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Etiological diagnostics of acute exacerbation of chronic obstructive pulmonary disease: an experience of combined use of bacteriological and virological methods of examination

Keywords: *etiology, pathogen, COPD, exacerbation.*

Etiological diagnostics of acute exacerbation of chronic obstructive pulmonary disease (AE COPD) still remains a challenging problem. This is true mainly to the limitation of our imagination regarding the nature of pathological process. Until now there was isolated more than 100 different species of microorganisms from the lung tissue of patients, who died of lung infection [8]. Regardless of the use of most advanced microbiological methods the etiology of disease can only be verified in half of the cases [7]. From one hand this fact points on limited opportunities of currently available diagnostics, and from another it allows to predict an existence of unknown pathogens. Recent findings in this field have changed current knowledge regarding the etiology of AE COPD.

These findings, mainly viral by nature, become available due to development and introduction into current laboratory practice of novel technologies and molecular methods such as non-isotope hybridization analyses, mass-spectroscopy, DNA-chips hybridization technique, polymerase chain reaction (PCR) and other methods of nucleic acid amplification, technologies which are very expensive, and can be reproduced in highly specialized laboratory facilities only by highly-qualified specialists.

On the other hand scientists work on development of other diagnostic technologies, which are able under conditions of limited resources to provide simple and fast identification of major pathogens without any loss in quality. High specificity and susceptibility, prompt response, simplicity and low cost are the basic requirement for such a techniques. These requirements are completely met in so called fast immunochromatography tests (ICT).

Considering all above mentioned it is evident that an emerging point at present is a choice of novel technological approaches and a development of balanced algorithm of etiological diagnostics of AE COPD.

The aim was to study a spectrum of bacterial and viral pathogens of AE COPD and to evaluate the effectiveness of diagnostics, which include both bacteriological and virological tests.

Materials and methods

165 patients with AE COPD, hospitalized at SO «National institute of phthisiology and pulmonology named after F.G. Yanovskiy NAMS of Ukraine» in 2007–2012 were enrolled. Diagnosis was established using GOLD recommendations [10]. In order to distinguish an infectious nature of exacerbation the criteria, developed by N. Antonisen et al., were used [9].

Bacteriological tests were done at microbiological laboratory of Principal Military hospital of Ministry of Defense of Ukraine (Head of laboratory – I. G. Kostenko). Sputum, spontaneously obtained from lower respiratory tract at deep expectoration before meals, was used for examination. Sputum was taken into sterile containers. Time from collection to processing of sputum didn't accede 1–2 hours at room temperature.

First, microscopy of non-stained sputum smears was done to evaluate quality of samples. A sample was considered usable if Gram-stained sputum smear contained not less than 25 white blood cells and no more than 10 epithelial cells per vision field (×100) [1].

Evaluation of microbial population in sputum was done by a quantitative method according to Dixon and Miller in L. Selina modification on solid media. An isolate was considered etiologically significant when potential pathogen titer reached 10^6 colony forming units per 1 ml [2, 3].

Primary culture was grown on blood and chocolate agar (Columbia agar base). In order to enhance growths of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* the blood and chocolate agar were enriched with 5 % RBC suspension. For identification of facultative flora (*S. aureus*, *Enterobacter spp.,* fungi) the samples were cultured on Saburo, Endo, biliary salts media using conventional methods of isolated colonies, pure cultures and their further identification. Additionally, patient samples were cultured on liquid media (sugar and serum bouillon). Incubation was done at 37 °C in 5 % CO_2 atmosphere.

Isolated strains were identified using API test systems by BioMerieux, France.

Antibiotic susceptibility testing was conducted by means of disc diffusion method on Muller-Hinton agar media by BioMeriex, France [3, 5]. Discs, produced in Russia and USA (BBL), were used. For the purpose of susceptibility testing NCCLS control strains were used (*S. pneumoniae* ATCC 49619, *H. influenzae* ATTC 49247, *S. aureus* ATC 25923, *E. coli* ATC 25922, *P. aeruginosa* ATC 27953 and others).

Atypical strains were identified using real-time PCR. To reveal DNA of *Mycoplasma pneumoniae, Chlamydophila pneumoniae* in clinical samples there were used «AmpliSens® *Mycoplasma pneumoniae/ Chlamydophila pneumoniae*-FL» PCR kits.

For express diagnostics of *S. pneumoniae* and *L. pneumophila* (serotype 1) infections the fast chromatographic tests by Alere Scarborough, Inc. (USA) were applied (respectively, *Streptococcus pneumonia* Antigen Test Kit and *Legionella* Urinary Antigen Test Kit NOW®).

For virology part of study to detect genomic RNA and DNA of respiratory viruses the following molecular biology tests were used: classic PCR, reverse transcription PCR, multiplex PCR, real-time PCR. All those test were performed at Department of virology P. L. Shupik National Medical Academy of Postgraduate Education (chief – Prof. I. Dziublyk).

Nasal smears were taken. Smears were obtained by means of dry cotton swabs with plastic handles. Afterwards, the probes were put into sterile single-use tubes 1,5–2 ml with cap, containing transport media 0,5 ml. To obtain nasal lavage fluid 3–5 ml of warm sterile of isotonic solution of sodium chloride were injected into each nostril. The nasal fluid was placed into sterile tube.

Clinical samples were transported to the lab into temperature-controlled containers at +4 °C.

Separation of nucleic acids (RNA and DNA) of respiratory viruses was conducted as described [6]. For detection of results the electrophoresis and hybridization-fluorescent methods were used. Gel-electrophoresis detection of results with subsequent documentation was done using GeiDoc equipment by BioRad, USA.

The following kits were used:

– for RNA and DNA separation – kits by AmpiSens, Russia;

– for reverce transcription – kits Reverta-L by AmpiSens, Russia;

– for detection and identification of influenzae A and B viruses – AmpiSens® Influenza virus A/B-FL, by AmpiSens, Russia;

– kit A (H_1N_1) 2009 AmpiSens® Influenza virus A/H_1swine-FL, by AmpiSens, Russia;

– for identification of avian flu – kits AmpliSens® Influenza virus A H_5N_1-FL, by AmpiSens, Russia;

– kit for identification of 12 respiratory viruses RV-12 SEE GENE (by ALT, Ukraine);

– kit Seeplex® RV12 ACE Detection (Seegen, Korea);

– kit Seeplex® FluA ACE Subtyping (Seegen, Korea)

– AmpiSens® AVRI-screen-FL (variant FRT), AmiSens, Russia.

To detect influenza A and B RNA AmpiSens® Influenza A/B-FL kit for hybridization-fluorescent detection was used. For reverse transcription PCR the Reverta L kits by AmpiSens, Russia were used. WHO recommendations were followed for PCR testing in the period of influenza and AVRI epidemic and influenza A (H_1N_1) California epidemic. A specific protocol «Influenza A specific simple and real-time PCR» as well as «Reverse transcription PCR in real time», produced by CDC for influenza virus A (H_1N_1) detection (2009 version).

Fast immunochromatographic tests «CitoTest Influenza A&B», «CitoTest ADENO RESPI», «CitoTest RSV Blister» (Farmasco, Ukraie) for express identification of influenza A and B viruses, respiratory adenoviruses and RS-virus were used.

Atypical pathogens (*M. pneumoniae, C. pneumoniae, L. pneumophila*) in clinical samples were identified using real-time PCR.

Results

Baceriological testing of sputum was performed in 165 AE COPD patients. Based on bacteriosopy the biological samples were suitable for analysis in 138 (83,6 %) cases. Sputum and BALF cultures helped to isolate in 92 (66,7 %) patients 103 bacterialcstarins in diagnostic significant concentration (Fig. 1). *H. influenzae*, the major pathogen, was isolated in (46,6 ± 4,9) % of patients. All other microbes were isolated with following rate: *S. pneumoniae* – (20,4 ± 4,0) %, *K. pneumoniae* – (13,6 ± 3,4) %, *M. catarrhalis* – (0,9 ± 0,8) %, *E. coli* – (10,7 ± 3,0) %, *S. aureus* – (4,9 ± 2,1) % and *P. aeruginosa* – (2,9 ± 1,6) % cases. In 11 ((10,7 ±3,0) %) patients an association of pathogens was detected: *H. influenzae* with *K. pneumoniae* – in 3 patients, *H. influenzae* with *S. aureus* – in 1 patient, *S. pneumoniae* with *E. coli* – in 3 patients, *P. aeruginosa* with *S. pneumoniae* – in 1 patient and *H. influenzae* with atypical bacteria (*M. pneumoniae* or *C. pneumoniae*) – in rest of patients.

In one of the most prevalent pathogen – *H. influenzae* – the rate of penicillin, aminopenicillins and chloramphenicol

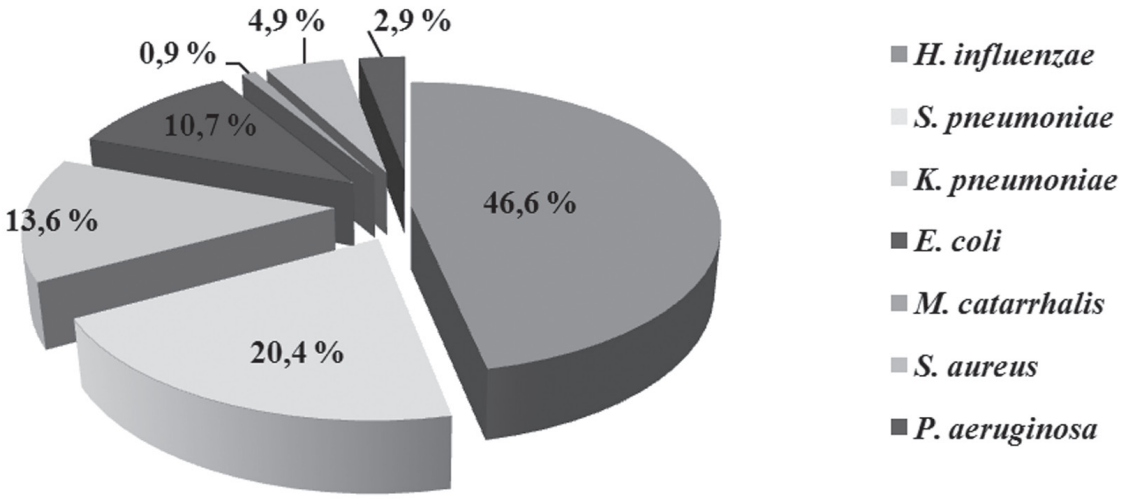

Figure 1. **Prevalence of bacterial pathogens in AE COPD patients**

resistance was (5,8 ± 3,2) % (Tab 1). In (20,4 ± 4,0) % cases the cause of AE COPD was *S. pneumoniae* with penicillin, aminopenicillins and protected aminipenicillins resistance rate of (9,5 ± 6,4) %, ciprofloxacin and chloramphenicol – (14,3 ± 7,6) %. It should be underlined, that (9,5 ± 6,4) % strains of *S. pneumoniae* also posesssed an associated resistance to microlides and 2nd generation fluoroquinolones. At the same time all strains of this pathogen were susceptible to 3rd generation fluoroquinolones (levofloxacin). *M. catarrhalis was isolated in* (0,9 ± 0,8) % of cases. This microorganism was resistant to natural and semisynthetic penicillins. In (13,6 ± 3,4) % of patients *K. pneumoniae* was isolated. (78,6 ± 11,0) % strains appeared resistant to penicillin and aminopenicillins. In (4,9 ± 2,1) % of patients *S. aureus* was detected with resistance to natural and synthetic penicillin of (80,0 ± 17,9) %. *E. coli* was the causative agent in (10,7 ± 3,0) % of COPD patients. A clinically significant penicillin and aminopenicillins resistance rate was found in (63,5 ± 14,5) % of strains, to chloramphenicol – (45,5±15,0) %. In (2,9 ± 1,6) % of patients we isolated *P. aeruginosa*.resistant to amvkacine and levofloxacin of (33,3 ± 27,2) %, and to ciprofloxacin – of (66,7 ± 27,2) % strains.

The presence of atypical pathogens (*M. pneumoniae, C. pneumoniae, L. pneumophila*) in patient' samples was evaluated by real-time PCR. Te samples from 27 AE COPD patients were tested. *M. pneumoniae* was identified in 1 (3,7 %) patient, *C. pneumoniae* – in 2 (7,4 %) patients.

Rapid test for evaluation of *S. pneumoniae* urine antigen was done in 18 COPD patients. The results were compared with the results of cultures. This test was positive in 27,8 % cases, while co-incidence with cultures reached 80,0 %.

Virology tests were carried out in autumn-winter season 2007–2008 years. Clinical samples of 52 AE COPD patients were tested.

Respiratory viruses were identified in 28,8 % of patients using molecular-biological tests. *Influenzae A* (seasonal) virus was found as mono-infection in 5 patients (9,6 %). Respiratory coronavirus (*hCov*) and *Parainfluenza virus* 2 were identified in 4 subjects (7,7 %). In 2 samples we identified rhinovirus (*hRv*): in 1 – mixt-infection «*hPiv* + *hRv*» (Fig. 2).

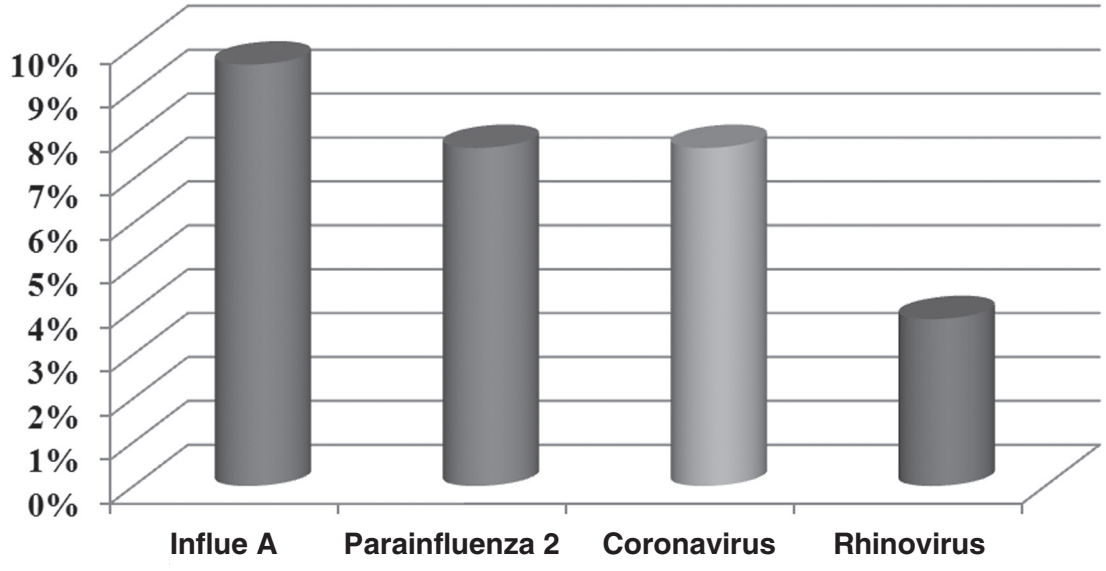

Figure 2. **Distribution of viruses in COPD patients (2007–2008 years)**

Table 1

Antibiotic resistance of AE COPD pathogens, % (M ± m)

Antibiotic	Microorganism						
	H. influenzae **(n = 48)**	***S. pneumoniae*** **(n = 21)**	***M. catarrhalis*** **(n = 1)**	***K. pneumoniae*** **(n = 14)**	***S. aureus*** **(n = 5)**	***E. coli*** **(n = 11)**	***P. aeruginosa*** **(n = 3)**
Penicillin	2 4,2 ± 2,9	2 9,5 ± 6,4	1 100,0	11 78,6 ± 11,1	4 80,0 ± 17,3	7 63,6 ± 14,2	–
Amicillin	2 4,2 ± 2,9	2 9,5 ± 6,4	1 100,0	11 78,6 ± 11,1	4 80,0 ± 17,3	7 63,5 ± 14,2	–
Oxacillin	–	–	1 100,0	–	0	–	–
Amoxicillin/ clvulanate	0	2 9,5 ± 6,4	0	0	0	0	–
Cloramfenikol	3 6,3 ± 3,5	3 14,3 ± 7,0	0	6 42,9 ± 13,2	–	5 45,5 ± 15,4	–
Azythromcin	0	1 4,8 ± 4,7	0	–	1 4,8 ± 4,7	–	–
Amykacin	0	–	0	0	0	0	1 33,3 ± 27,0
Cefuroxim	0	0	0	0	0	0	–
Cefatoxim	0	0	0	0	0	0	–
Ceftriaxone	0	0	0	0	0	0	–
Ciprofloxacin	0	3 14,3 ± 7,0	0	0	–	0	2 66,7 ± 27,6
Levofloxacin	0	0	0	0	0	0	1 33,3 ± 27,6

Markers of viruses (particularly *Influenza A* virus antigens) were identified by means of rapid tests in 5 patients (9,6 %). Respiratory adenoviruses were not identified at all.

The results of rapid tests closely correlated with the results of PCR tests only for seasonal *Influenza A virus.*

Thus, the use of technologically advanced bacteriological and virological tests helped to identify the pathogen in 84,9 % of AE COPD patients, which was 31,5% more effective than under conventional laboratory approaches application ($p < 0,05$).

Discussion of results and conclusions

Current study results one more time confirmed that the spectrum of bacterial pathogens of AE COPD is quite constant, comparing with data published by different authors from all over the world [7, 10]. Unexpected trends and threats considering resistance pattern of respiratory pathogens were not revealed.

As we have demonstrated the use of conventional microbiological tests only for identification of AE COPD causative agents was not sufficient. Hence, etiological diagnostics should include novel virological methods. Multiplex PCR belong to the advanced methods of diagnostics, developed for use of several primers in one tube in order to identify nucleic acids fragments of different germs. Currently, kits for multiplex PCR are available on the market, registered in Ukraine and indicated for identification and differentiation of wide spectrum of respiratory pathogens.

At the same time, clinical practice now require confirmation of etiology of disease under conditions of limited resources, when novel instrumental biological methods, costly equipment and highly qualified personnel are not available. Rapid test is the best solution of the challenge like this, allowing the results in shortest terms – 10–15 minutes. Among the advantages of this method are high specificity and sensitivity, high reproducibility of results. They are technically simple, requiring no special preparation of clinical samples, and easy to read. Rapid tests also have internal control, which can confirm the quality of test and minimize the chance of errors. Undeniable is the fact that these tests are much cheaper in comparison with a majority of classic and molecular-biological methods. But, depending on specificity/sensitivity current tests can sometimes be false-positive or false-negative. It is essential to consider verification of results for higher effectiveness of diagnostic algorithm. For this purpose the immune enzyme analysis or PCR can effectively serve.

In summary, the process of etiological diagnostics of AE COPD should include the following. The smears or nasal washings, sputum, BALF, blood and urine are tested. The patient samples should be sent to both microbiological and virological laboratories for application of bacteriological and molecular-biological tests. The rapid-tests could be performed elsewhere: at ER unit, the ward, medical office or laboratory. Clinical sample should be obtained prior to antibiotic therapy start.

Bacteriological method must include at minimum microscopy of Gram-stained sputum smears, and cultures (blood cultures in severe cases).

Molecular-biological tests (real-time PCR) should be used for identification of atypical bacteria and respiratory viruses. For instance, Seeplex® RV12 ACE Detection kits are used to identify nucleic acid fragments of *Humanadenovirus* (*AdV*), *influenza A virus (FluA)*, *Influenza B virus (FluB)*, *Human respiratory syncytial virus A (RSVA)*, *Human respiratory syncytial virus B* (*RSVB*), *Human metapneumovirus (MPV), Human parainfluenza virus 1 (PIV1), Humanparainfluenzavirus 2 (P1V2)*, *Humanparainfluenzavirus 3 (PIV3)*, *Human rhinovirus A/B (HRV)*, *Human coronavirus 229E/NL63 (229E/NL63)*, *Human coronavirus OC43/HKU1 (OC43/HKU1)* viruses.

Positive for influenza A virus samples must be additionally tested to identify pandemic influenza A (swine H_1N_1) virus, seasonal influenza A virus (human H_1N_1), seasonal influenza A virus (H_3N_2) and avian influenza A virus (H_5N_3).

Express testing at patient's bed should be done within 10–15 minutes using patented in Ukraine rapid-tests, which allows identification of *S. pneumoniae, L. pneumophila,* influenza viruses A and B, respiratory adenoviruses and RS-virus.

We established that simultaneous use of three different methodologies (classic bacteriology, rapid tests and PCR) for detection of respiratory pathogens is effective strategy in terms of confirmation of etiology of COPD exacerbation. Moreover, inclusion of advanced multiplex PCR commercial tests for detection of 6–12 and even more respiratory viruses in diagnostic algorithm gives an important information regarding possible mono- or co-infection (viral-viral or viral-bacterial) in very short time (6–8 h). In certain cases use of rapid tests alone helps to make a precise etiological diagnosis in 10–15 minutes.

Combined use of different diagnostic methods require further studies and validation of their efficacy considering casts and availability in healthcare network.

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References

1. *Борисов*, *Л. Б.* Медицинская микробиология, вирусология, иммунология [Текст] / Л. Б. Борисов. – М., 2002. – 736 с.

2. *Визначення* чутливості мікроорганізмів до антибактеріальних препаратів [Текст] / МОЗ України, Державна санітарно-епідеміологічна служба. – К., 2007. – 74 с.

3. *Кречиков*, *О. И.* Выделение, идентификация и определение чувствительности к антибиотикам *Streptococcus pneumoniae* [Текст] / О. И. Кречиков, Р. С. Козлов, Т. М. Богданович // КМАХ. – 2000. – Т. 2, № 1. – С. 88–98.

4. *Полімеразна* ланцюгова реакція в лабораторній діагностиці інфекційних хвороб [Текст] / За ред. І. В. Дзюблика, Н. Г. Горовенко. – К., 2012. – 219 с.

5. *Практические* аспекты современной клинической микробиологии [Текст] / Л. З. Скала [и др.]. – М. : ЛАБИНФОРМ, 1997. – 184 с.

6. *ПЦР* в реальном времени [Текст] / Под ред. Д. В. Ребриков. – М. : Бином, 2009. – 223 с.

7. *Синопальников*, *А. И.* Внебольничные инфекции дыхательных путей [Текст] / А. И. Синопальников, Р. С. Козлов. – М. : Премьер-МТ, 2007. – 354 с.

8. *Чучалин*, *А. Г.* Актуальные вопросы диагноза в пульмонологии [Текст] / А. Г. Чучалин // Пульмонология. – 2001. – № 1. – С. 6–11.

9. *Antonisen*, *N. R.* Antibiotic therapy in exacerbations of chronic obstructive pulmonary disease [Text] / N. R. Antonisen, J. Manfreda, C. P. Warren // Ann. Intern. Med. – 1987. – Vol. 106, Suppl. 2. – P. 196–204.

10. *Global* strategy for the diagnosis, management and prevention of chronic obstructive pulmonary disease (revised 2011) [Електронний ресурс]. – Режим доступу: http://www.goldcopd.org.

ЭТИОЛОГИЧЕСКАЯ ДИАГНОСТИКА ИНФЕКЦИОННОГО ОБОСТРЕНИЯ ХРОНИЧЕСКОГО ОБСТРУКТИВНОГО ЗАБОЛЕВАНИЯ ЛЕГКИХ: ОПЫТ КОМБИНИРОВАННОГО ИСПОЛЬЗОВАНИЯ БАКТЕРИОЛОГИЧЕСКИХ И ВИРУСОЛОГИЧЕСКИХ МЕТОДОВ ИССЛЕДОВАНИЙ

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Резюме. *В последнее время все большее внимание уделяется вирусным возбудителям обострений хронического обструктивного заболевания легких (ХОЗЛ).*

Целью *роботы было изучение спектра бактериальных и вирусных возбудителей инфекционного обострения ХОЗЛ и оценка эффективности этиологической диагностики, включающей в себя одновременное использование бактериологических и вирусологических методов исследования.*

Материалы и методы. *В исследование были включены 165 пациентов с инфекционным обострением ХОЗЛ, которые находились на стационарном лечении. Для определения этиологии инфекционного процесса одновременно использовали классические микробиологические, иммунохроматографические и вирусологические (в том числе и новейшие молекулярно-генетические) методы исследования.*

Результаты. *Применение высокотехнологичных бактериологических и вирусологических методов исследования дало возможность идентифицировать возбудителя у 84,9 % больных с инфекционным обострением ХОЗЛ, что на 31,5 % выше, чем при использовании традиционных подходов ($p < 0,05$).*

Выводы. *Одновременное использование трех различных методических подходов (классического бактериологического, быстрых тестов и ПЦР) для определения респираторных возбудителей является эффективным для получения окончательного результата и улучшает этиологическую диагностику инфекционного обострения ХОЗЛ.*

Ключевые слова: *этиология, патоген, ХОЗЛ, обострение.*

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ETIOLOGICAL DIAGNOSTICS OF ACUTE EXACERBATION OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE: AN EXPERIENCE OF COMBINED USE OF BACTERIOLOGICAL AND VIROLOGICAL METHODS OF EXAMINATION

Ya. O. Dziublyk

Abstract. *Viruses as the pathogens of acute exacerbation (AE) of chronic obstructive pulmonary disease (COPD) are now in focus of attention of scientific community.*

The aim *of current survey was to study a spectrum of bacterial and viral pathogens of COPD AE and to evaluate an effectiveness of etiological diagnostics, which combines both bacteriological and virological tests.*

Materials and methods. *One hundred and sixty five patients with AE COPD were enrolled into the study. In order to identify a causative pathogen the classic microbiological and novel virological tests (including molecular-genetic ones) were used simultaneously.*

Results. *By using of technologically advanced tests the AE etiology was identified in 84,9 % of patients, which was 31,5 % higher, comparing with the use of conventional methods ($p < 0,05$).*

Conclusion. *Simultaneous use of three different methodological approaches (classic bacteriology, quick tests and PCR) for detection of respiratory pathogens is an effective diagnostic tool improving the effectiveness of etiological diagnostics.*

Key words: *etiology, pathogen, COPD, exacerbation.*

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