A NEW PROCEDURE FOR THE RAPID DETERMINATION OF *PSEUDOMONAS AERUGINOSA* BACTERIA IN MEDICATIONS

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Introduction. *The microbiological quality of non-sterile products covers two relevant aspects: firstly, the assessment of microbial load, and secondly, the absence of pathogenic microorganisms. The absence of certain pathogenic microorganisms in a preparation depends on their route of administration. Oral pharmaceutical products require the absence of microorganisms such as Salmonella and coliform bacteria, such as Escherichia coli. On the other hand, topical products require the absence of species like Staphylococcus aureus and Pseudomonas aeruginosa. The methods commonly used for identifying microorganisms in medications are laborious and time-consuming. Considering these factors, the development of rapid methods for determining unacceptable microorganisms in medications is an urgent necessity.*

Material and methods. *This is an experimental study in which reference bacterial strains, medicinal preparations, components, ingredients, selectivity factors, and inhibition factors were utilized. For the preparation of the culture medium, high-purity reagents registered in the Republic of Moldova were used.*

Results. *Following the conducted studies, a rapid procedure has been developed for determining P. aeruginosa bacteria in medications. Detection of individual P. aeruginosa cells is possible after 9-12 hours of incubation, while results for concentrations of 104-105 CFU are obtained within 5-6 hours. The medium stands out for its high selectivity and specificity, predominantly for these bacteria, and is economical and straightforward to apply. The shelf life of the medium is two years (observation period).*

Conclusions. *The developed procedure is valuable for the rapid determination of P. aeruginosa bacteria, and the accuracy of the results will enable the timely implementation of microbiological monitoring strategies for large batches of medications.*

Cuvinte-cheie: *procedeu, determi-nare rapidă, Pseudomonas aeruginosa, mediu de cultură, medicament.*

UN NOU PROCEDEU DE DETERMINARE RAPIDĂ A BACTERIILOR *PSEUDOMONAS AERUGINOSA* ÎN MEDICAMENTE

Introducere. *Calitatea microbiologică a produselor nesterile acoperă două aspecte relevante: evaluarea încărcăturii microbiene și absența microorganismelor patogene. Absența unor microorganisme patogene într-un preparat este în funcție de calea lor de administrare. Astfel, în produsele farmaceutice de uz oral nu trebuie să existe microorganisme din genul Salmonella și bacterii coliforme, cum ar fi Escherichia coli, iar în produsele topice sunt inadmisibile speciile Staphylococcus aureus și Pseudomonas aeruginosa. Întrucât metodele actuale de identificare a microorganismelor în medicamente sunt laborioase și necesită mult timp, dezvoltarea de metode rapide este o necesitate stringentă.*

Material și metode. *În prezentul studiu experimental au fost utilizate tulpini bacteriene de referință, preparate medicamentoase, componente, ingrediente, factori de selectivitae și de inhibiție. Pentru prepararea mediului de cultură au fost utilizate reactive de puritate înaltă înregistrate în Republica Moldova.*

Rezultate. *În urma studiilor efectuate s-a elaborat un procedeu rapid pentru determinarea bacteriilor P. aeruginosa în medicamente. Indicarea celulelor unice de P. aeruginosa este posibilă peste 9-12 ore de incubare, iar la concentrații de 10^4-10^5 UFC rezultatele se obțin timp de 5-6 ore. Mediul de cultură se distinge prin selectivitate și specificitate înaltă preponderent pentru această specie bacteriană, este econom și simplu în aplicare și are un termen de păstrare de doi ani (termen de observare).*

Concluzii. *Procedeul elaborat este util pentru determinarea rapidă a bacteriilor P. aeruginosa în medicamente, iar veridicitatea rezultatelor permite aplicarea în timp util a strategiilor de monitorizare microbiologică a loturilor mari de medicamente.*

INTRODUCTION

The microbiological control of medication is one of the essential missions in ensuring the quality surveillance of human pharmaceuticals, in accordance with the provisions of the current analytical and normative documentation. Currently, medical practice and observations in the pharmaceutical field demonstrate that numerous pharmaceutical forms, often overlooked, may contain bacteria and/or fungi in significantly varied proportions and diverse species, mostly saprophytic. Their contamination with pathogenic, conditionally pathogenic, or even saprophytic microorganisms increasingly raises public, collective, or individual health concerns. These medications pose a significant risk, especially in the treatment of burns, wounds, and severe ulcerations on large denuded epidermal surfaces. Additionally, indigenous intravenous medications prepared in hospitals, which carry a high risk of microbial contamination, have a short shelf life, leading to incidents that have resulted in fatalities (1, 2).

The increasing number of pharmaceutical recalls in recent years has once again made us aware of how crucial the microbiological quality of medications is. The presence of microorganisms in medications can become a source of infectious disease spread through them, especially when contaminated products are processed industrially in large batches (3).

The stability of a medication, alongside efficacy, purity, and safety, is a crucial factor in ensuring its quality. A medication considered stable maintains the quality characteristics imparted during preparation within the limits specified by official standards for a specified period. The medication should exhibit physical, chemical, and microbiological stability. Any change in the medication, whether apparent or not, as long as it falls outside the specifications provided by the manufacturer, can compromise its efficacy and safety. Medication incompatibilities may arise, and the presence of unacceptable microorganisms can introduce sources of contamination (1, 4).

In turn, the metabolic activity of microorganisms can induce physico-chemical instabilities, leading to the production of pyrogenic metabolites, a decrease in pharmacokinetic properties, and the nullification of therapeutic effects. This can instill doubt in the patient regarding the benefits offered by the medication.

In the treatment of various diseases, various pharmaceutical forms are used based on their content, mode of administration, and therapeutic action. These forms may be exposed to the unfavorable factors mentioned above. These preparations fulfil the indispensable conditions for the development of microorganisms; hence, a primary concern is to avoid microbial pollution from the technological preparation phase to the administration phase. The increasing number of pharmaceutical product recalls in recent years has once again made us aware of how crucial the microbiological quality of medications is (2, 4).

The *aim* of the study is to develop a procedure for the rapid detection and identification of unacceptable microorganisms in medications focusing on *P. aeruginosa*, which involves the culture medium for rapid multiplication and detection, as well as a minimal set of tests for swift identification.

MATERIAL AND METHODS

Bacterial strains

In this study, seven bacterial strains from the American Type Culture Collection (ATCC) and the National Collection of Strains from the United Kingdom were utilized, belonging to four species: *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas aeruginosa* ATCC 49189, *Pseudomonas aeruginosa* NCTC 6750, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Staphylococcus epidermidis* ATCC 149990. Prior to the study, the strains were precultivated in Tryptic Soy Broth. All microbial strains were cultured on specific media for each species. The utilized media included Tryptic Soy Agar, Mannitol Salt Agar, MacConkey Agar, Blood Agar, Peptone Agar (BioMérieux, France), and MCS-Ps medium. The study also involved the use of medications labelled as "sterile," oral and topical preparations, components of formulations for micro-pellicle media, reagents, and chemical compounds for achieving selectivity, factors promoting multiplication, and inhibition factors obtained from official distributors.

A microbiological method was employed in the study, comparing known methods for the detection and identification of *Pseudomonas aeruginosa* with the proposed rapid determination proce-

dure, the investigation of the selectivity of the concentrated micro-pellicle medium, the examination of the specificity of the rapid procedure, a comparative study of established identification means with the developed one, and the microbiological control of the medication regulated by the European Pharmacopoeia compared with the control of the medication using the proposed procedure and algorithm (5).

Development of the culture medium

The proposed procedure consists of the micro-pellicle culture medium, which includes in its composition casein hydrolysate, gelatin, sodium malonate, cetyltrimethylammonium bromide, sodium chloride, water-soluble bromothymol blue, monopotassium phosphate, and disodium phosphate. The research was conducted using materials and high-purity reagents from "Sigma-Aldrich".

For the preparation of the medium, stock solutions of phosphates are prepared in advance to obtain the phosphate buffer solution at pH 6.9-7.0, a 10% gelatin solution, and a 0.5% bromothymol blue solution.

The preparation of phosphate stock solutions takes place using the following method: monopotassium phosphate (KH2PO4) – 9.078 g is dissolved in 1000 ml of distilled water, and disodium phosphate (Na2HPO4) – 11.876 g in 1000 mL of distilled water until complete dissolution.

The 10% gelatin solution is prepared in a sterile tube by introducing 1.0 g of crystallized gelatin and 9.0 mL of distilled water. It is left for 45-50 minutes to swell, then heated in a water bath at a temperature of 85-90°C, stirring with a glass rod until completely dissolved.

For the preparation of the 0.5% bromothymol blue solution, in a sterile tube, 10.0 mL of sterile distilled water is poured, and 0.05 g of water-soluble bromothymol blue is added. The tube is then heated for 45 minutes in a water bath at a temperature of 85-90°C.

The preparation of the culture medium involves introducing casein hydrolysate, sodium malonate, cetyltrimethylammonium bromide, sodium chloride, sterile distilled water, disodium phosphate, and monopotassium phosphate into a clean and sterile chemical retort. The mixture is stirred, and gelatin solution and bromothymol blue solution are added. The content is mixed to obtain a liquid culture medium, which is then dispensed in 0.2 ml portions into sterile bottles using a measured pipette. The bottles are dried at a temperature of +37°C for 24-48 hours and sterilized under the action of ultraviolet rays for 90-120 minutes. The tubes are sealed with sterile rubber stoppers and then with metal caps. Thus, the developed medium presents a sterile micro-pellicle fixed at the bottom of a 10.0 mL bottle.

RESULTS

For the determination of *P. aeruginosa* bacteria in the bottle with the medium, 2.0 ml of sterile distilled water and the strain of *P. aeruginosa* in a concentration of 10^1 .... 10^9 are applied. The medium dissolves within 2-3 minutes, and then it is incubated in a thermostat at a temperature of 37°C for up to 9 hours.

In the presence of *P. aeruginosa* bacteria in the examined material, the color of the mixture in the bottle changes from green to blue. Meanwhile, in the control bottle, the color remains unchanged (remains green).

The time for the detection of *P. aeruginosa* bacteria depends on the initial concentration of the germs in one milliliter of the examined material. The detection of individual P. aeruginosa cells is possible after 9-24 hours of incubation, while the detection of concentrations of 10^4-10^5 CFU/mL takes 5-6 hours of incubation at a temperature of 37°C (tab. 1).

We determined the sensitivity and selectivity of the proposed medium. To assess the selectivity of the medium, we conducted a series of experiments with four batches of microorganisms in association, in 32 repetitions. When forming the associations, we selected the microorganisms deemed unacceptable in the majority of medications.

I. *P. aeruginosa* ATCC 27853 (10^2) + *S. aureus* ATCC 25923 (10^6); *P. aeruginosa* ATCC 27853 (10^2) + *E. coli* ATCC 25922 (10^6); *P. aeruginosa* ATCC 27853 (10^2) + *S. epidermidis* ATCC 149990 (10^6).

II. *P. aeruginosa* ATCC 27853 (10^3) + *S. aureus* ATCC 25923 (10^6); *P. aeruginosa* ATCC 27853 (10^3) + *E. coli* ATCC 25922 (10^6); *P. aeruginosa* ATCC 27853 (10^3) + *S. epidermidis* ATCC 149990 (10^6).

III. *P. aeruginosa* ATCC 27853 (10^4) + *S. aureus* ATCC 25923 (10^6); *P. aeruginosa* ATCC 27853 (10^4) + *E. coli* ATCC 25922 (10^6); *P. aeruginosa* ATCC 27853 (10^4) + *S. epidermidis* ATCC 149990 (10^6).

IV. *P. aeruginosa* ATCC 27853 (10^5) + *S. aureus* ATCC 25923 (10^6); *P. aeruginosa* ATCC 27853 (10^5) + *E. coli* ATCC 25922 (10^6); *P. aeruginosa* ATCC 27853 (10^5) + *S. epidermidis* ATCC 149990 (10^6) (tab. 2).

Table 1. The time of detection of *P. aeruginosa* based on their initial concentration (CFU/mL).

Microorganism concentration (CFU/mL)	Detection time, hours									
	1	2	3	4	5	6	7	8	9	24
10^1	-	-	-	-	-	-	-	+	++	++
10^2	-	-	-	-	-	-	-	+	++	++
10^3	-	-	-	-	-	-	-	++	++	++
10^4	-	-	-	-	-	+	++	++	++	++
10^5	-	-	-	-	+	++	++	++	++	++
10^6	-	-	+	++	++	++	++	++	++	++
10^7	-	+	+	++	++	++	++	++	++	++
10^8	-	-	+	++	++	++	++	++	++	++
10^9	-	+	++	++	++	++	++	++	++	++
>2 mld.	++	++	++	++	++	++	++	++	++	++

Note: „++" – pronounced positive reaction; „+" – positive reaction; „-" – negative reaction.

Table 2. The selectivity of the culture medium.

Microbial species	Exp. No.	Detection, hours			p	
		6	9	24	6.9	9.24
***P. aeruginosa* (10^2) *S. aureus* (10^6)**	32	0	90.6±1.9	96.9±2.1	-	<0.05
***P. aeruginosa* (10^2) *E. coli* (10^6)**	32	0	93.8±2.0	100±0.0	-	<0.05
***P. aeruginosa* (10^2) *S. epidermidis* (10^6)**	32	0	87.5±1.7	96.9±2.1	-	<0.05
***P. aeruginosa* (10^3) *S. aureus* (10^6)**	32	0	93.8±2.0	100±0.0	-	<0.05
***P. aeruginosa* (10^3) *E. coli* (10^6)**	32	0	96.9±2.1	96.9±2.1	-	<0.05
***P. aeruginosa* (10^3) *S. epidermidis* (10^6)**	32	0	100±0.0	100±0.0	-	-
***P. aeruginosa* (10^4) *S. aureus* (10^6)**	32	9.4±0.6	100±0.0	100±0.0	<0.001	-
***P. aeruginosa* (10^4) *E. coli* (10^6)**	32	6.2±0.4	96,9±2.1	96.9±2.1	<0.001	<0.05
***P. aeruginosa* (10^4) *S. epidermidiss* (10^6)**	32	9.4±0.6	96.9±2.1	100±0.0	<0.001	<0.05
***P. aeruginosa* (10^5) *S. aureus* (10^6)**	32	28.1±1.1	96.9±2.1	100±0.0	<0.001	<0.05
***P. aeruginosa* (10^5) *E. coli* (10^6)**	32	31.3±1.2	100±0.0	100±0.0	<0.001	-
***P. aeruginosa* (10^5) *S. epidermidis* (10^6)**	32	28.1±1.1	96.9±2.1	100±0.0	<0.001	<0.05

Based on the obtained results, we have determined that the medium exhibits selectivity towards *P. aeruginosa* bacteria, depending on the initial concentration of pseudomonads and the microorganisms in association.

To determine the sensitivity of the medium, we conducted a series of experiments with four reference strains of *P. aeruginosa* at two concentrations, 10^4 and 10^5 CFU/mL, in 14 repetitions. In parallel, we carried out the cultivation of bacteria on blood agar and peptone agar media (tab. 3).

Experimentally, we determined that the proposed medium is much more sensitive compared to blood agar and peptone agar media, allowing for the detection of *P. aeruginosa* bacteria within 6 hours.

The composition and optimal ratio of ingredients create a medium in the form of a micro-pellicle fixed at the bottom of a bottle, which serves as both a container for preserving the medium and, at the same time, for conducting the analysis. The storage term of the medium is 2 years (observation period).

Table 3. The sensitivity of the detection of *P. aeruginosa* bacteria.

Microbial species	Exp. No.	The concentration of microorganisms CFU/ml and their detection after 6 hours of incubation at 37ºC					
		The micro-pellicle medium		Blood agar		Peptone agar	
		10^4	10^5	10^4	10^5	10^4	10^5
***P. aeruginosa* ATCC 27853**	14	71.4±1.3	100±0.0	0	0	0	0
***P. aeruginosa* ATCC 15442**	14	83.3±1.4	100±0.0	0	0	0	0
***P. aeruginosa* ATCC 49189**	14	70.0±1.4	100±0.0	0	0	0	0
***P. aeruginosa* NCTC 6750**	14	80.0±1.6	100±0.0	0	0	0	0

DISCUSSIONS

The control of "microbiological purity" carried out in support of pharmaceutical and biopharmaceutical production falls into three main categories: indication (qualitative), enumeration (quantitative), and characterization/identification. Traditional microbiological methods are listed in compendia and discussed using conventional techniques based on growth and multiplication, which are bulky and time-consuming. Generally, such tests require several days of incubation to determine microbial contamination, and therefore, managing them can lead to proactive corrective measures. Additionally, microbial growth is limited by the culture medium used and incubation conditions, influencing the sensitivity, specificity, and reproducibility of testing (6).

The most discussed topics revolve around the development of various technological platforms for rapid microbiological methods, and many have been readily adopted by microbiology laboratories and the pharmaceutical industry. Their use could enable drug companies to adapt to deadlines for manufacturing processes and product release. Some rapid methods also provide the possibility of real-time microbiological control, allowing management to respond to microbial contamination events over a more extended period and potentially offering cost savings and increased efficiency in quality control testing laboratories. Despite the numerous proven advantages in quality management and the initiatives of international associations to promote the use of analytical process technology, including rapid microbiological methods, the pharmaceutical and biopharmaceutical industry has been somewhat slow to embrace alternative microbiological control methodologies due to reported divergent results (6, 7).

The use of rapid methods is a dynamic field in applied microbiology that has garnered increased attention both nationally and internationally over time (8, 9, 10). This topic has been widely addressed in conferences and published documents worldwide. More recently, the use of alternative methods for microbiological quality control of

pharmaceutical products and materials used in pharmaceutical production has been extensively discussed in various guidelines and compendia in an attempt to facilitate the implementation of these technologies by pharmaceutical companies (6, 11).

Based on the above, the issue of developing procedures and means for the rapid detection and identification of bacteria from the *Pseudomonas spp., Staphylococcus, Candida*, and *Enterobacteriaceae* groups, as microorganisms unacceptable in medications, is current and aligns with the purpose of the proposed study.

The procedure pertains to culture media for the detection of *Pseudomonas aeruginosa* bacteria in various samples, environmental objects, including human pharmaceuticals. In technical essence, closer is the reagent for the detection of *P. aeruginosa* bacteria, which contains all optimal ingredients, with reference to the specific substrate of sodium citrate, used for rapid detection within 9 hours of incubation at a temperature of 37°C. The disadvantage of the known reagent is its lower specificity, as it allows the detection of other citrate-positive microbial species.

The problem addressed by the proposed procedure lies in the development of a culture medium that allows a significant improvement in the specificity of detecting *Pseudomonas aeruginosa* bacteria.

A new culture medium is proposed that includes all optimal ingredients for the detection of *P. aeruginosa* bacteria, with sodium malonate as a specific substrate, water-soluble bromothymol blue as an indicator, sodium hydrogen phosphate, and potassium dihydrogen phosphate. The results of the conducted studies have shown an increase in the specificity of the medium by including sodium malonate as a specific substrate, which *P. aeruginosa* uses as the sole carbon source for multiplication. The other ingredients are included to promote and facilitate the multiplication of P. aeruginosa. Detection occurs under the pH conditions formed by sodium hydrogen phosphate and potassium dihydrogen phosphate, and the cleavage products of sodium malonate are indicated using bromothymolblue.

CONCLUSIONS

1. The use of the developed procedure allows for the rapid and specific isolation of P. aeruginosa strains and facilitates timely monitoring of pharmaceutical preparations and resources to limit contamination throughout the pharmaceutical manufacturing process.
2. The conducted studies indicate a clear increase in the sensitivity and speed of detection of P. aeruginosa bacteria in pure culture, mixed culture, or other research materials.
3. The described procedure is simple to implement, cost-effective, and can be used in microbiological laboratories of various levels.

REFERENCES

1. Salem N, Elbarrawy M, Azzam N. Microbiological quality of non-sterile pharmaceuticals in Egypt. *Beni-Suef Univ J Basic Appl Sci.* 2021;10:38. doi:10.1186/s43088-021-00127-6
2. Kumari B, Kumar S, Thota P, Pandey MK, Raghuvanshi RS, Teotia AK. Prevalence of Microbial Contaminants in Non-sterile Pharmaceutical Antacids. *Biomed J Sci & Tech Res*. 2023;50(3) 41696-41700. doi:10.26717/BJSTR.2023.50.007959
3. Rauf A, Erum A, Noreen S, Shujaat J, Ashraf MU, Afreen S. Microbiological quality control of some non-sterile preparations commonly used in Pakistan. *Pak J Pharm Sci.* 2018;31:1237–1242. Available online: https://www.researchgate.net/profile/Alia-Erum/publication/326413775 [Acccessed on 23 Dec.2023].
4. Miller MJ. Rapid Microbiological Methods. *Pharmaceutical Microbiological Quality Assurance and Control.* 2019: 429–458. doi:10.1002/9781119356196.ch13
5. European Pharmacopeia (11th Edn.). Microbiological examination of nonsterile products: microbial enumeration tests and Examination of non-sterile products: test for specified microorganisms. Available online: https://www.edqm.eu/en/european-pharmacopoeia [Accessed on 06 Sep.2023].
6. Nemati M, Hamidi A, Maleki Dizaj S, Javaherzadeh V, Lotfipour F. An Overview on Novel Microbial Determination Methods in Pharmaceutical and Food Quality Control. *Adv Pharm Bull.* 2016;6(3):301-308. doi:10.15171/apb.2016.042.
7. Moldenhauer J. Overview of Rapid Microbiological Methods. In: Zourob, M., Elwary, S., Turner, A. (eds.) *Principles of Bacterial Detection: Biosensors,*

Recognition Receptors and Microsystems. Springer, New York, 2008. doi:10.1007/978-0-387-75113-9_4

8. Puşcaş N, Balan G, Burduniuc O. Micro-test system for rapid isolation and identification of Candida species in urinary tract infections. Anthropological Researches And Studies. 2017;7:64-70. doi:10.26758/7.1.7
9. Puscas N. Procedure for rapid determination of sterility of injectable solutions. *One Health and Risk Management.* 2023;2:78. Available online: https://ibn.idsi.md/sites/default/files/imag_file/78_23.pdf [Accessed on 11 Dec.2023].
10. Balan G, Puscas N, Rudic V, Borta V, Timbalari E. Sistemul microtest pentru diagnosticul microbiologic rapid al vulvovaginitei candidozice. *Curierul medical.* 2014;57(1):86-90. Available online: https://moldmedjournal.md/wp-content/uploads/2016/09/Cm-1-57-1.pdf [Accessed on 11 Dec.2023].
11. Louis M, Leblanc L, Montero Julian FA. Introduction to culture media in pharmaceutical microbiology for non-sterile products. *Pharmaceutical Microbiological Quality Assurance and Control.* 2019: 79–104. doi:10.1002/9781119356196.ch4