Isolation of Enterobacter cloacae in a pharmaceutical matrix containing amoxicillin and clavulanic acid

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Abstract

Background: in recent years, clinical importance has been detected for the Enterobacter cloacae Complex due to its high resistance to various antimicrobials and its association with their inactivation and degradation, and also for its nosocomial pathogenicity. **Objective :** to show the survival capacity of Enterobacter cloacae to a mixture of antimicrobial and beta-lactamase inhibitor. **Method:** the microbiological analysis method used in the present study corresponds to the direct method described in the general chapter 62 (Microbiological examination of non-sterile products: tests for specific microorganisms) of the Pharmacopoeia of the United States of America, which consists in placing the sample directly in the nutrient medium as preincubation, to complete the scheme with direct passages to broth and selective agar. **Results:** from the determination of objectionable microorganisms, microbial growth was obtained, which was purified to perform preliminary tests for identification, obtaining short gram-negative bacilli in staining; in the oxidase test, no color change was obtained on the strip test, which indicates a negative result, and in miniaturized galleries the identification gave Enterobacter cloacae with a probability percentage of 100%. The results were obtained in the microbiological analysis (objectionable microorganisms) of a pharmaceutical matrix evaluated in the Microbiological Analysis Quality Control Laboratory of the National Directorate of Medicines carried out in 2022. **Conclusion:** there is an evident resistance to antimicrobial drugs by Enterobacter cloacae, even when it comes to multi drugs, intended to block the action of beta-lactamases (which is the nature of the studied matrix) created by resistant microorganisms.

Keywords: Enterobacter cloacae Complex , bacterial resistance, isolation, microbiology, objectionable microorganisms.

Introduction

Bacteria over time have developed effective mechanisms to avoid the action of antimicrobials. These resistant infections are more difficult to treat, generating higher rates of morbidity and mortality, requiring more resources to control them, which generates a significant increase in health costs (García, C. 2018).

Bacteria of the genus Enterobacter are gram-negative bacilli belonging to the Enterobacteriaceae family, widely distributed in nature. They can be found in soil, water and as part of the microbiota of animals, insects and the human gastrointestinal tract. It includes 21 species within which the Enterobacter cloacae complex is defined (Silva and Martínez, 2018).

The amoxicillin-clavulanic acid association presents activity against a large number of enterobacteria, including those resistant to amoxicillin due to the production of broad-spectrum beta-lactamases such as TEM-1, TEM-2 or SHV-1. These beta-lactamases hydrolyze penicillins

(aminopenicillins [ampicillin, amoxicillin], carboxypenicillins [ticarcillin]) and, as mentioned, are sensitive to beta-lactamase inhibitors (Navarro, F. 2011).

The resistance of Enterobacteriaceae to antimicrobials has been increasing in the last 20 years. The main mechanism involved is the production of extended-spectrum ß-lactamases (ESBLs), which confers resistance to antimicrobials of different generations. The production rate of ESBLs by Enterobacteriaceae in Latin American countries is higher than in other regions of the world (García, C. 2018).

Among β-lactams, the Enterobacter cloacae complex is considered intrinsically resistant to aminopenicillins, first-generation cephalosporins and cefoxitin due to the presence of AmpC-type β-lactamases with different degrees of expression, which implies variable susceptibility to third and fourth generation cephalosporins. In addition, strains producing extended-spectrum β-lactamases have been described (Silva and Martínez, 2018).

Antimicrobial resistance (AMR), arises when bacteria change over time and stop responding to medications, making it more difficult to treat infections and increasing the risk of disease spread and the appearance of serious illnesses and even death. As a consequence of drug resistance, antibiotics and other antimicrobial medicines become ineffective, making infections increasingly difficult or impossible to treat (WHO, 2021).

Therefore, the need for the present study has the objective of demonstrating the survival of the Enterobacter cloacae complex against drugs intended to treat bacterial infections, even when these drugs have a deactivation mechanism against the resistance presented by bacteria such as clavulanic acid.

Materials and methods

-Glass bottle containing 90 mL of Casein and Soy Digested Broth
-Glass bottle containing 100 mL of MacConkey Broth
-Petri dishes containing 30 mL of MacConkey Agar
-10 µL serological loops
-Sterile 10 mL and 1 mL tips
-Biosafety cabin
-10 mL and 1 mL pipettor
-Incubator at 42 °C – 44 °C
-Incubator at 30 °C – 35 °C
-MICROGEN ID Identification Galleries

The method used for the microbiological analysis was: Tests of Specific Microorganisms, determination of Escherichia coli, direct method (FEUM, 2021).

This study was carried out using the data generated in the quality control analysis to verify the microbiological purity of a pharmaceutical matrix containing amoxicillin and clavulanic acid, evidencing the contamination of Enterobacter cloacae that is of clinical importance, requiring confirmation of the data in a second analysis with a second analyst, carried out in September 2022 with the same batch of the initial sample, the analyzes were carried out under controlled conditions according to optimal microbiological laboratory practices (USP, 2022a) in the microbiology laboratory of the National Directorate of Medicines.

Growth promotion: Prior to the execution of the tests carried out, the quality control of the different culture media used is carried out, both for nutritional media and for selective and differential media, performing the growth promotion test using the microorganisms presented in Table 1 (USP, 2022b) (Ph Eur, 2022) (BP, 2022a).

Table 1. Growth promotion, inhibitory and indicative properties of the culture media

	Medio	Propiedad	Cepa de prueba
Tests for bilis-tolerant gram-negative bacteria	Mossel broth for bacterial enrichment	Growth promotion	E. coli P. aeruginosa
		Inhibitor	S. aureus
	Red agar violet bilis glucose	Growth promotion + indicators	E. coli P. aeruginosa
Prueba para Escherichiacoli	MacConkey broth	Growth promotion	E. coli
		Inhibitor	S. aureus
	McConkey Agar	Growth promotion + indicator	E. coli
Prueba para Salmonella	Enrichment broth for Salmonella Rappaport Vassiliadis	Promoción de crecimiento	Salmonella enterica subsp. enterica serovar Typhymorium o Salmonella enterica subsp enterica serovar Abony
		inhibitory	S. aureus
	Agar Xylosa, lisina Deoxycolato	Growth promotion	Salmonella enterica subsp. enterica serovar Typhomorium or Salmonella enterica subsp. enterica serovar Abony
Pseudomonas aeruginosa test	Cetrimide Agar	promotes growth	P. aeruginosa
		inhibitory	E. coli

Selective and differential media for the determination of specific microorganisms, the following abbreviations correspond to the microorganisms presented in the different tests P. (Pseudomonas), E. (Escherichia), S. (Staphyloccocus) (BP, 2022 b) .

Sample preparation and previous incubation: the sample is prepared by diluting 10 grams of the pharmaceutical matrix containing amoxicillin and clavulanic acid in 90 mL of digested casein and soy broth with additives (see Table 2) , which function is to neutralize the antimicrobial action of the pharmaceutical matrix. Once the pharmaceutical matrix is well incorporated into the broth (1 in 10 dilution), a second dilution is carried out by taking 10 mL of the homogenized sample and placing it in 90 mL of digested casein and soy broth with additives (1 in 100 dilution) (USP, 2022b).

It is incubated under controlled conditions (continuous monitoring) of temperature between 30 °C to 35 °C for a period of 18 to 24 h (FEUM, 2021).

Negative control: to verify the conditions under which the test has been carried out, the diluent selected without sample preparation is used as a negative control. No growth of microorganisms should be observed and this negative control must be subjected to the same conditions that the sample dilution faces (USP, 2022b).

Table 2. Ingredients for preparation of initial nutrient broth for Escherichia coli determination test

Casein and soy broth digest	
Pancreatic casein digest	17,0 g
Papaic soybean digest	3,0 g
Sodium Chloride	5,0 g
Dibasic potassium phosphate	2,5 g
Monohydrate glucose	2,5 g
Tween 80	5,0 g
Soybean lecithin	0,7 g
Purified water	1000 mL

The additives Tween 80 and lecithin are added to neutralize the antimicrobial action of the pharmaceutical matrix analyzed; g is used to indicate grams and mL to indicate milliliters (USP, 2022b)

Selection and subculture: once the incubation period is over, the incubated sample is shaken (1 in 100 dilution), 1 mL of the casein and soy broth digest is transferred to 100 mL of MacConkey broth and incubated under controlled conditions (continuous monitoring) temperature of between 42 °C to 44 °C for a period of 24 to 48 h (BP, 2022a). At the end of the incubation period, the MacConkey broth is shaken and 10 µL are subcultured on a MacConkey agar plate and incubated under controlled conditions (continuous monitoring) of temperature between 30 °C to 35 °C for a period from 6 to 72h (BP, 2022a).

Interpretation: Colony growth indicates the possible presence of E. coli. This is confirmed by identification tests. The product complies with the test if no colonies develop or if identification test results are negative (USP, 2022b).

Identification method: for the identification of the microorganism detected in the pharmaceutical matrix containing amoxicillin and clavulanic acid, a miniaturized gallery of biochemical tests of the Microgen GnA+B ID type was used (Microgen Bioproducts Ltd, 2009a).

The miniaturized galleries use a combination of biochemical test results to perform a characterization of the microorganism based on its metabolism through the use of different substrates, which are described in Table 3.

Table 3. Substrates used in biochemical tests

Miniaturized Microgen Identification Substrate Kit GnA+B ID					
Lysine	Ornithine	H_2S	Glucose	Manitol	Xylose
Ortho-Nitrophenyl galactoside	Nitrate	Indole	Urease	Voges Proskauer	Citrate
Tryptophan deaminase	Gelatin	Malonate	Inositol	Sorbitol	Rhamnose
Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicine

Source: self made

It should be noted that all microorganisms detected during the execution of microbiological purity tests of specific microorganisms in the different pharmaceutical matrices are identified to the genus and species level with their respective probability percentage (Microgen Bioproducts Ltd, 2009a).

Statistical analysis: in the Microgen GnA+B ID report form, the substrates have been arranged in a set of 3 reactions, with each substrate being assigned a numerical value of 1, 2 or 4. The sum of the positive reactions for each triplet gives a single digit of the octal code used to determine the identity of the isolated microorganism (Microgen Bioproducts Ltd, 2009a).

The octal code is entered into the computer program to proceed with identification, which is a computer-assisted probability-based identification program. This determines the best range of tests to obtain maximum differentiation capabilities for the species under investigation. It generates a report of the five microorganisms in the database that are most likely to be found. The software generates an identification based on probability, percentage probability, and likelihood, with an analysis of the quality of differentiation, see Table 4 .

Table 4. Percent probability of differentiation analysis

Porcentaje de probabilidad		Interpretación
Primera elección	Segunda elección	
99.9	<0.01	The firt option is 10,000 times more likely than the second option, this means, both are well separated
99	<1	The first option is 100 times more likely than the second one, it means they are well separated
70	25	The first option is 3 times more likely than the second option, this means, both are badly separated.

This provides a measure of the extent of the 5 options as long as they are separated or differentiated from each other. (Microgen Bioproducts Ltd, 2009a)

Results

Growth promotion: The culture media used in the test where the presence of Enterobacter cloacae was detected were evaluated with fourth-passage ATCC mother strains, for which the different quality aspects of the culture medium were verified, determining: appearance of the medium of prepared culture, pH after sterilization, sterility test and growth promotion test see Table 5.

Table 5. Quality control results of the culture media used in the tests

Final opinion for the batch of culture medium												
Internal code	Parent Strain	ATCC number	Strain batch	Test batch	Lote de referencia	Appearance	Final pH	Sterility	Productivity	Selectivity	Specificity	Determination
B12	Pseudomonas aeruginosa	9027	70006683	260722-3-2	120722-1-1	C	7.3	Complies with the test`s requirements	2			A
	Staphyloccocus aureus	29737	61864407									
	Bacillus subtilis	6633	70005675									
B12	Pseudomonas aeruginosa	9027	70006683	300822-3-2	260722-3-2	C	7.3	Complies with the test`s requirements	2			A
	Staphyloccocus aureus	29737	61864407									
	Bacillus subtilis	6633	70005675									
B05	Escherichia coli	8739	48310201	050722-3-2	010622-1-1	C	7.2	Complies with the test`s requirements	2	SC		A
	Staphyloccocus aureus	29737	61864407									
A08	Escherichia coli	8739	48310201	120822-1-1	030622-1-1	C	7	Complies with the test`s requirements	1.2	SC	C	A
	Staphyloccocus aureus	29737	61864407									

Source: self made. The following abbreviations correspond to: C. (Complies), SC. (No Growth, A. (Approved), internal codes B12 (Casein and Soy Digested Broth), B05 (MacConkey Broth), A08 (MacConkey Agar)

Sample preparation and previous incubation: in the two analyzes carried out at different times and with different analysts, it was observed that, after the incubation period of the homogenized sample in casein and soy digested broth, it showed turbidity since the sample is of solid origin, therefore, we proceed to continue with the next phase of the analysis.

Negative control: the different culture media used in the different tests were evaluated at the same time as the samples, under the same conditions, and no growth was observed, resulting in the negative control being in compliance.

Selection and subculture: at this stage of the analysis, a color change was observed in the MacConkey broth to a light yellow color. This broth contains lactose, which, as indicated in the technical sheet with the presence of enterobacteria, degrades and produces acid and gas, the production of acid is detected by the purple bromocresol indicator, which turns yellow. Ox bile promotes the growth of several species of intestinal bacteria and inhibits that of microorganisms that do not live in the intestine (Merck, 2021a). When reading the MacConkey agar plates, a growth of circular, convex, pinkish colonies was observed, indicating suspicion for specific microorganisms.

Identification method: the suspicion was purified on nutrient agar, obtaining isolated, circular, cream-colored colonies. A colony was taken to perform preliminary tests for identification , obtaining Gram (-) short stains bacilli. While in the oxidase test, no color change was obtained on the reagent strip, which indicates a negative test so the miniaturized galleries were assembled. of Microgen GnA+B ID, see results in Table 6. The identification resulted in Enterobacter cloacae with a probability percentage of 100%.

Table 6. Identification tests of the microorganism isolated from a pharmaceutical matrix

Identification Tests	
Oxidase Test	Doesn't show blue/violet color in the oxidase detection strip
Gram Stain	Short Bacilli gram (-)
GnA+B galleries in first isolation	Enterobacter cloacae 100%
GnA+B galleries in second isolation	Enterobacter cloacae 100%
Octal code	27562745

Source: self made

Statistical analysis : Once the appropriate data table is created, it can be organized into a database (see Figure 1), based on the percentage of a given positive organism for a selected test.

Figure 1. Reaction pattern of the isolated strain

Results Introduction

Octal Code 27562745

-	LYS	Lysine Decarboxylase	+	ORN	Ornithine Decarboxylase	-	H2S	H2S Production
+	GLU	Acid from Glucose	+	MAN	Acid from Mannitol	+	XYL	Acid from Xylose
+	ONP	ONPG	-	IND	Indole	+	UR	Urea Hydrolysis
+	VP	Voges Proskauer	+	CIT	Citrate Utilization	-	TDA	Tryptophan Deaminase
-	GEL	Gelatin Liquefaction	+	MAL	Malonate Utilization	-	INO	Acid from Inositol
+	SOR	Acid from Sorbitol	+	RHA	Acid from Rhamnose	+	SUC	Acid from Sucrose
+	LAC	Acid from Lactose	-	ARA	Acid from Arabinose	-	ADO	Acid from Adonitol
+	RAF	Acid from Raffinose	-	SAL	Acid from Salicin	+	ARG	Arginine Dihydrolase

Source: own elaboration using Microgen ID software version 2.0.8.33

Figure 2 . Result obtained from the microorganism under study in the identification galleries of the GN-ID system panel A and B

GN-ID A+B PANEL
REPORT FORM

Lab. No.	Specimen Type: Lim356122 Agar Macconkey
ID-049122	Date: 25/08/22

Well Number				GN A wells										G N B wells											
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Reaction	Oxidase	Motility	Nitrate	Lysine	Ornithine	H2S	Glucose	Mannitol	Xylose	ONPG	Indole	Urease	V.P.	Citrate	TDA	Gelatine	Malonate	Inositol	Sorbitol	Rhamnose	Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicin	Arginine
Result				-	+	-	+	+	+	+	-	+	+	+	-	-	+	-	+	+	+	+	-	-	+	-	+
Reaction index	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1	4	2	1
Sum of positive Reactions	N/A			2			7			5			6			2			7			4			5		

Octal Code: 27562745

Final Identification: Enterobacter cloacea

The calculation of probabilities of an unknown isolated strain is carried out (binomial theorem) for each species in the database, see Figure 3.

A/100 * (1 – B/100) * C/100 = probability

Figure 3. Degree of differentiation of each identification option with respect to the other options

Identidication Analysis

	Enterobacter cloacae	Enterobacter dissolvens	Enteric Gp69	Enterobacter amnigenusbiogp	1 Enterobacter hormaechei
ID selected option	Sí	No	No	No	No
Probability	1/18,607	<1/10,000,000	<1/10,000,000	<1/10,000,000	<1/10,000,000
Probability percentage	100%	<0.01%	<0.01%	<0.01%	<0.01%
Likelyhood	0.03%	<0.01%	<0.01%	<0.01%	<0.01%
Human-isolated strain	Sí	No	No	Si	No
Test against					
Test 1		ARA (99.9%)	ARA (99.9%)	ARA (99.9%)	ARA (99.9%)
Test 2	ARA (99.99%)	SAL(99.9%)	SAL(99.9%)	UR(0.1%)	SOR(0.1%)
Test 3		LAC(0.1%)	UR (0.1%)	SOR (9%)	RAF(0.1%)
Additional Test	sI	SI	SI	SI	SI
Acid from Dulcitol	15%	0.1%	99.9%	0.1%	87%
Acid from Melibiose	90%	99.9%	99.9%	99.9%	0.1%
Motility (37C)	95%	0.1%	99.9%	92%	52%
Esculin Hydrolysis	30%	99.9%	99.9%	91%	0.1%
Acid from glycerol	40%	0.1%	0.1%	0.1%	4%
Additional comments		18			16

16. Formerly enteric group 75.J. Clin. Microbiol. (1989) 27: 2046-2049
18. Formerly Erwinia dissolvens.J. Clin. Microbiol. (1986) 23: 1114-1120

Source: own elaboration using Microgen ID software version 2.0.8.33

Discussion:

The presence of Enterobacter cloacae in an antimicrobial indicates the capacity of this microorganism to survive this agent which is because its genetic material has changed, this is why it becomes of pharmaceutical interest to study it at a molecular biology level, since this way the metabolites generated by these microorganisms can be known. Although this pharmaceutical matrix has an antimicrobial, it also has a beta-lactamases blocking agent, which does not allow microorganisms to survive the antimicrobials.

In order to have a broader picture of the impact that this isolation of Enterobacter cloacae may generate, it is necessary to evaluate the stability of the pharmaceutical matrix and verify throughout its useful life how this finding impacts, and thus, verify if this microorganism is capable of degrade an antimicrobial through the assessment of the different active ingredients present in it.

The limitation in the research, in order to expand and reinforce the study, was the lack of installed capacity to evaluate resistance tests to the different types of antimicrobials that follow various mechanisms of action, in order to verify the impact on AMR that this isolation can mean. This is the result of a microorganism that has been isolated from a matrix that has a mixture of drugs that are intended to block the resistance capacity of the microorganisms. There has also been a limitation in carrying out tests at the genetic level and to determine the causes of the defense mechanism presented by the isolated bacteria.

With this research, it has been possible to expand the collection of wild microorganisms that the DNM microbiology laboratory has, which is expanding with the different isolates obtained that in the future will support a research that can be carried out about the AMR it can present to different pharmaceutical products destined to bacteria eradication. This creation of strains could be used as a general plan in the making of a series of algorithms that allow us to confront this type of microorganisms in health emergency situations and at the same time have a good understanding of the root cause of the different phenomena that lead to the generation of resistant microorganisms to antimicrobials.

If resistance to multiple antimicrobials is confirmed, we could classify Enterobacter cloacae as a new super bacteria, having serious consequences for antimicrobial treatment regimens in health systems.

One of the main applications for which the present study can be used is to avoid recommending or administering mixtures of amoxicillin and clavulanic acid to patients who have proven clinical symptoms of Enterobacter cloacae infection .

Conclusion

In the microbiological purity test of specific microorganisms from a pharmaceutical matrix containing antimicrobial agents, Enterobacter cloacae was isolated, an opportunistic microorganism that is capable of populating the human intestine and causing infectious diseases.

For the analysis of pharmaceutical products containing antimicrobial agents, it cannot be assumed that they are free of contamination. In the present study it can be demonstrated that they can be contaminated for different reasons, but what is important is the ability of the analyst to detect and demonstrate them, the ability to neutralize the antimicrobial effect by the different methods that exist, such as the use of inhibitory substances or carrying out serial dilutions, which for this study both methods were chosen.

It is recommended for laboratories manufacturing antimicrobial products to carry out microbiological evaluations of all the raw materials involved in the manufacture of these products, as well as to carry out all quality controls, both on raw materials and the finished product, using the different forms of inactivation of the antimicrobial agents.

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