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Original

Characterization of the enzyme aac(3)-id in a clinical isolate of *Salmonella enterica* serovar Haifa causing traveler's diarrhea

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ABSTRACT

Introduction: The objective of this investigation was to identify the mechanism of decreased susceptibility to gentamicin in a *Salmonella* clinical isolate, leading to the detection of a aminoglycoside acetyltransferase gene found in a class 1 integron.

Methods: A multidrug-resistant *Salmonella* strain was recovered from feces of a traveler to Egypt. The antimicrobial susceptibility test to 12 antimicrobial agents was performed with the Kirby-Bauer method. The presence of class 1 integron was determined by PCR. The amplified product was recovered and sequenced in order to establish the genes carried. In addition, susceptibility to gentamicin C_{1a}, gentamicin C_1, sisomicin, neomycin, dibekacin, kanamycin, tobramycin, amikacin, netilmicin, apramycin, dactimicin, spectinomycin, streptomycin, lividomycin and butirosin, was established. The Champion™ pET101 Directional TOPO® Expression Kit was used to clone and express the *aac(3)-I* gene.

Results: The isolate was identified as *Salmonella enterica* serovar Haifa, showing resistance to nalidixic acid, tetracycline and decreased susceptibility to gentamicin. One integron with a size circa 1,500 bp, encoding an *aac*(3)-*Id* plus *aadA7* genes was observed. The analysis of the susceptibility to different aminoglycosides in the *E. coli* TOP10F' transformed with the vector carrying *aac*(3)-*Id* gene showed resistance to gentamicin C_{1a}, gentamicin C_1, and dactimicin, in accordance with the presence of this enzyme but, was susceptible to sisomicin. The homology of the amino acid and nucleotide sequences with the AAC(3)-Id enzyme was of 100%.

Conclusion: The presence of the AAC(3)-Id enzyme was described for the first time in a *S.* Haifa.

Caracterización del enzima AAC(3)-Id en un aislamiento clínico de *Salmonella* Haifa causante de diarrea del viajero

Palabras clave:
Aminoglucósido acetiltransferasa
Integrón
Salmonella
Diarrea del viajero

RESUMEN

Introducción: el objetivo de este estudio fue identificar el mecanismo de sensibilidad disminuida a gentamicina en un aislamiento clínico de *Salmonella*, lo que nos condujo a la detección de un gen que codifica una acetiltransferasa modificante de aminoglucósidos localizada en un integron tipo 1.

Métodos: la cepa multiresistente de *Salmonella* fue aislada de las heces de un viajero a Egipto. La susceptibilidad a 12 agentes antimicrobianos se determinó mediante Kirby-Bauer. La presencia de integron clase 1 se realizó mediante PCR. El producto de PCR amplificado del integrón fue recuperado y secuenciado para conocer los genes que contenía dicho integrón. Además se determinó la susceptibilidad a gentamicina C_{1a}, gentamicina C_1, sisomicina, neomicina, dibekacina, kanamicina, tobramicina, amikacina, netilmicina, apramicina, dactimicina, espectinomicina, estreptomicina, lividomicina y butirosina. El kit de expresión Champion™ pET101 Directional TOPO® fue utilizado para clonar y expresar el gen *aac(3)-I*.

Resultados: el aislamiento fue identificado como *Salmonella enterica* serovariedad Haifa, el cual presentaba resistencia al ácido nalidixico, tetraciclina y sensibilidad disminuida a gentamicina. Se observó la presencia de un integron tipo 1 con un tamaño de 1,500 bp en el que se encontraron dos genes (*aac*(3)-*Id* y *aadA7*). El análisis de la sensibilidad a diferentes aminoglucósidos de la cepa de *E. coli* TOP10F' transformada con el vector que contenia el gen *aac*(3)-*Id* demostró resistencia a gentamicina C_{1a}, gentamicina C_1, y dactimicina,

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en concordancia con la presencia del enzima pero era susceptible a sisomicina. La secuencia de aminoácidos presentaba un 100% de identidad con el enzima AAC(3)-Id.
Conclusión: la presencia del enzima AAC(3)-Id ha sido descrita por primera vez en *S.* Haifa.

Introduction

Traveler's diarrhea (TD) is a frequent health problem among travelers to developing countries. This illness may be due to a large variety of microorganisms, among these, *Salmonella* is one of the most frequent following diarrheogenic *Escherichia coli* and *Shigella* spp.[3,21]. Diarrhea associated with *Salmonella spp*. is usually self-limited and does not require antibiotic therapy. However, in specific cases, due to both the severity or the duration of the symptoms, antibiotic treatment is required. Unfortunately, antimicrobial resistance levels among diarrheagenic pathogens have increased in recent years, and *Salmonella* spp. is not an exception[3,21].

Acquisition of resistance may be related to two different mechanisms: 1. Transferable, such as plasmids or transposons, and 2. Non transferable, usually associated with chromosomal point mutations[7,19]. The gastrointestinal environment serves as a reservoir for integron-bearing strains and since integrons are carried on plasmids and transposons, antibiotic selective pressure can potentate the dissemination of antibiotic resistance genes through these genetic elements[15].

To date, nine classes of integrons have been described[16]. Of these, the most relevant at a clinical level are those belonging to classes 1 and 2. The integrons of these two aforementioned classes usually carry gene-cassettes encoding for antibiotic resistance mechanisms. Among these gene-cassettes, the aminoglycoside-modifying encoding genes are considered the most prevalent[6]. The aim of this work was to investigate the mechanism of decreased susceptibility to gentamicin in a clinical isolate of *Salmonella enterica* serotype Haifa.

Methods

Bacterial isolate

A *Salmonella* isolate recovered from feces of a traveler with diarrhea was identified by different typing methods, including biochemical tests and serotyping using somatic and flagella antiserum[11].

Antimicrobial susceptibility

A preliminary antimicrobial susceptibility test was performed, using an agar diffusion method with commercially available disks (Becton Dickinson) to the following antibiotics: ampicillin, amoxicillin plus clavulanic acid, nalidixic acid, tetracycline, trimethoprim/sulphametoxazole, chloramphenicol, gentamicin, amikacin, imipenem, norfloxacin, ciprofloxacin and ceftazidime. Interpretation of results was performed according to the Clinical Laboratory Standards Institute (CLSI) guidelines. *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls[4].

To assess an activity pattern, susceptibility to gentamicin C_{1a}, gentamicin C_1, sisomicin, neomycin, dibekacin, kanamycin, tobramycin, amikacin, netilmicin, apramycin, dactimicin, spectinomycin, streptomycin, lividomycin and butirosin, the disk diffusion method on Mueller-Hinton agar was used. These disks were manually prepared adding 30 μg of each antibiotic to 10 mm sterile blank filter disks. *E. coli* ATCC 25922 was used as a susceptible control strain. Reductions of the inhibition zone were considered as the result of aminoglycoside-modifing enzyme (AME) activity. Antimicrobial susceptibility levels of gentamicina were also established by the E-Test method following the manufacturer's instructions.

Detection of class 1 integrons

The presence of class 1 integrons was determined by PCR using the primers and conditions previously described[12]. The amplified products were gel recovered using the Wizard SV Gel and PCR Clean-up System Kit (Promega, Madison, USA) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer, Emeryville, USA).

Plasmid analysis

Plasmid DNA was isolated as described by Kado and Liu[8]. The plasmid extracted DNA was resolved by electrophoresis on 0.8% agarose gel and stained with ethidium bromide (0.5 mg/L).

Conjugation

Bacterial conjugation experiments were performed using *E. coli* J53 (F-, pro, Gm^S, Rif^R, Lac +) as the receptor strain as previously described[12], and were repeated three times.

DNA amplification and cloning of the aac(3)-I gene

The Champion™ pET101 Directional TOPO® Expression Kit (Invitrogen, USA) was used to clone and express the *aac(3)-I* gene, following the manufacturer guidelines. Briefly, the entire AAC(3)I encoding gene was amplified using the forward primer AAC3IF: 5′-CAC CGT GTC AGT CGA AAT CAT C-3′ and the reverse primer AAC3IR: 5′-GGC ATG ATT TTT ACT CTG C-3′. The amplification product was resolved by electrophoresis on a 2% agarose gel stained with ethidium bromide.

The PCR product was gel recovered, using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wi) and was directly cloned into the pET vector and transformed into *E. coli* TOP10F'. Transformed *E. coli* strain were spread on a selective plate with ampicillin and incubated overnight at 37 °C. Plasmids were isolated from several colonies and then analyzed by PCR using the AAC3IF primer and the specific vector primer T7 Reverse. The isolated plasmid was used to transform *E. coli* BL21 Star (DE3) for expression studies. The cloned insert was expressed in plates containing IPTG (1 mM).

Results and discussion

A *Salmonella enterica* serovar Haifa was isolated from feces of a traveler with diarrhea returning from Egypt. This strain showed resistance to nalidixic acid, tetracycline and decreased susceptibility to gentamicin, while remaining susceptible to ampicillin, amoxicillin plus clavulanic acid, ceftazidime, cotrimoxazole,

Table 1
Antimicrobial susceptibility of *S.* Haifa

Strain	Gm C1a	GmC1	Sisomicin	Dactimicin	Streptomycin	Spectinomycin
E. coli ATCC	27[a]	26	25	18	20	15
BL21	28	29	32	19	23	16
S. Haifa	13	15	25	0	0	0
AAC(3)-Id[b]	0	0	27	0	20	17

[a] Diameter of the inhibition zone in mm.
[b] BL21 *E. coli* strain transformed with the vector carrying the *aac*(3)-*Id* gene.

chloramphenicol, amikacin, imipenem, norfloxacin, and ciprofloxacin.

The isolate was investigated for the presence of class 1 integrons. One amplicon of circa 1500 bp was detected. The sequence of this amplicon revealed the association of the integron with *aac*(3)-*Id* plus *ant(3″)* (also named *aadA7*) aminoglycoside-resistance genes (Figure 1). The detected *aac*(3)-*Id* nucleotide sequence showed amino acid and nucleotide homologies of 100% both with the *aac*(3)-*Id* and *aac*(3)-*Ie* genes located in similar integrons in *Salmonella enterica* serovars Newport and Kentucky[5,9], as well as in *Vibrio fluvialis*[1] (GeneBank access: AY458224, AY463797 and AB114632). Meanwhile, the homology with *aac*(3)-*Ia* , *aac*(3)-*Ib* and *aac*(3)-*Ic* was lower. The *ant(3″)* did not show differences with other nucleotide sequences previously reported.

The *Salmonella enterica* serovar Haifa isolate showed a resistance pattern partially consistent with the presence of an AAC(3)-Id plus an ANT(3″) aminoglycoside nucleotidyltransferase, with resistance or decreased susceptibility to gentamicin C_{1a}, gentamicin C_1, dactimicin, streptomycin and spectinomycin, but susceptible to sisomicin an aminoglycoside also considered a substrate of the AAC(3)I-type enzymes. (Table 1)[10]. In order to establish the exact role of the *aac*(3)-*Id* in the aminoglycoside resistance pattern detected, the gene was cloned in an expression vector. In the presence of IPTG, the transforming strain showed resistance to gentamicin C_{1a}, gentamicin C_1 and dactimicin, but lost the resistance to streptomycin, spectinomycin (Table 1), remaining susceptible to sisomicin. When the MIC of gentamicin was established in this transforming strain the results shows that possess a MIC of 48 μg/ml irrespective of the presence or absence of IPTG.

Aminoglycoside modifying enzymes are commonly located within integrons in pathogens causing TD, such as *Shigella spp.*[13]. In fact, different reports both in pathogens causing TD or not, considered that cassettes encoding for these genes are the most frequently found among integrons[2]. To date, 5 different *aac*(3)-*I* encoding genes have been described in the literature: *aac*(3)-*Ia*[22], *aac*(3)-*Ib*[17], and *aac*(3)-*Ic*[14], *aac*(3)-*Id* (1) and *aac*(3)-*Ie*[9]. However, out of them, the *aac*(3)-*Id* and the *aac*(3)-*Ie* genes, showed the same nucleotide sequence. As in the above mentioned *aac*(3)-*I-like* genes, the *aac*(3)-*Id* described in the present study is located within an integron, together with the *ant(3″)* gene. Analysis of the transformed *E. coli* strain clearly showed that the *aac*(3)-*Id* gene product is responsible for the resistance to gentamicin C_{1a}, gentamicin C_1, and dactimicin, while resistance to streptomycin and spectinomycin was probably associated with the detected *ant(3″)* gene[20,23].

The genetic location of the genes encoding the different acetyltransferases varies widely; for instance, the *aac(2′)-I* gene is almost exclusively located in the chromosome in *Providencia* spp. and *Proteus* spp.[18], whereas most of the genes of the AAC(6) family are present in plasmids[18]. To establish the genetic location of the *aac(3)*-I gene, both plasmid analysis and conjugation experiments were performed, showing negative results. However, the PCR of the *aac*(3)-*Id* using chromosomal DNA extracted from an agarose gel as a DNA template was positive. The amplification of the *gyrA* gene was used as a control (data not shown). Therefore, our results suggest the chromosomal location of the integron.

The phenotypic characteristics of the strains analyzed (decreased susceptibility to gentamicin and susceptibility to sisomicin) suggested the presence of a new aminoglycoside acetyltransferase gene. Nevertheless, the genetic study show the presence of AAC(3)-Id enzyme, before described in *Salmonella enterica* serovar Newport. The phenotype (decreased susceptibility to gentamicin and susceptibility to sisomicin) shown by the strain may be explained by a posttranslational change in the conformation of the enzyme. However, structural studies would be needed to show this hypothesis.

A *Salmonella enterica* serovar Haifa carrying a *aac*(3)-*Id* gene was identified in a class 1 integron for the first time. This result shows the potential of integrons to carry and spread resistance genes.

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References

1. Ahmed A, Nakagawa MT, Arakawa E, Ramamurthy T, Shimoda S, Shimamoto T. New aminoglycoside acetyl transferase gene, aac(3)-*Id*, in a class 1 integron from a multirresistant strain of *Vibrio fluvialis* isolated from an infant aged 6 month. J Antimicrob Chemother. 2004;53:947–51.
2. Bissonette L, Roy PH. Characterization of InO of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of Gram-negative bacteria. J Bacteriol. 1992;174:1248–57.
3. Cabrera R, Ruiz J, Marco F, Oliveira I, Arroyo M, Aladueña A, et al. Characterization of the mechanism of resistance to several antimicrobial agents in *Salmonella* clinical isolates causing traveler's diarrhea. Antimicrob Agents Chemother. 2004;48:3934–9.
4. Clinical and Laboratory Standards Institute. 2006. Performance standards for antimicrobial susceptibility testing; 16th informational supplement. M100-S16. Clinical and Laboratory Standards Institute, Wayne, PA.
5. Doublet B, Weill FX, Fabre L, Chaslus-Dancla E, Cloeckaert A. Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster containing a 3′-N-aminoglycoside acetyltransferase gene cassette, *aac*(3)-*Id*, in *Salmonella enterica* serovar Newport. Antimicrob Agents Chemother. 2004;48:3806–12.
6. Hall RM, Collis CM, Kim MJ, Partridge SR, Recchia GD, Stokes HW. Mobile gene cassettes and integrons in evolution. Ann N Y Acad Sci. 1999;18:68–80.
7. Harbottle H, Thakur S, Zhao S, White DG. Genetics of antimicrobial resistance. Anim Biotechnol. 2006;17:111–24.
8. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol. 1981;145:1365–73.
9. Levings RS, Partridge SR, Lightfoot D, Hall RM, Djordjevic SP. New integron-associated gene cassette encoding a 3-*N*-aminoglycoside acetyltransferase. Antimicrob Agents Chemother. 2005;49:1238–41.
10. Miller GH, Sabatelli FJ, Hare RS, Glupczynski Y, Mackey P, Shlaes D, et al. The most frequent aminoglycoside resistance mechanisms—changes with time and geographic area: a reflection of aminoglycoside usage patterns? Aminoglycoside Resistance Study Groups. Clin Infect Dis. 1997;24:S46–62.
11. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Manual of clinical Microbiology. 6th ed. Washington, DC: ASM Press; 1995.
12. Navia MM, Ruiz J, Sánchez-Céspedes J, Vila J. Dispersión de una cepa de *Shigella flexneri* resistente a trimethoprim. Enferm Infecc Microbiol Clin. 2003;21:401–3.
13. Navia MM, Ruiz J, Vila J. Molecular characterization of the integrons in *Shigella* strains isolated from patients with traveler's diarrhoea. Diagn Microbiol Infect Dis. 2004;48:175–9.

14. Riccio ML, Docquier JD, Dell'Amico E, Luzzaro F, Amicosante G, Rossolini GM. Novel 3-N-Aminoglycoside acetiltransferase gene, *aac(3)-Ic*, from a *Pseudomonas aeruginosa* integron. Antimicrob Agents Chemother. 2003;47:1746–8.
15. Roe MT, Vega E, Pillai SD. Antimicrobial resistance markers of class 1 and class 2 integron-bearing *Escherichia coli* from irrigation water and sediments. Emerg Infect Dis. 2003;9:822–6.
16. Sabaté M, Prats G. Structure and function of integrons. Enferm Infecc Microbiol Clin. 2002;20:341–5.
17. Schwocho LR, Schaffner CP, Miller GH, Hare RS, Shaw KJ. Cloning and characterization of a 3-*N*-aminoglycoside acetyltransferase gene, aac(3)-*Ib*, from Pseudomonas aeruginosa. Antimicrob Agents Chemother. 1995;39: 1790–6.
18. Shaw KJ, Rather PN, Sabatelli FJ, Mann P, Munayer H, Mierzwa LR, et al. Characterization of The Chromosomal *aac(6′)-Ic* Gene from *Serratia marcescens*. Antimicrob Agents Chemother. 1992;36:1447–55.
19. Tenover FC. Mechanisms of antimicrobial resistance in bacteria. Am J Infect Control. 2006;34:3–10.
20. Tolmasky ME. Bacterial resistance to aminoglycosides and beta-lactams: The Tn*1331.* Transposon paradigm. Front Biosci. 2000;5:20–9.
21. Vila J, Gascón J, Abdalla S, Gómez J, Moreno A, Corachán M, et al. Antimicrobial resistance of nontyphoidal *Salmonella* isolates in Traveler's Diarrhea. J Travel Med. 1995;2:45–7.
22. Wohlleben W, Arnold W, Bissonnette L, Pelletier A, Tanguay A, Roy PH, et al. On the evolution of Tn*21*-like multirresistance transposons: sequence analysis of the gene (*aacC1*) for gentamicin acetyltransferase-3-I (AAC(3)-I), another member of the Tn*21*-based expression cassette. Mol Gen Genet. 1989;217:202–8.
23. Wolf E, Vassilev A, Makino Y, Sali A, Nakatani Y, Burley SK. Crystal structure of a gcn5-related n-acetyltransferase: *Serratia marcescens* aminoglycoside 3-n-acetyltransferase. Cell. 1998;94:439–49.