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GENERAL CHARACTERISTIC OF THE METHODS FOR DETECTION OF DIPHTHERIA TOXIN

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The main virulence factor produced by *Corynebacterium diphtheriae* is diphtheria toxin (DT), an antigenic extracellular protein that is highly toxic for susceptible animals and people. Therefore, the most significant test in the microbiological diagnosis of diphtheria is the detection of the potent and lethal exotoxin from a suspect clinical isolate as rapidly and accurately as possible.

Probably, some external factors such as immunization, use of antibiotics and others could favor the appearance of temporary weak toxin producing Corynebacteria strains. Under such conditions application of the routine methods for characteristic of Corynebacteria virulence potential may be not enough suitable for diphtheria toxin detection [1].

The characteristic of the tests for detection DT will give an opportunity for researchers and laboratory staff to find the correct method and obtain reliable results of toxigenicity investigation.

A number of in vitro (phenotypic or genotypic) and in vivo (biological) methods are used for DT detection. Depending of the aim of the research (to detect only toxin production function of bacteria or amount of exotoxin) the researchers can choose between qualitative and quantitative assays.

The phenotypic in vitro methods for DT detection include:

1. Elek immunoprecipitation test. The most widely used methods for the microbiological diagnosis of diphtheria are those based upon immunologic techniques. This qualitative test based on interaction between toxin produced by Corynebacteria and antitoxic antibody (antitoxin) in solid nutrient medium**.** In the agar's areas where exotoxin cross antitoxin immunoprecipitation lines arise. The Elek immunoprecipitation test is still used in many laboratories worldwide; however, this test is prone to misinterpretation, particularly when it is performed infrequently. Some strains may produce very weak immunoprecipitin lines in Elek tests, and the results for these strains could be misinterpreted. In addition, the clarity and accuracy of the test are dependent upon the constituents of the medium, the concentration of antitoxin, and the use of appropriate control strains [1, 2].

2. Flocculation (Ramon) test. The strength of diphtheria toxin is determined by the flocculation method. For general routine purposes a graduated series of quantities of antitoxin is added to a series of tubes of uniform bases containing toxin to be examined. These are shaken, incubated and examined at suitable intervals until a fine precipitate appears in one tube. This precipitate rapidly becomes more pronounced and flocculates. The rate at which a mixture of toxin and antitoxin flocculates is the essential feature of the Ramon test. Flocculation appears when toxin and antitoxin are mixed in different proportions but under any given conditions the rate of flocculation depends upon the relative proportion of toxin and antitoxin and a balanced mixture will precipitate before all others. The antitoxins against which the toxins are titrated must be standardized by flocculation methods. The test is moderately sensitive and is used in commercial production for determination of diphtheria toxin and anatoxin antigen strength which usually is very high [1].

3. Agglutination assays. These quantitative tests for DT detection based upon the application of erythrocytes (or latex) sensitized with the purified antitoxic antibodies. According to the kind of antibody bearers used for the assay we can differ reversed passive hemagglutination (RPHA) assay and reversed passive latex agglutination (RPLA) assay. In the presence of diphtheria toxin, agglutination of the sensitized red blood cells (latex) occurred – complete agglutination (an even blanket of agglutinated particles) or partial agglutination (a central button surrounded by a halo of agglutinated particles) as the end point. The titer was efined as the reciprocal of the highest dilution in which agglutination was observed. In control experiments without toxin, the antitoxin-sensitized erythrocytes (latex) formed compact buttons in microtiter trays. The RPHA assay can detect less than 20 pg of diphtheria toxin and is comparable in sensitivity to intracutaneous tests for toxin. The lowest concentration of diphtheria toxin detectable by the RPLA assay is about 5 ng/ml [3].

3. Enzyme immunoassay (EIA) developed for the phenotypic detection of diphtheria toxin among clinical isolates of corynebacteria. The assay uses equine polyclonal antitoxin as the capture antibody and an alkaline phosphatase-labeled monoclonal antibody, specific for fragment A of the toxin molecule, as the detecting antibody. The assay is rapid, sensitive, and specific: a final result is available within 3 h of colony selection, and the limits of detection are 0,1 ng of pure diphtheria toxin/ml. Toxigenicity could be detected with isolates grown on a diverse range of culture media, including selective agars [4].

4. Immunochromatographic strip (ICS) test. The assay based upon the using of equine polyclonal antibody as the capture antibody and colloidal gold-labeled monoclonal antibodies specific for fragment A of the diphtheria toxin molecule as the detection antibody. The ICS test has been fully optimized for the detection of toxin from bacterial cultures; the limits of detection are approximately 0,5 ng of diphtheria toxin per ml within 10 min [5].

5. Immunoblotting with a monoclonal antibody specific for the catalytic domain (fragment A) of the

toxin is used to assess the presence of toxin in whole-cell lysates of pathogenic corynebacteria. The highly sensitive method based upon the combination of electrophoresis and EIA. Whole-cell lysates are treated with sodium dodecyl sulfate (SDS) and reducing agent (b-mercaptoethanol) and are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12,5% acrylamide gels. The proteins are transferred onto nitrocellulose membranes and are detected with a monoclonal antibody specific for the catalytic domain of the toxin. The limit of detection of the immunoblot assay is in the range of 0,1 to 1,0 mg/ml; and therefore the assay used is mainly for qualitative determination of toxin production [1].

6. Tissue culture tests for toxigenicity of *Corynebacterium diphtheriae* detection. A number of these qualitative and quantitative assays based upon the DT cytotoxicity relatively to toxin-susceptible tissue cultures (HeLa, CHO, Vero cells). Cytotoxicity of DT can be observed microscopically by cells structural changes (the cells appear rounded and have become detached from the culture dish), visually (by naked eye) – toxin-affected areas of monolayer fail to stain or stain poorly compared with the unaffected areas or colorimetric – by absence of changes in the color of nutrient medium with indicator in the case of cells growth inhibition. The assays are specific, accurate, and reliable for the detection of biologically active DT produced by isolates of *C. diphtheriae*. The limitations of the tissue culture assays which hinder its use in the diagnostic laboratory are the time required for determination of a positive or a negative result and the need for specialized tissue culture facilities [6, 7].

Genotypic methods, based upon polymerase chain reaction (PCR**)**, offer many advantages over phenotypic techniques; they are rapid, simple, and easy to interpret and facilities are becoming increasingly available in many laboratories. The standard PCR assay detects sequences of the diphtheria toxin gene (tox) that code for the A and/or B subunits of diphtheria toxin due to selective amplification by the instrumentality of enzyme – thermostable DNA-polymerase. The new real-time PCR method for the detection of the A and B subunits of the tox gene, with the emergence of new fluorescent probe gene amplification technologies, provide the ability to improve the standard PCR assay by quantitative results, eliminating postamplification handling, and increasing sensitivity. The detection of the DT structural gene by PCR provides a rapid detection method with good sensitivity; results are available within 4 h from the time of selection of only a few bacterial colonies. This method, however, does not provide information on the ability of the organism to express fully functional DT. Any defects or mutations either in the structural gene or in genes coding for regulatory elements required for DT expression may not be detected by this method and it is therefore advisable to use PCR only as an adjunct to phenotypic tests, such as the Elek test. However, an accurate, negative PCR result is useful for the rapid exclusion of toxigenicity [8-11].

In vivo virulence bioassays have always been regarded as the "gold standard" tests for toxigenicity detection. All bioassays is performed using conventional animals which are susceptible to diphtheria toxin (commonly rabbits and guinea pigs). Virulence (toxigenicity) detection are performed by intradermal or subcutaneous injection of the *C. diphtheriae* strains or its cell-free cultures supernatants. For these assays it is preferably to use pure culture of bacteria, because it's quite possible to obtain controversal results by using mixed culture.

The intradermal test for DT detection is mainly reliable. The animals are observed for 48 h, and a positive reaction is assessed by the presence of specific dermonecrotic lesions which are absent in the animals administered preparations treated with diphtheria antitoxin. The skin test, based on the production of erythema at the site of intracutaneous inoculation of toxin in rabbits or guinea pigs, is one of the most sensitive assays. The minimal reactive dose of Standard Diphtheria toxin in skin tests is 0,000025 flocculating units, equivalent to approximately 50 pg of diphtheria toxin. Skin tests are a thousand times more sensitive than flocculation tests or gel diffusion tests.

The subcutaneous test for virulence is more exact than intradermal. The rabbits or guinea pigs are observed on a daily basis for clinical manifestations and systemic effects associated with the production of DT. If the test isolate produced DT, the unprotected animal died within 2 to 5 days; postmortem examination revealed the presence of hemorrhagic and swollen adrenal glands. The control animal remains alive [1, 7].

Thus, the methods for diphtheria toxin detection used in a microbiological laboratory are vary and will dependent upon the facilities and resources available, the expertise of personnel, and the availability of a diphtheria reference laboratory in the country.

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The detection of toxigenicity among *Corynebacterium diphtheriae* strains is the most important test for the microbiological diagnosis of diphtheria. Therefore, we describe the moderately sensitive phenotypic (in vitro) assays for exotoxin detection (Elek test, flocculation test) and more sensitive ones (agglutination assays, enzyme immunoassay, immunochromatographic strip test, immunoblotting, tissue culture tests), as well as genotypic methods, based upon polymerase chain reaction and bioassays (in vivo), which have always been regarded as the "gold standard" tests for toxigenicity detection.

Key words: *Corynebacterium diphtheriae,* diphtheria toxin, toxigenicity, laboratory tests.

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ЗАГАЛЬНА ХАРАКТЕРИСТИКА МЕТОДІВ ВИЯВЛЕННЯ ДИФТЕРІЙНОГО ТОКСИНУ

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Виявлення токсигенності серед штамів *Corynebacterium diphtheriae* є найбільш важливим тестом для мікробіологічної діагностики дифтерії. Тому, наша робота висвітлює як помірно чутливі фенотипічні (in vitro) методи (тест Елека, реакція флокуляції), так і більш чутливі (реакції аглютинації, імуноферментний метод, тест із імунохроматографічними стрічками, імуноблотинг, методи з використанням культур клітин), а також генетичні методи на основі полімеразної ланцюгової реакції та біологічні проби (in vivo), що завжди вважались "золотим стандартом" для виявлення токсигенності.

Ключові слова: *Corynebacterium diphtheriae,* дифтерійний токсин, токсигенність, лабораторні тести.

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ОБЩАЯ ХАРАКТЕРИСТИКА МЕТОДОВ ОБНАРУЖЕНИЯ ДИФТЕРИЙНОГО ТОКСИНА

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Определение токсигенности среди штаммов *Corynebacterium diphtheriae* является наиболее важным тестом для микробиологической диагностики дифтерии. Поэтому, наша работа освещает как умеренно чувствительные фенотипические (in vitro) методы (тест Элека, реакция флоккуляции), так и более чувствительные (реакции агглютинации, иммуноферментный метод, тест с иммунохроматографическими полосками, иммуноблотинг, тесты с использованием культур клеток), а также генетические методы на основе полимеразной цепной реакции и биологические пробы (in vivo), которые всегда считались "золотым стандартом" для определения токсигенности.

Ключевые слова: *Corynebacterium diphtheriae,* дифтерийный токсин, токсигенность, лабораторне тесты.