Prevalence and Molecular Characterization of Fluoroquinolone-Resistant Escherichia coli in Healthy Children

Qiang Zhao1,2*, Yueyun Shen3†, Gang Chen1, Yanping Luo1, Shenghui Cui4* and Yaping Tian2*

1 Department of Laboratory Medicine, The First Medical Center, Chinese PLA General Hospital, Beijing, China, 2 Birth Defects Prevention and Control Technology Research Center, Chinese PLA General Hospital, Beijing, China, 3 Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education/Beijing), Department of Clinical Laboratory, Peking University Cancer Hospital & Institute, Beijing, China, 4 Department of Food Science, National Institutes for Food and Drug Control, Beijing, China

Faecal E. coli can act as reservoirs for resistance genes. Here, we analyzed prevalence of drug resistance in faecal E. coli isolated from healthy children at a single kindergarten in Beijing, China, then used whole genome sequencing to characterize fluoroquinolone-non-susceptible strains. Our results revealed high resistance to ampicillin (54.0%), trimethoprim/sulphurmethoxazole (47.5%) and tetracycline (58.9%) among 576 faecal E. coli isolates, 49.2% of which exhibited multidrug resistance. A total of 113 E. coli isolates were not susceptible to ciprofloxacin, with four sequence types, namely ST1193 (25.7%), ST773 (13.3%), ST648 (8.8%) and ST131 (7.1%) found to be the most prevalent (54.9%). With regards to resistance to quinolones, we detected chromosomal mutations in gyrA, parC, and parE in 111 (98.2%), 105 (92.9%), and 67 (61.1%) isolates, respectively. blaCTX-M (37.2%) was the major ESBL gene, whereas blaCTX-M-14 (12.4%) and blaCTX-M-27 (11.5%) were the most frequent subtypes. A total of 90 (79.6%) ExPEC and 65 (57.5%) UPEC isolates were classified. Overall, these findings revealed clonal spread of certain prevalent STs, namely ST1193, ST773, ST648 and ST131 E. coli isolates in healthy children within a single kindergarten in Beijing, China, affirming the seriousness of the multidrug resistance problem and potential pathogenicity of E. coli isolates in healthy children. Therefore, there is an urgent need for increased surveillance to enhance control of this problem.

Keywords: ESBL, Escherichia coli, fluoroquinolone, multidrug resistance, whole genome sequencing

INTRODUCTION

Escherichia coli is an important foodborne opportunistic pathogen, that causes various extraintestinal infections, such as urinary tract infections and septicemia (Zhong et al., 2019; Bonten et al., 2021). Previous studies have reported a high prevalence of fluoroquinolone resistance and extended-spectrum β-lactamase (ESBL) production worldwide (Palma et al., 2017; Boll et al., 2020;
Stapleton et al., 2020). In China, more than 50% of E. coli strains isolated from community-acquired infections are resistant to fluoroquinolones (Zhao et al., 2015), while 16% of these strains are reported to be ESBL-producing (Ling et al., 2006). Faecal E. coli can act as reservoirs for resistance genes, and are also considered a useful indicator for the spread of acquired antibiotic resistance genes in the community (Nys et al., 2004; Salyers et al., 2004). While geographical variation had been demonstrated in resistance rates of E. coli isolates in feces of healthy children (Sahoo et al., 2012), resistances to commonly used antibiotics like ampicillin, tetracycline and trimethoprim/sulfamethoxazole were frequently observed, especially in developing countries (Bartoloni et al., 2006; Dyar et al., 2012; Shaky et al., 2013; Ferjani et al., 2018; Mahmodi et al., 2020). Over 90% of faecal samples from Chinese children tested positive for β-lactam, macrolide, tetracycline, and aminoglycoside resistance genes (Ravensdale et al., 2018), indicating the magnitude of the problem of antimicrobial drug resistance in China. To date, however, only a handful of reports have described antibiotic resistance in faecal E. coli among Chinese healthy children (Lester et al., 1990; Huang et al., 2018). In the present study, we focused on fluoroquinolone resistance in children, since it is restricted in the pediatric population due to concerns about significant adverse effects associated with its use (Jackson and Schutze, 2016). To this end, we determined prevalence of resistance genes in E. coli isolated from rectal swabs from healthy children in China, then applied whole genome sequencing to characterize fluoroquinolone-non-susceptible strains.

**MATERIALS AND METHODS**

**Bacterial Strains and Antimicrobial Susceptibility Testing**

A total of 596 nonduplicate E. coli strains were isolated from rectal swab samples collected as part of routine physical examination from 736 children, aged between 3 and 6 years, at a kindergarten in Beijing, China, in October 2018. The strains were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Vitek MS; bioMérieux, France). The minimal inhibitory concentrations (MICs) of 10 antimicrobial agents, namely ampicillin, ampicillin/sulbactam, trimethoprim/sulfamethoxazole, ciprofloxacin, chloramphenicol, gentamicin, tetracycline, cefotaxime, cefazidime, and imipenem, were assessed by the agar dilution method with E. coli strain ATCC 25922 as the control according to CLSI 2018. Multidrug resistance (MDR) was defined as resistance of an isolate to any antibiotic from at least three different antibiotic groups (Magiorakos et al., 2012).

**DNA Extraction and Whole Genome Sequencing**

E. coli isolates resistant to ciprofloxacin were selected for whole genome sequencing. Briefly, genomic DNA was extracted from the isolates using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), confirmed by agarose gel electrophoresis and quantified by a Qubit 4 Fluorometer (Thermo Fisher Scientific, Singapore, Singapore). Whole genome sequencing was performed on the Illumina HiSeq platform (Illumina, San Diego, CA, USA) to generate 2×150 bp pair-end reads. Raw reads were de novo assembled using SOAP (Li et al., 2008), and the genomes annotated using RAST v2.0 (Aziz et al., 2008) and prokka v1.12 (Seemann, 2014).

**Genome Analysis**

Resistance genes, chromosomal mutations defining quinolone resistance, serotype, fimH subtype, multilocus sequence typing (MLST), and virulence genes of ciprofloxacin-resistant E. coli strains were analyzed using ResFinder 4.0, PointFinder, SerotypeFinder 2.0, FimTyper 1.0, MLST 2.0, and VirulenceFinder 2.0 tools, from the Center for Genomic Epidemiology (CGE) (https://cge.cbs.dtu.dk/services/). E. coli phylogenetic grouping was performed using the ClermonTyping tool (Beghain et al., 2018). Insertion sequence elements were identified using ISfinder (https://isfinder.biotoul.fr/). All drafts and ST-relevant genomes from Enterobase (Zhou et al., 2020) were submitted to kSNP3.0 (Gardner et al., 2015) for single nucleotide polymorphism (SNP) identification, then used to construct a phylogenetic tree with 100 bootstraps which was later visualized using the iTOl website (https://itol.embl.de/). Sequence comparison and map generation were performed using BLAST and Easyfig v2.1 (Sullivan et al., 2011), respectively. Strains were classified as extraintestinal pathogenic E. coli (ExPEC) if they exhibited positivity ≥2 for the following: papAH and/or papC (P fimbriae), sfa-focDE (S and FIC fimbriae), afa-draBC (Dr-binding adhesins), iutA (aerobactin siderophore system), and kpsM II (group 2 capsules). Strains were classified as uropathogenic E. coli (UPEC) if they showed a positivity ≥2 for chuA (heme uptake), fyuA (yersiniabactin siderophore system), vat (vacuolating toxin), and yfcV (adhesin) (Malberg et al., 2020).

**Statistical Analysis**

Categorical variables were assessed using two-tailed chi-square or Fisher exact tests, where appropriate. Data followed by P ≤ 0.05 were considered statistically significant.

**RESULTS**

**Antimicrobial Susceptibility Testing**

The results of resistant rates of individual antibiotics after antimicrobial susceptibility testing among the studied isolates are shown in Table 1. Summarily, a majority of the isolates were highly resistant to tetracycline (58.9%), followed by ampicillin (54.0%) and trimethoprim/sulphamethoxazole (47.5%). On the other hand, low resistance was observed in the isolates to ceftazidime (0.3%), whereas no isolate was resistant to imipenem (0.0%). Almost half of the isolates (n= 293, 49.2%) were resistant to three or more different antibiotics, typical of multidrug resistance.
MLST and Phylogenetic Group
A total of 113 ciprofloxacin-non-susceptible E. coli isolates were selected for whole genome sequencing. Phylogenetically, these strains were broadly distributed into the following groups: B2 (38.1%), A (31.0%), F (8.8%), B1 (8.0%), D (8.0%), G (2.7%), C (1.8%), and E (1.8%). The results from in silico MLST analysis revealed 35 different STs, including 3 novel ones (ST11494, ST11495, and ST11496) which have been submitted to Enterobase. ST1193 revealed 35 different STs, including 3 novel ones (ST11494–ST11496). This diversity was primarily due to the presence of a large number of STs in the ST1193 isolates. ST131 and ST1193 belonged to phylogenetic group B2, while ST648 and ST773 belonged to groups F and G, respectively (Figure 1).

Serogroup and fimH Type
Among 113 E. coli strains, 25 O serogroups and 20 fimH types were identified. The most prevalent O serogroup was O75 (24.8%), followed by O1 (13.3%), O25 (9.7%), and O21 (9.7%). In addition, fimH allele subtyping analysis showed that fimH64 was the most frequent subtype (25.7%), followed by fimH27 (9.7%), fimH54 (6.2%), and fimH30 (5.3%). Notably, almost all of the ST1193 isolates belonged to the O75-fimH64 type, while most of the ST648 and ST773 isolates belonged to O1 and O21 serogroups, respectively. For ST131 isolates, there were two major serogroup-fimH types, namely O25-fimH30 and O16-fimH41.

Detection of Antimicrobial Resistance Genes
We adopted ResFinder and PointFinder tools for analysis of antimicrobial resistance genes, and detected resistance to eight groups, namely aminoglycosides, β-lactams, quinolones, macrolides, tetracyclines, phenicol, fosfomycin, and sulphonamides. Notably, all of the 113 ciprofloxacin-resistant E. coli strains carried one or more resistance genes, 103 (91.2%) out of which carried three or more resistances to different antibiotic groups. With regards to resistance to quinolones, we detected chromosomal mutations in gyrA, parC, and parE in 111 (98.2%), 105 (92.9%), and 67 (61.1%) isolates, respectively. The most prevalent chromosomal mutations included gyrA (p.S83L) (98.2%), parC (p.S80I) (90.3%), and gyrA (p.D87N) (81.4%). Moreover, the mutation rate of parE among the four most prevalent STs isolates (ST1193, ST773, ST648 and ST131) was significantly higher than that among other isolates (91.9% vs 23.5%, p=0.000). Notably, parE (p.L416F), parE (p.L529L) and parE (p.S458A) were the unique chromosomal mutation types of parE in ST1193, ST131 and ST648 isolates, respectively. Apart from chromosomal mutations, we also detected aac (6’)-Ib-cr (8.8%), qnrS1 (7.1%), opxAB (4.4%), qnrS2 (2.7%) and qnrB4 (1.8%). At least one candidate quinolone resistance gene or chromosomal mutation could be found in all of the 113 ciprofloxacin-resistant E. coli strains. With regards to ß-lactam resistance, blaCTX-M (37.2%) was the major ESBL gene, of which blaCTX-M-1 (12.4%) and blaCTX-M-14 (11.5%) were the most frequent subtypes. ISecp1/ISecp1Δ (100%) was found upstream of blaCTX-M in both CTX-M-1 and CTX-M-9 groups. IS903B/IS903A (100.0%) was always found downstream in the CTX-M-9 group, while orf477/orf477A (83.3%) was common in the CTX-M-1 group. Besides ISecp1 and IS903B, IS26 (57.1%) was also found to be adjacent to blaCTX-M genes frequently (Table 2). For aminoglycoside resistance, the main genes carried by these isolates were strA (55.8%), strB (57.5%), adaA5 (53.1%), and aac(3)-IId (40.7%). sul1 (56.6%) and sul2 (57.5%) were the most prevalent genes encoded sulphonamide resistance while fosA3 (4.4%) and fosA7 (1.8%) were the major genes for fosfomycin resistance. The main tetracycline resistance genes detected were tet(A) (61.9%) and tet(B) (17.7%). Neither mcr nor carbapenemase-encoding genes were detected.

Virulence Genes
The results from VirulenceFinder analysis revealed the presence of 90 (79.6%) ExPEC and 65 (57.5%) UPEC isolates among the 113 ciprofloxacin-resistant E. coli strains. All of the ST131 and ST1193 isolates, which belonged to phylogenetic group B2, could be classified as both ExPEC and UPEC owing to the fact that they harbored iutA, kpsMII/kpsMII-K1/kpsMII-KS, papA, chuA, and fyuA virulence genes. Despite belonging to phylogenetic group A, and different from ST131 and ST1193, all 15 ST773 isolates were identified as ExPEC, and harbored kpsMII-K1, papA, and papC virulence genes (Table 3).

Prevalence of E. coli ST773 Isolates
The phylogenetic tree obtained by core-genome SNP analysis corroborated the findings from MLST analysis and ClermonTyping phylogenetic group segregation (A, B1, B2, C, D, E, and F).
Notably, the four most prevalent STs, ST1193, ST773, ST648 and ST131, were clustered within a monophyletic clade (Figure 1). We also downloaded sequences of draft genomes of 54 ST773 strains isolated across 6 continents (Africa, Asia, Europe, North America, Oceania, and South America) from Enterobase, and incorporated them in our genome analysis to construct a phylogenetic tree comprising 69 ST773 isolates. The results showed that all ST773 isolates belonged to the phylogenetic group A, and revealed 4 serogroups. The most prevalent serogroup was O21:H52 (56.5%), followed by H52 (21.7%), O11:H52 (18.8%), and O11:H4 (2.9%). The phylogenetic tree further clustered the four serogroup isolates into three clades, designated A-C: O11:H52 and H52 serogroups belonged to clade B while O21:H52 serogroup belonged to clade A (Figure 2). Notably, we detected the same chromosomal mutations in all but three of the ST773 isolates, namely gyrA (p.S83L)-gyrA (p.D87N)-parC (p.S80I). In addition, the chromosomal mutation rate of parE (p.S458A) in O11:H52 and H52 isolates (clade B) was significantly higher than that of O21:H52 isolates (clade A) (100.0% vs 38.7%, p=0.000). With regards to resistance to β-lactams, blaCTX-M (37.7%) was the major ESBL gene, of which blaCTX-M-14 (26.1%) and blaCTX-M-15 (7.2%) were the most frequent types.

**DISCUSSION**

Antibiotic resistance is a major global public health concern. Faecal *E. coli* is considered a key indicator for the transmission of acquired antibiotic resistance genes in the community. For instance, a previous systematic review reported that resistance...
to many primary care prescribed antibiotics was common among *E. coli* from faecal matter, and this was carried by asymptomatic children, especially in non-Organization for Economic Co-operation and Development countries (Bryce et al., 2016). In addition, multidrug resistance and ESBL production of commensal *E. coli* isolates were observed in more than 36.2 and 11.7%, respectively, among healthy children under 3 years old in Iran (Mahmoodi et al., 2020). The results from the present study revealed that a majority of the *E. coli* strains isolated from faecal matter of Chinese healthy children were highly resistant to ampicillin, trimethoprim/sulphamethoxazole and tetracycline, while almost half of the isolates were multidrug resistant, which was consistent with previous studies (Bartoloni et al., 2006; Shkaya et al., 2013; Mahmoodi et al., 2020). Notably, although the use of fluoroquinolones is restricted in the pediatric population because of the associated musculoskeletal adverse effects (Jackson and Schutze, 2016), 17.6% of the *E. coli* strains exhibited resistance to ciprofloxacin in this study.

Next, we employed whole genome sequencing to determine genetic characteristics of the 113 ciprofloxacin-resistant *E. coli* strains. Overall, the results indicated that resistance to fluoroquinolones was associated with amino acid substitutions in quinolone-resistance-determining-regions (QRDR), including *gyrA*, *parC*, and *parE*. *blaCTX-M*, especially *blaCTX-M-14* and *blaCTX-M-27*, contributed to third-generation cephalosporin resistance. In addition, more than 90% of those strains harbored resistance genes for three or more antibiotic groups, while 80% and 60% of them were classified as ExPEC and UPEC strains, respectively. These findings affirmed the seriousness of the problem of multidrug resistance and potential pathogenicity

**TABLE 2 | Genetic environments of *bla*<sub>CTX-M</sub> in *Escherichia coli* isolates.**

| *bla*<sub>CTX-M</sub> | Genetic environment | No. |
|----------------------|---------------------|-----|
| CTX-M-1 group        | *bla*<sub>CTX-M-3</sub> | IS26–ISEcp1A–*bla*<sub>CTX-M-3</sub>–Tn903B | 2 |
|                     | *bla*<sub>CTX-M-15</sub> | IS26–ISEcp1A–*bla*<sub>CTX-M-15</sub>–Tn903B | 2 |
|                     | *bla*<sub>CTX-M-55</sub> | IS26–ISEcp1A–*bla*<sub>CTX-M-55</sub>–Tn903B | 1 |
| CTX-M-9 group        | *bla*<sub>CTX-M-14</sub> | IS26–ISEcp1A–*bla*<sub>CTX-M-14</sub>–Tn903B | 2 |
|                     | *bla*<sub>CTX-M-27</sub> | IS26–ISEcp1A–*bla*<sub>CTX-M-27</sub>–Tn903B | 2 |
|                     | *bla*<sub>CTX-M-65</sub> | IS26–ISEcp1A–*bla*<sub>CTX-M-65</sub>–Tn903B | 1 |

**TABLE 3 | Distribution of virulence genes associated with ExPEC/UPEC identification in 113 ciprofloxacin-non-susceptible *Escherichia coli* isolates (n, %).**

| Virulence factor | non-prevalent STs | ST1193B2 n = 29 | ST131B2 n = 8 | ST648F n = 10 | ST773A n = 15 | Total n = 113 |
|-----------------|------------------|----------------|--------------|--------------|--------------|---------------|
|                 | B2/D n = 15 | | | | | |
| ExPEC           | 14 (93.3) | 17 (47.2) | 29 (100.0) | 8 (100.0) | 7 (70.0) | 15 (100.0) | 90 (79.8) |
| *luxA*          | 13 (86.7) | 23 (63.9) | 29 (100.0) | 8 (100.0) | 7 (70.0) | 7 (64.6) | 87 (77.0) |
| *aafC*          | 3 (20.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (20.0) | 0 (0.0) | 5 (4.4) |
| *rfaE*          | 3 (20.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (20.0) | 0 (0.0) | 5 (4.4) |
| *kpaMI*         | 6 (40.0) | 4 (11.1) | 0 (0.0) | 2 (25.0) | 1 (10.0) | 0 (0.0) | 13 (11.5) |
| *kpaMI*K1       | 5 (33.3) | 2 (5.6) | 29 (100.0) | 0 (0.0) | 0 (0.0) | 15 (100.0) | 51 (45.1) |
| *kpaMI*K5       | 4 (26.7) | 3 (8.3) | 0 (0.0) | 6 (75.0) | 8 (80.0) | 0 (0.0) | 21 (18.8) |
| *kpaMI*K52      | 0 (0.0) | 1 (2.8) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.9) |
| *papA*          | 12 (80.0) | 15 (41.7) | 29 (100.0) | 8 (100.0) | 0 (0.0) | 15 (100.0) | 79 (69.9) |
| *papC*          | 6 (40.0) | 12 (33.3) | 0 (0.0) | 2 (25.0) | 0 (0.0) | 15 (100.0) | 35 (31.0) |
| *tocC*          | 0 (0.0) | 2 (5.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.9) |
| *sfA*           | 0 (0.0) | 1 (2.8) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (0.9) |
| *UPEC*          | 14 (93.3) | 4 (11.1) | 29 (100.0) | 8 (100.0) | 10 (100.0) | 0 (0.0) | 65 (57.5) |
| *chuA*          | 15 (100.0) | 5 (13.9) | 29 (100.0) | 8 (100.0) | 10 (100.0) | 0 (0.0) | 67 (59.3) |
| *fyxA*          | 13 (86.7) | 19 (52.8) | 29 (100.0) | 8 (100.0) | 10 (100.0) | 14 (93.3) | 93 (82.3) |
| *vat*           | 6 (40.0) | 1 (2.8) | 28 (96.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 35 (31.0) |
| *yfcV*          | 6 (40.0) | 0 (0.0) | 29 (100.0) | 7 (87.5) | 10 (100.0) | 0 (0.0) | 52 (46.0) |
of *E. coli* isolates in healthy children. Therefore, there is an urgent need for increased surveillance and control of this phenomenon.

The results from our phylogenetic and MLST analyses revealed existence of clonal spread of *E. coli* isolates, especially for the four most prevalent STs, namely ST1193, ST773, ST648 and ST131, consistent with previous studies that have demonstrated that *E. coli* strains can be shared within households (Madigan et al., 2015; Johnson et al., 2016). In fact, *E. coli* ST131 has emerged as a major pathogen of blood-stream and urinary tract infections worldwide (Chen et al., 2019; Birgy et al., 2020; Findlay et al., 2020; Holland et al., 2020). To date, several factors, including resistance to fluoroquinolones, affiliation to phylogroup B2, and high virulence gene contents, have been associated with its successful spread (Valenza et al., 2019). Petty et al. reported that most fluoroquinolone-resistant ST131 strains belonged to a single subclone, designated clade C. Surprisingly, most CTX-M-15-producing ST131 isolates were also derived from a single clade within clade C, named clade C2 (Petty et al., 2014). In the present study, we identified 8 ST131 strains among the 113 ciprofloxacin-resistant *E. coli* isolates, of which 2 were CTX-M-15 positive. Notably, 6 of the 8 ST131 isolates clustered in clade C (Figure S1), while the 2 CTX-M-15-producing ST131 belonged to clade C2 (Figure S2), which was consistent with the findings of Petty et al. (2014). Since 2012, a new fluoroquinolone-resistant clone namely ST1193, and belonging to phylogenetic group B2, has been reported worldwide (Zhao et al., 2015; Johnson et al., 2019; Tchesnokova et al., 2019). Results of the present study showed that ST1193 was one of the most prevalent STs, accounting for a quarter of all ciprofloxacin-resistant strains. Almost all of the ST1193 isolates belonged to the O75-fimH64 type, and exhibited a set of four conserved mutations in QRDR (gyrA S83L, gyrA D87N, parC S80I and parE L416F), which was consistent with the findings of Valenza et al. (2019). ST648 strains have been

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**FIGURE 2** | Phylogenetic tree and genomic characteristics of 51 *Escherichia coli* ST773 isolates. Solid and hollow signs indicate presence and absence of acquired resistance genes and chromosomal mutations in QRDR, respectively.
reported globally in human patients and more incidentally from animals, because of ESBL phenotype and ExPEC-association (Ewers et al., 2014; Fernandes et al., 2018). Apart from the well-known prevalence of ST131, ST1193 and ST648, ST773 has been considered another key ST, despite having been rarely reported in the past. Despite its affiliation with phylogenetic group A, all 15 ST773 isolates were identified as ExPEC. In fact, almost all of these isolates harbored three conserved mutations that were associated with fluoroquinolone resistance (gyrA D87N, gyrA S83L, parC S80I), while nearly one-third of the isolates carried the blaCTX-M gene. Our phylogenetic tree revealed that 36 ST773 strains isolated across 6 continents exhibited a high degree of homology with our isolates, suggesting the potential of the widespread dissemination of this clone.

In conclusion, we identified four prevalent STs, namely ST1193, ST773, ST648 and ST131, in E. coli isolates. These exhibited clonal transmission across healthy children within a single kindergarten in Beijing, China. Since multidrug resistance and potential pathogenicity of E. coli is a serious problem among healthy children, we envisage that our findings will stimulate relevant discussions to guide urgent development of surveillance approaches to help control this phenomenon.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in GenBank under BioProject no. PRJNA679380.

ETHICS STATEMENT

Neither ethics committee approval, nor informed consent were required as all collected data was fully anonymized, there was no contact with patients and/or their families and no interventions to treatment were made, in accordance with local guidelines.

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