RAPID POLYMYXIN NP

Detection of sensitivity and resistance to polymyxin of enterobacteria (from colonies and blood culture)

10 tests (REF 23000)

For *in vitro* diagnostic and professional use only

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CE

I - INTENTED USE

The Rapid Polymyxin NP test has been designed for the detection of *Enterobacteriaceae* susceptibility and resistance to polymyxins (polymyxin E or colistin, and polymyxin B) from colonies grown on agar plates or from positive blood cultures.

2 - INTRODUCTION

The development of multi-resistant bacteria to several families of antibiotics (multi-resistant bacteria or MRB) represents a public health issue through the dramatic reduction in therapeutic treatment options, as well as by increasing mortality rates in intensive care units.

Among the MRB bacteria, Enterobacteriaceae (*Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae* or other species) are the main pathogens of concern. They are responsible for the majority of community (urinary tract, lung, intra-abdominal, blood infections) and nosocomial infections.

Furthermore, there has been an increase in worldwide reports of cases of acquired resistance to β-lactamins (penicillin, cephalosporin, monobactam) and to the broad spectrum carbapenem antibotics (carbapenems, aminoglycosides and quinolones).

The development of MRB bacteria has renewed interest in an old class of antibiotics, the polymyxins (polymyxin E or colistin and polymixin B), which are generally considered to be molecules of last resort.

However today, the growing use of colistin is leading to the emergence and multiplication of new multi-resistant Enterobacteriaceae strains. Combined resistance to both colistin and carbapenems is a new threat for the lasting efficiency of our therapeutic arsenal.

Therefore, the management of cases that prove difficult to treat, as well as the problem of infection risks in clinical environments, require rapid assessment of colistin susceptibility and resistant profiles in bacterial strains.

The currently available methods for the determination of susceptibility and resistance to colistin are not particularly adapted to clinical and hospital requirements. They are time-consuming, long (24 hours, liquid Medium Minimum Inhibitory Concentration), or can be unreliable such as with agar diffusion methods.

The Rapid Polymyxin NP test is both sensitive and specific and enables the determination of Enterobacteriaceae resistance to colistin in less than 3 h. The test is rapid, easy-to-use, easy-to-read and suitable for all medical laboratories. It employs a liquid method of detection of all resistant phenotypes, which enables an appropriate antibiotic treatment to be administered immediately, or the identification of carriers of colistin resistant strains in order to limit the risk of an epidemic spread.

3 - PRINCIPLE

The Rapid Polymyxin NP test is based on the principle described by Nordmann, Jayol and Poirel (1-2-3).

This liquid method relies on the colorimetric detection of rapid glucose metabolization associated with bacterial growth in the presence of a defined concentration of colistin. Acidification of the culture medium following glucose metabolization, is demonstrated by a colour change (orange to yellow) of the pH indicator (phenol red).

4 – REAGENTS

Description	Amount
RP NaCl : Vial of 3mL NaCl solution (0.85 g/L) for inoculum preparation	12
RP Medium : Vial of 1.5mL culture Medium containing cation-adjusted Mueller Hinton broth (25 g/L), glucose (10 g/L) and phenol red (pH indicator)	10
RP colistin tray: Tray with a negative control well C-, a Test well with colistin at 2 µg/mL concentration and a bacterial growth control well or positive control well C+. Tray packed in an aluminium sachet with an integrated desiccant.	10
RP TC (Turbity Control): Vial of 3mL barium sulphate solution for turbidity control	1
Closing System: Protective translucent plastic tray lid for the inoculated tray	10

5 - PRECAUTIONS

The reagents are intended solely for *in vitro* use and must be handled by authorized personnel.

The patient samples and inoculated reagents are potentially infectious; they must be handled with caution, in observance of hygiene rules and the current regulations for this type of product in the country of use. The use of a microbiological security station is recommended.

Do not use reagents after the expiry date.

Reagents have to be stored between 2-8°C.

Do not use reagents that have been damaged or that have been poorly conserved before use.

Do not use RP Medium vials with apparent signs of leakage.

The results obtained with the Rapid Polymyxin NP test indicate the presence of colistin-resistant or sensitive Enterobacteriaceae in the specimen. However , these results cannont be used alone to make a clinical diagnosis. The diagnosis must be carried out by a doctor based on biological results and clinical signs.

6 - SAMPLE COLLECTION

The bacterial colonies used for performing the Rapid Polymyxin NP test should be grown on non-acidifying media such as Luria Bertani, Mueller-Hinton, Colombia Agar 5% sheep blood, Chocolate agar – PolyVitex, Eosin-methylene blue or chromogenic agars. Agar culture media such as Drigalski are excluded from this list of media. Young colonies (15 h to 24 h incubation) must be used to carry out the test.

Enterobacteriaceae from blood samples can be tested directly from aerobic and anaerobic blood cultures.

7 - PREPARATION AND STORAGE OF REAGENTS

Reagents are ready to use.

The kit and its content when stored at 2-8°C in their original state are stable until the expiry date indicated on the box.

RP Medium and RP NaCl vials are single-use reagents.

Should the RP TC vial be used as a control for inoculum calibration, this vial has to be kept until the last RP Medium vial of the kit has been inoculated.

During storage, the RP TC reagent must be protected from the light and maintained at 2-8°C.

8 - MATERIAL REQUIRED BUT NOT PROVIDED

Waste container for contaminated waste

Suspension turbidity detector (densitometer)

Pipettes and tips

Certified incubator at +36°C+/-2°C

9 - METHOD

9.1 Tests conducted on colonies isolated on agar plates

Gram-negative bacterial phenotype must be verified by performing a Gram stain. The test should be carried out only from colonies identified as enterobacteria and with the exclusion of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* species.

Allow the reagents to reach room temperature (18-25°C) for 10 min.

Pre-incubate the RP Medium for 10 min at 37°C.

Remove the adhesive label from wells 1, 2 and 3.

Preparation of the negative control:

Distribute into well n°1 (C-):

- 75 µL of non-seeded RP Medium
- 25 µL of non-seeded RP NaCl

Preparation of the bacterial suspension in the RP NaCl vial:

- Pick up three or four identical isolated colonies with a 10 µl wire loop or an occluded Pasteur pipette.
- Inoculate a vial of RP NaCl with the colonies. Mix well.

Standardization of the inoculum

It is recommended that the inoculum should be standardized with a densitometer. However, a vial of RP TC is available and can be used provided that good usage practices are followed (see paragraph RP TC vial).

-with a densitometer

Verify with a densitometer that the turbidity of the inoculated medium is between 3 and 3.5 Mac Farland (Mc F). The lowest turbidity value must be taken into account (obtained by turning the test vial in the densitometer). If the Mc F is too low (insufficient inoculum), the vial should be inoculated again until a Mc F between 3 and 3.5 is obtained. If the obtained Mc F is higher than 3.5 (inoculum too rich), dilute it with fresh RP NaCl from a newly opened vial until a Mc F between 3 and 3.5 is obtained. For this purpose, 2 extra vials of RP NaCl are included in the kit . They must be disposed of after use.

If the RP NaCl vial provided does not fit in the densitometer, it is recommended that:

- the contents should be transferred into a tube compatible with the device,
- a 0 Mc F value will be obtained.
- then colonies should be added to obtain a 3-3.5 Mc F.

-in relation to the RP TC vial

This visual reading method can be subjective and requires good laboratory practice to ensure the reliability of a 3-3.5 Mc F in the inoculated RP NaCl vial.

In order to guarantee that the expected optical density of the inoculated RP NaCl vial, compared to the optical density of the provited RP TC, is obtained, it is essential that the inoculum turbidity method be validated.

Methodology:

Adjust the opacity of the inoculated medium to that of the RP TC turbidity control with the aid of the black lines printed on the vial label.

If necessary, proceed as above to adjust the turbidity.

Preparation of the inoculum in the RP Medium and distribution in the tray:

Transfer 500 µL of the seeded NaCl solution into the RP Medium.

Homogenize the seeded RP Medium and transfer as follow:

- 100 µL into the Test well (well 2) containing colistin
- 100 µL into the growth control well (well 3, C+) without colistin

Cover the seeded tray with the Closing System.

Mark the Rapid Polymyxin NP tray to identify the tested specimen.

Incubate the tray at 36 ±2 °C for 2 to 3 hours.

An initial reading of the colour can be performed after 2 hours of incubation time (for reading and interpretation conditions see paragraph 10 “Reading and interpretation”).

9.2 Tests conducted on positive blood culture
The test should only be carried out from colonies identified (by MALDI TOF) as enterobacteria with the exclusion of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* species.
Allow the reagents to reach room temperature (+18 to 25°C) for 10 minutes.
Mark the RAPID POLYMIXIN NP (RP NP) tray to identify the tested specimen.
Remove the adhesive label from wells 1, 2 and 3.
Preparation of the negative control:
Distribute into well n°1 (C-):
- 75 µL of non-seeded RP MEDIUM
- 25 µL of non-seeded RP NaCl

Preparation of the bacterial suspension in the RP NaCl vial:
Inoculate the RP NaCl vial with 300 µL of mono-microbial Enterobacteriaceae positive blood culture
Mix Well
Preparation of the inoculum in the RP Medium and distribution in the tray:
Transfer 500 µL of seeded RP NaCl in the RP MEDIUM vial and homogenize the vial
Distribute the seeded RP Medium as follows:
- 100 µL into the Test well (well 2) containing colistin
- 100 µL into the growth control well (well 3, C+) without colistin

Cover the seeded tray with the Closing System.
Incubate the tray at +36 ±2°C for 2 to 4 hours.
An initial reading of the colour can be performed after 2 hours of incubation time (for reading and interpretation conditions see paragraph 10 “Reading and interpretation”).

10 - READING AND INTERPRETATION

The reading of the results relies on the identification and comparison of the colour of the Test well with those of the C+ and C- wells.

Reading of the negative control (negative control well C-, n°1):
The negative control well (C-) displays the original (orange) colour of the medium. Colour change in the Test well is assessed by comparison with this control.
A yellow coloration in the C- well invalidates the test. In this case, the result must not be interpreted and the test must be repeated.
Validation (positive control well C+, N°3):
Verify that the medium in the growth control well (C+) has turned yellow.

Reading and interpretation of the Test well n°2:
A colour change of the medium, initially orange to yellow/orange or yellow, indicates that the tested strain is able to grow in the presence of colistin at a concentration of 2 mg/L.
However, when no colour change is observed, growth of the strain is inhibited at a colistin concentration of 2 mg/L .

• Inoculum from isolated colonies
An initial reading of the colour can be performed after incubation for 2 hours.
If the medium in the positive control well C+ (well n°3) is yellow, then read the Test well:
1 / If the TEST well is yellow (or yellow / orange and clearer than the negative control well C-, well n°1) then the strain is colistin resistant
2 / If the TEST well is orange (with an orange coloration of equal intensity to the negative control well C-, well n°1) then re-incubate the tray for 1 hour and read the test again.
The final result is obtained after 3 hours of incubation.

• Inoculum from positive blood culture
An initial reading of the colour can be performed after incubation for 2 hours.
If the medium in the positive control well C+ (well n°3) is yellow, then read the Test well:
1 / If the TEST well is yellow (or yellow / orange and clearer than the negative control well C-, well n°1) then the strain is colistin resistant
2 / If the TEST well is orange (with an orange coloration of equal intensity to the negative control well C-, well n°1) then re-incubate the tray for 2 hours and read the test again.
The final result is obtained after 4 hours of incubation.

There is no critical concentration available for Enterobacteriaceae in the CLSI guidelines (4-5). Thus, the strains are categorized as colistin susceptible or resistant according to the EUCAST interpretation criteria (6):
- an Enterobacteriaceae strain with a colistin MIC ≤ 2 µg/mL is defined as susceptible (bacterial growth inhibited; orange coloration of the medium).
- an Enterobacteriaceae strain with a colistin MIC > 2 µg/mL is categorized as resistant (bacterial growth not inhibited; yellow/orange or yellow coloration of the medium).

11 - QUALITY CONTROL

Quality control can be carried out from reference strains:
- *Escherichia coli* ATCC 25922, colistin-**susceptible** strain after 2 hours of incubation (orange C- well, **orange Test well,** yellow positive control well)
- *Proteus mirabilis* ATCC 25933 colistin-**resistant** strain after 2 hours of incubation (orange C- well, **yellow Test well**, yellow positive control well)
- *Escherichia coli* NCTC 13846 (mcr1 positive), colistin-**resistant** strain after 2 hours of incubation (orange C- well, **yellow Test well,** yellow positive control well)

12 - CAUSES OF ERROR and PARTICULAR CASES

Seeding of the wells has to be carried out within 60 minutes following preparation of the bacterial suspension in RP NaCl solution and the turbidity check (3-3.5 McFarland).

Reading and interpretation of the results should not be conducted if a colour change in the positive control well (C+, well n°3) occurs before the first 2 hours of incubation.

Early reading of the colour (i.e. reading of the colour change before the first 2 hours of incubation) can lead to incorrect interpretation, such as a false susceptibility result for a colistin resistant strain.

If the positive control well C+ (well n°3) does not show the expected colour change (yellow) **after the required 2 hours incubation**, no test results can be interpreted. A new test has to be performed.
For the final reading of results, do not exceed the indicated incubation times: 3 hours for tests carried out from isolated colonies **and 4** hours **for tests performed from positive blood cultures.**
Results of the RP NP test carried out with a blood culture containing the simultaneous presence of a sensitive and a resistant strain of the same enterobacteria species does not mask the detection of the colistin resistant strain.
For blood cultures, it is essential to respect the recommended incubation time of 2 to 4 hours.
The simultaneous presence in the blood culture broth of a susceptible and a resistant strain belonging to the same enterobacteria spp does not mask the detection of the resistant strain.
The presence of a colistin sensitive enterobacteria strain may mask the detection of resistance of another naturally resistant strain to colistin also present in the blood culture broth.
Blood samples should be tested from monomicrobial blood cultures.

13 - LIMITATIONS OF THE PROCEDURE

Bacterial colonies grown on acidifying culture media such as Drigalski, Mac Conkey and bromocresol purple (BCP) agars are not compatible with the RP NP test. Subcultures in appropriate media (see paragraph 6. Sample collection) are required before performing the test.
When seeding from positive blood cultures, red blood cell sedimentation can occur at the bottom of the wells. This does not affect interpretation of the colour change.
When carrying out an RP NP test from isolated colonies, the performance of the test is guaranteed if the inoculum is correctly standardized between 3 and 3.5 Mc F.
The limit of detection (or analytical sensitivity) is 10^7 CFU/mL; this corresponds to the minimum bacterial load required to detect colistin-resistant strains in a blood culture. A bacterial density of less than 10^7 CFU/mL in the blood culture may present false negative results.

14 - PERFORMANCE

14 -1 Performance from tests conducted on isolated colonies

The performance evaluation of the performance of the Rapid Polymyxin NP test was conducted in the Emerging Antibiotic Resistance Unit (INSERM, Faculty of Sciences, University of Fribourg, Switzerland), and was compared with the method for determining the Minimum inhibitory concentration (MIC) in liquid medium (microdilution Mueller-Hinton cation adjusted broth - according to the guidelines of the Clinical Laboratory Standard Institute (4-5) - described as the reference method).
The bacteria used in this study come from different, international, clinical samples and include the following species:

Species	Tested number	Species	Tested number	Species	Tested number
Klebsiella pneumoniae	113	*Enterobacter absuriae*	2	*Proteus stuartii*	1
Escherichia coli	40	Morganella morganii	2	*Proteus vulgaris*	1
Enterobacter cloacae	16	*Proteus mirabilis*	2	*Salmonella concord*	1
Citrobacter freundii	8	*Proteus rettgeri*	2	*Salmonella isangi*	1
Citrobacter koseri	8	*Salmonella enterica*	2		
Enterobacter aerogenes	8	*Serratia marcescens*	2		
Klebsiella oxytoca	8	*Salmonella sp.*	2		

219 Enterobacteria strains were selected as the most representative species of Enterobacteriaceae.

These include 78 sensitive strains and 141 strains resistant to colistin. Of the resistant strains, different molecular mechanisms of resistance to polymyxins have been observed (chromosomal, plasmid, intrinsic or unknown). The MICs of the strains and their resistance mechanisms are as follows:

MIC (µg/mL)	0,125	0,25	0,5	<1	1	2	4	8	16	>16	32	64	128	>128
Number of strains	9	1	1	60	3	4	8	18	19	10	21	33	20	12

Resistance mechanisms			Number of strains
Intrinsic natural resistance			10
Acquired resistance	*Chromosomal*	Heterogeneous resistance	2
		MgrB gene mutation	70
		PhoP or Q gene mutation	2
		PmrA or B gene mutation	10
	Plasmid	mcr -1 gene mutation	30
Unknown mechanism			17

The bacterial strains were re-isolated on agar (Luria Bertani, Mueller Hinton, Columbia 5 % blood) for 18 - 24 hours in order to test the Rapid Polymyxin NP in parallel with the determination of the MIC via the CLSI liquid micro-dilution technique.

The Rapid Polymyxin NP test was read after 2 hours and 3 hours of incubation.
The percentage of clinical concordance of the Rapid Polymyxin NP test is 97.7% compared to the MIC method in liquid medium. The sensitivity of the test is 99.3% and the specificity is 94.9%.

There are 4 Major Errors (ME) (MIC between 1 and 2 µg/mL) and a Very Major Error (VME) (*Klebsiella pneumoniae* strain with an MIC of 8 µg/mL of which the resistance mechanism is unknown).
The percentage of clinical concordance, to within 1 dilution, is 99.1%. 1 ME and 1 VME remain.

As far as incubation time is concerned, all of the strains gave a readable result within 2 hours. Furthermore, the profile of sensitive strains is stable even after 3 hours of incubation.

14 - 2 Performance of tests conducted on blood cultures

Global performance obtained by combining both types of protocols from blood cultures:

Performance	Clinical blood cultures	Spiked* blood cultures	Global
Sensitivity	66.7%	98%	**96.3%**
Specificity	100%	100%	**100%**

***Spiked: an enriched blood culture with a colistin sensitive or resistant strain**

• Performance from tests conducted on clinical blood cultures:

27 clinical blood cultures with both aerobic and anaerobic vials (BD BACTEC™ Plus Aerobic/F and Plus Anaerobic/F Medium plastic vials) from non-duplicate patients detected by the automate blood system Becton Dickinson FX were analyzed in the laboratory of Pr. G. Greub. at the CHUV Lausanne, Switzerland,
The species distribution was 19 *Escherichia coli*, 2 *Klebsiella pneumoniae*, 2 *Klebsiella oxytoca*, 2 *Enterobacter aerogenes*, 1 *Proteus mirabilis* and 1 *Serratia marcescens*.

Two naturally-colistin R strains (*Proteus mirabilis, Serratia marcescens*) gave concordant positive results within 4 hours of incubation for each aerobic and anaerobic culture condition.
One *Klebsiella pneumoniae* strain colistin resistant (MIC = 8 mg/L) was not detected (false negative result) within 4 hours of incubation of the test.

For clinical blood cultures, the sensitivity of the test is 66.7% and the specificity is 100%.
The bacterial density of all the tested blood culture broths in this study was $\geq 10^7$ CFU/mL with the exception of 2 anaerobic vials with a bacterial load of 10^6 CFU/mL.

• Performance from tests conducted on spiked blood cultures:

In order to test more resistant strains, a spiked blood culture protocol was carried out at the CHUV.

Out of the 72 strains tested, 51 resistant strains with a range of different polymyxin resistance genotypes and 21 strains were susceptible to colistin. For each strain, both aerobic and anaerobic vials were tested.

Only one discrepancy was observed with an *E. coli* MCR-1 strain (false negative result) among the 51 resistant strains.

For spiked blood cultures, the sensitivity of the test is 98% and the specificity is 100%.

15 - WASTE ELIMINATION

Waste should be disposed of in accordance with the hygiene rules and current regulations for this kind of product in the country of use.

16 - BIBLIOGRAPHY

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The changes from the previous version are highlighted in grey.

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