Video Article

Screening Foodstuffs for Class 1 Integrons and Gene Cassettes

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Abstract

Antibiotic resistance is one of the greatest threats to health in the 21st century. Acquisition of resistance genes via lateral gene transfer is a major factor in the spread of diverse resistance mechanisms. Amongst the DNA elements facilitating lateral transfer, the class 1 integrons have largely been responsible for spreading antibiotic resistance determinants amongst Gram negative pathogens. In total, these integrons have acquired and disseminated over 130 different antibiotic resistance genes. With continued antibiotic use, class 1 integrons have become ubiquitous in commensals and pathogens of humans and their domesticated animals. As a consequence, they can now be found in all human waste streams, where they continue to acquire new genes, and have the potential to cycle back into humans via the food chain. This protocol details a streamlined approach for detecting class 1 integrons and their associated resistance gene cassettes in foodstuffs, using culturing and PCR. Using this protocol, researchers should be able to: collect and prepare samples to make enriched cultures and screen for class 1 integrons; isolate single bacterial colonies to identify integron-positive isolates; identify bacterial species that contain class 1 integrons; and characterize these integrons and their associated gene cassettes.

Video Link

The video component of this article can be found at https://www.jove.com/video/52889/

Introduction

The discovery of antibiotics was one of the greatest scientific achievements of the 20th century. However, the use and abuse of antibiotics has led to the rapid evolution of antibiotic resistant bacteria, and these now pose a serious threat to public health in the 21st century. The rise of bacterial strains resistant to most treatment options raises the possibility we are entering an era where antimicrobial drugs are no longer effective[1,2].

The genetic machinery that confers antibiotic resistance is an ancient system, predating humans and antibiotic selection pressures by millions of years[3]. Mobile genetic elements, such as plasmids, transposons, genomic islands, integrative conjugative elements and integrons can disseminate antibiotic resistance genes (ARG) both within and between bacterial species[4]. Of these, integrons have played a central role in the spread of ARG, despite the fact that they rely upon plasmids and transposons for mobilization and insertion into bacterial genomes[5]. Integrons capture gene cassettes using an integron-integrase, and then express cassettes using an integron encoded promoter[6,7] (**Figure 1**). Integron gene cassettes are small mobile elements consisting of single open reading frames (ORF) whose products can confer resistance to antibiotics or disinfectants[8]. Class 1 integrons are the integrons most commonly recovered from clinical isolates[5], where they have collectively acquired over 130 different antibiotic resistance gene cassettes[9].

The spread of class 1 integrons into human-associated commensal and pathogenic bacteria generates human waste streams that contain large numbers of these genetic elements[10]. An estimated 10^{19} bacteria that contain class 1 integrons are released via sewage sludge each year in the United Kingdom[11]. It is therefore not surprising that class 1 integrons conferring antibiotic resistances are now being detected in microbiota of wild birds, fish, and other native wildlife[12-14]. Releasing integrons back into the environment poses a significant public health threat, since acquisition of new gene cassettes and complex rearrangements with other mobile elements continues to occur, particularly in sewage treatment plants and other water bodies[15-18]. The natural environment then becomes a fertile recruiting ground for new resistance determinants and opportunistic pathogens[19,20]. Novel integron-containing bacteria and new ARGs can circle back into the human community through contaminated water and food[21,22]. Surveillance of environmental ARGs is a key strategy for understanding and managing antibiotic resistance in the future[23]. In particular, attention should be paid to foodstuffs that are eaten raw or lightly cooked, since these present the greatest threat for transmission of new mobile elements and pathogens.

In this protocol, a streamlined approach for detecting, identifying and characterizing class 1 integrons and their associated gene cassettes in foodstuffs are outlined (**Figure 2**). Using a combination of culturing and polymerase chain reaction (PCR), integrons can be rapidly detected in complex bacterial communities and individual isolates. Methods for identifying the species of bacteria and the conformation and identity of the integron-associated gene cassettes are given. The method is suitable for a broad range of plant and animal foods, and examples of typical workflows are given for each of these food types.

Protocol

Foodstuffs that are eaten raw or lightly cooked are of most concern for human health. Examples include salad vegetables, fruit, shellfish and crustaceans.

1. Sample Collection

1. Collect samples under conditions that minimize contamination, and stored in separate, clean bags during transport. Once collected, samples should be stored at 4 °C and processed within 24 hr.

2. Enriched Culture Preparation

1. Fruits and vegetables:
    1. Place approximately 10 g of material in a durable plastic bag. If processing larger material, concentrate on the skin of the fruit or vegetable. Add 90 ml 0.1 M sodium phosphate buffer, pH 7.0 and process in a paddle blender for 30 sec. Alternatively, the material can be manually agitated if a paddle blender is not available.
    2. Collect the liquid into two 50 ml Falcon tubes. Centrifuge tubes at 4,000 x g for 7 min to pellet bacteria.
    3. Carefully remove the supernatant and suspend the pelleted bacteria in 30 ml of sterile Luria Bertani (LB) broth.
    4. Incubate on a shaker O/N, holding one tube at 25 °C and the other at 37 °C. Cultures should be shaken at 200 oscillations per minute (opm). Store cultures at 4 °C until required.

2. Seafood:
    1. Dissect out the stomach (oysters) or digestive tract (prawns) using sterile forceps and tweezers and place into a sterile 1.5 ml tube containing 200 µl of 0.1 M sodium phosphate buffer, pH 7.0. Macerate the sample to create a homogenate.
    2. Dispense 5 ml of LB broth into two sterile 5 ml tubes, and inoculate each with the seafood homogenate.
    3. Incubate on a shaker O/N, holding one tube at 25 °C and the other 37 °C. Cultures should be shaken at 200 opm. Store cultures at 4 °C until required.

3. Screening Cultures for Integrons

NOTE: Standard PCR protocols are used throughout this methodology, using buffers supplied with the enzyme, and a final $MgCl_2$ concentration of 2.5 mM. If required, Lorenz (2011)[24] has outlined PCR optimization and troubleshooting methods in an earlier issue of this journal.

1. Boiled DNA preparation:
    1. Dispense 100 µl of enriched culture into a sterile 0.5 ml PCR tube. Heat the sample to 99 °C for 10 min, using a water bath or heat block. Snap chill on ice for 2 min.
    2. Microcentrifuge at 14,000 x g for 5 min to pellet cell debris. Return to ice. DNA samples can be frozen at -20 °C until required, but must be thawed on ice.

2. Screening for class 1 integrons using PCR:
    NOTE: Mixed cultures from the O/N incubation are screened for class 1 integrons using the HS463a/HS464 PCR protocol (**Table 1**).
    1. Thaw all PCR reagents on ice, away from any potential sources of contaminating DNA. Make up 48 µl PCR mastermix per sample, including negative controls (**Table 2**). Dispense 48 µl of PCR master mix to each tube, using barrier tips.
    2. Add 2 µl of thawed DNA to individual PCR tubes using barrier tips. Run appropriate program in the PCR thermocycler.
    3. To assess the results of the amplification, electrophorese 7 µl of PCR product on a 2% agarose gel poured and run in TBE buffer (90 mM TRIS-borate, 2 mM EDTA). Include a molecular weight marker, such as a 100 bp ladder.
    4. Post stain the gel with a DNA stain, and visualize using a UV transilluminator. Check that there has been no amplification in the negative control. A strong band at around 470 bp for any particular sample indicates that the mixed culture contains bacteria that carry a class 1 integron. Pure colonies will now be isolated from any mixed culture that returned a positive PCR.

4. Screening of Single Colonies for Class 1 Integrons

1. Serial dilutions and spread plates:
    NOTE: To isolate single colonies from the PCR-positive mixed cultures, the mixed culture is serially diluted 10-fold in sodium phosphate buffer, and then plated to recover single colonies.
    1. Add 1 ml of mixed culture to 9 ml of 0.1 M sodium phosphate buffer, pH 7.0 and mix by inversion. Add 1 ml of the dilution to a further 9 ml of phosphate buffer, and repeat until a serial dilution of 10^{-8} is reached.
    2. Spread 100 µl of the 10^{-4} to 10^{-8} dilutions onto LB agar, in duplicate. Incubate the plates O/N at the same temperature used for the initial mixed culture. Plates can then be stored at 4 °C until required, however it is best to process the single colonies fairly promptly.

2. Single colony selection and boiled DNA preparation:
    1. Pick single colonies from the serial dilution plates, selecting as many different colony types as possible, using criteria such as colony size, shape and color. Use sterile toothpicks to select single colonies from the spread plate.

2. Touch the toothpick to the colony, and then transfer into a PCR tube containing 100 µl of sterile water. If large numbers of colonies are to be screened, then colonies can be prepared in a microtiter tray. Spin the toothpick between the fingers to dislodge some of the cells into the water.
3. Using the same toothpick, inoculate an LB plate with a streak of the colony. Large numbers of colonies can be stored on labelled plates if each streak is about 1 cm long. Repeat steps 1 to 3 until you have selected an appropriate number of isolates.
4. Incubate the LB plates O/N, at the same temperature as that used for the initial enriched culture. Cultures can then be stored at 4 °C until required.
5. For preparing DNA from the bacterial suspensions, follow the steps as outlined in section 3.1. For screening of pure culture lysates, perform the HS463a/HS464 PCR as outlined in section 3.2. Isolates that return a positive PCR will be used for preparation of genomic DNA (section 5), and for further analyses.

5. Genomic DNA Extraction of Integron-positive Single Colonies Using Bead Beating[25]

1. Inoculate 5 ml of LB broth with a sample of the pure culture isolated in section 4.2. Incubate O/N, shaking at the same temperature that was used for the initial enriched culture.
2. Pellet cells at 4,000 x g for 7 min, then remove the supernatant. Resuspend the pelleted cells in 1 ml CLS-TC solution in a Lysing Matrix E fast prep tube. Using a bead beating machine, process the sample at 5.5 m/sec for 30 sec.
3. Centrifuge tube at 14,000 x g for 5 min and recover 700 µl of supernatant into a sterile 1.5 ml tube.
4. Add 700 µl of binding matrix diluted 1:5 with 6 M guanidinium thiocyanate and mix at RT for 5 min. Centrifuge at 14,000 x g for 1 min, discard supernatant.
5. Add 800 µl salt ethanol wash (70% ethanol, 0.1 M sodium acetate) and vortex until the pellet is fully resuspended. Wash for 5 min at RT, centrifuge at 14,000 x g for 1 min and discard supernatant. Ensure all supernatant has been removed, using a micropipette if necessary, then allow to air dry for 5 min.
6. Resuspend the pellet matrix in 200 µl TE (10 mM Tris-HCl, 1mM EDTA, pH 7.6) by pipetting up and down. Incubate at RT for 3 min. Centrifuge at 14,000 x g for 3 min and transfer 160 µl of the eluted DNA into a sterile tube. Run an aliquot of the genomic DNA extraction on a 2% w/v agarose gel to check the yield and integrity of the DNA extraction. The purified genomic DNA is stored at -20 °C and defrosted on ice when required for PCR testing.

6. Diagnostic PCRs and DNA Sequencing

NOTE: The genomic DNA prepared in section 5 will be used for all diagnostic PCRs, and to confirm the positive test for class 1 integrons.

1. Thaw genomic DNA on ice, briefly vortex the DNA sample and pulse spin at 14,000 x g for 20 sec. Repeat the PCR for the class 1 integron-integrase gene using HS463a/HS464 (**Table 1**), confirming that the isolate is positive for the 471 bp amplicon (Section 3.2).
2. Identification of positive cultures to species level is achieved by analysis of the small subunit rRNA gene (16S rDNA). Amplify the 16S gene using primers f27/r1492 (**Table 1**). Check the PCR products using electrophoresis. All bacterial targets should generate a 16S amplicon of about 1450 bp.
3. Sequence the 16S rRNA gene amplicon to determine species identity, but since there are likely to be multiple positive PCRs from step 6.2, and many of these positives will be the same bacterial species, we will first distinguish different species using restriction digestion of the 16S PCR product.
    1. Digest an aliquot of the 16S PCR with the restriction enzyme *Hinf*1. Any enzyme that recognizes 4 bp cleavage sites will do, but *Hinf*1 generates good diversity from 16S targets, and is a reliable, inexpensive enzyme.
4. Set up a 30 µl restriction digest mastermix (**Table 2**) in a sterile tube and add 20 µl of un-purfied 16S PCR product. Incubate in a 37 °C water bath O/N. Check the digest on a 2% agarose gel.
5. Score the digestion patterns produced by *Hinf*1. Isolates with the same 16S restriction profile are likely to be the same species. Rather than sequencing every 16S PCR product, sequence at most three representatives of each *Hinf*1 restriction pattern.
6. Purify the 16S PCR products using a commercial kit. A single stranded exonuclease kit can be used, but column based or precipitation methods also work well to remove unincorporated primers and single stranded DNAs. Sequence the 16S amplicons using primer r910 (**Table 1**), setting up the reactions as specified by the sequencing facility.
7. Use the resulting DNA sequence to interrogate the NCBI DNA database with the blastn function (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Greater than 97% nucleotide identity is usually taken as species identity.

7. Mapping and Characterization of Integrons and Cassette Arrays

NOTE: Class 1 integrons emanating from human-dominated ecosystems are likely to be the most common integrons in all your samples. These integrons all have a recent single origin, and therefore have a highly conserved DNA sequence[5].

1. Differentiate between a class 1 integrons emanating from human sources and those that occur naturally in environmental samples by performing a PCR with primer set intI1F165/intI1R476[10] (**Table 1**).
   NOTE: Class 1 integrons from clinical or commensal bacteria will generate a ~300 bp product, while environmental class 1 integrons will not generate any product.
2. Characterize the clinical integrons by amplifying the cassette array with primer set HS458/HS459. This PCR will amplify the region between *intI1* and the 3' conserved segment at the terminus of most cassette arrays. Because the number and identity of cassettes is variable, the size of the PCR product will also vary.
3. Purify amplicons and sequence the DNA using each of the amplification primers. Use sequences to interrogate DNA databases using the blastn function (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4. Ensure naming of cassettes follow standardized nomenclature[9], since many data depositions for integron gene cassettes use inconsistent or ambiguous names. Give particular attention to novel gene cassettes, since these may encode new phenotypes, including genes that confer increased transmissibility, pathogenicity or virulence[8,21].
NOTE: Preclinical forms of the class 1 integron still circulate in natural environments. In particular, those that are linked to active Tn*402* transposons are of most interest[26]. The cassette arrays of these integrons will not amplify with primer set HS458/HS459, but can be amplified with the MRG284/MRG285 primer set (**Table 1**), generating amplicons of variable size dependent on cassette content. PCR products should be purified and DNA sequenced as outlined in 7.2.

Representative Results

Screening of mixed cultures and bacterial isolates for intI1

Primer set HS463a/HS464 PCR can be used to detect the presence of the class 1 integron-integrase gene, *intI1* (**Figure 1**). This primer set works well for detecting *intI1* in mixed cultures, and is also used to screen bacterial colonies harvested from spread plates (**Figure 2**). Positive isolates should generate a single strong band at 471 bp using this primer set (**Figure 3A**). The majority of positive isolates will carry *intI1* that has originated from humans or their agricultural and domestic animals. These isolates will also be positive in a PCR using primers F165/R476, which should generate a 311 bp amplicon (**Figure 1**). Environmental sources of *intI1* will generally not be positive in this second assay.

Characterization of integron cassette arrays

Genomic DNA from pure isolates is used for characterization of integron cassette arrays. The cassette arrays of Tn*402*-associated class 1 integrons can be amplified using primers HS458/HS459. These primers respectively target the integron recombination site, and the 3' end of the cassette array, which normally terminates in the *qacEΔ sul1* gene fusion (**Figure 1**). The size of PCR products generated in this assay varies according to the number and identity of cassettes in the array (**Figure 3B**). Environmental class 1 integrons are often embedded in bacterial chromosomes, and their cassette arrays can be amplified by primers that target the proximal and most distal recombination sites (**Figure 1**). Primers MRG284/285 are designed to amplify this region, and again, because the cassette content varies, the size of the amplicons also varies (**Figure 3C**). Sequencing of HS458/459 PCR products will normally recover known antibiotic resistance determinants, while sequencing MRG284/285 PCR products will generally recover gene cassettes that encode polypeptides of unknown function.

Identifying Bacterial species

Bacteria are identified using 16S rDNA sequencing and database comparisons. Genomic DNA is used as a template for amplification of the 16S small subunit rRNA gene. Using the primers suggested, this should generate an amplicon of about 1450 bp. Because large numbers of colonies might be screened at any one time, a hierarchical screening method is employed. Amplified 16S PCR products are digested with the restriction enzyme *Hinf*1, and these digests are separated on agarose gels. Individual species will generate distinctive patterns after digest, such that isolates of the same species can be easily identified (**Figure 3D**). Sequencing the 16S rRNA gene PCR products from a maximum of three isolates representing any one restriction pattern allows efficient identification of all the likely species in a collection of integron positive isolates.

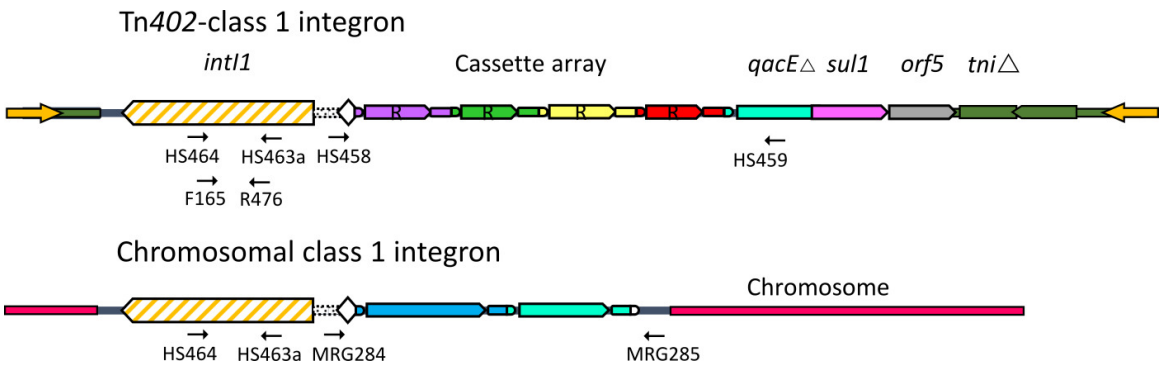

Figure 1. Structure of class 1 integrons. Schematic diagrams of clinical and environmental class 1 integrons, showing PCR primer binding sites referred to in the manuscript. Class 1 integrons consist of an integron-integrase gene (*intI1*) that catalyzes the capture and expression of gene cassettes to form a cassette array. Before the advent of antibiotic use, the majority of class 1 integrons were chromosomal, and carried gene cassettes whose functions are yet to be determined. Strong selection via antibiotic use has vastly increased the abundance of one sequence variant of class 1 integron associated with the Tn*402* transposon. These 'clinical' integrons have acquired arrays of cassettes that encode antibiotic resistance. It is these integrons that are currently polluting natural environments and the food production chain. Please click here to view a larger version of this figure.

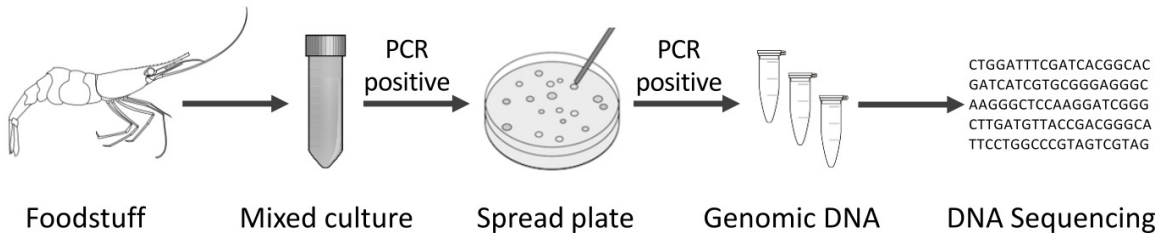

Figure 2. Schematic flowchart for detecting class 1 integrons in foodstuffs. Samples of foodstuffs are used to inoculate media and generate a mixed bacterial culture. These mixed cultures are screened for the presence of class 1 integrons, and positive cultures used to prepare spread plates. Individual colonies from the spread plates are rescreened for integrons. Positive cultures are purified, DNA extracted and characterized for cassette content by DNA sequencing. Sequencing of the 16S rRNA gene is used for species identification. Please click here to view a larger version of this figure.

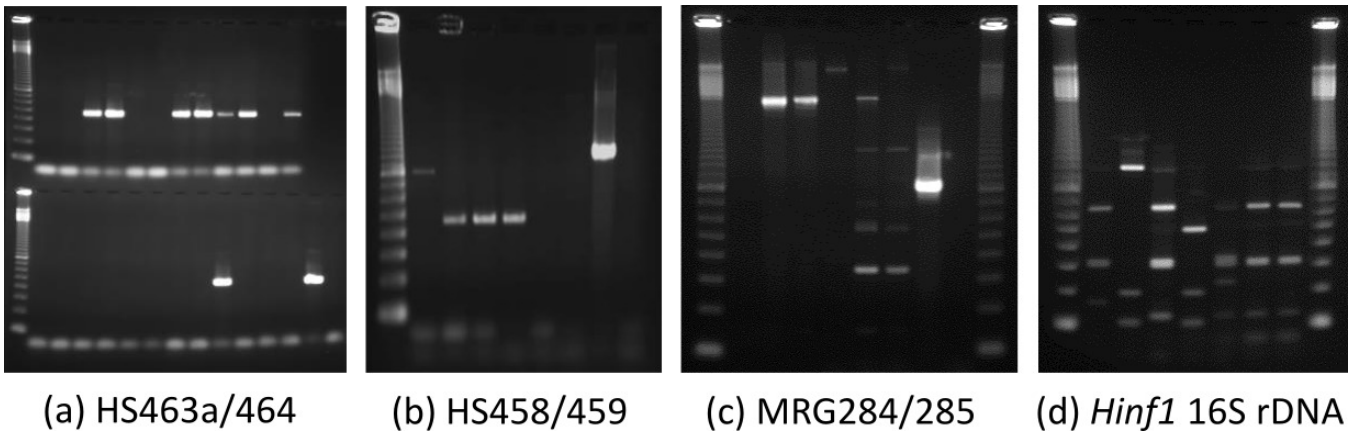

Figure 3. Representative electrophoretic analyses from the screening process. (**A**) Screening single colonies using *intI1* primers HS463a/464. Positive colonies generate a strong single band at 471 bp. (**B**) Amplification of cassette arrays from Tn*402* integrons using primers HS458/459. This PCR generates variable product sizes dependent on the identity and size of the component cassettes in the array. Identification of these cassettes requires DNA sequencing. (**C**) Amplification of cassette arrays from environmental class 1 integrons using primers MRG284/285. This PCR also generates variable product sizes dependent on the identity and size of the component cassettes in the array, however cassettes in environmental arrays are unlikely to encode antibiotic resistance. (**D**) Screening of 16S PCR products by digestion with *Hinf*1. Isolates with identical restriction patterns are likely to be the same species. If large numbers of a single restriction type are recovered, only a few of these need be sequenced. Please click here to view a larger version of this figure.

PCR	Gene target	Primer name	Direction	Sequence 5' – 3'	Cycling conditions	Reference
HS463a/HS464	Class 1 Integron	HS464	Forward	ACATGCGTGTAAATCATCGTCG	94 °C 3 min; 35 cycles 94 °C 30 sec, 60 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 5 min	Stokes *et al.*, 2006[28]
		HS463a	Reverse	CTGGATTTCGATCACGGCACG		
f27/r1492	16S rRNA	f27	Forward	AGAGTTTGATCMTGGCTCAG	94 °C 3 min; 35 cycles 94 °C 30 sec, 60 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 5 min	Lane, 1991[29]
		r1492	Reverse	TACGGYTACCTTGTTACGACTT		
intI1F165/ IntI1R476	Clinical class 1 integron	intI1F165	Forward	CGAACGAGTGGCGGAGGGTG	94 °C 3 min; 35 cycles 94 °C 30 sec, 55 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 5 min	Gillings, 2014[10]
		intI1R476	Reverse	TACCCGAGAGCTTGGCACCCA		
HS549/HS550	3'CS Clinical Integron	HS549	Forward	ACTAAGCTTGCCCCTTCCGC	94 °C 3 min; 35 cycles 94 °C 30 sec, 65 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 5 min	Stokes *et al.*, 2006[28]
		HS550	Reverse	CTAGGCATGATCTAACCCTCGG		
HS458/HS459	Clinical cassette array	HS458	Forward	GCAAAAAGGCAGCAATTATGAGCC	94 °C 3 min; 35 cycles 94 °C 30 sec, 55 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 5 min	Holmes *et al.*, 2003[30]
		HS459	Reverse	GTTTGATGTTATGGAGCAGCAACG		
MRG284/MRG285	Environmental cassette	MRG284	Forward	GTTACGCCGTGGGTCGATG	94 °C 3 min; 35 cycles 94 °C 30 sec, 55 °C 30 sec, 72 °C 1 min 30 sec; 72 °C 5 min	Gillings *et al.*, 2009[21]
		MRG285	Reverse	CCAGAGCAGCCGTAGAGC		

Table 1. Primer sequences and PCR conditions used for identifying Class 1 integrons and their associated gene cassettes.

PCR reagents	Volume (µl)	Restriction digest reagents	Volume (µl)
Sterile water	21.5	Sterile water	23
10x GoTaq White	25	Buffer B	5
RNAseA [1 mg/ml]	0.5	Bovine Serum Albumin [1 mg/ml]	1
Forward Primer [50 µM]	0.5	HinfI [10 units/µl]	1
Reverse Primer [50 µM]	0.5		
Mastermix volume	48	Mastermix volume	30
DNA template	2	PCR Template	20
Final PCR volume	50	Final restriction digest volume	50

Table 2. PCR and restriction digest mastermix constituents.

Discussion

The identification of integrons and their associated gene cassettes is potentially a key step in predicting the emergence of new opportunistic pathogens, tracking pathways for pathogens into the human food chain, and identifying new resistance and virulence determinants[8,21,26]. The aim of this paper was to describe a streamlined approach for screening samples for class 1 integrons, characterizing their cassette arrays and identifying the bacterial species in which they reside. Critical steps in the protocol involve good microbiological practice, and preventing PCR contamination that would generate false positives.

The protocol described here can be easily modified to detect other clinically relevant integrons, including the class 2 and class 3 integrons that are also found in human pathogens. It can also be modified to detect integrons in microbial communities from water, biofilms, soil or sediment. There are some limitations to this technique, which arise from a reliance on culturing of bacterial cells. Many environmental bacteria are not easily culturable, and the protocol described here would not detect these species. The range of species that are recovered could be expanded by using different bacterial media formulations and longer incubation times. Nevertheless, the majority of species of interest for human health are likely to grow under the conditions described here.

This protocol has some advantages over techniques that use plating on selective media. No assumptions need be made about the identity of resistance determinants carried by individual isolates, and new resistance genes can be recovered and characterized. In more general terms, the workflow can also be adapted to detect any element of the resistome[27] or mobilome that might be of interest[4]. Such assays are important for understanding the dynamics of the various DNA elements involved in antibiotic resistance, and critical for conserving the dwindling armory of antimicrobial compounds.

Disclosures

The authors have nothing relevant to disclose.

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