Genomic and functional characterisation of IncX3 plasmids encoding \( \text{bla}_{\text{SHV-12}} \) in \( \text{Escherichia coli} \) from human and animal origin

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The \( \text{bla}_{\text{SHV-12}} \) ß-lactamase gene is one of the most prevalent genes conferring resistance to extended-spectrum ß-lactams in Enterobacteriaceae disseminating within and between reservoirs, mostly via plasmid-mediated horizontal gene transfer. Yet, studies regarding the biology of plasmids encoding \( \text{bla}_{\text{SHV-12}} \) are very limited. In this study, we revealed the emergence of IncX3 plasmids alongside IncI1\(\alpha/\gamma\) in \( \text{bla}_{\text{SHV-12}} \) in animal-related \( \text{Escherichia coli} \) isolates. Four representative \( \text{bla}_{\text{SHV-12}} \)-encoding IncX3 plasmids were selected for genome sequencing and further genetic and functional characterization.

We report here the first complete sequences of IncX3 plasmids of animal origin and show that IncX3 plasmids exhibit remarkable synteny in their backbone, while the major differences lie in their \( \text{bla}_{\text{SHV-12}} \)-flanking region. Our findings indicate that plasmids of this subgroup are conjugative and highly stable, while they exert no fitness cost on their bacterial host. These favourable features might have contributed to the emergence of IncX3 amongst SHV-12-producing \( \text{E. coli} \) in the Netherlands, highlighting the epidemic potential of these plasmids.

The emerging IncX plasmid family consists of narrow host-range, self-transferable, iteron-containing plasmids with class A theta replication and sizes ranging approximately between 30 and 100 kb\(^1-3\). IncX plasmids have a highly syntenic backbone; yet based on phylogenetic analysis they can be assigned to six distinct subgroups, namely IncX1 to IncX6\(^4,5\). Although it has been demonstrated that IncX plasmids occurred only infrequently among commensal and pathogenic \( \text{E. coli} \) isolates\(^6\), plasmids of this family encoding various resistance genes were recently described in Enterobacteriaceae originating from diverse sources and geographical areas\(^2,4,7-11\). Among this plasmid family, the IncX3 subgroup mediates the spread of genes encoding resistance for clinically relevant first-line (fluoroquinolones and extended-spectrum cephalosporins) and last-resort (carbapenems) antibiotics. IncX3 plasmids have been reported to encode \( \text{qnrB} \), \( \text{qnrS} \)\(^2,8,12-15\), \( \text{bla}_{\text{CTX-M-1}} \)\(^1,11\), \( \text{bla}_{\text{KPC}} \)\(^2,20,21\), \( \text{bla}_{\text{NDM}} \)\(^1,18,19,23\), \( \text{bla}_{\text{NDM}} \)\(^4,24\), \( \text{bla}_{\text{NDM}} \)\(^3,25-28\), \( \text{bla}_{\text{NDM}} \)\(^15,26,29-33\), \( \text{bla}_{\text{NDM}} \)\(^13,16\), \( \text{bla}_{\text{NDM}} \)\(^1,17\), and \( \text{bla}_{\text{OXA}} \)\(^3,12-14,35,36\). Overall, these reports highlight the importance of this plasmid subgroup for the dissemination of antibiotic resistance genes within Enterobacteriaceae.

The \( \text{bla}_{\text{SHV-12}} \) gene ranks amongst the most predominant extended-spectrum ß-lactamases within Enterobacteriaceae of diverse origins\(^37\). Plasmid-mediated horizontal gene transfer constitutes a key mechanism by which this gene disseminates among bacterial populations, therefore monitoring the spread of plasmids is essential to track the transmission of the \( \text{bla}_{\text{SHV-12}} \) gene between different reservoirs\(^7\). Several plasmid replicon types have been associated with the worldwide dissemination of \( \text{bla}_{\text{SHV-12}} \), including \( \text{A/C} \), \( \text{colE} \), \( \text{F} \), \( \text{H} \), \( \text{I} \), \( \text{K} \), \( \text{L} \), \( \text{M} \), \( \text{N} \), \( \text{P} \), \( \text{R} \), as well as the recently emerging X3\(^7\). The few available data on the prevalence of \( \text{bla}_{\text{SHV-12}} \)-encoding

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plasmids in the Netherlands, report IncHI2 plasmids in human Salmonella enterica isolates, IncN and IncF plasmids in human Escherichia coli\(^{7,8}\), as well as IncK plasmids in E. coli from poultry\(^{9}\).

To understand bla\(_{SHV-12}\) diffusion in the Netherlands, also in light of the emerging role of IncX3 plasmids worldwide, we investigated a collection of previously uncharacterized SHV-12-encoding E. coli isolates. We report here the plasmid epidemiology of SHV-12-producing E. coli from different reservoirs in The Netherlands, the first fully assembled and annotated sequence of three bla\(_{SHV-12}\) encoding-IncX3 plasmids of animal origin, and the genetic and functional characteristics of IncX3 plasmids from both human and animal origin.

**Results**

**Plasmid epidemiology of bla\(_{SHV-12}\) and emergence of IncX3.** Among the 129 bla\(_{SHV-12}\) encoding E. coli isolates included in this study (Table 1), 49.6% (n = 64) was isolated from food-producing animals, 41.1% (n = 53) from retail meat and 9.3% (n = 12) from humans. Plasmid typing revealed that bla\(_{SHV-12}\) was encoded by nine different plasmid families: 11α/γ (n = 86; 66.7%), X3 (n = 21; 16.3%), X1 (n = 6; 4.7%), F (n = 5; 3.9%), B/O (n = 4; 3.1%), K (n = 4; 3.1%), N (n = 1; 0.8%), colE (n = 1; 0.8%) and multi-replicon F-X1 (n = 1; 0.8%). A gradual decrease from 90.0% to 55.6% of IncI1α/γ in parallel with a significant increase from 0.0% to 24.1% (p = 0.041) of IncX3 was documented between 2011 and 2014 among food-producing animals and retail meat (Fig. 1). As a result, IncX3 plasmids were among the predominant rep-types encoding bla\(_{SHV-12}\) in the Netherlands from 2012 onwards (Fig. 1).

To further study these emerging IncX3 plasmids, four of them (pEC-NRS18, pEC-393, pEC-125 and pEC-243) randomly selected from human-related E. coli ST69 and from diverse E. coli STs of animal origin (ST117, ST315 and ST410) were fully sequenced and functionally characterized in this study (Table 2).

**IncX3 plasmid backbone is highly syntenic and conserved.** Comparison between whole sequences of the four IncX3 plasmids from this study and twenty IncX3 plasmids available in GenBank revealed a highly conserved plasmid backbone and their organization into a number of distinct clades (Fig. 2). Plasmids of animal origin pEC-125 and pEC-243 were closely clustered together (MUMi distance 0.018) and grouped with the human-derived plasmid pEC-NRS18 that showed distance from 0.085 (pEC-125) to 0.093 (pEC-243). The animal-derived plasmid pEC-393 (turkey meat) clustered with a Klebsiella pneumoniae-encoded plmX-SHV from human source in Italy (MUMi distance 0.002). IncX3 plasmids recovered in the Netherlands clustered closely with pOXA181 (China) and pKS22 (Switzerland) encoding bla\(_{OXA-181}\) and qnrS1, respectively, with MUMi distances varying from 0.140 (pOXA181 with pEC-125) to 0.179 (pKS22 with pEC-393).

The four plasmids sequenced, assembled and fully annotated in this study had sizes varying from 43,506 (pEC-393) to 48,250 (pEC-NRS18) bp with an average GC content of 46.5% (Table 2). Similar to other IncX3 plasmids, they carried three putative origins of replication (oriV-α, oriV-β and oriV-γ), two origins of transfer (oriT-α and oriT-β), and approximately 6 iteron sequences. Nucleotide sequence analysis revealed 65 to 74 predicted open reading frames (Fig. 3). Comparative genomic analysis of all four IncX3 plasmids (Fig. 3) revealed high synteny among them, encoding genes for replication (pir: replication initiation protein and bis: replication accessory protein), partitioning (parAB), entry exclusion (exc), maintenance (topB and hns), transcriptional activation (actX), and conjugative transfer [piIX1-11 (type IV secretion system) and taxA-C]. In addition, a mosaic variable region containing resistance genes as well as intact and/or defective insertion sequences (i.e. IS21, ISKpn19, Tn3, and IS26) was identified in all four plasmids upstream of the partitioning gene parA (Fig. 3).

The variable region of pEC-NRS18 contained bla\(_{TEM-1}\), embedded in a Tn3 transposon, as well as genes bla\(_{SHV-12}\) and qnrS1 associated with the upstream presence of IS26 in the opposite and same orientation, respectively. Similarly, pEC-125 and pEC-243 contained both bla\(_{SHV-12}\) and qnrS1 genes, whereas pEC-393 encoded only bla\(_{SHV-12}\) associated to IS26. The genetic environment surrounding bla\(_{SHV-12}\) was characterized by two flanking copies of IS26 distributed in opposite orientation to form a composite IS26-IS26 transposon (3,633 bp); this structure was conserved in three of the four IncX3 plasmids (pEC-NRS18, pEC-125 and pEC-243; Fig. 3). BLAST analysis revealed that this composite transposon is 100% identical to previously described transposons located on plasmids of K. pneumoniae (IncFIB; GenBank accession no. CP019048.1) and Aeromonas veronii (IncA/C2; GenBank accession no. CP014775.1), as well as into the genome of Pseudomonas aeruginosa (GenBank accession no. GU592828.1). The flanking region of bla\(_{SHV-12}\) on pEC-393 showed a partial 2,484-bp overlap with corresponding regions of pEC-NRS18, pEC-125 and pEC-243 (Fig. 3) and encoded genes deoR, ygbL and truncated ygbK also present on several K. pneumoniae chromosomes (GenBank accession no. CP000647, CP002910 and CP008831). This 4,783-bp region exhibits 100% identity to a fragment of a bla\(_{SHV}\)-harboring IncR plasmid of K. pneumoniae (GenBank accession no. KP954150.1).

**IncX3 plasmid transfer is temperature-dependent.** Conjugation frequencies of the four IncX3 plasmids was determined and results are shown in Fig. 4. Transfer rates differed between solid matings at different temperatures. Geometric mean frequencies ranged below the detection limit (≤ 1 × 10^-9, pEC-393) to 3.73 × 10^-5 (pEC-NRS18) at 25 °C, from 6.36 × 10^-6 (pEC-393) to 7.16 × 10^-5 (pEC-243) at 30 °C, and from 1.33 × 10^-6 (pEC-393) to 1.46 × 10^-4 (pEC-NRS18) at 37 °C. The analysis showed a significant difference between conjugation frequencies at different temperatures (p = 0.027), mainly due to lower frequencies at 25 °C compared with frequencies at 30 °C. The difference in conjugation frequencies between different plasmids was nearly significant (p = 0.054). Comparisons of single plasmids at 30 °C and 37 °C indicated differences between the plasmids at 37 °C, with lower conjugation frequencies for animal-derived plasmids (pEC-125, pEC-243 and pEC-393), and higher frequencies for the human-derived plasmid pEC-NRS18. Overall, plasmid of animal origins seem to transfer better at 30 °C, conversely to the human-derived plasmid (Fig. 4).
| Isolate   | Date of isolation | Origin                        | ESBL gene(s) | Inc/rep-type of β-lactamase-encoding plasmid |
|-----------|-------------------|-------------------------------|--------------|--------------------------------------------|
| 1954014   | 2009              | Livestock (pig)               | β-lact/SHV-12 | IncF                                       |
| 35474     | 2009              | Livestock (poultry)           | β-lact/SHV-12 | IncF                                       |
| 36289     | 2009              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 36278     | 2009              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 313768    | 2009              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 37318     | 2009              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 35078     | 2009              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 37881     | 2009              | Livestock (poultry)           | β-lact/γFR-M1 (IncI1/IncI1/γ) | IncX3                                       |
| 35659     | 2009              | Livestock (poultry)           | β-lact/γFR-M1 (IncI1/IncI1/γ) | IncI1/IncI1/γ                              |
| 36498     | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 36700     | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 36809     | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 66191451  | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 35568     | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 636942    | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 1026302   | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 1025601   | 2010              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 55833907  | 2011              | Livestock (pig)               | β-lact/SHV-12 | IncF                                       |
| 55927588  | 2011              | Livestock (pig)               | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 55927758  | 2011              | Livestock (pig)               | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 37156     | 2011              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 36239     | 2011              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 55727422  | 2011              | Livestock (cattle)            | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 884       | 2011              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 1105      | 2011              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 1109      | 2011              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 984       | 2011              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 65268442  | 2012              | Livestock (pig)               | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 36788     | 2012              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 29062012-02 | 2012           | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 55580200139 | 2012        | Livestock (poultry)           | β-lact/SHV-12, β-lact/TEM-532 | IncK                                       |
| 65094754  | 2012              | Livestock (cattle)            | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 36458     | 2013              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 35658     | 2013              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 13999001  | 2013              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 859       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 219       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 1041      | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 900       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 570       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 500       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncB/O                                      |
| 374       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 287       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 738       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 1424      | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 71        | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 828       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX1                                       |
| 1083      | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 11        | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 240       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncF-X1                                     |
| 386       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 990       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 1341      | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |
| 1420      | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncX3                                       |
| 118       | 2014              | Livestock (poultry)           | β-lact/SHV-12 | IncI1/IncI1/γ                              |

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| Isolate | Date of isolation | Origin | ESBL gene(s)* | Inc/rep-type of bla<sub>TEM</sub>-<sub>1</sub>-encoding plasmid |
|----------|------------------|--------|---------------|-----------------------------------------------|
| 139      | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncX3                                        |
| 864      | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncX1                                        |
| 1296     | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 876      | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 20       | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncX1                                        |
| 229      | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncX3                                        |
| 1096     | 2014             | Livestock (poultry) | bla<sub>TEM</sub>-12 | IncF                                         |
| 1433     | 2014             | Livestock (pig)     | bla<sub>TEM</sub>-12 | IncX3                                        |
| 1116     | 2014             | Livestock (cattle)  | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69438407 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 (IncI1α/γ), bla<sub>TEM</sub>-12 | IncX3                                        |
| 69606962 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncX3                                        |
| 76495084 | 2012             | Meat (beef)         | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 76495084 | 2012             | Meat (beef)         | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69843324 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69210023 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncK                                         |
| 69843409 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69438105 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncX3                                        |
| 69585604 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69064655 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69770576 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12, bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69477895 | 2012             | Meat (poultry)      | bla<sub>TEM</sub>-12, bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69927807 | 2013             | Meat (poultry)      | bla<sub>TEM</sub>-12 (IncK), bla<sub>TEM</sub>-12 | IncB/O                                       |
| 698975250004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncK                                         |
| 699561810004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 699819760004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69986056 | 2013             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 693784120004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 693785440004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 693562060004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 699898610004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 699229530004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 6998980410004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 699600219 | 2013             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69960316 | 2013             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69960316 | 2013             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69345093 | 2013             | Meat (pork)         | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 69799639 | 2013             | Meat (beef)         | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 699799710004 | 2013 | Meat (beef) | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 694658380004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12, bla<sub>TEM</sub>-12, bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 699813050004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12, bla<sub>TEM</sub>-12 (IncI1α/γ) | IncI1α/γ                                    |
| 699990020004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncK                                         |
| 693503480004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncX1                                        |
| 699953490004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncX1                                        |
| 699081950004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncX1                                        |
| 699952170004 | 2013 | Meat (poultry) | bla<sub>TEM</sub>-12 | IncX3                                        |
| 69960189° | 2013             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncX3                                        |
| 79158224 | 2014             | Meat (beef)         | bla<sub>TEM</sub>-12 | IncF                                         |
| M1470112 | 2014             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 79197637 | 2014             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 79059443 | 2014             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 79230383 | 2014             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncB/O                                       |
| 79469536 | 2014             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 79194778 | 2014             | Meat (pork)         | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |
| 79195006 | 2014             | Meat (poultry)      | bla<sub>TEM</sub>-12 | IncI1α/γ                                    |

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IncX3 plasmids exert no fitness cost to the bacterial host and are highly stable. The cost of IncX3 plasmid presence on the host cell fitness was assessed in the absence and in the presence of cefotaxime by comparing the exponential growth rate of *E. coli* DH10b with and without plasmid (Fig. 5). The exponential growth rates of *E. coli* DH10b harbouring each of the IncX3 plasmids singularly varied from 0.91 (95% CI 0.84–0.99; DH10b::pEC-125) to 1.22 (95% CI 1.13–1.32; DH10b::pEC-NRS18) in the absence of antibiotic selective pressure, and from 1.48 (95% CI 1.14–1.83; DH10b::pEC-NRS18) to 1.94 (95% CI 1.77–2.12; DH10b::pEC-243) in the presence of cefotaxime. In the absence of cefotaxime, DH10b::pEC-125 showed significantly lower (\( p = 0.04 \)) and DH10b::pEC-NRS18 significantly higher (\( p < 0.001 \)) exponential growth rates compared to *E. coli* DH10b control strain. Pairwise comparison of the relative growth rates for *E. coli* DH10b harbouring each of the IncX3 plasmids in the absence and presence of cefotaxime indicated significantly higher rates in the presence of selective pressure (\( p < 0.001 \)) except for DH10b::pEC-NRS18.

**Table 1.** Characteristics of the 129 non-duplicate *bla*<sub>SHV</sub>-encoding *E. coli* isolates included in the study. *When known the inc/rep-type of the plasmid encoding ESBL genes (excluding the *bla*<sub>SHV</sub>-12) is given in parenthesis. ¥In bold the 4 plasmids sequenced and functionally characterized.

| Isolate | Date of isolation | Origin | ESBL gene(s)* | Inc/rep-type of *bla*<sub>SHV</sub>-12 encoding plasmid |
|---------|------------------|--------|---------------|---------------------------------------------------------|
| 79156655| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 79207004| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 79626295| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncB/O |
| 79207101| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 79352292| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncK |
| 79771872| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncX3 |
| 79445126| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 79042587| 2014             | Meat (poultry) | *bla*<sub>SHV</sub>-12 | IncX3 |
| 1190900169 ¥ | 2009 | Human (urine) | *bla*<sub>SHV</sub>-12 | IncX3 |
| 11909000881 | 2009 | Human (urine) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 1190900890 | 2009 | Human (urine) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 306 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 1.1 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 1.58 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 2.12 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 2.25 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 2.48 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 2.49 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 2.52 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncI1<sub>α</sub>/γ |
| 1.71 | 2014 | Human (faeces) | *bla*<sub>SHV</sub>-12 | IncN |

**Figure 1.** Prevalence of plasmid encoding *bla*<sub>SHV</sub>-12 in *E. coli* isolated between 2009 and 2014 in the Netherlands. Isolates were recovered from food-producing animals, retail meat and humans during national antimicrobial resistance monitoring programmes or national projects. Plasmids belonging to N, colE and F-X1 replicon types were recovered with prevalence of 0.85% and therefore were omitted from the figure.
E. coli DH10b transformed strains were also propagated without positive antibiotic selective pressure as a measure of plasmid stability. After approximately 180 generations of growth, the percentage of plasmid-harbouring cells in each population (for all four plasmids singularly) was determined. All plasmids were found to be stably maintained in the E. coli population, ranging from 99.9% (95% CI 99.98–99.99) (pEC-NRS18) to 100% (95% CI 99.9–100) (pEC-393, pEC-125 and pEC-243) plasmid-harbouring cells per generation.

IncX3 plasmids do not contribute to bacterial pathogenicity. Annotation of the four IncX3 plasmids revealed the presence of a Type 4 Secretion System (pilX1-11) and several ORFs with unknown function that could potentially act as virulence effectors (Fig. 3). The Galleria mellonella in vivo infection model was employed to evaluate the impact of harbouring an IncX3 plasmid on bacterial pathogenicity. The LD_{50} value after 24 h was determined to be 10^7 CFU/larvae and survival curves were compared between the isogenic control E. coli DH10b strain and DH10b transformed strains harbouring each of the IncX3 plasmids (Fig. 6). All four transformed strains carrying IncX3 plasmids displayed comparable virulence to the plasmid-free control strain (mortality = 40–86%), with no significant difference in the 96 h survival curves (Fig. 6). In both control groups all larvae survived.

### Table 2. IncX3 plasmids included in this study and their characteristics.

| Plasmid ID | Year | Host | Host source | Resistance gene(s) | Size (bp) | GC Content % | Open reading frames |
|------------|------|------|-------------|--------------------|-----------|--------------|---------------------|
| pEC-NRS18  | 2009 | E. coli | ST69/CC69 Human UTI | *bla*SHV-12,*bla*TEM-1,*qnrS1 | 48,250 | 46.4 | 74 |
| pEC-393    | 2013 | E. coli | ST410/CC23 Turkey meat | *bla*SHV-12 | 43,506 | 46.8 | 65 |
| pEC-125    | 2014 | E. coli | ST117 Chicken faeces | *bla*SHV-12,*qnrS1 | 46,338 | 46.4 | 73 |
| pEC-243    | 2014 | E. coli | ST315/CC38 Chicken faeces | *bla*SHV-12,*qnrS1 | 46,338 | 46.4 | 73 |

*Urinary tract infection.
Discussion
Our results confirmed that IncI1α/γ plasmids are the major facilitators of the \( \text{bla}_{\text{SHV-12}} \) diffusion in \( \text{E. coli} \) of human and animal origin and mirrored the global plasmid repertoire associated with \( \text{bla}_{\text{SHV-12}} \). However, a gradual decrease in the prevalence of IncI1α/γ and a parallel increase in IncX3 plasmids encoding \( \text{bla}_{\text{SHV-12}} \) was documented mostly in animal-related commensal \( \text{E. coli} \). Previously, the IncX3 plasmid subgroup was only incidentally associated with \( \text{bla}_{\text{SHV-12}} \) and/or \( \text{qnrS1} \) among clinically recovered \( \text{E. coli} \) isolates, and very recently it was identified among poultry isolates in Germany. IncX3 plasmids have been documented in other Enterobacteriaceae worldwide in association with multi-resistance, including to carbapenems.

Figure 3. Linear comparison in scale of IncX3 plasmids. The open reading frames identified in each sequence are represented with arrows, with the arrowhead indicating the direction of transcription. Their involvement in replication, partitioning, transfer, or antibiotic resistance, their association to mobile genetic elements, as well as other known or unknown functions and pseudogenes are colour-coded. Areas shaded in grey indicate nucleotide identity.

Figure 4. Conjugation frequencies of IncX3 plasmids. The reported values represent the average of three independent solid mating experiments (25°C, 30°C and 37°C) and the error bars the standard deviation.
Nevertheless, no association between IncX3 plasmids and other resistance genes (apart from bla\textit{SHV-12}, bla\textit{TEM-1} and qnrS\textit{1}) in Enterobacteriaceae was found in the Netherlands (data not shown).

The high degree of synteny and conservation in the backbone of IncX3 plasmids among \textit{E. coli} isolates of both human and animal origin reflects the ecological success of this plasmid subgroup\textsuperscript{2}. In addition to encoding genes essential for their maintenance and dissemination, IncX3 plasmids contained a variable region encoding resistance to clinically important antimicrobial agents (fluoroquinolones and/or extended spectrum cephalosporins). Our findings confirm the potential of this subgroup for accumulation of resistance genes via IS-mediated transposition, with the likely consequence of limiting effective treatment options for possible human infections\textsuperscript{9,13,15,16,18,21}. As previously described\textsuperscript{41}, the presence of IS\textit{26} linked to bla\textit{SHV-12} and other co-linear genes originating from the chromosome of \textit{K. pneumoniae} was documented within the IncX3 variable region, confirming the hypothesis of IS\textit{26}-mediated mobilization of a bla\textit{SHV} ancestor gene from the chromosome of \textit{K. pneumoniae}\textsuperscript{42}. Although we cannot prove how bla\textit{SHV-12} was integrated into IncX3 plasmids, the documented ability of IS\textit{26} to participate in both replicative transposition and self-targeted transposition creating IS\textit{26}-bounded transposons\textsuperscript{43} could have facilitated the formation of the composite bla\textit{SHV-12}-encoding IS\textit{26}-IS\textit{26} transposon seen in the majority of the IncX3 plasmids studied here. The presence of identical composite transposons, mostly on plasmids of diverse replicon types, indicates its mobilization ability preferentially onto plasmids rather than the chromosome, as previously suggested\textsuperscript{44}. In addition to the contribution of IS26 to the mobilization on conjugative plasmids and the subsequent dissemination of bla\textit{SHV-12}, it has been shown that IS26 supplies a promoter −35 box that can be coupled with a −10 box in the adjacent DNA\textsuperscript{45}, possibly also contributing to the expression of this resistance gene.

IncX3 plasmids, as well as the archetypal R6K plasmid of the IncX family, have been investigated for their ability to conjugate\textsuperscript{13,15,16,18,19,46}. We documented the temperature-dependent transfer of animal-derived IncX3 plasmids with frequencies higher at 30 °C, suggesting a more efficient transfer in the environment than in the animal gut. Similarly to other conjugative plasmids, IncX3 plasmids hold a gene encoding a H-NS-like protein\textsuperscript{2,47–51}. Several studies demonstrated the inhibitory role of H-NS-like proteins on gene thermoregulation owing to their ability to polymerize along and bridge adjacent DNA regions at 37 °C and to derepress H-NS-regulated genes at lower temperatures\textsuperscript{47,48,52}. The involvement of these proteins in the temperature-dependent conjugative transfer of
IncHI1 plasmids suggests a similar role for IncX3 plasmids that can only be speculated here. The ability of IncX3 plasmids to replicate and be stably maintained in α-, β- and γ-Proteobacteria, in combination with a higher conjugation frequency at 30 °C, underscore a potential mesophilic Proteobacteria reservoir of this plasmid subgroup.

In contrast with studies describing that plasmids impose fitness cost on their bacterial hosts, growth kinetics obtained over 24 h showed no evidence of fitness cost on the bacterial E. coli host. It has been demonstrated that H-NS-like proteins are able to silence newly introduced foreign sequences (including plasmids), based on increased adenine and thymine (AT) content in comparison with the chromosome. Taking into consideration the high AT content of the IncX3 plasmids, we hypothesize that the H-NS gene present on these plasmids allows them to invade bacterial hosts with a minimal impact on their fitness, ensuring the future competitiveness of the new plasmid-host combination even without the presence of antibiotic selective pressure. The significant IncX3 plasmid-mediated fitness enhancement of E. coli under antibiotic selective pressure highlights the ecological advantage and subsequent successful proliferation of these plasmids in antibiotic-rich reservoirs.

All IncX3 plasmids encoded the widespread partitioning system ParAB ensuring the correct inheritance of these plasmids to the daughter cells. The observed high stability of IncX3 plasmids is potentially due to their high conjugation frequency and absence of a fitness burden, as well as to a low rate of segregational loss.

Our data show that IncX3 plasmids encode a type IV secretion system (T4SS, pilX1-11), typically used for the exchange of genetic material within bacteria, toxin secretion and the translocation of virulent effector proteins into eukaryotic host cells. Yet, a virulence potential of IncX3-carrying E. coli DH10b was not observed, suggesting that T4SS does not play a role in the virulence of E. coli, at least in the G. mellonella model, conversely from other Gram-negative pathogens.

In conclusion, we report the first genetic characterisation of IncX3 plasmids of animal origin, as well as the first functional analysis of human- and animal-derived plasmids of this subgroup, including their conjugation frequencies, stability, fitness cost and virulence potential. IncX3 plasmids are highly conserved, syntenic, conjugative and highly stable, while they exert no fitness cost on their bacterial host, independent of their origin. Although clonal expansion of E. coli strains could also play a role as suggested by our finding in The Netherlands of E. coli from the same clonal complex and carrying the same IncX3 plasmid (data not shown), the favourable plasmid functional features potentially contributed to their emergence amongst SHV-12-producing E. coli in the Netherlands, highlighting the epidemic potential of this plasmid subgroup.

Materials and Methods

Bacterial strains, transformants and plasmids. A total of 129 non duplicate blaSHV-12 encoding E. coli, consecutively recovered during national antimicrobial resistance monitoring programmes or national projects between 2009 and 2014 were included in the study. Identification of the isolates was performed by MALDI-TOF Mass spectrometry (Brucker, Coventry, UK). Genes conferring an ESC phenotype were sought by microarray analysis followed by PCR amplification and sequencing. Plasmid location of blaSHV-12 was determined using a transformation-based approach. Briefly, plasmids encoding blaSHV-12 were extracted from the parental strain using a miniprep method and transformed into E. coli DH10b cells (Invitrogen, Van Allen Way, CA USA) by electroporation under the following conditions: 1.25 kV/cm, 200 Ω, 25 μF. Transformants were selected on Luria-Bertani (LB) agar plates supplemented with cefotaxime (1 mg/L) and confirmed for the presence of blaSHV-12 gene. The presence of a single plasmid in the transformants was confirmed by SI-PFGE on the transformants, followed by Southern blot hybridization using DIG-labelled probe (DIG DNA Labeling and Detection Kit, Roche, Mannheim, Germany) targeting the blaSHV-12 gene, as previously described. Plasmid typing was confirmed by PCR-based replicon typing (PBRT KIT, DIATEVA, Fano, Italy) on the transformants. Host E. coli sequence type were assigned by MLST based on the allelic profiles of seven housekeeping genes (adk, fumC, gyrB, icd, mdh, purA and recA).

Plasmid sequencing, assembly and analysis. Four genetically and epidemiologically unrelated IncX3 plasmids encoding blaSHV-12 were randomly selected for further analysis from E. coli isolates belonging to the single ST of human origin (ST69) and diverse STs of animal origin, including the predominant animal-related ST (ST117). The relevant characteristics of the selected plasmids are specified in Table 1. Plasmid DNA from transformants was isolated using the QIAfilter Plasmid Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s recommendations. Deep sequencing of the plasmid genomes was performed using 300 bp paired-end sequencing libraries (Nextera TAG-mentation sequencing kits [Epicentre]) on an Illumina MiSeq sequencer. High-quality filtered reads were subsequently assembled de novo using SPAdes algorithm (SPAdes version 3.7.1) for illumina-derived reads and then manually curated to close the gaps. Putative open reading frames (ORFs) were identified by RAST version 2.0 and manually curated when necessary. BLASTP analyses of the putative ORFs against the NCBI non-redundant proteins (NR) database, Pfam, and Interpro scan were used to assess their putative functions by identification of structural features and motifs. ResFinder (version 2.1), PlasmidFinder (version 1.3) and ISfinder were used to determine the presence of resistance genes, replicon types and insertion sequences, respectively. Plasmid sequences were hierarchically clustered and displayed as a phenogram using the BioNJ algorithm, where the underlying distance matrix was calculated from the pairwise non-overlapping maximal unique matches (MUMs) using Nucmer version 3.07. Relative pairwise distances were obtained by dividing the pairwise MUMs’ sum by the average genome size of the two paired genomes (MUMi genomic distance). BioNJ trees were generated from the MUMi distance matrix using SplitsTree. BLAST analysis was used to assess sequence identity between the blaSHV-12-containing region and nucleotide sequences deposited to NCBI.

Mating assays. Plasmid conjugation was assessed in solid mating assays at 25 °C, 30 °C and 37 °C conducted in triplicate. Chloramphenicol resistant (chlorR) E. coli MG1655::pACYC184 was used as a recipient strain in 1:1 ratio
with donor *E. coli* DH10b transformed strains carrying the different IncX3 plasmids, as previously described\(^7\). Overnight cultures of recipient and donor strains in mid-exponential phase were co-incubated (100 µl each) onto sterile nitrocellulose filters of 0.45 µm pore size (Schleicher and Schuell GmbH, Dassel, Germany) for 4 h at 25 °C, 30 °C and 37 °C. Transconjugants were selected on LB agar supplemented with chloramphenicol (32 mg/L) and cefotaxime (1 mg/L). Positive transconjugants were confirmed by PCR amplification for the resistance and *yfp* genes. Conjugation frequency was calculated as the number of transconjugants per donor cell, with the absence of transconjugants suggesting either non-conjugative plasmids or conjugation frequencies below the detection limit (<1 × 10\(^{-4}\)). For statistical analysis, conjugation frequencies were transformed to log10 values, the differences between the temperatures and plasmids were tested using a non-parametric Kruskal–Wallis test, and a p value < 0.05 was considered to be statistically significant. All analysis was performed using R and RStudio (version 1.0.143)\(^7\).

**Fitness cost assays.** Liquid cultures of *E. coli* DH10b transformed strains carrying different IncX3 plasmids were incubated overnight in 3 mL LB medium at 37 °C with 180 rpm shaking for 10 days (~180 generations) to determine their stability in an *E. coli* population. Cultures of each strain were daily diluted 1000-fold into 3 mL of fresh pre-warmed LB medium with and without antibiotic (1 mg/L of cefotaxime) and incubated under the same conditions until mid-exponential phase (OD\(_{600}\) of ≈0.5). 200 µL of each culture were loaded in triplicate in wells of a 100-well honeycomb plate and incubated at 37 °C with shaking for 24 h. Growth rates were obtained by measuring optical density at 600 nm every 30 min by using a Bioscreen C Reader (Oy Growth Curves, Helsinki, Finland). Assays were performed in triplicate. Relative growth rates were calculated by dividing the generation time of each DH10b transformed strain by the generation time of the wild-type DH10b strain which was included in each individual assay\(^8\). Growth rates between strains were compared using the Wilcoxon rank sum test with a Bonferroni adjustment for multiple comparisons. All statistical analysis were performed in R studio (version 1.0.143)\(^7\).

**Stability assays.** *E. coli* DH10b transformants carrying different IncX3 plasmids were propagated in antibiotic-free LB medium at 37 °C with 180 rpm shaking for 10 days (~180 generations) to determine their stability in an *E. coli* population. Cultures of each strain were daily diluted 1000-fold into 3 mL of fresh pre-warmed LB medium without antibiotics. On day 10, cultures were plated onto antibiotic-free LB agar and 100 randomly chosen colonies of each evolved line were replica-plated onto antibiotic-free and antibiotic-containing (1 mg/L of cefotaxime) LB agar plates. Plasmid presence was confirmed by colony PCR targeting the *taxC* gene of the IncX3 plasmids\(^5\). Colony growth on antibiotic-free but not on antibiotic-containing plates indicated the proportion of bacteria that lost the plasmid. Assays were performed in triplicate. The chance of *E. coli* DH10b keeping the plasmid was estimated for every plasmid using @Risk 6.3.1 (Palisade Corporation, Newfield, NY, USA), and the proportions of plasmid-harbouring colonies for each plasmid were compared.

**Galleria mellonella survival assays.** *Galleria mellonella* caterpillars in the final-instar larval stage were obtained in bulk from Livefood UK Limited (Rooks Bridge, Somerset, United Kingdom) and stored at 15 °C in the dark on wood shavings prior to use. Ten randomly chosen larvae weighing between 250 mg and 350 mg were employed for each group of an experiment. Strains included in the assay were grown overnight in LB broth and washed twice in sterile phosphate-buffered saline (PBS). The optimal bacterial inoculum was determined by injecting 10 larvae with 10 µl of bacterial suspensions containing 10\(^4\) to 10\(^7\) CFU/larva of organism in PBS. Bacterial inoculum concentration was determined by viable bacterial count on LB agar identifying the inoculum which killed 50% of larvae after 24 hours incubation at 37 °C (LD\(_{50}\)). The optimal inoculum was then injected into the hemocoels of the caterpillars via a left proleg using 25-µl Hamilton syringes (Cole-Parmer, London, United Kingdom). Following injection, larvae were incubated in petri dishes lined with filter paper at 37 °C for 96 h and scored for survival by 2 independent observers daily. Larvae were considered dead when they displayed no movement in response to touch. Two control groups were used per experiment, including larvae that were inoculated with PBS to control for any lethal effects of the injection process and larvae that received no injection. All *G. mellonella* survival assays were performed in triplicate using different batches of larvae. Survival curves were plotted using the Kaplan–Meier method and differences in survival were calculated by the log-rank test using R studio (version 1.0.143)\(^7\).

**Data availability.** All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

**Accession codes.** The reported plasmid sequences are deposited in GenBank under the following accession numbers: KX618696 (pEC-NRS18), KX618697 (pEC-393), KX618698 (pEC-243) and KX618703 (pEC-125).

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Author Contributions
A.L. and D.M. designed the study. A.L., A.K. and J.B. acquired the data. A.L., J.v.G., A.B and D.C. performed the data analysis. A.L. prepared the first draft of the manuscript. All authors interpreted the data, read, contributed to, and approved the final manuscript.

Additional Information
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