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REVIEW ARTICLE

Current and emerging polymyxin resistance diagnostics: A systematic review of established and novel detection methods

Tumisho Mmatumelo Seipei Leshaba | **Nontombi Marylucy Mbelle** | **John Osei Sekyere**

Department of Medical Microbiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

Correspondence
John Osei Sekyere, Department of Medical Microbiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa.
Emails: jod14139@yahoo.com; u18392467@tuks.co.za

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Abstract

The emergence of polymyxin resistance, due to transferable *mcr* genes, threatens public and animal health as there are limited therapeutic options. As polymyxin is one of the last-line antibiotics, there is a need to contain the spread of its resistance to conserve its efficacy. Herein, we describe current and emerging polymyxin resistance diagnostics to inform faster clinical diagnostic choices. A literature search in diverse databases for studies published between 2016 and 2020 was performed. English articles evaluating colistin resistance methods/diagnostics were included. Screening resulted in the inclusion of 93 journal articles. Current colistin resistance diagnostics are either phenotypic or molecular. Broth microdilution is currently the only gold standard for determining colistin MICs (minimum inhibitory concentration). Phenotypic methods comprise of agar-based methods such as CHROMagar™ Col-*APSE*, SuperPolymyxin, ChromID® Colistin R, LBJMR and LB medium; manual MIC-determiners viz., UMIC, MICRONAUT MIC-Strip and ComASP Colistin; automated antimicrobial susceptibility testing systems such as BD Phoenix, MICRONAUT-S, MicroScan, Sensititre and Vitek 2; MCR-detectors such as lateral flow immunoassay (LFI) and chelator-based assays including EDTA- and DPA-based tests, that is, combined disk test, modified colistin broth-disk elution (CBDE), Colispot, and Colistin MAC test as well as biochemical colorimetric tests, that is, Rapid Polymyxin NP test and Rapid ResaPolymyxin NP test. Molecular methods only characterize mobile colistin resistance; they include PCR, LAMP and whole-genome sequencing. Due to the faster turnaround time (≤3 h), improved sensitivity (84%–100%) and specificity (93.3%–100%) of the Rapid ResaPolymyxin NP test and Fastinov®, we recommend this test for initial screening of colistin-resistant isolates. This can be followed by CBDE with EDTA or the LFI as they both have 100% sensitivity and a specificity of ≥94.3% for the rapid screening of *mcr* genes. However, molecular assays such as LAMP and PCR may be considered in well-equipped clinical laboratories.

KEYWORDS
colistin, colistin resistance, detection methods, diagnostics, *mcr*, polymyxins

Tweet: Colistin resistance is a global threat to bacterial infections' management. Being a last-resort antibiotic for treating of MDR Gram-negative infections, it is important to establish an efficient diagnostic method for routine use in clinical microbiology laboratories. Herein, current & emerging diagnostics for detecting colistin resistance are described to inform clinical diagnostic options.

INTRODUCTION

The rapid dissemination of multidrug-resistant (MDR) *Acinetobacter* sp., *Pseudomonas* sp. and carbapenemase-producing Enterobacterales has been of vital significance to public and veterinary health (Teo et al., 2018; Vasoo, 2017). Of particular concern are carbapenem-resistant infections caused by these organisms (Li et al., 2018), as they are associated with high mortality rates owing to limited therapeutic options (Jousset et al., 2019; Osei Sekyere et al., 2020). The limited pipeline of new antibiotic classes has led to increased use of polymyxin E (colistin) alone or in combination with tigecycline or fosfomycin for the treatment of MDR Gram-negative infections (Dortet et al., 2020; Liu et al., 2016).

Polymyxin is of particular value as a last-line antibiotic for treating MDR and carbapenem-resistant Gram-negative infections as it is bactericidal unlike tigecycline, which is bacteriostatic and is not readily available in many countries (Bialvaei & Samadi Kafil, 2015). Polymyxin consists of hydrophilic and lipophilic moieties that allow it to have stable polar and hydrophobic interactions with the lipopolysaccharide (LPS) membrane of Gram-negative bacteria (Poirel et al., 2017; Sun et al., 2018). These interactions result in the destruction of the LPS membrane, causing the cytoplasmic content to leak out, ultimately killing the cell (Poirel et al., 2017). There are two types of polymyxins, B and E, but this review shall focus on polymyxin E, also known as colistin.

Increased use of polymyxin to treat MDR Gram-negative infections has led to the emergence of acquired colistin resistance (Nordmann et al., 2016a). Several mechanisms that mediate acquired colistin resistance have been identified, the most common being chromosomal mutations and plasmid-borne colistin resistance (Osei Sekyere, 2019; Poirel et al., 2017; Rodriguez, Maza, et al., 2019). Chromosomal mutations result in modification(s) of the LPS membrane using different mechanisms: (i) the addition of 4-amino-4-deoxy-L-arabinose (L-Ara-4N), phosphoethanolamine (pETN) or galactosamine moieties at the 4′ or 1′ position of lipid A, which reduces the overall anionic charge of the LPS; (ii) overexpression of efflux pumps systems; (iii) overproduction of capsule polysaccharide that reduces the LPS membrane's permeability (Bialvaei & Samadi Kafil, 2015; Osei Sekyere, 2019).

Plasmid-borne colistin resistance involves the acquisition of a mobile colistin resistance (*mcr*) gene that encodes a pETN transferase (Dortet et al., 2020). Since the discovery of the first plasmid-borne *mcr*-1 gene in *Escherichia coli* in China, other *mcr* variants viz., *mcr*-2, *mcr*-3, *mcr*-4, *mcr*-5, *mcr*-6, *mcr*-7, *mcr*-8, *mcr*-9 and *mcr*-10, have been described worldwide (AbuOun et al., 2017; Borowiak et al., 2017; Carattoli et al., 2017; Carroll et al., 2019; Liu et al., 2016; Wang et al., 2018; Wenjuan et al., 2017; Xavier et al., 2016; Yong-Qiang et al., 2018). Currently, the confirmation of polymyxin E resistance relies on the broth microdilution (BMD) (Furniss et al., 2019a). Although BMD is the gold standard for colistin susceptibility testing, and is used in many clinical laboratories, it is not suitable for routine clinical use as it is time-consuming and associated with methodological issues (Bardet & Rolain, 2018). Transmissible colistin resistance makes it imperative to establish rapid and reliable methods that will efficiently detect colistin resistance (Liu et al., 2016). As *mcr*-containing plasmids are capable of transfer between epidemic strains of Enterobacterales, rapid detection of colistin resistance could manage the dissemination of colistin resistance in human and animal populations (Bardet & Rolain, 2018; Liu et al., 2016).

There has been an increasing interest in discovering alternative methods of detecting resistance to colistin arising from both chromosomal mutations and plasmid-borne *mcr* genes (Furniss et al., 2019b; Vasoo, 2017). These methods can be categorized as either phenotypic or molecular methods (Bardet & Rolain, 2018; Osei Sekyere, 2019). This review aims to summarize and analyse clinical diagnostic methods that are currently available for detecting colistin resistance.

Evidence before this review

Methods used to detect polymyxin resistance have been reviewed (Bardet & Rolain, 2018; Osei Sekyere, 2019; Vasoo, 2017). Bardet and Rolain (2018) narratively described methods used to detect colistin resistance, focusing mainly on their efficiency to detect all mechanisms of colistin resistance. They also analysed methods specifically used to detect plasmid-mediated colistin resistance. Osei Sekyere (2019) provided a comprehensive description of polymyxin resistance and *mcr*-detecting diagnostic methods up to 2018. The review included the composition of culture media, primers and cycling conditions of PCR methods. Osei Sekyere (2019) summarized the sensitivities, specificities, turnaround time (TAT), skill, relative cost, essential agreement (EA), categorical agreement (CA), major error (ME) and very major error (VME) of polymyxin resistance detection methods. Since 2018, new evaluation studies have been reported, broadening our understanding and conclusions of the best colistin resistance diagnostics/methods. Therefore, we provide a comprehensive update and an expanded review, based on broader evaluation studies, of all the current diagnostic methods designed to detect colistin resistance.

Literature search strategy

A comprehensive literature search was performed using Pubmed, Web of Science and ScienceDirect. Articles published in English, from January 2016 to September 2020, were retrieved and screened using the following keywords:

'colistin AND resistan*', 'polymyxin AND resistan*' in permutation and combination with 'detection' and 'diagnostics', in a factorial order. The search was based on articles that were evaluating methods that are currently used for the detection of colistin resistance and *mcr* genes. Studies based on epidemiology, risk factors, surveillance, non-English language articles, other reviews, case reports or case studies were excluded. The inclusion and exclusion methods used in this review are demonstrated in Figure 1. The following data were extracted from the included articles and summarized in Table 1: Diagnostic methods used, types and sample size (in numbers) of bacterial species used in the evaluation, sensitivity, specificity, EA, CA, ME, VME, relative cost and TAT.

PHENOTYPIC TESTS

Broth microdilution

The Clinical and Laboratory Standard Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have jointly recommended that the MIC (minimum inhibitory concentration) testing of colistin be performed according to the ISO-20776 standard BMD method (Chew et al., 2017; Jayol, Nordmann, Andre, et al., 2018). Diffusion and agar dilution methods have been ruled out as it was shown that the large molecular size of colistin makes it poorly diffusible through agar (Jayol, Nordmann, Andre, et al., 2018; Simar et al., 2017; Vasoo, 2017).

It has been recommended that BMD be used with plain polystyrene trays and colistin sulphate salt without the addition of any surfactants (Haeili et al., 2019; Jayol, Nordmann, Andre, et al., 2018). The CLSI had initially recommended the addition of polysorbate-80 to alleviate the binding of polymyxin to negatively charged polystyrene surfaces (binding of colistin to polystyrene reduces the concentration of colistin) (Vasoo, 2017). However, there were concerns that the surfactant would act in a synergistic manner with colistin, and is therefore not recommended at this time (Haeili et al., 2019). Moreover, several studies have suggested that the loss of colistin concentration could be reduced by minimizing contact with unexposed pipette tips and by storing colistin solution in glass tubes (Haeili et al., 2019).

The CLSI and EUCAST have established a colistin susceptible breakpoint of ≤2 mg/L and a resistant breakpoint of >2 mg/L for *Acinetobacter* sp. and *Pseudomonas aeruginosa* (Chew et al., 2017). However, only the EUCAST has the same breakpoints for Enterobacterales while the CLSI has an epidemiological cut-off value of 2 mg/L that defines *E. coli*, *Klebsiella pneumoniae*, *Raoultella ornithiolytica*, *Enterobacter aerogenes* and *Enterobacter cloacae* as either wildtype or non-wildtype (Chew et al., 2017; Vasoo, 2017).

Broth microdilution performed by the reference ISO-20776 method is currently the only gold standard for

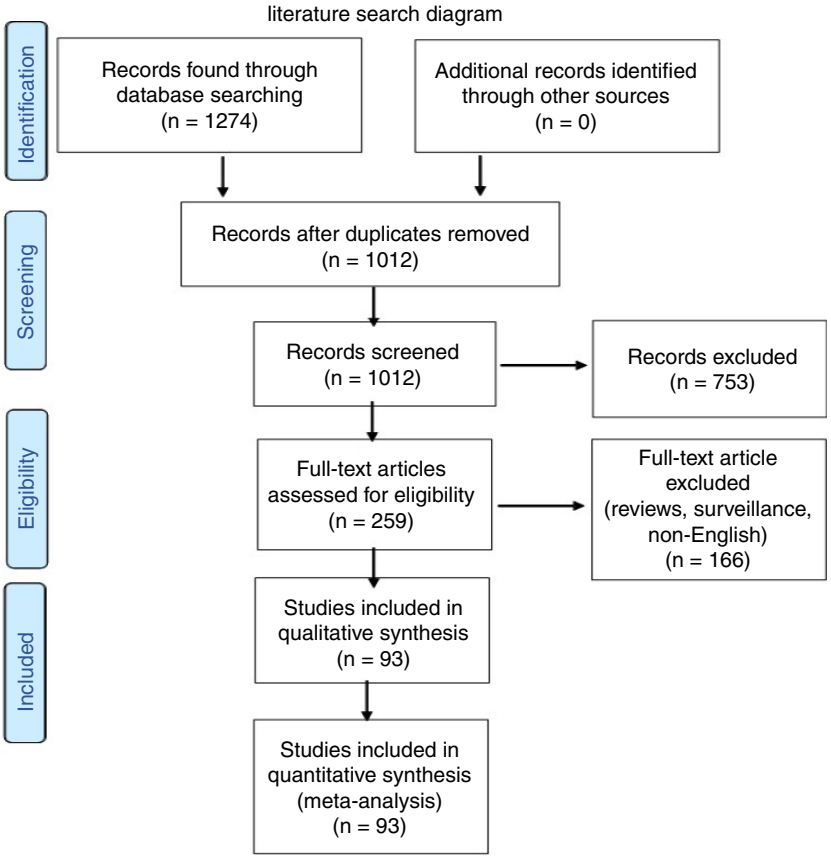

FIGURE 1 Literature search strategy and sorting techniques used to obtain articles for inclusion in the systematic review

determining colistin MIC and evaluating CA, EA, ME and VME; yet, it is laborious and rarely performed in routine clinical microbiology laboratories (Vasoo, 2017). Instead, diffusion methods and automated antimicrobial susceptibility testing (AST) systems are more commonly used (Vasoo, 2017). Chew et al. (2017) evaluated the detection of *mcr*-1-positive Enterobacterales by BMD in comparison with commercial automated AST systems viz., Sensititre, Microscan and Vitek 2, and a gradient diffusion test, that is, E-test. This study found that none of the commercial testing methods meet the CLSI recommendation standard for commercial antimicrobial susceptibility testing (AST) systems: EA ≥90%, CA ≥90%, VME ≤1.5% and ME ≤3.0%. Even so, the Sensititre and MicroScan were shown to detect 100% of the *mcr*-1-positive isolates (Refer to Automated AST systems below) (Chew et al., 2017). The BMD's overall sensitivity could be improved by reducing the susceptible breakpoint to ≤1 mg/L and using microtitre plates that were manufactured to reduce adsorption (Chew et al., 2017; Haeili et al., 2019).

The methodological challenges surrounding the standard BMD have led to interest in finding alternative means for detecting polymyxin resistance in Gram-negative bacteria (Haeili et al., 2019; Vasoo, 2017). Broth macrodilution method has been shown to be an obvious alternative, as it employs the use of glass tubes instead of polystyrene (Haeili et al., 2019). Notwithstanding, the broth macrodilution method is not a popular alternative because it requires the same preparation and TAT as the standard BMD. Hence, commercially available selective media and rapid colorimetric assays have become popular for screening (Table 1) (Haeili et al., 2019).

Diffusion and agar dilution methods

Diffusion methods for colistin susceptibility testing are still commonly used despite being disapproved by the CLSI and EUCAST (Lutgring et al., 2019; Matuschek et al., 2018; Simar et al., 2017; Vasoo, 2017). Two of the commercial gradient tests, E-test and MIC Test Strip, performed poorly for colistin-resistant isolates in one study (Matuschek et al., 2018). Disk diffusion is not an MIC determiner, although it generates high levels of VMEs (false susceptible) similarly to gradient diffusion while agar dilution has a tendency of generating higher MICs (which results in high MEs) than the reference method (Chew et al., 2017; Haeili et al., 2019; Jayol et al., 2017; Matuschek et al., 2018; Mitton et al., 2019; Simar et al., 2017; Vasoo, 2017). These findings further support the conclusion that polymyxins are poorly diffused in agar and therefore corroborates the CLSI and EUCAST recommendations to abandon diffusion and agar dilution methods (Jayol et al., 2017; Matuschek et al., 2018; Simar et al., 2017).

Manual commercial MIC determiners: UMIC, MMS and ComASP Colistin

UMIC (Biocentric), MICRONAUT MIC-Strip (MMS) (Merlin Diagnostika GmbH) and ComASP Colistin (formerly SensiTest™; Liofilchem) are non-automated BMD-based tests (Matuschek et al., 2018). UMIC and MMS both consist of a plastic device with 12 wells that allow for different colistin concentrations to be tested for a single isolate without the need for any additional equipment (Bardet et al., 2019; Jayol, Nordmann, Andre, et al., 2018; Matuschek et al., 2018). The ComASP (SensiTest) Colistin consists of a compact panel for four isolates with freeze-dried antibiotics in seven twofold dilutions (Carretto et al., 2018; Osei Sekyere et al., 2020). Matuschek et al. (2018) evaluated all three tests, where ComASP had a poor EA for *Acinetobacter* sp., and UMIC was poor for *Acinetobacter* sp. and *P. aeruginosa*. The overall performance of ComASP was improved (as shown in Table 1) when certain species were removed and *K. pneumoniae* and *E. coli* were tested, suggesting that ComASP is not suitable for testing all species (Osei Sekyere et al., 2020).

UMIC was generally found to lower the MIC of some colistin-resistant isolates, which may result in failure to detect colistin-resistant isolates with low MICs (≤8 mg/L) (Bardet et al., 2019; Jayol, Nordmann, Andre, et al., 2018). More so, UMIC failed to detect four *Stenotrophomonas maltophilia* isolates with MICs ranging from 8 to 32 mg/L in one study, although in another study all *S. maltophilia* isolates were detected accordingly (Bardet et al., 2019; Jayol, Nordmann, Andre, et al., 2018). Although MMS had the best correlation to the BMD among the three tests; there are not sufficient studies evaluating this test (Table 1) (Matuschek et al., 2018).

Automated AST systems: BD Phoenix, MICRONAUT-S, MicroScan, Sensititre and Vitek 2

Automated AST systems are of particular interest for colistin susceptibility testing due to their ease of use than the reference BMD (Jayol, Nordmann, Andre, et al., 2018; Matuschek et al., 2018). Several studies have shown that some automated AST systems can achieve results that are relatively similar to those of the reference BMD (Chew et al., 2017; Hong et al., 2019; Jayol, Nordmann, Andre, et al., 2018, 2017; Matuschek et al., 2018). The Sensititre recorded a high rate of agreement with the reference BMD in several studies, particularly demonstrating the highest potential for detecting *mcr*-1-producing Enterobacterales together with MicroScan in one study (Chew et al., 2017; Jayol, Nordmann, Andre, et al., 2018; Matuschek et al., 2018). However, MicroScan has a tendency of overestimating MICs (which may result in false-resistant isolates) of *E. cloacae*, *Salmonella* sp. and

TABLE 1 Comparative diagnostic efficiencies of colistin resistance diagnostics

Diagnostic	Species (*n*)	Sensitivity (%)	Specificity (%)	Relative cost	Relative skill required
Broth macrodilution (BMAD)	GNB (109)	100	100	Cheap	Low
Diffusion and agar dilution methods					
Disc diffusion (Rosco)	GNB (109)	76.19	96.59	Cheapest	Low
Disc diffusion (BBL Sensi-Disc; Becton Dickinson)	GNB (109)	76.19	100	Cheapest	Low
Disc diffusion	*Hafnia* (25)	0	100	Cheapest	Low
Agar dilution	GNB (109)	100	60.23	Cheap	Low
Selective agar-based media					
Superpolymyxin™	Enterobacterales (94, 700, 231, 1430, 385)	86.8, 87.5, 95.2, 100, 88.9	97.5, 99.5, 95.3, 0, 81.6	Cheap	Low
CHROMagar™ COL-APSE	*E. coli* (158) GNB (89)	96, 82.05	97, 66.67	Cheap	Low
CHROMID® Colistin_R	*E. coli* 158 Enterobacterales (94)	99, 86.8	97, 100	Cheap	Low
LB Medium	Enterobacterales (9)	100	100	Cheap	Low
Chelator and non-chelator-based assays					
Rapid Polymyxin NP (Commercial)	Enterobacterales (132)	100	96.7	Expensive	Low
In-house Rapid Polymyxin NP	*K. pnuemoniae* (131) *Hafnia* sp. (25) Enterobacterales (200, 339)	99, 100, 98, 98.21, 98.1, 71.1, 100	82, 100, 98, 100, 98.2, 88.6, 95.9	Cheapest	Low
ASAT	Enterobacterales (300)	90.7	100	Cheapest	Low
Rapid *Acinetobacter*	*A. baumannii* (21)	100, 93.3	100, 86.8	Expensive	Low
Rapid *Pseudomonas*	*P. aeruginosa* (17)	100, 100	100, 95	Expensive	Low
Rapid Resapolymyxin NP	*A. baumannii* (82, 43, 165) Enterobacterales (20, 32) *P. aeruginosa* (49) Gram-negative bacilli (253)	93.3, 100, 100, 100, 100, 84	93.3, 100, 97, 100, 99, 100	Cheap	Low
EDTA-CDT	Enterobacterales (48, 92)	12, 100	65.2, 100	Cheap	Low
EDTA-CMR/BMD	Enterobacterales (92)	93.2	54.2	Cheap	Low
Colistin MAC test	Enterobacterales (92) GNB (84)	84.1, 100	100	Cheap	Low
CBDE	GNB (172)	100	97.76	Cheap	Low
CBDE+EDTA	Enterobacterales (85)	100	95.8, 94.3	Cheap	Low
CBM	Enterobacterales (68) NF (17)	95.35, 87.5	84, 66.67	Cheap	Low
MPT	Enterobacterales (68) Non-fermenters (17)	88.37, 75	80, 77.78	Cheap	Low
CSTT	Enterobacterales (68) NF (17)	93.02, 62.5	88, 100	Cheap	Low

Turnaround time (hr)	CA (%)	EA (%)	ME (%)	VME (%)	LOD (CFU/ml or reaction)	References
16–20	100	100	0	0	NS	Haeili et al. (2019)
16–24	92.6	NA	3.4	23.8	NS	Haeili et al. (2019)
16–24	95.4	NA	0	23.8	NS	Haeili et al. (2019)
16–24	0	NA	0	100	NS	Jayol et al. (2017)
16–20	67.9	23.8	39.77	0	NS	Haeili et al. (2019)
24–48	NA	NA	NA	NA	NS	Girlich, Bernabeu, et al. (2019); Girlich, Naas, et al. (2019); Germ, Poirel, et al. (2019); Germ, Seme, et al. (2019); Jayol et al. (2018); Przybysz et al. (2018); van Hout et al. (2020)
18–20	NA	NA	NA	NA	NS	Thiry et al. (2019); Osei Sekyere et al. (2020)
18–24	NA	NA	NA	NA	NS	Thiry et al. (2019); Girlich, Bernabeu, et al. (2019); Girlich, Naas, et al. (2019)
48	NA	NA	NA	NA	10^2–10^3	Turbett et al. (2019)
2–3	NS	NA	3.48	0	NS	Malli et al. (2019)
2–4	NS	NA	18.18, 0, 2, 0, 0, 1.8, 7.4, 0	1.02, 0, 2, 1. 79, 1.9, 10.2 ,3.85	10^8	Malli et al. (2018); Jayol et al. (2017); Dalmolin et al. (2019); Jayol et al. (2016); Mitton et al. (2019); Przybysz et al. (2018); Yainoy et al. (2018)
3	NS	NA	0	10.17	NS	Rodriguez, Maza, et al. (2019)
3–4	100	NA	0,	0,	NS	Lescat et al. (2019); Malli et al. (2019)
3–4	100	NA	0, 5	0	NS	Lescat et al. (2019); Sadek et al. (2020)
3–4	95.1, 100, cal, 99.2, cal	NA	3.7, 0, 4, 34, 0, 1.01, 0	1.2, 0, 0, 0, 0, 16	NS	Germ, Poirel, et al. (2019); Germ, Seme, et al. (2019); Lescat, Poirel, Tinguely, et al. (2019); Jia et al. (2020); Rodriguez et al. (2019)
18–24	NS,100	NA	NS, 0	NS, 0	NA	Clément et al. (2018); Yauri Condor et al. (2019)
18–24	NS	NS	6.8	45.81	NS	Budel et al. (2019)
18–24	NA	NA	0	15.9, 0	NS	Budel et al. (2019); Osei Sekyere et al. (2020)
16–20	98	99	0	8	NS	Simner et al. (2019)
16–20	NS	NS	4.1, 5.75	0	NS	Bell et al. (2019); Fenwick et al. (2020)
16–20	91.18, 76.47	95.59, 82.35	16, 33.33	4.65, 2.5	NS	Dalmolin et al. (2020)
16–20	85.29, 76.47	98.53, 76.47	20, 22.22	11.63, 25	NS	Dalmolin et al. (2020)
16–20	91.18, 82.35	NA	12	6.98, 37.5	NS	Dalmolin et al. (2020)

(Continues)

TABLE 1 (Continued)

Diagnostic	Species (*n*)	Sensitivity (%)	Specificity (%)	Relative cost	Relative skill required
Lateral Flow immunoassay (NG-TEST MCR-1)	Enterobacterales (298, 138) *A. baumannii* (50) *P. aeruginosa* (50)	100	98, 99	Expensive	Low–High
Rapid-flow cytometry method (FASTinov R®)	Enterobacterales (*137*), *Pseudomonas* sp. (*35*), *Acinetobacter baumannii* (*32*)	NS	NS	Expensive	Low
Commercial MIC determiners					
UMIC	GNB (235)	100	100	Expensive	High
ComASP Colistin (Sensitest)	GNB (84)	87.18	73.33	Expensive	High
BD Phoenix M50	GNB (131)	100	94.29	Very expensive	High
BD Phoenix 100	*Hafnia* sp. (25) *A. baumannii* (165) *K. pnuemoniae* (131)	100, 48, 68, 95	100, 100, 100, 94	Very Expensive	High
MicroScan	Enterobacterales (110) GNB (84)	89.29, 85.71	93.90, 76.19	Very expensive	High
Vitek 2	*K. pnuemoniae* (131)	98	88	Very expensive	High
Molecular assays					
Amplidiag® Carba-R+MCR assay	GNB	92–100	86–100	Expensive	High
AusDiagnostic MT CRE EU assay	GNB (393)	95.5–100	99.8	Expensive	High
LAMP-*mcr*-1	GNB (22)	100	100	Expensive	High
Multiplex-LAMP	Enterobacterales (58)	100	100	Expensive	Very high
MALDI-TOF MS	*K. pneumonia* (139)	78	89	Expensive	Very high
MALDIxin	*A. baumannii* (17) *E. coli* (40) *K. pneumoniae* (81)	100	100	Expensive	Very high
MCDA-LFB	GNB (59)	100	100	Expensive	High
Multiplex PCR	*E. coli* (50) *K. pneumoniae* (41) *S. enterica* (12, 407) *Aeromonas* sp. (82) *Shewenella* sp. (10)	100	100	Very expensive	High
Real-time PCR	*E. coli* (1110)	32–98	100	Very expensive	High
Real-time PCR (SYBR Green)	*E. coli* (332, 1062)	100	100	Very expensive	High
Real-time PCR (Taqman)	GNB (23)	100	100	Very expensive	High
Recombinase polymerase amplification	Enterobacterales (23)	100	100	Expensive	High
Whole genome sequence	*Citrobacter werkmanii* (5)	100	100	Most expensive	Highest

The bold values show the various diagnostic tests categories.

Abbreviations: CA, categorical agreement; EA, essential agreement; LOD, limit of detection; ME, minor error; VME, very major error.

Turnaround time (hr)	CA (%)	EA (%)	ME (%)	VME (%)	LOD (CFU/ml or reaction)	References
<0.25	NA	NA	0	0.34	2.10^8	Volland et al. (2019); Fenwick et al. (2020)
1–2	99.0	NS	0.0	NS	NS	Fonseca e Silva et al. (2019)
18–24	100	94	0	0	NS	Bardet et al. (2019)
16–24	78.57	78.57	26.67	12.82	NS	Osei Sekyere et al. (2020)
16–24	98.5	98.5	5.7	0	NS	Hong et al. (2019)
16–24	100,	100,	0, 0, 0, **6.06**	0, 52, 32, **5.1**	NS	Jayol et al. (2017); Rodriguez et al. (2019); Malli et al. (2018)
16–24	92.7, 80.95	NS	6.1, 23.81	10.7, 14.29	NS	Lutgring et al. (2019); Osei Sekyere et al. (2020)
18–24	NS	NS	12.12	2.04	NS	Malli et al. (2018)
<3	NA	NA	NA	NA	NS	Girlich, Bernabeu, et al. (2019)
<4	NA	NA	NA	NA	NS	Meunier et al. (2018)
<1	NA	NA	NA	NA	10^8	Zou et al. (2017)
<1	NA	NA	NA	NA	10^4–10^5	Zhong et al. (2019)
?	NA	NA	NA	NA	NS	Giordano and Barnini, (2018)
0.25–0.5	NA	NA	NA	NA	NS	Furniss et al. (2019); Dortet et al. (2020); Dortet et al. (2018)
<1	NA	NA	NA	NA	600 fg	Gong et al. (2019)
<2–3	NA	NA	NA	NA	NS	Jousset et al. (2019); Borowiak et al. (2020)
<3	NA	NA	NA	NA	0.1–2200 cfu/ml	Chandler et al. (2020)
<3	NA	NA	NA	NA	54 cfu/ml	Chalmers et al. (2018); Tolosi et al. (2020)
<3	NA	NA	NA	NA	10^3	Daniels et al. (2019)
<0.5	NA	NA	NA	NA	100fg	Xu et al. (2018)
<48	NA	NA	NA	NA	NS	Peter et al. (2018)

non-fermenters (*A. baumannii*, *P. aeruginosa* and *S. maltophilia*) (Jayol, Nordmann, Andre, et al., 2018; Osei Sekyere et al., 2020). Vitek 2 had the highest rate of VMEs at 36% for *mcr*-1-producing isolates and failed to detect colistin resistance in eight *mcr*-negative *E. cloacae* complex isolates (Chew et al., 2017). However, another study found that Vitek 2 had VMEs of 2.04% for *K. pneumoniae* isolates that were *mcr*-negative (Malli et al., 2018). MICRONAUT-S (Merlin Diagnostika GmbH) performed similarly to the Sensititre although it had two VMEs, whereas the Sensititre had none (Matuschek et al., 2018). The Sensititre test is unique as it can be performed manually or semi-automated unlike MicroScan, MICRONAUT-S, Vitek 2 and BD Phoenix that require an automated inoculation delivery system (Jayol, Nordmann, Andre, et al., 2018; Matuschek et al., 2018).

The detection of colistin resistance by BD Phoenix highly agreed with that of the reference BMD method (Hong et al., 2019; Jayol et al., 2017). Nevertheless, the BD Phoenix 100 system failed to detect colistin-resistant isolates that may have hetero-resistance; the BD Phoenix M50 had 5.7% MEs, all of which were within twofold dilutions, as compared to the reference BMD (Hong et al., 2019; Jayol, Nordmann, Lehours, et al., 2018; Rodriguez, Traglia, et al., 2019). Notably, the BD Phoenix M50 has not been evaluated on strains that exhibit hetero-resistance; hence, its evaluation is limited compared to BD Phoenix 100 (Table 1) (Hong et al., 2019).

Chelator-based and non-chelator-based tests

Rapid Polymyxin NP

The Rapid Polymyxin NP is a colorimetric test that is based on glucose metabolism to detect the growth of Enterobacterales at a given concentration of a polymyxin (colistin or polymyxin B) (Nordmann et al., 2016a). Resistance to polymyxins is demonstrated by a colour change (orange to yellow) of a pH indicator, that is, phenol red, as a result of acid formation associated with the metabolism of glucose (Nordmann et al., 2016a; Poirel et al., 2018; Yainoy et al., 2018). Rapid Polymyxin NP test is commercially available (ELITechGroup) and can also be performed in-house with the preparation of two solutions (Jayol, Kieffer, et al., 2018; Nordmann et al., 2016a). The in-house Rapid Polymyxin NP test is prepared with stock solutions of polymyxins and a Rapid Polymyxin NP solution, which consists of cation-adjusted Mueller-Hinton broth powder, phenol red indicator and D(+)-glucose (Nordmann et al., 2016a). This test has demonstrated an excellent detection of colistin resistance in several studies, including detecting colistin resistance directly from blood cultures with a sensitivity of 100% (Jayol et al., 2016; Malli et al., 2019; Yainoy et al., 2018). However, in a study using ComASP as the reference, the Rapid Polymyxin NP test had a lower specificity than BD Phoenix, Vitek 2 and E-test with *K. pneumoniae* isolates (Table 1) (Malli et al., 2018). In another study, Rapid Polymyxin NP test recorded a lower sensitivity and specificity (71.1% and 88.6%) than E-test, which had a sensitivity and specificity of 80.4% and 95.8%, respectively (Przybysz et al., 2018). However, 10 isolates that were included in the calculations of the E-test performance were excluded from the Rapid Polymyxin NP test as they were considered non-evaluable due to no growth in the growth control (Przybysz et al., 2018).

Although the Rapid Polymyxin is limited to use on Enterobacterales, the test is easy to perform and the final results can be read in no more than 4 h, with the majority of the results being positive in 2 h (Dalmolin et al., 2019; Jayol et al., 2016, 2017; Mitton et al., 2019).

Andrade Screening Antimicrobial Test

Following the Rapid Polymyxin NP test, another colorimetric assay for detecting colistin resistance in Enterobacterales was developed (Rodriguez, Maza, et al., 2019). The Andrade Screening Antimicrobial Test (ASAT) was developed using an in-house broth consisting of 10 g peptone, 5 g sodium chloride, 3 g beef extract and 10 ml Andrade indicator made with 0.5 g acid fuchsin and 16 ml NaOH in 100 ml water (Rodriguez, Maza, et al., 2019). The evaluation of the ASAT was performed in tubes containing 175 µl of Andrade broth and colistin at a concentration of 3.8 mg/L (Rodriguez, Maza, et al., 2019). The test achieved a sensitivity and specificity of 90.7% and 100%, respectively, where a positive result in the presence of colistin was shown by a change in colour of the Andrade indicator (light pink to magenta) (Rodriguez, Maza, et al., 2019).

The ASAT test was further evaluated against the BD Phoenix using Enterobacterales clinical isolates (Rodriguez, Maza, et al., 2019). However, this evaluation demonstrated discrepancies between the two methods where 10 *E. coli*, seven *K. pneumoniae* and one *E. cloacae* colistin-resistant isolates were accurately detected by ASAT and not by BD Phoenix (Rodriguez, Maza, et al., 2019). Most of the isolates that were not detected by BD Phoenix had colistin MIC values ranging between 4 and 8 µg/ml and carried *mcr*-1 genes (Rodriguez, Maza, et al., 2019). Although most of the colistin-resistant *K. pneumoniae* isolates had colistin MIC values >16 µg/ml and carried bla_{KPC} genes, only three isolates with colistin MIC values ≤16 µg/ml showed discrepancies between the two methods (Rodriguez, Maza, et al., 2019).

Rapid Polymyxin Acinetobacter, Rapid Polymyxin Pseudomonas and Rapid Resapolymyxin NP

ElitechGroup introduced Rapid Polymyxin tests for *Acinetobacter* sp. and *Pseudomonas* sp. in October 2018

(Lescat, Poirel, Jayol, et al., 2019; Malli et al., 2021). Both tests use the same principle as the Rapid Polymyxin NP test as they rely on the colorimetric detection of bacterial metabolism in the presence of a defined concentration of colistin (Lescat, Poirel, Jayol, et al., 2019). A positive result by Rapid Polymyxin *Acinetobacter* was read by a change in colour of a pH indicator, phenol red (red to orange or yellow), whereas the Rapid Polymyxin *Pseudomonas* uses bromocresol purple pH indicator (green-yellow to violet) (Lescat, Poirel, Jayol, et al., 2019; Sadek et al., 2020). Sadek et al. (2020) evaluated the in-house version of the Rapid Polymyxin *Pseudomonas* test, which agreed with Lescat et al. (2019), recording 100% sensitivity and a lower specificity of 95% (Lescat, Poirel, Jayol, et al., 2019; Sadek et al., 2020). However, the Rapid Polymyxin *Acinetobacter* had discrepancies, recording no errors in one study and eight errors (three VMEs and five MEs) in another (Lescat, Poirel, Jayol, et al., 2019; Malli et al., 2021).

The Rapid Resapolymyxin NP test was also developed to detect colistin resistance in all colistin-resistant Gram-negative bacteria including Enterobacterales, *A. baumannii* and *P. aeruginosa* (Lescat, Poirel, Tinguely, et al., 2019). The test was carried out by inoculating 20 µl standardized bacterial suspension (3.5 McFarland) in Mueller-Hinton broth containing a final concentration of 3.75 mg/L of colistin sulphate (Lescat, Poirel, Tinguely, et al., 2019). A 10% concentration of resazurin PrestoBlue was added after 3 h of incubating the medium and the results were read over a period of 1 h after the addition of resazurin PrestoBlue (Lescat, Poirel, Tinguely, et al., 2019). The detection of colistin resistance is based on the reduction of blue resazurin to pink resorufin by metabolically active cells in the presence of a defined concentration of colistin (Lescat, Poirel, Tinguely, et al., 2019).

The evaluation of Rapid ResaPolymyxin NP test showed reliable detection of colistin resistance in non-fermenters and 100% accuracy in Enterobacterales (Germ, Poirel, et al., 2019; Jia et al., 2020; Lescat, Poirel, Tinguely, et al., 2019). Although the growth of *Pseudomonas* sp. may be delayed, Enterobacterales and *Acinetobacter* sp. should be detectable in less than 4 h (Jia et al., 2020; Rodriguez, Traglia, et al., 2019). The Rapid ResaPolymyxin NP test makes up for the limitations of Rapid Polymyxin NP test in testing polymyxin resistance in Gram-negative bacilli (Jia et al., 2020; Lescat, Poirel, Tinguely, et al., 2019). Therefore, this test is more suitable for general categorization of colistin-resistant and colistin-susceptible isolates than the Rapid Polymyxin NP test (Jia et al., 2020).

EDTA/DPA-based colistin resistance tests

The MCR catalytic domain, PEtN transferase, is a zinc metalloprotein, where zinc deficiency reduces colistin MICs in MCR-producing *E. coli* (Clement et al., 2018; Esposito et al., 2017; Yauri Condor et al., 2019). The combined disk test (CDT), colistin MIC reduction (CMR) test, modified Rapid Polymyxin NP (MPNP) test and alteration zeta potential are four tests that are based on the inhibition of MCR activity by EDTA and have been strategically developed to detect *mcr* genes (Esposito et al., 2017).

The CDT uses 10 µl of 100 mM EDTA solution, which is impregnated into one of two 10 µg colistin disks (Esposito et al., 2017). Results are read as positive if there is an increase of ≥3 mm in inhibition zone around the colistin disk containing EDTA as compared to the colistin disk without EDTA (Esposito et al., 2017). The CDT recorded a sensitivity and specificity of 96.7% and 89.6%, respectively (Esposito et al., 2017). However, a latter study recorded a sensitivity and specificity of 12% and 65.2%, respectively. Hence, further evaluation studies are necessary to confirm the CDT's results (Clement et al., 2018).

A pre-diffusion method of the CDT was evaluated, where two colistin disks were placed and allowed to diffuse for 2 h on MH agar (Yauri Condor et al., 2019). The disks were removed and the plates were left at room temperature for 18–24 h after which two disks containing 1 µmol of EDTA were strategically placed (one exactly where the colistin disk had been placed and the other, at least 30 mm away) (Yauri Condor et al., 2019). Diameters of inhibition zones were measured after 18 h of incubation and colistin-resistant MCR-positive isolates demonstrated a ≥5 mm increase in inhibition zone around the disks (Yauri Condor et al., 2019). The pre-diffusion method, using a cut-off value of ≥5 mm, improved the CDT to 100% accuracy for *mcr* detection (Yauri Condor et al., 2019); it has however not been extensively evaluated to ensure reproducibility and seems more complicated, laborious and time-consuming than the CDT.

Colistin MIC reduction test was performed by BMD using MH broth without cation supplementation but with 80 µg/ml EDTA solution instead (Esposito et al., 2017). It was considered that cation supplementation with calcium and magnesium would impair the inhibitory activity of EDTA; moreover, calcium could favour the activity of putative PEtN transferases in *E. coli* (Esposito et al., 2017). Even so, this method did not efficiently detect *mcr*-producers among Enterobacterales isolates although different concentrations of EDTA were used (Budel et al., 2019; Esposito et al., 2017).

Innovatively, EDTA has been added to the Rapid Polymyxin NP test in the MPNP test to enable it to identify MCR producers. MPNP is the Rapid Polymyxin NP test with the addition of two wells filled with colistin-free solution and colistin-containing solution, both with 80 µg/ml EDTA (Esposito et al., 2017). Results were read as positive for the production of MCR-1 PEtN transferase if there was no change in colour of red phenol in the presence of colistin and EDTA (Esposito et al., 2017). The presence of EDTA in the MPNP

test successfully detected MCR-1-positive colistin-resistant *E. coli* isolates as demonstrated by a sensitivity and specificity of 96.7% and 100%, respectively (Esposito et al., 2017).

Finally, addition of EDTA results in an alteration of zeta potential of membrane charge, which is measured to determine MCR expression (Esposito et al., 2017). Particle size and zeta potential of colistin-susceptible and colistin-resistant bacterial cells grown in MH broth with or without 80 μg/ml EDTA was measured using a ZETAPALS zeta potential analyser (Esposito et al., 2017). Colistin-susceptible and colistin-resistant MCR-1 Enterobacterales demonstrated zeta potential values between −21.54 and −44.21 mV while colistin-resistant MCR-1 positive had ≤-20 mV (−4.20 to −19.34 mV) (Esposito et al., 2017). In the presence of EDTA, an alteration of zeta potential ranging from −21.13 to −40.81 was observed in colistin-resistant MCR-1-positive *E. coli* isolates (Esposito et al., 2017). A zeta potential ratio ($R_{zp} = ZP_{+EDTA}/ZP_{-EDTA}$) was calculated for all isolates and a cut-off value of $R_{zp} \geq 2.5$ as a criterion for the presumed detection of MCR-1-positive *E. coli* isolates was established (Esposito et al., 2017). Alteration of zeta potential yielded a sensitivity and specificity of 95.1% and 100%, respectively. However, EDTA had no inhibitory effect on *mcr*-1-positive *K. pneumoniae* isolates (Esposito et al., 2017).

Colistin broth-disk elution

Simner et al. (2019) developed the colistin broth-disk elution method (CBDE), which was performed on a collection of Enterobacterales, *P. aeruginosa* and *A. baumannii* isolates: four tubes were assigned to each isolate (Simner et al., 2019). The four tubes contained 10 ml of CA-MHB with colistin disks to yield final concentrations of 0, 1, 2 and 4 μg/ml, respectively (Simner et al., 2019). The tubes were incubated for 30 min at room temperature, allowing colistin to dissolve into the broth, after which 50 μl aliquot of 0.5 McFarland standard bacterial suspensions were added to each tube (Simner et al., 2019). Colistin MIC values were visually read after 16–20 h of incubation at 35°C in ambient air (Simner et al., 2019). In this study, CBDE was compared to the reference BMD and Sensititre, where the CBDE had a CA and EA of 98% and 99%, respectively, as compared to both BMD methods (Simner et al., 2019). Three *mcr*-1-producing *E. coli* isolates resulted in a VME rate of 8% due to one dilution difference by CBDE and BMD; however, no errors were observed when CBDE was compared to broth macrodilution (Simner et al., 2019).

Three studies have evaluated the modified version of the CBDE (Bell et al., 2019; Dalmolin et al., 2020; Fenwick et al., 2020). Bell et al. (2019) were the first to describe a modified CBDE method. The method was performed as previously described; however, 1 mM EDTA was used by adding 20 μl of 0.5 M EDTA to each tube containing CA-MHB and 10 μg colistin disks (Bell et al., 2019; Fenwick et al., 2020). Fenwick et al. (2020) added a fifth tube to generate CBDE+EDTA with colistin concentrations of 0, 0.4, 1, 2 and 4 μg/ml, respectively. The CBDE+EDTA method has shown overall sensitivity and specificity of 100% and 94.3%–95.8%, respectively, for screening the presence of MCR in Enterobacterales and *P. aeruginosa* (Bell et al., 2019; Fenwick et al., 2020).

Dalmolin et al. (2019) evaluated the CBDE method using final volumes of 1 ml and 200 μl in colistin broth microelution (CBM) and the microelution test (MPT), respectively. The two methods were evaluated on Gram-negative bacterial isolates from human and animal samples (Dalmolin et al., 2020). Both CBDE methods were performed as previously described; however, the CBDE mixture was fractioned in 1 ml tubes for the CMB test and 200 μl in microtiter plates for MPT test (Dalmolin et al., 2020). Additionally, this study evaluated the colistin susceptibility test tube, which was performed using one tube with 5 ml CA-MHB and 10 μg colistin disk to yield a final concentration of 2 μg/ml (Dalmolin et al., 2020). All three methods presented unsatisfactory MEs and VMEs; particularly, they performed poorly for non-fermenters (Dalmolin et al., 2020).

Colistin broth-disk elution methods are performed using reagents that are readily available at low cost (Bell et al., 2019; Simner et al., 2019). However, CBDE with EDTA could be more suitable for screening *mcr*-positive isolates as CBDE alone tends to underestimate MICs of *mcr*-positive isolates (Bell et al., 2019; Fenwick et al., 2020; Simner et al., 2019).

Colistin-MAC test

The colistin-MAC test was designed to detect *mcr* genes on the basis of CMR by a fixed concentration (900 μg/ml) of dipicolinic acid (DPA) (Coppi et al., 2018). The test was carried out in 96-well microtitre plates using CA-MHB with DPA stock solution prepared in dimethyl sulfoxide (Coppi et al., 2018). Coppi et al. (2018) established a cut-off value of ≥eightfold CMR in the presence of DPA for the presumptive identification of *mcr*-positive isolates. However, a latter study used a different cut-off value, where ≥3 twofold MIC reduction in the presence of DPA indicated a positive result (Budel et al., 2019). The Colistin MAC test was found to perform well for *E. coli* isolates and ineffective in detecting *mcr* genes of *K. pneumoniae* and *Salmonella* sp. (Budel et al., 2019; Coppi et al., 2018; Osei Sekyere et al., 2020). The lack of inhibitory effect of DPA in *K. pneumoniae* isolates can be attributed to a decrease in DPA permeability or the existence of other mechanisms of colistin resistance in these strains (Coppi et al., 2018). Moreover, the addition of DPA resulted in reductions and increments in MICs of some

isolates, although these adjustments did not affect the accurate sensitivity classification of the isolates (Osei Sekyere et al., 2020).

Colispot

Colispot is a test developed by Jouy et al. (2017), and it consists of applying a single drop of 8 mg/L colistin solution on MH agar to detect colistin resistance. The test was initially carried out by applying 10 µl drop of colistin (two-fold concentrations ranging from 0.25 to 256 mg/L) on MH agar inoculated with 10^5 *E. coli* suspensions (Jouy et al., 2017). Each drop was strategically placed so that their centres were at least 2 cm away from each other to allow for an inhibition zone of >5 mm (Jouy et al., 2017). The colispot test was evaluated on *E. coli* isolated from veterinary faecal samples and 35 *mcr*-1-positive *E. coli* isolates from bovine samples (Jouy et al., 2017). Susceptible isolates had a clear inhibition zone around colistin drops with concentrations ranging from 0.25 to 4 mg/L although the size of inhibition zone was dependent on the colistin concentration tested (Jouy et al., 2017). A clear inhibition zone of 8–10 mm was observed with all the susceptible isolates when a single concentration of colistin solution with CLSI/EUCAST bacterial inoculum size and incubation temperature were used (Jouy et al., 2017).

Agar-based screening medium

Superpolymyxin

Superpolymyxin is a selective medium for polymyxin-resistant Gram-negative bacteria that is based on eosin methylene blue agar (Abdul Momin et al., 2017). The medium was developed with the optimal colistin concentration of 3.5, 10 µg/ml of daptomycin (to inhibit potential growth of Gram-positive strains) and 5 µg/ml of amphotericin B as an antifungal. Nordmann et al. (2016) designed Superpolymyxin for screening intrinsic and acquired polymyxin-resistant Gram-negative bacteria as previous screening media containing deoxycholic acids and a high concentration of colistin inhibited the growth of strains with acquired resistance and low MIC values (Table 1).

The use of eosin Y and methylene blue dyes helped distinguish lactose fermenters (dark brown to purple) from non-fermenters (colourless) (Nordmann et al., 2016a). This medium distinguishes lactose-fermenting *E. coli* (metallic green sheen) from other Enterobacterales, including non-fermenting *E. coli* (dark brown to purple) (Girlich, Naas, et al., 2019; Nordmann et al., 2016b). However, studies evaluating other selective media against Superpolymyxin have shown a weaker detection of non-fermenters by Superpolymyxin (Abdul Momin et al., 2017; Bardet et al., 2017).

This medium was able to detect colistin-resistant Gram-negative bacteria directly from bacterial culture and clinical samples (i.e. rectal swabs and stool samples) with high sensitivity and specificity (van Hout et al., 2020; Jayol, Poirel, et al., 2018; Przybysz et al., 2018). However, direct inoculation from clinical swabs may result in the growth of colistin-susceptible isolates on the medium, therefore resulting in a poor specificity (as low as 80.45%) (van Hout et al., 2020; Przybysz et al., 2018). The poor specificity of Superpolymyxin for clinical samples was suspected to be due to sample storage conditions and bacterial inoculum effect ($\geq 10^6$ CFU per ml) (Jayol, Poirel, et al., 2018; Przybysz et al., 2018).

Two studies have recorded a low sensitivity (≤77.3%) for *Enterobacter* sp., which may be due to hetero-resistant phenotypes (i.e. may have a small population of bacterial cells with colistin resistance) (Germ, Seme, et al., 2019; Jayol, Poirel, et al., 2018). In both studies, the Superpolymyxin plate was inoculated with 10 µl of a 0.5 McFarland bacterial suspension. Therefore, a higher inoculum for *Enterobacter* sp. was suggested (Germ, Seme, et al., 2019; Jayol, Poirel, et al., 2018).

CHROMagar™ COL-APSE

CHROMagar™ COL-*APSE* by CHROMagar (Paris, France) is the first selective medium designed to detect and differentiate all *Acinetobacter* sp., Enterobacterales, *Pseudomonas* sp. and *Stenotrophomonas* sp. (Abdul Momin et al., 2017). The agar plates were prepared in-house using a dehydrated CHROMagar base medium and supplements (S1 and X192) containing colistin sulphate and oxazolidinones to enhance the growth of colistin-resistant Gram-negative bacteria and inhibit that of Gram-positive bacteria (Abdul Momin et al., 2017). Swarming by *Proteus* sp. was inhibited by adding p-nitrophenyl glycerol to the medium preparation, which did not disrupt the medium's performance. This makes CHROMagar™ COL-*APSE* suitable for screening mixed specimens (Abdul Momin et al., 2017).

The accuracy in detecting and differentiating colistin-resistant Gram-negative species was evaluated by Osei Sekyere et al. (2020), where the morphological appearance of the detected strains was as described by the manufacturer. Moreover, three studies that have evaluated CHROMagar™ COL-*APSE* agreed that the medium had a high sensitivity in detecting isolates harbouring *mcr* genes (Abdul Momin et al., 2017; Osei Sekyere et al., 2020; Thiry et al., 2019). However, there was significant difference in the sensitivity and specificity recorded by Abdul Momin et al. (2017) and Osei Sekyere et al. (2020) (Table 1). The poor performance

in the recent study could be due to the use of cultured bacteria instead of using serial dilutions in broth (Table 1) (Abdul Momin et al., 2017; Osei Sekyere et al., 2020).

ChromID® Colistin R

ChromID® Colistin R is a chromogenic selective medium that is primarily used for isolating colistin-resistant Enterobacterales from clinical stools and rectal swab samples (Garcia-Fernandez et al., 2019). Similar to CHROMagar™ COL-*APSE*, the medium can differentiate between bacterial species based on morphological appearance of bacterial colonies, that is, *E. coli* (pink to burgundy), *Klebsiella* sp., *Enterobacter* sp., *Serratia* (blue to green), *Salmonella* sp. (white or colourless) and *Proteeae* tribe (beige-brown) (Table 1) (Garcia-Fernandez et al., 2019; Girlich, Naas, et al., 2019).

The assessment of ChromID® Colistin R and Superpolymyxin using stool and rectal swab samples resulted in an overall better performance by ChromID® Colistin R (Girlich, Naas, et al., 2019). The lower limit of detection (LOD) of this medium being at least one log lower in 69.2% of the isolates detected on both media, whereas Superpolymyxin only had a better LOD for 7.7% isolates (Girlich, Naas, et al., 2019). Nonetheless, Superpolymyxin could be directly inoculated with stool or rectal swab samples without a 4–5 h enrichment step required by ChromID® Colistin R; and the final sensitivity (84.9%–86.8%) recorded for this medium was achieved after extending the TAT from 24 to 48 h (which also allowed for the detection of an *mcr*-1-producing *E. coli* isolate) (Girlich, Naas, et al., 2019).

A study by Thiry et al. (2019) evaluated this medium against CHROMagar™ COL-*APSE* on 158 colistin-resistant bovine *E. coli* isolates. Half (48/96) of the isolates considered to be intermediate to the disk diffusion test had MIC >2 and were able to grow on both media, with two more isolates growing on ChromID® Colistin R alone (Thiry et al., 2019). Although both media could support the growth of (21/22) *mcr*-1-positive and (13/14) *mcr*-2-positive isolates, CHROMagar™ COL-*APSE* has an advantage over ChromID® Colistin R as it is not limited to (isolating and differentiating) Enterobacterales (Table 1) (Abdul Momin et al., 2017; Thiry et al., 2019).

LBJMR medium

Lucie-Bardet-Jean-Marc-Rolain (LBJMR) medium, a polyvalent medium based on Purple agar, has been designed for the isolation of colistin-resistant Gram-negative bacteria as well as vancomycin-resistant Gram-positive bacteria (Bardet et al., 2017). The medium was developed by adding glucose (7.5 g/L), colistin sulphate (4 μg/ml) and vancomycin (50 μg/ml) to 31 g/L purple agar base (Bardet et al., 2017). In all, 143 bacterial isolates, including colistin-resistant Enterobacterales and non-fermentative Gram-negative bacilli, were used to evaluate this medium, where the specificity and sensitivity were 100% (Bardet et al., 2017). The medium was further evaluated on 56 *mcr*-1-positive and 10 *mcr*-1-negative chicken and human stool samples as well as two clinical rectal swabs (Bardet et al., 2017). The study found that the LBJMR could detect *mcr*-1-positive isolates with high sensitivity, particularly showing a higher sensitivity for colistin-resistant non-fermenters than Superpolymyxin (Bardet et al., 2017). Furthermore, the LBJMR medium does not contain daptomycin and amphotericin B, which are used in some of the agar-based media, that is, Superpolymyxin, to inhibit the growth of Gram-positive bacteria, including vancomycin-resistant *Enterococcus* (Bardet et al., 2017).

Luria-Bertani medium

A selective medium for detecting colistin-resistant Enterobacterales (including those with *mcr*-1 genes) in spiked stools was evaluated (Turbett et al., 2019). The medium was developed by adding 4 mg/ml colistin, 10 mg/ml vancomycin and 5 mg/ml amphotericin B to agar medium made with 25 g of Luria-Bertani (LB) powder (Turbett et al., 2019). Each of the isolates was spiked into faecal samples and serially diluted to final concentrations of 10^2 or 10^3 CFU per ml; 0.5 ml of the stool mixture was spiked in 4.5 ml of Enterobacterales enrichment broth and incubated for 24 h at approximately 35℃ (Turbett et al., 2019). Afterwards, 10 μl of the spiked Enterobacterales enriched broth was inoculated onto the LB medium and incubated at 35℃ ± 2℃ for 48 h in ambient air (Turbett et al., 2019). The selective LB medium demonstrated a sensitivity of 100% (Table 1) (Turbett et al., 2019).

Lateral flow immunoassay

Monoclonal antibodies were used to develop lateral flow assays to detect MCR-1-producing bacterial isolates (Volland et al., 2019). Bacterial colonies were isolated from agar plates, suspended in extraction buffer and dispensed on the Monoclonal antibodies-containing cassette where they were allowed to migrate for 15 min (Volland et al., 2019). All MCR-1-producing isolates were detected accordingly as shown by a pink band on the test line and control line of the assays. Furthermore, this test was able to detect MCR-2-producing isolates (Volland et al., 2019). The same assay is currently marketed as the NG-Test MCR-1 by NG Biotech in France (Fenwick et al., 2020). Initially, the evaluation of

the NG-Test MCR-1 resulted in eight false-positive results that were ultimately resolved to negatives apart from one isolate that was found to be an MCR-2 producer (Fenwick et al., 2020). Although the detection of the MCR-2 product by the NG Test MCR-1 further confirms the results of the developers, cross-reactivity with MCR-2 limits the accuracy of the assay for the MCR-1 producers (Fenwick et al., 2020). Nonetheless, the lateral flow immunoassays were found to be highly sensitive, easy to use and cost-effective for detecting MCR-1/-2 (Fenwick et al., 2020; Volland et al., 2019).

Rapid flow cytometry method (FASTinov®)

FASTinov® (Porto, Portugal) recently developed a flow-cytometry-based assay that enables the direct detection of colistin-susceptible and colistin-resistant Gram-negative bacteria in blood culture or on plates within 1–2 h. This assay works by measuring the fluorescence levels of cells ruptured by colistin, which acts by destroying the cell membrane. In colistin-susceptible cells, colistin ruptures the cell membranes and allows the entry of fluorescent probes, allowing susceptible cells to emit stronger fluorescence that is detected and analysed by dedicated software (BioFast) developed by FASTinov®. Meanwhile, resistant cells emit little or no fluorescence as the cell membrane remain intact, preventing the entry of the fluorescent probes. This functional assay does not depend on the growth of bacteria, but on their cellular membrane reaction to colistin, allowing for a faster detection of colistin susceptibility than culture-based tests. This assay, which requires less skill to operate and analyse, produced a CA of 99.0% with no MEs (Fonseca E Silva et al., 2019).

MOLECULAR TESTS

Amplidiag Carba-R+ MCR assay

The Amplidiag Carba-R+ MCR assay is a multiplex nucleic-acid-based test developed for detecting carbapenemase and *mcr*-1/-2 genes from rectal swabs and bacterial culture (Girlich, Bernabeu, et al., 2019). The assay was performed on 215 Gram-negative bacilli and 51 Enterobacterales isolates (Girlich, Bernabeu, et al., 2019). The Amplidiag Carba-R+MCR assay did not detect one GES carbapenemase-producing *P. aeruginosa*. However, all *mcr*-1- and *mcr*-2-producing isolates were accurately detected (Table 1) (Girlich, Bernabeu, et al., 2019).

Additionally, this assay was performed on DNA extracted from 100 rectal swabs, including 40 carbapenemase-positive samples: the sensitivity was 92.5% (Girlich, Bernabeu, et al., 2019). Two NDM and one OXA-48 producers were not detected due to a low concentration of bacteria; therefore, the samples were subjected to an overnight enrichment in brain heart infusion with 0.5 μg/ml ertapenem (Girlich, Bernabeu, et al., 2019). The enrichment step allowed for the detection of two of the three samples that were not previously detected. One of the samples was an OXA-48-producing *E. cloacae* with an AcOXA (*Acinetobacter* oxacillinases with carbapenemase activity) gene (Girlich, Bernabeu, et al., 2019). The overall performance of this assay was acceptable, demonstrating sensitivity and specificity ranging from 92% to 100% and 86% to 100%, respectively (Girlich, Bernabeu, et al., 2019). Moreover, the assay can be performed on cultured bacteria as well as DNA extracted from rectal swabs in no more than 3 h (Table 1) (Girlich, Bernabeu, et al., 2019).

AusDiagnostic MT CRE EU assay

AusDiagnostics MT CRE EU assay is a two-step nested multiplex-tandem PCR assay by AusDiagnostics (Meunier et al., 2018). One study evaluated the AusDiagnostics MT CRE EU assay for detecting carbapenemase, *mcr*-1 and *mcr*-2 genes (Meunier et al., 2018). A collection of Enterobacterales, *Pseudomonas* sp. and *Acinetobacter* sp., including carbapenemase or *mcr*-1/-2 producers, were used to evaluate the performance of this assay (Meunier et al., 2018). The assay was performed by suspending two to three bacterial colonies grown overnight on Columbia blood or cystine lactose electrolyte agar in tubes with a sample buffer (Meunier et al., 2018). The tubes were loaded onto the AusDiagnostics MT processor platform for template extraction and the first round of PCR (Meunier et al., 2018). Lastly, the nested RT-PCR was performed by loading a 384-PCR plate containing the reaction mix onto the AusDiagnostic MT analyser (Meunier et al., 2018). The results were automatically read using the AusDiagnostics MT assay software. The assay failed to detect four out of the 22 *mcr* genes; however, the *mcr* genes were also not detected by the reference PCR (Meunier et al., 2018).

Evaluation of the assay resulted in eight and 18 false-positive results (Meunier et al., 2018). An overall sensitivity and specificity of 95.5% and 99.8%, respectively, were obtained, which improved to 100% following repeats of the assay (Meunier et al., 2018). The AusDagnostic MT CRE EU assay detected *mcr*-1/-2 genes as well as carbapenemase genes with minimal hands-on time (Table 1) (Meunier et al., 2018).

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that allows autocycling strand displacement DNA synthesis at constant temperature

using Bst DNA polymerase (Zhong et al., 2019). The use of LAMP for detecting *mcr*-1 gene was first described by two studies (Imirzalioglu et al., 2017; Zou et al., 2017). Zou et al. (2017) established a LAMP assay to detect *mcr*-1 gene from cultured bacteria and spiked human stools. In this study, the LAMP assay was performed in 25 µl reaction mixtures that contained 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.8 M betaine, 0.1% Tween-20, 1.4 mM of each deoxyribose nucleotide triphosphate and 8 U Bst DNA polymerase (Zou et al., 2017). Each reaction mixture had specified amounts of forward and backward inner primers, outer forward and backward primers, loop primers as well as the appropriate amount of DNA template (Zou et al., 2017). The mixture was covered with 25 µl wax and incubated in a dry bath incubator for 60 min at constant temperature, the amplification products were read visually and by turbidimetry (Zou et al., 2017). Visual detection was by colour change of a fluorescent metal indicator, a positive reaction was demonstrated by the formation of a magnesium pyrophosphate precipitate, which changed the reaction mixture from orange to green during amplification (Zou et al., 2017).

Imirzalioglu et al. (2017) evaluated the eazyplex SuperBug *mcr*-1 kit (Amplex Biosystems GmbH) for the rapid detection of *mcr*-1 gene. The *mcr*-1-detecting LAMP assays were found to detect *mcr*-1 genes accurately, and the in-house LAMP assay was stated to be more sensitive than conventional PCR assays (Table 1) (Imirzalioglu et al., 2017; Zou et al., 2017).

However, *mcr*-1-detecting LAMP assays cannot detect other potential target genes (Imirzalioglu et al., 2017; Zhong et al., 2019; Zou et al., 2017). Therefore, Zhong et al. (2019) developed a restriction endonuclease-based multi-LAMP for the detection of multiple *mcr* genes. Two separate LAMP systems were established, a double-LAMP (*mcr*-2 and *mcr*-5) and triple-LAMP system (*mcr*-1, *mcr*-3 and *mcr*-4) which were performed in 25 µl reaction mixtures (Zhong et al., 2019). The 25 µl reaction mixture consisted of 12.5 µl LAMP-reaction mix, 1 µl Bst 2.0 polymerase, 1.25 µl primer mix, 8.25 µl nuclease-free water and 2 µl DNA lysate (Zhong et al., 2019). Amplification products were detected visually by change in colour of SYBR Green I, which changed from yellow to orange for a positive reaction (Zhong et al., 2019). Amplification products were also stained with GoldView TM and analysed by electrophoresis on 2% agarose gel (Zou et al., 2017). Multiplex detection of *mcr*-1 to *mcr*-5 genes was established through restriction digestion of the LAMP products based on band numbers and fragment lengths using Hind restriction enzyme (Zhong et al., 2019).

An advantage of LAMP assays over conventional PCR in that LAMP is more sensitive, has a shorter processing time of <60 min, is relatively easier to run and multiplex detections can be conducted in the same detection system (Table 1) (Imirzalioglu et al., 2017; Zhong et al., 2019; Zou et al., 2017).

Matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS)

Matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF MS) is a technique that is based on the production of mass spectra from whole cells and their contrast to a reference spectrum (Giordano & Barnini, 2018). This method is widely used for species identification of pathogens in clinical microbiology laboratories. Giordano and Barnini (2018) were the first to evaluate the possibility of detecting colistin resistance using MADI-TOF MS. In this study, genomic DNA of 139 *K. pneumoniae* isolates from clinical samples was used for identification using MALDI-TOF MS (Bruker Daltonik GmbH) (Giordano & Barnini, 2018). The MICs of the isolates were determined by Sensititre using a range of antibiotics including colistin (Giordano & Barnini, 2018). First, a training set for mass peak analysis was established using 50/139 of the *K. pneumoniae* isolates from which 400 spectra were obtained and used for database entry (Main Spectrum Profile) as well as to classify algorithm models (Giordano & Barnini, 2018). Finally, the remaining 89/139 isolates were used to conduct the test; 712 spectra were collected from this set (Giordano & Barnini, 2018). However, from the 712 spectra, 158 were excluded as they constituted flat-line spectra or outliers, demonstrating identification score below 2.3 (Giordano & Barnini, 2018). Based on the mass signals and intensities of the bacterial protein samples, two-dimensional peak distribution classified the training set spectra into two main groups viz., colR-KPn (colistin-resistant *K. pneumoniae*) and colS-KPn (colistin-susceptible *K. pneumoniae*) isolates (Giordano & Barnini, 2018).

The newly created database entry consisted of using *MALDI Biotyper RTC* and *MALDI Biotyper* v3.0 to identify *K. pneumoniae* isolates and for the automatic detection of colistin resistance, respectively (Giordano & Barnini, 2018). The automatic classification of the test set resulted in the correct classification of 71% colR-KPn and 40% colS-KPn (Giordano & Barnini, 2018). Furthermore, different algorithm models were tested using ClinProTools v3.0 (Bruker Daltonics). The three algorithms tested included the Genetic Algorithm, Supervised Neural Network and Quick Classifier (Giordano & Barnini, 2018). However, the tested algorithms either had good recognition capability and cross-validation but poor classification of colistin resistance or poor recognition capability and acceptable classification of colistin resistance (Giordano & Barnini, 2018). The Genetic Algorithm

seemed more promising as it was better suited for biological samples (Giordano & Barnini, 2018). Therefore, a reliable classification model was created by combining the most relevant peaks detected from the Genetic Algorithm (Giordano & Barnini, 2018). The resulting peak combination of 4507.28/5142.84 Da from Genetic Algorithm demonstrated a sensitivity and specificity of 78% and 89%, respectively (Giordano & Barnini, 2018).

Three studies have evaluated MALDIxin, a MALDI-TOF-based assay (Dortet et al., 2020; Dortet et al., 2018; Furniss et al., 2019a). The MALDIxin test was developed to detect pETN modification in lipid A directly from bacterial colonies in <15 min (Dortet et al., 2018; Furniss et al., 2019a). Dortet et al. (2018) evaluated MALDIxin on *A. baumannii* isolates, where the mass spectrum in colistin-susceptible isolates was characterized by two sets of peaks at the centre of *m/z* 1728.1 and *m/z* 1910.3. The peaks were assigned to bis-phosphorylated hexa-acyl and bis-phosphorylated hepta-acyl lipid A that had 12–14 carbons making up the acyl chain, respectively (Dortet et al., 2018). The mass spectrum in colistin-resistant isolates was observed by two sets of peaks at the centre of *m/z* 1935.3 and *m/z* 2033.3, showing *m/z* +25 and *m/z* +123 shifts of mass unit of the bis-phosphorylated hepta-acyl lipid A at *m/z* 1910.3 (Dortet et al., 2018). The peaks observed at *m/z* 2033.3 and *m/z* 1935.3 were assigned to pETN-modified-bis-phosphorylated hepta-acyl and pETN-modified-mono-phosphorylated hepta-acyl lipid A, respectively, with an acyl chain of 12 carbons in length (Dortet et al., 2018). The peaks (*m/z* 2033.3 and *m/z* 1935.3) associated with pETN-modified lipid A were observed in all colistin-resistant isolates and were not observed in any of the colistin-susceptible isolates (Dortet et al., 2018).

Furniss et al. (2019) and Dortet et al. (2020) described the optimization of the MALDIxin test for detecting colistin resistance in clinical *E. coli* and *K. pneumoniae* isolates, respectively. Furniss et al. (2019) optimized the MALDIxin test by adopting the low-resolution linear mode used by the MALDI Biotyper Sirius system. The optimization was achieved by adding a mild-acid hydrolysis step, which is required for analysis of clinical isolates in negative ion mode (Dortet et al., 2020). The mild-acid hydrolysis step was performed by resuspending a single bacterial colony grown on MH agar for 18–24 h in 200 µl distilled water (Dortet et al., 2020; Furniss et al., 2019a), after which 50–100 µl of 2% acetic acid was added to double-distilled water containing bacterial suspension and heated for 5–15 min at 98–100℃ (Dortet et al., 2020; Furniss et al., 2019a). For MALDI-TOF analysis, Furniss et al. (2019) used a MALDI Biotyper Sirius system, whereas Dortet et al. (2020) used a 4800 Proteonic Analyzer. The optimization of the MALDIxin allowed for the identification of L-Ara4N- and pETN-modified lipid A in *E. coli* and *K. pneumoniae* isolates. Moreover, the optimized methods were able to distinguish between chromosome-encoded and MCR-mediated colistin resistance (Dortet et al., 2020; Furniss et al., 2019a).

Microarray

A commercial CT103XL microarray system that allows for the simultaneous detection of *mcr*-1/-2 and β-lactamase genes was evaluated (Bernasconi et al., 2017). The study was conducted on 106 Enterobacterales isolates including *mcr*-1- and *mcr*-2-positive strains, as well as carbapenemase and extended-spectrum β-lactamase-producing strains (Bernasconi et al., 2017). The CT103XL microarray, which uses a multiplex ligation detection reaction, was performed following bacterial DNA extraction from bacterial cultures (Bernasconi et al., 2017). The commercial CT103XL microarray was confirmed to simultaneously detect *mcr*-1/-2 and β-lactamase genes with accuracy, although it failed to detect *mcr*-3, which shares 45% and 47% identity to *mcr*-1 and *mcr*-2, respectively (Bernasconi et al., 2017).

Multiple cross-displacement amplification coupled with gold nanoparticles-based lateral flow biosensor

A multiple cross-displacement amplification (MCDA) method, coupled with gold nanoparticles-based lateral flow biosensor (LFB) assay, for detecting *mcr*-1 gene was developed (Gong et al., 2019). The MCDA reaction was performed on extracted DNA from 59 bacterial isolates, where each 25 µl reaction consisted of 12.5 µl reaction buffer, 1 µl Bst DNA polymerase 2.0, 1 µl colorimetric indicator, 1.6 µM of each cross primers, 0.4 µM of each displacement primers, 0.4 µM amplification primers and 1 µl DNA template (Gong et al., 2019). The MCDA reaction systems were then subjected to isothermal temperature (63℃) for 40 min, after which the amplification products were analysed using 2% agarose gel electrophoresis, colorimetric indicator and LFB (Gong et al., 2019). For *mcr*-1 detection by LFB, 0.2 µl of the amplicons was added to the well of the sample pad, followed by the addition of three drops of running buffer (1% Tween 20 and 0.01 mol/L phosphate-buffered saline) (Gong et al., 2019). The results were visually read after 1–2 min; a positive result was demonstrated by two red bands, one at the test-line and the other at the control line (Gong et al., 2019). The results were positive for all *mcr*-1-positive isolates and negative for all non-*mcr*-1 isolates (Gong et al., 2019).

The MCDA-LFB assay was further applied to stool samples spiked with 100 µl dilutions of bacterial strains (Gong et al., 2019). The resulting detection limit was 600 fg of *mcr*-1 plasmid DNA per microliter in bacterial culture and 4.5×10^3 CFU per ml in the spiked faecal samples (Gong

et al., 2019). The MCDA-LFB has demonstrated the same sensitivity as the *mcr*-1 LAMP, which is more sensitive than the conventional PCR. Furthermore, the MCDA-LFB has demonstrated a shorter reaction time (Gong et al., 2019; Zou et al., 2017).

Recombinase polymerase amplification (RPA)

The rapid detection of *mcr*-1, using a recombinase polymerase amplification (RPA), has been described (Xu et al., 2018). RPA is a novel isothermal amplification method, which can be performed in no more than 30 min at body temperature without the need for thermal cycling instruments (Xu et al., 2018). This study used basic RPA (B-RPA) and RPA with lateral flow (LF-RPA) on 23 genomic DNA extracted from 20 *mcr*-1-positive and three *mcr*-1-negative Enterobacterales (Xu et al., 2018). The B-RPA was based on the TwistAmp Basic kit reaction system, which was incubated at room temperature for 30 min, after which the amplicons were extracted by phenol/chloroform solution or purified using an amplicon purification kit (Xu et al., 2018). The LF-RPA reaction required primers and a probe, which were labelled with biotin and fluorescence (Xu et al., 2018). The LF-RPA was based on TwistAmp Nfo kit reaction system, which was incubated as described for the B-RPA (Xu et al., 2018). The amplification products for the LF-RPA were diluted at 1:50 with running buffer, after which a downstream operation was carried out (Xu et al., 2018).

The results for the B-RPA assay were read by agarose gel electrophoresis, whereas the results for the LF-RPA assay were visually read using Hybridetect 2T dipsticks (Xu et al., 2018). A positive *mcr*-1 detection by LF-RPA was demonstrated by two purple bands at the test line and the quality control line (Xu et al., 2018). Both assays detected the *mcr*-1-positive and *mcr*-1-negative DNA samples accordingly; therefore, both assays are equally suitable for detecting *mcr*-1 genes (Xu et al., 2018).

Conventional and real-time PCR and whole-genome sequencing

The presence or absence of *mcr* genes is determinable by PCR assays and whole-genome sequencing (WGS) as standard (Imirzalioglu et al., 2017). Although WGS is able to characterize the mechanism of resistance and determine the molecular evolutionary trajectory of colistin-resistant isolates, PCR is only able to characterize resistance genes (Hua et al., 2017; Peter et al., 2018) However, WGS technology is limited in settings that lack adequate resources and therefore PCR assays are widely adopted for detecting *mcr* genes (Rebelo et al., 2018; Zhong et al., 2019).

Nijhuis et al. (2016) were the first to design a real-time PCR assay for detecting *mcr*-1 from clinical isolates. The assay was validated on 26 *mcr*-1-positive *E. coli* isolates, where the presence of *mcr*-1 was detected in all 26 isolates (Nijhuis et al., 2016). Additionally, the assay was evaluated on spiked stool samples and the efficiency of the PCR was 102.6% and the LOD was 3–30 CFU per reaction (Nijhuis et al., 2016). However, *mcr*-1 genes were not detected in other colistin-resistant strains, that is, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Acinetobacter*, etc.

A multiplex PCR (M-PCR) assay for the simultaneous detection of *mcr*-1 and carbapenem-resistant genes, bla_{KPC}, bla_{NDM}, bla_{IMP}, $bla_{OXA-48-like}$, was described (Hatrongjit et al., 2018). The assay was validated on reference strains including *E. coli* A434-59, which contains *mcr*-1 and bla_{NDM-1} (Hatrongjit et al., 2018). Evaluation of the M-PCR on 127 carbapenem-resistant, eight *mcr*-1-positive and 62 carbapenem-susceptible Enterobacterales found the assay to be 100% sensitive and specific (Hatrongjit et al., 2018).

Additionally, three studies designed M-PCR assays to detect *mcr*-1 to *mcr*-5 genes (Lescat et al., 2018; Rebelo et al., 2018; Jousset et al., 2019). The assay designed by Rebelo et al. (2018) allowed for the simultaneous detection of *mcr* genes and their variants in bovine and porcine isolates (Rebelo et al., 2018). This study did not use internal amplification controls as they were incompatible with DreamTaq Green PCR Master Mix (Rebelo et al., 2018). The master mix contains DNA polymerase synthesized in *E. coli* and thus would produce amplicons if 16S rRNA primers are used (Rebelo et al., 2018).

However, Lescat et al. (2018) designed a more rapid (<2 h) M-PCR assay that was compatible with internal controls. Recently, Jousset et al. (2019) designed and evaluated an M-PCR assay on 50 *E. coli*, 41 *K. pneumoniae* and 12 *Salmonella enterica* isolates (from which a total of 40 were MCR-producers), which was 100% accurate in detecting *mcr*-positive isolates. The assay was additionally performed on 82 *Aeromonas* sp. and 10 *Shewanella* sp. that were previously described as potential originators of *mcr*-3 and *mcr*-4, respectively (Jousset et al., 2019). None of the *Aeromonas* sp. were *mcr*-positive, although two *Shewanella* sp., *S. bicestrii* JAB-1 strain and *S. woody* S539 with MICs of 0.25 and <0.12 mg/L respectively, were *mcr*-4 positive (Jousset et al., 2019). However, cloning *S. bicestrii* JAB-1 genes into *E. coli* TOP10 resulted in an *mcr*-4 positive outcome by the PCR assay with a colistin MIC of 4 mg/L (Jousset et al., 2019).

Borowiak et al. (2020) described the detection of *mcr*-1 to *mcr*-9 in colistin-resistant *S. enterica* isolates using an M-PCR (*mcr*-1 to *mcr*-5) designed by Rebelo et al. (2018) and a newly designed M-PCR assay (*mcr*-6 to *mcr*-9). The assay was performed on 407 colistin-resistant *S. enterica* isolates from animals, animal feed, food and the environment (Borowiak et al., 2020). In all, 254 of the isolates had *mcr*

genes. Moreover, the assay detected *mcr*-9 in isolates carrying *mcr*-1 (Borowiak et al., 2020). However, two separate frameshift mutations of *mcr*-9 were shown to have occurred in the respective isolates as demonstrated by WGS analysis; the mutations are believed to have contributed to non-functional MCR-9 proteins (Borowiak et al., 2020).

Two studies have described methods for broth enrichment of colistin-resistant *E. coli* followed by real-time PCR to detect *mcr* genes (Chalmers et al., 2018; Chandler et al., 2020). Chalmers et al. (2018) were the first to describe a SYBR Green-based real-time PCR method for *mcr*-1 and *mcr*-2 following enrichment with *E. coli* (EC) broth containing colistin (1 μg/ml). All the porcine faecal and chicken caecal samples were screened by real-time PCR after 16 h of culture in EC broth (Chalmers et al., 2018). However, none of the *mcr*-1 and *mcr*-2 genes were detected by PCR in any of the samples after 16 h of enrichment (Chalmers et al., 2018). As well, the method described by Chandler et al. (2020) for detecting *mcr*-1 included enrichment using EC broth containing colistin (1 μg/ml) and vancomycin (8 μg/ml). The method was evaluated on 100 feral swine faecal samples, which were inoculated with one of five different *mcr*-1-positive *E. coli* strains (Chandler et al., 2020). The bacteria was inoculated at concentrations ranging between 0.1–9.99, 10–49.99, 50–99, 100–149 and 200–2200 CFU per gram from which *mcr*-1 was detected with 32%, 72%, 88%, 95% and 98% accuracy by real-time PCR, respectively (Chandler et al., 2020).

Four SYBR Green-based real-time PCR assays have been developed for *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5 detection (Bontron et al., 2016; Dona et al., 2017; Li et al., 2017; Tolosi et al., 2020). Bontron et al. (2016) designed a SYBR Green-based real-time PCR assay for detection of *mcr*-1 from cultured bacteria and stools. The assay was validated on 20 Enterobacterales, where it was found to accurately detect the presence or absence of *mcr*-1 at a LOD of 10^2 cultured bacteria (Bontron et al., 2016). Furthermore, Dona et al. (2017) described a SYBR Green real-time PCR assay to also detect *mcr*-1 from human faecal samples. However, in this study, 20 μg of the stool samples was enriched overnight in 10 ml LB broth containing 2 μg/ml colistin and plated on four selective agar plates prior to DNA extraction (Dona et al., 2017). The real-time PCR accurately identified *mcr*-1 harbouring *E. coli* isolates with an LOD of 10^1 and PCR efficiency of *c*. 106% (Dona et al., 2017).

Li et al. (2017) also designed a multiplex SYBR Green-based real-time PCR assay for *mcr*-1, *mcr*-2 and *mcr*-3 detection. The assay was validated on 25 isolates including *mcr*-1-positive and *mcr*-3-positive strains; the *mcr*-2 gene was synthesized in the study due to a lack of *mcr*-2-positive isolates (Li et al., 2017). Although the *mcr* genes were detected with 100% accuracy with a LOD of 10^2, *mcr*-2 was not validated on cultured bacteria (Li et al., 2017). However, in this study, all three *mcr* genes could not be simultaneously detected in one reaction unlike when the Taqman probe was used (Li et al., 2017). A more recent study evaluated the SYBR Green-based real-time PCR method proposed by Li et al. (2017) in detecting and quantifying *mcr*-1 to *mcr*-3 as well as newly designed assays for *mcr*-4 and *mcr*-5 (Tolosi et al., 2020). The optimized *mcr*-1 to *mcr*-5 PCR assays were validated on bacterial isolates (Tolosi et al., 2020). The study found that SYBR Green real-time PCR, followed by melting curve analysis, was more efficient in detecting and quantifying *mcr*-1 to *mcr*-5 genes in both bacterial isolates (Tolosi et al., 2020). The described assays detected all five *mcr* genes with a lower limit of 10^2. Moreover, the assays enabled screening of five individual samples in a single reaction (Tolosi et al., 2020).

The parallel detection of *mcr*-1, *mcr*-2 and *mcr*-8 by real-time PCR using Taqman® probes has been described (Chabou et al., 2016; Daniels et al., 2019; Nabti et al., 2020). Chabou et al. (2016) designed two quantitative real-time PCR assays with TaqMan® probes for the rapid detection of *mcr*-1 gene. Primers and probes were designed to develop the two PCR assays, designated PE1 and PE2 (Chabou et al., 2016). The assays were evaluated on 100 bacterial isolates (18 of which were colistin resistant) and 833 broiler faecal samples (Chabou et al., 2016). The sensitivity and specificity of both assays were 100%, with a calibration curve that was linear from 10^1 to 10^8. However, the PE1 assay was recommended for initial screening of *mcr*-1 followed by PE2 assay for confirming the results (Chabou et al., 2016).

Daniels et al. (2019) developed a multiplexed real-time PCR with TaqMan® probes to detect *mcr*-1 and *mcr*-2. The assay was validated on 25 bacterial isolates, some of which were *mcr*-positive (Daniels et al., 2019). The sensitivity and specificity of the assay was 100%, being able to detect *mcr*-1 and *mcr*-2 from dilutions containing 8.5×10^3 and 7.7×10^3 CFU per ml, respectively (Daniels et al., 2019). A specific real-time PCR assay using TaqMan probes to identify *mcr*-8 was designed for the first time by Nabti et al. (2020). The specificity and sensitivity of the assay were evaluated on 290 bacterial isolates from clinical samples and 250 metagenomic DNA from human stools (Nabti et al., 2020). The PCR assay accurately detected *mcr*-8 from the one positive *K. pneumoniae* isolate with an overall efficiency of 92.64% and a LOD of 55 CFU per ml (Nabti et al., 2020).

CONCLUSION

The rapid dissemination of colistin resistance, mediated by chromosomal mutations and *mcr* genes, poses a threat to public and veterinary health as colistin is one of the last-line antibiotics. Currently, the gold standard for colistin susceptibility testing is the BMD method, which is not suitable for routine clinical use as it is time-consuming and is associated

with methodological issues. Even so, the transmissibility of colistin resistance makes it imperative to establish rapid and reliable methods that will efficiently detect colistin resistance. A suitable method should be cost-effective and should not discriminate against the mechanism of colistin resistance as well as take into consideration hetero-resistant isolates. Among the available diagnostic assays, the Rapid Resapolymyxin NP test is a promising initial screening method as it can be performed in-house, therefore making it relatively cheap; it is easy to perform, and it is not limited to glucose-fermenting colistin-resistant Gram-negative bacteria. Other colorimetric screening methods such as ASAT, Rapid Polymyxin NP, Rapid Polymyxin *Acinetobacter* and Rapid Polymyxin *Pseudomonas* are species-specific and cannot be used for general screening in high-capacity clinical laboratories. The recent rapid flow cytometry method (FASTinov®), albeit expensive, has a rapid TAT of 1–2 h and is simpler to use.

Likewise, agar-based methods are cheap and can be used as initial screening tools in poorer settings, although most of the agar-based assays fail to detect isolates with hetero-resistance. They also have a longer TAT of 24 h. Should there be a need to use agar-based assays, CHROMagar COL-*APSE* was designed to detect and differentiate all colistin-resistant isolates, although it might be relatively expensive than Superpolymyxin and the LBJMR medium, which can be performed in-house. However, the LBJMR medium was found to detect hetero-resistance better than Superpolymyxin. ChromID® Colistin R and LB media can only be used to screen for Enterobacterales.

MIC determiners are of particular interest in determining the optimal dosage for colistin treatment and pharmacokinetics as colistin is associated with nephrotoxic and neurotoxic side effects. Thus, MIC-determining methods could be used for initial screening in highly resourced laboratories. Although they relatively require a higher skill than the agar-based tests and the biochemical colorimetric tests. The non-automated MIC strips, that is, UMIC, MMS and ComASP are cheaper than automated MIC determiners, that is, Microscan, Sensititre and BD Phoenix. However, automated MIC determiners could be available in most well-resourced clinical laboratories as they are generally used for AST. Non-automated MIC strips are cheaper, require less skill and recommendable for less-resourced laboratories.

A second screening can be performed to mainly detect *mcr*-production using chelator-based phenotypic assays, which are more suitable although most are subjected to >16 h incubation. Moreover, the lateral flow assay that detects MCR-1/-2 production could be used for rapid detection. Molecular methods could be considered to detect *mcr* genes in well-resourced clinical microbiology laboratories. Particularly, the LAMP assays could be used as they were found to be more sensitive than PCR methods. More so, LAMP requires less equipment and has a shorter TAT than PCR and WGS methods.

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CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

ORCID

John Osei Sekyere https://orcid.org/0000-0002-9508-984X

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