In-Depth Genomic Characterization of a Meropenem-nonsusceptible *Pseudomonas otitidis* Strain Contaminating Chicken Carcass

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ABSTRACT

Background: The indiscriminate use of antibiotics in food-animal production has a major impact on public health, particularly in terms of contributing to the emergence and dissemination of antimicrobial resistant bacteria in the food-animal production chain. Although *Pseudomonas* species are recognized as important spoilage organisms in foodstuff, they are also known as opportunistic pathogens associated with hospital-acquired infections. Furthermore, *Pseudomonas* can play a role as potential reservoirs of antimicrobial resistance genes, which may be horizontally transferred to other bacteria. Considering that cephalosporins (3rd and higher generations) and carbapenems are critically important beta-lactam antimicrobials in human medicine, this study reports the occurrence and genomic characterization of a meropenem-nonsusceptible *Pseudomonas otitidis* strain recovered from a chicken carcass in Brazil.

Materials, Methods & Results: During the years 2018-2019, 72 frozen chicken carcasses were purchased on the retail market from different regions in Brazil. Aliquots from individual carcass rinses were screened for Extended Spectrum Beta-lactamase (ESBL)-producing bacteria in MacConkey agar supplemented with 1mg.L^{-1} cefotaxime. Phenotypically resistant isolates were further tested for resistance to other antimicrobials and confirmed as ESBL-producers by means of disk-diffusion method using Müller-Hinton agar. Only one meropenen-nonsusceptible isolate was detected and submitted to whole genome sequencing (WGS) in Illumina Miseq. The strain was identified as *Pseudomonas otitidis* by local alignment of the 16S rRNA sequence using BLASTn and confirmed by Average Nucleotide Identity (ANI) analysis using JspeciesWS database. Genes encoding for antimicrobial resistance were detected by means of Resfinder and Comprehensive Antibiotic Resistance Database (CARD) databases. The phenotypic non-susceptibility to meropenen was attributed to the gene bla_{POM-1}. A total of 192 different genes encoding for quorum sensing system, antiphagocytosis, iron uptake, efflux pump, endotoxin and toxin, adherence, and secretion systems were detected by means of Virulence Factor Database (VFDB). *Pseudomonas otitidis*-pan genome was built using Roary-rapid large-scale prokaryote pan genome analysis using the present strain (K_25) and other two *P. otitidis* genomes (PAM-1, DSM 17224) publicly available at the NCBI. The core genome analysis of the two human strains resulted in similar percentages.

Discussion: Carbapenems are critically important drugs for human health and bacterial strains resistant to these antimicrobials pose a public health problem. The bla_{POM-1} gene harbored by the *Pseudomonas otitidis* K_25 strain encodes a metallo-beta-lactamase (MBL) conferring resistance to carbapenems. *Pseudomonas otitidis* was the first confirmed pathogenic *Pseudomonas* species expressing MBL constitutively in the absence of inducible beta-lactamase genes. Furthermore, the several virulence genes associated with the capacity of the *P. otitidis* K_25 to colonize, evade the immune system and cause lesions in the human host confirm this strain as a potential opportunistic pathogen contaminating foodstuff. These reinforce the need to address antimicrobial resistance in a One Health perspective, in which resistant bacteria and resistance determinants circulate among environment, animals and humans.

Keywords: carbapenem-resistance, POM-1, *Pseudomonas otitidis*, Brazil.

Descritores: resistência bacteriana, Meropenem, *Pseudomonas otitidis*, Brasil.

INTRODUCTION

Pseudomonas are opportunistic pathogens often related to complicated nosocomial infections in humans. Moreover, *Pseudomonas* species harbor intrinsic and acquired antimicrobial resistance mechanisms, leading to hard-to-treat infections [16] with severe disease outcome as a result of various virulence factors such as flagellum, pili, protein secretion systems, exoenzymes, lectins, quorum sensing and biofilm matrix synthesis [9,10,24].

In foods, *Pseudomonas* species are recognized as important spoilage bacteria [22] causing significant economic losses in the food industry [18]. Among these species, *Pseudomonas otitidis* was first described in patients diagnosed with acute otitis [6], and has thereafter been isolated from environmental sites and foodstuffs [15,23,28,29].

The majority of the studies about antimicrobial resistance in foods address only food-borne pathogens and microbial indicators, such as *Escherichia coli* and *Enterococcus* sp. Therefore, the role of spoilage bacteria as potential reservoirs of antimicrobial resistance genes that can be transmitted to other bacteria is practically untapped. Among the antimicrobials of the beta-lactam class, cephalosporins (3rd and higher generation) and carbapenems are critically important drugs in human medicine, as bacterial pathogens that are resistant to these antimicrobial agents pose a public health concern globally [26]. Therefore, information regarding the mechanisms associated with bacterial resistance against these drugs is critical to tackle the emergence and dissemination of antimicrobial resistance. This study aimed at providing in-depth genomic characterization of a meropenem-nonsusceptible *Pseudomonas otitidis* recovered from chicken carcass in Brazil.

MATERIALS AND METHODS

Samples

During the years 2018-2019, 72 frozen chicken carcasses were purchased on retail market from different cities in Brazil in order to investigate the occurrence of Extended Spectrum Beta-lactamases (ESBL) producing- and carbapenem-resistant enterobacteria. Frozen chicken carcasses were collected in their original packaging and kept refrigerated at 4°C for defrosting before processing. After the removal of the plastic package and the giblets, the carcasses were placed into sterile plastic bags and weighted. Four hundred milliliters of buffered peptone water (BPW 1%) were added into the bag, and the carcass was rinsed for approximately one minute.

Screening for ESBL and resistance to carbapenems

Aliquots (30 mL) from the carcass-rinses were cultured in non-selective enrichment broth (BPW 1%) at 37º for 18-22h. Thereafter, one loopful (10 µL) of the overnight culture was transferred to MacConkey[1] agar plates containing 1 mg.L^{-1} of cefotaxime[2] (CTX agar) and incubated at 44°C ± 0.5°C for 18-22 h. Up to three colonies per sampled carcass were individually picked, streaked onto CTX agar and cultivated at 37°C ± 1°C for 18-22 h. In order to screen for ESBL producing- and carbapenem resistant bacteria, each isolate was tested for antimicrobial susceptibility to cefotaxime (30 µg), ceftazidime (30 µg) and meropenem (10 µg) disks[1] by means of disk-diffusion method using Müller-Hinton[1] agar [7,8]. *Escherichia coli* ATCC© 25922 was used for quality control purposes.

Isolates identification and antimicrobial susceptibility test

The isolates were phenotypically identified by means of biochemical procedures as previously described [17]. Further antimicrobial resistance profiling was determined by disk-diffusion test, against the following drugs[1]: amikacin (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), gentamicin (10 µg), levofloxacin (5 µg), norfloxacin (10 µg) and tobramycin (10 µg). The minimum inhibitory concentration (MIC) of colistin[2] was determined by broth microdilution method using cation-adjusted Muller-Hinton broth[1] (CAMHB) [7].

Sequencing and analysis

Genomic DNA was extracted using Mag-MAX™ CORE Nucleic Acid Purification Kit[3] according to the manufacturer's instructions. DNA integrity was visually assessed on 1% agarose gel and quantified by fluorometry (Qubit)[4]. The genomic library was prepared by means of Nextera XT DNA Library[5]. Fragment sizes were evaluated using a capillary electrophoresis system (Fragment Analyzer)[6], and paired-end sequenced in Illumina MiSeq[5] using a 2x250 cycle V2 kit[5].

Fastqc tool (bioinformatics.babraham.ac.uk/ projects/fastqc) was used to check the quality of the reads, followed by trimming of Illumina adapters and low-quality reads (Phred score < 30) using Trimmomatic (available from usadellab.org/cms/index). Genome assembly was performed using SPAdes [1] and Abyss [21]. The assembly quality was assessed in QUAST [11] and the best result was obtained using SPAdes with a k-mer of 77. In order to improve the assembly metrics in terms of N50, we used the Aligngraph tool [2]. Before running AlignGraph tool, a dotplot similarity matrix by means of pairwise alignments was obtained using the web platform D-GENIES [5].

The strain was identified by local alignment of the 16S rRNA sequence using BLASTn and confirmed by Average Nucleotide Identity (ANI) analysis using JspeciesWS database [19] (available from jspecies. ribohost.com). Barrnap (http://www.vicbioinformatics. com/software.barrnap.shtml) was used to extract the 16S rRNA sequence for identification. The genome annotation was performed under the automatic NCBI pipeline submission system. Roary-rapid large-scale prokaryote pan genome analysis (academic.oup.com/ bioinformatics/article-lookup/doi/10.1093/bioinformatics/btv421) was used to build a pangenome.

Genes encoding for antimicrobial resistance were screened by means of ResFinder 3.2 (available from cge.cbs.dtu.dk/services/ResFinder/) and CARD (available from https://card.mcmaster.ca/analyze/rgi). Virulence factors were investigated using the Virulence Factor Database (VFDB) [13].

RESULTS

From the 72 frozen chicken carcasses, 55 (76.4%) were positive for microbiological isolation in CTX agar. A total of 164 colonies were screened for resistance to meropenem and cephalosporins. After screening, only one isolate showed non-susceptibility to meropenem, while it was susceptible to all tested cephalosporins. This isolate was phenotypically assigned within the *Pseudomonas* genus. Further determination of antimicrobial resistance profiling demonstrated that the isolate was susceptible to amikacin, ciprofloxacin, imipenem, gentamicin, levofloxacin, norfloxacin and tobramycin. The MIC of colistin was determined at 2 mg.L^{-1}, below the resistance breakpoint (≥ 4 mg.L^{-1}) [8].

Since meropenem-resistance is not a frequent feature among bacteria from foodstuffs, we decided to further characterize the strain, which was registered in the culture collection as K_25. The whole genome sequencing (WGS) was performed and the genome sequence was estimated as 6,406,500 bp long with 67.06% of GC (Table 1).

The local alignment of the 16S rRNA sequence using BLASTn identified the strain K_25 as *Pseudomonas otitidis*. The Average Nucleotide Identity (ANI) resulted in a 0.99 cutoff value compared with the reference *P. otitidis*, indicating that the strain was correctly identified. The genome annotation was performed under the automatic NCBI pipeline submission system. The draft genome sequence of *P. otitidis* K_25 is available at GenBank under the accession no. NZ_WTFN00000000.

There were only two available *Pseudomonas otitidis* strains (PAM1 and DSM17224) available at the NCBI database: DSM 17224 - FOJP00000000 isolated in 2016 in the United States; and PAM-1-PXJI00000000 isolated in 2018 in Lebanon, both from humans. The results demonstrated that the *P. otitidis* K_25 genome was not very close to any of the two references. As expected, AlignGraph results did not produce any extension of the contigs.

The pangenome was built with the present strain (K_25) and the other two reference strains (PAM-1, DSM 17224). The obtained pangenome consisted of a total of 7,463 genes while the core genome presented a total of 4,595 genes, which comprised 71.25% of the entire genome of the K_25 strain. The analysis of the two human strains resulted in similar percentages. In addition, *P. otitidis* K_25 was found to harbor a 2,389 bp plasmid. The cryptic plasmid had a GC content of 60.7% and four open reading frames (ORFs), one encoding for replication protein (rep) and the others encoding hypothetical proteins.

The resistome analysis identified the gene bla_{POM-1} (98.78% id - accession number GU002295). No other antimicrobial resistance gene was found. One hundred and ninety-two (192) virulence factors were identified by VFDB. Genes encoding for quorum sensing system, antiphagocytosis, iron uptake, efflux pump, endotoxin and toxin, adherence, and secretion systems were detected (Table 1). From the 192 genes carried by strain *P. otitidis* K_25, 84.3% are also present in *P. aeruginosa*.

Table 1. Genomic and epidemiological features of the first meropenen-nonsusceptible *Pseudomonas otitidis* from chicken carcass at retail market in Brazil.

Features	*Pseudomonas otitidis* K_25
Genome data	
Genome size (Mb)	6,4
Contigs (> 200 bp)	735
GC contente (%)	67,06
Number of genes	6.449
CDSs	6.379
tRNAs	58
Epidemiological Genomic Data	
Virulence factors[a] Class	Number of related genes
Adherecence	95
Antiphagocytosis	29
Enzyme	2
Iron uptake	16
Protease	1
Quorum sensing	1
Regulation	6
Secretion system	25
Toxin	2
Amino acid and purine metabolism	1
Efflux pump	1
Endotoxin	1
Glycosylation system	1
Immune evasion	4
Invasion	3
Iron acquision	1
Magnesium uptake	1
Serum resistance	1
Stress adaptation	1
Total	192
Resistome[b,c]	
Beta-lactam[b]	bla_{POM-1}

[a]http://www.mgc.ac.cn/VFs/ ; [b]https://cge.cbs.dtu.dk/services/ResFinder/ ; [c]https://card.mcmaster.ca/analyze/rgi

DISCUSSION

The genomic analysis revealed bla_{POM-1} gene as the mechanism of meropenem non-susceptibility harboured by the *P. otitidis* strain (K_25) isolated from chicken carcass. The resistance gene bla_{POM-1} encodes for the production of a metallo-beta-lactamase (MβL), which is known to confer resistance to carbapenems [4]. The gene bla_{POM} seems to be highly conserved and constitutively expressed in *P. otitidis* [25]. While the expression of POM MBL does not necessarily confers a carbapenem-resistant phenotype in some *P. otitidis* strains, the strain *P. otitidis* K_25 reported in this study showed a reduced susceptibility to meropenem. Other resistance genes were not identified, and the strain was phenotypically susceptible to all other tested antimicrobials. As previously reported in other POM MBL-expressing strains [12], K_25 was susceptible to cephalosporins. This beta-lactam resistance profile is unusual in the *Pseudomonas* genus [12]. *Pseudomonas otitidis* was the first pathogenic *Pseudomonas* species constitutively expressing MBL in the absence of inducible beta-lactamase genes [25]. It has been suggested that the gene bla_{POM} was originally acquired by horizontal transfer mechanisms, followed by re-combination into the chromosome downstream of the conserved phosphonate operon, after divergence of *P. otitidis* from the other species [25].

Carbapenems are not administered to food animals [27] and resistance to this antimicrobial group is still low in bacteria isolated from foodstuffs and environment [3]. Still, spoilage *Pseudomonas* species showing resistance to carbapenems have been reported in pork, chicken and dairy products [18,28]. In *Pseudomonas*, carbapenem resistance is usually attributable to self-induced physiological changes triggered by several factors. The exposure to other antimicrobials has been suggested as a possible driver for the emergence of carbapenem resistance [3]. The origin and relevance of these carbapenem-resistant spoilage bacteria are still unclear. Most probably they are environmental bacteria that contaminate foodstuffs during processing and can reach the consumer through retail market. In the great majority of the cases, heat treatment of foods can destroy these contaminants and the proper handling of foods during preparation can mitigate their transmission to humans. Despite these aspects, the detection of viable *P. otitidis* harboring several virulence factors in chicken carcass at retail

market highlights the potential role of spoilage bacteria as opportunistic pathogens [6]. Interestingly, *P. otitidis* K_25 shared 84.3% of the virulence genes harbored by *P. aeruginosa*, which is the reference pathogenic species that is able to colonize, evade the immune system and cause lesions in the human host [20]. These findings, corroborate the potential of *P. otitidis* K_25 to cause disease in humans.

The findings of the present study shed light on the potential role of spoilage bacteria in foodstuffs as opportunistic pathogens and reservoirs of antimicrobial resistance genes. Antimicrobial resistance should be addressed in a One Health perspective, as resistant bacteria and resistance determinants circulate among environment, animals and humans. In any of these interconnected compartments, bacteria are exposed to different selective drivers that affect fitness and evolution of strains, and these events may later reflect in the other compartments [14]. Moreover, in-depth genomic information of organisms in foodstuff, such as those provided in this investigation, can support evolutionary and epidemiologic studies addressing beta-lactam resistance among Gram-negative species, which can ultimately contribute to mitigate antimicrobial resistance in the animal-food industry.

CONCLUSION

The in-depth genomic characterization of a bla_{POM-1}-mediated meropenen-nonsusceptible *P. otitidis* K_25 strain from a chicken carcass highlights that spoilage bacteria in foodstuff can serve as reservoirs of genes conferring resistance against critically important antimicrobial drugs in human medicine.

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