# Prevalence, Molecular Characterization, and Phenotypic Confirmation of Extended-Spectrum Beta-Lactamases in *Escherichia coli*, *Klebsiella pneumoniae*, and *Klebsiella oxytoca* at the Radboud University Nijmegen Medical Centre in The Netherlands

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The prevalence and molecular types of extended-spectrum beta-lactamases (ESBLs) were determined during a 1-year period in unselected clinical nonduplicate isolates of *Escherichia coli* ($n = 1,738$), *Klebsiella pneumoniae* ($n = 436$), and *Klebsiella oxytoca* ($n = 208$), cultured at the University Medical Centre Nijmegen, The Netherlands. Isolates identified as ESBL producer by the Phoenix automated system were collected prospectively and subjected to molecular analysis for the most common ESBLs TEM, SHV, and CTX-M, as well as OXA and GES. Both the Etest ESBL and double-disk synergy test were performed as confirmatory tests. The estimated prevalence of ESBLs was 2.1% in *E. coli*, 5.2% in *K. pneumoniae*, and 2.4% in *K. oxytoca*. TEM-12 and -26, SHV-5 and -12, and CTX-M groups 1 and 9 were the most frequent ESBLs found. Isolates identified as ESBLs by the Phoenix were confirmed by polymerase chain reaction (PCR) in only 42%. In ESBL PCR-positive *E. coli* and *K. pneumoniae*, both confirmatory tests were positive in 95% of the isolates. In 28% of the Etest and 13% of the double-disk synergy test-positive isolates, PCR could not detect any ESBL gene. In these cases, other resistance mechanisms may play a role. Confirmatory tests were unreliable for *K. oxytoca*. A previously described mutation in the K1 enzyme was detected in one ceftazidime-resistant *K. oxytoca*. The prevalence of ESBLs in The Netherlands is increasing. The predominant molecular types of ESBLs detected were comparable to other studies. Phoenix ESBL results need to be confirmed as advocated by ESBL detection guidelines.

## Introduction

**W**ORLDWIDE BETA-LACTAM RESISTANCE among Enterobacteriaceae, mediated by extended-spectrum beta-lactamases (ESBLs), is increasing. Data on ESBLs from The Netherlands are, however, limited to only two older studies, and it is expected that the prevalence of ESBLs is increasing in The Netherlands, despite restrictive antimicrobial policies.[1,23] Moreover, surveillance programs also report trends of rising third-generation cephalosporin resistance in *Escherichia coli* and *Klebsiella pneumoniae*.[8,24] The aim of this study was to determine the prevalence of ESBLs and to characterize the ESBL genes in consecutive unselected clinical isolates of *E. coli*, *K. pneumoniae*, and *Klebsiella oxytoca* collected at the University Medical Centre Nijmegen, The Netherlands. In addition, the detection of ESBLs using the Phoenix automated microbiology system (Becton Dickinson, Sparks, MD) as a first screen with confirmation by phenotypic tests was evaluated.

## Materials and Methods

### Bacterial isolates

All *E. coli*, *K. pneumoniae*, and *K. oxytoca* isolated from clinical specimens in the routine microbiology laboratory during a 1-year period were identified using the digital laboratory system. From each patient only the first isolate indicated by the Phoenix as ESBL and the first isolate not indicated by the Phoenix as ESBL were selected. A total of 1,738 *E. coli*, 436 *K. pneumoniae*, and 208 *K. oxytoca* were identified, of which 128 isolates were indicated as ESBLs by the BD Phoenix, including 65 *E. coli*, 37 *K. pneumoniae*, and 26 *K. oxytoca*. Although it was instructed to the routine clinical

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laboratory to collect and store all isolates that were ESBL positive by the Phoenix, 26 isolates were not stored. Therefore, 102 isolates, 50 *E. coli*, 29 *K. pneumoniae*, and 23 *K. oxytoca*, were collected prospectively and stored at $-20^\circ$C for further analysis. Polymerase chain reaction (PCR) and phenotypic confirmation tests were performed after two overnight subcultures.

### Molecular characterization of beta-lactamases

The isolates were analyzed for the presence of TEM, SHV, CTX-M, GES, and OXA by PCR. Total DNA was extracted using the MagNa Pure Total NA isolation kit (Roche, Almere, The Netherlands). PCRs were set up to analyze for the presence of the ESBL genes in a final volume of $50\,\mu$l with the PCR Master mix (Roche) and primers as described previously.[2,3,16,22] After one step at $95^\circ$C for 5 min, the samples were cycled 40 times (denaturation at $94^\circ$C for 1 min, annealing at $50^\circ$C for 1 min, and extension at $72^\circ$C for 2 min). The presence of $bla_{CTX-M}$, $bla_{GES}$, and $bla_{OXA}$ were analyzed by gel electrophoresis. $bla_{TEM}$, $bla_{SHV}$, and $bla_{CTX-M}$ genes were further analyzed by sequence analysis. After purification, the PCR product was sequenced with the BigDye Terminator v3 kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

### Phenotypic confirmation tests

ESBL confirmatory tests were performed by the Etest ESBL (AB BIODISK, Solna, Sweden) as well as the cephalosporin–clavulanate double-disk synergy (DDS) test using both cefotaxime and ceftazidime (Becton Dickinson) as described by the Clinical and Laboratory Standards Institute [CLSI] guidelines.[6] In brief, Etest strips and antimicrobial disks were applied to Mueller–Hinton agar (Becton Dickinson) inoculated with a 0.5 McFarland bacterial suspension and incubated at $37^\circ$C in ambient air overnight for 16–18 hr. Etest ESBL was positive if the minimum inhibitory concentration (MIC) for cefotaxime was $>0.5\,\mu$g/ml and the MIC ratio cefotaxime/cefotaxime + clavulanic acid was $>8$, if the MIC for ceftazidime was $>1\,\mu$g/ml and the MIC ratio ceftazidime/ceftazidime + clavulanic acid was $>8$, if there was a deformation of the growth inhibition zone around cefotaxime or ceftazidime, or if a phantom zone was present. The DDS test was positive if the growth inhibition zone around the antimicrobial disk containing only the cephalosporin increased to $>5$ mm in the presence of clavulanic acid.

### AmpC detection

The presence of AmpC enzymes was investigated by determining susceptibility to cefoxitin using disk diffusion according to CLSI criteria.[6] A growth inhibition zone of $<18$ mm was considered suspect for AmpC production.[10]

## Results

### Molecular characterization

ESBL was confirmed by PCR and sequence analysis in 43 of the 102 isolates (42%) that were identified as ESBLs by the Phoenix (Table 1). ESBL genes were detected in 23 *E. coli* (46%), 14 *K. pneumoniae* (48%), and 4 *K. oxytoca* (19%). Two *K. oxytoca* were later identified by sequence analysis as *Klebsiella ornithinolytica* and both contained SHV-5 (see below). TEM-derived ESBLs were present in 20 isolates,

SHV-derived ESBLs in 14, and CTX-M enzymes in 11. GES, OXA, and AmpC enzymes were not detected.

CTX-M and TEM enzymes were the predominant ESBLs in *E. coli*. In *K. pneumoniae*, TEM- and SHV-derived ESBLs were detected at a similar rate, and in *K. oxytoca* all four ESBLs were TEM derived (Table 1).

CTX-M enzymes were only detected in *E. coli* and belonged to the CTX-M-1 and CTX-M-9 groups. TEM-12 and TEM-26, and SHV-12 and SHV-5 were the predominant TEM and SHV ESBLs (Table 1).

Of the 59 isolates with no ESBL gene detected, 48 harbored wild-type beta-lactamase genes or genes with mutations not previously described as ESBL. CTX-M PCR was positive in all *K. oxytoca* isolates, but in all cases it was due to the presence of the chromosome-encoded K1 enzyme as was confirmed by sequence analysis. This has been described previously using these primers with *K. oxytoca*.[15] Two indole-positive isolates that were identified as *K. oxytoca* by the Phoenix did not harbor K1, but both isolates were confirmed to be *K. ornithinolytica* by sequence analysis of the 16S RNA DNA.

### Phenotypic confirmation

In *E. coli* and *K. pneumoniae*, 95% of the 37 isolates with positive PCR results for ESBLs were positive with both confirmatory tests. Both confirmatory tests were negative in 71% of 42 *E. coli* and *K. pneumoniae* with negative ESBL PCR results, thereby confirming the occurrence of false-positive results using the Phoenix. However, 29% (12 of 42) of ESBL PCR-negative *E.coli* and *K. pneumoniae* were positive by confirmatory tests. Eleven were positive with the Etest. Five of these isolates were *E. coli* with a TEM-1 enzyme ($n=2$) or no beta-lactamase ($n=3$), and six isolates were *K. pneumoniae* harboring non-ESBL SHV ($n=4$), LEN-3 ($n=1$), and no beta-lactamase ($n=1$). Using DDS test, five isolates of ESBL PCR-negative *E.coli* and *K. pneumoniae* were positive. Two of these isolates were *E. coli*, both with positive Etest results as well, one harboring a TEM-1 enzyme and one with no beta-lactamase detected. Two isolates were *K. pneumoniae* that also tested positive with the Etest, one with SHV-11 and one with no beta-lactamase detected. One was a *K. pneumoniae* with combined SHV-11 and TEM-1 and was negative with Etest.

In *K. oxytoca*, all four isolates that were ESBL PCR positive were also positive by Etest but negative with DDS test. However, with the Etest, 88% of the 17 PCR-negative isolates were positive as well. In contrast, only three (18%) of these isolates were positive by the DDS test. Of note, these three isolates were all resistant to ceftazidime (MIC $> 32$ mg/L), not in keeping with hyperproduction of chromosomal K1 enzyme, whereas all other isolates, including the four isolates with positive ESBL PCR, were susceptible to ceftazidime (MIC $< 0.5$ mg/L). In one of the ceftazidime-resistant isolates, the sequence analysis of the K1 enzyme showed a mutation resulting in a proline-to-serine substitution at position 167 which was previously shown to be responsible for hydrolysis of ceftazidime by the K1 enzyme.[13,18] In the other cases, no correlation with K1 mutations was found.

### ESBL prevalence

Extrapolating the PCR results obtained with the isolates that were available for molecular analysis to the total number of isolates identified by the Phoenix as ESBL, the prev-

TABLE 1. BETA-LACTAMASE GENES AND RESULTS OF TWO PHENOTYPIC CONFIRMATORY TESTS
(ETEST AND DOUBLE DISK SYNERGY TEST) IN 102 ISOLATES IDENTIFIED AS EXTENDED-SPECTRUM
BETA-LACTAMASES BY THE PHOENIX AUTOMATED MICROBIOLOGY SYSTEM

| | | Etest | | DDS test | |
|---|---|---|---|---|---|
| | bla *gene* | *Positive* | *Negative* | *Positive* | *Negative* |
| *Escherichia coli* 50 | | | | | |
| ESBL 23 | | | | | |
| TEM 9 | **TEM-6** | 1 | | 1 | |
| | **TEM-12** | 3 | 1 | 3 | 1 |
| | **TEM-26** | 3 | 1 | 3 | 1 |
| SHV 4 | **SHV-5** | 1 | | 1 | |
| | **SHV-5**, TEM-1 | 2 | | 2 | |
| | **SHV-2**, TEM-52 | 1 | | 1 | |
| CTX-M 10 | **CTX-M-1** | 2 | | 2 | |
| | **CTX-M-9** | 2 | | 2 | |
| | **CTX-M-1**, TEM-1 | 3 | | 3 | |
| | **CTX-M-9**, TEM-1 | 3 | | 3 | |
| Non-ESBL 27 | | | | | |
| | TEM-1 | 2 | 15 | 1 | 16 |
| | None | 3 | 7 | 1 | 9 |
| *Klebsiella pneumoniae* 29 | | | | | |
| ESBL 14 | | | | | |
| TEM 7 | **TEM-12**, LEN2 | 1 | | 1 | |
| | **TEM-12**, LEN3 | 2 | | 2 | |
| | **TEM-26**, SHV-26 | 3 | | 3 | |
| | **TEM-26**, SHV-11 | 1 | | 1 | |
| SHV 7 | **SHV-5** | 1 | | 1 | |
| | **SHV-12** | 3 | | 3 | |
| | **SHV-12**, TEM-1 | 3 | | 3 | |
| Non-ESBL 15 | | | | | |
| | SHV-1 | 2 | | | 2 |
| | SHV-11 | 2 | 3 | 1 | 4 |
| | SHV-11, TEM-1 | | 1 | 1 | |
| | SHV-26 | | 4 | | 4 |
| | SHV-36 | | 1 | | 1 |
| | LEN-3 | 1 | | | 1 |
| | None | 1 | | 1 | |
| *Klebsiella oxytoca* 21 | | | | | |
| ESBL 4 | | | | | |
| TEM 4 | **TEM-7** | 1 | | | 1 |
| | **TEM-12** | 1 | | | 1 |
| | **TEM-26** | 1 | | | 1 |
| | **TEM-116** | 1 | | | 1 |
| non-ESBL 17 | | | | | |
| | K1 | 12 | 1 | 2 | 11 |
| | K1, TEM-1 | 3 | 1 | 1 | 3 |
| *Klebsiella ornithinolytica* 2 | | | | | |
| ESBL 2 | | | | | |
| SHV 2 | **SHV-5** | 2 | | 2 | |

ESBL genes are shown in bold.
DDS, double-disk synergy; ESBL, extended-spectrum beta-lactamase.

alence of ESBLs in the total population was estimated to be 2.1% in *E. coli*, 5.2% in *K. pneumoniae*, and 2.4% in *K. oxytoca*.

## Discussion

Despite the strict antimicrobial policy in The Netherlands, resistance mediated by ESBLs is increasing as in other parts of the world. The increasing prevalence of ESBLs threatens the use of the majority of beta-lactam antibiotics in future treatment of infections. The prevalence of ESBL-mediated resistance in The Netherlands in *E. coli* and *K. pneumoniae* was less than 1% in a study in 1995.[23] In a study in 2004, however, the prevalence of ESBLs was estimated to be as high as 5.6% in *E. coli*, 2.1% in *K. pneumoniae*, but none in *K. oxytoca*.[1] In our study the estimated prevalence in *E. coli*, *K. pneumoniae*, and *K. oxytoca* was 2.1%, 5.2%, and 2.4%, respectively, based on the PCR results. The long prospective study period and the large number of isolates investigated in our study as well as local epidemiology may account for the small differences found when compared with the contem-

porary study in 2004.[1] The increasing prevalence of ESBLs is reflected in the reports by the national surveillance program coordinated by the Dutch Foundation of the Working Party on Antibiotic Policy (Stichting Werkgroep AntibioticaBeleid, or SWAB) which shows that third-generation cephalosporin resistance was between 2% and 3% in *E. coli* and *K. pneumoniae* collected from unselected hospital wards in 2007.[24] The European Antimicrobial Resistance Surveillance System that collects resistance data from invasive isolates throughout Europe shows that third-generation cephalosporin resistance in The Netherlands has increased from <1% in 2001 to 4% in 2007 in *E. coli*, and from 4% in 2004 to 7% in 2007 in *K. pneumoniae*.[8] Compared with other parts of the world, ESBL prevalence in The Netherlands remains low. The European Antimicrobial Resistance Surveillance System data show marked differences between countries, but in 2007 the majority of the European countries, including The Netherlands, have increasing ESBL prevalence compared with previous years, but most countries already have ESBL prevalence rates of >5%, and some eastern countries have extremely high rates (>40%). A recent global study that evaluated tigecycline susceptibility in isolates from hospital-associated infections showed that the prevalence of ESBLs in *E. coli* and *K. pneumoniae* was higher in Europe (7.6% and 13.3%) than in North America (2.2% and 7.5%) but lower compared with the Asia/Pacific Rim (12% and 22.4%) and Latin America (13.5% and 44%).[17]

The large genetic diversity in this study among the ESBLs (11 different types) from a single hospital illustrates the extent of the ESBL problem. Taking into account the species in which ESBLs were detected and the concurrent enzymes present, 22 unique profiles were present among the 43 ESBL isolates. Still, transmission between patients and plasmid transfer may contribute to high frequencies of certain types of ESBLs.

The ESBL genes detected were similar to those detected in other studies from Europe and the United States. TEM- and SHV-derived ESBLs, the first ESBLs recognized in the early 80s, were most frequently detected. In this study, most TEM-derived ESBLs were TEM-12 and TEM-26. These were the most frequent TEM ESBLs encountered in the United States as well and have been reported regularly in European studies.[4,19,21] The high frequency of SHV-5 and SHV-12 among SHV-derived ESBLs is also in line with other studies from Europe and the United States.[4,7] In *E. coli*, 43% of the ESBLs were CTX-M enzymes. The CTX-M enzymes are emerging throughout the world, especially in community-acquired *E. coli* with a predominance of group 1 and 9 enzymes as observed in this study.[7] The previously mentioned study from Amsterdam, The Netherlands, showed that CTX-M enzymes overall were the predominant ESBL type in that region, with a marked absence of TEM-derived ESBLs. In that study the ESBL screen was performed with cefpodoxime only and included all species of Enterobacteriaceae as well as nonfermenters with only a limited number of *E. coli* and *Klebsiellae* spp. isolates which may account for these different findings.[1]

In 58% of the isolates identified as ESBLs by the Phoenix, the presence of ESBL genes was not confirmed by PCR. Initially, the detection of ESBLs by the Phoenix was reported to be highly sensitive and specific using preselected well-defined isolates.[20] However, recent large studies evaluat-

ing the Phoenix ESBL detection in the routine laboratory reported a sensitivity of 93–100% in *E. coli* and *K. pneumoniae* but specificities of only between 67% and 72%, respectively.[9,25] Although the more common ESBL genes were investigated in this study, the presence of ESBL genes not detected by the primers used, including unknown or rare ESBL genes, in a number of the Phoenix-positive but PCR-negative isolates can obviously not be excluded.

Similar to other studies that evaluated ESBL detection by the Phoenix and that showed positive confirmatory tests in ESBL PCR-negative isolates in 14% and 20%, in our study the apparent phenotype for ESBL using Etest (*n* = 7), DDS test (*n* = 1), or both (*n* = 4) was present in 12 of 42 isolates (29%) of *E. coli* and *K. pneumoniae* that were negative for the ESBL genes investigated.[9,25] In four of these isolates, no beta-lactamase gene was detected at all with the methods used, thus suggesting the presence of an undetected beta-lactamase gene. Besides undetected ESBL genes, the ESBL phenotype in the remaining eight isolates with non-ESBL enzymes detected may also be explained by hyperproduction of their beta-lactamases or coexisting changes in the outer membrane.[14,26]

In contrast to the Phoenix ESBL detection, PCR and confirmatory tests were performed after storage of the isolates at −20°C. It is unlikely that the positive Phoenix results with negative PCR and/or confirmatory test results were caused by the storage conditions. Although plasmids may be lost as a result of stress, loss of plasmids from Enterobacteriaceae containing resistance genes as a result of freeze–thawing was not detected in two studies that specifically addressed this issue.[11,12] In addition, Wiegand *et al.* did perform Phoenix ESBL detection simultaneously with PCR and confirmatory testing and also found a low specificity for the Phoenix.[25]

Thus, considering the low specificity of the Phoenix reported in other studies and the finding that in the majority (71%) of the ESBL PCR-negative isolates both confirmatory tests were negative, most of these ESBL PCR-negative isolates very likely represent false-positive results obtained with the Phoenix.[9,25] Hence, there is a need for confirmatory testing after screening using the Phoenix or other methods as advocated in the CLSI guidelines for ESBL detection to prevent inappropriate reporting of resistance for valuable therapeutic antimicrobials.[6] Indeed, confirmatory testing using either Etest or DDS test detected 95% of the ESBL PCR-positive isolates.

ESBL genes were detected in only 4 of the 21 *K. oxytoca* isolates and were all TEM derived. In addition, three ESBL PCR-negative isolates showed high MICs for ceftazidime. In one of these isolates, sequence analysis of the K1 enzyme showed a mutation resulting in a proline-to-serine substitution at position 167 which has been described twice earlier and results in resistance to ceftazidime.[13,18] The phenotypic detection of ESBLs in *K. oxytoca* is known to be difficult because of the presence of the chromosomal K1 enzyme that causes false-positive screen and confirmatory test results, and all tests were unreliable in our study as in other studies.[25] *K. oxytoca* with no ESBL gene detected, susceptibility to ceftazidime, a negative ESBL phenotype using ceftazidime, and a positive ESBL phenotype using cefotaxime is the phenotype consistent with hyperproduction of the K1 enzyme.[25] This was the case in 12 of the 21 *K. oxytoca* isolates using Etest. Interestingly, the DDS test using ceftazidime and cefotaxime did not result in false positives as described re-

cently, but instead could not detect the four TEM-derived ESBLs.[25] The phenotypic confirmation of ESBLs in *K. oxytoca* that showed increased MIC for cefotaxime but not ceftazidime remains problematic. As Carter *et al.* showed good performance of the DDS test using cefpodoxime, we repeated this test with all *K. oxytoca* isolates; however, the results remained unchanged (data not shown), and the previously reported good performance may be explained by the selection of the isolates tested.[5]

The prevalence of ESBL in The Netherlands is increasing but still relatively low compared with most other European countries, and this may change rapidly. The variety of ESBL genes detected in the isolates illustrates the extent of ESBL-mediated resistance in our setting. The ESBL genes detected were comparable to other international studies. Current molecular and phenotypic detection methods have important shortcomings complicating epidemiological studies and the routine detection of ESBLs by the clinical laboratory. While overdetection results in inappropriate use of broad-spectrum antibiotics further complicating the resistance problem, underdetection may result in treatment failure and uncontrolled spread.

## Disclosure Statement

No competing financial interests exist.

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