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(54) Title: NEW DIAGNOSTIC METHOD FOR SPECTROSCOPIC DETECTION OF TUBERCULOSIS

(57) Abstract: The procedure for stabilising and individual substrate for spectroscopic detection of tuberculosis according to this invention, having as starting components 18.1-20.0 ml exudate from pleural effusions, 0.2-1.5 ml of stabiliser for separating turbid and clear centrifugates, 0.3-0.7 ml of a transparent solvent, such as ethanol, dioxane or tetrahydrofuran, of spectroscopic purity, with a novelty in taking from human body in a sterile manner and as customary the quantity of 18.1-20.0 ml as exudate, composed of pleural effusions, to which 0.2-1.5 ml of 10 mass% aqueous solution of guanidinium sulphate, cesium sulphate, potassium dihydrogen phosphate, ammonium sulphate or urea is added, and the obtained mixture is centrifuged for 720-1200 s at 1200-2400 rpm, whereby the obtained clear content is aliquoted to 2.0-3.00 ml, to which 0.3-0.7 ml of transparent solvent (spectroscopic purity), ethanol, is added and the individual substrate sample obtained is frozen at 248-257°K, until use or immediately used for spectroscopic detection of tuberculosis. A new diagnostic method for detecting tuberculosis is defined in this manner.



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NEW DIAGNOSTIC METHOD FOR SPECTROSCOPIC DETECTION OF TUBERCULOSIS

a) Technique field of the invention

The invention belongs to new, spectroscopic screening diagnostic methods in medicine and it refers to a new, additional UV-VIS spectroscopic screening diagnostic method for detection of tuberculosis in pleural effusion through analysis of the individual substrate for spectroscopic tuberculosis detection. According to the international patent classification it has the following designation: A61B-05/00, C12Q01/68 and C12R1/32.

b) Technical problem

Technical problem within this subject matter represents providing such a UV-VIS spectroscopic screening diagnostic method which indicates the presence of tuberculosis in the stabilised substrate *in vitro*, in a new, so far not recorded manner in the professional literature referring to pulmonology. Besides, when tuberculosis is concerned, so far adopted methods in pulmonology, except for the clinical picture and x-rays, are either long-lasting or unreliable (unspecific and with low sensitivity) or invasive or expensive. Clinical picture and x-rays are indicative, but the final diagnosis is made through application and combining of the specified methods which have serious drawbacks.

Making a diagnosis is the common clinical and technical problem. Every additional information in diagnostics, in terms of additional screening diagnostic methods is precious, because it complements the picture of a pathologic process in the organism, facilitating thereby the right final diagnosis.

The basic problem of every diagnostic method (classical and supplementary) is how to make it the least possible invasive or non-invasive, quick and reliable. The illustration of the difficulty of this task is the diagnosis of hypertension, i.e. the fact that the most precise way of measuring blood pressure is the invasive method by using the intra-arterial catheter. Due to invasiveness, technical limitations and financial reasons, it is not suitable for every-day use and screening (Momčilo Babić, Skrinig u medicini, Markom-publik, Beograd, 2001).

Unfortunately, the diagnostic method precision is often in direct proportion to its invasiveness, therefore as such it is the last one to be used. Therefore, the work is being done on developing and finding of supplementary diagnostic methods which are less invasive, but usually less precise as well.

Regarding that all the methods have their advantages and disadvantages and that none of them is either 100% reliable or uniform, the final diagnosis is always made by using and referring to several different diagnostical methods. The problem of making a right diagnose is solved by

developing new additional, quick, screening diagnostic methods which facilitate, complement and speed up the usual standard clinical procedure.

The new diagnostic method must be faster and more informative than the existing ones, less invasive and economically more favourable instrumentation has to be available in its use.

c) Technique status

The success of etiologic diagnosis of lower respiratory pathways diseases depends on the number of micro-organisms in respiratory secretion, then on the fact whether such micro-organism is found in normal oropharyngeal flora composition, as well as on how easily is the agent cultivated. For example, if there is a typical clinical picture of tuberculosis, isolation of tuberculosis bacillus from the sputum is sufficient to diagnose the disease, because the indicated micro-organism is not normally found in the respiratory tract. The drawback of such method is that one must wait six to eight weeks from taking sputum to the final diagnosis. This method is almost useless for diagnostics of tuberculosis in pediatrics, because tuberculosis bacilli which can be isolated are rarely found in the sputum. Therefore antibiotic therapy is often used for the purpose of diagnosing tuberculosis in children in the course of 2 months and if drastic increase of body weight occurs in children, tuberculosis diagnosis can be established (Šićević S., Pluća-Razvoj, anatomija i klinička fiziologija, Institut za zaštitu majke i deteta, RS, Beograd, 1972). Tuberculosis is difficult to diagnose in pediatrics because even Polymerase chain reaction (PCR) method is unreliable (it mainly gives false positive result) therefore, all additional, new fast diagnostic methods are necessary as information which facilitates uncertain diagnosis.

Genus *Mycobacterium* is represented by pathogen micro-organisms (tuberculosis and leprosy causative agents), which are conditionally pathogen (mycobacteriosis causative agents) and of saprophyte kind. Up till now, more than 80 species of mycobacteria have been identified, 30 of which cause diseases in humans. Mycobacteria are straight or slightly bent, slim bacilli, $0.2 - 0.6 \times 10^{-6}$ m of size. Cell wall contains great quantity of lipids which cause acid alcohol resistance, slow growth, virulence and resistance of mycobacteria to action of detergents, bases, acids and antibiotics. Mycobacteria are hard to colour as per Gram (Gram-positive bacilli), but they are easily coloured by carbol fuchsin technique as per Ziel-Nielsen and then they cannot be coloured by alcohol and acid (acid alcohol resistance). The majority of mycobacteria grow slowly (two to eight weeks and longer) on special culture media in aerobic conditions. Beside slowly growing mycobacteria there are also fast growing ones (they form colonies in less than 7 days). Causative agents of tuberculosis are *M. tuberculosis*, *M. bovis* and *M. africanum*. The specified species, together with *M. microti* (does not cause diseases in humans), represent *M. tuberculosis* complex. Of opportunistic species, lungs diseases are caused by *M. avium* complex (*M. avium* and *M. intracellulare*), *M. kansasii*, *M. xenopii*, and rarely *M. scrofulaceum*, *M. chelonae*, *M. fortuitum* and *M. mageritense*. (Gabriel Virella, Microbiology and Infectious Diseases, 3rd edition, Williams&Wilkins, The Science of Review, 1997).

Bacteriological diagnostics of tuberculosis

Bacteriological diagnostics of tuberculosis lasts long (in most cases longer than two months) and it requires repeated taking of patient's material (3 to 5 samples). For routine diagnostics it is sufficient to examine microscopic, cultural and biochemical properties of tuberculosis bacilli. (Simone PM, Cook JL, Upper and lower respiratory tract infections, In: Mahon CR, Manuselis G (Eds.) Textbook of Diagnostic Microbiology, WB Saunders Company, 1995).

Materials from which tuberculosis bacilli can be isolated are the following:

- sputum – morning sputum is taken, 5 to 10 ml,
- bronchoalveolar lavage – if the patient is unable to cough up by himself, material for bacteriological examination can be taken in the course of bronchoscopy,
- laryngeal smear – it is taken in small children and persons who cannot cough up,
- gastric rinse – it is used in the cases of lungs tuberculosis when the sample cannot be obtained in other manner, and not for the diagnosis of stomach tuberculosis. Gastric rinse should be neutralised immediately. Direct preparations are not made of gastric content due to existence of saprophytic acid alcohol resistant (AAR) bacilli contained therein. It is sometimes more sensitive than the bronchoalveolar lavage.
- pleural fluid – anticoagulant agents are added to samples containing fibrinogen (e.g. pleural fluid) in classical clinical biochemical procedure.

Identification of tuberculosis bacillus is performed on the basis of microscopic, cultural and biochemical characteristics of isolated bacterium. (Gabriel Virella, Microbiology and Infectious Diseases, 3rd edition, Williams & Wilkins, The Science of Review, 1997). Biological experiment can be conducted as well if needed.

Microscopic preparation – It is tested directly or from the culture medium.

Direct preparation – It can be prepared before or after material processing and they are coloured by carbol fuchsin technique as per Ziehl –Nielsen (ZN) or fluorescent colours (auramine, rhodamine). On the preparation which has been performed before material processing and coloured as per ZN, straight or slightly bent, slim, red coloured acid alcohol resistant (AAR) bacilli are detected, while all other elements on the preparation are coloured blue (accompanying flora, desquamated cells, leukocytes etc.). On the sediment preparation, after material processing, acid alcohol resistant bacilli are seen as well as the background made of cell detritus. It is necessary to examine the direct preparation cautiously, whole surface and greater number of visual fields. If the preparation is coloured by carbol fuchsin, 300 visual fields should be examined, for which it takes about 15 minutes. In fluorochrome coloration 30 visual fields should be examined (in 1.5 minute) because objective with smaller magnifying possibility is used. The

presence of AAR bacilli can be detected through inspection of direct preparation, Koch's bacillus (BK) cannot be morphologically distinguished from other mycobacteria. In the result of direct microscopy it should be indicated whether AAR bacilli are present and which technique has been used to colour the preparation. The number of AAR bacilli should be determined in positive findings. This method is therefore unspecific and the presence of Koch's bacillus cannot be established on the basis of the same.

Testing of cultural characteristics – Rough colonies similar to cauliflower can be observed on Löwenstein-Jensen medium (LJ) after 3 or more weeks. Media should be cultivated for 8 weeks at least, which is great drawback of this method.

Gas-liquid chromatography can be used for the purpose of identifying mycobacteria, as a fast and simple method for analysis of fat acids and cell wall alcohol composition of these micro-organisms. Drawback of this method is that it is extremely expensive and clinics mainly do not have HPLC for routine analyses and diagnosis making.

Biological experiment. – Guinea pig is a laboratory animal sensitive to BK to which processed material is inoculated into inguinal area. Generalised tuberculosis process and death of guinea pig occur in 8 weeks. This method is long-lasting and it is not applicable because it requires available laboratory animals.

Serodiagnosis. – The application of serologic tests for tuberculosis diagnosis is restricted due to low specificity and weak sensitivity of these tests.

Tuberculin test. – Purified protein derivative (PPD) is used for testing late oversensitivity in tuberculosis. It is most commonly used method in diagnosis of tuberculosis in pediatrics. PPD is administered intradermally by injecting. Results are read after 48-72 hours. The occurrence of indurated erythematous reaction which has value greater than 10 mm in diameter is considered to be positive test result. Positive result does not mean that patient has tuberculosis but only that he was previously exposed to bacterium infection. Vaccination can in such cases contribute to the positive result of the previous exposure to bacterium. False positive result can be obtained in the case of exposure to the action of some other mycobacterium. False negative result is also possible, e.g. in the case of immunosuppression. (Gabriel Virella, Microbiology and Infectious Diseases, 3rd edition, Williams & Wilkins, The Science of Review, 1997; Đurić i sar.: Tuberkuloza, Savremena administracija, Beograd, 1996). Due to everything indicated, although it is the most frequently used method in pediatrics, it cannot be considered reliable but only indicative.

Methods for fast diagnosis of mycobacterial diseases

The identification of mycobacteria on the basis of their cultural and biochemical characteristics is long-lasting due to their slow growth. Fast, specific and sensitive diagnostic methods should be developed in order to control mycobacterial diseases successfully. The following methods can be used in order to gain time (Srboljub Sekulić, Plućne bolesti, Elit Medica, Belgrade, 2000):

1. microscoping of direct preparations,
2. selective liquid culture media with sensitive growth detectors,
3. identification of mycobacteria cell wall components,
4. serodiagnosis, and
5. molecular techniques which include:

(a) typification of mycobacteria species, (b) multiplication of nucleic acids and detection of *M. tuberculosis* from the patient's material, (c) identification of genes responsible for resistance to antituberculous drugs, and (d) standardisation of *M. tuberculosis* – restriction fragment length polymorphism (RFLP).

Microscoping of direct preparations, that is finding of AAR bacilli on direct preparation is the fastest and cheapest method for mycobacteria detection. Identification of mycobacteria on the species level is not possible on the basis of this method and the presence of great number of bacilli in the patient's material (>10000 bacilli/ml of material) is necessary for the positive findings, therefore, this method is unspecific and low sensitive.

Selective liquid culture media with sensitive growth detectors. – The time necessary for detection, identification and testing of BK sensitivity to antituberculous drugs is significantly reduced through usage of selective liquid culture media with sensitive growth detectors. There are two groups of new liquid culture media (radioactive and nonradioactive) into which the growth of mycobacteria is detected on the basis of CO₂ production or O₂ consumption. The presence of mycobacteria is for example detected by radiometric BACTEC method on the basis of their metabolism and not their observable growth. Through metabolising of palmitic acid marked by radioactive carbon being the integral part of the medium, marked CO₂ is being released which is detected by special instrument. The presence of *M. tuberculosis* is detected through application of BACTEC system in approximately 10 days. Method has a serious drawback because it is expensive and because clinical personnel is not trained to perform routine analyses with radioactive media or radioactive carbon on the pulmonology level.

Invasive material collecting for tuberculosis diagnosis represents other diagnostic possibility, i.e. direct collecting of material from lungs through application of methods with which oropharynx is avoided. This is applied in persons when the sample cannot be obtained by coughing up, as well as in cases when the results of inspection of coughed up sputum are diagnostically useless. Basic techniques which are applied are: a) bronchoalveolar lavage, b) transtracheal aspiration, c) pleural puncture, d) percutaneous transthoracic aspiration and e) open biopsy. The first method is not completely reliable because bronchoscope passes through upper respiratory pathways in bronchoalveolar lavage and there is a possibility of contamination of the obtained sample with the oropharyngeal secretion (Srboljub Sekulić, Plućne bolesti, Elit Medica,

Beograd, 2000; Bošnjak-Petrović B: Hronična bolest pluća i TBC, Zbornik radova za XXXVI savetovanje pulmologa Srbije 1992), and other mentioned methods are very invasive.

Routine, clinical diagnostics which is performed through collection of pleural effusions.

A sample of pleural effusion is obtained through pleural puncture, i.e. thoracentesis. The protein content in the effusion gives an approximate information whether transudate (<30 g/L) or exudate (>30 g/L) is in question. The existence of exudate requires additional examination for the purpose of establishing the cause of local disease. In macroscopic terms, exudate can be serous, hemorrhagic, suppurative and rarely – chylous. Serous ones generally occur in specific and unspecific infections, but often in malignities as well; hemorrhagic one occurs mainly in malignity, lungs infarct and injuries, suppurative one in purulent process on pleura. At least one of three criteria must be met for precise routine clinical diagnosis of exudate: ratio of protein content in pleural effusion and in serum > 0.5, ratio of LDH level in pleural effusion and in serum >0.6, LDH level in effusion is greater than 2/3 of normal upper limit in serum. Further analyses of exudate include: description of liquid appearance, glucose level (low in tuberculosis, carcinoma, RA), amylase level (high in pancreatitis effusion), pH value (<7.30), microbiological findings and cytological findings (differential and compulsory inspection to malignant cells). For UV-VIS diagnosis of exudate it is important that it is not hemorrhagic and suppurative.

Pleura biopsy. – Biopsy can provide for histological diagnosis in etiologically unclarified effusion, although regarding that blind needle biopsy is in question, in case untypical results of biopsate have occurred twice, pleuroscopy with targeted biopsy of visible changes or open biopsy through explorative thoracotomy can be considered.

Special requirement for development of additional diagnostic methods when tuberculosis is in question has appeared in infective diseases clinics where HIV positive patients very often have also tuberculosis, therefore, timely and accurate diagnosis is necessary in such cases.

Through detailed inspection of previously specified scientific and professional literature it has been established that there is a very small number of references related to the problem of tuberculosis diagnosis, which at the same time strive to overcome the drawbacks of known invasive, expensive, unspecific, low sensible and long-lasting diagnostic methods. Karl Storz GmbH&Co. specifies in its patent application of 26/09/1996 (EP0861044A1) the invention of an instrument for photodynamic diagnosis *in vivo*, directly from the biological tissue. The problem of diagnosis is present in oncology in almost identical manner where additional diagnostic methods are used in order to overcome the same. Biomax Technologies Inc., describes for example in its patent application of 24/06/1999 (WO 9966830, CA 9900586) an instrument and procedures which enable direct observation of induced tissue by fluorescence by means of endoscope without the need for heavy and expensive auxiliary instruments. On the other side, Board of Regents, The University of Texas System, indicates in its application of 24/12/1997 (WO 97/48329, PCT/US 97/10204) NIR Raman spectroscopy for *in vitro* and *in vivo* detection of cervical precancer. None of these patent applications describes such simple procedure for early diagnosis of malignity as well as the confirmation of malignity diagnosis made through so far routine clinical methods in such a way as does the application of Hemofarm Koncern A.D.

Pharmaceutical-chemical Industry Vršac of 11/12/2001 (P-877/01), by means of relatively simple UV-VIS spectroscopy, which indicates the presence of malignity in stabilised substrate *in vitro*.

Leopold et al. describe in their patent application of 20/08/1998 (WO 98/36089) test kit for diagnosis of tuberculosis through determination of alanine dehydrogenase. Pasteur Institute, Guesdon, Jean-Luc; Hevrier, Daniele indicates in his patent application of July 16, 1998 (W098/30699) polynucleotide coding for 27kd polypeptide of mycobacterium which belongs to the complex of tuberculosis mycobacterim for the purpose of diagnosis and prevention of tuberculosis. Corixa Corporation describes in its patent application of 23/04/1998 (WO 98/6646) compounds which can be incorporated into vaccines or drugs against tuberculosis and which are based on the strengthening of immunity against this disease. Medical Research Council presented in its patent application of 26/05/1992 (WO 92/21697) peptides which have been isolated from the proteins of M.tuberculosis bacilli and which sequence serves for recognition of tuberculosis. K. Archana et al. indicate in their patent application of 06/01/1994 (WO94/00493) immunogenic mycobacteria which serve for development and strengthening of PCR method in diagnostic procedure of tuberculosis. Aktiebolaget Astra describes in its patent application of 22/03/1991 (WO91/14448) new proteins, peptides and corresponding DNA and RNA sequence and trials useful for tuberculosis diagnosis. Cogent Limited specifies in its patent application of 22/02/1990 (WO 90/10085) trials, kits and methods for detection and differentiation of mycobacteria. All these diagnostic procedures are very specific, relatively complicated and expensive. The procedure by means of UV-VIS spectroscopy *in vitro* has been applied in fast and early tuberculosis diagnosis in stabilised substrate and its essence has been indicated in further text.

d) Presenting the invention essence

The invention essence lies in the fact that the disease present in the organism changes the chemism of body fluids, and the changed chemism can be read by screening UV-VIS spectroscopy of the taken and stabilised individual substrate *in vitro*, being recognised by the position (wave length) and number and form of the obtained peaks on UV-VIS spectrum of the recorded effusion (of the stabilised individual substrate).

The procedure of individual substrate stabilising *in vitro* for spectroscopic tuberculosis detection comprises, according to our invention the addition of 0.2-1.5 ml of 10 mass% aqueous solution stabiliser in the fluid taken *in vivo* (pleural punctate) and the obtained mixture is centrifuged in the course of 720-1200s with rotation of 1200-2400 revolutions/min, in the course of which the aliquoted part of 2.0-3.0 ml is taken from the overall volume of the obtained clear content sample, in the course of which 0.3-0.7 ml of transparent solvent of spectroscopic purity is added, and the individual substrate sample obtained is frozen at 248-257°K until use or immediately recorded on UV-VIS spectroscope.

According to preferred embodiment of our invention, the stabilisers having the function of denaturing and stabilising of body fluid, which contains beside other proteins are the following: ammonium sulphate, cesium sulphate, potassium dihydrogen phosphate, guanidinium sulphate, guanidinium chloride, carbamide, lithium chloride, sodium perchlorate sulphate, potassium thiocyanate, potassium II chloride and guanidinium thiocyanate.

According to the most preferred embodiment of our invention, the aqueous solutions of carbamide, ammonium sulphate, cesium sulphate, potassium dihydrogen phosphate and guanidium sulphate are used as stabilisers.

All UV-VIS spectra were recorded in the wave length range of 200 to 800 nm. The spectrum obtained is characteristic for tuberculosis the presence of which was uniquely detected in the analysed pleural effusions.

A broad band with a lot of noise at the top is observed in the spectrum ranging from 230 nm to 280 nm. The absorbance value is between 4 and 5. That broad band is typical of bodily fluids which are a consequence of benign diseases in the organism and the occurrence of intense noise is a characteristic of pleural effusions of patients suffering from tuberculosis. There is a very high plateau in the range from 380 nm to 599 nm which is four times greater than the plateau in the case of malignant disease. Only in the case of tuberculosis the broad band within the range from 380 nm to 500 nm demonstrates 2 peaks. These two peaks are typical of tuberculosis.

The advantage and essence of the invention comparing to the existing diagnostic methods such as all indicated methods in the technique status, is that it is the fastest, informative in the sense that it gives clear indications about the direction of application of the other known additional diagnostic methods, financially affordable and by its nature non-invasive because it uses pleural effusion taken from the diseased human organism as a standard part of the clinical protocol and it also facilitates patient's breathing. Besides, it is confirmative in the sense of coinciding with other clinical methods, e.g. with bacteriological diagnostics (from sputum, bronchoalveolar lavage, laryngeal swab, gastric rinse, pleural punctate), biological experiment, serodiagnosis, tuberculin test, PCR technique etc.

e) Short description of figures

Figure 1 represents typical UV-VIS spectrum characteristic of tuberculosis presence in the patient's organism (miliary tuberculosis), diagnosis confirmed by bacteriological routine procedure.

Figure 2 represents typical UV-VIS spectrum characteristic of tuberculosis presence in the organism when the pleural effusion has been prepared as an individual substrate as in example B.

Figure 3 represents typical UV-VIS spectrum characteristic of tuberculosis presence in the organism when the pleural effusion has been prepared as an individual substrate as in example A.

Table 1 represents all the data related to the selected sample of the certain number (15) of patients whose stabilised bodily fluid has been analysed, where the diagnosis made by UV-VIS spectroscopy uniquely matches the clinical diagnoses obtained by different standard clinical methods.

Table 1

Patient Code	Age/sex	Exudate	Clinical diagnosis	UV/VIS diagnosis
XX02	1950/f	pleural punctate	TBC, bacteriological findings, clinical picture	T
XX05	1963/f	pleural punctate	TBC, PCR, bacteriological findings	T
XX07	1962/f	pleural punctate	TBC, bacteriological findings, PPD	T
XX08	1940/m	pleural punctate	TBC, bacteriological findings, x-rays	T
XX09	1952/f	pleural punctate	TBC, bacteriological findings, PCR	T
XY10	1976/m	pleural punctate	TBC, clinical picture, x-rays	T
XX13	1967/f	pleural punctate	TBC, bacteriological findings, PPD	T
XX14	1937/f	pleural punctate	TBC, bacteriological findings, clinical picture	T
XX22	1942/f	pleural punctate	TBC, bacteriological findings, clinical picture, x-rays	T
XY24	1951/m	pleural punctate	TBC, bacteriological findings, clinical picture, PPD	T
XY27	1983/m	pleural punctate	clinical picture, PPD	T
XX28	1938/m	pleural punctate	bacteriological findings (sputum)	T
XX29	1947/f	pleural punctate	bacteriological findings, x-rays	T
XX30	1962/m	pleural punctate	bacteriological findings, PPT	T
XY32	1958/f	pleural punctate	bacteriological findings, clinical picture	T
XX33	1924/m	pleural punctate	bacteriological findings, clinical picture	T

The patient's age, type of the analysed bodily fluid (pleural punctate) and clinical diagnosis were presented in the table 1. At the end of the table there is findings (diagnosis) obtained by UV-VIS spectroscopic method and tuberculosis presence is marked with T.

Table 1. clearly demonstrates (on the selected sample of 15 patients) that the clinical diagnosis i.e. the presence of tuberculosis uniquely matches the diagnosis made by UV/VIS spectroscopy.

Regarding that pleural effusion is a manifestation of great number of different genesis diseases, all other tests in accordance with doubt based on the clinical picture are carried out for the purpose of establishing the cause of pleural effusion incidence. In view of that, our additional UV-VIS spectroscopic diagnostic method for establishing the tuberculosis presence, as well as our previous patent application (Hemofarm Koncern, Patent Application, P-877/01 of 11/12/2001) for diagnosis of malignity in patients with pleural effusion or ascites or pericardial effusion are of great help for understanding the occurrence of effusion for other reasons, such as e.g.: heart insufficiency, pericardium diseases, cirrhosis of the liver, nephritis syndrome, peritoneal dialysis, mixedem, lungs embolism, sarcoidosis.

It is known that transudate pleural effusions accompany many diseases and conditions, and those are mainly extrapulmonary diseases, mostly diseases of heart, liver and kidneys. Heart

insufficiency is known to represent the most common cause of pleural effusion incidence. Regarding that increase of hydrostatic pressure either in systemic or in lung circulation results in effusion generation, pleural effusion can occur both in left and right heart insufficiency. The signs of this disease are dominant in clinical terms, while arresting changes in lungs, increased heart shadow and reciprocal pleural effusions are evident radiographically in 88%. In 20 cases of all analysed pleural effusions by means of UV-VIS spectroscopy it turned out that heart insufficiency was in question which facilitated the procedure of further patient cardiological treatment.

f) Detailed invention description

Information on the disease of all analysed patients were collected in accordance with the standard clinical protocol and form, which includes age, sex, profession, occurrence of first symptoms; smoking habit, body weight and results of other analyses. It is characteristic for all analysed patients with pleural effusion that they did not have the diagnosis at the moment of UV-VIS spectroscopy and 90% of them were not in therapy (except in cases when in the course of anamnesis taking, they did not reveal which medications they were taking beyond doctor's knowledge and control). Up till now 107 patients with pleural effusions were analysed. Ninety four patients had positive UV-VIS findings, i.e. spectrum of stabilised individual substrate which is characteristic of tuberculosis presence in the organism 87.85%). Six patients had heart insufficiency, 4 of them pneumonia (figure 3b) and the diagnosis which was contrary to other clinical diagnoses was made in 3 patients. Reliability degree was above 87%.

The following part comprises detailed examples of procedures for obtaining the individual substrate for spectroscopic tuberculosis detection (Examples A-S):

Example A. Sterilely take 19 ml of pleural effusion as an exudate in the usual manner from the human body and add 1 ml of stabiliser. 10 mass% aqueous solution of cesium sulphate is used as a stabiliser. Centrifuge the obtained mixture in the course of 900s by the speed of 1500 revolutions in min. Aliquote the obtained clear content to 2.5 ml, add 0.5 ml of transparent spectroscopic 95% ethanol solvent and mix well. Freeze the obtained sample of the individual substrate at 253°K until use or use it immediately for spectroscopic tuberculosis detection.

Example B. Sterilely take 18.5 ml of pleural effusion as an exudate in the usual manner from the human body and add 1.5 ml of stabiliser. 10 mass% aqueous solution of carbamide is used as a stabiliser. Centrifuge the obtained mixture in the course of 1200s by the speed of 2400 revolutions in min. Aliquote the obtained clear content to 2.3 ml, add 0.7 ml of transparent spectroscopic tetrahydrofuran solvent and mix well. Freeze the obtained sample of the individual substrate at 248°K until use or record immediately on UV-VIS spectrophotometer.

Example C. Sterilely take 19.2 ml of pleural effusion as an exudate from the human body in the usual manner and add 0.2 ml of stabiliser. 10 mass% aqueous solution of carbamide is used as a

stabiliser. Centrifuge the obtained mixture in the course of 720s by the speed of 1200 revolutions in min. Aliquote the obtained clear content to 2.7 ml, add 0.3 ml of transparent spectroscopic tetrahydrofuran solvent and mix well. Freeze the obtained sample of the individual substrate at 257°K until use or record immediately on UV-VIS spectrophotometer.

Example D. Sterilely take 18.5 ml of pleural effusion as an exudate from the human body in the usual manner and add 1 ml of stabiliser. 10 mass% aqueous solution of sodium perchlorate is used as a stabiliser. Centrifuge the obtained mixture in the course of 1080s by the speed of 1800 revolutions in min. Aliquote the obtained clear content to 2.8 ml, add 0.5 ml of transparent spectroscopic tetrahydrofuran solvent and mix well. Freeze the obtained sample of the individual substrate at 248°K until use or record immediately on UV-VIS spectrophotometer.

Example E. The procedure is the same as in the example A, the fact that ammonium sulphate has been used as a stabiliser instead of cesium sulphate being the only difference.

Example F. The procedure is the same as in the example A, the fact that guanidinium sulphate has been used as a stabiliser instead of cesium sulphate being the only difference.

Example G. The procedure is the same as in the example A, the fact that potassium dihydrogen phosphate has been used as a stabiliser instead of cesium sulphate being the only difference.

Example H. The procedure is the same as in the example D, the only difference is that dioxane is used as a transparent spectroscopic solvent.

Example J. The procedure is the same as in the example D, the only difference is that tetrahydrofuran is used as a transparent spectroscopic solvent.

Example I. The procedure is the same as in the example A, the only difference is that equivalent quantity of equimolar mixture of ammonium sulphate and potassium dihydrogen sulphate is used as a stabiliser instead of cesium sulphate.

Example K. The procedure is the same as in the example G, the only difference is that tetrahydrofuran is used as a transparent solvent.

Example L. The procedure is the same as in the example G, the only difference is that dioxane is used as a transparent solvent.

Example M. Individual substrate for spectroscopic tuberculosis detection is obtained from 2.5 ml of clear content which was obtained from the centrifuged exudate from the human body to which 1 ml of stabiliser had been previously added and it was mixed with 0.5 ml of transparent solvent and freezed at 253°K until use.

Example N. The procedure is the same as in the example B, the only difference is that calcium II chloride is used as a stabiliser.

Example O. The procedure is the same as in the example B, the only difference is that lithium chloride is used as a stabiliser.

Example P. The procedure is the same as in the example B, the only difference is that guanidium thiocyanate is used as a stabiliser.

Example Q. The procedure is the same as in the example C, the only difference is that dioxane is used as a solvent.

Example R. The procedure is the same as in the example Q, the only difference is that carbamide is used as a stabiliser.

Example S. The procedure is the same as in the example Q, the only difference is that tetrahydrofuran as a solvent and ammonium sulphate as a stabiliser.

Further details of the given invention will be presented in the following examples (1-8), without limitation of the invention range only to these examples.

Example 1: Pleural effusion extraction was performed with the patient XY01 (1955/m) by means of classic thoracocentesis and 2 ml of effusion (without additional finishing) was transferred into quartz civet for UV-VIS spectroscope. The UV-VIS spectrum was recorded on the UV-VIS spectrophotometer: Hewlett Packard, model 8452A in the range from 200 nm to 800 nm. Reference solution was distilled water. The figure 1 presents the obtained UV-VIS spectrum. A broad band with a lot of noise is observed within the range from 230 nm to 275 nm. The peaks absorbance value is between 4 and 5. A plateau with high absorbance value, about 4 is clearly observed in the range between 380 nm and 500 nm and 2 peaks are clearly recognizable.

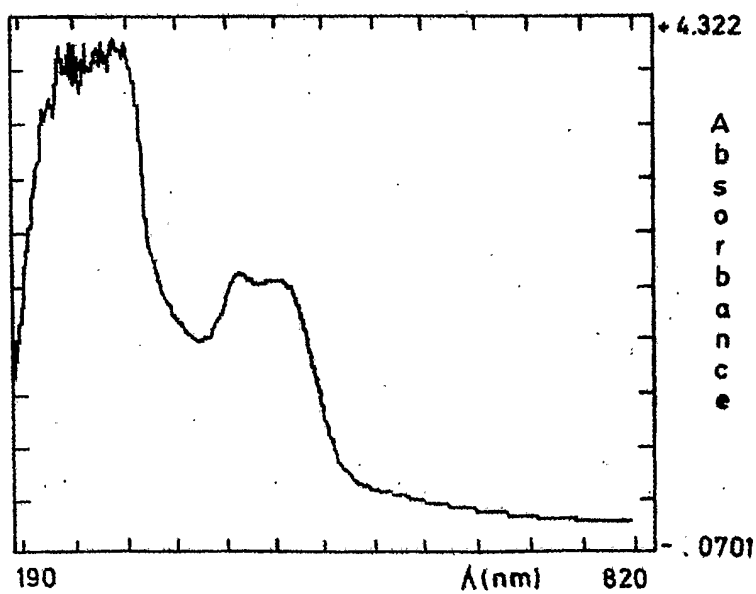


Figure 1

It is the usual spectrum type for human liquid which is the consequence of tuberculosis present in the organism, in this case UV-VIS spectrum of the recorded effusion indicates clearly to tuberculosis. Clinical diagnosis made by means of other diagnostic methods is as follows: primary tuberculosis on the basis of clinical picture, bacteriological findings and x-rays.

Example 2: Pleural effusion was extracted from the pleural region with the patient XX01 (1952, m) and in the moment of extraction there was no clinical diagnosis. 2 ml of pleural effusion of light yellow colour were taken, the individual substrate was prepared according to the procedure analogous to the example B, and it was transferred to the quartz civet for UV-VIS spectroscopy. Reference solution was distilled water. The spectrum was recorded at UV-VIS spectrophotometer: Hewlett Packard, model 8452A within the range from 200 nm to 800 nm. Reference solution was distilled water. The figure 2 presents the obtained UV-VIS spectrum. A broad band with a lot of noise which absorbance value is between 4 and 5 is observed in the range from 230 nm to 280 nm. The broad band with a lot of noise is typical for presence of tuberculosis in the organism. A high plateau distinguished in 2 peaks which is also characteristic of the tuberculosis presence occurs within the range from 380 nm to 599 nm. Clinical diagnosis which was obtained later by other classical diagnostic methods is miliary tuberculosis on the basis of clinical picture and bacteriological findings.

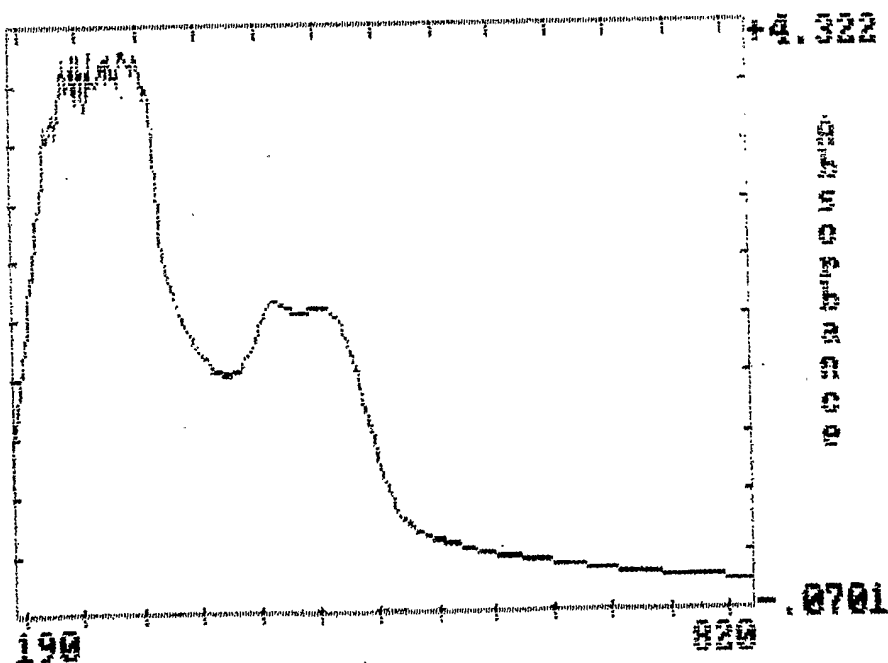


Figure 2

Example 3: The pleural effusion was extracted in the patient XX02 (1946/f). 2 ml of pleural effusion were taken and individual substrate was prepared as in the example A and it was transferred in the quartz civet for UV-VIS spectroscopy. The spectrum was recorded on UV-VIS spectrophotometer: Hewlett Packard, model 8452A in the range from 200 nm to 800 nm. Reference solution was distilled water. The figure 3a presents the obtained UV-VIS spectrum. A broad band with a lot of noise is observed within the range from 230 nm to 275 nm. The peak absorbance value is between 3 and 4. A plateau with high absorbance value distinguished in 2 peaks is clearly observed in the range between 380 nm to 500 nm. It is the usual spectrum type for human fluid which is the consequence of tuberculosis (figure 3a). For the purpose of comparison, the picture 3b presents the spectrum obtained by means of UV-VIS spectroscopy of the stabilised individual substrate of the patient with pneumonia. A broad band with little noise, without distinguished peaks is observed within range from 230 to 275 nm. Peaks absorbance value is between 3 and 4. A flat plateau with high absorbance value is observed within range from 380 nm to 500 nm. It is UV-VIS spectrum characteristic for human liquid which is a consequence of benign disease e.g. of pneumonia or heart insufficiency and it is a confirmation that there is no malign process or tuberculosis in the diseased organism. The recorded UV-VIS spectrum of the pleural effusion in this case clearly indicates to tuberculosis. It was established by means of different diagnostic methods that patient XX02 suffers from primary tuberculosis.

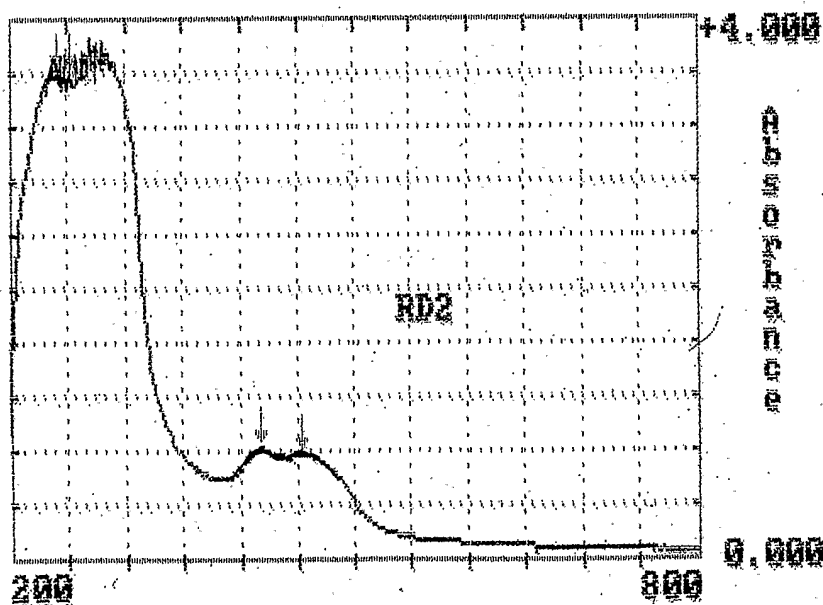


Figure 3a

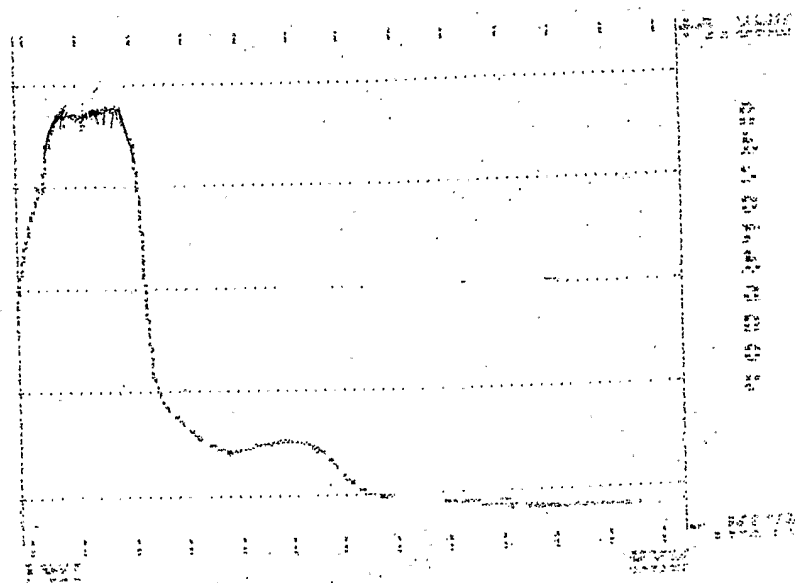


Figure 3b

Example 4: 19 ml of pleural effusion is taken as an exudate from the human body in a sterile manner and as customary and 1 ml of stabiliser is added. 1 ml of 10mass% aqueous solution of cesium sulphate is used as stabiliser. The obtained mixture is centrifuged in the course of 950 s at 1500 revolutions per minute. An aliquot part of 2.5 ml is taken from the obtained clear content, to which 0.5 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 253°K until use or immediately used for spectroscopic detection of tuberculosis.

Example 5: 19.5 ml of pleural effusion is taken as an exudate from the human body in a sterile manner and as customary and 0.5 ml of stabiliser is added. 0.5 ml of 10mass% aqueous solution of ammonium sulphate is used as a stabiliser. The obtained mixture is centrifuged in the course of 800 s at 1600 revolutions per minute. An aliquot part of 2.4 ml is taken from the obtained clear content, to which 0.6 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 250°K until use or immediately used for spectroscopic detection of tuberculosis.

Example 6. 20 ml of pleural effusion is taken as an exudate from the human body in sterile manner and as customary and 0.3 ml of stabiliser is added. 0.3 ml of 10mass% aqueous solution of potassium dihydrogen phosphate is used as a stabiliser. The obtained mixture is centrifuged in the course of 1000 s at 1400 revolutions per minute. An aliquot part of 2.6 ml is taken from the obtained clear content, to which 0.4 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 257°K until use or immediately used for spectroscopic detection of tuberculosis.

Example 7. 18.7 ml of pleural effusion is taken as an exudate from the human body in sterile manner and as customary and 0.8 ml of stabiliser is added. 0.8 ml of 10mass% of aqueous solution of guanidium sulphate is used as stabiliser. The obtained mixture is centrifuged in the course of 1050 s at 1370 revolutions per minute. An aliquot part of 2.7 ml is taken from the obtained clear content, to which 0.3 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 248°K until use or immediately used for spectroscopic detection of tuberculosis.

Example 8. 18.4 ml of pleural effusion is taken as an exudate from the human body in sterile manner and as customary and 1.2 ml of stabiliser is added. 1.2 ml of 10mass% aqueous solution of ammonium sulphate is used as a stabiliser. The obtained mixture is centrifuged in the course of 850 s at 1550 revolutions per minute. An aliquot part of 2.2 ml is taken from the obtained clear content, to which 0.7 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 250°K until use or immediately used for spectroscopic detection of tuberculosis.

Example 9. 18.0 ml of pleural effusion is taken as an exudate from the human body in sterile manner as customary and 1.0 ml of stabiliser is added. 1.0 ml of 10mass% aqueous solution of ammonium sulphate is used as a stabiliser. The obtained mixture is centrifuged in the course of 880 s at 1500 revolutions per minute. An aliquot part of 2.5 ml is taken from the obtained clear content, to which 0.9 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 250°K until use or immediately used for spectroscopic detection of tuberculosis.

Example 10. 18.0 ml of pleural effusion is taken as an exudate from the human body in a sterile manner as customary and 1.0 ml of stabiliser is added. 1.0 ml of 10mass% of aqueous solution of ammonium sulphate is used as a stabiliser. The obtained mixture is centrifuged in the course of 880 s at 1500 revolutions per minute. An aliquot part of 2.5 ml is taken from the obtained clear content, to which 0.9 ml of spectroscopic solvent, 95% ethanol is added and mixed well. The obtained individual substrate sample is frozen at 250°K until use or immediately used for spectroscopic detection of tuberculosis.

Statement of the best usage of the registered invention:

The procedure of stabilising individual substrate for spectroscopic tuberculosis detection, which consists of 2.0-3.0 ml of clear content from the centrifuged exudate from the human body, consisting of 18.1-20 ml of pleural effusion and 0.2-1.5 ml of 10 mass% aqueous solution of potassium dihydrogen phosphate or ammonium sulphate, and which is mixed with 0.3-0.7 ml of transparent spectroscopic solvent, 95% ethanol and which is frozen at 248°K to 257°K until use, according to this invention is best used in a way that the liquid individual substrate is recorded by UV-VIS spectroscopy in the range from 200 to 800 nm, whereby the spectrum characteristic for tuberculosis has, in the range from 230 nm to 275 nm in the response characteristics a broad band of high absorbance with distinct noise, and there is a flat plateau with two easily recognisable peaks in the range from 380 nm to 500 nm, whereby the reliability degree of diagnostics is over 87%.

PATENT CLAIMS

1. The procedure of individual substrate stabilising for spectroscopic tuberculosis detection, is characterised that the substrate taken sterile from the human body as an exudate, pleural effusion is stabilised by adding to the quantity, (0.2-1.5 ml) of 10 mass% aqueous solution of stabiliser, and the obtained mixture is centrifuged in the course of 720-1200s at the rotation of 1200-2400 revolutions/min, whereby an aliquot part of 2.0 – 3.0 ml is taken from the obtained clear content, to which 0.3-0.7 ml of transparent solvent of spectroscopic purity is added, and the obtained sample of individual substrate is frozen at 248-257°K until use or recorded immediately.
2. The procedure according to the claim 1, characterised that compound of the general formula I is used as stabiliser

$$M^+ N^- \quad (I)$$
 wherein,

M^+ - represents guanidium, lithium, calcium, sodium, ammonium, cesium or potassium cation, and

N^- - represents sulphate, chloride, thiocyanite, perchlorate or dihydrogen phosphate anion, or carbamide is used as a stabiliser.
3. The procedure according to the claims 1 and 2 and variant I, characterised that 95% ethanol, dioxane or tetrahydrofuran are used as transparent solvents of spectroscopic purity.
4. The procedure according to the claims 1 and 2 and variant II, characterised that dioxane is the transparent solvent.
5. The procedure according to the claims 1 and 2 and variant III, characterised that tetrahydrofuran is a transparent solvent.
6. The procedure according to the claims 1 and 2 and variant IV, characterised that ammonium sulphate is a stabiliser.
7. The procedure according to the claims 1 and 2 and variant V, characterised that cesium sulphate is a stabiliser.
8. The procedure according to the claims 1 and 2 and variant VI, characterised that potassium dihydrogen phosphate is a stabiliser.

9. New diagnostic method for spectroscopic detection of tuberculosis, characterised that the stabilised individual substrate obtained according to the claim 1 is used in the way that it is recorded by UV-VIS spectroscopy in the range from 200 to 800 nm, whereby the spectrum characteristic for tuberculosis has, in the range from 230 nm to 275 nm, in the response characteristic a broad band with a lot of noise with high absorbance value and a plateau with clearly recognisable two peaks and high absorbance value is present within the range from 380 nm to 500 nm, whereby the reliability degree of diagnostics is over 87%.