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A quantitative polymerase chain reaction method for the detection in avian faeces of salmonellas carrying the *spvR* gene

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SUMMARY

A quick, semi-quantitative method of detecting *Salmonella* species which contain the virulence plasmid has been developed using the polymerase chain reaction (PCR). A pair of primers have been synthesized encompassing a 500 bp fragment of the *spvR* virulence gene. Competitor DNA consisting of the *spvR* gene with a 94 bp deletion situated between the primer recognition sequences, was cloned into a plasmid vector. Co-amplification of the 'unknown' target salmonella DNA with known quantities of competitor DNA in the same reaction tube gave PCR products of 500 and 406 bp respectively. Visual assessment of the ratio of the two products on ethidium bromide stained agarose gels provided an estimate of the approximate number of salmonella cells present in avian faeces.

The technique could be applied to detect quantifiably any non-host DNA in clinical samples if a suitable DNA sequence for primer construction is available.

INTRODUCTION

Pathogenic micro-organisms in clinical, food and environmental samples are usually detected by time-consuming bacteriological methods. For example, testing for the presence of salmonella can take up to 3 days, and longer to identify the serotype [1]. There is an increasing need for more rapid tests which are sensitive, reliable and can be used in routine analysis.

Polymerase chain reaction methods for the detection of different pathogenic organisms in clinical samples, food stuffs and water have been published [2–5], but these are usually used only for research purposes. The sensitivity and rapidity of PCR detection have been its main attraction; the method has consistently proved to detect as few as 4–10 cells [6, 7]. This is particularly useful for food samples which will contain relatively few organisms compared with clinical samples.

There are various problems which can affect interpretation of PCR results. The sensitivity could potentially cause problems with samples which contain only a few cells but which might not be considered dangerous in terms of disease or carrier status. False positives can be detected by the use of the correct negative controls and 'clean' laboratory practice. The presence of dead organisms in a sample can also be misleading. This applies especially to foods which have undergone irradiation treatment or some other form of processing which renders

the bacteria harmless but leaves behind detectable DNA. False negatives are mainly due to the presence of Taq polymerase inhibitors. This situation occurs in both clinical and food samples [3, 8, 9]. Any other failure of the reaction can usually be detected by the use of positive controls.

Attempts to quantify the number of pathogens using PCR have been mainly restricted to HIV virus detection by reverse transcription followed by PCR [10–12]. There are difficulties inherent in PCR which make quantification potentially unreliable – the exponential increase in product will similarly increase any contamination, and small differences due to pipetting errors or temperature differences across the heating block of the thermal controller can greatly alter the amount of end product [13, 14]. It is generally accepted that the most reliable method is to use an internal competitor DNA as a quantifying standard which is amplified in the same tube as the unknown DNA and is as near as possible the same sequence, utilizing the same primers [15–17]. To accomplish this, several dilutions of the competitor are used, adding to each an identical amount of the sample containing the unknown number of target molecules. Following PCR amplification, the same starting amounts of competitor and target will give bands of equal intensity when subjected to electrophoresis through an agarose gel. whereas a greater initial number of molecules of competitor will give a competitor band of greater intensity and *vice versa*. As it is the ratio between the amounts of the two products that is important, any non-specific binding of the primers to non-target sequences will not affect the result.

Our aim was to create a test which could be used directly on clinical samples without the need for pre-enrichment. The test should be quick, sensitive and give some idea of the amount of pathogenic organisms present. The present investigation involved the detection of salmonella organisms in chicken faeces. The recent increases in frequency of isolation of salmonella, especially *S. enteritidis* from poultry together with the large number of cases and outbreaks of salmonella-associated food poisoning has resulted in regular statutory testing of breeder flocks [18].

MATERIALS AND METHODS

Bacterial strains

Sixty-seven bacterial strains were tested with the PCR primers spvR1 and 2. These comprised 15 salmonella serotypes including 22 *S. enteritidis* strains and 28 non-salmonella isolates from closely related genera. Table 1 lists these strains and presence of a salmonella virulence-associated plasmid if known. All strains were stored on Dorset egg slopes at 4 °C. Bacterial strains were provided by P. W. Jones or P. A. Barrow, Institute for Animal Health, Compton. All bacteria were grown in Luria-Bertani (LB) broth (Oxoid) overnight with agitation at 37 °C. Viable bacterial counts were made on LB agar using a modification of the method of Miles, Misra and Irwin [19].

Primers and probe

Suitable *spvR* primers were selected using published sequence of the *spvR* gene of the 8 kb virulence-associated fragment on the *S. typhimurium* virulence plasmid [20]. Figure 1 shows the relative positions of the primers and probe on the *spvR*

gene. The PCR primers designated spvR1 and spvR2 were 20mers encompassing a 500 bp region of *spvR*. SpvR1 was a forward primer 5′-ACAGGTTC-CTTCAGTATCGC-3′ (NT 130–149) and spvR2 was a reverse primer 5′-CTGTT-GATATCAGGTTT-3′ (NT 629–610). SpvR3 (5′-TCTGAAATAACCCTGCTCAG-3′, NT 201–182) was also a reverse primer which, when used for PCR in conjunction with spvR1 produced a 72 bp product which was utilized as a probe. All oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer.

DNA extraction and PCR amplification

Plasmid DNA for use as an internal competitor in PCR and that used as a positive control, was extracted using a lithium chloride method from cultures of *Escherichia coli* HB101 grown in LB broth [21]. The DNA concentration of these extracts was determined by measuring the absorbance spectrophotometrically at 260 nm.

Whole genomic DNA from salmonella cells in broth-grown cultures used for the sensitivity, specificity and whole cell quantitative PCR assays (QPCR), was extracted *in situ* in the PCR reaction tube. In order to test the ability of primers spvR1 and 2 to anneal to different bacterial strains during the PCR, approximately 2 μl of an overnight culture was added to each reaction tube. For assessment of sensitivity and whole cell QPCR, volumes of 50 μl of culture dilutions were centrifuged in 0·5 ml microfuge tubes and the supernatant discarded. DNA was amplified by PCR using a final concentration of 1 μM of each primer, 0·2 μM of each deoxynucleotide triphosphate (dNTP), 1·5 mM-$MgCl_2$, 50 mM-KCl, 10 mM-Tris-HCl (pH 8·8), 0·1 % Triton X-100, 0·1 U Taq DNA polymerase (Promega) in a final volume of 50 μl. The reaction mixtures were overlaid with 40 μl of light mineral oil (Sigma) and amplified on a M J Research programmable thermal controller. After an initial denaturation period of 5 min at 94 °C, amplification was carried out for 40 cycles of 1 min at 94 °C (denaturation), 1 min at 60 °C (annealing of primers to template) and 2 min at 72 °C (extension). A finishing period of 10 min at 72 °C was carried out to ensure complete extension. Lysis of whole cells took place during the initial heat denaturation step.

Several methods were used to extract DNA from chicken faecal samples to which *S. typhimurium* had been added at 10^8 cells/g of faecal material (wet weight). In the first method, samples (0·3 g) were boiled in 1 ml 2 % dithiothreitol for 10 min (M. J. Woodward, personal communication). In the second method, DNA was extracted by a proteinase K and phenol/chloroform technique [9]. In the third method, DNA was released from faecal samples using 'Geneclean' (Stratech Scientific Ltd). Sodium Iodide (0·8 ml) and 5 μl 'glassmilk' were added directly to 0·3 g faeces, mixed and incubated at room temperature for 10 min. The procedures outlined in the manufacturer's protocol for washing and eluting DNA were followed (J. H. Kusters, personal communication). The resultant DNA from these methods was diluted 1:10, 1:100, 1:1000 and 1:10000 in distilled water and 40 μl of these dilutions subsequently used in the PCR alongside the undiluted sample. Amplification of DNA was carried out as described above. Some faecal samples were dialysed against TE buffer (10 mM-Tris-HCl pH 8·0, 1 mM-EDTA) overnight at 4 °C before extraction by any of the various methods.

PCR products were separated by electrophoresis on 2 % agarose gels in 1XTBE

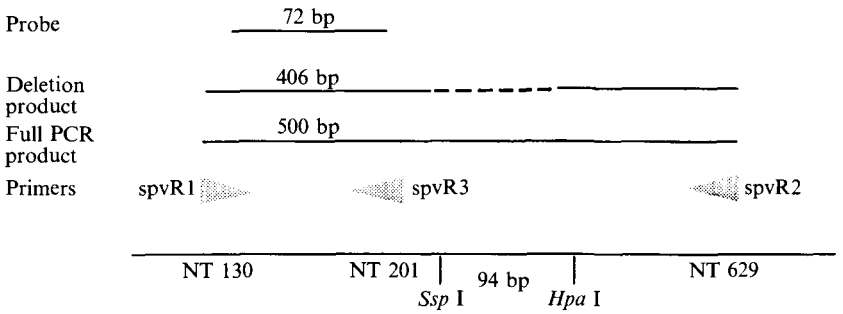

Fig. 1. Relative positions of the primers spvR1, 2 and 3 on the *spvR* gene and their respective PCR products. The 72 bp probe used to confirm the sequence of the PCR products was constructed by amplifying DNA between primers spvR1 and 3. The plasmid DNA used as a competitor for PCR was created by a deletion between *Ssp* I and *Hpa* I sites. The positions of the primers on the *spvR* gene are indicated by published nucleotide number (NT) [20].

buffer containing ethidium bromide at 0·5 mg/l and visualized on a u.v. transilluminator.

Hybridization of PCR product with digoxygenin (DIG)-labelled probe

The primers spvR1 and spvR3 were used to amplify a 72 bp fragment from the *spvR* gene of *S. dublin* using dNTPs at 0·5 mM which included digoxygenin-2′-deoxyuridine 5′-triphosphate in place of 2′-deoxythymidine 5′-triphosphate. Labelled fragment was purified by electroelution and used to probe Southern blots of gels of PCR products. DNA from gels was transferred to nylon membranes (Amersham International) by the method of Southern and hybridized overnight with the denatured probe [21]. The DIG label was detected using a DIG luminescent detection kit (Boehringer-Mannheim UK) and subjected to autoradiography for up to 1·5 h using X-ray film (Fuji).

Restriction digestion of PCR product

DNA from PCR reactions was extracted with phenol/chloroform, precipitated with isopropanol and the pellet vacuum-dried and redissolved in 15 μl of sterile distilled water. Seven microlitres were digested with *Taq* I and *Ava* II (Boehringer-Mannheim UK), following manufacturer's directions. Digestion products were analysed by electrophoresis on 2% agarose gels.

Construction of competitor DNA for quantification

The competitor DNA used for quantification was derived from pATspvR, a plasmid vector containing the *Eco*R I-*Sal* I fragment of the essential virulence region which contains the *spvR* gene, provided by J. M. Spink (Institute for Animal Health, Compton). pATspvR was cut with *Hpa* I and *Ssp* I (at positions 1448 and 1542, [20]) eliminating a 94 bp section from between the primer sites of spvR1 and spvR2 (Fig. 1), and re-ligated in a blunt end ligation reaction using T4 ligase (Promega).

RESULTS

Detection of salmonella *using the polymerase chain reaction*

Sixty-six bacterial strains, both salmonella and non-salmonella, were tested using the spvR1 and spvR2 primers in the polymerase chain reaction, in order to establish the presence of the *spvR* gene in plasmid containing strains and to ascertain the specificity of the primers for *Salmonella* species. A PCR product of 500 bp was obtained from all strains thought to contain the 8 kb virulence region from colony blot hybridization studies [22] and other serotypes containing a large plasmid of approximately 54 kb (P. A. Barrow, personal communication), presumed to be a salmonella virulence plasmid. Plasmid-free salmonella strains, salmonella strains known to contain a plasmid not containing the 8 kb virulence region and other related members of the Enterobacteriaceae were not detectable with these primers. All strains were tested twice. The results are shown in Table 1.

In order to confirm that the correct sequence was being amplified, and that the PCR products obtained were not the result of non-specific binding of the primers, the PCR products obtained from the above salmonella strains were investigated using Southern blot and restriction enzyme analysis. A DIG-labelled probe whose sequence corresponded to part of the *spvR* gene hybridized to the PCR products amplified from *Salmonella* species containing the virulence plasmid (results not shown). Restriction digests were carried out on DNA from pooled PCR product from salmonella serotypes producing the 500 bp fragment, and on the 406 bp product from the competitor plasmid. The restriction enzymes *Taq* I and *Ava* II were selected because both enzymes cut the PCR product sequence once upstream of the 94 bp deletion, generating two detectable fragments (Fig. 2). When the resultant digests were separated on an agarose gel, bands of about the expected sizes were seen; from the 500 bp fragment the predicted bands were 173 and 327 bp from the *Taq* I digest and 203 and 297 bp from the *Ava* II digest, and from the 406 bp product the predicted bands were 173 and 233 bp for *Taq* I and two bands of 203 bp for *Ava* II.

The sensitivity assay using whole *S. enteritidis* cells could detect down to 3 cells using 40 cycles of amplification (results not shown).

Quantitative PCR

Initially the quantification of the assay was tested on a vector plasmid containing the *spvR* gene. A known amount of the target DNA was amplified in the presence of a series of 10-fold dilutions of the 406 bp competitor plasmid. Figure 3*a* shows that an approximately equal amount of competitor and target appeared on the gel as bands of equal intensity.

A similar assay to that used above for the plasmid was used to assess the accuracy of quantification on whole, broth-grown salmonella cells. A dilution series of the competitor DNA was made and added to a known number of log phase salmonellas. These experiments gave a consistently high estimation of molecules of this gene fragment per cell with an average (logarithmic average of eight experiments) of 190 molecules per cell, within a range 40–700 molecules/cell (an example of these results is shown in Fig. 3*b*). As there is only presumed to be one copy of this gene per plasmid, and the published copy number for the

Table 1. *Bacterial strains used to determine the specificity of PCR products*

	Plasmid	Amplification with spvR1 & 2
Salmonella strains		
*S. abortusovis**	+	+
*S. derby**†	+	−
*S. dublin**	+	+
S. enteritidis (20 isolates)‡	+	+
S. gallinarum	+	+
S. give	−	−
S. heidelburg†	+	−
*S. infantis**	−	−
*S. nagoya**†	+	−
*S. newport**	−	−
*S. paratyphi C**	+	+
S. pullorum	+	+
*S. schwarzengrund**†	+	−
*S. stanley**	−	−
S. typhimurium (5 isolates)	+	+
Non-salmonella strains		
Citrobacter diversus		−
C. freundii		−
C. sp.		−
Enterobacter cloacae		−
E. agglomerans		−
Escherichia coli (11 isolates)		−
Klebsiella sp. (3 isolates)		−
Proteus sp. (3 isolates)		−
Pseudomonas aeruginosa (3 isolates)		−

* Salmonella strains used in colony hybridization studies with the 8 kb virulence region [22].

† Some of these strains have plasmids which do not contain the 8 kb virulence region.

‡ Of these *S. enteritidis* isolates, 8 were phage type (PT) 4 and 1 was PT6; the rest were undetermined. An extra PT4 isolate which did not contain a plasmid was tested with the primers and proved negative as expected.

salmonella virulence plasmid is 1–2 [23], this result implies that the quantification. while proving accurate in estimating numbers of molecules of the gene present in a sample when the gene is incorporated into a plasmid vector, is less so when whole cells are used.

Samples of chicken faeces were inoculated with a known number of *S. typhimurium* cells. These samples had no initial infection with salmonella as confirmed by control samples without added salmonella. The samples were presumed to contain the normal gut flora, none of which produced false positive PCR results.

Due to the presence of one or more inhibitors of Taq polymerase present in the faecal samples, the DNA extracted by all three methods had to be diluted to between 1:100 and 1:10000 before a PCR product could be detected on the gel.

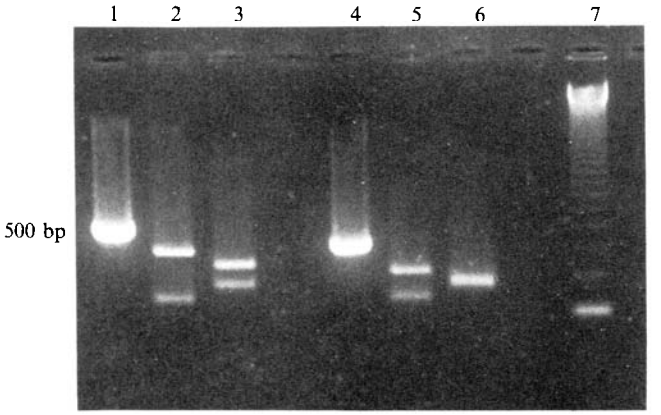

Fig. 2. Restriction enzyme analysis of PCR products. Lanes: 1. 500 bp PCR product using primers spvR1 and 2, from pooled product of *S. enteritidis* and *S. typhimurium*, 2. Same product after digestion with *Taq* I (bands of 173 and 327), 3. Same product after digestion with *Ava* II (bands of 203 and 297 bp), 4. 406 bp PCR product using the same primers from competitor DNA, 5. Same product after digestion with *Taq* I (bands of 173 and 233 bp), 6. Same product after digestion with *Ava* II (two co-migrating bands of 203 bp), 7. 123 bp ladder molecular weight marker.

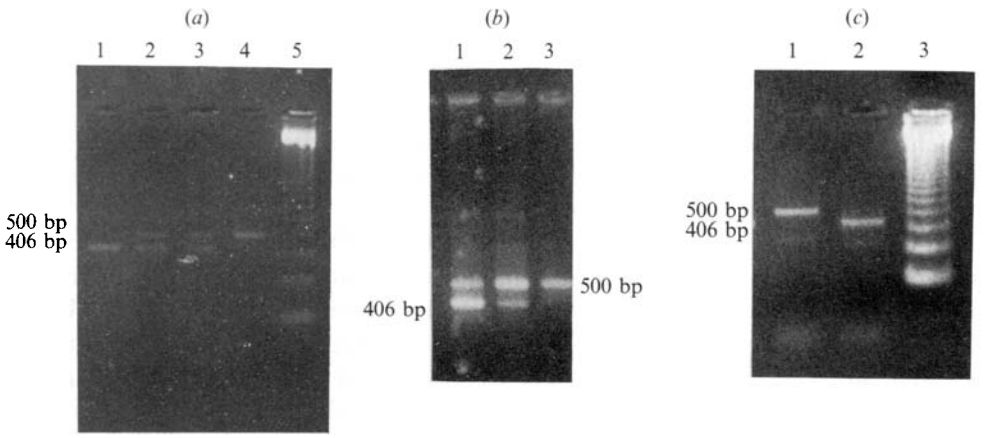

Fig. 3. QPCR results.

(*a*) A constant amount of pATspvR (2×10^6 molecules) was co-amplified with decreasing ten fold dilutions of competitor DNA (pATspvR with a 94 bp deletion). Lanes: 1. 7×10^8 molecules competitor DNA, 2. 7×10^7 molecules, 3. 7×10^6 molecules, 4. 7×10^5 molecules, 5. 123 bp ladder molecular weight marker.

(*b*) A constant amount of whole *S. typhimurium* 4 cells ($1{\cdot}8 \times 10^4$) were co-amplified with decreasing tenfold dilutions of competitor DNA. Lanes: 1. $2{\cdot}7 \times 10^6$ molecules competitor DNA, 2. $2{\cdot}7 \times 10^5$ molecules, 3. $2{\cdot}7 \times 10^4$ molecules.

(*c*) A constant amount of competitor DNA ($2{\cdot}7 \times 10^3$ molecules) was co-amplified with DNA extracted from chicken faeces by the 'Geneclean' method, to which different amounts of *S. typhimurium* 4 had been added. Lanes: 1. 50000 cells. 2. 500 cells. 3. 123 bp ladder molecular weight marker.

This obviously reduced the sensitivity of the assay on these samples. No method of DNA extraction appeared to be better than the other in terms of removing polymerase inhibitors. Indeed, any differences in dilution required in order to reduce the inhibition to acceptable levels, varied from sample to sample (results

not shown). Dialysis of the faecal sample before DNA extraction did not reduce the degree of dilution needed to visualize PCR products.

It was possible to determine whether a given faecal sample had more or less salmonella cells present than a pre-set standard using the QPCR technique. The competitor DNA was added at a level of $2{\cdot}7 \times 10^3$ molecules and samples with more than this amount of salmonella gave a product of 500 bp, whereas samples with less than this amount gave product of 406 bp (Fig. 3*c*).

DISCUSSION

A semi-quantitative method for the detection of salmonella which is both quick and sensitive in broth-grown cultures has been developed. A result can be obtained from faecal samples in the course of one working day using quantitative PCR (QPCR). This method was developed as a model of a technique using primers to a salmonella virulence gene, *spvR*, which has been sequenced from several serotypes [24]. Although most virulent serotypes of salmonella contain the virulence plasmid there are several notable exceptions (e.g. *S. virchow*), and the method could be expanded to detect all salmonellae by using primers from DNA common to all serotypes. This method could quantitatively detect salmonellas in broth cultures, although the calculated plasmid copy number (190) did not agree with the published value of 1–2 [23] which is widely held to be a reasonable estimate. The reason for this discrepancy is unclear, but could be due to a slight mismatch in one or both of the primer sequences with the non-competitor target which influences the reaction in favour of the competitor DNA. The degree of accuracy achieved is an improvement on the current detection methods which do not estimate the numbers of cells.

In addition to standard bacteriological culture, detection methods reported for salmonella include ELISA, utilizing monoclonal antibodies [25, 26], fluorogenic detection using the enzyme substrate 4-methylumbrelliferylcaprylate (MUCAP) [27, 28], a latex slide agglutination test (Mercia Diagnostics) and a conductance test [29, 30]. These tests are quicker than the conventional bacteriological method but they are neither quantitative nor sensitive enough to detect small numbers of bacteria [31]. Non-quantitative detection of salmonellas by PCR after cell isolation by a magnetic-immuno system has been described [2]. This PCR method was sensitive enough to detect 100 cfus of salmonella, compared to 3 cfus in this study. The magnetic-immuno technique has since been used on human faecal samples with some success but has not yet been tested with avian faeces [32]. In our study there did not appear to be any significant non-specific binding of the primers with DNA from faeces, as shown by negative controls using faeces which had not been inoculated with salmonella.

The QPCR method allows the detection of false negatives caused by technical failure or error as the competitor DNA acts as an internal control. The absence of product from competitor DNA in a reaction confirms inhibition or failure of the PCR [33]. QPCR also enables a pre-set number of bacterial cells to be selected, allowing discrimination between samples which are either slightly or heavily contaminated.

One of the main problems preventing the widespread application of PCR

methods to routine laboratory testing is the lack of a standard DNA extraction method for clinical samples. The method described here has been shown to work on avian faecal specimens, but the degree of dilution of DNA needed to ensure the removal of polymerase inhibitors resulted in a loss of sensitivity of 10^2–10^4-fold. This problem is usually counteracted by extensive DNA purification [3, 9], although this may affect accuracy and sensitivity, as DNA is lost at every stage of purification. The addition of T_4 gene 32 DNA binding protein to the PCR has been shown to reduce the inhibition in some cases [32]. Particular difficulty was experienced by us in using chicken faeces. This may be due to the differing composition of avian and mammalian faeces in that the former contains urates and urine is known to inhibit PCR [34]. Dialysis of faecal samples did not remove the inhibitory substance present. It is possible that the relatively insoluble urates present in avian faeces could be possible candidates for the polymerase inhibitor. The inhibitory problems encountered with faecal samples were not fully resolved. Nevertheless, the method represents a significant step toward a rapid quantitative detection method for clinical samples.

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