

Detection of the C protein gene among group B streptococci using PCR

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Abstract

Aim—To develop a polymerase chain reaction (PCR) for the specific detection of the C protein gene in strains of group B *Streptococcus*.

Methods—A single primer pair derived from the nucleotide sequence of the IgA binding β antigen of the C protein complex permitted the specific amplification of a 592 base pair DNA fragment from the C protein gene. After 35 cycles of amplification this product could be detected by agarose gel electrophoresis. Southern blot hybridisation confirmed that this product was the C protein gene.

Results—PCR detected the C protein gene in 75 (63%) of 119 strains of group B streptococci analysed. The product was not detected in other Gram positive organisms, showing that this PCR assay was highly specific. The sensitivity of the assay was satisfactory to a dilution of 1 in 10 000 of extracted DNA.

Conclusions—The C protein of group B streptococci is associated with neonatal sepsis. The specific detection of the C protein gene by PCR may help identify which strains are likely to be associated with infection by the organism.

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Group B streptococci are facultative Gram positive organisms with an ultrastructure similar to other Gram positive cocci.¹ They may cause serious infection in adults,² especially in post-partum women and in diabetics, but it is in the context of neonatal sepsis that the most serious consequences of infection occur.³ Infection in the first seven days of life (early onset disease) results in septicaemia, pneumonia, meningitis and more rarely, osteomyelitis.⁴ Late onset disease (7 days to 3 months of age) more commonly results in meningitis.⁵ Risk factors for infection are known to include prematurity, prolonged rupture of membranes, and maternal fever during labour, in the presence of vaginal or rectal carriage of the organism.⁶

Immunity to group B streptococci is associated with the presence of antibody against the six well described type specific capsular polysaccharides which form the basis for the current serotyping system of the organism.⁷ We recently developed a typing scheme based on the separation of radiolabelled proteins of

the organism.⁸ There is evidence that antibodies produced against protein components of the organism are also important in conferring protective immunity.^{9,10} In particular, antibodies to a protein complex consisting of four antigenic components (α , β , γ and δ), known as the C protein complex, have been associated with the development of protective immunity.^{11,12} Part of the C protein complex consists of two distinct acid extractable antigenic components, the α antigen and a β antigen, both of which are expressed on the bacterial surface.¹³ It has been suggested that α and β antigen positive strains are more virulent in the context of the complex interactions of immunoglobulins, complement, and polymorphonuclear cells and that these strains are more resistant to opsonisation and to intracellular killing. C proteins seem to contribute to resistance to opsonisation and intracellular killing by phagocytes.¹⁴ About 60% of group B streptococci isolates of human origin contain the C protein complex and there is increasing evidence that the complex may contribute to the virulence of this organism.¹⁵

The sequence determination of the IgA binding β antigen of the C protein complex¹⁶ enabled us to develop oligonucleotide primers specific for the C protein gene. The polymerase chain reaction (PCR) can selectively amplify the copy number of a target gene more than 10⁶ fold,¹⁷ and is being used increasingly as a diagnostic tool to indicate the presence of a potentially infecting organism.¹⁸ Because the presence of the C protein may relate to virulence associated with invasive strains of the organism, the development of an assay to identify rapidly those strains carrying the gene of this protein could be helpful.

Methods

BACTERIAL STRAINS AND CULTURE MEDIA

One hundred and nineteen group B streptococci isolates obtained from routine clinical high vaginal swabs were initially cultured and purified by inoculation on to pre-reduced blood agar plates and Islam's selective media plates (Oxoid, Basingstoke). The pure cultures were stored in glycerol broth at -70°C. Nine freeze dried NCTC strains representing different group B streptococci serotypes (Public Health Laboratory Service, Colindale), clinical isolates of strains of streptococci groups A, C, D and G, and a clinical strain of *Staphylococcus epidermidis* and

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the NCTC strain of *Staphylococcus aureus* (Oxford Staph) were also included in the study and stored at -70°C in glycerol broth. Working stocks of all strains were obtained by culturing reconstituted strains on blood agar plates at 37°C under anaerobic conditions for 48 hours.

SYNTHETIC OLIGONUCLEOTIDES

All oligonucleotides were synthesised on an Applied Biosystems synthesiser (391-EP) by the automated phosphoramidite coupling method and purified as described by the manufacturer. Oligonucleotide concentrations were determined spectrophotometrically. Specific oligonucleotide primers were designed from sequence data of the IgA binding B antigen of the C protein complex. Primers IgAagGBS (5'CTATTTTTGATA-TTGACAATGCAA3') and RgAag GBS (5'GTCGTTACTTCCTTGAGATGTAA-C3') amplified a 592 base pair product.

Internal primers (Int GBS; 5'CCGCAT-GTTCGGGAATCACCAAAG3' and Int GBSR; 5'CTTTGGT GATTCGGGAATGCGG3') amplified a 96 base pair fragment which was internal to the 592 base pair product and were used both as internal primers and as probes in Southern hybridisation.

PREPARATION OF TARGET DNA

Extraction of target DNA

A standard loop of group B streptococci was inoculated into 20 ml Todd Hewitt broth (Oxoid, Basingstoke) and grown to log phase in a 37°C incubator. Cultures were centrifuged at $4000 \times g$ for 10 minutes. Cells were washed three times in phosphate buffered saline to remove excess polysaccharides. The cells were resuspended in 200 μl mutanolysin to a final concentration of 100 U/ml of TRIS-EDTA (0.5M). Suspensions were incubated for 1 hour at 37°C . DNA was isolated using a rapid method with guanidium thiocyanate salt. The cell material was resuspended in 500 μl GES reagent (guanidium thiocyanate 0.5M and EDTA 0.1M). Ammonium acetate (250 μl) was added and the samples incubated on ice for 10 minutes. Chloroform reagent (500 μl) was added (chloroform:pentanol 24:1) and the sample spun at $13\,000 \times g$ for 5 minutes. Isopropanol (0.54 volumes) was added to precipitate the DNA. The pellet was washed in 70% ethanol. DNA concentrations were determined spectrophotometrically and the purity determined by electrophoresis on a 0.8% agarose gel at 100 volts for 2 hours.

Crude DNA extraction

Group B streptococci were prepared as above by growth to log phase in Todd Hewitt broth. After centrifugation the cell pellet was treated with mutanolysin (100 μl) and incubated for 1 hour at 37°C . This preparation was then boiled for 10 minutes and the resulting boilate was centrifuged (13 000 rpm). The supernatant fluid was then used directly for the PCR assay (boilate).

PCR ASSAY

The prepared target DNA (1–10 μl) was used in the amplification assay. This target DNA was added to a 100 μl reaction mixture containing 10 mM TRIS-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.01% w/v gelatin, 0.2 mM of each deoxynucleotide, dNTP, AGCT (Pharmacia Ltd, Milton Keynes, England) and 0.5 μl of each oligonucleotide primer. *Taq* polymerase (2.5 units) (Amersham UK) was added and the reaction mix was overlaid with 50 μl mineral oil. PCR was performed using an automatic thermal cycle (Hybaid Ltd, Twickenham, Middlesex).

The amplification cycle consisted of an initial denaturation of target DNA at 98°C for 5 minutes, followed by denaturation of 94°C for 1 minute. Primer annealing was carried out at 45°C for 1 minute and extension at 72°C for 1 minute. The final cycle included 7 minutes at 72°C to ensure full extension. Samples were amplified through 35 consecutive cycles. Negative control reactions were performed with each batch of amplifications, consisting of tubes containing distilled water in place of DNA. To minimise the occurrence of false positive results, the procedures outlined by Kwok and Higuchi were followed.¹⁹

The amplified product (25 μl) and 2 μl gel loading buffer (40% weight/volume sucrose, 0.25% weight/volume bromophenol blue) were visualised on an agarose gel stained with ethidium bromide and photographed with filtered ultraviolet illumination on Polaroid type 665 film.

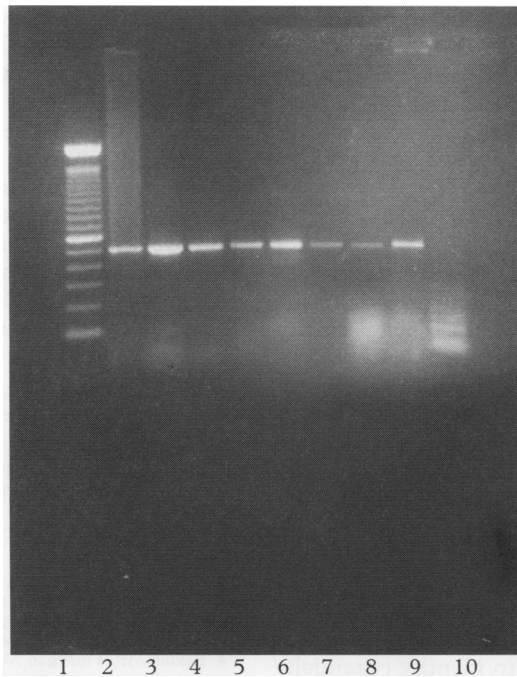
SOUTHERN HYBRIDISATION

Oligonucleotide primer Int GBS was labelled at the 5' end with ^{32}P (^{32}P] ATP; Amersham UK) and 25 U of T4 polynucleotide kinase in a buffer containing 50 mM TRIS-HCl (pH 7.4), 10 mM MgCl_2 , and 1 mM dithiothreitol. The solution was incubated at 37°C for 1 hour, and the labelled probe was separated from the unincorporated isotope by ethanol precipitation.

Amplified DNA was transferred from an agarose gel to a nylon membrane (Hybond N; Amersham Ltd) by the method of Southern.¹⁸ The membrane was prehybridised for 4 hours at 42°C in a hybridisation buffer containing $6 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15M NaCl, 0.015M sodium citrate), $10 \times \text{Denhardt's}$ solution (0.2% weight/volume bovine serum albumin, 0.2% weight/volume polyvinylpyrrolidone, 0.2% Ficoll weight/volume) and 50 μg of denatured salmon sperm DNA per ml.

The labelled oligonucleotide probe was added to the hybridisation buffer at a concentration of 1 ng/ml and hybridisation was carried out for 14 hours at 5°C below the melting temperature of the probe. After hybridisation, the membrane was washed twice for 5 minutes in $6 \times \text{SSC}$ – 0.1% (weight/volume) sodium dodecyl sulphate at room temperature and once for 5 minutes at the melting temperature of the probe. It was exposed to x ray film at -70°C for 14 hours and the film was developed.

Figure 1 Agarose gel electrophoresis of the 592 base pair PCR product of a number of group B streptococci isolates.



Lane 1, 123 base pair ladder was used as a molecular weight marker; lanes 2-6 and 9, 592 base pair products from purified DNA; lanes 7 and 8, 592 base pair products from supernatant fluids of mutanolysin treated cells; lane 10, 96 base pair product obtained using internal primers in nested PCR.

Results

DEVELOPMENT OF THE PCR ASSAY

The positions of primers IgAagGBS and RIgAagGBS within the nucleotide sequence of the β antigen predicted that a 592 base pair fragment would be generated following PCR of group B streptococci chromosomal DNA. Extracted DNA from 119 group B streptococci strains yielded a 592 base pair product in 75 (63%) of strains. Figure 1 shows the 592 base pair fragment which was the PCR product obtained by both direct extraction of the DNA (lanes 2-6 and 9), and by the boilate methodology (lanes 7 and 8). Lane 10 shows a 96 base pair product obtained by the preparation of internal primers from within the 592 base pair product.

SPECIFICITY AND SENSITIVITY OF THE PCR ASSAY

None of the eight other different Gram positive strains examined produced an amplified product, suggesting a high specificity of this PCR assay (table 1). Figure 2 shows (lanes 4-11) that a PCR product of the appropriate

Specificity of PCR assay

Bacterial species	Source	Presence (+) or absence (-) of the 592 base pair product
<i>Staphylococcus epidermidis</i>	Clinical isolate	-
<i>Staphylococcus aureus</i>	NCTC	-
<i>Streptococcus milleri</i>	Clinical isolate	-
<i>Staphylococcus warneri</i>	NCTC	-
<i>Staphylococcus saprophyticus</i>	NCTC	-
Group A streptococci	Clinical isolate	-
Group G streptococci	Clinical isolate	-
Group D streptococci	Clinical isolate	-
Group B streptococci	Clinical isolate	+

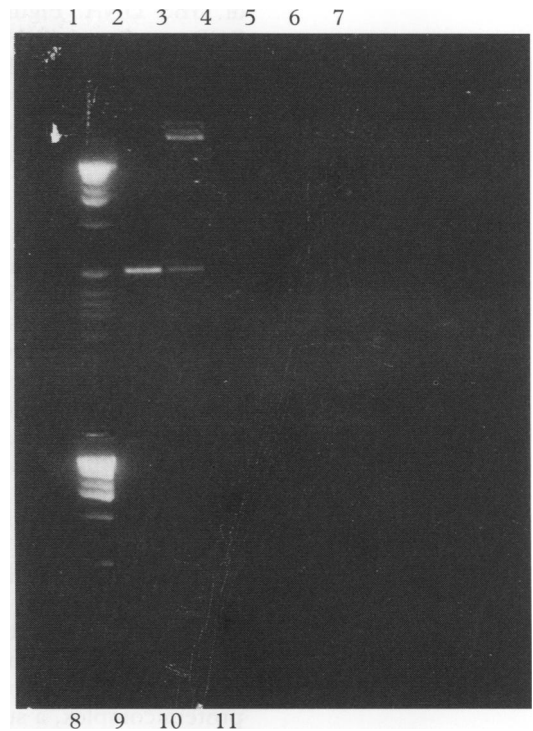


Figure 2 Agarose gel of the sensitivity and specificity of the PCR assay.

Lanes 1 and 8, molecular weight markers; lanes 2 and 3, 10^0 and 10^{-4} dilutions of GBS DNA; lane 4, *Staphylococcus epidermidis*; lane 5, *Staphylococcus aureus*; lane 6, *Streptococcus milleri*; lane 7, *Staphylococcus warneri*; lane 9; group A streptococci; lane 10, group G streptococci; lane 11, group D streptococci.

fragment size could not be detected in these strains. Additional specificity is shown in fig 3 in which there is specific labelling of the internal oligonucleotide primers with radioactive ^{32}P , resulting in a probe which hybridised to the amplified PCR product.

The sensitivity of the assay was examined by assaying with a series of 10-fold dilutions

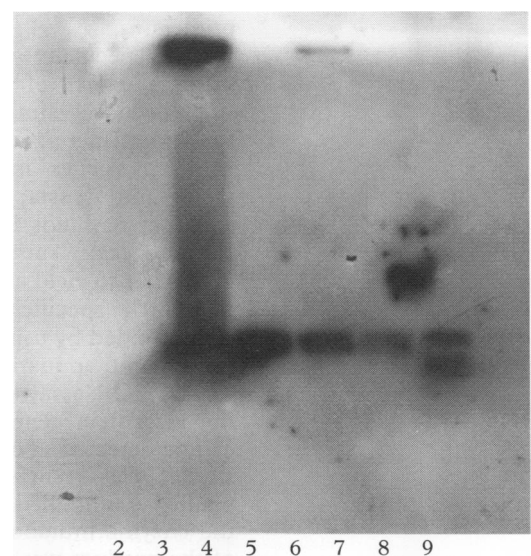


Figure 3 Southern hybridisation of PCR products of group B streptococci DNA with internal oligonucleotide primers.

Lanes 2-6, 9, hybridised products of group B streptococci DNA; lanes 7-8, hybridised products of mutanolysin prepared supernatant fluids.

of GBS DNA. Figure 2 (lane 2) shows the PCR product with undiluted DNA; lane 3 shows the product obtained at a 1 in 10 000 dilution. At dilutions of 10^{-5} and 10^{-6} a faint PCR product was detectable, but at 10^{-7} no product could be seen.

Discussion

PCR DNA amplification is a technology that has been adapted to identify micro-organisms involved in infectious diseases. The specificity and sensitivity of the assay makes the technique very attractive as a diagnostic method. It is particularly suitable for the detection of pathogens present in small numbers, or those which are difficult to culture.^{20,21}

Group B streptococci are organisms which may colonise the vaginal tract of up to 25% of pregnant women.²² In about 1% of infants who become colonised with the organism invasion may occur, producing life-threatening sepsis. The PCR assay outlined here was designed in an attempt to identify potentially pathogenic strains of the organism. The C protein complex, a surface protein of group B streptococci, has been shown to elicit the production of protective antibodies.^{12,23} Moreover, the β antigen of the complex binds to human immunoglobulin A (IgA),²⁴ which may play a critical role in bacterial resistance to host mucosal defences. Elucidation of the sequence of the IgA binding β antigen, which may have an important role in the pathogenesis of group B streptococci infections, facilitated the development of this PCR assay to detect the presence of the C protein gene.

PCR products of the correct size (592 base pairs) were obtained with both purified DNA and mutanolysin treated supernatant fluids. Confirmation of the product as the C protein gene was given using internal primers in nested PCR which yielded a 96 base pair product and by Southern hybridisation with radioactive probes of these internal oligonucleotide primers.

The specificity of the assay was tested using a panel of other streptococcal and staphylococcal strains. It was necessary to show that there was no sequence homology with any genes in these species. Purified DNA and lysostaphin, mutanolysin treated supernatant fluids in the staphylococcal and streptococcal species, respectively, were found not to yield amplified products indicating 100% specificity. False positive results were avoided by using rigorous precautions to prevent contamination.¹⁹

The PCR assay described has shown that the C protein gene of group B streptococci can be detected in 63% of such strains. Initial studies used purified DNA, but the assay could be simplified by boiling the cells with the enzyme mutanolysin, although the signal of the product was not as consistently strong as with purified DNA. We are currently studying the standard strains of group B streptococci identified by ³⁵S-methionine typing⁸ to assess whether detection of the pro-

tein genes by PCR correlates with the pathogenic strains identified by the new typing scheme. PCR may be useful in identifying all invasive strains of group B streptococci because although the C protein is only found in 63% of group B streptococci strains, the gene for the C protein may be present but not expressed in invasive strains. Collections of invasive strains of the organism are currently under analysis to assess this hypothesis. If it proves valid, this PCR assay will be useful in distinguishing colonising strains from potentially invasive strains of the organism.

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