Polymerase chain reaction screening for integrons can be used to complement resistance surveillance programs

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Abstract

Integrons have been recognised as important contributors to the acquisition and dissemination of antibiotic resistance in Gram-negative bacteria. In a collection of 19 multi-antibiotic resistant Gram-negative clinical isolates, 47 per cent (9/19) of strains were found to contain one or more integron, using a polymerase chain reaction (PCR) based screening method. Resistance gene cassettes within the integrons were amplified, sequenced and characterised. Antibiotic susceptibility testing demonstrated that resistance phenotypes correlated with the resistance conferred by gene cassettes identified. PCR-screening for integrons and gene cassettes provides a rapid technique for the identification of genetic determinants of resistance in Gram-negative bacteria. Such screening could assist in guiding treatment regimens and complement existing antibiotic resistance surveillance programs by providing information on molecular mechanisms of both resistance and resistance dissemination. *Commun Dis Intell* 2003;27 Suppl:S103–S110.

Keywords: antibiotic resistance, polymerase chain reaction screening, integrons resistance surveillance programs

Introduction

Antibiotic resistance is a serious clinical problem worldwide. Acquisition of resistance genes in Gram-negative bacteria is facilitated by mobile genetic elements called integrons, which are associated with resistance plasmids and transposons.[1] Integrons encode an enzyme, termed integrase, which allows them to capture antibiotic resistance gene cassettes (Figure).[2,3] Over 80 cassettes have been identified to date, conferring resistance to almost all classes of antibiotic. The length of gene cassettes varies considerably from 262 base pairs (bp) to 1,549 bp,[4,5] however, a common feature of all gene cassettes is a specific recombination site [termed 59-base element (59–be)], located downstream of the gene. The 59–be is recognised by the integron-encoded integrase (IntI),[6] which enables the gene cassette to be inserted into the integron at a second recombination site (*attI*), located immediately upstream of the integrase gene (*intI*) (Figure).[7,8,9,10] Gene cassette arrays in integrons can consist of up to nine cassettes,[11] which are expressed from an upstream promoter (Figure).[2,12,13] Integrons involved in antibiotic resistance can be divided into three classes, class 1, 2 and 3, based on the amino acid sequence of their respective integrases.

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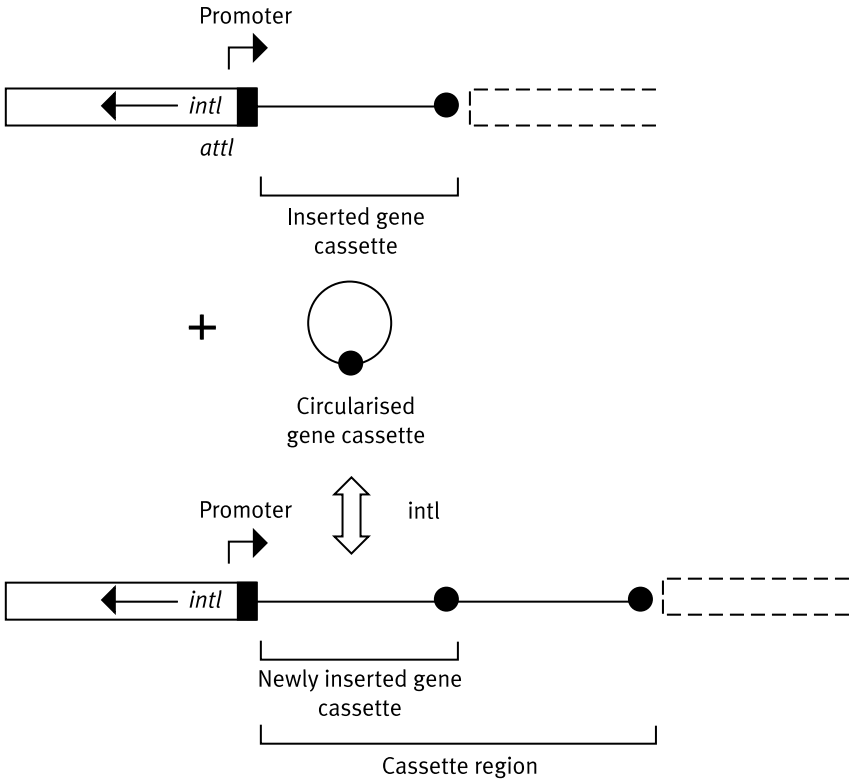
Figure. The insertion of a gene cassette into an integron



The preferential integration of gene cassettes at the *attI* recombination site (indicated by a black box), is catalysed by the product of *intI*, the integrase gene (open box). Two gene cassettes are indicated in the cassette region after the integration event. The filled circle represents the recombination site (59–be) of the gene cassette. A promoter for the expression of integrated gene cassettes, found upstream of the cassette region is also shown.

Integrons are prevalent amongst Gram-negative bacteria and have been associated with antibiotic resistance in clinical isolates.[14,15,16,17,18,19] In a previous study we showed that integrons are significantly associated with multi-resistance in urinary isolates of *Enterobacteriaceae*.[14] Investigations into the prevalence of integrons and characterisation of gene cassettes in clinical isolates provide information on the evolution of multiple-antibiotic resistant strains, the prevalence of antibiotic resistance genes and the molecular mechanisms of antibiotic resistance. This is important when considering strategies for effective antibiotic treatment of bacterial infections.

The present study investigates integrons and gene cassettes in a random selection of Gram-negative clinical isolates that were identified as multi-drug resistant. The presence of antibiotic resistance gene cassettes was correlated with the phenotypic antibiotic resistance profiles to evaluate the contribution of integrons to resistance.

Materials and methods

Clinical isolates

Nineteen randomly selected multi-resistant strains of Gram-negative bacteria from a laboratory collection of clinical isolates were examined (Table 1). All isolates were obtained in 2000 and were considered multi-resistant if resistant to more than four classes of antibiotic. *Escherichia coli* Top 10 and *E. coli* NCTC 10418 were used as integron negative controls, while strains containing class 1, 2 and 3 integrons were also included as positive controls in all experiments.

Table 1. Integron status of bacterial strains studied

Strain	Organism*	Source of isolation†	Integron
INSJ04	*Proteus mirabilis*	N/A	+
INSJ07	*Klebsiella* sp.	Urine	–
INSJ08	*Klebsiella* sp.	Urine	–
INSJ09	*Klebsiella* sp.	Urine	–
INSJ10	*Proteus mirabilis*	Urine	+
INSJ11	*Klebsiella* sp.	Blood	+
INSJ12	*Enterobacter cloaceae*	Wound	–
INSJ14	*Stenotrophomonas maltophilia*	Wound	–
INSJ15	*Acinetobacter baumanii*	Sputum	–
INSJ16	*Klebsiella* sp.	Urine	+
INSJ17	*Acinetobacter baumanii*	Urine	–
INSJ18	*Acinetobacter baumanii*	Urine	–
INSJ19	*Acinetobacter baumanii*	Sputum	–
INSJ20	*Proteus mirabilis*	Urine	+
INSJ21	*Escherichia coli*	Blood	+
INSJ22	*Pseudomonas* sp.	Sputum	–
INS95	*Salmonella typhimurium*	Stool	+
INSTR2	*Citrobacter freundii*	Urine	+
INSTR5	*Enterobacter cloaceae*	Urine	+

* Classification based on standard biochemical criteria.

† N/A: information not available.

Antibiotic susceptibility testing

Susceptibility to antimicrobial agents was determined using the Calibrated Dichotomous Sensitivity method.[20] The antibiotics tested included: aminoglycosides (amikacin, gentamicin, kanamycin, netilmicin, streptomycin and tobramycin), ß-lactams (ampicillin, augmentin, cefotaxime, cefotetan, cephalexin, imipenem and timentin), quinolones (nalidixic acid, norfloxacin), chloramphenicol, nitrofurantoin, sulphafurazole, tetracycline, and trimethoprim.

Detection and classification of integrons and gene cassettes

Methods used to extract bacterial DNA and detect integrons were as previously described by our group.[14,21] Briefly, integrons were detected using polymerase chain reaction (PCR), with primers targeting conserved regions of integron-encoded integrases *intI1*, *intI2*, and *intI3*.[14,21] Integrase PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis, using *Hin*fI and *R*sal to determine integron class as previously described.[14] Gene cassette regions were amplified by PCR and characterised by sequencing and RFLP.[14,21] Analysis of sequence data was performed using programs provided in WebANGIS, by the Australian National Genomic Information Service.

Results

Resistance profiles

All strains tested were resistant to ampicillin, while 18 of 19 (95%) organisms were resistant to cefotaxime, 17 of 19 (89%) organisms were resistant to cephalexin, streptomycin, tobramycin and nitrofurantoin (Table 2). Most strains were susceptible to imipenem, with only 37 per cent of organisms resistant to this antibiotic (Table 2). All strains were resistant to at least 50 per cent of the range of antibiotics tested and one strain, *Acinetobacter baumanii* INSJ18, was resistant to all antibiotics tested (data not shown).

Table 2. Percentage of organisms resistant to antibiotics tested

Antibiotic	Resistant organisms %
Amikacin	63
Gentamicin	79
Kanamycin	84
Netilmicin	68
Streptomycin	89
Tobramycin	89
Ampicillin	100
Augmentin	74
Cefotaxime	95
Cefotetan	47
Cephalexin	89
Imipenem	37
Timentin	84
Nalidixic acid	68
Norfloxacin	58
Chloramphenicol	84
Nitrofurantoin	89
Sulphafurazole	74
Tetracycline	79
Trimethoprim	74

Integron detection and classification

Organisms were screened for the presence of integrase genes by PCR in order to determine the prevalence of integrons in the multi-resistant collection. Nine of 19 (47%) strains contained at least one integron. RFLP analysis revealed that six isolates contained a single class 1 integron, one strain contained two class 1 integrons, one strain contained both a class 1 and a class 2 integron, and in one other strain, a single class 2 integron was detected (Table 3). Class 3 integrons were not detected in this study.

Table 3. Integron cassette arrays and integron-associated antibiotic resistance profiles of integrase positive organisms

Strain	Cassettes contained by integron 1†	Cassettes contained by integron 2†	Resistant to antibiotics*								
			AK	CN	K	NET	S	TOB	AMP	SF	W
P. mirabilis INSJ04	*aacA4, aacC1, orfXa, orfXb, aadA1*	***dfrA1, sat1, aadA1‡***	R	R	R	R	R	R	R	R	R
P. mirabilis INSJ10	***dfrA1, sat1, aadA1‡***	–	R	R	R	R	R	R	R	R	R
Klebsiella sp. INSJ11	*oxa1, aadA1*	–	S	R	R	R	R	R	R	R	R
K. pneumoniae INSJ16	*aadB*	–	R	R	R	R	S	R	R	R	S
P. mirabilis INSJ20	*aacA4, oxa2, orfD*	*aadB, aadA1*	S	R	R	R	R	R	R	R	R
E. coli INSJ21	*dfrA1, aadA1*	–	R	S	S	S	R	S	R	R	R
S. typhimurium INS95	*aadA2*	–	S	R	S	S	R	R	R	R	S
C. freundii INSTR2	NA	–	R	S	R	R	R	R	R	R	R
E. cloaceae INSTR5	*aadA1*	–	R	R	R	R	R	R	R	S	S

* Abbreviations given as standard Calibrated Dichotomous Sensitivity codes: AK amikacin; CN gentamicin; K kanamycin; NET netilmicin; S streptomycin; TOB tobramycin; AMP ampicillin; SF sulphafurazole; W trimethoprim. Resistance (R) and sensitivity (S) are based on interpretation of the Calibrated Dichotomous Sensitivity method of sensitivity testing. Resistance phenotypes encoded by integron-associated resistance genes are underlined.

† Arbitrarily denoted as integron 1 and 2 in the case of strains containing more than one integron. Cassettes are indicated as inserted into the integron from 5' to 3'. NA: not amplified.

‡ Class 2 integrons are indicated in bold type.

Gene cassette characterisation

Analysis of the cassette regions revealed class 1 integrons contained between one and five different gene cassettes (Table 3). Class 2 integrons contained the three gene cassettes associated with Tn*7*, namely *dfrA1* (trimethoprim resistance),[22] *sat1* (streptothricin resistance)[4] and *aadA1* (streptomycin/spectinomycin resistance).[23] A predominance (15/23) of gene cassettes were identified that confer resistance to the aminoglycosides (Table 3). These cassettes were identified as *aadA1* and *aadA2,*[24] *aadB* (resistance to gentamicin, kanamycin and tobramycin),[12] *aacA4* (resistance to amikacin, netilmicin and tobramycin),[25] and *aacC1* (resistance to gentamicin, astromicin and sisomicin).[26] Gene cassettes conferring resistance to trimethoprim (*dfrA1*) and the ß-lactams (*oxa1* and *oxa2*) (i.e., oxacillin and ampicillin resistance)[27,28] were also identified (Table 3). Resistance conferred by the gene cassette correlated with phenotypic resistance as determined by susceptibility testing (Table 3). In addition, all strains containing a class 1 integron with the exception of *Enterobacter cloaceae* INSTR5, were resistant to sulphonamides (Table 3). This sulfonamide resistance is probably due to the presence of a *sul1* gene that is nearly always found downstream of the cassette array in class 1 integrons.

Discussion

Integrons have been recognised as important contributors to antibiotic susceptibility profile of Gram-negative isolates.[14,15,16,17,18,19] Nine of 19 (47%) multi-resistant Gram-negative clinical isolates contained at least one integron, and 2 of 19 (11%) strains contained two integrons. The proportion of strains in this collection of bacteria carrying integrons is comparable to other studies. For example, 49 per cent of 120 urinary isolates of *Enterobacteriaceae* in Sydney were found to carry integrons,[14] 52 per cent of 54 clinical isolates of *E. coli* in Taiwan,[15] and 43 per cent of 163 Gram-negative isolates in European hospitals contained class 1 integrons.[18]

Integrons have not only been found in isolates from human infection. They have also been reported in environmental and animal isolates, for example, integrons have been reported in bacteria from diseased poultry,[29] fish,[30] pigs and cattle[31] and retail ground meats.[32] Thus, there is potential for the transfer of integron-carrying bacteria from these sources to humans.

Up to 50 per cent of multi-resistant strains of *A. baumanii* have been previously reported to carry class 1 and 2 integrons.[33] However, integrons were not detected in the four strains of *Acinetobacter baumanii* in this study although they were resistant to most antibiotics tested (data not shown). These strains, along with the 53 per cent of bacteria that were integron negative, demonstrate that although integrons are significantly associated with a multi-resistance phenotype,[14] multi-resistance in some isolates appears to be mediated by other mechanisms.

In 2000, an outbreak of shigellosis spread rapidly through a community of homosexual men in Sydney and was attributed to a *Shigella sonnei* biotype g strain.[34] Retrospective PCR screening of outbreak strains by our group revealed that they all harboured class 2 integrons that contain resistance genes to streptomycin, streptothricin and trimethoprim.[34] This study highlighted the need for improved control of the spread of resistance-carrying bacteria and demonstrates usefulness of molecular screening techniques for rapid identification of resistance genes. Information provided could have been used to alter the continuation of ineffective antibiotic treatments that occurred during the outbreak.

Integron-screening and gene cassette characterisation can potentially be utilised as a rapid PCR-based method of resistance profile analysis that allows the identification of genetic resistance determinants. Integrons are a marker for multi-resistance, hence integron screening can be used to predict phenotypic antibiotic resistance. The presence of integrons in clinical isolates is of concern due to their ability to capture further gene cassettes. This gives the host organism the potential to acquire resistance against a wide variety of antibiotics, since gene cassettes exist to nearly all classes of antibiotic. Additionally, integron-screening provides the potential for identification of new

resistance gene cassettes, demonstrated by characterisation of two novel gene cassettes *aadA5* and *dfrA17* by our group in 2000.[35] In the present study of Gram-negative multi-resistant bacteria, we have found that integrons contribute considerably to the resistance profiles of nearly 50 per cent of these organisms. This information complements antibiotic resistance surveillance programs, providing information on the molecular mechanisms of resistance in addition to elucidating means of resistance gene acquisition.

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