13 and 22 is about 80%. The polymorphisms in intron 7 and exons 14 and 26 may bring further information in some cases. Highly informative extragenous VNTR St14 (heterozygocity up to 90%) is burdened by high risk of recombination (3-5%) and is used in exceptional cases only.

***5. Detection of non-random lyonization of chromosome X***

The investigation is based on the fact that DNA of the inactivated X chromosome is modified by site specific methylation. This can be detected by restriction endonucleases sensitive to presence of the methylation. Besides the methylation site, the investigated genomic region should possess high frequency sequence variations, so that both chromosomes could be distinguished from each other in the majority of the tested carriers. The HUMARA gene (human androgen receptor) localised at Xq11 has been found to be a suitable locus (37). The investigation has to be carried on the genomic DNA.

### *6. Sex determination*

The foetal sex can be determined by PCR based methods, utilising either Y chromosome specific sequence - SRY (38a) or a sequence in the amelogenin gene (38b)*, which is present on both sex chromosomes but differs in length. Advantage of the amelogenin locus is that in samples of either sex specific amplification is obtained. In contrary, lack of specific amplification may be either due to female sex or technical failure when SRY locus is employed. However, the SRY (but not amelogenin) gene can be utilised in highly sensitive real-time quantitative PCR (RQ-PCR) to detect trace amounts of Y chromosome in blood of a pregnant woman carrying male foetus (23).

### *7. Factor VIII determination in foetal blood*

For the interpretation of factor VIII level (activity and antigen) in umbilical chord blood sample it is necessary to consider its developmental changes; factor VIII level is physiologically low (FVIII:C 25–80%, FVIII:Ag 11-46%) at the time of sampling (39, 40). Contamination of the sample with the mother's blood or amniotic fluid has to be controlled. In the latter case, high amount of thromboplastin precludes correct determination of the factor VIII activity, while the factor VIII antigen remains unaffected. Estimation of the mother's blood admixture in the foetal blood sample is based on differential acidoresistance of foetal and adult red cells (Kleihauer test) and the foetal and adult hemoglobin levels are determined.

### *8. Preimplantation genetic diagnosis*

For the preimplantation genetic diagnosis (PGD), 1–2 blastomers are taken from the embryo at eight cells stage (24). Sex determination of the individual blastomer is performed by fluorescent *in situ* hybridisation (FISH). The development of reliable single-cell techniques suitable for preimplantation diagnosis of haemophilia is under way. The current approaches try to utilise the principles and molecular markers of the indirect testing and utilise the polymorphic and employ highly sensitive detection systems (nested PCR, fluorescent detection) (41).

## CONCLUSION

The principles of diagnostic tests are based on the knowledge of molecular pathogenesis of haemophilia A (16). Diagnosis at the molecular level is necessary for genetic counselling and helpful for rational treatment. The methods and procedures are accessible in the Czech Republic to provide complex and reliable diagnosis of haemophilia A.

**Abbreviations**

CCM - chemical cleavage missmatch
CNS - central nervous system
CSGE - conformation-sensitive gel electrophoresis
CVS - chorionic villus sampling
DGGE - denaturing gradient gel electrophoresis
DNA - deoxyribonucleic acid
FISH - fluorescent *in situ* hybridisation
HPLC - high performance liquid chromatography
HUMARA - human androgen receptor
LD-PCR - long-distance PCR
M-PCR - multiplex PCR
mRNA - messenger ribonucleic acid
PCR - polymerase chain reaction
PGD - preimplantation genetic diagnosis
PTT - protein truncation test
RFLP - restriction fragment length polymorphism
RQ-PCR - highly sensitive real-time quantitative PCR
RT-PCR - reverse transcription PCR
SRY - sex-determining region Y
SSCP - single stand conformation polymorphism
ÚHKT - Institute of Hematology and Blood Transfusion (Prague)
VNTR - variable number of tandem repeats
vWD - von Willebrand disease
vWF - von Willebrand factor

## REFERENCES


1. **Over, J.:** Methodology of the one-stage assay of Factor VIII (VIII:C). Scand. J. Haematol., 1984 (Suppl. 41), pp. 13-24.
2. **Vorlová, Z., Sikorová, J.:** [Coagulation activity of factor VIII. A study by 10 laboratories.] Čas. Lék. čes., 1982, 121, pp. 518-1520.
3. **Barrowcliffe, T. W.:** Methodology of the two-stage assay of Factor VIII (VIII:C). Scand. J. Haematol., 1984, 41 (Suppl. 41), pp. 25-38.
4. **Rosen, S.:** Assay of factor VIII:C with a chromogenic substrate. Scand. J. Haematol., 1984, 40 (Suppl. 40), pp. 139-145.
5. **Rosen, S., Andersson, M., Blomback, M. et al.:** Clinical application of a chromogenic substrate method for determination of factor VIII activity. Thromb. Haemost., 1985, 54, pp. 818-823.
6. **Hemker, H. C., Al Dieri, R., Beguin, S.:** Thrombin generation assays: accruing clinical relevance. Curr. Opin. Hematol., 2004, 11, pp. 170-175.
7. **Barrowcliffe, T. W.:** Monitoring haemophilia severity and treatment: new or old laboratory tests? Haemophilia, 2004, 10, pp.109-114.
8. **Mazurier, C., Parquet-Gernez, A., Gaucher, C. et al.:** Factor VIII deficiency not induced by FVIII gene mutation in a female first cousin of two brothers with haemophilia A. Br. J. Haematol., 2002, 119, pp. 390-392.
9. **Mazurier, C.:** von Willebrand disease masquerading as haemophilia A. Thromb. Haemost., 1992, 67, pp. 391-396.
10. **Mazurier, C., Meyer, D.:** Factor VIII binding assay of von Willebrand factor and the diagnosis of type 2N von Willebrand disease - results of an international survey. On behalf of the Subcommittee on von Willebrand Factor of the Scientific and Standardization Committee of the ISTH. Thromb. Haemost., 1996, 76, pp. 270-274.
11. **Hay, C. R.:** Why do inhibitors arise in patients with haemophilia A? Br. J. Haematol., 1999, 105, pp. 584-590.
12. **Vermylen, J.:** How do some haemophiliacs develop inhibitors? Haemophilia, 1998, 4, pp. 538-542.
13. **Gill, J. C.:** The role of genetics in inhibitor formation. Thromb. Haemost., 1999, 82, pp. 500-504.
14. **Sikorová, J., Vorlová, Z.:** [DDAVP and its effect on factor VIII activity (author's transl.)]. Čas. Lék. čes., 1981, 120, pp. 553-556.
15. **Goodeve, A. C., Peake, I. R.:** The molecular basis of hemophilia A: genotype-phenotype relationships and inhibitor development. Semin. Thromb. Hemost., 2003, 29, pp. 23-30.
16. **Habart, D.:** Molekulární patogeneze hemofilie A. Čas. Lék. čes., 2005, 144, pp. 727-732.
17. **Nisen, P. D., Waber, P. G.:** Nonrandom X chromosome DNA methylation patterns in hemophiliac females. J. Clin. Invest., 1989, 83, pp. 1400-1403.
18. **Belmont, J. W.:** Genetic control of X inactivation and processes leading to X-inactivation skewing. Am. J. Hum. Genet., 1996, 58, pp. 1101-1108.
19. **Peake, I. R., Newcombe, R. G., Davies, B. L. et al.:** Carrier detection in haemophilia A by immunological measurement of factor VIII related antigen (VIIIRAg) and factor VIII clotting antigen (VIIICAg). Br. J. Haematol., 1981, 48, pp. 651-660.
20. **Oyesiku, J. O., Turner, C. F.:** Reproductive choices for couples with haemophilia. Haemophilia, 2002, 8, pp. 348-352.
21. **Sedláček, Z., Macek, M., Malcolm, S. et al.:** [Initial experience with the diagnosis of hemophilia A using molecular genetics]. Čas. Lék. čes., 1987, 126, pp. 682-686.
22. **Shetty, S., Ghosh, K., Mohanty, D.:** Prenatal diagnosis in a haemophilia A family by both factor VIII activity and antigen measurements. J. Assoc. Physicians India, 2003, 51, pp. 916-918.
23. **Hromadniková, I., Houbová, B., Hridelová, D. et al.:** Replicate real-time PCR testing of DNA in maternal plasma increases the sensitivity of non-invasive fetal sex determination. Prenat. Diagn., 2003, 23, pp. 235-238.

24. **Sermon, K., van Steirteghem, A., Liebaers, I.:** Preimplantation genetic diagnosis. Lancet, 2004, 363, pp. 1633-1641.
25. **Caron, C., Mazurier, C., Goudemand, J.:** Large experience with a factor VIII binding assay of plasma von Willebrand factor using commercial reagents. Br. J. Haematol., 2002, 117, pp. 716-718.
26. **Habart, D., Komrska, V., Smejkal, P. et al.:** Národní registr nemocných s vrozenými prouchami koagulace. XX. olomoucké hematologické dny s mezinárodní účastí, 2005, P73, Abstr. PP. 109-110.
27. **Antonarakis, S. E., Rossiter, J. P., Young, M. et al.:** Factor VIII gene inversions in severe hemophilia A: results of an international consortium study. Blood, 1995, 86, pp. 2206-2212.
28. **Bagnall, R. D., Waseem, N., Green, P. M., Giannelli, F.:** Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. Blood, 2002, 99, pp. 168-174.
29. **Habart, D., Kalabová, D., Hrachovinová, I., Vorlová, Z.:** Significant prevalence of the intron 1 factor VIII gene inversion among patients with severe hemophilia A in the Czech Republic. J. Thromb. Haemost., 2003, 1, pp. 1323-1324.
30. **Bowen, D. J., Keeney, S.:** Unleashing the long-distance PCR for detection of the intron 22 inversion of the factor VIII gene in severe haemophilia A. Thromb. Haemost., 2003, 89, pp. 201-202.
31. **Berg, L. P., Wieland, K., Millar, D. S. et al.:** Detection of a novel point mutation causing haemophilia A by PCR/direct sequencing of ectopically-transcribed factor VIII mRNA. Hum. Genet., 1990, 85, pp. 655-658.
32. **Oldenburg, J.:** Mutation profiling in haemophilia A. Thromb, Haemost., 2001, 85, pp. 577-579.
33. **Kemball-Cook, G., Tuddenham, E. G. D.:** HAMSTeRS. The Haemophilia A Mutation, Structure, Test and Resource Site, 1999, http://europium.csc.mrc.ac.uk/WebPages/Main/road_map.htm
34. **Krepelová, A., Vorlová, Z., Acquila, M., Mori, P.:** GAA(Glu)272 – – – AAA(Lys) and CGA(Arg)1941 – – – CAA(Gln) in the factor VIII gene in two haemophilia A patients of Czech origin [letter]. Br. J. Haematol., 1992, 81, p. 458.
35. **Habart, D., Kalabová, D., Novotný, M., Vorlová, Z.:** Thirty-four novel mutations detected in factor VIII gene by multiplex CSGE: modeling of 13 novel amino acid substitutions. J. Thromb. Haemost., 2003, 1, pp. 773-781.
36. **Liu, Q., Sommer, S. S:** Subcycling-PCR for multiplex long-distance amplification of regions with high and low GC content: application to the inversion hotspot in the factor VIII gene. Biotechniques, 1998, 25, pp. 1022-1028. *
37. **Allen, R. C., Zoghbi, H. Y., Moseley, A. B. et al.:** Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am. J. Hum. Genet., 1992, 51, pp. 1229-1239.

38a. **Nakahori, Y., Hamano, K., Iwaya, M., Nakagome, Y.:** Sex identification by polymerase chain reaction using X-Y homologous primer. Am. J. Med. Genet., 1991, 39, pp. 472-473.

38b. **Nakahori, Y., Hamano, K., Iwaya, M., Nakagome, Y:** Sex identification by polymerase chain reaction using X-Y homologous primer. Am. J. Med. Genet., 1991, 39, pp. 472-473.*

39. **Mibashan, R. S., Rodeck, C. H., Thumpston, J. K. et al.:** Plasma assay of fetal factors VIIIC and IX for prenatal diagnosis of haemophilia. Lancet, 1979, 1, pp. 1309-1311.
40. **Mibashan, R. S., Peake, I. R., Rodeck, C. H. et al.:** Dual diagnosis of prenatal haemophilia A by measurement of fetal factor VIIIC and VIIIC antigen (VIIICAg). Lancet, 1980, 2, pp. 994-997.
41. **Fiorentino, F., Magli, M. C., Podini, D. et al.:** The minisequencing method: an alternative strategy for preimplantation genetic diagnosis of single gene disorders. Mol. Hum. Reprod., 2003, 9, pp. 399-410.

** Added for e-version 30 Nov. 2005.*

*Acknowledgement*

*This work was supported by Internal Grant Agency of the Ministry of Health of the Czech Republic, Grant number: NH/7615-3. The work is dedicated to Zdena Vorlová, MD., CSc., who served for many years as a Head of the Haemophilia Centre at ÚHKT. Many thanks to Prof. Radim Brdička, MD. D.Sc., for critical reading of the manuscript.*

Translation: Oldřich Louthan