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Molecular basis of Carbapenem-Resistant *Acinetobacter baumannii* from Southwest, Nigeria

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ABSTRACT

Information on the molecular basis of resistance to carbapenem resistant *Acinetobacter baumannii* (CRAB) in Nigeria is sparse. This study was aimed at charaterising the molecular basis of CRAB isolated from tertiary hospitals in Southwest Nigeria. Identity of clinical isolates was confirmed using Oxoid 12E Microbact™ Gram-negative identification system, $bla_{OXA\text{-}51\text{-}like}$ primers, and *16S-rRNA* sequencing. Antimicrobial susceptibility was performed by disk diffusion method using 34 antimicrobial agents belonging to 8 classes of antibiotics. Molecular characterization was conducted on 21 isolates with resistance phenotype to imipenem and meropenem. Detection and characterization of carbapenem resistance determinants were achieved by PCR. Reference strains, *A. baumannii* type strain ATCC 17978 and *Acinetobacter baylyi* ADP1 were used as controls. All isolates were resistant to ≥14 antimicrobial agents tested, with 95.9% isolates resistant to 20-34 antibiotics. Resistant phenotype of 78.7% and 57.4% were recorded for meropenem and imipenem respectively, with ciprofloxacin (36.1%), amikacin (37.9%) and polymyxin B (39.3%). Oxacillinase gene, $bla_{OXA\text{-}51\text{-}like}$ was detected in all isolates, and $bla_{OXA\text{-}23}$ carrying *ISAba1* element upstream of gene within genomic resistant island (GRI) Tn*AbaR1* and cephalosporinase gene, $bla_{ADC\text{-}7}$ were also detected in (18/21) of the isolates. Carbapenem resistant *A. baumannii* isolates from Southwest Nigeria encode genes that code for resistance to carbapenem.

Key words: *Acinetobacter baumannii*, Carbapenem resistance, Oxacillinase genes and Nigeria.

INTRODUCTION

Acinetobacter baumannii (*A. baumannii*) is a bacterial species which can subsist on moist and dry environments. This characteristic has allowed for the widespread in clinical facilities, colonizing diverse surfaces including, medical instrumentation and clothing of hospital staff and patients, thriving as commensal on skins or hair of medical personnel as well as patients. The aptitude of this bacterium to acquire multiplicity of virulence factors and thrive in health care facilities for protracted periods has led to its emergence as a successful global opportunistic hospital acquired pathogen. Apart from bacteremia and secondary meningitis, *A. baumanni* is implicated in various infections of urinary and respiratory tracts, skin, soft tissues, solid organ transplant, and burn wounds (Vijayakumar *et al*., 2018; Huang *et al*., 2019). Antibiotic resistance mechanisms of *Acinetobacter* strains include efflux pumps, β-lactamases, and modifications in porin proteins. *A. baumannii* expresses aminoglycoside-modifying enzymes (AMEs) making them resistant to aminoglycoside antibiotics. Again, mutations in the *gyrA* and *parC* genes confer on them resistance to quinolones (Munoz-Price and Weinstein, 2008). Also important are the findings of Fournier *et al*. (2006) who detected genomic resistance island, AbaR1 a region with 86 kb and encompassing a cluster of resistance genes, coding for tetracycline efflux pumps, several AMEs, AmpC, and OXA-10 β-lactamases. Genetic analysis of this region also revealed the presence of transposons and genes formerly identified in *Salmonella* spp. and *E. coli* (Fournier *et al*., 2006). The rapid emergence of carbapenem-resistant *A. baumannii* (CRAB) within the past decade has been reported around the world as a public health issue. A United States national surveillance study conducted in 2010 reported a prevalence of 44.7% and 49.0% among *A. baumannii* strains resistant to imipenem and meropenem, respectively (Queenan *et al*., 2012; Huang *et al*., 2019). A recent publication by Huange *et al*., (2019), revealed that mortality rate of infections caused by CRAB has risen to 76.0% from 16.0%. Similarly, findings on mortality rate regarding Korea and China concerning blood infections revealed 79.8% and 70.0% respectively to infections caused by CRAB (Kim *et al*., 2012; Lee *et al*., 2014). Enzymatic degradation by β-lactamases is the most prevalent mechanism of β-lactam resistance in *A. baumannii*. According to the Ambler classification, carbapenemases mainly belong to class A, B, and D β-lactamases. Serine oxacillinases an Ambler class D

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β-lactamases are most promiscuous and commonly disseminated in *A. baumannii*, which are comprised of OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, and OXA-23, and is the primary cause of carbapenem resistance in *A. baumannii* (Higgins *et al*., 2013; Huang *et al*., 2019). Insertion sequence (IS) elements are the smallest bacterial transposons that play an essential role in antimicrobial resistance genes acquisition in bacteria and bla_{OXA} carbapenemase genes may be regulated by these mobile genetic elements, especially ISAba1 (Yazdansetad *et al*., 2019). ISAba1 belongs to the IS4 family and has11-bp inverted repeat sequences flanked by 9-bp direct repeats of the target sequence (Pagano *et al*., 2016; Yazdansetad *et al*., 2019). Presence of the ISAba1 promoter sequence in association with bla_{OXA} genes strongly provides carbapenem resistance in *A. baumannii* (Turton *et al*., 2006; Bahador *et al*., 2015; Yazdansetad *et al*., 2019). Usually, OXA-51-like is intrinsic to *A. baumannii* and naturally exists in all strains and can be overexpressed when flanked by IS elements (Yazdansetad *et al*., 2019). Mobile elements that are incorporated into bacterial chromosomes at specific locations via the action of a site-specific recombinase, which is known as integrative or integrating elements, playing an important role in the spread of virulence determinants and antibiotic resistance determinants (Hamidian *et al*., 2015). Accumulating studies have revealed that the chromosomes of some *A. baumannii* strains harbour large clusters of horizontally transferred genes conferring resistance to multiple antibiotics and heavy metals, which are integrated at a specific site in a particular ATPase (*comM*) gene (Fournier *et al*., 2006; Post *et al*., 2010, Krizova *et al*., 2011). Nine such genomic resistance islands (*A. baumannii* resistance islands [AbaRs]) have been fully characterized, eight of which were found in strains of EU clone I, including AbaR1, AbaR3, AbaR5, AbaR6, AbaR7, AbaR8, AbaR9, and AbaR10 (Krizova *et al*., 2011). Some strains of EU clone I were also found to harbour a bla_{OXA-23} gene-carrying island known as AbaR4, which was integrated at a chromosomal site different from that of the *ATPase* gene (Krizova *et al*., 2011; Turton *et al*., 2011) and the backbone of which is formed by a Tn6021 transposon (Krizova *et al*., 2011). In this study, we carried out an in-depth characterization of extremely resistant non-duplicated *A. baumannii* strains recovered from clinical sources of patients admitted into the tertiary hospitals in Southwest Nigerian.

MATERIALS AND METHODS

Study population

Multi-drug resistant suspected *A. baumannii* isolates, 78.7% and 57.4% resistant to meropenem and imipenem respectively, were collected from 72 patients from southwest tertiary hospitals. Isolates were collected from the six southwest states as follows: Ekiti (N = 6), Lagos (N = 25), Ogun (N = 6), Ondo (N = 3), Osun (N = 6), and Oyo (N = 26). The isolates were collected between April, 2011 and May, 2013 from blood (N = 22), urine (N = 26), and wound swab (N = 24) samples.

Bacterial isolates identification

The recovered *A. baumannii*, isolates were cultured on constituted multidrug resistant Leeds Acinetobacter medium (MDR-LAM) and all isolates were presumptively designated as *A. baumannii* based on 12E Microbact™ Gram-nagative identification system (Oxoid Ltd, Basingstoke, UK). Putatively, isolates were confirmed by polymerase chain reaction (PCR) amplification of the $bla_{OXA-51-like}$ carbapenemase gene and the *16S-rRNA* gene sequencing (Figure 1).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing against 34 antimicrobial agents consisting of 18 β-lactams/cephalosporins, 2 macrolides, 4 fluoroquinolones, 3 aminoglycosides, chloramphenicol, tetracycline, nitrofurantoin, nalidixic acid, trimethoprim-sulfamethoxazole, polymyxin B, and colistin were ordered from Oxoid Ltd, Basingstoke, UK outlet in Lagos and were carried out according to Clinical and Laboratory Standards Institute (CLSI) procedures (CLSI, 2017). *A. baumannii* isolates were categorized as multidrug-resistant (MDR), according to the definition provided by Magiorakos *et al*. (2012). Reference strains, *A. baumannii* type strain ATCC 17978 and *Acinetobacter baylyi* ADP1 were used as controls.

PCR and DNA sequencing

Genomic DNA used as a template for PCR assays was obtained from bacterial suspension grown overnight in Luria broth with shaking incubator at 37 °C using Qiagen mini-preps kit. Primers used for detection of OXA-23-like, OXA-51-like, ADC-7, ISAba1 element, and 5' and 3' *comM* junctions of AbaR1 are listed in Table 1. PCRs were performed in a final volume of 25 µL. PCR mix component was as follows; 5 µL of 10X PCR buffer [0.1 mol/L Tris-HCl (pH 8.8), 0.5 mol/L KCl, 1% Triton X-100], 3 µL of 0.025 mol/L $MgCl_2$, 5 µL of 10X dNTP (0.002 mol/L dATP, dCTP, dGTP and dTTP), 2 µL each of primer (25 pmol/µL), 34 µL deionised sterile water, 1

U of *Taq* DNA polymerase and 5 µL of template DNA. PCR amplification condition was as follows: initial denaturation at 94 °C for 3 min, 94 °C for 45 seconds, 55 °C for 1 min, 72 °C for 3 min followed by 34 cycles and 5 min at 72 °C with a final extension (Lévesque *et al*., 1995; Rosser and Young, 1999). PCR products were fractionated by agarose gel electrophoresis and then visualized under ultraviolet illumination at a wavelength of 312 nm using the Kodax imaging system (Kodax, USA). PCR products from $bla_{OXA\text{-}51\text{-}like}$ and *16S-rRNA* was sequenced using an AB I3730XL DNA Analyzer (Applied Biosystem Inc., Foster City, CA) using Sanger (dideoxy chain termination) technology. Analysis of the sequenced PCR products was carried out using the Mega5.2 (http://www.megasoftware.net /mega5.2/mega.html) software and online Blastn (http://www.ncbi.nlm.nih.gov/blast/). Sequences of some known *Acinetobacter* genospecies prototype strains retrieved from the GenBank were aligned using ClustalW (version 1.81).

Table 1: PCR oligonucleotide primers used in this study

Primers/targets	Amplicon Size (bp)	sequence (5' to 3')
16 S-rRNA	1600	F: AGAGTTTGATCMTGGCTCAG R: GGTTACCTTGTTACGACTT
$bla_{OXA\text{-}51\text{-}like}$	353	F: TAATGCTTTGATCGGCCTTG R: TGGATTGCACTTCATCTTGG
OXA23- ISAba1-$bla_{OXA\text{-}23}$*-ATPase*	1065	F: GATGTGTCATAGTATTCGTCG R: TCACAACAACTAAAAGCACTG
$bla_{ADC\text{-}7}$	1152	F: ATGCGATTTAAAAAAATTTCTTGT R: TTATTTCTTTATTGCATTCAG
5' junction of AbaR1	633	F: ATATCTATAAACCACTCGAC R: TTATGCTGAGCTTGCTGGC
	796	R: CCCAAATACTGCCATGTTGA
3' junction of AbaR1	651	F: CAACCCTGTCTTTGCATTTG R: CTGTTTATGGGAGTATTTCG
	845	R: GTGCAGTTTTCAAGCTCGAA
5' junction in ADP1	variable	F: TTCACTGGATCTGGCTGATG
3' junction in ADP1		R: TTCGCTTCTAAGGGTTGACG

RESULTS

Identity of isolates

Based on biochemical and genetic characterizations, 100% (n = 72) and 95.8% (n = 69) of the isolates were identified as *A. baumannii* strains respectively. Figure 1 shows the electrophoretic verification of $bla_{OXA\text{-}51\text{-}like}$ intrinsic gene and *16S-rRNA* gene.

Antimicrobial susceptibility profile of the A. baumannii isolates

All the 69 *A. baumannii* isolates were resistant to ≥14 antimicrobial agents tested, with 66 (95.1%) isolates resistant to 20-34 antibiotics. The 69 (100%) isolates were also resistant to amoxicillin, amoxicillin clavulanic acid, ampicillin, cefpodoxime, ceftazidime, ceftriaxone, cefuroxime, and cloxacillin. Significantly high rates of resistance were observed for cephazolin, erythromycin (98.4%); aztreonam, (93.4%); tetracycline (91.8%); cephalothin, kanamycin, trimethoprim-sulfamethoxazole (88.5%); gentamicin, ticarcillin (83.6%); piperacillin, nitrofurantoin (84.4%); chloramphenicol (80.3%); ofloxacin (70.5%); colistin (60.7%). High rates of carbapenem resistance were detected for meropenem (78.7%) and imipenem (57.4%). The least resistance was observed for ciprofloxacin (36.1%), amikacin, (37.9%) and polymyxin B (39.3%). Figure 2 shows the susceptibility patterns of 19 antimicrobial agents against 69 CRAB isolates. Resistance determinants, ISAba1 element, and TnAbaR1genomic Resistance Island. Of the 21 CRAB isolates subjected to molecular characterization, 85.7% (n = 18) harboured $bla_{OXA\text{-}23\text{-like}}$ gene carrying *ISAba1* element upstream of the gene within genomic resistance island, Tn*AbaR1*. Similarly, 85.7% (n = 18) isolates of CRAB were found to be positive for $bla_{ADC\text{-}7}$, encoding cephalosporinases. Chromosomally encoded intrinsic gene, $bla_{OXA\text{-}51\text{-}like}$ was detected in all CRAB isolates authenticating their identity as *A. baumannii*. The 5' and 3' junctions flanking Tn*AbaR1* within the well-conserved *comM* gene were detected in 80.0% (n = 17) of CRAB isolates using primer pairs specific for these junctions (Table 1). The expected size for 5' junction (845 bp) and for 3' junction (796 bp) was amplified for Tn*AbaR1* demonstrating that they harboured a typical Tn*AbaR1* transposon in the *comM* gene. However, repeated attempts to amplify same junctions in the negative control strain *Acinetobacter baylyi* ADP1 proved unsuccessful demonstrating the absence of Tn*AbaR1* transposon in an environmental strain of *A. baylyi* ADP1.

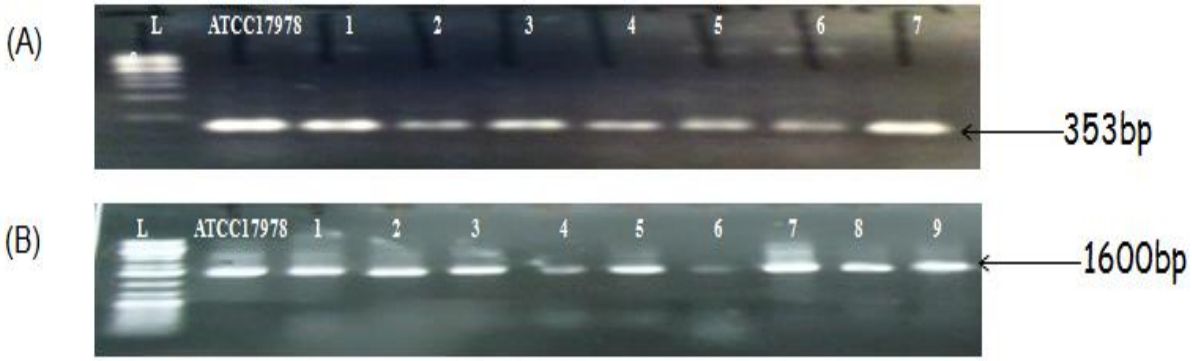

Figure 1. PCR-based analysis. (A) PCR based interrogation of $bla_{OXA\text{-}51\text{-}like}$ gene and (B) a *16S-rRNA* gene analysis of *A. baumanni* isolates.

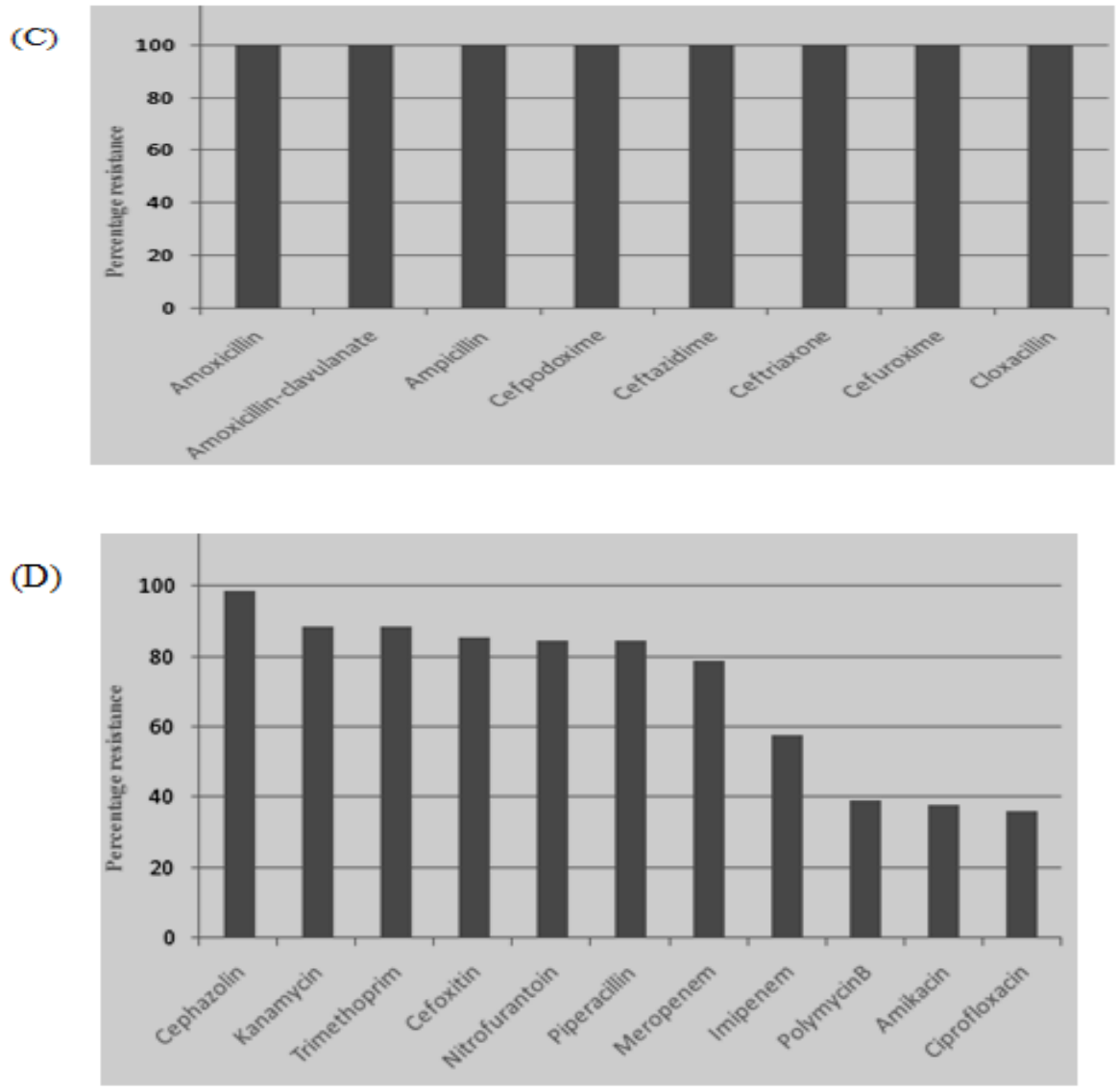

Figure 2. Antimicrobial susceptibility analysis. (C) All *A. baumannii* strains were resistant to a subset of the antibiotics and (D) illustrates other strains that were susceptible to a subset.

DISCUSSION

Carbapenems remain the antibiotic of choice for the treatment of *A. baumannii* related infections and other Gram-negative infections due to both a wider spectrum of antibacterial activity and less frequent side effects. However, their overuse and misuse have selected for nosocomial isolates presenting intrinsic and acquired multidrug resistance determinants (Kuo *et al*., 2015) Resistance against carbapenems has been considered in itself, sufficient to define an *A. baumannii* as highly resistant (Fonseca *et al*., 2013). To the best of our knowledge, this is the first epidemiological survey revealing CRAB isolates harboring *ISAbaR1-bla*$_{OXA-23}$-like gene, and Tn*AbaR1* genomic resistant island in clinical *A. baumannii* strains from Nigeria. In this study, all the isolates showed resistance phenotype to ≥14 antibiotics with 95.7% of the isolates resistant to 20-34 of the 34 antibiotics tested. Interestingly, high rate of resistance to carbapenems observed in this study has also been reported in previous studies (Moniri *et al*., 2010; Rahbar *et al*., 2010; Japoni *et al*., 2011; Mohajeri *et al*., 2013; Safari *et al*., 2013; Shoja *et al*., 2013; Bagheri *et al*., 2015). Polymyxin B, and colistin are last drugs of choice for treating infection by *A. baumannii*; however, reports show that Polymyxin B and colistin resistant *A. baumannii* has emerged around the globe (Bagheri *et al*., 2015). In this study, results showed a resistance phenotype of 60.7% and 39.3% to colistin and Polymyxin B respectively. These results are in agreement with those of previous reports from Saudi Arabia, Kuwait and Egypt (Al-Agamy *et al*., 2014; Bagheri *et al*., 2015). There is a growing concern about the global spread of carbapenem-hydrolyzing class D β-lactamases (CHDLs) and, to a lesser extent, of metallo-β-lactamases (MBLs) which has been reported as the common cause of carbapenem resistance in Acinetobacter species. In *A. baumannii*, CHDLs can be intrinsic (Oxa-51-like) or acquired (OXA-23-like, OXA-24-like, and OXA-58-like) (Evans and Amyes 2014; Zhao *et al*., 2019). Of these oxacillinase genes, the most common mechanism for *A. baumannii* resistance to carbapenem is the existence of the OXA-23 type (Lee *et al*., 2011; Cicek *et al*., 2014). In the current study, 85.7% of CRAB isolates harbored *bla*$_{OXA-23}$ gene with *ISAba1* promoter flanked upstream of the gene and this combination has been shown to confer high level of carbapenem resistance (Turton *et al*., 2006; Cicek *et al*., 2014). In addition, our genetic analysis revealed high prevalence of cephalosporin resistance gene variant; *Acinetobacter* derived cephalosporinase (ADC-7) gene in 85.7% of *A. baumannii* strains. This finding is a verification of the resistance phenotype observed against all the third generation cephalosporin antibiotics (cefuroxime, cefpodoxime, ceftazidime, ceftriaxone) tested using the disk diffusion method. These data suggest that cephalosporins are no longer effective in the treatment of infections caused by *A. baumannii* strains in Nigeria hospitals. Existence of *ISAba1* upstream of the *bla*$_{ADC-7}$ gene was not investigated; moreover, the result is suggestive of *ISAba1* upstream of the gene. This finding is in tandem with other similar studies (Al-Agamy *et al*., 2014; Joshi *et al*., 2017; Zhao *et al*., 2019) with high resistance rate against third generation cephalosporin antibiotics among *A. baumannii* strains harboring *bla*$_{ADC-7}$ gene in Nepal. Fournier *et al.* (2006) reported an *ATPase* gene (subsequently renamed *comM*) of the MDR *A. baumannii* strain AYE which was truncated by an 86 kb resistance island named AbaR1. The genome sequence of this bacterium has been identified to contain 45 putative resistance genes. According to Rose (2010), AbaR1 is remotely related to a highly promiscuous Tn7 transposon, and has been renamed *TnAbaR1*. In this study junctions of 3' and 5' ends of *TnAbaR1* were probed and detected in 80.0% of the CRAB strains with sequence homology of 100% to partial *comM* gene. Repeated attempts to amplify the entire 86 kb island was unsuccessful, a clear cut evidence of the presence of AbaR1 island (Rose 2010).

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