Semirapid Detection of Piperacillin/Tazobactam Resistance and Extended-Spectrum Resistance to β-Lactams/β-Lactamase Inhibitors in Clinical Isolates of Escherichia coli

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ABSTRACT Piperacillin/tazobactam (TZP) is a β-lactam/β-lactamase inhibitor (BL/BLI) recommended for the empirical treatment of severe infections. The excessive and indiscriminate use of TZP has promoted the emergence of TZP-resistant Escherichia coli isolates. Recently, we demonstrated that TZP may contribute to the development of extended-spectrum resistance to BL/BLI (ESRI) in E. coli isolates that are TZP susceptible but have low-level resistance to BL/BLI (resistance to amoxicillin/clavulanic acid [AMC] and/or ampicillin/sulbactam [SAM]). This raises the need for the development of rapid detection systems. Therefore, the objective of this study was to design and validate a method able to detect TZP resistance and ESRI in E. coli. A colorimetric assay based on β-lactam ring hydrolysis by β-lactamases was designed (ESRI test). A total of 114 E. coli isolates from bloodstream and intra-abdominal sources, characterized according to their susceptibility profiles to BL/BLI, were used. Detection of the three most frequent β-lactamases involved in BL/BLI resistance (bla_{TEM}, bla_{OXA-1}, and bla_{SHV}) was performed by PCR. The ESRI test was able to detect all the TZP-intermediate/-resistant isolates, as well as all the TZP-susceptible isolates with a capacity for ESRI development. Their median times to results were 5 and 30 min, respectively. All the isolates without resistance to BL/BLI displayed a negative result in the ESRI test. bla_{TEM} was the most frequent β-lactamase gene detected, followed by bla_{SHV} and bla_{OXA-1}. These results demonstrate the efficacy of the ESRI test, showing great clinical potential which could lead to reductions in health costs, ineffective treatments, and inappropriate use of BL/BLI.

IMPORTANCE TZP is a BL/BLI recommended for the empirical treatment of severe infections. The excessive use of TZP has promoted the emergence of TZP-resistant Escherichia coli isolates. We recently reported that TZP may contribute to the development of ESRI in E. coli isolates that are TZP susceptible but have low-level resistance to BL/BLI. This raises the need for the development of rapid detection systems. Here, we demonstrated that the ESRI test was able to detect the TZP-intermediate or -resistant isolates and the TZP-susceptible isolates with the capacity for ESRI development. All the isolates without BL/BLI resistance were negative for the ESRI test and did not harbor β-lactamase genes. For ESRI developers and TZP-intermediate or -resistant isolates, bla_{TEM} was the most frequent β-lactamase gene detected, follow by bla_{SHV} and bla_{OXA-1}. The sensitivity, specificity, and positive and negative predictive values were all 100%. These data demonstrate the efficacy of the ESRI test and show that it has great clinical potential.

KEYWORDS ESRI, Escherichia coli, beta-lactamase, beta-lactamase inhibitor, piperacillin, resistance, tazobactam
Excessive and indiscriminate use of antibiotics has accelerated the emergence and spread of antimicrobial resistance and increased the inefficacy of available antimicrobial treatments. Within the wide range of bacterial species able to produce infection in humans, the order Enterobacterales holds a prominent place, with Escherichia coli being the species with the highest clinical relevance (1). Among the antimicrobials used for the treatment of infections by E. coli, we found a group of β-lactams combined with β-lactamase inhibitors (BL/BLI), including ampicillin/sulbactam (SAM), amoxicillin/clavulanic acid (AMC), and piperacillin/tazobactam (TZP). The latter is efficacious against E. coli and other Gram-negative bacteria producing β-lactamases (mainly TEM enzymes) (2).

TZP is used especially in severe infections and health care-associated infections (3, 4). However, the abusive use of this antibiotic has led to the appearance of resistant strains (5, 6).

In Spain, data extracted from the Study for Monitoring Antimicrobial Resistance Trends (SMART) from 2002 to 2010 and 2016 to 2017 revealed an increase in the rate of TZP resistance in intra-abdominal E. coli isolates, from 7.7% to 10.0% (7, 8). A similar increase was observed in England between 2011 and 2015 in bacteremic E. coli isolates (9).

The presence of TEM, inhibitor-resistant TEM (IRT), OXA-1, SHV, and/or AmpC β-lactamases is a common cause of E. coli resistance to BL/BLI (10–14). Of these, TEM-1 hyperproduction and IRT are the most frequent mechanisms involved in the resistance to BL/BLI, especially to SAM and AMC and, in some cases, to TZP (15, 16).

In E. coli, the pattern of BL/BLI resistance is a gradual and unidirectional process that extends from SAM, to TZP, through AMC (17). Thus, one isolate may be resistant to SAM but susceptible to AMC and TZP (RSS phenotype), resistant to SAM and AMC but susceptible to TZP (RRS phenotype), or resistant to all three combinations (RRR phenotype) (17). Recently, we demonstrated both in vitro and in patients with intra-abdominal infections that E. coli isolates with the RSS or RRS phenotype, classified as having a low level of resistance to BL/BLI, and carrying the blaTEM gene, are able to acquire stable resistance to TZP and to the other two combinations if they are exposed to TZP at sub-MIC levels, indicating that extended-spectrum resistance to BL/BLI (ESRI), from SAM (RSS) to TZP (RRR), can be developed (17). Early detection of both resistance to TZP and the ability to develop ESRI in E. coli isolates with low-level resistance to BL/BLI (RRS or RRS) is essential to establish adequate initial and early antimicrobial treatment of severe infections, the moment in which the TZP treatment has special relevance. In clinical practice, when E. coli is isolated in a clinical sample, the Microbiology Service of hospital system reports only the susceptibility or resistance to the different BL/BLI, among other antimicrobials, but not the potential development of resistance to those agents. Therefore, there is no system that allows us to detect this possible ESRI when E. coli isolates present low-level resistance to BL/BLI (RSS or RRS). In this study, we developed a semirapid detection system to detect resistance to TZP and the potential development of ESRI in clinical isolates of E. coli (Fig. 1).

RESULTS

Antimicrobial susceptibility in clinical isolates of E. coli. Susceptibility profiles to SAM, AMC, and TZP obtained through MicroScan were analyzed, allowing the selection of 114 E. coli clinical isolates with different profiles (susceptible to all three drug combinations [SSS], RSS, RRS, and RRR) in order to develop and validate the ESRI test (Tables 1 to 3). On the other hand, the TZP MICs obtained by broth microdilution classified 67 (58.7%) of the 114 E. coli isolates as susceptible, 6 (5.2%) as intermediate, and 41 (35.9%) as resistant to TZP, according to the breakpoint established by CLSI (18). The use of MicroScan and the standard broth microdilution method showed the same TZP susceptibility profiles for the majority of clinical isolates. However, some exceptions were observed: the C1-8 (RRR) and C1-102 (RRR) isolates presented MICs of 4 mg/liter and were classified as susceptible (Table 2), while the C1-136 (RRS), C1-166 (RSS), and...
C1-189 (RSS) isolates showed MICs of >512, >512, and 128 mg/liter, respectively, and were classified as resistant (Table 3).

**Detection of ESRI.** The 67 isolates classified as susceptible to TZP were exposed to TZP pressure in order to reveal any ability to develop ESRI. Of these, 22 (32.8%) did not develop ESRI (Table 1), maintaining their TZP MICs or increasing their MICs slightly but without becoming resistant. Of the remaining isolates, 45 (67.2%) were able to develop ESRI, increasing their MICs to TZP at least 8-fold (Table 2).

**Detection and sequencing of ß-lactamases.** None of the *bla*TEM, *bla*OXA-1, and *bla*SHV genes were detected in the 22 isolates that were susceptible to TZP and without the ability to develop ESRI (Table 1). However, of the 45 isolates susceptible to TZP and with the ability to develop ESRI, *bla*TEM, *bla*OXA-1, and *bla*SHV genes were detected in 38 (84.4%), 1 (2.2%), and 6 (13.3%) isolates, respectively. Simultaneous detection of *bla*TEM and *bla*SHV was found in four isolates (8.8%). However, there were two *E. coli* isolates

### TABLE 1 Clinical isolates of Escherichia coli that were non-ESRI developers and had negative results in the ESRI test

| Isolate (n = 22) | Origin | BL/BLI resistance profilea | TZP MIC (mg/liter)b | Resistance mechanismc | ESRI testd | Result at 6–8 h of growth | Time to result (min) | Result at 24 h of growth | Time to result (min) | TYP pressure testd | New TZP MIC (mg/liter) |
|------------------|--------|----------------------------|---------------------|----------------------|------------|---------------------------|---------------------|------------------------|---------------------|-------------------|----------------------|
| C1-74 Blood      | RRS    | 16                         | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 32                   |
| C1-83 Blood      | RRS    | 8                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 8                    |
| C1-90 Blood      | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 2                    |
| C1-95 Blood      | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 8                    |
| C1-97 Blood      | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 16                   |
| C1-100 Blood     | SSS    | 2 to 4                     | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-110 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-111 Blood     | SSS    | 2 to 4                     | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 8                    |
| C1-112 Blood     | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 16                   |
| C1-113 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-114 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-146 Blood     | SSS    | 2 to 4                     | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-152 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-155 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-157 Blood     | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-168 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-169 Blood     | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-172 Blood     | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C1-177 Blood     | SSS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |
| C2-4 Bile        | SSS    | 2                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 2                    |
| C2-8 Bile        | SSS    | 8                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 8                    |
| C2-12 Bile       | RRS    | 4                          | Neg                 | --                   | >120       | --                        | >120                 | --                     | --                  | --                | 4                    |

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aSSS, susceptible to ampicillin/sulbactam, amoxicillin/clavulanic acid, and piperacillin/tazobactam; RSS, resistant to ampicillin/sulbactam and susceptible to amoxicillin/clavulanic acid and piperacillin/tazobactam; RRS, resistant to ampicillin/sulbactam and amoxicillin/clavulanic acid and susceptible to piperacillin/tazobactam.

bTZP, piperacillin/tazobactam.

cNeg, negative result in relation to studied resistance mechanisms (TEM type, OXA-1 type, and SHV type).

d−, negative result in ESRI detection or antimicrobial pressure test.
TABLE 2 Clinical isolates of *Escherichia coli* that were ESRI developers and had positive results in the ESRI test

| Isolate (n = 45) | Origin | BL/BLI resistance profilea | TZIP MIC (mg/liter)b | Resistance mechanismc | Result at 6–8 h of growth (min) | Time to result (min) | Result at 24 h of growth (min) | Time to result (min) | TZIP pressure testd (mg/liter) | New TZIP MIC (mg/liter) |
|------------------|--------|---------------------------|---------------------|----------------------|-------------------------------|-------------------|-------------------------------|-------------------|-------------------------------|----------------------|
| C1-8             | Blood  | RRR 4                      | Neg                 | +                    | 25                             | 60                | +                             |       | +                             | 256                  |
| C1-31            | Blood  | RRS 8                      | TEM-40              | +                    | 25                             | 60                | +                             |       | +                             | 64                   |
| C1-38            | Blood  | RRS 16                     | TEM-35              | +                    | 1                              | 1                 | +                             |       | +                             | 256                  |
| C1-48            | Blood  | RRS 4                      | TEM-1               | +                    | 20                             | 30                | +                             |       | 128                           |
| C1-65            | Blood  | RRS 8                      | TEM-1               | +                    | 60                             | 60                | +                             |       | 512                           |
| C1-72            | Blood  | RRS 16                     | TEM-1               | +                    | 30                             | 60                | +                             |       | 512                           |
| C1-81            | Blood  | RRS 16                     | TEM-30              | +                    | 2                              | 1                 | +                             |       | 512                           |
| C1-91            | Blood  | RRS 1                       | Neg                 | +                    | 40                             | 20                | +                             |       | 256                           |
| C1-93            | Blood  | RSS 2                       | SHV                 | +                    | 20                             | 10                | +                             |       | 512                           |
| C1-99            | Blood  | RSS 8                       | TEM-1               | +                    | 40                             | 30                | +                             |       | >512                          |
| C1-102           | Blood  | BRR 4                       | TEM-1               | +                    | 1                              | 2                 | +                             |       | 32                             |
| C1-103           | Blood  | RSS 8                       | TEM-1               | +                    | 40                             | 100               | +                             |       | 512                           |
| C1-106           | Blood  | RSS 8                       | TEM-1               | +                    | 40                             | 25                | +                             |       | 512                           |
| C1-108           | Blood  | RSS 2                       | TEM-1               | +                    | 40                             | 30                | +                             |       | 512                           |
| C1-118           | Blood  | RRS 16                      | OXA-1               | +                    | 30                             | 10                | +                             |       | 256                           |
| C1-120           | Blood  | RRS 8                       | TEM-1               | +                    | 40                             | 20                | +                             |       | >512                          |
| C1-121           | Blood  | RRS 8                       | TEM-1/SHV           | +                    | 30                             | 30                | +                             |       | 512                           |
| C1-126           | Blood  | RRS 8                       | TEM-1               | +                    | 40                             | 25                | +                             |       | 256                           |
| C1-129           | Blood  | RRS 16                      | TEM-1               | +                    | 40                             | 25                | +                             |       | >512                          |
| C1-130           | Blood  | RRS 8                       | TEM-1               | +                    | 40                             | 25                | +                             |       | 256                           |
| C1-137           | Blood  | RRS 8                       | TEM-1               | +                    | 40                             | 25                | +                             |       | >512                          |
| C1-139           | Blood  | RRS 8                       | TEM-1               | +                    | 30                             | 120               | +                             |       | >512                          |
| C1-141           | Blood  | RRS 4                       | TEM-1/SHV           | +                    | 30                             | 10                | +                             |       | >512                          |
| C1-142           | Blood  | RRS 4                       | TEM-1/15/SHV        | +                    | 10                             | 10                | +                             |       | 512                           |
| C1-143           | Blood  | RRS 8                       | TEM-1               | +                    | 30                             | 60                | +                             |       | >512                          |
| C1-153           | Blood  | RRS 8                       | TEM-1/SHV           | +                    | 50                             | 90                | +                             |       | >512                          |
| C1-159           | Blood  | RRS 2                       | TEM-1               | +                    | 100                            | 30                | +                             |       | 512                           |
| C1-160           | Blood  | RRS 4                       | TEM-1               | +                    | 50                             | 60                | +                             |       | 512                           |
| C1-161           | Blood  | RRS 16                      | TEM-1               | +                    | 100                            | 60                | +                             |       | >512                          |
| C1-162           | Blood  | RSS 4                       | Neg                 | +                    | 30                             | 30                | +                             |       | 512                           |
| C1-164           | Blood  | RSS 8                       | TEM-1               | +                    | 30                             | 30                | +                             |       | 512                           |
| C1-170           | Blood  | RSS 4                       | TEM-1               | +                    | 30                             | 60                | +                             |       | >512                          |
| C1-171           | Blood  | RRS 8                       | TEM-1               | –                    | >120                           | 40                | +                             |       | 512                           |
| C1-174           | Blood  | SSS 2                       | TEM-1               | +                    | 120                            | 120               | +                             |       | >512                          |
| C1-175           | Blood  | RRS 16                      | TEM-1               | +                    | 30                             | 60                | +                             |       | 512                           |
| C1-183           | Blood  | RRS 8                       | TEM-1               | +                    | 30                             | 40                | +                             |       | >512                          |
| C1-187           | Blood  | RRS 2                       | TEM-1               | +                    | 100                            | 40                | +                             |       | >256                          |
| C1-190           | Blood  | RRS 16                      | SHV                 | –                    | >120                           | 120               | +                             |       | >512                          |
| C1-191           | Blood  | RRS 8                       | TEM-1               | +                    | 60                             | 100               | +                             |       | 256                           |
| C1-306           | Blood  | RRS 16                      | TEM-1               | +                    | 30                             | 30                | +                             |       | 128                           |
| C2-14            | Bile   | RRS 8                       | TEM-1               | +                    | 10                             | 10                | +                             |       | 512                           |
| C2-47            | Intra-abdominal abscess      | RSS 8               | TEM-1               | +                    | 20                             | 30                | +                             |       | 512                           |
| C2-49            | Peritoneal fluid            | RSS 8               | TEM-1               | +                    | 15                             | 30                | +                             |       | 512                           |
| C2-54            | Intra-abdominal abscess      | RSS 8               | TEM-1               | +                    | 15                             | 30                | +                             |       | 256                           |
| PT3             | Blood  | RRS 8                       | TEM-1               | +                    | 30                             | 50                | +                             |       | 256                           |

*a*SSS, susceptible to ampicillin/sulbactam, amoxicillin/clavulanic acid, and piperacillin/tazobactam; RRS, resistant to ampicillin/sulbactam and susceptible to amoxicillin/clavulanic acid and piperacillin/tazobactam; RRS, resistant to ampicillin/sulbactam and amoxicillin/clavulanic acid and susceptible to piperacillin/tazobactam; RRR, resistant to ampicillin/sulbactam, amoxicillin/clavulanic acid, and piperacillin/tazobactam.

bTZIP, piperacillin/tazobactam.

cNeg, negative result in relation to studied resistance mechanisms (TEM type, OXA-1 type, and SHV type).

dNew TZIP MIC, negative result in ESRI detection or antimicrobial pressure test; +, positive result in ESRI detection or antimicrobial pressure test.
| Isolate (n = 47) | Origin          | BL/BLI resistance profile | TZP MIC (mg/liter) | Resistance mechanism | ESRI test \(^d\) | Time to result (min) | Result at 24 h of growth | Time to result (min) |
|-----------------|-----------------|---------------------------|--------------------|---------------------|------------------|-----------------------|-------------------------|------------------------|
| C1-23 Blood     | RRR             | >256 TEM-1                | +                  | 2                   | +                | 10                    |                         |                        |
| C1-82 Blood     | RRS             | 32 TEM-1                 | +                  | 10                  | +                | 10                    |                         |                        |
| C1-94 Blood     | RRS             | 32 TEM-1                 | +                  | 15                  | +                | 10                    |                         |                        |
| C1-109 Blood    | RRR >256 SHV    | TEM-1/SHV                | +                  | 20                  | +                | 25                    |                         |                        |
| C1-116 Blood    | RSS >512 TEM-1  | TEM-1                    | +                  | 100                 | +                | 30                    |                         |                        |
| C1-189 Blood    | RSS 128 TEM-1   | TEM-1                    | +                  | 10                  | +                | 10                    |                         |                        |
| C1-239 Blood    | RRR >256 TEM-1  | TEM-1                    | +                  | 2                   | +                | 2                     |                         |                        |
| C1-436 Blood    | RRR >256 TEM-1  | TEM-1                    | +                  | 3                   | +                | 3                     |                         |                        |
| C2-23 Bile      | RRR >256 TEM-1  | TEM-1                    | +                  | 10                  | +                | 10                    |                         |                        |
| C2-45 Peritoneal fluid | RRR 256 TEM-1 | TEM-1                    | +                  | 10                  | +                | 60                    |                         |                        |
| C2-48 Intra-abdominal abscess | RRR >256 TEM-1 | TEM-1                    | +                  | 2                   | +                | 10                    |                         |                        |
| C2-74 Peritoneal fluid | RRR 256 TEM-1 | TEM-1                    | +                  | 5                   | +                | 3                     |                         |                        |
| C2-82 Hepatic abscess | RRR >256 TEM-1/SHV | TEM-1/SHV                | +                  | 3                   | +                | 3                     |                         |                        |
| C2-90 Peritoneal fluid | RRR 256 TEM-1 | TEM-1                    | +                  | 4                   | +                | 2                     |                         |                        |
| C2-95 Peritoneal fluid | RRR 64 TEM-1 | TEM-1                    | +                  | 15                  | +                | 15                    |                         |                        |
| C2-103 Peritoneal fluid | RRR 256 TEM-1 | TEM-1                    | +                  | 2                   | +                | 3                     |                         |                        |
| C2-106 Peritoneal fluid | RRR 128 OXA-1 | TEM-1/SHV                | +                  | 30                  | +                | 25                    |                         |                        |
| C2-113 Intra-abdominal abscess | RRR 256 TEM-1/SHV | TEM-1/SHV                | +                  | 2                   | +                | 3                     |                         |                        |
| C2-116 Intra-abdominal abscess | RRR 256 TEM-1 | TEM-1                    | +                  | 2                   | +                | 3                     |                         |                        |
| C2-117 Peritoneal fluid | RRR 128 TEM-12 | TEM-12                   | +                  | 5                   | +                | 3                     |                         |                        |
| C2-136 Peritoneal fluid | RRR 128 TEM-84 | TEM-1/SHV                | +                  | 5                   | +                | 1                     |                         |                        |
| C2-146 Peritoneal fluid | RRR >256 TEM-1 | TEM-1                    | +                  | 1                   | +                | 1                     |                         |                        |
| C2-147 Intra-abdominal abscess | RRR >256 TEM-1 | TEM-1                    | +                  | 3                   | +                | 1                     |                         |                        |
| PT4 Blood       | RRR             | 256 TEM-1                | +                  | 10                  | +                | 15                    |                         |                        |
| PTR1 Blood      | RRR 64 OXA-1    | TEM-1/SHV                | +                  | 10                  | +                | 5                     |                         |                        |
| PTR2 Blood      | RRR 256 TEM-1   | TEM-1/SHV                | +                  | 10                  | +                | 1                     |                         |                        |
| PTR3 Blood      | RRR 256 Neg     | TEM-1/SHV                | +                  | 10                  | +                | 1                     |                         |                        |
| PTR4 Blood      | RRR >256 Neg    | TEM-1/SHV                | +                  | 3                   | +                | 1                     |                         |                        |
| PTR5 Blood      | RRR >256 TEM-1  | TEM-1/SHV                | +                  | 3                   | +                | 1                     |                         |                        |
| PTR7 Blood      | RRR 256 Neg     | TEM-1/SHV                | +                  | 15                  | +                | 1                     |                         |                        |
| PTR8 Blood      | RRR 128 OXA-1   | TEM-1/SHV                | +                  | 30                  | +                | 2                     |                         |                        |
| PTR9 Blood      | RRR 128 TEM-1/1 OXA-1 | TEM-1/SHV                | +                  | 3                   | +                | 5                     |                         |                        |
| PTR10 Blood     | RRR >256 SHV    | TEM-1/SHV                | +                  | 1                   | +                | 1                     |                         |                        |
| PTR11 Blood     | RRR 128 TEM-1   | TEM-1/SHV                | +                  | 1                   | +                | 1                     |                         |                        |
| PTR12 Blood     | RRR 256 Neg     | TEM-1/SHV                | +                  | 3                   | +                | 1                     |                         |                        |
| PTR13 Blood     | RRR 256 TEM-1   | TEM-1/SHV                | +                  | 3                   | +                | 10                    |                         |                        |
| PTR14 Blood     | RRR 32 Neg      | TEM-1/SHV                | +                  | 50                  | +                | 40                    |                         |                        |
| PTR15 Blood     | RRR 128 OXA-1   | TEM-1/SHV                | +                  | 10                  | +                | 10                    |                         |                        |
| PTR16 Blood     | RRR 128 TEM-1   | TEM-1/SHV                | +                  | 15                  | +                | 15                    |                         |                        |
| PTR17 Blood     | RRR >256 TEM-1/1 OXA-1 | TEM-1/SHV                | +                  | 10                  | +                | 3                     |                         |                        |
| PTR18 Blood     | RRR 128 TEM-1   | TEM-1/SHV                | +                  | 10                  | +                | 10                    |                         |                        |
| PTR19 Blood     | RRR 64 OXA-1    | TEM-1/SHV                | +                  | 10                  | +                | 10                    |                         |                        |
| PTR20 Blood     | RRR >256 TEM-1  | TEM-1/SHV                | +                  | 10                  | +                | 3                     |                         |                        |

\(^a\)RSS, resistant to resistant to ampicillin/sulbactam and susceptible to amoxicillin/clavulanic acid and piperacillin/tazobactam; RRS, resistant to ampicillin/sulbactam and amoxicillin/clavulanic acid and susceptible to piperacillin/tazobactam; RRR, resistant to ampicillin/sulbactam, amoxicillin/clavulanic acid, and piperacillin/tazobactam.

\(^b\)TZP, piperacillin/tazobactam.

\(^c\)Neg, negative result in relation to studied resistance mechanisms (TEM type, OXA-1 type, and SHV type).

\(^d\)#, positive result in ESRI detection test.
with the ability to develop ESRI (4.4%) in which bla\textsubscript{TEM}, bla\textsubscript{OXA-1}, and bla\textsubscript{SHV} were not detected. Regarding the type of bla\textsubscript{TEM} detected, 34 isolates expressed TEM-1 and the other 4 isolates each expressed TEM-30, TEM-35, TEM-40, or TEM-135 (Table 2).

In the case of the 47 isolates that were intermediate or resistant to TZP, bla\textsubscript{TEM}, bla\textsubscript{OXA-1}, and bla\textsubscript{SHV} genes were detected in 34 (72.3%), 7 (14.9%), and 5 (10.6%) isolates, respectively. bla\textsubscript{TEM} was found together with bla\textsubscript{SHV} in three isolates and with bla\textsubscript{OXA-1} in two other isolates. There were six isolates (12.7%) in which bla\textsubscript{TEM}, bla\textsubscript{OXA-1}, and bla\textsubscript{SHV} were not detected. All the bla\textsubscript{TEM} genes detected expressed TEM-1, except for isolates C2-117 and C2-136, which expressed TEM-12 and TEM-84, respectively (Table 3).

**ESRI test validation.** All the isolates that were susceptible to TZP and without an ability to develop ESRI presented with a negative result in the ESRI test (Table 1). Regarding the isolates susceptible to TZP and potentially ESRI developers, 43 isolates (95.5%) gave a positive result for the ESRI test at 6 to 8 h of bacterial growth. The two isolates that gave a negative result for the ESRI test became positive at 24 h of bacterial growth (Table 2). Similarly, the isolates that were intermediate/resistant to TZP were 100% positive in the ESRI test, both at 6 to 8 and 24 h of bacterial growth (Table 3).

Statistically significant differences between the two groups (ESRI developer isolates and TZP-intermediate/-resistant isolates) were found in the times for positivity of the ESRI test. In the first group, the median times and interquartile ranges (IQRs) were 30 min (25 to 40 min) at 6 to 8 h of growth and 30 min (25 to 60 min) at 24 h of growth. However, in the second group, the median times and IQRs were 5 min (3 to 10 min) at 6 to 8 h ($P < 0.001$) and 3 min (2 to 10 min) at 24 h ($P < 0.001$) (Fig. 2). The sensitivity, specificity, positive predictive value (PPV), and negative predictive value

![FIG 2 Times of ESRI test positivity in TZP-resistant and ESRI developer E. coli isolates. S, susceptible; R, resistant; TZP, piperacillin/tazobactam.](image-url)
(NPV) for the detection of isolates intermediate or resistant to TZP were 100% at both 6 to 8 h and 24 h of growth (Table 4). However, in the group of ESRI developers, the sensitivity and NPV decreased to 96% and 92%, respectively, at 6 to 8 h (Table 5).

Regarding the initial *E. coli* isolates, we have calculated the sensitivity and specificity of the ESRI test based on the initial resistance patterns (RSS, RRS, and RRR). The results of this analysis show a global sensitivity and specificity of 97.8% and 94.7%, respectively.

**ESRI detection in paired isolates of *E. coli*.** In a previous study (17), we analyzed the clinical and microbiological data from two *E. coli* clinical isolates (C2-49 and C2-54) belonging to sequence type 69 (ST69) and recovered from intra-abdominal abscesses of the same patient before and after 10 days of TZP treatment (4 g/0.5 g each 8 h) without clinical improvement. Similarly, we analyzed two other *E. coli* clinical isolates (PT3 and PT4) belonging to ST88 and recovered from blood cultures in a second patient with a perianal abscess and persistent bacteremia before and after 8 days of TZP treatment (4 g/0.5 g each 8 h) without clinical improvement. Source control was performed surgically for both patients after the first positive culture. The first isolates from both patients, C2-49 and PT3, presented an RSS resistance pattern to BL/BLI and were ESRI positive, and the second isolates, C2-54 and PT4, presented with RRS and RRR resistance patterns, respectively, and were ESRI positive (Table 6).

**DISCUSSION**

In the present study, we designed a semirapid detection system, called the ESRI test, for detecting TZP resistance or the ability to develop ESRI in *E. coli*. We characterized a collection of 114 clinical isolates of *E. coli* that were used to validate the ESRI test.

Discordant results in TZP MICs between MicroScan and standard broth microdilution were observed for five (4.3%) of the *E. coli* isolates studied. Two replicates were performed and showed the same results. This difference could be due to technical problems associated with the development of the MicroScan method.

This study showed that *bla*<sub>TEM</sub> was the most frequently detected β-lactamase gene, both in TZP-resistant and ESRI developer *E. coli* isolates. In contrast, *bla*<sub>OXA-1</sub> was the least frequently detected β-lactamase gene in both groups, with a clear difference between TZP-resistant (14.9%) and ESRI developer (2.2%) isolates. Similar data were observed in another study, in which OXA-1 was strongly associated with TZP resistance in *E. coli* (13). Moreover, in some isolates, none of the studied β-lactamases were detected, probably indicating the involvement of other β-lactamases, such as AmpC (19).

It is noteworthy that the ESRI test is very sensitive and specific and has high PPV and NPV, being able to detect all of the TZP-resistant and nearly all of the ESRI devel-

**TABLE 4 Validation of the ESRI test in the detection of TZP-resistant isolates (n = 69)**

| Growth (h) | No. of isolates whose ESRI results matched their phenotypic profiles/no. of isolates with phenotypic profile (%)<sup>a</sup> | Sensitivity | Specificity | PPV | NPV |
|------------|-------------------------------------------------|-------------|------------|-----|-----|
| 6–8        | 47/47 (100)                                     | 22/22 (100) | 47/47 (100) | 22/22 (100) |
| 24         | 47/47 (100)                                     | 22/22 (100) | 47/47 (100) | 22/22 (100) |

<sup>a</sup>n = 69 isolates, corresponding to a total of 22 and 47 isolates susceptible and resistant to TZP, respectively. PPV, positive predictive value; NPV, negative predictive value.

**TABLE 5 Validation of the ESRI test in the detection of ESRI developer isolates (n = 67)**

| Growth (h) | No. of isolates whose ESRI results matched their phenotypic profile/no. of isolates with phenotypic profile (%)<sup>a</sup> | Sensitivity | Specificity | PPV | NPV |
|------------|-------------------------------------------------|-------------|------------|-----|-----|
| 6–8        | 43/43 + 2 (96)                                  | 22/22 (100) | 45/45 (100) | 22/22 + 2 (92) |
| 24         | 45/45 (100)                                     | 22/22 (100) | 45/45 (100) | 22/22 (100) |

<sup>a</sup>n = 67 isolates, corresponding to a total of 22 and 45 isolates susceptible to TZP and developers of ESRI, respectively. PPV, positive predictive value; NPV, negative predictive value; + 2, 2 isolates negative for ESRI test at 6–8 h.
oper E. coli isolates analyzed in this study. Only two ESRI developer isolates were not detected by the ESRI test at 6 to 8 h. One possible explanation is that the growth rates of both isolates were lower than those of the rest of the isolates. Another possibility is that both isolates did not produce high enough quantities of β-lactamase to be detected by the test at 6 to 8 h of bacterial growth. More studies are needed to confirm this hypothesis.

Other similar tests have been developed and/or commercialized for the detection of extended spectrum β-lactamases (ESBL) or carbapenemases, such as the ESBL NDP test (20), the Rapidec Carba NP test (21), the Nitro-Carba test (22), and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (23), among others. In addition, manual and automatic methods like rapid antimicrobial susceptibility testing (RAST) and the automated commercial laser-scattering-based in vitro system Alfred 60AST have been developed to identify TZP-resistant E. coli from positive blood culture bottles within 4 to 6 h (24, 25). The main difference from these interesting tests is that the ESRI test was designed to detect not only TZP-resistant isolates but also ESRI developer isolates, which are primarily susceptible to TZP.

The ESRI test combines many advantages in relation to conventional clinical methods after positivity of a blood culture. It is semirapid, simple, and inexpensive. We demonstrated that the ESRI test allows us to differentiate with a high probability between isolates resistant to TZP (≤10 min of detection time) and ESRI developers (>10 to 120 min of detection time). These early detection times (less than 3 h) are an advantage compared with the 24 h needed by the conventional methods. We propose to use this test for all E. coli-positive blood cultures prior to the MIC testing (Fig. 3).

In addition, we would like to highlight that the ESRI test cannot be used to test resistance to SAM because SAM-susceptible isolates that developed ESRI (e.g., C1-174 isolate) and SAM-resistant isolates that did not develop ESRI (e.g., C1-74, C1-83, C1-138, and C2-12 isolates) were found. This discrepancy will limit the antimicrobial treatment options. We believe that the present ESRI test will overcome this limitation and allow differentiation between TZP-susceptible isolates and ESRI developer and TZP-resistant isolates where TZP should not be used for patient treatment.

The introduction of the ESRI test into clinical practice would have clinical implications. The rapid detection of ESRI in E. coli would be essential to establish an adequate antibiotic treatment for intra-abdominal infections, especially during the first 24 h, when the use of TZP is especially relevant. This might avoid a possible therapeutic failure due to the development of resistant bacteria, as well as an inappropriate use of antibiotics that in turn will contribute to increasing the rates of resistance of BL/LBI. Further clinical data are needed to assess the impact of the use of the ESRI test on the clinical prognosis of patients with intra-abdominal infections by E. coli. To this end, we need to determine the impact of the ESRI test on the duration of symptoms, clinical cure, complications of infection, need for subsequent surgical intervention, length of hospital stay, and mortality.

**TABLE 6** Paired isolates with positive results in ESRI test

| Isolate (n = 4) | Origin | ST | BL/BLI resistance profilea | TZP MIC (mg/liter)b | Resistance mechanism | Result at 6–8 h growth | Time to result (min) | Result at 24 h growth | Time to result (min) | T Zap pressure testc | New TZP MIC (mg/liter) |
|----------------|--------|----|---------------------------|--------------------|---------------------|-----------------------|----------------------|----------------------|----------------------|---------------------|----------------------|
| C2-49          | Peritoneal fluid | ST69 | RSS 8 TEM-1              | +                  | 15                  | +                     | 30                   | +                    | 30                   | +                   | 512                  |
| C2-54          | Intra-abdominal abscess | ST68 | RSS 8 TEM-1              | +                  | 15                  | +                     | 30                   | +                    | 30                   | +                   | 256                  |
| PT3            | Blood | ST88 | RSS 256 TEM-1        | +                  | 10                  | +                     | 15                   | NPd                  | NP                  |                     |                      |
| PT4            | Blood | ST88 | RRR 256 TEM-1          | +                  | 10                  | +                     | 15                   | NPd                  | NP                  |                     |                      |

*RSS, resistant to ampicillin/sulbactam and susceptible to amoxicillin/clavulanic acid and piperacillin/tazobactam; RRS, resistant to ampicillin/sulbactam and amoxicillin/clavulanic acid and susceptible to piperacillin/tazobactam; RRR, resistant to ampicillin/sulbactam, amoxicillin/clavulanic acid, and piperacillin/tazobactam.

TZP, piperacillin/tazobactam.

a, positive result in ESRI detection or antimicrobial pressure test.

a, NP, not performed.
As limitations, the ESRI test was designed only for *E. coli* isolates growing in hemoculture bottles, unlike the above-mentioned tests that have also been tested with other genera of the *Enterobacterales* and from agar plates. However, it should be noted that *E. coli* is the main Gram-negative bacterium isolated from blood, peritoneal fluid, and bile samples (1), which are usually processed in hemoculture bottles in clinical microbiology laboratories. Future analyses of the ability of the ESRI test to detect TZP resistance in other bacterial genera and from different clinical samples are welcomed.

In conclusion, the ESRI test is a powerful weapon for the fight against antimicrobial resistance. This system may be an innovative contribution to microbiological diagnosis, allowing us to obtain information on the possible development of resistance to BL/BLI in less than 24 h and a better clinical prognosis, both in terms of survival and the absence of recurrence of resistant microorganisms, and also to achieve an economic impact associated with the improvement of the quality of health care.

**MATERIALS AND METHODS**

**Bacterial isolates.** One hundred fourteen *E. coli* clinical isolates were obtained from bloodstream and intra-abdominal samples of patients with suspected bacteremia or intra-abdominal infections at the Hospital Universitario Virgen del Rocío, Seville (Spain). The *E. coli* ATCC 25922 strain was the control in all experiments. The study was approved by the Ethics Committee of the Hospital Universitario Virgen del Rocío of Seville (approval no. 0023-N-16).

**Antimicrobial susceptibility testing.** The SAM, AMC, and TZP antimicrobial susceptibility profiles were initially tested by broth microdilution using the MicroScan WalkAway NM44 panels (Beckman Coulter, Inc., USA). Isolate selection was made taking into account their BL/BLI susceptibility profiles (SSS, RSS, RRS, and RRR). The MICs of TZP were subsequently confirmed using the standard broth microdilution method (18). MICs were determined for the original isolates and isolates subjected to TZP pressure.

**Antimicrobial selection pressure.** The antimicrobial selection pressure experiment was performed as described previously (17). Overnight bacterial cultures grown in 10 ml of Mueller-Hinton broth (MHB) at 37°C were adjusted to 0.5 McFarland standard (10^8 CFU/ml) and diluted to a final inoculum level of 10^7 CFU/ml. The diluted inocula were incubated with subinhibitory concentrations of TZP (8:1 ratio) corresponding to 1-fold dilutions below the MICs at 37°C for 24 h. Positive bacterial growth was readjusted to 10^5 CFU/ml, and adjusted cultures were incubated with a 2-fold-increased concentration of TZP. These steps were repeated until reaching a TZP concentration of 256/32 mg/liter or until a TZP concentration that did not allow bacterial growth was reached. At the end of the process, MICs were determined for all of the pressured isolates.

**Detection and sequencing of bla\textsubscript{TEM}, bla\textsubscript{OXA-1}, and bla\textsubscript{SHV} genes.** The bla\textsubscript{TEM}, bla\textsubscript{OXA-1}, and bla\textsubscript{SHV} genes were analyzed in all studied isolates by PCR using the following primers: for bla\textsubscript{TEM}, forward,
S'-ATGAGTTATCAGACTTCCCG, and reverse, S'-CTGACAGTACAATGCTTA (17); for bla<sub>CRE</sub>, forward, S'-GGATAACCCCCCAAGGAG-3', and reverse, S'-TGACAGTTTTTTCTCCATAC-3' (26); and for bla<sub>SB</sub> forward, S'-GGGATTCTTTATTGTGC CG, and reverse, S'-TTAGCTTGCCAGTGCTC (27). All positive PCR products were screened by Sanger sequencing using an ABI 3500 genetic analyzer (Applied Biosystems, Foster, VA, USA) and analyzed using SnapGene Viewer 2.7.2 software and BLAST internet services (www.ncbi.nlm.nih.gov/BLAST).

**ESRI test.** The ESRI test is based on colorimetry- and pH-based detection of the hydrolysis of the β-lactam ring produced by the β-lactamase enzyme. The clinical isolates were inoculated into different blood culture vials (Bactec standard/10 aerobic/F and Bactec lytic/10 anerobic/F; Becton Dickinson, USA) at concentrations of 10<sup>4</sup> and 10<sup>5</sup> CFU/ml and incubated at 37°C with agitation at 180 rpm for 6 to 8 and 24 h in order to test the method's efficacy in the time interval in which a patient blood culture with E. coli bacteremia is usually positive. After this time, 1.5 ml of the bacterial suspension grown in the blood culture was taken, transferred to an Eppendorf tube, and centrifuged at 9,600 × g for 2 min at room temperature. Then, the pellet was resuspended in 100 μl of B-PER II lysis buffer (bacterial protein extraction reagent; Thermo Fisher Scientific, USA) and incubated at 37°C for 30 min. Afterwards, the sample was centrifuged again at 9,600 × g for 2 min at room temperature. Subsequently, 30 μl of the supernatant was added to a 195-μl solution consisting of 190 μl of ampicillin (3 mg/ml) (Sigma, Spain)-sulbactam (4 μg/ml) (Sigma, Spain) plus 5 μl of 0.5% (vol/vol) phenol red (Sigma, Spain) on a flat-bottom microplate. Finally, the plate was incubated at 37°C for 120 min, with readings every minute during the first 10 min and every 10 min for the rest of the time until 120 min (Fig. 1). In order to interpret the results, those samples with changes in color with respect to the negative control were considered positive results. Negative test controls were performed in culture medium without the addition of ampicillin in order to check the stability of the color medium.

We should mention that, although the test is designed to detect TZP resistance and the possible development of ESRI, SAM was used in the test instead of TZP. This was for two reasons: (i) TZP resistance mediated by β-lactamases also leads to SAM resistance, and (ii) ESRI development always starts with SAM resistance, allowing us to detect SAM hydrolysis even if the isolate is susceptible to TZP and to avoid false-negative isolates.

**ESRI test validation.** The statistical parameters sensitivity, specificity, PPV, and NPV were used to determine the validity of the detection method developed and fine-tuned in this work. To calculate these parameters, we included the data for the isolates susceptible to TZP.

**Statistical analysis.** Descriptive analysis was performed for the times of positivity of the ESRI test, with the medians and interquartile ranges (IQR) being reported. Times of positivity of the ESRI test were analyzed using the Mann-Whitney U test. Differences were considered significant at a P value of <0.05. All statistical analyses were performed using SPSS software, version 23.0 (SPSS).

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Y.S. conceived the study and designed the experiments. A.R.-V., A.G.L., and L.G.-B. performed experiments and interpreted data. J.P. and J.A.L. revised the manuscript, and Y.S. wrote the manuscript with the input of all the other authors. We declare no conflicts of interest.

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