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Prolonged carriage of OXA-244-carbapenemase-producing *Escherichia coli* complicates epidemiological investigations

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ABSTRACT

The rapid increase of OXA-244-producing *Escherichia coli*, predominantly driven by genetically clustered isolates of sequence type (ST)38, has been observed in at least nine European countries, including Germany. However, the reasons for the spread of OXA-244-producing *E. coli* remain unclear. Here, we aim to evaluate the possibility of prolonged carriage. We identified a total of six different patients with repeated detection of OXA-244-producing *E. coli* isolates, which were subjected to both short and long-read whole-genome sequencing (WGS). Besides allelic differences using core genome multilocus sequence typing (cgMLST) analyses, we obtained numbers of single-nucleotide polymorphisms (SNPs) to calculate individual base-pair substitution (BPS) rates. To assess possible re-exposure and risk factors for prolonged carriage, case interviews were conducted. The time between detections ranged from eleven months to more than three years. Initial isolates originated in three+ out of six cases from clinical samples, whereas remaining samples were from screening, mostly in the inpatient setting. As expected, cgMLST analyses showed low numbers of allelic differences between isolates of each case ranging from 1 to 4, whereas numbers of SNPs were between 2 and 99 (mean = 36), thus clearly highlighting the discrepancy between these different bacterial typing approaches. For five out of six cases, observed BPS rates suggest that patients can be colonized with OXA-244-producing *E. coli*, including ST38 cluster isolates, for extensively long times. Thus, we may have previously missed the epidemiological link between cases because exposure to OXA-244-producing *E. coli* could have occurred in a time frame, which has not been evaluated in previous investigations. Our results may help to guide future epidemiological investigations as well as to support the interpretation of genetic diversity of OXA-244-producing *E. coli*, particularly among ST38 cluster isolates.

1. Introduction

Carbapenemase-producing Enterobacterales (CPE) are of major public health concern. Their enzyme-mediated resistance substantially limits available treatment options for patients suffering from severe infections. Different types of carbapenemases have been described, of which the *Klebsiella pneumoniae* carbapenemase (KPC), the New Delhi metallo-ß-lactamase (NDM), the Verona Integron-metallo-ß-lactamase (VIM) and the Oxacillinase-48 (OXA-48) are the most common. A variant of the latter is OXA-244, which differs from OXA-48 by a single amino-acid substitution showing reduced activity against carbapenems, thus making its detection particularly challenging (Oteo et al., 2013, Hoyos-Mallecot et al., 2017). It was first described in 2013 in a *Klebsiella pneumoniae* isolate from Spain and has also been identified in *K. aerogenes* and *Proteus mirabilis* from Russia as well as in sporadic cases of *Escherichia coli* from Germany, France and the Netherlands (Oteo et al., 2013, Fursova et al., 2015, van Hattem et al., 2016, Hoyos-Mallecot et al., 2017, and references therein). However, unlike its plasmid localization in the Enterobacterales species, OXA-244 has predominantly been found to be integrated into the chromosome in *E. coli*,

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including isolates belonging to the sequence type (ST)38 (Oteo et al., 2013, Fursova et al., 2015, Hoyos-Mallecot et al., 2017, Potron et al., 2016). Indeed, besides other global lineages of extraintestinal pathogenic *E. coli* (ExPEC) carrying $bla_{OXA-244}$, such as ST10, ST69, ST131, ST167 and ST361, the emergence and rapid dissemination of OXA-244-producing *E. coli* in Europe was predominantly due to genetically clustered isolates of ST38, as revealed by a rapid risk assessment coordinated and published by the European Centre for Disease Prevention and Control (ECDC) (ECDC, 2020, Hammerum et al., 2020, Welker et al., 2020). However, outbreak investigations in Denmark (Hammerum et al., 2020), Germany (Kremer et al., 2020) and France (Emeraud et al., 2021) revealed only limited overlap in place and time of patients with OXA-244-producing *E. coli* ST38 cluster isolates. Thus, it remains unclear whether this is due to community transmission or a common source. Following an urgent inquiry owing to an outbreak in Norway in 2021, the ECDC published an update on the rapid risk assessment to call attention on healthcare-associated transmission and the continued spread of OXA-244-producing *E. coli* in Europe (ECDC, 2021). For further outbreak investigations, the ECDC issued a questionnaire, focusing on the month before detection regarding nutrition habits and the last 12 months for possible expositions addressing both medical history and travel. However, *E. coli* can persist for over 12 months in the gastrointestinal tract, what might cause a time gap between detection of and exposure to OXA-244-producing *E. coli* (e.g. Titelman et al., 2014, Nakane et al., 2016). The objective of the present study was to verify the possibility of prolonged carriage of patients with OXA-244-producing *E. coli* isolates as well as to conduct a comprehensive investigation of these cases.

2. Material and methods

2.1. Background and samples

All diagnostic laboratories in Germany are encouraged to send isolates suspected of being carbapenemase-producing to the National Reference Centre (NRC) for multidrug-resistant Gram-negative bacteria, which provides the verification and genotyping as a free service. Along with the sample, diagnostic laboratories are asked to provide certain epidemiological data, including pseudonymized patient information, such as sex and date of birth. Details on the phenotypic and molecular methods applied for the identification of carbapenemases can be found elsewhere (Pfennigwerth et al., 2020). Among our sample collection, we identified a total of six different patients with multiple OXA-244-producing *E. coli* isolates with a minimum of 11 months between sampling dates.

2.2. Epidemiological investigation

In Germany, reporting of infections or colonisations with carbapenemase-producing or carbapenem-resistant Enterobacterales is mandatory according to the German Infection Protection Act. Notifications in the reporting system at the Robert Koch Institute corresponding to the cases of prolonged carriage were identified by using an automated probabilistic matching strategy based on birth month and year, sex, federal state, sample material, date of analysis and the type of reported carbapenemase. Identified notifications were confirmed manually in contact with local health authorities.

In a retrospective cohort study design, confirmed cases were contacted and in case of informed consent, a semi-structured interview was conducted by an epidemiologist and a medical doctor. We used an adapted version of the questionnaire developed by ECDC and investigated past travel, hospitalizations, contact to other cases and animals, diet as well as individually possible re-exposure to the suspected source of transmission and the medical history including risk factors for prolonged carriage like immunosuppression, implanted devices, and antibiotic treatment (Ljungquist et al., 2019).

2.3. Sequencing and data analyses

Bacterial cultures were grown overnight in LB medium (PanReac AppliChem ITW Reagents, Darmstadt, Germany) supplemented with ampicillin (100 mg/ml). Genomic DNA from 1 ml bacterial cultures was extracted using the Easy-DNA gDNA Purification Kit (Invitrogen, Thermo Fisher Scientific, Schwerte, Germany) and prepared for whole-genome sequencing (WGS) using the Nextera XT DNA Library Preparation Kit (Illumina, Eindhoven, Netherlands) following the manufacturer's instructions. Libraries were paired-end sequenced either on a HiSeq (2 ×251 bp) or a MiSeq instrument (2 ×301 bp) (Illumina, San Diego, United States) and quality of raw sequences was checked using FastQC v0.11.9 (Andrews, 2010). Mash Distance v2.1 and Mash Screen v2.1 were applied on raw reads for species identification and contamination check, respectively (Ondov et al., 2016, 2019). Reads were de novo assembled using SPAdes v3.10.1 with default parameters in the careful mode (Bankevich et al., 2012). Quality metrics of assemblies were assessed using QUAST v5.0.2 without a reference sequence (Gurevich et al., 2013). Assemblies were uploaded to the SeqSphere+ software version 9.0.3 (Ridom, Muenster, Germany), which was used for multilocus sequence typing (MLST) following the Achtman scheme and core genome (cg)MLST based on the Enterobase *E. coli* scheme (2513 loci). Between isolates of each case and isolates of the same sequence type from our previous study (Kremer et al., 2020), pairwise allelic differences were computed and used to construct a neighbour-joining tree, which was illustrated in Interactive Tree Of Life (iTOL) version 6.5.7 (Letunic and Bork, 2021).

In addition, high molecular weight DNA of the first isolate of each case was extracted from 1 ml bacterial cultures using the Wizard HMW DNA Extraction Kit (Promega, Walldorf, Germany) following the manufacturer's instructions. As described in the applicable protocols, DNA extracts of three isolates were subjected to multiplex long read sequencing using the Rapid Barcoding Kit (SQK-RBK004) followed by loading onto a FLO-MIN106D flow cell and a ONT MinION device (Oxford Nanopore Technologies, Oxford, United Kingdom). Embedded in the MinKNOW software package, Guppy (6.5.7) was used for basecalling and demultiplexing of reads, which were directly converted into the fastq file format. Raw reads from the Illumina and the ONT sequencing platforms were combined as input for Unicycler v0.5.0 (Wick et al., 2017) to generate a hybrid assembly, of which the circularised chromosome was used as reference for subsequent single nucleotide polymorphism (SNP) calling. Prior to SNP calling, raw Illumina reads generated from the second isolate were trimmed using Trimmomatic v0.39 to remove potential adapters and low-quality bases (Bolger et al., 2014). Snippy (4.6.0), which is based on the BWA-MEM and Samtools pipeline, was used with default parameters to call high-quality SNPs from the trimmed Illumina data by covering the mutational base by at least 10 reads, which corresponds to a depth of coverage of 10x (Seemann, 2015). In this context, however, it has to be noted that the pipeline applied also identifies nucleotide insertions and deletions (indels), which were not used for subsequent analyses because (i) these might not only reflect mutational alterations but also correspond to sequencing errors of the ONT technology and (ii) there is scarce information on values for indel rates specifically measured for *E. coli*. Numbers of SNPs were used to individually calculate the base-pair substitution (BPS) rate for isolates of each case in order to evaluate the possibility of prolonged carriage of strains. In addition, circularised chromosomes were annotated using Rapid Annotation Using Subsystem Technology (RAST)(Aziz et al., 2008) and the chromosomal region harbouring the $bla_{OXA-244}$ gene was visualized using Easyfig (version 2.2.2) (Sullivan et al., 2011) in the context of other *E. coli* genomes publicly available (Aziz et al., 2008, Sullivan et al., 2011), which were also used for genome comparison using BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011).

Table 1
Characteristics of cases with repeated detection of OXA-244 producing *Escherichia coli* and interview results. Isolates in bold were used as reference sequence for subsequent analyses (see text for details).

Case Nr. & Matching Information	Isolate	Sampling date	Colonisation Period[a]	Age (first detection) & Sex	Material of isolate	Infection status	Hospitalisation (at detection)	Antibiotic treatment (prior detection)	Previous hospitalisation (< 12 months)	Travel exposition (< 24 months)	Comorbidities of patient
Case 1(matched)	**NRZ-11542a**	2014-01-13	3 years, 3 months and 19 days	29 Male	Screening (rectal)	-	-	-	-	-	-
	NRZ-34242	2017-05-02			Screening (rectal)	-	-	-	-	-	-
Case 2(matched and interviewed)	NRZ-37217[b]	2017-08-04	1 year, 2 months and 23 days	0	Blood	-	-	Unknown	Yes	No	Percutaneous endoscopic gastrostomy tube
	NRZ-36584	2017-08-22		Male	Screening (rectal)	Colonised	No				
	NRZ-47214	2018-11-14			Screening (rectal)	-	No	Unknown	No[a]	No[a]	
Case 3(matched and interviewed)	**NRZ-50242**	2019-03-25	11 months and 1 day	56	Screening (rectal)	Colonised	Yes	Yes	No	Turkey	Urolithiasis
	NRZ-59076	2020-02-26		Male	Screening (rectal)	Colonised	Yes	Yes	No[a]	No[a]	Urinary catheter
Case 4(matched and interviewed)	**NRZ-50602**	2019-04-19	1 year, 6 months and 29 days	68 Male	Blood	Infected	Yes	Yes	Yes	Morocco	Metastatic lung cancer, stroke
	NRZ-64296	2020-11-17			Screening (rectal)	Colonised	Yes	No	No[a]	United Arab Emirates[a]	See above
Case 5(matched and interviewed)	**NRZ-51765**	2019-06-01	1 year, 10 months and 26 days	79 Male	Tracheobronchial secretion	Infected	Yes	Unknown	Yes	No	COPD, encephalitis
	NRZ-67572	2021-04-27			Screening (rectal)	Colonised	Yes	Unknown	Yes[a]	No[a]	COPD
Case 6(matched)	**NRZ-51464**	2019-05-16	2 years, 4 months and 26 days	0 Male	Screening (rectal)	-	Yes	-	-	-	-
	NRZ-71760	2021-10-12			Screening (other than rectal)	-	-	-	-	-	-

[a] between first and second detection
[b] date of sampling was not available at the time the study was conducted

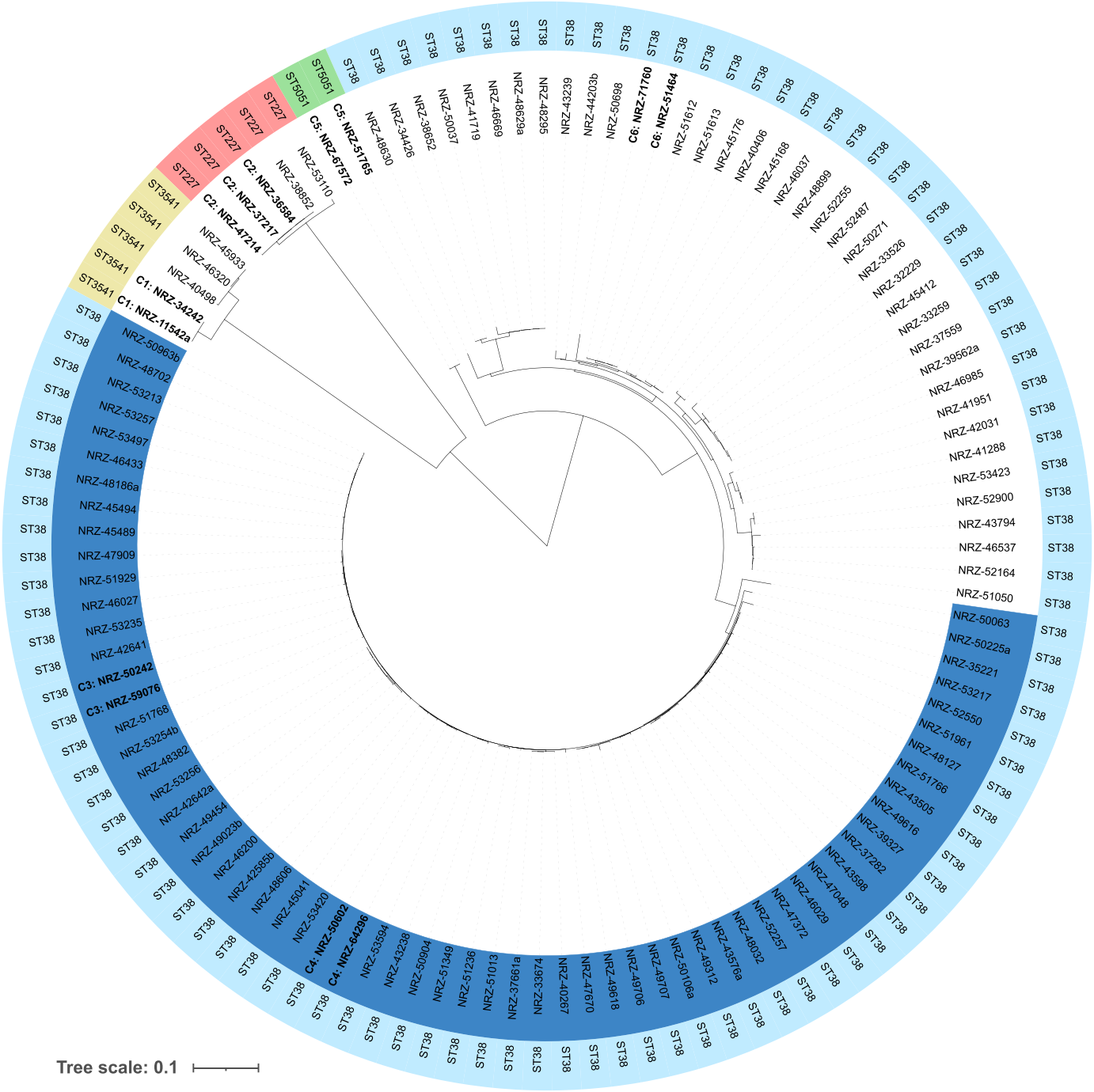

Fig. 1. Phylogenetic tree of OXA-244 producing *Escherichia coli*, including isolates from our previous study (Kremer et al., 2020). Numbers in bold correspond to isolates of cases from the present study. Highlighted in dark blue is the ST38 cluster.

2.4. Ethics statement

A formal ethical review was not required for the outbreak investigation in accordance with Article 25, Section 1 of the German Infection Protection Act of 2001. Informed consent for the interview was obtained from the patient or his or her legal guardian.

3. Results

3.1. Epidemiological investigation of cases

With a total of 13 OXA-244-producing *E. coli* isolates, we identified six different cases suggestive for prolonged carriage (Table 1). All cases were detected in different facilities in 4 federal states. The timespan between sampling dates of the first and the last isolate of the same patient ranged from 11 months to 3 years and 3 months. All patients could be identified in the mandatory reporting system. Thereby, additional information on the patient as well as infection and hospitalization status were added to the laboratory data.

We were able to conduct case interviews with four out of the six identified cases. All cases were male and all interviewed cases had comorbidities prior detection. Two of the six cases were infants at the time of detection, one patient was 29 years old, while the others were above 55 years. The cases were hospitalised for 7 out of 9 detections, for

Table 2
Summary of sequencing results of OXA-244 producing *Escherichia coli* from the six cases.

	MLST	Complex Type	Allelic differences (cgMLST)	Genome size (Mbp)	No. of SNPs	No. of SNPs/ year
Case 1	ST3541	494	1	4.74	2	1
Case 2	ST227	7016	4	4.74	23	19
Case 3	ST38 Cluster	2883	1	5.19	99	107
Case 4	ST38 Cluster	2883	4	5.23	31	20
Case 5	ST5051	7224	4	4.95	16	8
Case 6	ST38 Non-cluster	7221	4	5.35	43	18

which information was available. As a result, screening was the most frequent sampling material (n = 10). Three out of four interviewed cases reported hospitalization within 12 months and two reported travel to Turkey or Morocco within 24 months before the first detection. Information on diet and contacts to animals were without any noticeable clustering. Regular antibiotic treatment two times per year was reported by case 3.

3.2. Genetic analyses

Illumina WGS data of the seven OXA-244-producing *E. coli* isolates from the six cases were subjected to MLST and cgMLST analyses and compared to OXA-244-producing *E. coli* isolates of the same ST from our previous study (Kremer et al., 2020). The isolates from the present study belonged to four different sequence types: ST3541 (C1: case 1), ST227 (C2: case 2), ST5051 (C5: case 5), and ST38 (C3: cases 3; C4: case 4; and C6: case 6), including isolates of cases 3 and 4, which grouped into the ST38 cluster (Fig. 1 and Table 2). Besides overall low numbers of pairwise allelic differences (AD) between isolates of the same case ranging from 1 to 4 AD, cgMLST analysis showed that isolates from the same case clustered closely together within the phylogenetic analyses indicating their close genetic relatedness (Fig. 1 and Table 2).

To further evaluate the possibility of prolonged carriage of patients with OXA-244-producing *E. coli*, we combined ONT and Illumina reads to generate a hybrid assembly from the first isolate of each case, which was used as high-quality reference for subsequent SNP calling (Table 1). Overall numbers of SNPs ranged from 2 to 99 (mean = 36) (Table 2). However, to account for the different sampling intervals of cases, we also calculated the numbers of SNPs per year with values ranging from 1 to 107 SNPs/year (mean = 29 SNPs/year). While cases 1, 2, 4, 5 and 6 showed overall low numbers of annual SNPs (1 – 20 SNPs/year), we detected substantially higher numbers of SNPs per year for case 3 (107 SNPs/year).

Consistent with previous findings, we identified the chromosomal integration of $bla_{OXA-244}$ in all isolates analyzed, as illustrated in Fig. 2. However, comparison of sequences encompassing flanking

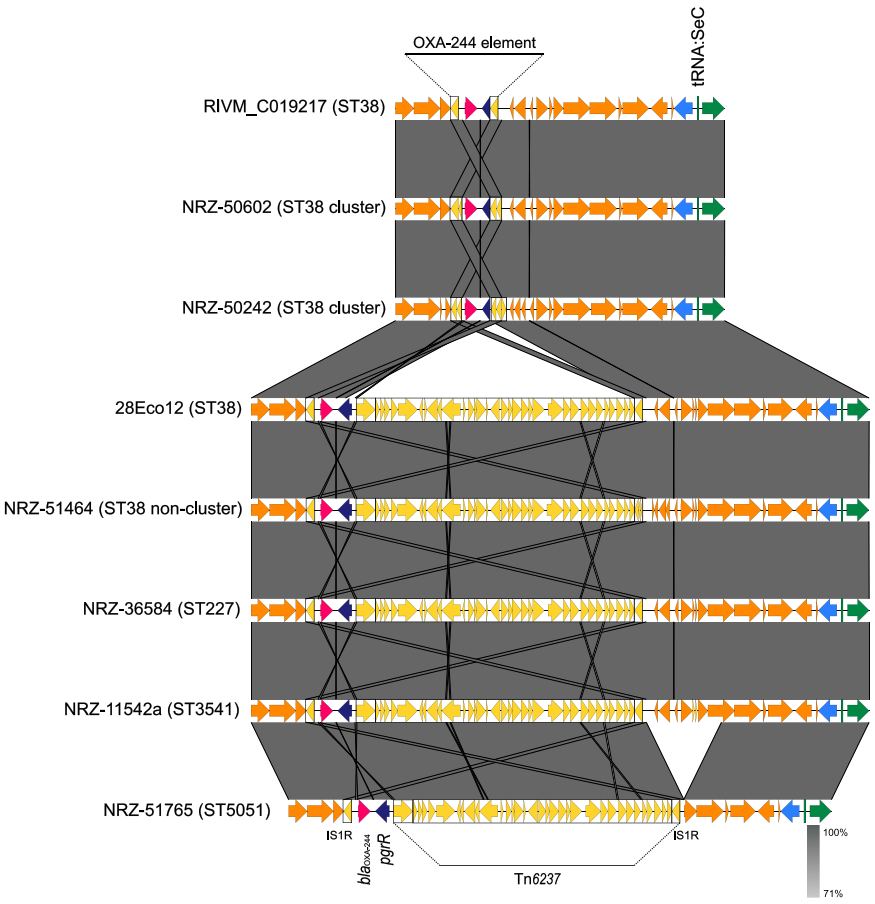

Fig. 2. Comparison of the chromosomal region containing the $bla_{OXA-244}$ gene in isolates from the present study with selected isolates of ST38 from public databases. Shown is the chromosome region upstream of tRNA:SeC highlighted in green. Arrows represent annotated coding sequences with the $bla_{OXA-244}$ gene highlighted in pink, the transcriptional regulator gene *pgrR* highlighted in dark blue, the prophage integrase gene *intS* highlighted in turquoise, the transposon Tn*6237* highlighted in yellow, the genomic island highlighted in orange. Mobile genetic elements are framed and pairwise sequence similarities are indicated by the grey colour code.

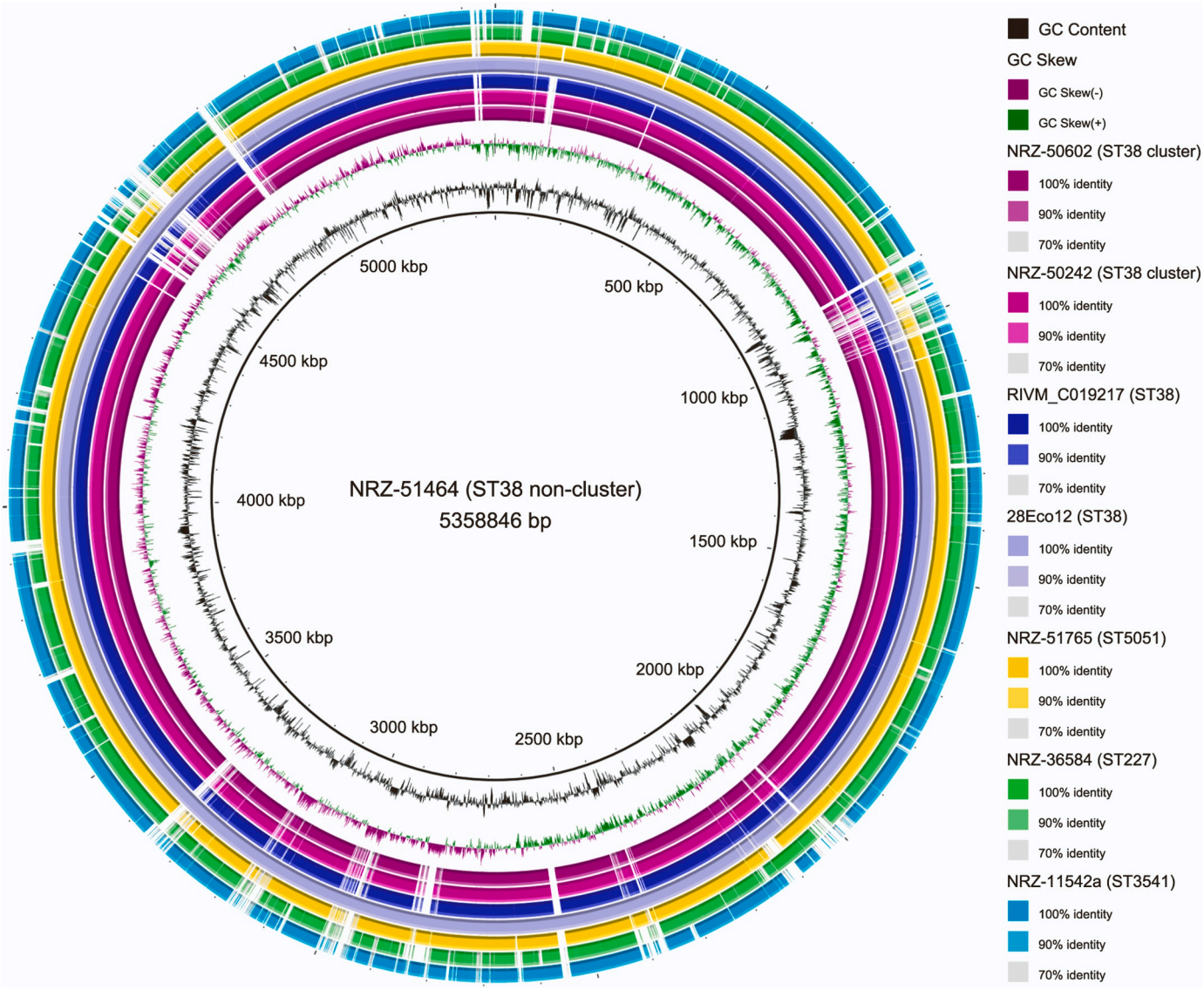

Fig. 3. BLASTn comparison of complete *Escherichia coli* chromosomes harbouring the $bla_{OXA-244}$ gene. The isolate NRZ-51464 (ST38 non-cluster) from the present study was used as reference. Order of strains from internal to external is as follows: NRZ-50602 (ST38 cluster), NRZ-50242 (ST38 cluster), RIVM_C019217 (ST38), 28Eco12 (ST38), NRZ-51765 (ST5051), NRZ-36584 (ST227), and NRZ-11542a (ST3541).

chromosomal regions revealed three different variants. In three isolates, comparative analyses showed that the $bla_{OXA-244}$ gene was embedded in the transposon Tn*6237* inserted into a genomic island within the tRNA: SeC gene, as has previously described in the isolate 28Eco12 (Abril et al., 2019). Although an identical localization of $bla_{OXA-244}$ within Tn*6237* was identified, we detected a partial loss of flanking sequences in one isolate corresponding to the second variant. In comparison to the ST38 cluster isolates from the present study, however, we identified a complete genome of an *E. coli* isolate (RIVM_C019217) from the Netherlands showing a similar $bla_{OXA-244}$ element suggestive of being a remnant of the Tn*6237* transposon (Notermans et al., 2022, Lindemann et al., 2023). Overall, we identified substantial differences between chromosome sizes of strains belonging to the different ST ranging between 4.74 to 5.35 Mb (Table 2). In particular, strains of ST38 showed consistently larger genomes as compared to the other strains which is in accordance to other completed genomes of OXA-244-producing *E. coli* ST38 isolates obtained from public databases (Fig. 3).

4. Discussion

Until 2022, OXA-244 has become the most prevalent carbapenemase among *E. coli* isolates in Germany indicating the rapid and ongoing increase of OXA-244-producing *E. coli* (Pfennigwerth et al., 2023). Despite extensive outbreak investigations to track the source and understand transmission routes of OXA-244-producing *E. coli*, an epidemiological link could hitherto not be identified, which at least in part may also be due to bacterial strain characteristics (Titelman et al., 2014). Here, we show that patients may carry the same strain of OXA-244-producing *E. coli* for over three years. Our study focussed on 13 isolates from six patients with repeated detection of OXA-244-producing *E. coli*. For five out of six cases, including one ST38 cluster and one ST38 non-cluster strain, obtained BPS rates seem to support our hypothesis of prolonged carriage of OXA-244-producing *E. coli*. In this context, however, it has to be noted that we cannot provide definitive mutation rates due to the difficulties to unambiguously measure bacterial generation times in the human host. Nevertheless, by applying a doubling time of 15 h, which has previously been suggested for *E. coli* in the wild, our extrapolated BPS rates of 10^{-9} per site per generation would roughly agree

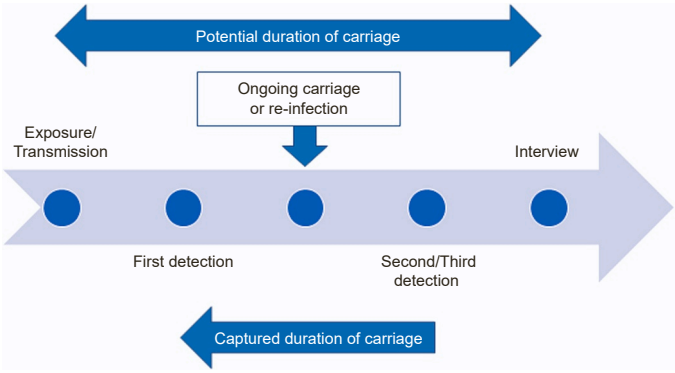

Fig. 4. Time lag between exposure and detection and interview compared to captured time of carriage. Detection refers to isolates that were sent to the NRC.

with previously published values, thus further supporting prolonged carriage of patients with OXA-244-producing *E. coli* (Gibson et al., 2018, Drake, 1991, Lee et al., 2012, Jee et al., 2016). Case 3, however, carried an OXA-244-producing *E. coli* ST38 cluster isolate, for which higher rates have been observed. Although this might suggest two genetically different OXA-244-producing *E. coli* isolates, we did not find indications for re-exposure because neither hospitalisation nor contact to another known case or travel between detections was reported by case 3. Instead, repeated antibiotic treatment of case 3 might account for the higher BPS rate observed as a result from continuous selective pressure against OXA-244-producing *E. coli* (Long et al., 2016). This, however, needs experimentally to be evaluated. Nevertheless, in accordance with our findings, prolonged carriage of OXA-244-producing *E. coli* has also been reported from Dutch travellers (van Hattem et al., 2016). Furthermore, in a prospective cohort study, 15% of patients with community acquired urinary tract infection caused by ESBL-producing *E. coli* or *K. pneumoniae* still had fecal carriage after 3 years or more (Jørgensen et al., 2017). Thus, the duration of carriage might even be longer, considering the time between exposure and first detection as well as lacking information on negative samples or later positive samples that were not sent to the NRC (Fig. 4). Our results suggest that continued colonization is most probable in cases with prolonged carriage, whereas re-exposure and re-infection with the same pathogen seems unlikely although we cannot rule out the possibility of community transmission of OXA-244-producing *E. coli*. In this context, healthcare-associated transmission of OXA-244-producing *E. coli* has also been reported (ECDC, 2021). However, the overall high proportion of outpatients are suggestive for another frequent source of infection (ECDC, 2020) and isolation and risk precautions in patients with OXA-244-producing *E. coli* make a nosocomial re-infection unlikely. Amongst common epidemiological links, travel to North Africa or the Middle East has been suggested as relevant exposure and was reported in two out of four interviewed cases but none of the interviewed cases reported travel to the same region (as a potential place of re-infection) before the second detection.

5. Limitations

We identified only a small number of isolates, which were predominantly detected in hospitalized patients with additional comorbidities and either of very young or older age. Thus, our collection of isolates as well as the results obtained may not be representative for the general population. In addition, we neither can infer the frequency of cases with prolonged carriage nor their duration because repeatedly detected isolates are commonly not sent to the NRC. Finally, the data quality of the interviews may be influenced by a recall bias since the interviews were conducted after the second detection.

6. Conclusions

In summary, our results suggest that patients can be colonized with OXA-244-producing *E. coli* for several years without obvious re-exposure. This emphasizes the impact of these pathogens on patients and public health as well as the importance of surveillance and compliance with infection and prevention control measures. Outbreak investigations referring only to the past 12 months may need to be reconsidered towards an extended timeframe. The recall bias that is implicated in such an extended timeframe calls for a focus on nutrition habits instead of specific food consumption in past weeks in investigations of possible foodborne transmission, and a One Health surveillance approach in order to be able to find links to food items independently from individual memory. Finally, our estimated base-pair substitution rate of OXA-244-producing *E. coli* may support interpretation of genetic diversity within cluster isolates.

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CRediT authorship contribution statement

Brinkwirth Simon: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Reichert Felix:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Hans Jörg B.:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gatermann Sören:** Data curation. **Werner Guido:** Data curation. **Eckmanns Tim:** Data curation. **Fritsch Lena Sophie:** Formal analysis, Investigation, Methodology. **Haller Sebastian:** Data curation. **Niels Pfennigwerth:** Data curation.

Declaration of Competing Interest

No conflict of interest by the authors.

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