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Identification of $bla_{OXA-51-like}$, bla_{OXA-58}, bla_{DIM-1}, and bla_{VIM} Carbapenemase Genes in Hospital *Enterobacteriaceae* Isolates from Sierra Leone

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We describe the results of a molecular epidemiological survey of 15 carbapenemase-encoding genes from a recent collection of clinical isolates from Mercy Hospital in Bo, Sierra Leone. The most salient findings revealed that (i) 60% of the isolates harbored multiple carbapenemase genes; (ii) the bla_{DIM-1} gene, which has previously only been reported in The Netherlands, is also circulating in this environment; and (iii) $bla_{OXA-51-like}$ and bla_{OXA-58} genes, which were thought to reside exclusively in *Acinetobacter* species, can also be found in members of the *Enterobacteriaceae*.

Carbapenem antibiotics are currently the most potent β-lactam antibiotics clinically available and are used as a last resort to treat infections caused by multidrug-resistant Gram-negative pathogens. The significance and usage of this family of antibiotics have risen dramatically over the last decade, especially in hospital settings, due to the global spread and increasing prevalence of bacterial pathogens that harbor extended-spectrum β-lactamase genes that confer resistance to all β-lactam compounds except for carbapenems (1). Not surprisingly, the positive selection pressure provided by the increased usage of carbapenems has resulted in the emergence and expansion of carbapenem resistance in a number of common nosocomial pathogenic species (1).

Carbapenem resistance is mediated mostly by β-lactamase enzymes that are capable of hydrolyzing carbapenem compounds (carbapenemases) and often differ in host microorganism range, substrate specificity, and β-lactamase inhibitor sensitivity (2, 3). Furthermore, carbapenemases are frequently found in pathogenic strains that contain additional genetic determinants that confer resistance to aminoglycosides, tetracyclines, β-lactams, and fluoroquinolones and can result in intractable infections with high mortality rates (4, 5). The spread of Ambler class A, B, and D carbapenemase genes, which were encountered only rarely less than 2 decades ago, has been facilitated by conjugative plasmids, transposons, and integrons to such an extent that carbapenemase genes have now been reported worldwide (2). Despite this fact and the clinical impact of these genes, the true incidence and prevalence of carbapenem resistance and carbapenemase genes are still unknown, as many countries do not track and report antibiotic resistance rates; this is particularly true in many African countries (1).

To better understand the level, distribution, and evolution of multidrug resistance in environments that currently lack a national antibiotic stewardship policy, we initiated a molecular epidemiology surveillance program at Mercy Hospital in Bo, Sierra Leone. The preliminary evidence, obtained using an antimicrobial resistance determinant microarray as a screening tool (6), indicated the presence of class B metallo-β-lactamase (MBL) and class D carbapenem-hydrolyzing β-lactamase (CHDL) genes in this environment. In this study, we utilized PCR and DNA sequencing to target 15 carbapenemase genes (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, $bla_{OXA-51-like}$, $bla_{OXA-58-like}$, bla_{AIM}, bla_{BIC}, bla_{DIM}, bla_{GIM}, bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{SIM}, bla_{SMP}, and bla_{VIM}) from a recent collection of Mercy Hospital isolates to better understand the carbapenemase content of actively circulating strains. Ethical approval was obtained from the Njala University Institutional Review Board. All clinical samples were obtained from Mercy Hospital as preexisting diagnostic specimens that had been stripped of all identifiers. The Gram-negative clinical isolates analyzed ($n = 20$) were collected from November 2010 to April 2011 on agar media and then identified based on PCR amplification and sequencing of a 16S rRNA gene amplicon that spanned the V3 and V4 variable regions (7) and of the *rpoB* and/or *gyrA* gene, as previously described (8, 9). For a preliminary identification, the obtained 16S rRNA gene sequences were used to classify each isolate by using the naive Bayesian classifier available through the Ribosomal Database Project (release 10) (http://rdp.cme.msu.edu/classifier/classifier.jsp), with ≥80% confidence as the identification threshold (10). This analysis identified 70% of the isolates as members of the *Enterobacteriaceae*, with the remaining isolates belonging to the *Pseudomonadaceae*, *Burkholderiaceae*, or *Comamonadaceae*. Secondary identifications based on *rpoB* or *gyrA* gene sequencing not only confirmed the 16S rRNA gene identifications but also provided genus- and species-level resolution to reveal well-known

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TABLE 1 Summary of carbapenemase gene-containing hospital isolates in Bo, Sierra Leone

Strain	16S rRNA gene identification (% confidence)[a]	*rpoB* or *gyrA* identification (% identity to reference sequence)	Presence of gene			
			$bla_{OXA-51-like}$[d]	$bla_{OXA-58-like}$[e]	bla_{VIM}	bla_{DIM}
SL-1	*Enterobacteriaceae* (100)	*Enterobacter cloacae*[b] (100)	64	58	−	+
SL-3	*Enterobacteriaceae* (100)	*Enterobacter cloacae*[b] (100)	−	−	+	−
SL-4	*Pseudomonas* (93)	*Comamonas testosteroni*[c] (100)	−	58	−	+
SL-5	*Escherichia/Shigella* (98)	*Escherichia coli*[b] (100)	65	58	+[f]	+
SL-6	*Escherichia/Shigella* (100)	No amplification	−	−	−	−
SL-7	*Enterobacteriaceae* (80)	*Enterobacter cloacae*[b] (98)	−	58	−	+
SL-8	*Enterobacteriaceae* (100)	No amplification	−	−	+[f]	−
SL-9	*Enterobacteriaceae* (100)	*Enterobacter cloacae*[b] (100)	98	58	+	+
SL-10	*Enterobacter* (80)	*Enterobacter* sp.[c] (90)	−	58	−	−
SL-11	*Klebsiella* (99)	*Klebsiella pneumoniae*[b] (100)	71[g]	58	+[f]	−
SL-12	*Klebsiella* (98)	*Klebsiella pneumoniae*[b] (100)	71 or 241	−	+[f]	−
SL-13	*Klebsiella* (98)	*Klebsiella pneumoniae*[b] (99)	98	58	+	−
SL-14	*Providencia* (100)	No amplification	98	58	+	−
SL-15	*Enterobacteriaceae* (100)	*Enterobacter cloacae*[b] (97)	98	58	+[f]	−
SL-16	*Klebsiella* (100)	*Klebsiella pneumoniae*[b] (99)	−	58	+	−
SL-18	*Pseudomonas* (100)	No amplification	−	58	+	+
SL-19	*Burkholderia* (100)	No amplification	−	58	−	+[f]
SL-20	*Delftia* (97)	*Delftia acidovorans*[c] (99)	−	58	−	−
SL-21	*Burkholderia* (100)	No amplification	−	58	−	−
SL-22	*Pseudomonadaceae* (100)	No amplification	−	58	+	+

[a] Derived from 16S rRNA gene sequencing. All identifications are family- or genus-level identifications.
[b] Derived from *rpoB* gene sequencing using the methods of Mollet et al. (8). All identifications are genus- and species-level identifications.
[c] Derived from *gyrA* gene sequencing using the methods of Tayeb et al. (9). All identifications are genus- and species-level identifications.
[d] Numbers indicate the identified OXA-51-like variant. The variants were identified using 94% of the complete gene sequence in all cases except for SL-11 and SL-12, for which ~30% of the gene sequence was obtained.
[e] Numbers indicate the identified OXA-58-like variant. The variants were identified using 56% of the complete gene sequence in all cases.
[f] The obtained amplicon was not sequenced. All other amplicons lacking this designation were confirmed by DNA sequencing.
[g] Identified as a new variant of OXA-71.

nosocomial pathogens (*Enterobacter cloacae*, *Escherichia coli*, and *Klebsiella pneumoniae*). In addition, the genomic DNA from one strain (SL-1) was subjected to low-pass whole-genome sequencing using an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA), and the assembled and annotated draft sequences were mined for carbapenemase genes and their flanking regions.

Targeted PCR analyses were performed using previously described primers and conditions (11, 12), and selected amplicons were sequenced for allele confirmation. Importantly, 11 of the 15 targeted carbapenemase genes (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-48}, bla_{AIM}, bla_{BIC}, bla_{GIM}, bla_{IMP}, bla_{KPC}, bla_{NDM}, bla_{SIM}, and bla_{SMP}) were not detected in any of the isolates tested. However, the remaining targeted Ambler class B (bla_{VIM} and bla_{DIM-1}) and D ($bla_{OXA-51-like}$ and $bla_{OXA-58-like}$) carbapenemase genes were detected and codetected in a surprising number of strains (Table 1). In fact, 60% of the tested isolates harbored multiple carbapenemase genes.

Perhaps even more surprising was the detection of the $bla_{OXA-58-like}$ and $bla_{OXA-51-like}$ CHDL genes in 85% and 40% of the isolates, respectively, as these genes were thought to reside exclusively in *Acinetobacter* species. While $bla_{OXA-58-like}$ genes are known to reside on conjugative plasmids in *Acinetobacter* spp., the $bla_{OXA-51-like}$ genes have been considered exclusively chromosomally encoded, intrinsic oxacillinase genes of *Acinetobacter baumannii* and are used by many investigators for species identification and strain typing (2, 13, 14). However, a number of recent reports indicate that the $bla_{OXA-51-like}$ genes have been mobilized and are spreading to other *Acinetobacter* spp. by conjugative plasmids (15, 16). Our findings bolster this contention and corroborate a recent meeting abstract that describes the presence of $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ genes in *Klebsiella pneumoniae* and *Escherichia coli* isolates, respectively (17). Furthermore, an analysis of the SL-1 draft genome sequence identified this strain as an *Enterobacter cloacae* isolate that harbored a complete bla_{OXA-58} open reading frame surrounded by flanking sequences that were 100% identical to IS*Aba3*, found in a number of *Acinetobacter* plasmids (GenBank accession no. KC004135). Taken together, the results demonstrate the presence of the bla_{OXA-58} and $bla_{OXA-51-like}$ carbapenemase genes in non-*Acinetobacter* Gram-negative genera and suggest that these genes should no longer be considered genetic determinants that can be used for the specific identification of *Acinetobacter* species.

The $bla_{OXA-51-like}$ genes were detected in eight isolates (Table 1). Previously published primers (primers OXA-51-likeF and OXA-51-likeR [12] and primers 5′OXA-51-like-all-F and 3′OXA-51-like-all-R [18]) were used to amplify and sequence these genes for allelic identification and to determine the presence of flanking IS*Aba1* sequences (using primers ISAba1F [18] and OXA-51-likeR). The obtained sequences were screened against a database of >80 currently known $bla_{OXA-51-like}$ genes, and a total of five different $bla_{OXA-51-like}$ variants were found (see Fig. S1 in the supplemental material). Nearly full-length sequences (94% of the entire gene) of the $bla_{OXA-51-like}$ variants revealed the presence of OXA-64 (strain SL-1), OXA-65 (strain SL-5), and OXA-98 (strains SL-9, SL-13, SL-14, and SL-15) genes. For two of the strains (SL-11 and SL-12), it was only possible to amplify and sequence ~30% of the gene. Although greater sequence coverage is needed for unambiguous identification of these variants, the

obtained sequence information was sufficient to determine that the $bla_{OXA-51-like}$ gene in SL-11 contained a novel combination of single nucleotide polymorphisms (SNPs) that warranted its classification as a new variant of the OXA-71 gene. The same amount of sequence information also revealed that the $bla_{OXA-51-like}$ gene found in strain SL-12 matched either the OXA-71 or OXA-241 gene. IS*Aba1* insertion sequences were detected in each of the $bla_{OXA-51-like}$ gene-containing isolates, but they were not found flanking the $bla_{OXA-51-like}$ genes. Similar analyses were performed to determine the allelic identity of the 16 $bla_{OXA-58-like}$ genes that were detected in these isolates. Only two different sequence variants were identified, with one present in strains SL-1 and SL-11 and the other in all of the remaining positive samples. The variants differed by two SNPs (see Fig. S2), both of which generated silent mutations. The deduced amino acid sequences for both variants were identical and matched exactly with the OXA-58 sequence.

In addition to the CHDL genes, two MBL genes were also identified among the tested isolates. Sixty percent of the isolates carried identical bla_{VIM} sequences (which, based on the amplicon sequence, were consistent with bla_{VIM-2} or four other, less frequently observed variants: bla_{VIM-10}, bla_{VIM-16}, bla_{VIM-17}, and bla_{VIM-30}), and 40% of the isolates were found to contain bla_{DIM-1}. While bla_{VIM} (and especially bla_{VIM-2}) is acknowledged as one of the most commonly isolated MBLs in Africa and throughout the world (19–22), the presence of bla_{DIM-1} has been reported only once before, for a *Pseudomonas stutzeri* isolate from The Netherlands (23). In this study, bla_{DIM-1} was found in hospital isolates belonging to the families *Enterobacteriaceae*, *Pseudomonadaceae*, *Burkholderiaceae*, and *Comamonadaceae*. Importantly, the complete bla_{DIM-1} gene sequence was also found within the SL-1 draft genome and was found to be flanked by sequences consistent with a type 1 integron (GenBank accession no. KC004136).

In this study, we have presented the first molecular epidemiological survey of carbapenemase genes in West Africa. With respect to antimicrobial resistance determinants, this is a wholly uncharacterized environment. It is also an especially challenging environment in that the lack of surveillance coincides with easy over-the-counter access to the most commercially available antibiotics, little to no usage oversight, low-level awareness of infection control practices, and the absence of a national antibiotic stewardship policy. To begin to ascertain whether these factors may have played a role as the selection pressure that led to these findings, we conducted a voluntary survey of 15 independent pharmacies and 5 hospital dispensaries (including Mercy Hospital) to determine the availability of antimicrobial compounds in Bo, Sierra Leone. While none of the hospital dispensaries issued antibiotics without a prescription, 87% of the pharmacies that had access to the same collection of antibiotics as the hospital dispensaries acknowledged readily dispensing antimicrobial drugs without a prescription. Interestingly, none of the survey participants acknowledged issuing the carbapenem antibiotics imipenem-cilastatin, meropenem, ertapenem, and doripenem, and the availability and use of carbapenem antibiotics have not previously been reported for this region. However, β-lactam antibiotics such as amoxicillin, ampicillin, cloxacillin, and, to a lesser extent, penicillin, cefuroxime, and ceftriaxone were readily issued (as were antibiotics from other classes, such as chloramphenicol, ciprofloxacin, clarithromycin, cotrimoxazole, doxycycline, erythromycin, metronidazole, gentamicin, rifampin, tetracycline, ofloxacin, and nitrofurantoin). Overall, the survey suggested that antibiotics in general are available and in use for self-treatment without a prescription or any professional supervision. Thus, while the selection pressure for the maintenance of the carbapenemase genes identified in this study does not appear to be due to the indiscriminate use of carbapenem antibiotics *per se*, it is possible that these alleles are maintained in this population due to the use of β-lactam antibiotics or their genetic linkage to other elements that may provide a more direct selective advantage. Furthermore, similar queries of antibiotic usage for veterinary medicine and food animal production have not been conducted in this environment and thus cannot be dismissed as the source of such selection pressure.

The serious implications of the dissemination of carbapenemase genes has led to the assertion that the early identification of strains containing these molecular determinants is necessary for effective infection control measures and informed therapeutic options (4, 24). Although it cannot be presumed that the presence of class D carbapenemase genes within genetic backgrounds other than *Acinetobacter* will result in high resistance to carbapenem antibiotics (13, 25, 26), these findings clearly demonstrate a broadening reservoir for these resistance determinants. Taken together and considering the needs, resources, and level of existing characterization of this environment, our findings warrant the continued use of molecular tools to better understand the scope, severity, and evolution of carbapenemase gene circulation and multidrug resistance in this part of the world.

Nucleotide sequence accession numbers. Newly determined nucleotide sequences have been deposited in GenBank under accession numbers KC004135 and KC004136.

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There are no conflicts of interest to declare.

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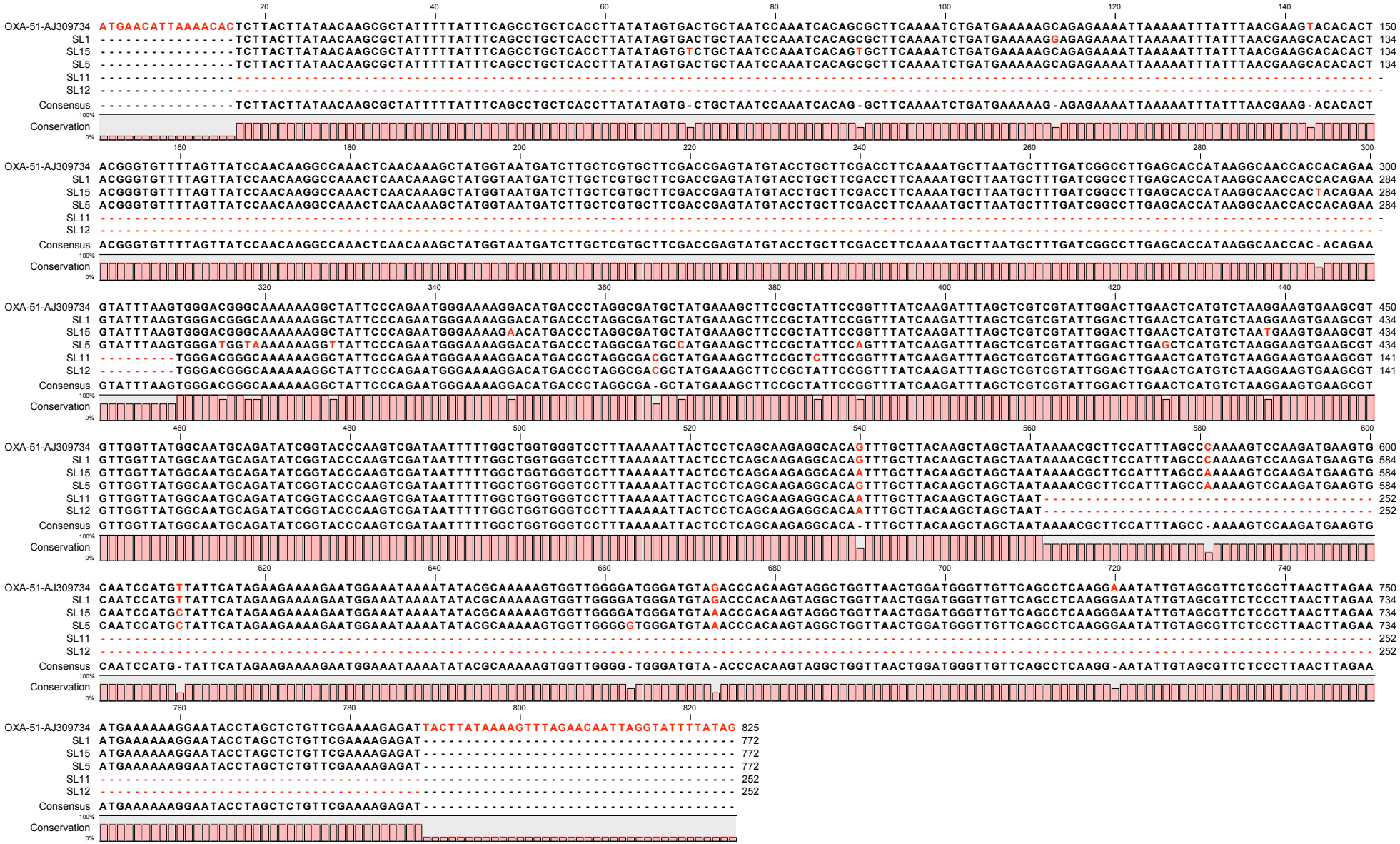
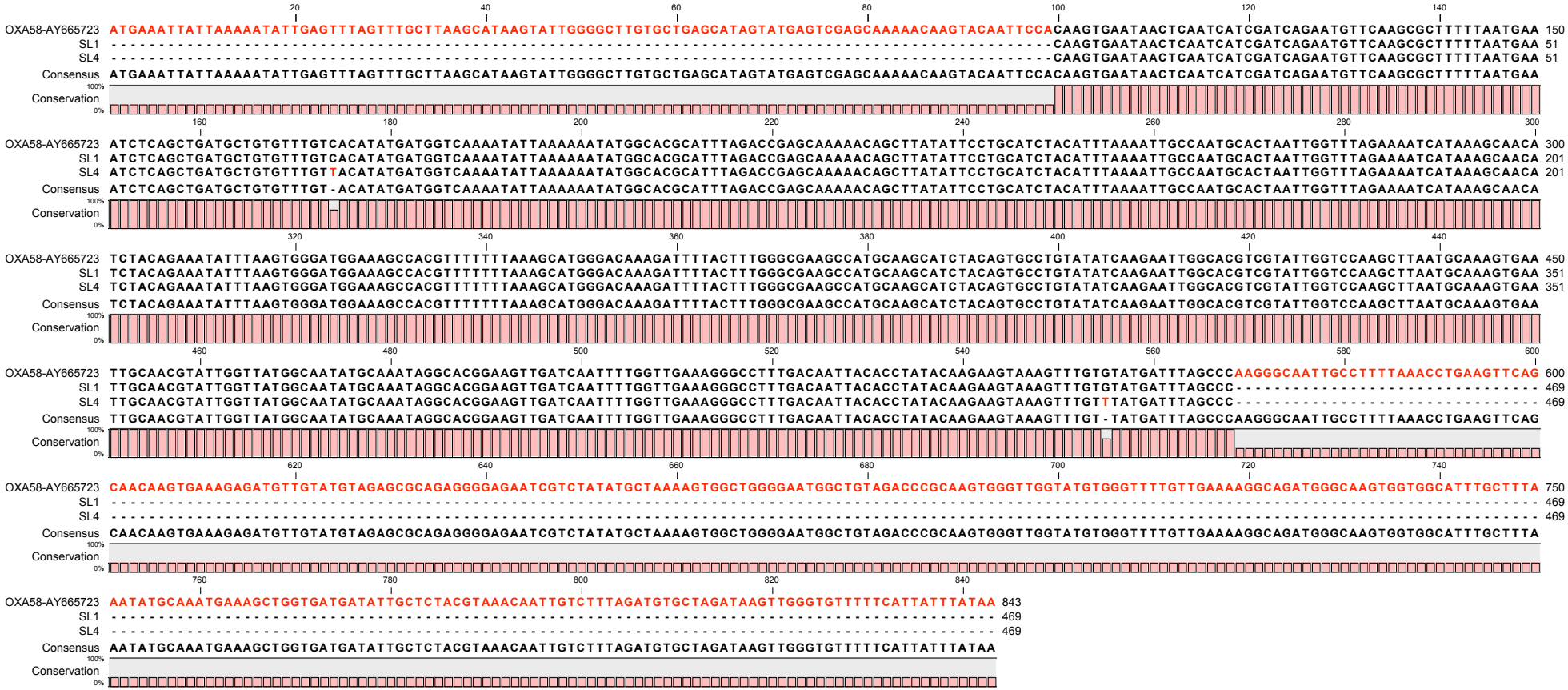
Supplemental Figure S1. Alignment of *bla*$_{OXA51}$-like genes



The amplicons of *bla*$_{OXA-51-like}$ genes were sequenced in both directions, trimmed to the equal length and aligned together with reference *bla*$_{OXA-51}$ gene (accession no. AJ309734). Only one sequence for each variant was selected for alignment. The aligned fragments span a gene fragment that corresponds to nucleotide positions 17 to 788 (94% sequenced variants) and 310 to 561 (partial 30% sequence only variants) of the *bla*$_{OXA-51}$ gene. Single nucleotide polymorphisms (SNPs) in sequences of detected variants are highlighted using red font, a dash "-" in consensus sequence and lower bars in histogram below sequence alignment.

Supplemental Figure S2. Alignment of *bla*$_{OXA58}$-like genes



The amplicons of *bla*$_{OXA-58}$ genes were sequenced in both directions, trimmed to the equal length and aligned together with reference *bla*$_{OXA-58}$ gene (accession no. AY665723). Only one sequence for each variant was selected for alignment. The aligned fragments span a gene fragment that corresponds to nucleotide positions 100 to 568 (approximately 56%) of the *bla*$_{OXA-58}$ gene. Single nucleotide polymorphisms (SNPs) in sequences of detected variants are highlighted using red font, a dash "-" in consensus sequence and lower bars in histogram below sequence alignment.