GENETIC STABILITY AND WORLDWIDE DISSEMINATION OF INTEGRON INSERT ENCODING TRIMETHOPRIM RESISTANCE

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Abstract. A hospital-based surveillance study of class 1 integron-positive *Acinetobacter* isolates in the Urals and Siberia (Russia) demonstrated that dihydrofolate reductase gene (*dfrA5*) was located in a functional gene cassette insertion driven from a promoter of a weak type. This insertion is an example of the integron-associated trimethoprim resistance disseminated worldwide from the free-living animals to human. Despite the long-term persistence as an integron insert any loss or modifications in the nucleotide sequence especially at *attC* site was not observed. Nucleotide sequence previously referred as a weak type promoter may be considered as a wild-type configuration of an integron promoter region.

Key words: *Acinetobacter*, class 1 integron, trimethoprim resistance, Russia

INTRODUCTION

Synthesized antifolate trimethoprim (TMP) had a start in the medical practice in 1962 and now is in use worldwide. A rise in TMP resistance among bacterial populations associated with humans has been traced to both selective pressure and to a lack of evident contact with TMP. It has been shown that resistance to TMP and trimethoprim/sulfonamide combination (TMP/SXT) are common in the Asian countries. In 1988, most of *Shigella flexneri* strains isolated from patients with acute diarrhea in Thailand are resistant to TMP (Lolekha *et al*, 1991). Strains of *Escherichia coli, Klebsiella pneumoniae, Enterococcus* spp, and *Proteus mirabilis* among the numbers of pediatric uropathogens in Taiwan are TMP/SXT-resistant (48.9% in 1991-2000 and 46.6% in 2001-2005) (Tseng *et al*, 2008). It has been reported that *Acinetobacter* strains isolated from water sediment and animal manure on the integrated fish farms situated near Bangkok also demonstrate TMP- and TMP/SXT-resistant phenotypes (Petersen *et al*, 2002).

Resistant strains can express genes of *dfr* (*dhfr*) *A* and *B* families encoding dihydrofolate reductase enzyme insensitive to competitive inhibition by TMP. A significant proportion of different *dfrA* genes (in the form of gene cassettes) appear to be inserted into class 1 and 2 integrons (Alonso and Gready, 2006). It has been shown that

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class 1 integron-positive *E. coli* strains isolated from healthy volunteers and outpatients in southern Thailand are more resistant to TMP/SXT (77% to 15% of strains tested, $p<0.001$) (Phongpaichit *et al*, 2008).

We have revealed that representative isolates of *Acinetobacter* genus recovered from hospital wards and community in Russia are TMP/SXT-resistant (Solomennyi, 2008). The aim of the present study was to test the hypothesis that TMP-resistance determinants may be connected with the mobilizable class 1 integron(s) disseminated worldwide. Genetic stability of integron-associated sequences is also discussed.

MATERIALS AND METHODS

A total number of 36 isolates of *Acinetobacter baumannii* group isolates was recovered from colonized or infected patients admitted to a major teaching hospital in Perm City (Russia, the Urals on "invisible border between Europe and Asia") in 2003-2004 and to a municipal hospital in Krasnoyarsk (Siberia, geographical center of Russia) in 2000-2001. Four environmental isolates were also recovered from medical equipments in Perm Hospital. All isolates were routinely identified using biochemical tests and re-tested by means of Microscan Dried Gram Negative MIC/Combo Panels system (Dade Behring) Antimicrobial susceptibility data were simultaneously recorded.

DNA templates for PCR were obtained from bacterial colonies grown on Mueller-Hinton agar by hot-lysis method. Primer sequences for the amplification of both of integron-inserted cassettes and promoter region were 5'-TCT CGG GTA ACA TCA AGG (nucleotide target of class 1 integron situated inside integrase-containing segment) and 5'-AAGCAGACTT GACCTGA (nucleotide target in 3'-conserved segment). PCR was conducted in a thermal cycler (MyCycler, BioRad). Themocycling regimen started with a 4 minute initial denaturation step at 94ºC followed by 30 cycles of 1 minute of denaturation at 94ºC, annealing at 56ºC (1 minute), elongation at 72ºC (4 minutes) and a final elongation at 72ºC for 10 minutes. Electrophoreses of amplified fragments were conducted using standard protocols in ethidium bromide-stained agarose gel with Bio-Rad mini-sub® cell GT apparatus. DNA bands were visualized by exposure to UV light in a transilluminator and images were recorded for computer comparison with standard DNA size markers.

Amplified nucleotide fragments were purified with DYEnamic ET dye terminator cycle sequencing kit (GE Healthcare, UK), sequenced in both directions in a capillary sequencer (Amersham Biosciences, MegaBASE 2000 system) and, after alignment, were compared with GenBank data using BLAST search algorithm.

RESULTS

PCR amplification with primers complementary to the inner part of integrase gene and 3'-conserved segment of class 1 integron resulted in a single amplicon of approximately 2.7 kb in two *Acinetobacter* strains isolated in Perm: one of *A. baumannii* persisting in hospital ward for 9 months and one of *Acinetobacter* isolate 21 (failed to grow at 44ºC, the principal character for *A. baumannii*) isolated from one patient (Fig 1). No amplification bands were visualized among all twelve isolates originating from Krasnoyarsk Hospital (data not shown).

TMP minimum inhibitory concentration value of more than 8 μg/ml was

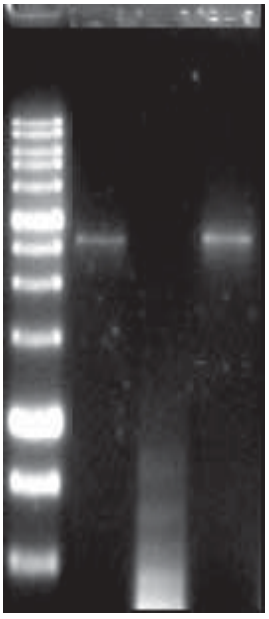

Fig 1–Electrophoretic detection of PCR-amplified class 1 integron sequence. Lane M, DNA ladder (1 kb); lane 1, *A. baumannii* strain 19; lane 2, T7 phage DNA (negative control); lane 3, *Acinetobacter* isolate 21.

determined for both integron-positive cultures. Direct sequencing of amplicons revealed two integron-inserted gene cassettes, *dfrA5*, encoding dihydrofolate reductase (Fig 2) and downstream located *ereA2*, encoding erythromycin esterase. Upstream *dfrA5* two putative promoters were recognized: P1 of weak type and P2 characterized by non-optimal spacer sequence (from 14-bp only) between -35 and -10 hexamers (Fig 3).

DISCUSSION

Similar insertion of gene cassettes in class 1 integron(s) was earlier investigated in bacterial strains worldwide including Asia-Pacific region. Available data list includes the *E.coli* strains originated from Japan (GenBank accession number AB188269), Australia (White *et al*, 2001), Central African Republic (Frank *et al*, 2007) and Spain (Machado *et al*, 2007); non-O1, non-O139 *Vibrio cholerae* strain from India (accession number AF512546) and *Aeromonas veronii* isolated at Taiwan island (accession number EU085376). In addition, this combination of gene cassettes was found in the genome of *Enterobacter* strain circulating in a large hospital in The Netherlands (Peters *et al*, 2001) and in *Salmonella* Wien strain (isolation in 1988) from the global Salmonella Reference Collection B (accession number AY827837). Probably, the same insert was also amplified from DNA from coliform water strain isolated on Scotland's shore (Rosser and Young, 1999). Promoter region sequence in integrons enumerated has been published only for *Enterobacter* isolate originated from infected patients at Utrecht, The Netherlands. In this study we could detect minor mutations in the -35 and extended -10 hexamers of P1 promoter as shown in Fig 3.

Search for nucleotide sequences similar to *dfrA5* gene cassette resulted in fourteen sequences deposited in GenBank under criterion of maximal identity ≥ 98% and under 100% coverage (from start to stop codons of *dfrA5*). Most of bacterial hosts are related to *Enterobacteriaceae* and *Salmonella* but in this list no non-fermenting bacteria (*Acinetobacter*, *Pseudomonas*, etc) are present.

A single integron-inserted *dfrA5* gene cassette has been amplified (Waturangi *et al*, 2003; accession number AJ419169) in different *E. coli* isolates recovered from feces of free-living Indonesian monitor lizards (*Varanus* spp). Variations in the nucleotides for a functional part of gene in tandem class 1 integron insertion existed as individual A↔T nucleotide substitutions

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1   GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA   60
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   GGCATCCAAGCAGCAAGCGCGTTACGCCGTGGGTCGATGTTTGATGTTATGGAGCAGCAA   60

1   CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAATGTTAACCCGGAACCAAAATTGTGA  120
    |||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
2   CGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAACCCGGAACCAAAATTGTGA  120

1   AAGTATCATTAATGGCTGCAAAAGCGAAAAACGGAGTGATTGGTTGCGGTCCACACATAC  180
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   AAGTATCATTAATGGCTGCAAAAGCGAAAAACGGAGTGATTGGTTGCGGTCCACACATAC  180

1   CCTGGTCCGCGAAAGGAGAGCAGCTACTCTTTAAAGCCTTGACGTACAACCAGTGGCTTT  240
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   CCTGGTCCGCGAAAGGAGAGCAGCTACTCTTTAAAGCCTTGACGTACAACCAGTGGCTTT  240

1   TGGTGGGCCGCAAGACGTTCGAATCTATGGGAGCACTCCCTAATAGGAAATACGCGGTCG  300
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   TGGTGGGCCGCAAGACGTTCGAATCTATGGGAGCACTCCCTAATAGGAAATACGCGGTCG  300

1   TTACTCGCTCAGCCTGGACGGCCGATAATGACAACGTAGTAGTATACCCGTCGATCGAAG  360
    |||||||||||||||||||||||||||||||||||| |||||| ||||||||||||||||
2   TTACTCGCTCAGCCTGGACGGCCGATAATGACAACGTAATAGTATTCCCGTCGATCGAAG  360

1   AGGCCATGTACGGGCTGGCTGAACTCACCGATCACGTTATAGTGTCTGGTGGCGGGGAGA  420
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   AGGCCATGTACGGGCTGGCTGAACTCACCGATCACGTTATAGTGTCTGGTGGCGGGGAGA  420

1   TTTACAGAGAAACATTGCCCATGGCCTCTACGCTCCATATATCGACGATTGATATTGAGC  480
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   TTTACAGAGAAACATTGCCCATGGCCTCTACGCTCCATATATCGACGATTGATATTGAGC  480

1   CGGAAGGAGATGTTTTCTTTCCGAATATTCCCAATACCTTCGAAGTTGTGCGAGAGCAAC  540
    ||||||||||||||||||||||||||||||||||||||||||||||||    |||||||
2   CGGAAGGAGATGTTTTCTTTCCGAATATTCCCAATACCTTCGAAGTTGTTTTTGAGCAAC  540

1   ACTTTAGCTCAAACATTAACTATTGCTATCAAATTTGGCAAAAGGGTTAACAAAGCTATG  600
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   ACTTTAGCTCAAACATTAACTATTGCTATCAAATTTGGCAAAAGGGTTAACAAAGCTATG  600

1   CAATTGACGGTAAAAAGCTTCGTTCGCTTCGCTTGCTACGCTTCTTACCGCAATTGATAACGGCGTTA 668
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
2   CAATTGACGGTAAAAAGCTTCGTTCGCTTCGCTTGCTACGCTTCTTACCGCAATTGATAACGGCGTTA 668
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Fig 2–Alignment of *dfrA5* gene cassette in class 1 integron of *Acinetobacter baumannii*-like strain 21 (1, this study) and *E. coli* (2, a single cassette insert, GenBank accession number AJ419169). Identical nucleotides are connected with vertical lines; start and stop codons are viewed against black background; and 1R and 2R sequences of the *attC* site are underlined.

5'-ACGGATTAAGGCACGAACCCAG**T**G**GACA**TAAGCCTGTTCGGTT**C**G**TAAGCT**
GTAATGCAAGTAGCGTATGCGCTCACGCAACTGGTCCAGAACCTTGACCGAAC
GCAGCGGTGGTAACGGCGCAGTGGCGGTTTTCATGGCTTGTTATGACTGTTTTT
TTGTACAGTCTATGCCTCG*GGCATCCAAGCAGCAAG*-3'

Fig 3–Promoter region sequence (P1 and P2 promoters) for *dfrA5-ereA2* gene cassette insertion into class 1 integron of *Acinetobacter* 21 strain. The -35 and -10 hexamers are underlined; for P1 these are typed in bold letters. Potential "hot spots" for nucleotide substitutions (**G** to **T** and **C** to **G** according to Peters *et al*, 2001) are viewed against black background. Nucleotide sequence shown in italics is the start sequence shown in Fig 2.

except for 4 nucleotides located close to the stop codon (Fig 2, positions 530-533). *AttC* site possesed 100% similarity in nucleotide sequence when compared with other gene cassettes.

Dfr-positive isolates are commonly present in number of enteric commensals and pathogens (Alonso and Gready, 2006; Ahmed *et al*, 2007). We showed the possibility of the transfer of *dfrA5* gene cassette as class 1 integron insert to the "water-borne" opportunistic pathogen *Acinetobacter*. Representatives of this genus probably are naturally competent and may be transformed with DNA fragments originating from enteric bacteria including plasmids, genome islands or integrons (Fournier *et al*, 2006).

Attention should be paid to the origin of integron-associated TMP resistance encoded by *dfrA* gene family. It has been earlier postulated that gene cassettes exist as small autonomous DNA (Labbate *et al*, 2009) but insertion into different integron platforms allows promoterless cassettes to be expressed immediately. Essential role of folic acid as growth factor of *Lactobacillus*, *Propionibacterium*, and *E. coli* may be a moving force of natural selection of *dfr* gene in form of a cassette for expression this useful metabolic trait in carnivores and especially herbivores intestinal flora. Molecular data supporting this hypothesis in part is that the mol% G+C content of *dfrA5* gene (excluding *attC* site sequence) is about 47% compared with the value of whole genome of *E. coli* of 50.8% (for *Acinetobacter baumannii* group of 40-43%). Based on the investigation of the *dfrA5*-positive integron-positive strain isolated from animal inhabiting remote areas it could be suggested that free-living animals are the undiscovered primary host of mobilizable TMP resistance. *DfrA1* cassette commonly found in the resistance integrons was found located at a superintegron of the marine fish pathogen *Vibrio salmonicida* (GenBank accession number AJ277063) and the gene cassette drift may have occurred from superintegron to resistance integron between bacterial commensals and pathogens.

Weak promoter configuration was present in cassette-free class 1integron (Bissonnette and Roy, 1992) and this sequence appears to be a wild-type promoter conserved in the resistance integrons although an opposite notion has been presented (Fonseca *et al*, 2008). Single or even second *dfrA* gene cassette location(s) can provide a sufficient level of gene expression driven from weak type promoter only. As a result, TMP resistance determinants should not be waiting re-location after turning out as an integron insert.

In summary, *dfrA*5 and the other TMP resistance gene cassettes are able to disseminate globally as class 1 integron inserts where these are found in connection with sulfonamide resistance genes. Therefore, TMP preparations should not be used routinely for prophylaxis and treatment of "travellers" diarrhea in tourists visiting sub-tropical and tropical regions.

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REFERENCES

Alonso H, Gready JE. Integron-sequestered

dihydrofolate reductase: a recently redeployed enzyme. *Trends Microbiol* 2006; 14: 236-42.

Ahmed AM, Motoi Y, Sato M, *et al.* Zoo animals as reservoirs of gram-negative bacteria harboring integrons and antimicrobial resistance genes. *Appl Environ Microbiol* 2007; 73: 6686-90.

Bissonnette L, Roy PH. Characterization of In0 of *Pseudomonas aeruginosa* plasmid pVS1, an ancestor of integrons of multiresistance plasmids and transposons of gram-negative bacteria. *J Bacteriol* 1992; 174: 1248-57.

Fonseca EL, Freitas FS, Vieira VV, Vicente ACP. New *qnr* gene cassettes associated with superintegron repeats in *Vibrio cholerae* O1. *Emerg Infect Dis* 2008; 14: 1129-31.

Fournier P-E, Vallenet D, Barbe V, *et al.* Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genetics* 2006; 2: e7.

Frank T, Gautier V, Talarmin A, Bercion R, Arlet G. Characterization of sulphonamide resistance genes and class 1 integron gene cassettes in Enterobacteriaceae, Central African Republic (CAR). *J Antimicrob Chemother* 2007; 59: 742-5.

Labbate M, Case RJ, Stokes HW. The integron/gene cassette system: an active player in bacterial adaptation. *Methods Molec Biol* 2009; 532: 103-25.

Lolekha S, Vibulbandhitkit S, Poonyarit P. Response to antimicrobial therapy for shigellosis in Thailand. *Rev Infect Dis* 1991; 13 (suppl 4): S342-46.

Machado E, Ferreira J, Novais Â, *et al*. Preservation of integron types among *Enterobacteriaceae* producing extended-spectrum β-lactamases in a Spanish hospital over a 15 year period (1988 to 2003). *Antimicrob Agents Chemother* 2007; 51: 2201-04.

Peters EDJ, Leverstein-van Hall MA, Box ATA, Verhoef J, Fluit AC. Novel gene cassettes and integrons. *Antimicrob Agents Chemother* 2001; 45: 2961-64.

Petersen A, Andersen JS, Kaewmak T, Somsiri T, Dalsgaard A. Impact of integrated fish farming on antimicrobial resistance in a pond environment. *Appl Environ Microbiol* 2002; 68: 6036-42.

Phongpaichit S, Wuttananupan K, Samasanti W. Class 1 integrons and multidrug resistance among *Escherichia coli* isolates from human stools. *Southeast Asian J Trop Med Public Health* 2008; 39: 279-87.

Rosser SJ, Young H-K. Identification and characterization of class 1 integrons in bacteria from an aquatic environment. *J Antimicrob Chemother* 1999; 44: 11-18.

Solomennyi AP. ISABa1 insertion sequence in genome of epidemically relevant *Acinetobacter strains. Zh Mikrobiol Epidemiol Immunobiol* 2008; 5: 98-100 (in Russian).

Tseng M-H, Lo W-T, Lin W-J, Teng C-S, Chu M-L, Wang C-C. Changing trend in antimicrobial resistance of pediatric uropathogens in Taiwan. *Pediatrics Int* 2008; 50: 797-800.

Waturangi DE, Suwanto A, Schwarz S, Erdelen W. Identification of class 1 integrons-associated gene cassettes in *Escherichia coli* isolated from *Varanus* spp in Indonesia. *J Antimicrob Chemother* 2003; 51: 175-7.

White PA, McIver CJ, Rawlinson WD. Integrons and gene cassettes in the *Enterobacteriaceae*. *Antimicrob Agents Chemother* 2001; 45: 2658-61.