Medical technologies used in the diagnosis conflict serological

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Abstract

The leakage of foetal Rh-positive red blood cells into the bloodstream of the Rh-negative mother is the most common cause of severe hemolytic disease of foetuses and newborns in Poland. It is extremely important, therefore, to introduce appropriate diagnostics. This paper presents multi-step diagnostics of prenatal serological conflict. It primarily concerns the assessment of antibody titers against the foetal blood cells in the cardiovascular system of the mother. Furthermore, the use of ultrasound methods to determine the gestational age, foetal middle cerebral arterial Doppler assessment and echocardiography. The article also describes the importance of the father's blood group determination. In addition, invasive methods are mentioned, such as foetal cordocentesis and amniotic fluid spectrophotometry.

Key words:

Rh incompatibity, foetal circulation, prenatal deveopment

Introduction

Serological conflict (*colisio serologia*), also called *hemolytic disease of the newborns* (HDN) [*latin morbus hemolyticus naeonatorum*], is a constantly present, very important topic in the field of perinatology medicine. This condition applies to both the foetus and the newborn, who inherit the D antigen from the father. Clinical disorders are caused by alloimmunisation pathomechanism, which is the result of previously occurring sensitization of the mother not having the D antigen (RHD-negative) [1]. In addition to

the D antigen, haemolytic disease of the newborns can be caused by 43 other antigens. These antigens are for example c, *C, E, Kell, Fy.* However, the D antigen is most common and has the highest immunogenic properties – estimated as approximately 50 times more potent compared to other antigens. That is why, serological conflict in terms of this antigen is considered to be the leading cause of morbidity and mortality when haemolytic disease of the newborns is concerned. [1,2,3,4,5].

The occurrence of hemolytic disease is most often associated with the following pregnancy because the sensitization of the mother not having the D antigen to the foetus's Rh-positive antigens occurs during the first pregnancy. It happens this way because the first immunization is weak. As a result, IgM antibodies are produced which have no capacity to pass through the placental barrier. IgG antibodies have the ability to cross the placenta, the production of which occurs at a later time [3]. Already in 1953, Bruce Chown demonstrated that the Rh system immunization is caused by a leak of red blood cells of the Rh-positive foetus to the circulation of the Rh-negative mother. The use of a cytometric method made it possible to determine the presence of microscopic leaks between the foetus and the mother in every developing pregnancy [6]. Immunization can occur regardless of the period of pregnancy, however, the probability of its occurrence increases in direct proportion to the duration of pregnancy. Thus, in the first trimester it is approx. 3%, in the second approx. 43%, while in the third it is approx. 64% (Fig. 1). A directly proportional dependence also occurs between the risk of immunization and the amount of blood introduced into the mother's bloodstream as a result of a foetal-maternal leak. With the blood volume <0.1 ml the risk of immunization is approx. 3%, with the volume of > 0.1 ml it rises to approx. 22% [3]. Sensitization by foetal-maternal leaks can also occur as a result of performed obstetrical procedures or a miscarriage [3].

According to Bowman, serological conflict occurs after 4-5% of induced miscarriages, and approx. 2% of spontaneous miscarriages. It has also been proved that multiple small leaks between the foetal and maternal circulation carry a greater risk of antibody production by the mother than a single, larger volume leak. A different than obstetric cause of the conflict might be transfusion of another person's blood [1,2,3].

The initial stage of the diagnostic procedure is:

- carrying out an ultrasound examination during the first trimester of pregnancy facilitating the identification of the gestational age,
- determination of antibody titers,
- a detailed interview about the complications of the conflict in the Rh system in the course of previous pregnancies [2,3,7].

In addition, the Doppler tests of flow in the foetus's middle cerebral artery and echocardiography are performed [7,8]. The father's blood group determination also has a diagnostic significance. Among invasive methods, spectrophotometry of amniotic fluid and foetal cordocentesis should be enumerated [2].

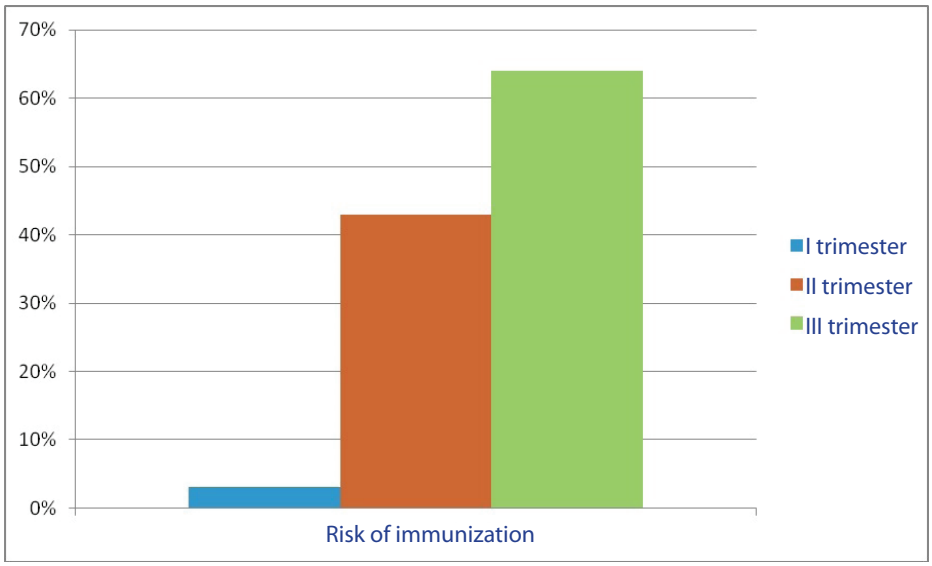
Ultrasound examination

It is a non-invasive test which can be repeated many times and combined with other methods of assessing the foetus's health. It allows you to capture the moment when it becomes necessary to introduce invasive procedures. As already mentioned, this examination is done in early pregnancy to accurately determine the gestational age. This is crucial in further diagnosis. It conditions the correct estimation of the volume of blood circulating in the foetus and bilirubin concentration denoted in the spectrophotometric examination of amniotic fluid. The parameters that facilitate the precise determination of the occurrence of foetal anaemia include:

- the placenta thickness,
- the umbilical vein diameter,
- the liver measurement parameters,
- the spleen circumference,
- the volume of amniotic fluid (polyhydramnios) [2].

Small pericardial effusion, as well as enlarged heart chambers, observed by an ultrasound, are the earliest signs of foetal decompensation of the cardiovascular system of the foetus. Swelling of the scalp, and pleu-

Fig. 1.
Percentage immunization risk depending on the trimester of pregnancy



ral effusions are later signs. Generalized swelling of the foetus is a far-advanced symptom of hemolytic disease. It should be noted, however, that most of the above symptoms appear less reliable in clinical practice of diagnosing severe foetal hemolytic disease[2].

Determination of foetal antigens

The answer to the question of whether the foetus has an antigen to which antibodies produced in the mother's body are directed, is a very important element in the diagnosis of serological conflict. It makes it possible to reduce the group of women who will require close monitoring and possible treatment. In the case of the most common conflict pertaining to the RhD system, knowledge of the foetal D status is very important also from the point of view of non-immunized women. In the case of the foetus which does not have the D antigen, there is no need to supply the anti-RhD immunoglobulin to the pregnant woman. This allows you to save the expensive, scarce preparation [9].

Until some time ago, the determination of D, C, or E antigens of the foetus was done by serological methods, which examined erythrocytes obtained from the foetus by means of cordocentesis. In addition, genetic methods – the examination of amniocytes and placental villi were used. The above methods are burdened with high risk of complications in the form of deepening of the mother's immunization of or even pregnancy loss [4,9].

The latest developments make it possible to examine foetal antigens on the basis of a DNA analysis, using material taken from the mother. This is possible thanks to the discovery by Lo et al. in 1997, of cell free foetal DNA in the plasma of a pregnant woman. It is detectable from 5-7 weeks of gestation and constitutes from 3 to 6% of DNA isolated from the plasma. Its concentration increases in direct proportion to the duration of pregnancy and disappears quickly after its completion [4,9].

Foetal DNA in the plasma in the first half of pregnancy is too low to make it possible to examine foetal genes, and for this reason it is done in the second half of pregnancy, and in fact it can be performed as early as from the 16th week of gestation. For the test 10-15 ml of maternal blood are taken to 2-3 vacuum tubes with a gel barrier and sprayed EDTA. This protection prevents the blood samples from contamination by foreign DNA. 2 ml of the father's blood are also collected – the principle of collection – like for morphology. Maternal blood should be centrifuged within 5 hours from the time of collection. This allows for separation of the plasma from the cellular components by means of the gel barrier. Centrifugation has to be carried out strictly following the tube manufacturer's instructions. They must not be opened. Failure to follow the instructions creates the

risk of depletion of the plasma in foetal DNA. The father's blood should not be centrifuged. The collected samples have to be sent to a molecular biology laboratory within 24 hours, or failing that time – submitted in a frozen state. Defrosting and re-freezing are not recommended, as they cause rapidly progressing degradation of foetal DNA in the plasma. Along with the samples, a referral must be provided indicated the following data: name, surname, social security number, address, hbd, consent for genetic testing, contact telephone number, potential interview, and moreover the information about the patient's race or her partner's, if it is different than Caucasian, and the pregnancy attending doctor's name, phone number and address. In order to isolate foetal DNA, automatic methods are used which are characterized by high efficiency. A high sensitivity *real-time* PCR method makes it possible to translate the presence of the gene to a fluorescent signal. The test consists in finding the gene (allele), which comes from the foetus, while it is absent in the mother's genome [9].

The described examination is quantitative, allowing you to determine whether the positive result is the result involving DNA of the mother or the foetus. The determination of the foetal genotype, like all diagnostic tests, involves risks of false or negatively positive results. False positive results may result from the contamination of the sample with foreign DNA. Their consequence is the unnecessary monitoring of immunized pregnant women by invasive techniques. On the other hand, in the case of women not having anti-RhD antibodies, anti-RhD immunoglobulin is administered unnecessarily (the child is RhD-negative). False negative results are particularly dangerous and they are usually the result of the lack of foetal DNA in the specimen. Assuming that the doctor relies on such a result, we can reckon with the fact that he/she will not undertake the treatment of the foetus with HDN of the mother with antibodies. When it comes to women without antibodies – they will not be given anti-D immunoglobulin. In order to avoid the risk of non-detection of the foetal gene, it has to be proved that foetal DNA is present in the sample. In the case of a male foetus, a gene from chromosome Y is tested. When it comes to female foetuses, there is a need to look for polymorphism inherited from the father and not present in the mother. Such examinations are complicated and conducted in many stages [4,9].

Non-invasive examination of foetal RHD genotype

The RHD gene, which is present in the genome of RhD-positive people, is encoded by the D antigen. The RHD gene is present in 85% of Caucasian, 92% of Negroid and 99% of Mongoloid race (Fig. 2). As regards the phenotype RhD-negative, the basis is highly diverse, and different for different ethnic groups. Ninety-nine percent of RhD-negative Caucasians are devoid of the RHD gene. This makes it possible to directly examine the presence of the RDH gene to determine the RHD genotype. In the case of other races, as well as in a small percentage of Caucasians, the nonfunctional RHD gene is taken as the basis of RhD negative phenotype. Thus, if in the plasma of the RhD-negative woman the RHD gene is detected, this means that it comes from the Rh-positive foetus. The complex construction of the RHD gene and the presence of its many variants make it necessary to use in the real-time PCR examination more than one primer pair coming from various portions of the RHD. Three fragments of the RHD gene are sought, i.e. intron 4 and exon 7 and 10 [4,9].

Non-invasive examination of RHCE gene alleles

Although less well known, serological conflict arising with regard to the antigen *c* of the Rh system is equally dangerous. It is the most common cause of HDN/N immediately after the RhD antigen. HDN/N resulting from the production of anti-c antibodies is usually severe, foetal and/or exchange transfusions are required. A non-invasive examination of the RHCE gene alleles is performed just like the *RHD* examination but specific primers and probes are used [9].

Anti-E antibodies, quite often found in pregnant women, rarely are the cause of HDN/N. However, in perinatology and neonatology cases with a very severe course are recorded [9].

Foetal middle cerebral arterial Doppler assessment

In pregnant women with immune antibodies the ultrasound examination of the middle cerebral artery (MCA) flow of the foetus makes it possible to specify the degree of anaemia of the foetus [2,7]. In the case of the development of foetal anaemia, accelerated blood flow is observed in all the blood vessels of the foetus. It is secondary to reduced blood viscosity, which is also the reason for the increased venous return and increased cardiac output [3]. Due to special characteristics, a measurement of peak systolic velocity (PSV) is used in the MCA. The execution of such an examination is relatively easy. The accuracy of the speed measurement is conditioned by maintaining the 0° angle between the ultrasound beam and the direction of blood flow. Smaller variability between successive measurements, as well the examinations carried out by different people, ensure the performance of the measurement in the MCA proximal section [7].

The influence of gestational age on the measurements is corrected by the use of multiples of the median (MoM) for the MCA-PSV value. One hundred percent sensitivity in detecting moderate to severe anaemia is characterized by the MCA-PSV > 1.5 MoM, assuming a 12% rate of false positive results. The authors' observations show that more important is the trend which persist in the MCA-PSV, rather than a single measurement. Therefore, it is recommended to perform MCA periodically at seven day intervals [2,7].

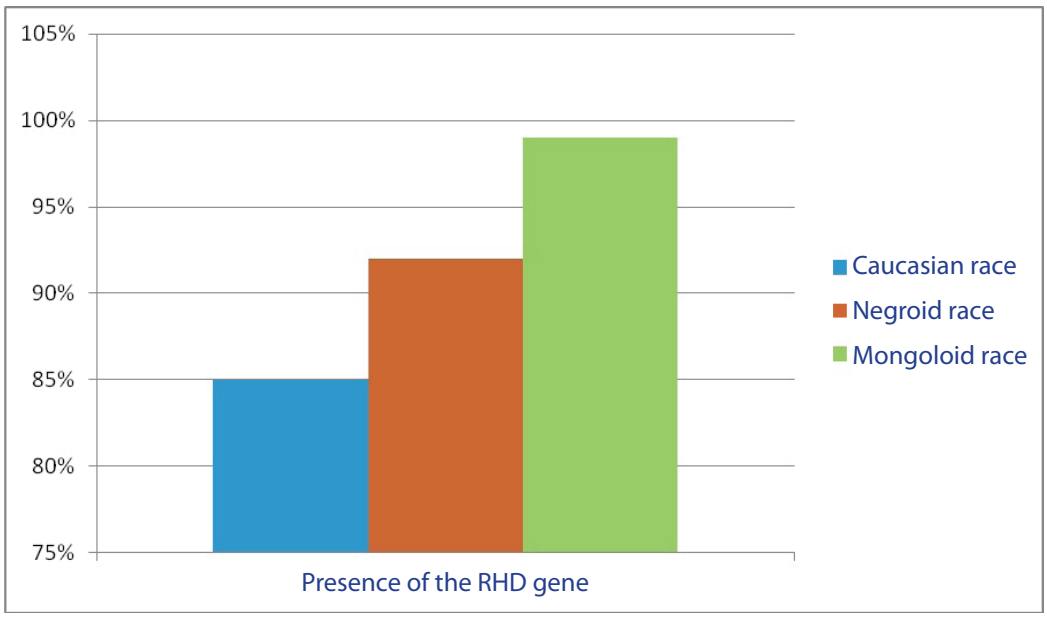
The publication of a first large-scale study, the aim of which was to evaluate Doppler ultrasound, by Mari et al. (2000), resulted in the loss of importance of amniocentesis in detecting foetal anaemia. Reducing the need for invasive procedures is assessed as approximately 2/3 [2,7].

Echocardiography

In the case of serological conflict echocardiography presents the following symptoms:

- increased foetal heart,
- cardiomegaly,
- tricuspid regurgitation,
- accelerated flow rate in the pulmonary artery [8].

FIg. 2.
The presence of the RHD gene depending on human races



Determination of the father's blood group

Given that the risk of serological conflict in terms of the RHD system exists only in the case where the D antigen can be passed to the offspring by the father, a very important element is the definition of the father's blood group genotype. Each child of a father who is a Rh-positive homozygote will have a Rh-positive blood group. On the other hand, children from the relationship of a man who is a Rh-positive homozygote (Dd) and a Rh-negative women have a 50% chance of inheriting either a Rh-positive or Rh-negative blood group [3]. The father's blood group should be determined as soon as possible. In cases where the father is a heterozygote, and maternal serum shows clinically significant value of antibody titers and it is not possible to perform tests which would determine the foetal blood group as a result of identification of foetal DNA isolated from maternal blood, the determination of the foetus's genotype, using CVS test or amniocentesis, should be considered. It is necessary to estimate the potential benefits and risks associated with performing invasive diagnostics [2].

Non-invasive tests – the prospects for the future

There are still technical difficulties in the case of the test of the antigen K from the Kell system, which is encoded by Kel*1 allele and the HPA-1 antigen of blood cells, which appears to be the most important from the clinical point of view. The continuation of research towards the improvement of non-invasive methods depends on the analysis of different physico-chemical properties of DNA coming from the foetus and the mother pertaining to the differences in size of the DNA fragments and methylation [9].

Fetal cordocentesis

Cordocentesis (percutaneous umbilical cord blood sampling – PUBS) facilitates foetal blood sampling under the control of an ultrasound and the assessment of hemolytic disease advancement from week 17/18 of gestation. A sample of foetal blood is usually collected from a transplacental puncture or intra-hepatic vessels. A puncture of the free loop of the umbilical cord is performed only in special cases. Morphology, reticulocytosis, the phenotype of erythrocytes can be determined using the collected blood sample [3]. A regularly repeated diagnostic cordocentesis had been a standard procedure before the effectiveness of estimating the severity of foetal anaemia by means of Doppler flow in the middle cerebral artery of the foetus was proven. The cordocentesis procedure should be performed only by experienced professionals in specialized centres. It is necessary to meet this condition because the risk of pregnancy loss associated with the cordocentesis performance amounts to from 1 to 2% in the case of foetuses without swelling and up to about 15%, especially before hbd 20, in foetuses with generalized swelling [3]. In addition, diagnostic cordocentesis should be performed only in the case of women who have abnormal results of foetal middle cerebral arterial Doppler assessment or spectrophotometric examination of amniotic fluid. The complications of cordocentesis include: foetal bradycardia, hematoma, bleeding into the amniotic sac, the increase of anti-D titers, premature rupture of the amniotic sac, premature delivery, infection [2].

Amniotic fluid spectrophotometry

The amniotic fluid spectrophotometric method is described in this article because of its historical significance, because it should be noted that in most developed and developing countries it has been successfully replaced by non-invasive methods [2].

The assessment of bilirubin concentration in amniotic fluid has been usually performed in the following situations:

- confirming the presence of critical antibody titers in the mother's blood test,
- bad obsteric history,
- other clinical data.

The method was introduced by Liley in 1961, when he made a spectrophotometric examination of bilirubin using a light wave with the length of 450 nm. The test involved 101 Rh-negative immunized patients who were in hbd 27-41. Factors confounding the correct evaluation of spectrophotometry are erythrocytes, meconium and porphyrins decomposition products present in amniotic fluid. In order to eliminate the risk of interference of the above factors with the results, the extinction of amniotic fluid with chloroform is carried out. Liley used the collected data to separate three ranges depending on the gestational age. Zone 1 (lower range) corresponds to the absence of disease or its mild form. Zone 3 (upper range) indicates severe hemolytic disease and high probability of death of the foetus within 7 to 10 days [2].

Whidfield et al., used the determination of bilirubin concentration in amniotic fluid, which indicates the presence and progress of foetal erythrocyte hemolysis, to determine the point at which it is necessary to introduce an invasive intervention in the form of labour or foetus transfusion [2]. The repetition frequency of the determination of bilirubin concentration in amniotic fluid has not been clearly defined yet. It is recommended to repeat the determinations every 3-4 weeks for the results found in zone 1 and every 1 to 4 weeks for the results in zone 2 [2].

The development of diagnostic and therapeutic possibilities in the postnatal intensive care units led to the creation of so-called Liley's "modified" curves, which allow the assessment of bilirubin in amniotic fluid before hbd 27. However, as Queenan et al., demonstrated in their study, in order to be able to talk about reliable spectrophotometric test results one cannot rely on a single measurement, the assessment has to be repeated. For this and many other reasons, including a serious risk of complications such as the amniotic sac damage and premature outflow of amniotic fluid, the described method has not received universal acceptance, and – as mentioned at the beginning of the description – it has mostly historical significance [2].

Summary

It is reasonable that in order to be able to speak about the only appropriate procedure in the case of the threat of serological conflict, special emphasis should be placed on accurate, detailed diagnostics because it is the basis for the implementation of appropriate prevention, and thus, it minimizes complications posed by hemolytic disease of foetuses and newborns. Currently, diagnostics of serological conflict pins high hopes on non-invasive methods of testing of the Rh-positive foetal blood leak into the bloodstream of the Rh-negative mother. We should believe that the application of these methods will become in the near future a standard of care in the case of the threat of this conflict.

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