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CarbAcineto NP Test for Rapid Detection of Carbapenemase-Producing *Acinetobacter* spp.

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Multidrug-resistant *Acinetobacter baumannii* isolates, particularly those that produce carbapenemases, are increasingly reported worldwide. The biochemically based Carba NP test, extensively validated for the detection of carbapenemase producers among *Enterobacteriaceae* and *Pseudomonas* spp., has been modified to detect carbapenemase production in *Acinetobacter* spp. A collection of 151 carbapenemase-producing and 69 non-carbapenemase-producing *Acinetobacter* spp. were tested using the Carba NP test and a modified Carba NP protocol (the CarbAcineto NP test) in this study. The CarbAcineto NP test requires modified lysis conditions and an increased bacterial inoculum compared to those of the original Carba NP test. The Carba NP test detects metallo-β-lactamase producers but failed to detect the production of other carbapenemase types among *Acinetobacter* spp. In contrast, the newly designed CarbAcineto NP test, which is rapid and reproducible, detects all types of carbapenemases with a sensitivity of 94.7% and a specificity of 100%. This cost-effective technique offers a reliable and affordable technique for identifying carbapenemase production in *Acinetobacter* spp., which is a marker of multidrug resistance in those species. Its use will facilitate the recognition of these carbapenemases and prevent their spread.

Acinetobacter spp., and particularly the *A. baumannii-A. calcoaceticus* complex, are opportunistic pathogens frequently involved in nosocomial outbreaks that occur mostly in intensive care units (ICU). Those infections range from septicemia to pneumonia and urinary tract infections (1). Due to their ability to develop rapid resistance to new antibiotics, multidrug-resistant strains belonging to the *A. baumannii-A. calcoaceticus* complex have been increasingly reported during the last decade (2). Consequently, carbapenems are often considered to be the antibiotic of last resort for treating infections caused by those strains. Therefore, resistance to carbapenems in those species is considered to define the isolate as highly resistant.

In recent years, the spread of carbapenem-resistant *Acinetobacter* spp. has become a worldwide issue. In *Acinetobacter* spp., resistance to carbapenems may result from (i) decreased permeability of the outer membrane due to the loss or modification of porins, (ii) modification of penicillin-binding proteins (which is rare), and (iii) production of a carbapenemase (most of the cases) (2). Those carbapenemases identified in *Acinetobacter* spp. belong to Ambler class A, B, or D. Some class A carbapenemases have been identified as being of the KPC or GES types (3, 4). Whereas KPC-producing isolates have rarely been described (4), the dissemination of GES-11- and GES-14-producing isolates was recently reported in the Middle East (5). Metallo-β-lactamases (MBLs) (Ambler class B) of the VIM, IMP, SIM, and NDM types have been also reported in *Acinetobacter* spp. (2, 6). Apart from SIM, those MBLs have all been reported in *Enterobacteriaceae* and *Pseudomonas* spp. (7). In addition, carbapenem-hydrolyzing Ambler class D β-lactamases (CHDLs) constitute the first source of acquired carbapenem resistance in *Acinetobacter* spp. Those CHDLs are divided into five different subgroups, namely, OXA-23, OXA-40, OXA-51, OXA-58, and OXA-143 (2, 8, 9). As opposed to KPC and MBLs, these CHDLs have been identified among *Acinetobacter* spp. isolates only. OXA-51-like enzymes are intrinsic and chromosomally encoded in the *A. baumannii-calcoaceticus* complex; they possess a weak carbapenemase activity and share a weak amino acid identity with the other known class D β-lactamases (10–12). Although the bla_{OXA-51}-like genes are usually not expressed in a wild-type isolate, the insertion of IS*Aba1* at the 5′ end of bla_{OXA-51}-like genes may lead to overexpression of the corresponding β-lactamase gene (13). On the other hand, genes encoding OXA-23, OXA-40, OXA-58, and OXA-143 CHDLs have been mainly identified on transferable genetic structures, such as plasmids or transposons, which contribute to their dissemination among the *Acinetobacter* genus (2).

Since most of the carbapenemases identified in *Acinetobacter* spp. are located on mobile genetic elements that may be transferred to other clinical relevant species (i.e., other *Acinetobacter* species, *Enterobacteriaceae*, and *P. aeruginosa*), it is critical to identify those carbapenemase-producing *Acinetobacter* organisms and consequently differentiate them from isolates that are carbapenem resistant due to nontransferable mechanisms (i.e., permeability defects and overexpression of efflux pumps). The rapid identification of the NDM-producing *A. baumannii-A. calcoaceticus* complex may help to limit the dissemination of carbapenemase genes not only in the *Acinetobacter* genus but also in *Enterobacteriaceae* through the rapid identification of their potential reservoirs (7).

Due to its intrinsic low permeability, the detection of carbapenemase production in the *Acinetobacter* genus is considered to be more difficult than in *Enterobacteriaceae* and *Pseudomonas* spp. Several phenotypic techniques have been proposed to detect

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carbapenemase-producing *Acinetobacter* spp. (14). The modified Hodge test has largely been used for this purpose. It is based on the *in vitro* detection of carbapenemase activity, therefore inactivating the antibiotic effect. Although this test is efficient for detecting IMP and VIM producers, NDM and CHDL producers may remain undetected, leading to false-negative results (15). Several techniques using the inhibition properties of EDTA were also proposed for detecting MBL-producing *Acinetobacter* spp. Those techniques include the combined disk test and the Etest MBL strip (15, 16). However, those techniques are not highly sensitive or specific, and they require an additional period of growth of 24 h.

Biochemical detection of carbapenemase production using ultraviolet (UV) spectrophotometry has also been proposed as a suitable method (17). This technique efficiently detects VIM, IMP, and SIM producers, but NDM and CHDL producers remain difficult to detect (15). Recently, the detection of carbapenemase production using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry has been proposed for *Acinetobacter* spp. (18–20). It is based on the detection of the spectra of imipenem and of its hydrolyzed product. It shows good sensitivity and specificity but requires trained microbiologists and expensive equipment. Finally, molecular-based techniques using specific primers are useful for identifying carbapenemase genes. Simplex or multiplex PCR methods are available (21), as well as real-time PCR approaches that have the advantage of providing a result in 3 h (22). More recently, a DNA microarray has been developed to detect 91 target sequences associated with antibiotic resistance within 4 h from bacterial culture to results (23). Although those molecular-based tests are highly sensitive and specific, they fail to detect unknown carbapenemase genes or those that are not included in the panel of the test used.

One of the most promising techniques for the rapid and accurate detection of any carbapenemase producer is the Carba NP test. This test is based on the biochemical detection of the hydrolysis of the β-lactam ring of imipenem (24, 25). Although the Carba NP test has been extensively validated worldwide for the detection of carbapenemase producers among *Enterobacteriaceae* and *Pseudomonas* spp. (24, 26), it has not been validated for the detection of carbapenemase-producing *Acinetobacter* spp. The imipenem hydrolysis rates actually obtained with those CHDLs are too low to be detected by the Carba NP test in its original version. The aim of this study was to further evaluate the Carba NP test for detecting carbapenemase producers in *Acinetobacter* spp. and to settle a modified version of the Carba NP test, the CarbAcineto NP test, for optimal detection of this activity in *Acinetobacter* spp.

MATERIALS AND METHODS

Strain collection. A total of 220 strains were used to evaluate the performance of the CarbAcineto NP test. They were from various clinical origins (blood culture, urine, and sputum samples) and of worldwide origins. Those strains had previously been characterized at the molecular level for their carbapenemase content (Tables 1 and 2). This strain collection included the most frequently acquired carbapenemases identified in human *A. baumannii* clinical isolates (Table 1). Those carbapenemases were OXA-23 ($n = 68$), OXA-24/OXA-40-like ($n = 19$), OXA-58-like ($n = 26$), OXA-143-like ($n = 3$), GES ($n = 8$), IMP ($n = 2$), VIM ($n = 1$), SIM ($n = 1$), and NDM ($n = 14$). Nine isolates coproduced two carbapenemases: GES-11 plus OXA-23 ($n = 6$) and NDM-1 plus OXA-23 ($n = 3$). The negative controls ($n = 69$) included (i) wild-type strains ($n = 51$), (ii) narrow-spectrum β-lactamase producers ($n = 3$), and (iii) extended-spectrum β-lactamase producers ($n = 12$) (Table 2). Three strains overexpressing their chromosome-encoded bla_{OXA-51}-like carbapenemase genes were also tested (Table 2).

Susceptibility testing. Susceptibility testing was performed by determining MIC values using the Etest (bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates at 37°C, and the results were recorded according to U.S. guidelines (CLSI), as updated in 2013. The breakpoints for imipenem and meropenem were ≤4 μg/ml for susceptibility (S) and ≥16 μg/ml for resistance (R).

Carba NP test. The Carba NP test is based on the colorimetric and pH-based detection of hydrolysis of the β-lactam ring of imipenem. The updated protocol of the Carba NP test was performed on bacterial isolates grown on Trypticase soy (TS) agar (bioMérieux), as previously described (25).

CarbAcineto NP test. The CarbAcineto NP test was adapted from the updated version of the Carba NP test used for the detection of carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas* spp. (25), in order to use the test for *Acinetobacter* spp. In this updated version, the lysis buffer was replaced by a 5 M NaCl solution, avoiding any buffer effect, and the bacterial inoculum was doubled from one-third to one-half of a calibrated loop (10 μl) to a full calibrated loop in order to increase the enzyme quantity. Briefly, a full calibrated loop (10 μl) of the tested strain was recovered from TS agar plates and resuspended in two 1.5-ml Eppendorf tubes (A and B) containing 100 μl of 5 M NaCl. In tube A (internal control), 100 μl of the revealing solution containing a pH indicator (phenol red) was added. In tube B (test tube), 100 μl of an extemporaneously prepared revelation solution supplemented with 6 mg/ml imipenem was added. Tubes A and B were incubated at 37°C for a maximum of 2 h. Optical reading of the color change of each tube was performed. In tube B, the carbapenemase activity was detected by a color change of phenol red solution (red to yellow/orange) resulting from the hydrolysis of imipenem into a carboxylic derivative, leading to a decrease of the pH value. The results of the CarbAcineto NP test were interpreted as follows: (i) tube A red and tube B remaining red indicated a non-carbapenemase-producing isolate, (ii) tube A red and tube B turning yellow/orange indicated a carbapenemase-producing strain, and (iii) tube A and tube B both turning yellow/orange indicated a noninterpretable result. The phenol red revealing solution was prepared as previously described (24). The 5 M NaCl solution was prepared from a dilution of NaCl powder (Sigma-Aldrich, Saint-Quentin-Fallavier, France) in distilled water.

RESULTS

Using the updated protocol of the Carba NP test set up for the detection of carbapenemase activity in *Enterobacteriaceae* and *Pseudomonas* spp., positive results were obtained for MBL-producing *Acinetobacter* spp. only (Table 1). The Carba NP test failed to detect *Acinetobacter* spp. isolates producing carbapenemases of the GES and/or OXA types (Table 1). This test did not detect any carbapenemase activity among strains found to be resistant to carbapenems due to the overexpression of their intrinsic OXA-51-like enzyme (Table 2). Accordingly, the specificity and sensitivity of the Carba NP test for the detection of *Acinetobacter* spp. isolates producing an acquired carbapenemase were found to be 100% and only 11.9%, respectively.

Conversely, the CarbAcineto NP test detected all isolates that produced an acquired carbapenemase, with the exception of some GES-type producers (Table 1 and Fig. 1B). Isolates that were carbapenem resistant due to overexpression of their chromosome-encoded OXA-51-like β-lactamase (Table 2, Fig. 1C) or to non-carbapenemase-mediated mechanisms, such as combined mechanisms of resistance (outer membrane permeability defect and/or associated with the overproduction of cephalosporinase

TABLE 1 Results of the Carba NP and CarbAcineto NP tests when performed on *Acinetobacter* spp. producing an acquired carbapenemase

Carbapenemase	*Acinetobacter* species	No. of isolates	MIC (μg/ml) of[a]: IMP	MER	Carba NP test result	CarbAcineto NP test result
Ambler class A						
GES-type						
GES-11	*A. baumannii*[b]	3	2	4	−	−
GES-14	*A. baumannii*	5	24–32	16–32	−	−
Ambler class B						
NDM-type						
NDM-1	*A. baumannii*	13	>32	>32	+	+
NDM-2	*A. baumannii*	1	>32	>32	+	+
IMP-type						
IMP-1	*A. baumannii*	1	4	6	+	+
IMP-4	*A. baumannii*	1	24	16	+	+
VIM-type						
VIM-4	*Acinetobacter* sp. genomospecies 16	1	>32	>32	+	+
SIM-type						
SIM-1	*A. baumannii*	1	>32	>32	+	+
Ambler class D						
OXA-23 group						
OXA-23	*A. baumannii*	68	24 to >32	8 to >32	−	+
OXA-40 group						
OXA-24/OXA-40	*A. baumannii*	8	>32	>32	−	+
OXA-25	*A. baumannii*	1	>32	>32	−	+
OXA-26	*A. baumannii*	1	>32	>32	−	+
OXA-72	*A. baumannii*	9	>32	>32	−	+
OXA-58 group						
OXA-58	*A. baumannii*	24	16 to >32	8 to >32	−	+
OXA-58	*A. haemolyticus*	1	>32	8	−	+
OXA-97	*A. baumannii*	1	>32	>32	−	+
OXA-143 group						
OXA-143	*A. baumannii*	2	>32	>32	−	+
OXA-253	*A. baumannii*	1	>32	>32	−	+
Multiple						
GES-11 + OXA-23	*A. baumannii*	6	32	>32	−	+
NDM-1 + OXA-23	*A. baumannii*	3	>32	>32	+	+

[a] IMP, imipenem; MER, meropenem.
[b] *A. baumannii* indicates *A. baumannii-A. calcoaceticus* complex.

and/or extended-spectrum β-lactamases [ESBLs]) also remained negative with this test (Table 2 and Fig. 1A). In most cases, positive results gave a frank color change from red to yellow (Fig. 1B). For MBL producers, a positive result was always obtained in <15 min. For ca. 13% (9/68) of the OXA-23 producers and ca. 23% (6/22) of the OXA-58-like producers, the color in the test tube (tube B) turned from red to orange but only after 2 h of incubation (Fig. 1B). However, this color change was easy to detect when looking at the upper part of the tubes after vortexing (Fig. 1B). Among the 211 tested strains of *Acinetobacter* spp., only two gave noninterpretable results (Table 2 and Fig. 1D). The specificity and sensitivity of the CarbAcineto NP test were therefore estimated to be 100% and 94.7%, respectively.

DISCUSSION

The Carba NP test, initially set up for the detection of carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas* spp., efficiently detects MBL-producing *Acinetobacter* spp. However, it fails to detect OXA-type carbapenemases, which are the most frequently identified carbapenemases among *Acinetobacter* spp. Since those CHDLs found in *Acinetobacter* spp. (OXA-23, OXA-24/OXA-40-like, OXA-58-like, and OXA-143-like) possess weaker carbapenemase activity than that usually measured in *Enterobacteriaceae* (mostly KPC, MBLs, and OXA-48) and *Pseudomonas* spp. (mostly MBLs), we made the hypothesis that the buffer used in the Carba NP test may buffer at a too high level and counteract the detection of any weak carbapenemase activity.

The specificity and sensitivity of the CarbAcineto NP test were found to be 100% and 94.7%, respectively. The CarbAcineto NP test efficiently detected OXA-type carbapenemase producers, leading to a significant improvement of the sensitivity (94.7% for the CarbAcineto NP test versus 11.9% for the Carba NP test). The better detection of CHDLs is due to two independent factors. First, the bacterial inoculum used in the CarbAcineto NP test was doubled compared to that in the Carba NP test, leading to an increased amount of enzyme released in the revealing solution. Second, the lysis buffer used for the Carba NP test (Bacterial Pro-

TABLE 2 Results of the Carba NP and CarbAcineto NP tests when testing *Acinetobacter* spp. strains that do not possess acquired carbapenemases

Acquired β-lactamases	*Acinetobacter* species	No. of isolates	MIC (μg/ml) of[a]: IMP	MER	Carba NP test result	CarbAcineto NP test result
None						
	A. baumannii[b]	36	0.19–2	0.12–3	−	−
	A. junii	3	0.05–0.09	0.05–0.09	−	−
	A. lwoffii	4	0.09–0.19	0.09–0.19	−	−
	A. ursingii	2	0.06–0.09	0.06–0.09	−	−
	A. johnsonii	4	0.06–0.09	0.06–0.09	−	−
	A. baumannii	1	0.5	0.75	NI[c]	−
	A. johnsonii	1	0.09	0.09	NI	−
Narrow-spectrum β-lactamases						
SCO-1	*A. baumannii*	1	0.38	0.5	−	−
RTG-4	*A. baumannii*	1	0.38	0.38	−	−
OXA-21	*A. baumannii*	1	0.75	0.5	−	−
Extended-spectrum β-lactamases						
SHV-5	*A. baumannii*	1	6	4	−	−
PER-1	*A. baumannii*	1	1.5	1.5	−	−
VEB-1	*A. baumannii*	9	0.38–1	0.5–1.5	−	−
GES-12	*A. baumannii*	1	32	32	−	−
Overexpressed chromosome-encoded OXA-51-like β-lactamases						
IS*Aba1* + OXA-51	*A. baumannii*	2	2–3	3	−	−
IS*Aba1* + OXA-66	*A. baumannii*	1	4	6	−	−

[a] IMP, imipenem; MER, meropenem.
[b] *A. baumannii* indicates *A. baumannii-A. calcoaceticus* complex.
[c] NI, not interpretable.

tein Extraction Reagent [B-PER II]; Thermo Scientific Pierce, Villebon-sur-Yvette, France) has been replaced by an hyperosmotic solution of 5 M NaCl. The use of the 5 M NaCl solution provides two main advantages. It does not buffer enough to interfere with slight pH changes, and its hyperosmotic properties lead to an efficient lysis of the bacteria. Water has also been tested as a substitute for the B-PER-II lysis buffer, but the color changes were less clear and the test failed to detect 19 carbapenemase producers (nine OXA-23 and six OXA-58-like), giving orange results (data not shown). This lack of detection might be explained by the weak lysis of the bacteria in water compared to the hyperosmotic solution of NaCl, resulting in lower enzyme release. Recently, a derivative version of the Carba NP test was developed. This test used water instead of the B-PER buffer and bromothymol blue instead of red phenol as the pH indicator (27). The authors claimed that the use of water instead of B-PER buffer led to the detection of all carbapenemase-producing *Acinetobacter* spp. However, in their study, only 14 OXA-23 and six OXA-58-like producers were tested, which is not sufficient to assess the reliability of that test, considering that only 13.2% of the OXA-23 producers and 23.1% of the OXA-58-like producers were detected when using water instead of NaCl. As previously observed using the Carba NP test with *P. aeruginosa* strains, the GES-type carbapenemase producers were not detected using the CarbAcineto NP test (26). The GES-type carbapenemases are point mutant analogues of the ESBL GES-1 and possess an extended but very weak carbapenemase activity that might explain this lack of detection. This lack of detection of GES-type carbapenemases in *P. aeruginosa* may be also due to a weaker release or production of GES-type carbapenemases in *P. aeruginosa* (26), since a GES-5-producing *Enterobacter cloacae* isolate was identified with the Carba NP test (24).

Although the number of tested strains was low (3 isolates), the CarbAcineto NP test gave negative results when testing carbapenem-resistant *Acinetobacter* spp. overexpressing their chromosome-encoded OXA-51-like β-lactamase (Table 2). In fact, the lack of detection of OXA-51-like overproducers may be interesting when considering the usefulness of the CarbAcineto NP test. Indeed, those chromosomally encoded resistance mechanisms are not supposed to be transferable to other organisms, in contrast to plasmid-encoded mechanisms, which therefore constitutes a much less important clinical issue. Thus, clearly distinguishing acquired from nonacquired carbapenemase producers provides an added value to the test.

In conclusion, the CarbAcineto NP test offers a rapid and cost-effective solution for detecting acquired carbapenemase producers in *Acinetobacter* species. It might contribute to preventing the spread of those multidrug-resistant strains and also of several carbapenemase genes, considering, for example, that bla_{NDM-1}-like genes that initially spread among *Acinetobacter* spp. then targeted *Enterobacteriaceae* and *Pseudomonas* spp. (7). The use of the CarbAcineto NP test will be interesting particularly for ICU patients, for whom multidrug-resistant isolates belonging to the *A. baumannii-A. calcoaceticus* complex are a common source of severe infections. It particularly makes sense to test for carbapenemase production considering that the acquisition of a carbapenemase in *A. baumannii* is a marker of multidrug resistance.

Finally, by using both the Carba NP and CarbAcineto NP tests, any microbiology laboratory worldwide may have the opportunity to efficiently identify one of the most important clinical resis-

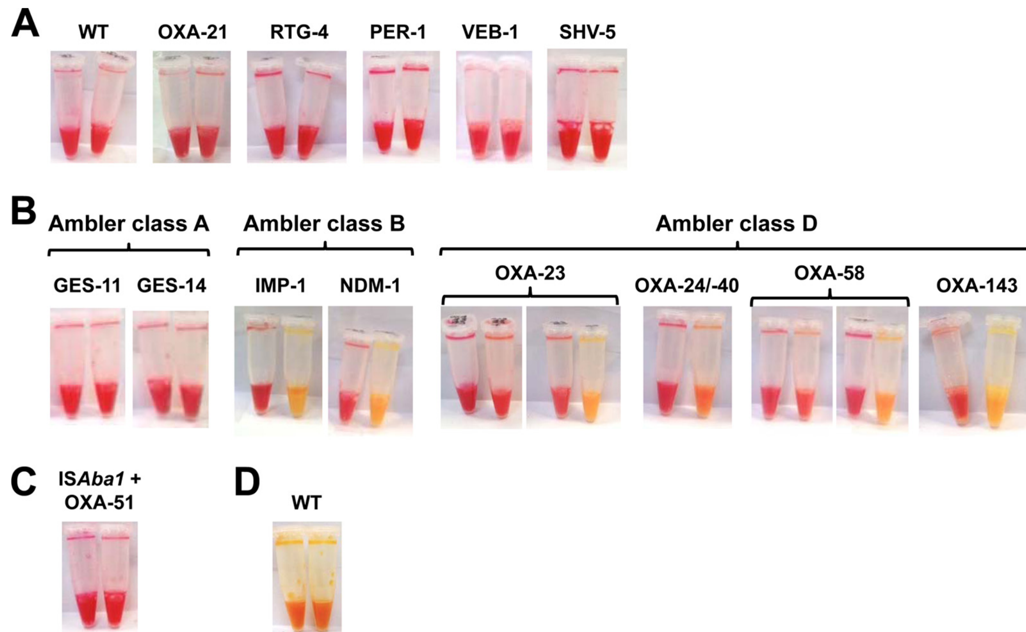

FIG 1 Representative results obtained using the CarbAcineto NP test on *Acinetobacter* spp. isolates. (A) Representative results obtained using the CarbAcineto NP test on wild-type (WT), narrow-spectrum β-lactamase-producing (OXA-21 and RTG-4), and extended-spectrum β-lactamase-producing *Acinetobacter* spp. (PER-1, VEB-1, and SHV-5). (B) Representative results obtained using the CarbAcineto NP test on acquired carbapenemase-producing *Acinetobacter* spp. isolates. Ambler class A (GES-types), Ambler class B (IMP-type and NDM-type), and Ambler class D (OXA-23, OXA-24/OXA-40, OXA-58, and OXA-143) carbapenemases are represented. (C) Representative result obtained using the CarbAcineto test on a carbapenem-resistant *Acinetobacter* sp. overexpressing its chromosome-encoded OXA-51 β-lactamase. (D) Representative result of a noninterpretable result using the CarbAcineto NP on a wild-type *Acinetobacter* isolate.

tance traits of modern microbiology, i.e., carbapenem resistance-mediated mechanisms leading to multidrug or pandrug resistance in clinically significant Gram negatives.

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An international patent form for the Carba NP test (that included further developments, such as the CarbAcineto NP test) has been filed on behalf of INSERM Transfert (Paris, France).

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