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Detection of expanded-spectrum cephalosporin hydrolysis by lateral flow immunoassay

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Summary

Early detection of expanded-spectrum cephalosporin (ESC) resistance is essential not only for an effective therapy but also for the prompt implementation of infection control measures to prevent dissemination in the hospital. We have developed and validated a lateral flow immunoassay (LFIA), called LFIA-CTX test, for the detection of ESC (cefotaxime) hydrolytic activity based on structural discrimination between the intact antibiotic and its hydrolysed product. A single bacterial colony was suspended in an extraction buffer containing cefotaxime. After a 30-min incubation, the solution is loaded on the LFIA for reading within 10 min. A total of 348 well-characterized Gram-negative isolates were tested. Among them, the 38 isolates that did not express any cefotaxime-hydrolysing β-lactamase gave negative results. Of the 310 isolates expressing at least one cefotaxime-hydrolysing β-lactamase, all were tested positive, except three OXA-48-like producers, which were repeatedly detected negative. Therefore, the sensitivity was 99.1% and the specificity was 100%. The LFIA-CTX test is efficient, fast, low-cost and easy to implement in the workflow of a routine microbiology laboratory.

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Introduction

The discovery of antibiotics has revolutionized medicine by enabling effective treatment of many life-threatening bacterial infections. During the 20th century, described as the golden age of antibiotics, the entire spectrum of antibiotics was discovered allowing efficient treatment of most bacterial infections (Silver, 2011; Bbosa *et al*., 2014). The latter products were obtained by chemical modification of pre-existing antibiotics to produce different generations with improved efficacy and broadened spectrum of activity (Silver, 2011). However, the abundance of antibiotics in the environment, water and medicine and the recurrent use of the same classes of antibiotics over the past decades have led to the emergence of resistant bacteria to these antibiotics and to difficult-to-treat bacterial infections, which constitute a significant problem to the global health (Silver, 2011). Today, c.a. 7000 molecules have been described with antibacterial activity, but only about 100 have made it into human therapy, as some are toxic to human cells, not very effective in vivo or chemically unstable (Awad *et al*., 2012). Thus, the fight against bacteria is again turning into one of the greatest challenges faced by our societies, especially with the spread of multi-drug-resistant (MDR) bacteria (O'Neill, 2014).

Acquisition of antibiotic resistance mechanisms is a natural process that is amplified by overuse and misuse of antibiotics and inadequate or absent epidemiological surveillance in human and veterinary medicine, which has led to the dissemination of these MDR bacteria.

β-Lactams, due to their safety, reliable killing properties and clinical efficacy, are among the most frequently prescribed antibiotics used to treat bacterial infections. However, their utility is being threatened by the worldwide proliferation of β-lactamases (BL) with broad hydrolytic capabilities, especially in MDR Gram-negative

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bacteria (GNB; Bush and Bradford, 2016). As the incidence of GNB infections increases, for which few effective treatments are available, so does the contribution of ß-lactam-hydrolysing enzymes, the BL, to this serious clinical problem. Currently, BL-mediated resistance does not spare even the newest and most powerful β-lactams (i.e. expanded-spectrum cephalosporins (ESC) and carbapenems; Munita and Arias, 2016). Their dissemination is a matter of great clinical concern given the importance of these pathogens as causes of nosocomial infections and of community-acquired infections, resulting in difficult-to-treat infections associated with high mortality rates and increased costs (Li and Webster, 2018).

The production of BL is a commonly encountered mechanism of resistance in Gram-negative bacteria, especially in Enterobacterales (Bush and Bradford, 2016; Bonomo, 2017). In healthcare settings, the activity of ESCs is challenged by extended-spectrum β-lactamases (ESBLs) and plasmid-encoded cephalosporinases and carbapenemases, but ESBLs are by far the most prevalent mechanism in Enterobacterales (Jacoby, 2006; Munita and Arias, 2016). The CDC has estimated that ESBL-producing Enterobacterales (ESBL-E) account for 19% of healthcare-related infections annually and that infections involving ESBL-E are also associated with increased mortality and cost of care (Centers for Disease Control and Prevention, 2013). ESBLs hydrolyse most β-lactams, including penicillins and ESCs (e.g. cefotaxime, ceftazidime), and as their genes are carried by plasmids, ESBL producers are often resistant to other families of antibiotics, thus resulting in an MDR phenotype (Paterson, 2006), and in some cases, resistance extends to the entire repertoire of available therapeutic agents (the so-called pan-drug-resistant phenotypes), posing a formidable challenge to antimicrobial therapy. Within the ESBLs, the CTX-M family appeared in the early 1980s and now represent the most prevalent ESBLs worldwide. They are divided into five groups based on amino acid sequence identity: the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 groups (Cantón *et al.*, 2012; Bevan *et al.*, 2017).

The rapid and effective detection of antibiotic resistant bacteria is a critical step for antibiotic stewardship and infection control. Despite technological improvements, the identification of pathogenic bacteria, as well as the detection of antibiotic resistance, remains complex and time-consuming, with time to results often in the range from 24 h to 48 h (Shanmugakani *et al.*, 2020).

Detection is mainly based on phenotypic approaches with antimicrobial susceptibility using disc diffusion methods in the presence and absence of BL inhibitors (Dijk *et al.*, 2014; Benkova *et al.*, 2020). These conventional screenings are usually slow, requiring overnight culture, and labour intense and require expertise for interpretation. The molecular methods, DNA microarray-based and nucleic acid amplification-based, either home-made or commercially available, remain the gold standard for the detection of CTX-Ms and other ESBLs (Rood and Li, 2017). Molecular methods are usually slow (1–4 h), labour intense and expensive, and require specific equipment and trained staff in order to be implemented in any clinical laboratory (ResearchGate). Lateral flow immunoassays (LFIAs) have proven to be useful as easy, rapid and reliable confirmatory tests for detection of antibiotic resistance mechanisms, especially for β-lactamases in Gram-negatives (Boutal *et al.*, 2018; Takissian *et al.*, 2019). The NG test CTX-M MULTI showed 100% sensitivity and specificity with isolates cultured on agar plates and was able to detect 98% of the ESBL producers identified in a clinical setting in France, either from colonies or from positive blood cultures (Bernabeu *et al.*, 2020). These assays detect the presence of enzymes that confer resistance to ESCs, and even though CTX-Ms account for most of the resistance mechanisms leading to ESC resistance, some rare enzymes may be missed. Biochemical confirmatory tests have been developed, which evaluate the enzyme's ability to hydrolyse β-lactams (ESBL NPD test; MALDI-TOF in mass spectrometry; UV spectroscopy; electrochemical test; β-lacta™ test; Bernabeu *et al.*, 2020). Even though these tests display good analytical performances, the ESBL NDP test and β-lacta™ test are sometimes difficult to interpret, and MALDI-TOF, UV spectroscopy and the electrochemical tests require expertise, hands-on time and specific equipment.

Here, we have developed an assay that takes advantage of the ease of LFIA and the universality of biochemical tests and that detects the enzymatic activity of ESC hydrolysis. This LFIA, called the LFIA-CTX test, is based on the structural discrimination of cefotaxime and its hydrolytic product (see Fig. 1A and B). It uses a monoclonal antibody recognizing only the non-hydrolysed cefotaxime. This makes it possible to detect the presence of BLs that are capable of hydrolysing 'cefotaxime', such as hyper-producers of the chromosomally encoded cephalosporinases (cAmpC), plasmid-encoded cephalosporinases (pAmpC), ESBLs and some of the carbapenemases. The aim is to develop a new tool that is fast, of low-cost and easy to use for the detection of BLs that hydrolyse ESCs to overcome the disadvantages of other detection tests.

Results

Test development

Our test is based on the use of monoclonal antibodies specific to cefotaxime. In order to obtain these antibodies, several steps were necessary. Biozzi mice (10 weeks old) were immunized with the immunogen cefotaxime–BSA,

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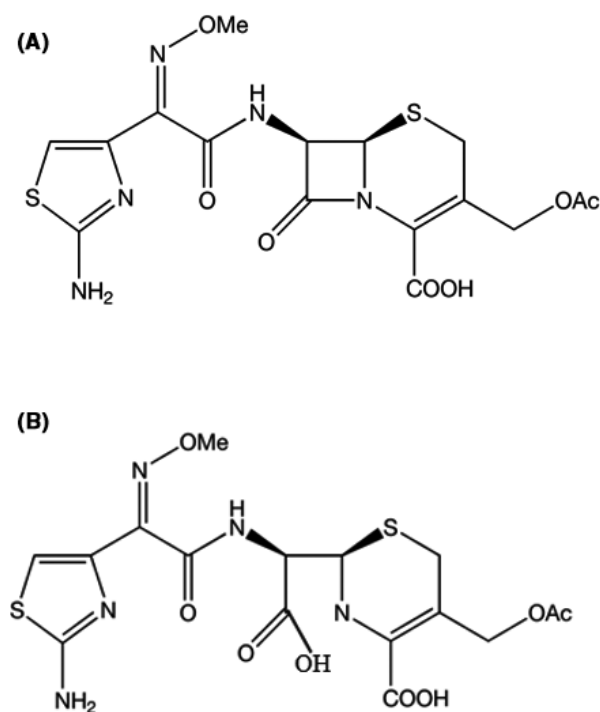

Fig. 1. (A) Intact cefotaxime, recognized by the antibody of the LFIA. (B) Hydrolysed cefotaxime, not recognized by the antibody LFIA.

previously produced. The immune response was followed by immunoenzymatic tests (Grassi *et al.*, 1988), measuring the anti-cefotaxime antibodies in sera using biotin-labelled cefotaxime as antigen. The two mice with the highest antibody titres were selected for mAb production. Spleen cells were fused with NS1 mouse myeloma cells, and specific anti-cefotaxime antibodies in myeloma culture supernatants were detected using the same immunoenzymatic test as the one previously used for the polyclonal response evaluation. At this step, one hundred hybridomas were selected, and then, a second round of selection was based on total recognition of the intact form of cefotaxime and non-recognition of the hydrolysed form (cross-reactions $< 0.1\%$). A third round of selection was performed with the twenty monoclonal antibodies previously selected using a competitive immunoassay in order to evaluate their relative affinity for cefotaxime and hydrolysed cefotaxime. At the end of this step, five monoclonal antibodies were selected. These five antibodies were evaluated in an LFIA test, named LFIA-CTX. In this LFIA, there is competition between the cefotaxime present in the sample and the cefotaxime immobilized on the membrane of the strip at the test line for recognition by the monoclonal antibody labelled with colloidal gold (Fig. 2). If the cefotaxime in the sample is intact, it will occupy all the sites of the antibody, which will not be able to bind to the immobilized cefotaxime. If cefotaxime is hydrolysed, it will not be recognized by the monoclonal antibody, which will be able to bind to the immobilized cefotaxime at the test line (Fig. 3; video available on https://youtu.be/F2PM9uKjXN0).

Thus, to select the best mAbs for the LFIA, each previously selected antibody was coupled with colloidal gold (Feraudet-Tarisse *et al.*, 2021). The strips (0.5 cm in width and 4.5 cm in length) were composed of three parts: (i) a sample pad (Standard 14; Whatman, Munich, Germany; 0.5 cm in length), (ii) a nitrocellulose membrane (Prima 40; 2.5 cm in length) and (iii) an absorption pad (Cellulose grade 470; Whatman; 1.5 cm in length), all attached to a backing card. The detection zone contained immobilized cefotaxime–BSA as a test line (1 mg ml^{-1}) dispensed at 1 μl cm^{-1} using an automatic dispenser (Biojet XYZ 3050; BioDot, Westergate, England). After drying for 1 h at 37°C in an air oven, the membrane was incubated with a blocking solution containing BSA for

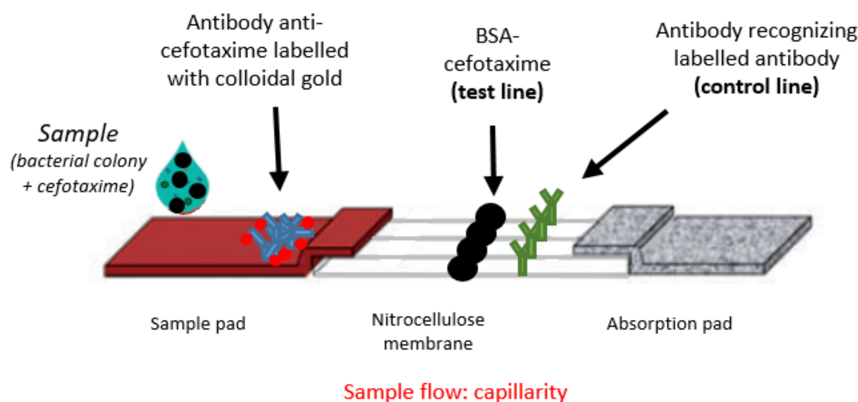

Fig. 2. Structure of the strips. The sample containing cefotaxime and the bacterial strain is deposited upstream of the sample paper. Downstream of the sample pad, the colloidal gold-labelled antibody recognizing the intact cefotaxime (also called mAb tracer) is dried. On the nitrocellulose membrane, we made a test line consisting of intact cefotaxime coupled with BSA and a control line consisting of antibodies recognizing the colloidal gold-labelled antibodies. Finally, the absorption pad allows the sample to migrate along the strip.

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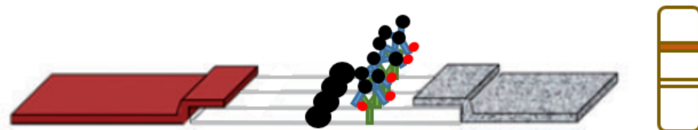
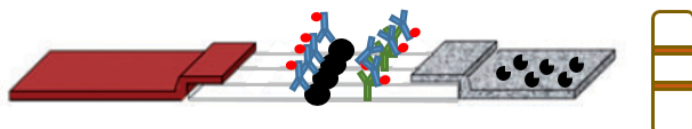
Case 1 :
- The control line appears: the test is correct
- No test line appears: negative test for expanded-spectrum cephalosporinase



Case 2 :
- The control line appears: the test is correct
- The test line appears: positive test for expanded-spectrum cephalosporinase



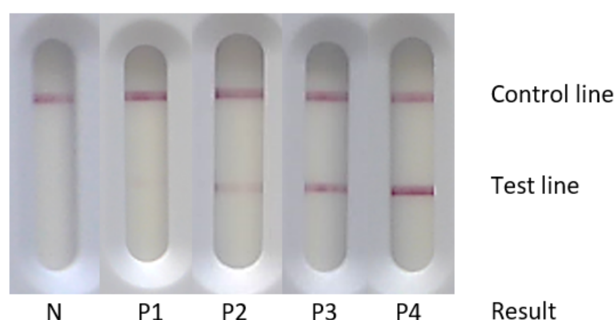


Fig. 3. Test procedure. One colony resuspended in 150 μl of extraction buffer with 25 ng ml^{-1} of cefotaxime, after a 30-min incubation, 100 μl loaded on the cassette. Then, the results are obtained after 10 min of migration. Data acquisition by a naked eye reading. Case 1: In the absence of enzymatic activity, all the mAb's paratopes are occupied by the cefotaxime added to the sample before the test. The mAb tracer is captured by the goat antibodies on the control line: 'N', equal to a negative result. Case 2: in the presence of enzymatic activity, the hydrolysed cefotaxime is not recognized by the mAbs, which are able to react with the cefotaxime immobilized on the test line. A signal appears on the test line and the control line: 'P', defined as a positive result, when cephalosporinase-expressing strains are present. The associated number of 'P' is relative to the increasing intensities observed on the test line

30 min at RT. The membrane was washed twice with deionized water and then dried for 1 h at 37°C in an air oven. After the absorption pad and the sample pad were fixed to the top and bottom of the membrane, respectively, the card was cut into strips 5 mm in width using an automatic programmable cutter (CM4000 Guillotine Cutting System; BioDot). Each mAb was evaluated in the LFIA format using serial dilutions of cefotaxime and hydrolysed cefotaxime (1000, 100, 10, 1 and 0 ng ml^{-1}) in extraction buffer. One hundred μl of this solution was mixed with 10 μl of the conjugate antibody with colloidal gold (tracer) before dipping the strip.

In this experiment, the aim is to identify the antibody among those selected for which the signal is maximal whatever the concentration of hydrolysed cefotaxime and absent for the lowest concentration of intact cefotaxime. Using the selected antibody, several optimizations were made. First, the tracer was dried on a conjugate pad, which is placed between the sample pad and the nitrocellulose membrane. Different amounts of tracer were tested with a defined concentration of cefotaxime. The $OD_{600\ nm}$ is 1.75, which is the amount of tracer before an apparition of signal on the test line. Second, the concentration of intact cefotaxime in the sample to inhibit any signal on the test was optimized. To obtain the best competition, the minimum concentration of intact cefotaxime to inhibit any signal on the test line must be found. Different amounts of cefotaxime were tested with the amount of tracer fixed. The 25 ng ml^{-1} concentration was selected. Finally, different amounts of BSA–cefotaxime on the test line were optimized: 1, 0.6, 0.3, 0.1 and 0.03 mg ml^{-1}. The 0.1 mg ml^{-1} was selected with not lose of signal when all the amount of tracer fix the test line.

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FOR the validation with isolated colonies, the tests were manufactured by NG Biotech. A control line was added on the strip by immobilizing antibodies recognizing the labelled monoclonal antibody (Fig. 2), and the strips were enclosed in a plastic cassette to facilitate execution of the test.

Test validation

Three hundred and forty-eight GNB isolates with PCR-characterized β-lactamase content were tested for the validation of the LFIA-CTX test. This collection included 38 isolates unable to hydrolyse cefotaxime, as revealed by routine antibiotic susceptibility testing (fully susceptible bacteria) and whole-genome sequencing used as negative controls (see Table 1), and 310 isolates able to hydrolyse cefotaxime (see Table 2). One single colony of these bacteria grown on MH plates was resuspended in an extraction buffer supplemented with cefotaxime and incubated for 30 min at room temperature (between 20 and 25°C), prior to loading on the LFIA-CTX test. The test was read by eye after 10 min of migration. The ESC (cefotaxime) hydrolytic activities are identified by associating the positive result with a signal on the test line.

Isolates unable to hydrolyse cefotaxime

None of the 38 negative control isolates gave a positive signal (Table 1). These isolates corresponded to 21 Enterobacterales that were either wild-type (WT) or expressed various BLs unable to hydrolyse cefotaxime (low-level cAmpC, OXA-23, OXA-58, IMI-like, SME-like, narrow-spectrum penicillinases [TEM, CHV], CTX-M-93), 12 *Pseudomonas* spp. (WT), impermeability mutants, narrow-spectrum β-lactamase producers (CARB-4, OXA-32) poorly expressed ESBLs (PME-1) and carbapenemases lacking ESC hydrolytic activity (OXA-198, AIM-1) and 5 *Acinetobacter baumannii* either WT or expressing narrow-spectrum enzymes (RTG-4, OXA-13 and OXA-21).

LFIA-CTX test results with known cefotaxime-hydrolysing enzymes

Of the 310 isolates expressing at least one cefotaxime-hydrolysing β-lactamase, all were tested positive, except three OXA-48-like producers alone that were repeatedly detected negative (Table 2). These isolates belonged to Enterobacterales ($n = 209$), *P. aeruginosa* ($n = 50$) and *A. baumannii* ($n = 51$). All overexpressed AmpCs, ESBLs and carbapenemases can be detected by the test. All CTX-Ms were detected, as well as all the different carbapenemases known to hydrolyse ESCs (KPC, NDM, VIM and IMP). Even OXA-48 producers were detected, except three of them. However, the tested isolates contained all either an ESBL, a pAmpC or another carbapenemase. Likewise, *A. baumannii* producing OXA-23, OXA-24, OXA-40, OXA-51, OXA-58, OXA-66, OXA-72, OXA-97, OXA-143 and OXA-253 were efficiently detected, as a consequence of the natural cAmpC present in these isolates.

Test performance on OXA-48-like producers alone

OXA-48 has moderate hydrolytic activity for cefotaxime (Docquier *et al*., 2009; Oviaño *et al*., 2019; Oueslati *et al*., 2020). In order to evaluate the property of OXA-48-like enzymes alone to hydrolyse cefotaxime in our assay, we tested 4 K. *pneumoniae* isolates producing OXA-48 along with its chromosomally encoded broad-spectrum SHV enzyme and 13 *E. coli* producing either OXA-48 (5), OXA-181 (4), OXA-244 (3) or OXA-484 (1). Three out of the 4 OXA-48-producing *K. pneumoniae* were detected, and 11 out of the 13 OXA-48-like *E. coli* producers were detected (OXA-48 (5/5), OXA-181 (3/4), OXA-244 (2/3) and OXA-484 (1/1)). Thus, the LFIA-CTX test was able to detect 82% of the OXA-48 producers in which OXA-48 was the only acquired ß-lactamase.

Table 1. LFIA-CTX test results with negative control isolates.

	Acquired enzymes	Positive results
Enterobacterales ($n = 21$)		0/21
Escherichia coli ($n = 5$)	WT (4); CTX-M-93	0/5
Proteus mirabilis ($n = 3$)	WT (1); OXA-58; OXA-23	0/3
Citrobacter freundii ($n = 1$)	WT (1)	0/1
Enterobacter spp. ($n = 6$)	WT (2); IMI-1/2/17 (4)	0/6
Salmonella spp. ($n = 4$)	WT (4)	0/4
Serratia marcescens ($n = 2$)	SME-1/2 (2)	0/2
Pseudomonas spp. ($n = 12$)	WT (5); Overexpressed efflux pumps (2); CARBA-4; OXA-32; OXA-198; PME-1; AIM-1	0/12
Acinetobacter baumannii ($n = 5$)	WT (2); RTG-4; OXA-13; OXA-21	0/5

Table 2. LFIA-CTX test results with isolates having known cefotaxime-hydrolysing enzymes.

	Acquired enzymes	Positive results
Enterobacterales (n = 209)		206/209
AmpCs (n = 17)	Overexpressed AmpC (12), DHA-1/2 (2); ACC-1 (2); CMY-136	17/17
Broad-spectrum penicillinases (n = 2)	SHV-11/38 (2)	2/2
ESBLs (n = 75)	SHV-2/2a/12/36 (5); TEM-3/24/52 (6); CTX-M-1/2/3/8/10/14/15/17/18/19/24/27/32/37/55/57/65/71/82/100/101/182 (58); GES-1/6 (2); OXA-48-like (4)	75/75
Carbapenemases (n = 46)	KPC-2/3 (9); NDM-1/7/9/19 (5); IMP-1/8/14 (3); VIM-2 (2); OXA-48-like (17); TMB-1; GIM-1; FRI-1; GES-5; NDM-1 + VIM-2; KPC-28 + OXA-48; KPC-4 + NDM-7; KPC + VIM; VIM-4 + OXA-48; NDM-5 + VIM-1 + OXA-181	43/46
AmpCs + ESBLs (n = 6)	Overexpressed AmpC + TEM-3 (2); overexpressed AmpC + CTX-M-15; DHA-2/1 + SHV-2/75 (2); CTX-M-13 + CMY-2;	6/6
AmpCs + carbapenemases (n = 10)	CMY-16 + NDM-1; CMY-2/13 + VIM-1/4 (2); CMY-2/4 + OXA-48-like (2); CMY-150 + LMB-1; CMY-135 + MOX-9 + OXA-372; overexpressed AmpC + IMI-3; overexpressed AmpC + NMCA; overexpressed AmpC + SME-2	10/10
ESBLs + carbapenemases (n = 46)	SHV-5/70 + VIM-1 (3); SHV-5/12 + IMP-1/8/10 (5); SHV-5/11/12 + OXA-48 (3); CTX-M-15 + GES-5; CTX-M-1/15 + OXA-48-like (19); CTX-M-15 + KPC-2/3 (2); CTX-M-15 + NDM-1/6/19 (4); CTX-M-3 + VIM-19; CTX-M-15 + NDM-4 + KPC-2; CTX-M-15 + NDM-1/5 + OXA-48-like (6); CTX-M-15 + VIM-1 + OXA-48	46/46
AmpCs + ESBLs+ carbapenemases (n = 7)	CTX-M-15 + CMY-2/6 + NDM-1/4 (2); CTX-M-15 + CMY-2/4/48 + OXA-48-like (5)	7/7
Pseudomonas spp. (n = 50)		50/50
ESBLs (n = 10)	SHV-2a/5 (2); TEM-4; GES-2/9 (2); PER-1/2 (2); CTX-M-2; BEL; VEB-1	10/10
Carbapenemases (n = 36)	GES-5 (1); SPM-1; KPC-2 (4); NDM-1 (2); VIM-1/2/4 (9); IMP-1/2/7/13/15/19/26/29/31/39/46/56/63/71 (18); DIM-1	36/36
AmpCs + impermeability (n = 3)	Overexpressed AmpC + porin deficiency + overexpressed efflux pumps (3)	3/3
Broad spectrum penicillinase + carbapenemases (n = 1)	OXA-2 + OXA-395 + GIM-1	1/1
Acinetobacter spp. (n = 51)		51/51
AmpCs (n = 1)	Overexpressed AmpC	1/1
ESBLs (n = 18)	SHV-5 (2); GES-11/12/14 (10); PER-1; CTX-M-15; SCO-1; VEB-1; OXA-14; VEB-1 + OXA-10	18/18
Carbapenemases (n = 16)	SIM-1; NDM-1/2 (5); IMP-1/4 (3), VIM-1/4 (2); NDM-1 + OXA-23 (5)	16/16
AmpCs + ESBLs (n = 1)	Overexpressed AmpC + OXA-18/20	1/1
AmpCs + carbapenemases (n = 13)	Overexpressed AmpC + OXA-23 (5); overexpressed AmpC + OXA-51-like (2); overexpressed AmpC + OXA-58-like (2); overexpressed AmpC + OXA-143-like (2); overexpressed AmpC + OXA-24-like (2)	13/13
AmpCs + ESBLs + carbapenemases (n = 2)	Overexpressed AmpC + GES-11/14 + OXA-23 (2)	2/2

Overall performance

The results obtained during this validation of the LFIA-CTX test revealed a sensitivity of 99.1% and a specificity of 100%. Culture on chromogenic media such as URISe-lectTM Agar 4 (Bio-Rad, Marnes-La-Coquette, France) or LB Agar (Sigma-Aldrich, St. Quentin Fallavier Cedex, France) did not alter the test results. Of the 348 isolates tested, 44 of them expressed at least one OXA-48-like equivalent to 12.6% of isolates. In 61.3% of the cases, they were at least associated with an overexpressed cAmpC, a pAmpC, an ESBL or a carbapenemase (27/44). The number of isolates that expressed OXA-48 as the only ß-lactamase was high in this study with 17/44 isolates. Nevertheless, the LFIA-CTX test was able to detect 82% of them, even though cefotaxime hydrolysis is low (14/17). It is worth noting that the proportion of the different β-lactamases in this study do not reflect the prevalence of β-lactamases in clinical samples, and in particular those of OXA-type β-lactamases. Indeed, the prevalence of OXA-48-like producers alone is very low in clinical samples. If our results are mitigated with the actual prevalence of the different isolates, the sensitivity of the test reaches nearly 100%. Detailed results are available in Appendix S1.

Discussion

The spread of GNB isolates expressing BLs capable of hydrolysing ESC, such as cephalosporinases (cAmpC and pAmpC), ESBLs and a large proportion of carbapen-emases, is associated with a major public health and economic burden. The rapid identification of these iso-lates is essential not only to adapt treatment in the case of an infection but also to prevent their spread in the hospital environment. The earlier the enzyme activity is detected, the more effective is the treatment and the likelihood of nosocomial spread is reduced (Thomson,

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2010). In this sense, the LFIA-CTX test allows the detection of BLs capable of hydrolysing ESCs such as cefotaxime. After 30 min of incubation, positive results were interpretable within 10 min of migration. All the BLs in this study known to hydrolyse cefotaxime were correctly detected. No false positives and three false negatives were obtained. As a result, the analytical performance of the LFIA-CTX test reached a sensitivity of 99.1% and a specificity of 100%.

The choice of cefotaxime as substrate for the test LFIA-CTX was dictated by the fact that it is hydrolysed by most AmpCs, ESBLs and a large proportion of carbapenemases. This contrasts with ceftazidime, another expanded-spectrum cephalosporin, which is not hydrolysed by all of CTX-M type ESBLs (large class of ESBLs).

The LFIA-CTX test was validated on Enterobacterales (n = 230), *Pseudomonas* spp. (n = 62) and *Acinetobacter* spp. (n = 56). Despite the natural resistance to ESC by *Pseudomonas* spp. and *Acinetobacter* spp (Lupo *et al*., 2018) due to impaired outer membrane, their β-lactamase resistance could be detected. As a result, this test detects any significant hydrolytic activity leading to enzyme-mediated resistance, even making it possible to differentiate between basal levels of AmpC expression (WT) and increased levels of AmpC (as a consequence of overexpressed cAmpC or plasmid-encoded AmpC). These increased levels of enzymes may lead to hydrolysis of expanded-spectrum cephalosporins such as cefotaxime. This selectivity allows the distinction between sensitive bacteria that naturally produce AmpC and the resistant bacteria that overproduce AmpC (Jacoby, 2009).

The most worrying BLs are ESBLs and carbapenemases. The majority of ESBLs can be divided into four groups: TEM, SHV, CTX-M and minor ESBLs (GES, PER, VEB,...; Naas *et al*., 2008). Most TEM-ESBLs are derived from the penicillinase TEM-1. TEM-ESBL variants are usually more active against ceftazidime than cefotaxime or ceftriaxone. TEM-10 and TEM-26 are still highly prevalent in the United States but seem to be replaced by CTX-Ms in many European countries (Villegas *et al*., 2004). SHV-type ESBLs are all derived by point mutations from the narrow-spectrum penicillinase SHV-1 (Naas *et al*., 2017). Today, SHV-5 and SHV-12 are among the most represented members of this family (Bauernfeind *et al*., 1992), but have also been outcompeted by CTX-M enzymes.

CTX-M type ESBLs presently comprise known 246 enzymes (Naas *et al*., 2017). These enzymes are more widespread than the classic TEM- and SHV-type ESBLs, with CTX-M-14 and CTX-M-15 being the most common variants (Govinden *et al*., 2007). Minor ESBLs, such as the GES-type ESBLs, have emerged from the shadows to become a recognized resistance threat (Weldhagen, 2006). GES-type ESBLs have now spread worldwide, especially in *P. aeruginosa*, where detection is very difficult, as GES enzymes are not targeted by commercially available molecular assays. In this study, a wide variety of SHV, TEM, CTX-M and GES variants was tested, which is not representative of the prevalence that may be observed in different countries. Nevertheless, the LFIA-CTX test was capable of detecting most ESBLs expressed in Enterobacterales, *Pseudomonas* spp. (except PME-1) and *Acinetobacter* spp. (except RTG-4).

PME-1 and RTG-4 have the particularity of hydrolysing cefotaxime or ceftazidime very weakly, unlike cefepime, which is strongly hydrolysed by these two ESBLs (Potron *et al*., 2009; Tian *et al*., 2011). ESBLs that have low hydrolysis for cefotaxime or are associated with other resistance mechanisms (i.e. impermeability) could be detected. Also, when broad-spectrum penicillinases are overexpressed, the test is able to differentiate them from those expressed at a basal level, such as CTX-M-93 (loss of activity for cefotaxime due to a mutation), CARB-4, OXA-13/21/13.

The LFIA-CTX test also allowed detection of some carbapenemases, e.g. KPC-like, MBLs and even of OXA-48, which has a moderate hydrolytic activity for cefotaxime (Oueslati *et al*., 2015). In more than 80% of the cases, OXA-48 is associated with ESBLs that will hydrolyse cefotaxime efficiently (https://maquette.cnr-resistance-antibiotiques.fr/ressources/pages/Rapport_CNR_2018v1.pdf, T. Naas, personal communication). For the remaining, 20% the outcome of the LFIA-CTX test is unpredictable. Indeed, 3 of 17 OXA-48 producers tested yielded a negative result. Previous studies showed that the catalytic activity (Kcat/Km) of OXA-48 for cefotaxime is moderate, whereas it is practically null for ceftazidime or cefepime (Docquier *et al*., 2009; Oviaño *et al*., 2019; Oueslati *et al*., 2020). This is the case for most OXA-48 variants, except OXA-405, OXA-247 and OXA-163 that hydrolyse strongly ESCs, but lack carbapenemase activity (Oueslati *et al*., 2020). This inconsistent detection probably reflects different expression levels and activities at the limit of detection of the assay. These results are in line with in vivo experiments in mice that evidenced effective therapy with ceftazidime against OXA-48-producing Enterobacterales lacking ESBLs or AmpC-like ß-lactamases, whereas the therapy with cefotaxime had little impact in reducing lethality of the rodents (Mimoz *et al*., 2012; Wiskirchen *et al*., 2013). Also, some carbapenemases were not detected because of their lack of expanded-spectrum hydrolysing activity (including cefotaxime) such as IMI-1/2/17, SME-1/2, AIM-1 and OXA-198, which are still relatively rare.

There are currently several other tests for the detection of ESC-hydrolysing BLs, such as the ESBL NDP test

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(Nordmann *et al.*, 2012) and β-lacta test (Renvoisé *et al.*, 2013), which are sometimes difficult to interpret, MALDI-TOF-based assays, which require expensive equipment and kits, and UV spectrophotometry, which is highly complex and reserved for reference laboratories. These different assays have drawbacks that can be overcome by the LFIA-CTX test (Váradi *et al.*, 2017; Bernabeu *et al.*, 2020). Indeed, the LFIA-CTX test is fast, low-cost and easy to use in a routine workflow of a clinical microbiology laboratory for the detection of BLs that hydrolyse ESCs. Moreover, unlike tests based on a colour change (ESBL NDP test and β-lacta test), LFIA-CTX is based on the easily interpretable appearance of a signal. It can also be used in other fields such as the food industry, animal medicine and environmental studies.

Thus, this test is a new tool that further demonstrates the usefulness of LFIA for the detection of antibiotic resistance. This innovative test format will allow the development of tests with new features. Indeed, we observed that anti-cefotaxime antibodies did not recognize other antibiotics, and by producing antibodies specific to other antibiotics, we could therefore develop tests allowing simultaneous detection of different lactamase activities. It is also possible to carry out a test allowing the detection on the same strip of β-lactamase activity associated with the identification of enzymes responsible for this activity to have a unique test.

Experimental procedures

Ethics statement

All experiments were performed in compliance with French and European regulations on the care of laboratory animals (European Community Directive 86/609, French Law 2001-486, 6 June 2001) and with the agreements of the Ethics Committee of the Commissariat à l'Energie Atomique (CEtEA 'Comité d'Ethique en Experimentation Animale' n°44) nos. 12-026 and 15-055 delivered to S. S. by the French Veterinary Services and CEA agreement D-91-272-106 from the Veterinary Inspection Department of Essonne (France).

Methods

A brief description was given in the first part of the results. Full details of the methods used are available in Appendix S2.

Conflict of interest

A patent has been filed (B252171FR D41054) to protect this invention, in the name of three inventors: Dr. Hervé Volland, Université Paris-Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SPI, 91191 Gif-sur-Yvette, France; M. Christian Moguet, Université Paris-Saclay, CEA, INRAE, Département Médicaments et Technologies pour la Santé (DMTS), SPI, 91191 Gif-sur-Yvette, France; and Dr. Thierry Naas, Team Resist, UMR1184, Université Paris-Saclay-INSERM-CEA, LabEx Lermit, Associated French National Reference Center for Antibiotic Resistance: Carbapenemase-producing Enterobacterales, Le Kremlin-Bicêtre, France.

Author contributions

H.V., S.S. and T.N. conceptualized the study. A.S., S.G., C.G. and C.M. contributed to methodology. C.G. and C.M. performed validation. C.G., T.N. and C.M. performed formal analysis. H.V., T. N., C.G. and C.M. performed investigation. T.N., H.V. and S.S. provided resources. H.V., T.N. and C.M. wrote the original draft preparation. T.N., H.V., S.S. and X.X. underwent supervision. H.V., S.S. and T.N contributed to project administration. H.V., S.S. and T.N performed funding acquisition. All authors wrote, reviewed and edited the manuscript, and have read and agreed to the published version of the manuscript.

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Supporting information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Results for 348 isolates in the validation of the LFIA-CTX-test.

Appendix S2. Steps for the test development.

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