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Detection of Colistin-Resistant MCR-1-Positive *Escherichia coli* by Use of Assays Based on Inhibition by EDTA and Zeta Potential

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ABSTRACT The emergence and rapid dissemination of colistin-resistant *Escherichia coli* carrying the plasmid-mediated *mcr-1* gene have created an urgent need to develop specific screening methods. In this study, we evaluated four assays based on the inhibition of MCR-1 activity by EDTA: (i) a combined-disk test (CDT) comparing the inhibition zones of colistin and colistin (10 μg) plus EDTA (100 mM); (ii) reduction of colistin MIC (CMR) in the presence of EDTA (80 μg/ml); (iii) a modified rapid polymyxin Nordmann/Poirel test (MPNP); and (iv) alteration of zeta potential ($R_{ZP} = ZP_{+EDTA}/ZP_{-EDTA}$). We obtained encouraging results for the detection of MCR-1 in *E. coli* isolates recovered from human, food, and animal samples, using the following assay parameters: ≥3 mm difference in the inhibition zones between colistin disks without and with EDTA; ≥4-fold colistin MIC decrease in the presence of EDTA; R_{ZP} of ≥2.5; and the absence of metabolic activity and proliferation, indicated by unchanged color of phenol red in the presence of colistin-EDTA, in the MPNP test. In this regard, the CDT, CMR, R_{ZP}, and MPNP assays exhibited sensitivities of 96.7, 96.7, 95.1, and 96.7% and specificities of 89.6, 83.3, 100, and 100%, respectively, for detecting MCR-1-positive *E. coli*. Our results demonstrate that inhibition by EDTA and zeta potential assays may provide simple and inexpensive methods for the presumptive detection of MCR-1-producing *E. coli* isolates in human and veterinary diagnostic laboratories.

KEYWORDS polymyxins, colistin resistance, combined disk test, polymyxin NP test, charge modification

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Colistin (polymyxin E) and polymyxin B belong to a group of polypeptide antibiotics classified as polymyxins, which are considered one of the last lines of therapy for the treatment of lethal infections caused by multidrug-resistant Gram-negative pathogens (1, 2). Th antibacterial activity of polymyxins is based on an electrostatic interaction between cationic polypeptide antibiotics and negatively charged moieties present on the lipid A portion of the lipopolysaccharide (LPS) that form the outer membrane of Gram-negative bacteria (1–3). Consequently, the outer membrane is destabilized, increasing its permeability and leading to leakage of the cytoplasmic content, with subsequent lysis and bactericidal activity. Polymyxin resistance is usually caused by LPS modifications (3). In most resistant strains, 4-amino-4-deoxy-L-arabinose (L-Ara4N), phosphoethanolamine (PEtN), or galactosamine moieties are enzymatically added to

the lipid A or the LPS core (1–3). These modifications result in a decrease in a net negative charge of phosphate residues, leading to a reduction in polymyxin affinity (2, 3). Some species are naturally resistant to polymyxins, including *Proteus* spp., *Morganella morganii*, *Providencia* spp., *Serratia marcescens*, and nonfermentative *Burkholderia mallei*, and *Burkholderia cepacia* (2, 4, 5), whereas in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*, the two-component regulatory systems (TCSs) PmrA/PmrB and PhoP/PhoQ have been identified as regulatory systems involved in resistance to polymyxins, where PmrAB activates L-Ara4N synthesis leading to polymyxin resistance. Additionally, the insertional inactivation of the PhoQ/PhoP *mgrB*-encoding regulator has also been associated with colistin resistance (2, 3).

Recently, the plasmid-encoded polymyxin resistance determinant MCR-1 has been identified in clinically significant *Enterobacteriaceae* (particularly *E. coli*), and new plasmid-borne colistin resistance genes, *mcr-2*, *mcr-3*, and *mcr-4*, were further described (6–12). *mcr*-type genes encode phosphoethanolamine transferases that add PEtN to the phosphate group of the lipid A moiety (at the 4′ position) anchored on LPS, reduce negative charges that are present in LPS, and consequently confer resistance to polymyxins (4, 9–12). The *mcr-1* gene has been identified as a plasmid-mediated resistance mechanism being widely disseminated among human, animal, food, and environmental *E. coli* isolates (6–8, 13–19).

Recent structural studies have revealed that the catalytic domain of the MCR-1 phosphoethanolamine transferase resembles a zinc metalloprotein, where zinc deprivation has reduced colistin MICs in MCR-1-producing *E. coli* isolated from different sources, revealing the importance of zinc to MCR-1 activity and supporting the notion that assays under zinc-limiting conditions could represent a strategy for phenotypic detection of MCR-1 (9, 20–23). Since the emergence of both intrinsic and transferable mechanisms of polymyxin resistance is becoming a critical issue worldwide, the development of rapid and reliable methods to determine the susceptibility and resistance to polymyxins is an urgent need for clinical laboratories. In addition, phenotypic tests for screening colistin-resistant *Escherichia coli* carrying the plasmid-mediated *mcr-1* gene are highly desirable (24, 25). In this study, we evaluated four specific assays based on the inhibition of the MCR-1 activity by EDTA: (i) a combined-disk test (CDT) comparing the inhibition zones of colistin and colistin (10 μg) plus EDTA (100 mM); (ii) reduction of colistin MIC (CMR) in the presence of EDTA (80 mg/liter); (iii) a modified rapid polymyxin Nordmann/Poirel test (MPNP); and (iv) alteration of zeta potential ($R_{ZP} = ZP_{+EDTA}/ZP_{-EDTA}$).

RESULTS

MCR-1 detection by CDT. From the different EDTA concentrations tested, 100 mM EDTA was chosen for inhibition activity of MCR-1 in the CDT, since this concentration showed no inhibitory activity across the bacterial growth of all screened isolates when sterile blank disks impregnated with 10 μl of 100 mM EDTA were tested. When all colistin-resistant (MIC, >2 μg/ml) MCR-1-positive *E. coli* isolates were analyzed, an increase of $\geq$3 mm in the size of inhibition zones around the 10 μg of colistin–100 mM EDTA in comparison to the inhibition zones of colistin without EDTA was observed. Table 1 summarizes the results of the CDT, which was performed three times on distinct dates. Under these conditions, the sensitivity (SN) and specificity (SP) of CDT were 96.7 and 89.6%, respectively. In this regard, a colistin-susceptible (MIC 1 μg/ml) MCR-1-positive *E. coli* isolate (strain ICBEC 146) and a colistin-resistant MCR-1-positive *K. pneumoniae* isolate (CCBH24080) were not identified by the CDT (Table 1). On the other hand, five colistin-resistant MCR-1-negative *E. coli* isolates displayed an increase of $\geq$3 mm in the size of inhibition zones around colistin-EDTA.

CMR in the presence of EDTA. For CMR assays, the final concentration of EDTA was fixed at 80 μg/ml, since this concentration showed no antibacterial activity against all colistin-resistant screened isolates, allowing us to observe a $\geq$4-fold colistin MIC decrease among MCR-1-positive *E. coli* isolates in the presence of EDTA. In Table 2, the results of reproducible replicates, performed three times on 3 distinct occasions, are

TABLE 1 Evaluation of CDT using EDTA for detection of MCR-1-producing *Escherichia coli*

Isolate[a]	Species	Source	Inhibition zone diam (mm)[b]									Mechanism of colistin resistance
			Assay 1			Assay 2			Assay 3			
			−E	+E	Increase	−E	+E	Increase	−E	+E	Increase	
Colistin-resistant isolates												
50H	*E. coli*	Human	9	13	4	10	13	3	10	14	4	Plasmid-mediated *mcr-1* gene
51H	*E. coli*	Human	10	14	4	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
77H	*E. coli*	Human	11	14	4	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
200H	*E. coli*	Human	10	13	3	9	13	4	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 72H	*E. coli*	Human	10	13	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 79H	*E. coli*	Human	10	13	3	9	12	3	9	12	3	Plasmid-mediated *mcr-1* gene
ICBEC 2.6	*E. coli*	Chicken	11	14	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 3.6	*E. coli*	Chicken	10	13	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 5.2.1	*E. coli*	Chicken	9	12	3	9	12	4	9	13	4	Plasmid-mediated *mcr-1* gene
ICBEC 5.3	*E. coli*	Chicken	9	12	3	8	12	4	8	12	4	Plasmid-mediated *mcr-1* gene
ICBEC 5.5	*E. coli*	Chicken	10	14	4	10	14	4	10	14	4	Plasmid-mediated *mcr-1* gene
ICBEC 6.3	*E. coli*	Chicken	10	13	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 9.3	*E. coli*	Chicken	11	14	3	11	14	3	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 9.6	*E. coli*	Chicken	10	14	4	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 11.3	*E. coli*	Chicken	11	14	3	10	13	3	11	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 11.8	*E. coli*	Chicken	11	14	3	10	13	3	11	14	3	Plasmid-mediated *mcr-1* gene
ICBEC 12.3	*E. coli*	Chicken	9	13	4	10	14	4	10	14	4	Plasmid-mediated *mcr-1* gene
ICBEC 12.6	*E. coli*	Chicken	10	13	3	11	14	3	11	14	3	Plasmid-mediated *mcr-1* gene
96	*E. coli*	Chicken	10	13	3	11	14	3	10	13	3	Plasmid-mediated *mcr-1* gene
662	*E. coli*	Chicken	11	14	3	11	14	3	11	14	3	Plasmid-mediated *mcr-1* gene
284	*E. coli*	Bovine	8	12	4	8	12	4	9	13	4	Plasmid-mediated *mcr-1* gene
946	*E. coli*	Bovine	8	12	4	9	13	4	8	12	4	Plasmid-mediated *mcr-1* gene
CF 1.2	*E. coli*	Chicken meat	9	12	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
CF 101	*E. coli*	Chicken meat	10	13	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
CF 111	*E. coli*	Chicken meat	9	12	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
CF 131	*E. coli*	Chicken meat	9	12	3	9	13	4	9	12	3	Plasmid-mediated *mcr-1* gene
CF 132	*E. coli*	Chicken meat	10	13	3	9	13	4	9	12	3	Plasmid-mediated *mcr-1* gene
CF 341	*E. coli*	Chicken meat	9	13	4	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
CF 351	*E. coli*	Chicken meat	10	13	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
28	*E. coli*	Turkey	9	12	3	9	12	3	10	14	4	Plasmid-mediated *mcr-1* gene
69	*E. coli*	Turkey	9	12	3	11	14	3	10	13	3	Plasmid-mediated *mcr-1* gene
71	*E. coli*	Turkey	10	13	3	8	11	3	10	13	3	Plasmid-mediated *mcr-1* gene
73	*E. coli*	Turkey	11	14	3	10	13	3	9	12	3	Plasmid-mediated *mcr-1* gene
75	*E. coli*	Turkey	10	13	3	10	13	3	10	13	3	Plasmid-mediated *mcr-1* gene
77	*E. coli*	Turkey	10	13	3	10	14	4	9	12	3	Plasmid-mediated *mcr-1* gene
79	*E. coli*	Turkey	10	14	4	10	13	3	10	14	4	Plasmid-mediated *mcr-1* gene
84	*E. coli*	Turkey	10	13	3	10	13	3	9	13	4	Plasmid-mediated *mcr-1* gene
93	*E. coli*	Turkey	10	13	3	10	13	3	9	12	3	Plasmid-mediated *mcr-1* gene
02	*E. coli*	Swine	10	13	3	10	14	4	10	13	3	Plasmid-mediated *mcr-1* gene
06	*E. coli*	Swine	9	12	3	9	13	4	9	12	3	Plasmid-mediated *mcr-1* gene
08	*E. coli*	Swine	10	13	3	9	13	4	10	14	4	Plasmid-mediated *mcr-1* gene
10	*E. coli*	Swine	9	12	3	10	14	4	10	13	3	Plasmid-mediated *mcr-1* gene
12	*E. coli*	Swine	8	11	3	10	13	3	9	12	3	Plasmid-mediated *mcr-1* gene
14	*E. coli*	Swine	10	13	3	9	12	3	10	13	3	Plasmid-mediated *mcr-1* gene
18	*E. coli*	Swine	8	11	3	10	13	3	9	13	4	Plasmid-mediated *mcr-1* gene
24	*E. coli*	Swine	10	13	3	10	14	4	10	13	3	Plasmid-mediated *mcr-1* gene
26	*E. coli*	Swine	10	13	3	10	13	3	9	12	3	Plasmid-mediated *mcr-1* gene
29	*E. coli*	Swine	11	14	3	9	13	4	10	14	4	Plasmid-mediated *mcr-1* gene
33	*E. coli*	Swine	10	13	3	11	14	3	10	13	3	Plasmid-mediated *mcr-1* gene
35	*E. coli*	Swine	9	13	4	8	13	5	8	12	4	Plasmid-mediated *mcr-1* gene
53	*E. coli*	Swine	10	13	3	9	12	3	10	13	3	Plasmid-mediated *mcr-1* gene
55	*E. coli*	Swine	10	14	4	10	14	4	9	12	3	Plasmid-mediated *mcr-1* gene
60	*E. coli*	Swine	11	15	4	10	14	4	12	15	3	Plasmid-mediated *mcr-1* gene
86	*E. coli*	Swine	10	13	3	10	14	4	10	13	3	Plasmid-mediated *mcr-1* gene
88	*E. coli*	Swine	11	14	3	10	13	3	9	12	3	Plasmid-mediated *mcr-1* gene
95	*E. coli*	Swine	9	12	3	8	11	3	8	12	4	Plasmid-mediated *mcr-1* gene
98	*E. coli*	Swine	10	13	3	10	14	4	10	13	3	Plasmid-mediated *mcr-1* gene
ICBEC 171	*E. coli*	Swine	9	13	4	9	13	4	10	14	4	Plasmid-mediated *mcr-1* gene
7P	*E. coli*	Penguin	10	13	3	10	13	3	9	12	3	Plasmid-mediated *mcr-1* gene
HC113	*E. coli*	Human	8	11	3	9	12	3	8	11	3	Unknown
HC629	*E. coli*	Human	12	17	5	10	13	3	10	13	3	Unknown
M6	*E. coli*	Wild bird	9	11	2	10	12	2	9	11	2	Unknown
M37A	*E. coli*	Wild bird	8	12	4	8	12	4	8	12	4	Unknown
M51	*E. coli*	Wild bird	8	12	4	8	12	4	8	12	4	Unknown
M55	*E. coli*	Wild bird	8	12	4	9	13	4	10	13	3	Unknown

(Continued on next page)

TABLE 1 (Continued)

Isolate[a]	Species	Source	Inhibition zone diam (mm)[b]									Mechanism of colistin resistance
			Assay 1			Assay 2			Assay 3			
			−E	+E	Increase	−E	+E	Increase	−E	+E	Increase	
Δ806 mutant	*E. coli*	Human	10	12	2	10	11	1	10	12	2	PmrB P92T
CCBH24080	*K. pneumoniae*	Human	9	10	1	10	10	0	9	11	2	Plasmid-mediated *mcr-1* gene
Alerta 06	*K. pneumoniae*	Human	13	14	1	13	13	0	13	13	0	*phoQ* overexpression[c]
Alerta 08	*K. pneumoniae*	Human	14	14	0	14	14	0	13	14	1	Unknown
Alerta 09	*K. pneumoniae*	Human	14	14	0	13	14	1	13	13	0	Decreased *mgrB* expression[c]
Alerta 10	*K. pneumoniae*	Human	9	9	0	10	11	1	10	10	0	*phoP*/*phoQ* overexpression[c]
Alerta 12	*K. pneumoniae*	Human	12	12	0	10	10	0	12	12	0	MgrB IS*903*-like
Alerta 13	*K. pneumoniae*	Human	9	9	0	9	9	0	9	9	0	MgrB IS*903b*-like
Alerta 14	*K. pneumoniae*	Human	8	8	0	9	9	0	8	8	0	MgrB truncated
Alerta 15	*K. pneumoniae*	Human	9	10	1	8	9	1	9	10	1	MgrB IS*Kpn13*
Alerta 16	*K. pneumoniae*	Human	10	10	0	9	10	1	10	10	0	*mgrB* promoter IS*1* family
Alerta 17	*K. pneumoniae*	Human	10	12	2	10	10	0	11	11	0	Unknown
Alerta 31	*K. pneumoniae*	Human	11	11	0	12	12	0	11	11	1	PmrB T246C-R256G
Alerta 32	*K. pneumoniae*	Human	12	12	0	12	12	0	12	12	0	PmrB T246A-R256G-A282T-V290G-E291K
Alerta 33	*K. pneumoniae*	Human	11	12	1	10	11	1	10	11	1	Unknown
Alerta 35	*K. pneumoniae*	Human	10	11	1	10	11	1	10	11	1	Unknown
Alerta 36	*K. pneumoniae*	Human	12	12	0	12	13	1	11	12	1	Unknown
Alerta 37	*K. pneumoniae*	Human	11	12	1	10	11	1	10	11	1	Unknown
Alerta 38	*K. pneumoniae*	Human	10	11	1	11	12	1	11	12	1	PhoP W84C
Alerta 39	*K. pneumoniae*	Human	11	12	1	12	13	1	11	12	1	MgrB IS*903b*-like
Kp 148	*K. pneumoniae*	Human	0	0	0	0	0	0	0	0	0	Unknown
BL-II-04(2)	*M. morganii*	Human	0	0	0	0	0	0	0	0	0	Intrinsic
SM 26	*S. marcescens*	Human	0	0	0	0	0	0	0	0	0	Intrinsic
25933	*P. mirabilis*	ATCC	0	0	0	0	0	0	0	0	0	Intrinsic
Colistin-susceptible isolates												
31	*E. coli*	Turkey	13	13	0	12	13	1	13	13	0	
51	*E. coli*	Turkey	12	13	1	11	13	2	11	11	0	
91	*E. coli*	Turkey	12	13	1	11	12	1	11	13	2	
04	*E. coli*	Swine	12	13	1	11	13	2	10	12	2	
49	*E. coli*	Swine	12	14	2	10	12	2	11	13	2	
58	*E. coli*	Swine	13	13	0	13	14	1	10	12	2	
62	*E. coli*	Swine	13	14	1	11	13	2	11	12	1	
64	*E. coli*	Swine	14	15	1	14	15	1	13	14	1	
65	*E. coli*	Swine	11	12	1	13	13	0	13	13	0	
89	*E. coli*	Swine	13	14	1	12	14	2	11	12	1	
100	*E. coli*	Swine	12	13	1	10	10	0	12	12	0	
60198	*E. coli*	Human	12	13	1	11	12	1	11	12	1	
ICBEC 146	*E. coli*	Swine	9	10	1	10	10	0	10	10	1	Plasmid-mediated *mcr-1* gene
25922	*E. coli*	ATCC	13	13	0	14	14	0	13	13	0	
Alerta 26	*K. pneumoniae*	Human	10	11	1	10	12	2	11	12	1	
Alerta 27	*K. pneumoniae*	Human	11	12	1	10	11	1	11	12	1	
Alerta 28	*K. pneumoniae*	Human	12	12	0	12	12	0	10	11	1	
Alerta 29	*K. pneumoniae*	Human	10	11	1	11	12	1	11	12	1	
Alerta 30	*K. pneumoniae*	Human	10	11	1	11	12	1	10	12	2	
13883	*K. pneumoniae*	ATCC	13	13	0	14	14	0	13	13	0	

[a]PFGE and/or MLST data obtained from earlier studies revealed that most *mcr-1*-positive *E. coli* isolates were clonally unrelated (8, 13–17, 33).

[b]The combined disk test method was performed in triplicate. Two 10-μg colistin disks without (−E) and with (+E) EDTA (10 μl of a 100 mM solution [pH 8]) were used. An increase of ≥3 mm in the inhibition zone diameter in the presence of EDTA was considered a positive result.

[c]Although a variation in the gene expression was verified compared to the *K. pneumoniae* MGH 78578 strain, no mutations in amino acid or nucleotide sequences were detected in any studied genes.

shown. Under these conditions, the SN and SP of CMR were 96.7 and 83.3%, respectively. Nevertheless, for both colistin-susceptible MCR-1-positive *E. coli* strain ICBEC 146 and colistin-resistant MCR-1-positive *K. pneumoniae* strain CCBH24080, only a 2-fold colistin MIC decrease was recorded (Table 2). Moreover, two MCR-1-negative colistin-susceptible (isolates 58 and 89) and six MCR-1-negative colistin-resistant *E. coli* strains (isolates HC113, HC629, M6, M37A, M51, and M55) exhibited a ≥4-fold colistin MIC decrease in the presence of EDTA.

MPNP. The MPNP was based on the original NP test proposed for the rapid identification of polymyxin-resistant and -susceptible *Enterobacteriaceae* (26, 27). Inter-

TABLE 2 Alteration of zeta potential, MPNP test results, and CMR induced by EDTA for detection of MCR-1-producing *Escherichia coli*

Isolate	Species	Zeta potential (mean [SD]) (mV)[a]			Colistin MIC (μg/ml)[b]		MIC reduction (fold change)[c]	MPNP test result[d]	*mcr-1* confirmation
		−E	+E	R_{ZP}	−E	+E			
Colistin-resistant isolates									
50H	*E. coli*	−7.09 (0.54)	−38.21 (1.09)	5.39	16	2	8	+	+
51H	*E. coli*	−4.23 (0.56)	−37.65 (1.78)	8.90	8	1	8	+	+
77H	*E. coli*	−5.98 (0.45)	−38.09 (1.08)	6.37	8	0.5	16	+	+
200H	*E. coli*	−5.32 (0.97)	−32.09 (1.65)	6.03	16	1	16	+	+
ICBEC 72H	*E. coli*	−4.91 (0.53)	−34.06 (1.02)	6.94	8	1	8	+	+
ICBEC 79H	*E. coli*	−4.88 (0.46)	−39.50 (0.39)	8.09	8	1	8	+	+
ICBEC 2.6	*E. coli*	−4.88 (0.90)	−40.48 (0.29)	8.30	8	1	8	+	+
ICBEC 3.6	*E. coli*	−4.23 (0.89)	−36.52 (0.91)	8.63	8	1	8	+	+
ICBEC 5.2.1	*E. coli*	−5.73 (1.00)	−37.24 (0.41)	6.50	8	2	4	+	+
ICBEC 5.3	*E. coli*	−7.19 (0.63)	−40.62 (0.57)	5.65	8	0.5	16	+	+
ICBEC 5.5	*E. coli*	−8.19 (1.72)	−39.85 (1.61)	4.87	8	0.5	16	+	+
ICBEC 6.3	*E. coli*	−5.28 (0.95)	−35.71 (0.87	6.76	8	1	8	+	+
ICBEC 9.3	*E. coli*	−6.05 (0.34)	−40.26 (0.98)	6.65	8	0.5	16	+	+
ICBEC 9.6	*E. coli*	−7.20 (0.91)	−33.30 (0.95)	4.63	8	1	8	+	+
ICBEC 11.3	*E. coli*	−8.92 (0.48)	−34.93 (2.57)	3.92	8	2	4	+	+
ICBEC 11.8	*E. coli*	−7.24 (0.84)	−38.74 (1.16)	5.35	8	0.5	16	+	+
ICBEC 12.3	*E. coli*	−5.19 (0.47)	−40.81 (1.20)	7.86	16	2	8	+	+
ICBEC 12.6	*E. coli*	−7.66 (0.92)	−34.71 (2.26)	4.53	8	2	4	+	+
96	*E. coli*	−5.04 (0.81)	−35.26 (1.02)	7.00	4	0.25	16	+	+
662	*E. coli*	−4.37 (1.38)	−38.86 (0.81)	8.89	4	1	4	+	+
284	*E. coli*	−6.40 (0.52)	−36.42 (1.71)	5.69	4	1	4	+	+
946	*E. coli*	−4.39 (0.90)	−39.96 (0.76)	9.10	4	0.5	8	+	+
CF 1.2	*E. coli*	−6.08 (1.87)	−31.65 (0.98)	5.21	8	2	4	+	+
CF 101	*E. coli*	−6.31 (0.85)	−34.89 (0.35)	5.53	8	2	4	+	+
CF 111	*E. coli*	−8.76 (0.74)	−31.89 (1.43)	3.64	4	0.25	16	+	+
CF 131	*E. coli*	−9.43 (0.23)	−34.50 (0.29)	3.66	8	1	8	+	+
CF 132	*E. coli*	−7.21 (0.83)	−33.03 (0.98)	4.58	8	0.25	32	+	+
CF 341	*E. coli*	−4.99 (0.67)	−32.01 (0.39)	6.41	4	1	4	+	+
CF 351	*E. coli*	−5.24 (0.87)	−38.67 (0.65)	7.34	8	1	8	+	+
28	*E. coli*	−6.86 (1.71)	−35.99 (1.02)	5.25	4	0.5	8	+	+
69	*E. coli*	−8.92 (0.65)	−31.09 (0.74)	3.49	4	0.5	8	+	+
71	*E. coli*	−4.22 (0.32)	−21.13 (1.02)	5.01	4	1	4	+	+
73	*E. coli*	−5.67 (0.98)	−37.27 (0.36)	6.57	8	1	8	+	+
75	*E. coli*	−9.03 (1.16)	−35.27 (0.57)	3.91	4	0.5	8	+	+
77	*E. coli*	−8.20 (0.99)	−38.19 (1.39)	4.66	4	0.25	16	+	+
79	*E. coli*	−9.87 (1.67)	−36.78 (0.67)	3.73	4	1	4	+	+
84	*E. coli*	−7.41 (1.08)	−34.47 (1.32)	4.65	4	0.25	16	+	+
93	*E. coli*	−6.25 (0.17)	−36.96 (0.96)	5.91	4	0.5	8	+	+
02	*E. coli*	−6.08 (0.54)	−37.65 (1.03)	6.19	4	1	4	+	+
06	*E. coli*	−4.75 (0.36)	−38.64 (1.31)	8.13	4	0.5	8	+	+
08	*E. coli*	−8.57 (1.96)	−37.57 (2.07)	4.38	4	0.125	32	+	+
10	*E. coli*	−5.68 (0.73)	−37.11 (1.74)	6.53	8	1	8	+	+
12	*E. coli*	−6.14 (0.20)	−37.97 (1.69)	6.18	4	0.5	8	+	+
14	*E. coli*	−4.28 (1.98)	−35.83 (0.54)	8.37	4	0.125	32	+	+
18	*E. coli*	−7.56 (0.84)	−38.51 (1.23)	5.10	8	2	4	+	+
24	*E. coli*	−6.13 (0.51)	−37.18 (0.84)	6.10	4	0.25	16	+	+
26	*E. coli*	−6.39 (0.79)	−36.58 (0.38)	5.72	4	1	4	+	+
29	*E. coli*	−6.54 (0.71)	−37.56 (1.47)	5.74	4	1	4	+	+
33	*E. coli*	−4.20 (0.87)	−39.20 (1.46)	9.33	4	0.5	8	+	+
35	*E. coli*	−7.38 (0.95)	−37.02 (0.82)	5.01	4	0.125	32	+	+
53	*E. coli*	−3.34 (1.78)	−36.79 (0.09)	11.01	4	0.06	67	+	+
55	*E. coli*	−8.71 (0.37)	−37.56 (1.63)	4.31	4	0.5	8	+	+
60	*E. coli*	−5.89 (1.19)	−35.73 (0.61)	6.07	8	0.5	16	+	+
86	*E. coli*	−6.14 (2.10)	−35.85 (0.38)	5.78	4	0.5	8	+	+
88	*E. coli*	−6.96 (0.33)	−38.11 (0.63)	5.48	4	0.5	8	+	+
95	*E. coli*	−5.21 (1.96)	−32.28 (0.66)	6.20	4	1	4	+	+
98	*E. coli*	−6.32 (0.81)	−30.65 (0.12)	4.85	4	0.25	16	+	+
ICBEC 171	*E. coli*	−5.32 (0.64)	−32.41 (1.23)	6.09	8	0.5	16	+	+
7P	*E. coli*	−19.34 (0.58)	−30.42 (0.52)	1.57	8	2	4	+	+
HC113	*E. coli*	−15.03 (1.27)	−17.04 (1.78)	1.13	8	1	8	−	−

(Continued on next page)

TABLE 2 (Continued)

Isolate	Species	Zeta potential (mean [SD]) (mV)[a]			Colistin MIC (μg/ml)[b]		MIC reduction (fold change)[c]	MPNP test result[d]	*mcr-1* confirmation
		−E	+E	R_{ZP}	−E	+E			
HC629	*E. coli*	−24.07 (0.84)	−42.30 (0.88)	1.75	4	1	4	−	−
M6	*E. coli*	−3.52 (1.10)	−7.08 (1.02)	2.01	4	1	4	−	−
M37A	*E. coli*	−23.34 (1.58)	−22.51 (1.10)	0.96	4	0.5	8	−	−
M51	*E. coli*	−21.54 (0.24)	−16.02 (0.84)	0.74	8	1	8	−	−
M55	*E. coli*	−28.32 (0.70)	−29.15 (0.20)	1.03	8	1	8	−	−
Δ806 mutant	*E. coli*	−23.34 (1.58)	−21.53 (1.05)	0.92	4	4	—	−	−
CCBH24080	*K. pneumoniae*	−31.9 (0.48)	−28.51 (0.69)	0.89	8	4	2	−	+
Alerta 06	*K. pneumoniae*	−34.85 (1.42)	−37.51 (0.28)	1.07	16	16	—	−	−
Alerta 08	*K. pneumoniae*	−38.59 (1.78)	−39.83 (2.23)	1.03	4	4	—	−	−
Alerta 09	*K. pneumoniae*	−35.45 (0.93)	−39.28 (1.49)	1.11	8	4	2	−	−
Alerta 10	*K. pneumoniae*	−36.69 (2.38)	−39.25 (0.66)	1.07	4	2	2	−	−
Alerta 12	*K. pneumoniae*	−29.23 (0.82)	−34.36 (3.22)	1.18	4	4	—	−	−
Alerta 13	*K. pneumoniae*	−30.59 (0.77)	−34.19 (1.07)	1.12	16	16	—	−	−
Alerta 14	*K. pneumoniae*	−34.28 (2.28)	−47.21 (1.13)	1.38	8	8	—	−	−
Alerta 15	*K. pneumoniae*	−40.96 (0.52)	−35.83 (0.54)	0.87	4	4	—	−	−
Alerta 16	*K. pneumoniae*	−30.56 (2.37)	−33.52 (1.23)	1.10	8	8	—	−	−
Alerta 17	*K. pneumoniae*	−34.26 (0.88)	−35.52 (1.64)	1.04	>32	>32	—	−	−
Alerta 31	*K. pneumoniae*	−31.09 (2.10)	−33.11 (1.24)	1.06	8	8	—	−	−
Alerta 32	*K. pneumoniae*	−37.65 (1.09)	−38.08 (0.34)	1.01	8	4	2	−	−
Alerta 33	*K. pneumoniae*	−36.03 (1.24)	−35.49 (1.93)	0.99	8	8	—	−	−
Alerta 35	*K. pneumoniae*	−33.44 (2.47)	−32.10 (0.25)	0.96	8	4	2	−	−
Alerta 36	*K. pneumoniae*	−38.09 (1.40)	−39.89 (1.05)	1.05	4	4	—	−	−
Alerta 37	*K. pneumoniae*	−39.20 (0.56)	−35.98 (1.86)	0.92	16	16	—	−	−
Alerta 38	*K. pneumoniae*	−38.76 (0.98)	−39.21 (1.90)	1.01	8	8	—	−	−
Alerta 39	*K. pneumoniae*	−34.54 (1.03)	−33.23 (0.10)	0.96	8	4	2	−	−
Kp 148	*K. pneumoniae*	−30.91 (0.61)	−33.91 (0.26)	1.10	32	32	—	−	−
BL-II-04(2)	*M. morganii*	−36.91 (1.05)	−36.99 (0.98)	1.00	512	512	—	−	−
SM 26	*S. marcescens*	−36.85 (1.81)	−33.43 (1.40)	0.91	512	512	—	−	−
25933	*P. mirabilis*	−32.90 (1.42)	−35.06 (0.59)	1.07	512	512	—	−	−
Colistin-susceptible isolates									
31	*E. coli*	−36.57 (2.08)	−36.08 (1.26)	0.99	2	1	2	−	−
51	*E. coli*	−30.10 (3.10)	−35.93 (0.52)	1.19	0.06	0.06	—	−	−
91	*E. coli*	−33.13 (0.64)	−37.01 (0.57)	1.12	0.25	0.25	—	−	−
04	*E. coli*	−31.91 (0.51)	−37.33 (1.81)	1.17	1	0.5	2	−	−
49	*E. coli*	−29.60 (1.03)	−34.36 (2.71)	1.16	0.06	0.06	—	−	−
58	*E. coli*	−31.05 (2.48)	−36.38 (2.36)	1.17	1	0.06	16	−	−
62	*E. coli*	−32.14 (1.88)	−35.31 (1.14)	1.10	1	1	—	−	−
64	*E. coli*	−29.93 (2.63)	−36.48 (0.94)	1.22	1	1	—	−	−
65	*E. coli*	−30.23 (0.87)	−30.96 (1.03)	1.02	1	0.5	2	−	−
89	*E. coli*	−32.85 (1.04)	−37.37 (0.90)	1.14	0.25	0.06	4	−	−
100	*E. coli*	−35.08 (1.22)	−39.45 (0.93)	1.12	0.25	0.25	—	−	−
60198	*E. coli*	−44.21 (0.69)	−39.54 (2.70)	1.11	2	1	2	−	−
ICBEC 146	*E. coli*	−28.21 (2.74)	−30.29 (2.78)	1.08	1	0.5	2	−	+
25922	*E. coli*	−32.01 (1.75)	−37.53 (0.45)	1.17	1	1	—	−	−
Alerta 26	*K. pneumoniae*	−30.23 (1.32)	−31.14 (0.98)	1.03	0.5	0.5	—	−	−
Alerta 27	*K. pneumoniae*	−38.90 (1.98)	−38.21 (0.35)	0.98	0.25	0.25	—	−	−
Alerta 28	*K. pneumoniae*	−39.24 (1.47)	−38.98 (0.64)	0.99	1	0.5	2	−	−
Alerta 29	*K. pneumoniae*	−32.35 (1.65)	−31.29 (0.87)	0.97	0.5	0.5	—	−	−
Alerta 30	*K. pneumoniae*	−34.29 (2.09)	−35.60 (1.09)	1.04	1	1	—	−	−
13883	*K. pneumoniae*	−32.60 (1.03)	−32.04 (0.92)	0.98	1	1	—	−	−

[a]Bacterial surface charge (in millivolts) measures were determined for colistin-resistant and colistin-susceptible strains grown in Mueller-Hinton broth without (−E) and with (+E) EDTA (80 μg/ml). After 18 h of incubation at 37°C, cells were washed in 1.0 mM NaCl adjusted to 1×10^4 CFU/ml. Zeta potential (in millivolts) measures were determined using a Zeta Potential Analyzer (ZETAPALS; Brookhaven). The zeta potential ratio (R_{ZP}) is calculated as ZP_{+EDTA}/ZP_{-EDTA}, where ZP_{+EDTA} and ZP_{-EDTA} correspond to zeta potential values obtained for bacterial suspensions grown in the presence or absence of 80 μg/ml EDTA, respectively. Each value represents the mean of at least 5 individual measurements ± the standard deviation.

[b]Colistin MICs were determined by microdilution broth method according to EUCAST guidelines (32). Colistin MIC reduction was evaluated in the presence of EDTA at a final concentration of 80 μg/ml, and the values represent the results of reproducible replicates, performed three times on 3 distinct occasions.

[c]Dashes in empty cells indicate no MIC reduction (fold change) in the presence of of 80 μg/ml EDTA.

[d]MPNP was performed in triplicate. The MPNP test was considered positive to MCR-1 production when the growth of colistin-resistant *E. coli* in wells containing colistin solution (3.75 μg/ml) was inhibited by the addition of EDTA (80 μg/ml), as indicated by the unchanged color of phenol red in the NP solution (i.e., absence of metabolic activity and proliferation).

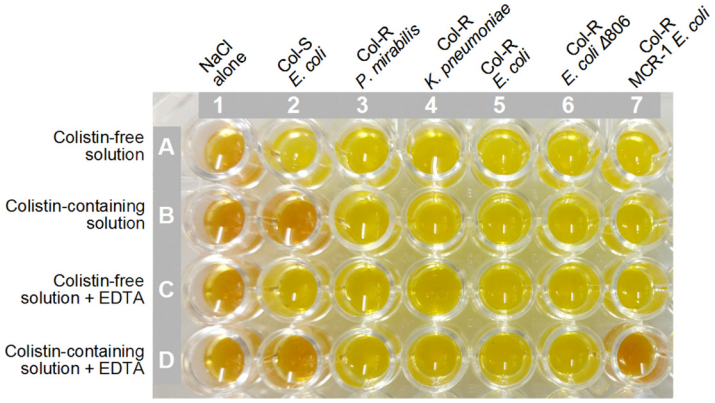

FIG 1 MCR-1 detection by the modified rapid polymyxin NP test (MPNP). Modification of the NP test was based on incorporation of two additional wells, which were filled with colistin-free solution plus EDTA (80 μg/ml) and colistin-containing (5 μg/ml) solution plus EDTA, respectively. Wells A1 to A7 were filled with 150 μl of a colistin-free NP solution. Wells B1 to B7 were completed with 150 μl of NP solution supplemented with 5 μg/ml colistin sulfate. Wells C1 to C7 were filled with 150 μl of colistin-free NP solution supplemented with 80 μg/ml EDTA. Wells D1 to D7 were added with 150 μl of NP solution containing 5 μg/ml colistin sulfate and 80 μg/ml EDTA. Wells in column 1 were filled with 50 μl of 0.85% NaCl (negative sterility control), whereas for each isolate, 50 μl of a 3.0 to 3.5 McFarland bacterial suspension ($\sim 10^9$ UFC/ml) was dispensed and mixed with 150 μl of reaction solution contained in each of the wells in columns 2 to 7. Columns 2 to 7 represent the MPNP test performed for *E. coli* ATCC 25922, *P. mirabilis* ATCC 25933, colistin-resistant (Col-R) *K. pneumoniae* Alerta 16 (*mgrB* promoter IS*1* family), colistin-resistant *mcr-1*-negative *E. coli* strain HC113, colistin-resistant *mcr-1*-negative *E. coli* Δ806 mutant strain, and *mcr-1*-positive *E. coli* strain ICBEC72H, respectively. The plates were incubated at 35 ± 2°C under aerobic conditions for 4 h, and visual changes in the color of the wells were monitored each hour. In wells with added colistin (B1 t oB7), a color change from orange to yellow was considered positive to colistin resistance, whereas the MPNP test was considered positive to MCR-1 phosphoethanolamine transferase production when the colistin-containing solution supplemented with EDTA (wells D1 to D7) remained orange (i.e., absence of glucose metabolization); this shows that growth of the colistin-resistant *E. coli* (*mcr-1*-positive) in the well containing colistin solution (well D7) was inhibited by EDTA.

estingly, while all colistin-resistant isolates showed a positive NP test result, only colistin-resistant MCR-1-positive *E. coli* isolates were inhibited by EDTA, as depicted in Fig. 1, where an absence of metabolic activity and bacterial proliferation (negative NP test indicated by unchanged color of phenol red in the well containing colistin-EDTA) was observed after a 2.5-h incubation at 37°C. The SN and SP were 96.7 and 100%, respectively. Indeed, the colistin-susceptible MCR-1-positive *E. coli* strain ICBEC 146 was not detected by the NP or the MPNP test. On the other hand, the colistin-resistant MCR-1-positive *K. pneumoniae* CCBH24080 strain showed positive and negative NP and MPNP results, respectively (Table 2).

Alteration of zeta potential. The zeta potential results for all strains evaluated in this study are summarized in Table 2. The replacement of lipid A with the PEtN-4′-lipid A, mediated by MCR-1, reduced the negative membrane charge of all colistin-resistant *E. coli* isolates to less than or equal to −20 mV (−4.20 to −19.34 mV), whereas for the *K. pneumoniae* strain CCBH24080, no charge reduction was observed. On the other hand, with the exception of two colistin-resistant MCR-1-negative *E. coli* strains (M6 and HC113), most colistin-susceptible and colistin-resistant (MCR-1-negative) *Enterobacteriaceae* presented zeta potential values between −21.54 and −44.21 mV (Fig. 2). For colistin-resistant MCR-1-positive *E. coli* isolates, bacterial growth in the presence of EDTA (80 μg/ml) resulted in an alteration of zeta potential ranging from −21.13 to −40.81 mV (Fig. 2), with an R_{ZP} value of ≥1.5. However, since two colistin-resistant MCR-1-negative *E. coli* strains (HC629 and M6) presented R_{ZP} values of 1.75 and 2.01, respectively, we have established an R_{ZP} value of ≥2.5 as the cutoff criterion for the presumptive identification of MCR-1-positive *E. coli* isolates. The SN and SP of R_{ZP} were 95.1 and 100%, respectively. For the colistin-susceptible MCR-1-positive *E. coli* strain ICBEC 146, an R_{ZP} of 1.08 was obtained, which was interpreted as a false-negative result.

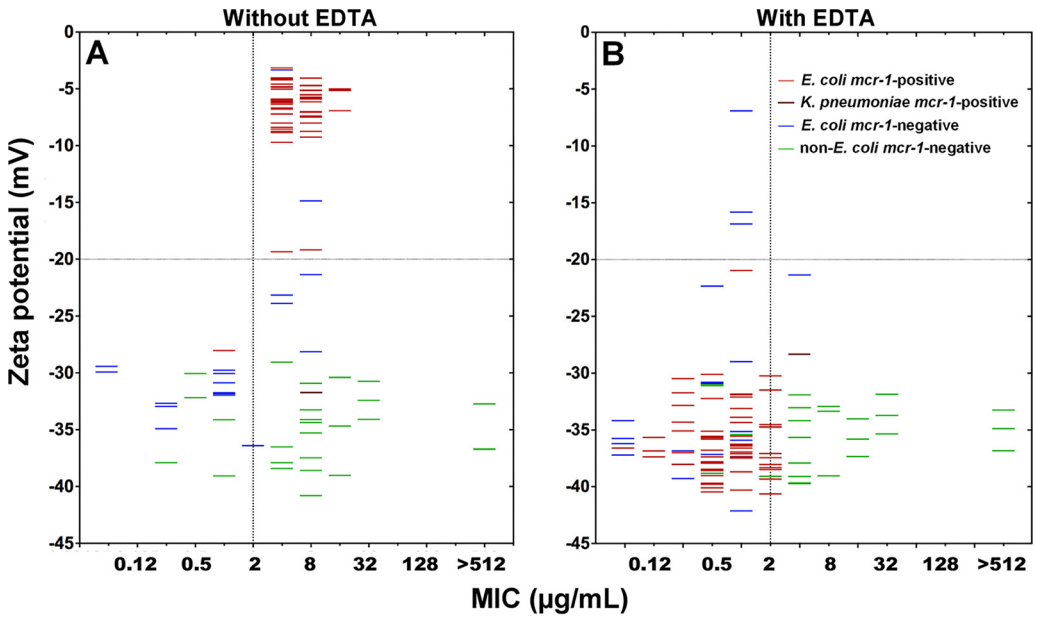

FIG 2 Bacterial surface charge distribution as a function of colistin MIC (A) and alteration of zeta potential induced by EDTA (B). Zeta potential was determined for colistin-resistant and colistin-susceptible strains grown in Mueller-Hinton broth without (A) and with (B) EDTA (80 μg/ml). After 18 h of incubation at 37°C, cells were washed in 1.0 mM NaCl adjusted to 1 $\times$ 10^4 CFU/ml. Zeta potential (millivolts) measures were determined using a Zeta Potential Analyzer (ZETAPALS; Brookhaven). *mcr-1*-positive *E. coli*, *mcr-1*-positive *K. pneumoniae*, *mcr-1*-negative *E. coli*, and non-*E. coli* strains are represented by red, brown, blue, and green dashes, respectively.

DISCUSSION

In this study, we have evaluated distinct phenotypic tests for the detection of colistin-resistant MCR-1-positive *E. coli* from human, food, and animal samples, based on the inhibition of the MCR-1 phosphoethanolamine transferase by EDTA. Microbiological assays relying on the synergy between EDTA and carbapenems have been previously developed and standardized for the screening of metallo-β-lactamase-producing Gram-negative bacteria, since it is well known that EDTA inhibits metallo-β-lactamase activity (28). Similarly, molecular and structure studies of the catalytic domain of MCR-type proteins have supported that phosphoethanolamine transferases can be assigned as a member of the alkaline phosphatase metalloenzyme superfamily, with zinc being required for MCR activity (9, 20–23). In fact, for MCR-1-positive strains, a clear reduction in colistin MIC was observed in the presence of EDTA, supporting the idea that zinc-limiting conditions induced by EDTA represent a good alternative for phenotypic identification of MCR-1-producing *E. coli* (20). Moreover, previous studies have demonstrated that the activity of putative Ca^{2+}-induced PEtN transferases, known to modify the other Kdo (3-deoxy-D-manno-octulosonic acid) residue of *E. coli* LPS, can be strongly inhibited by EDTA (29, 30). More recently, the inhibition of MCR-1 by dipicolinic acid (another metalloenzyme chelator) was reported as a useful method (named the colistin-MAC test) for phenotypic screening of *mcr-1*-positive colistin-resistant *E. coli* strains (31).

The CDT method was based on the utilization of disks containing colistin (10 μg), which were impregnated with 10 μl of 100 mM EDTA. Although colistin-resistant *E. coli* strains could be accurately classified as MCR-1 using as an interpretative criterion an increase of $\geq$3 mm in the inhibition zone of colistin disks plus EDTA in comparison to those observed for colistin disks without EDTA, this method may fail to detect MCR-1-positive isolates with colistin MICs of $\leq$2 μg/ml. In fact, a single strain was not identified as MCR-1 positive or colistin resistant by CDT, CMR, or MPNP, suggesting that *mcr-1* might not be expressed in this isolate. On the other hand, five colistin-resistant MCR-1-negative *E. coli* strains displayed a positive result, whereas a colistin-resistant

MCR-1-positive *K. pneumoniae* strain was not identified by the CDT method. In this regard, in a previous study of MCR-1 inhibition induced by dipicolinic acid (DPA), the disk diffusion format presented no significant differences in inhibition zones between *mcr-1*-positive and *mcr-1*-negative colistin-resistant strains, probably due to the low and variable diffusibility of colistin from disks into the Mueller-Hinton agar, as previously reported by Coppi et al. (31).

Although the colistin MICs of MCR-1-positive *E. coli* strains in the presence of EDTA resulted in a ≥4-fold colistin MIC reduction, this reduction was more evident for *E. coli* exhibiting high-level resistance to colistin. On the other hand, under this experimental condition, the specificity of MCR-1 detection was affected by the effect of the chelating agent against two colistin-susceptible *E. coli* strains. Moreover, this colistin MIC reduction in the presence of EDTA was also observed among colistin-resistant MCR-1-negative *E. coli* strains, with exception of the mutant *E. coli* strain.

The rapid polymyxin NP test was originally proposed for the detection of polymyxin-resistant and -susceptible *Enterobacteriaceae*, regardless of the resistance mechanism exhibited (26, 27). This method has been reported as an easy-to-perform, rapid, sensitive, and specific test. Interestingly, in this study, modification of the NP test based on the incorporation of two wells containing colistin-free solution plus EDTA, and a colistin-containing solution plus EDTA, resulted in the specific detection of MCR-1-positive colistin-resistant *E. coli* isolates, enhancing the accuracy of this method.

In order to develop colistin resistance, Gram-negative pathogens have developed multiple mechanisms to modify the lipid A structure of the LPS (2–4). In this study, we confirm that the biochemical mechanism by which MCR-1-positive bacteria acquire resistance to colistin is dependent on the reduction of the net negative charge of the bacterial outer membrane, which consequently decreases the binding affinity of colistin to the bacterial surface (2–4). Furthermore, under limited conditions of zinc for the MCR-1 biochemical function, as caused by EDTA, an increase in the net negative charge for colistin-resistant MCR-1-positive *E. coli* strains was observed. On the other hand, colistin MIC values in the presence of EDTA were reverted to a susceptible category, as interpreted according to the EUCAST breakpoint (32). The increase in net negative charge in the presence of EDTA reached zeta potential values identical to those of colistin-susceptible bacterial isolates. Thus, alteration of the zeta potential allowed us to generate an R_{ZP} index, where a value of ≥2.5 was associated with the MCR-1 phenotype. Finally, the lack of inhibitory effect of EDTA on the *mcr-1*-positive *K. pneumoniae* strain was also reported for DPA, which indeed could be due to a reduced permeability and/or additional unknown mechanisms of resistance to polymyxins (31).

There are certain limitations of this study, such as the reduced number of MCR-1-positive *K. pneumoniae* isolates investigated and the lack of other *Enterobacteriaceae* species (i.e., *Salmonella* spp., *Enterobacter* spp., and *Citrobacter* spp.) encoding MCR-1 and any isolates carrying other variants of the MCR-like PEtN transferase (10–12). We also should be aware of EDTA chelator activity that could act to nonspecifically affect other bacterial processes. On the other hand, the coproduction of an additional mechanism of colistin resistance, as related to the activation of TCSs, could interfere in the assays of inhibition by EDTA. Therefore, the results should be cautiously interpreted and confirmed with a reference method. Finally, all screening methods evaluated here fail in detecting the colistin-susceptible *E. coli* strain carrying the *mcr-1* gene, confirming that gene detection by molecular methods continues to be the gold standard.

In summary, our results demonstrate that assays of inhibition by EDTA may provide simple and inexpensive methods for detecting MCR-1-producing *E. coli* in human and veterinary diagnostic laboratories, mainly under the conditions described for the MPNP and zeta potential methods, which displayed the highest SN and SP values. However, additional studies are necessary to confirm the accuracy of these methodologies for phenotypically detecting MCR-producing isolates by testing other *Enterobacteriaceae* species or MCR variants isolated worldwide.

MATERIALS AND METHODS

Bacterial isolates. A total of 109 isolates belonging to the *Enterobacteriaceae* family were tested in this study (Table 1). Colistin-resistant *Escherichia coli* ($n = 66$) and *Klebsiella pneumoniae* ($n = 20$) and colistin-susceptible *E. coli* ($n = 13$) and *K. pneumoniae* ($n = 5$) isolates were recovered from humans, food, and animals. Intrinsically colistin-resistant *Morganella morganii* and *Serratia marcescens* (which were isolated from clinical specimens) and *Proteus mirabilis* ATCC 25933 were included. Additionally, the *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 strains were also evaluated. Among colistin-resistant *E. coli*, a total of 59 isolates were *mcr-1* positive, as confirmed by PCR and sequencing (6, 8, 10–17, 33), whereas colistin resistance in *K. pneumoniae* was related to MCR-1 production (8), activation of the two-component regulatory systems PhoPQ/PmrAB, or *mgrB* inactivation. In this regard, *K. pneumoniae* isolates were submitted to PCR amplification, followed by DNA sequencing (ABI 3500 genetic analyzers; Applied Biosystems, Foster City, CA) to the *pmrA*, *pmrB*, *pmrD*, *phoP*, *phoQ*, and *mgrB* genes (34, 35). Mutational analysis was performed by SeqManII version 5.0 and the BLASTn tool using *K. pneumoniae* MGH 78578 as the reference strain. Insertion sequences were confirmed by ISfinder (https://www-is.biotoul.fr). For those isolates that did not show any mutation, a reverse transcription-quantitative PCR (qRT-PCR) assay was performed to quantify the relative expression of the above-mentioned genes (35, 36). Additionally, a colistin-resistant mutant *E. coli* strain (Δ806) was developed under selective colistin pressure, as previously described (37).

CDT. The combined-disk test was adapted from methods described for the detection of metallo-β-lactamase-producing isolates (38, 39). Initially, in order to select an EDTA concentration displaying no antibacterial activity against all screened isolates, different concentrations and volumes of EDTA solution (Sigma-Aldrich, St. Louis, MO, USA) were added to both blank disks and 10-μg colistin disks (Oxoid, Basingstoke, UK). In brief, we tested combinations of 5, 10, and 20 μl at 50, 80, 90, 100, 150, 200, 300, 400, and 500 mM EDTA (pH 8.0). In this regard, the use of 10 μl of a 100 mM EDTA solution was selected for further tests. In this way, for each screened bacterium, two 10-μg colistin disks without and a blank disk with EDTA and a blank disk were placed onto Mueller-Hinton agar plates (Becton Dickinson, Le Pont de Claix, France) inoculated with a 0.5 McFarland bacterial suspension. Inhibition zone diameters around the colistin disks (with and without EDTA) were measured and compared after 18 to 24 h of incubation at 37°C.

CMR in the presence of EDTA. The MIC for colistin was performed by the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (32). However, for evaluation of the reduction in colistin MIC in the presence of EDTA, cation supplementation of Mueller-Hinton broth was not performed, since further addition of calcium and magnesium would impair the inhibitory activity of EDTA. Therefore, nonspecific binding of EDTA to excess of calcium and magnesium could reduce the concentration of free EDTA needed to chelate zinc ions required to MCR-1 activity (17). Moreover, it has been shown that calcium supplementation could favor the activity of putative PEtN transferases in *E. coli* (29, 30). In order to select an EDTA concentration exhibiting no antibacterial activity against all screened isolates, five different concentrations of EDTA (64, 80, 100, 128, and 256 μg/ml) were evaluated. The 80 μg/ml EDTA solution was selected for further tests. In brief, bacterial inocula were adjusted to a 0.5 McFarland turbidity standard and diluted to a ratio of 1:100 in Mueller-Hinton broth (Becton Dickinson, Le Pont de Claix, France). All isolates were tested in serial dilutions of colistin sulfate (Sigma-Aldrich, St. Louis, MO, USA) ranging from 0.06 to 32 μg/ml, except for the intrinsically colistin-resistant isolates, which were tested using serial dilutions of colistin sulfate ranging from 0.06 to 512 μg/ml. In this regard, *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 13883 were used as susceptible controls, whereas *P. mirabilis* ATCC 25933 was used as a resistant control (Table 2). MIC interpretation was performed according to EUCAST breakpoints (32).

MPNP. The MPNP rapid colorimetric test was based on the detection of bacterial growth in the presence of colistin sulfate at a final concentration of 3.75 μg/ml (26), where bacterial growth detection was supported by glucose metabolism with acid formation related to glucose metabolism in *Enterobacteriaceae*, resulting in a color change of a pH indicator (26, 27). In this study, modification was based on incorporation of two additional wells, which were filled with a colistin-free solution plus EDTA (80 μg/ml) and a colistin-containing (5 μg/ml) solution plus EDTA. Using sterile 96-well polystyrene plates (Sarstedt, Newton, NC, USA), the following experimental conditions were established in the MPNP test: (i) wells A1 to A12 were filled with 150 μl of colistin-free NP solution; (ii) 150 μl of NP solution supplemented with 5 μg/ml colistin sulfate was added to wells B1 to B12; (iii) wells C1 to C12 were filled with 150 μl of colistin-free NP solution supplemented with 80 μg/ml EDTA; (iv) 150 μl of NP solution supplemented with 5 μg/ml colistin sulfate and 80 μg/ml EDTA was deposited into wells D1 to D12; (v) except for the wells in column 1 (filled with 50 μl of 0.85% NaCl [negative sterility control]), for each isolate, 50 μl of a 3.0 to 3.5 McFarland bacterial suspension (~10^9 CFU/ml) was dispensed and mixed with 150 μl of reaction solution contained in each of the wells in columns 2 to 12. Therefore, the plate was organized so that each column represented the MPNP test performed for each strain. Finally, the plates were incubated at 35 ± 2°C under aerobic conditions for 4 h, and visual changes in the color of the wells were monitored each hour (26, 27). In this regard, in wells with added colistin-containing solution (B1 to B12), a color change from orange to yellow was considered positive to colistin resistance, whereas the MPNP test was considered positive to MCR-1 phosphoethanolamine transferase production when the colistin-containing solution supplemented with EDTA (wells D1 to D12) remained orange (i.e., absence of glucose metabolization) (Fig. 1). *E. coli* ATCC 25922 and *Proteus mirabilis* ATCC 25933 strains were used as controls.

Zeta potential measurement. Particle size (mean diameter in nanometers) and zeta potential (millivolts) of bacterial cells were measured with a ZetaPALS ZetaPotential Analyzer (Brookhaven

Instruments Corporation, Holtsville, NY), which was equipped with a 677-nm laser and dynamic light-scattering (PCS) at 90° for particle sizing (40). Zeta potential was determined from electrophoretic mobility μ at 25°C in 1 mM NaCl and using Smoluchowski's equation $\zeta = \mu\eta/\varepsilon$, where η is the medium viscosity and ε the medium dielectric constant (40–42). Prior to sample analysis, bacterial suspensions grown in the absence or presence of 80 μg/ml EDTA were centrifuged (5,000 rpm for 5 min at 5°C) and pellets were washed twice, being suspended in 2 ml of sterile 1 mM NaCl solution and adjusted to the turbidity of a 0.5 McFarland standard solution. For each bacterial sample, an additional 1:4 dilution was performed in 1 mM NaCl, and particle size and zeta potential were determined in 2-ml aliquots. Each value is shown as a mean of at least 5 individual measurements ± the standard deviation. Alterations of zeta potential induced by EDTA were calculated from the zeta potential ratio ($R_{ZP} = ZP_{+EDTA}/ZP_{-EDTA}$), where ZP_{+EDTA} and ZP_{-EDTA} correspond to zeta potential values obtained for bacterial suspensions grown in the presence or absence of 80 μg/ml EDTA, respectively.

Statistical analysis. Sensitivity (SN) and specificity (SP) were calculated for CDT, CMR, MPNP, and R_{ZP}. PCR and direct sequencing for the *mcr-1* gene were considered the gold standard. SN and SP were calculated with the formulas $a/(a + b)$ and $d/(c + d)$, respectively, where a is the number of isolates correctly identified as MCR-1 by the tested methods, b is the number of true MCR-1 positives that were incorrectly assigned non-MCR-1 by the tested methods, d is the number of true isolates that are non-MCR-1 producers that were correctly identified by the tested methods, and c is the number of isolates that were incorrectly identified as MCR-1 producers.

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We declare no conflicts of interest.

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