

## An overview of broad-range 16S rDNA PCR in the routine Microbiology Laboratory

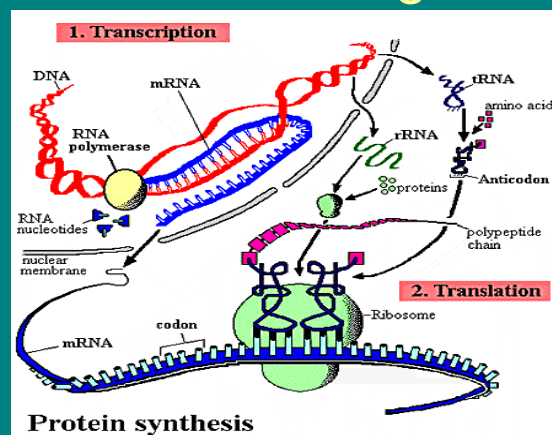
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- Overview of broad-range 16S rDNA PCR
- Re-cap of 2003 paper (validation)
- Ongoing use of the assay
- Organism specific real-time PCR
- Future Direction

## Broad-range PCR

- ◆ Multiple targets are detected with a single primer set.
- ◆ Time and cost efficient
- ◆ You don't need to know what you are looking for
- ◆ Novel organisms can be identified
- ◆ Ideal for Bacteria as most culture-based methods are also "broad-range".

## Broad range primers target the ribosomal genes



## The 16S rRNA gene

0 200 400 600 800 1000 1200 1400 1542



Variable regions



Conserved regions

## Theory

- Broad-range primers amplify a fragment of the 16S rDNA gene from any bacteria present in specimen.
- 340-bp PCR product is sequenced.
- Comparison of sequence to public databases (Genbank or The Ribosomal Database Project) or in-house database reveals identity of bacterium

## Theory (2)

- ◆ Can detect any bacterium
- ◆ Not as sensitive as a species specific PCR
- ◆ Less expensive and time consuming than multiple species specific PCRs
- ◆ Technique only works on samples from normally sterile sites.
- ◆ Mixed flora can be analysed by incorporating an additional cloning step

## 'Contaminating DNA'- reagents

Methods to reduce:

- ◆ UV irradiation
- ◆ Restriction digest
- ◆ Ultrafiltration
- ◆ Purchase 'Low DNA' reagents – We now use Taq, buffer and water from Molzym.

## 'Contaminating' DNA

- ◆ When you can't reduce it - Avoid detection by reducing the number of PCR cycles
- ◆ In 2003 we were doing 26 cycles. We now do 32 because of the Molzym reagents.

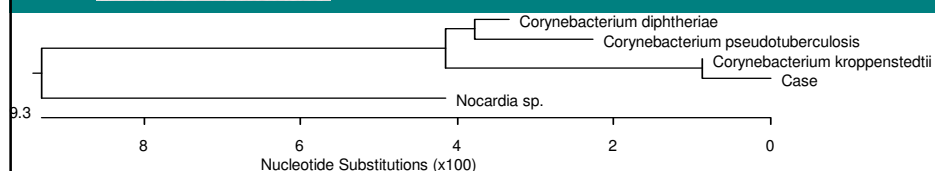
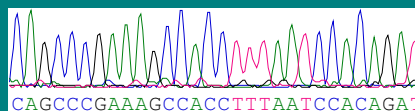
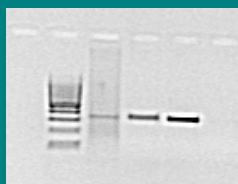
## Final sensitivity

- ◆ 10 – 100 cfu of *S. aureus* or *E. coli* input into PCR
- ◆ This is around 100 times less sensitive than a well-designed specific real-time PCR.

## Identification by sequencing

- Sequence data is analysed in 2 ways:
  - ❖ BLAST search against the Genbank or other databases
  - ❖ In- house phylogenetic analysis.

### *Corynebacterium kroppenstedtii*



#### Top hits on BLAST:

CP001620 *Corynebacterium kroppenstedtii* DSM 44385, complete genome

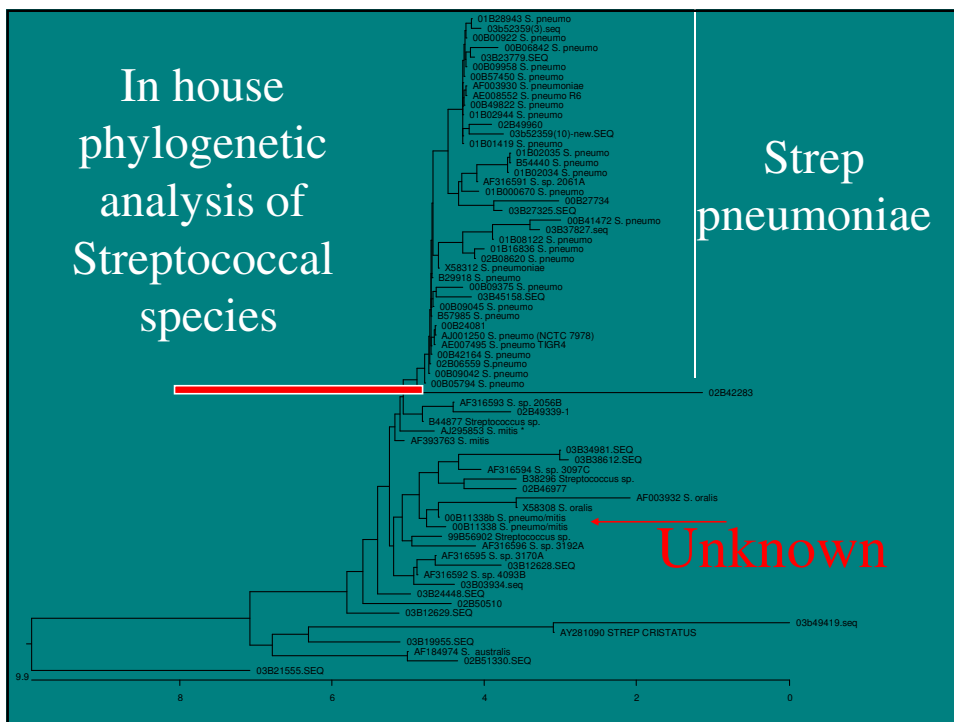
Score = 614 bits (332), Identities = 332/332 (100%), Gaps = 0/332 (0%)

Other organisms lower down list with lower % ID.

## A confusing blast!

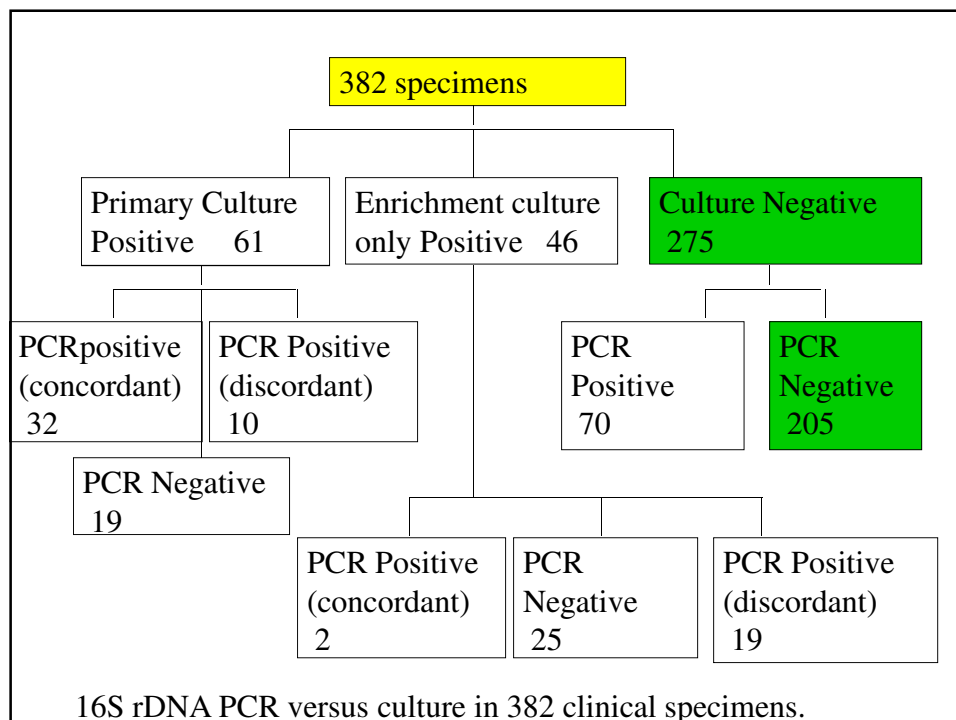
- [gi|2342538|emb|AJ001248.1|SP7466RR5](#)  
 Streptococcus pneumonia... 478 e-132  
 Identities = 247/249 (99%)
- [gi|2183313|gb|AF003929.1|AF003929](#) Streptococcus  
 mitis 16S r... 478 e-132 Identities = 247/249  
 (99%)

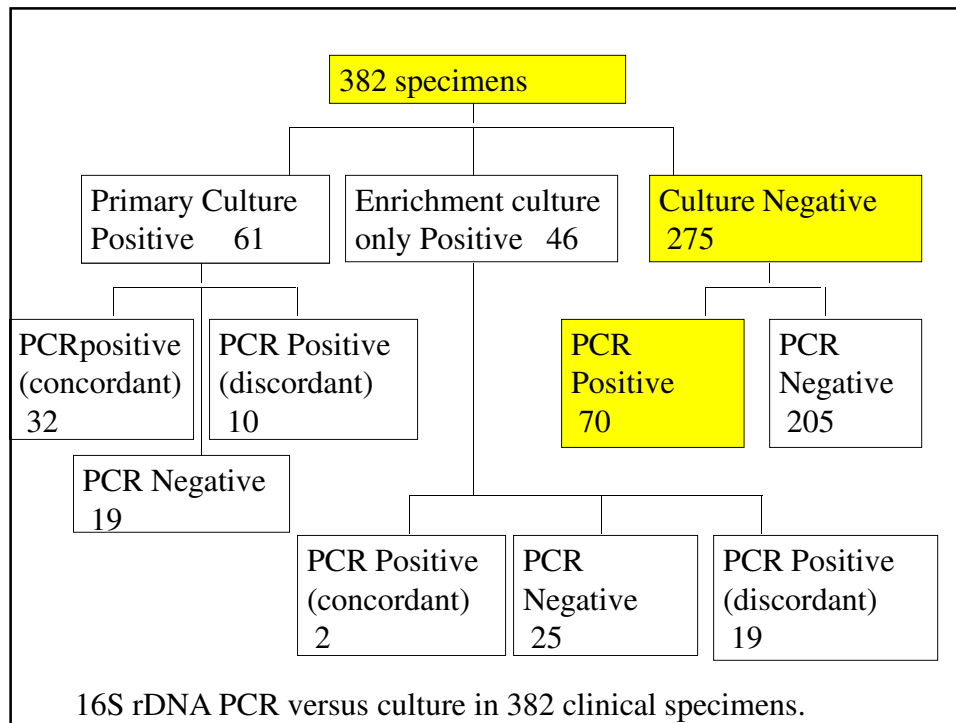
Resolve with in-house phylogenetic analysis.....



Harris K A and Hartley J C Development of broad-range 16S rDNA PCR for use in the routine diagnostic clinical microbiology service (2003) J. Med. Microbiol. 52: 685-691.

- This study looked at 382 specimens sent to the Microbiology laboratory between 31 October 1999 and 1 November 2001.
- Culture positive and culture negative specimens all ?bacterial infection
- Validation of the technique for routine use.
- Results were used in patient management





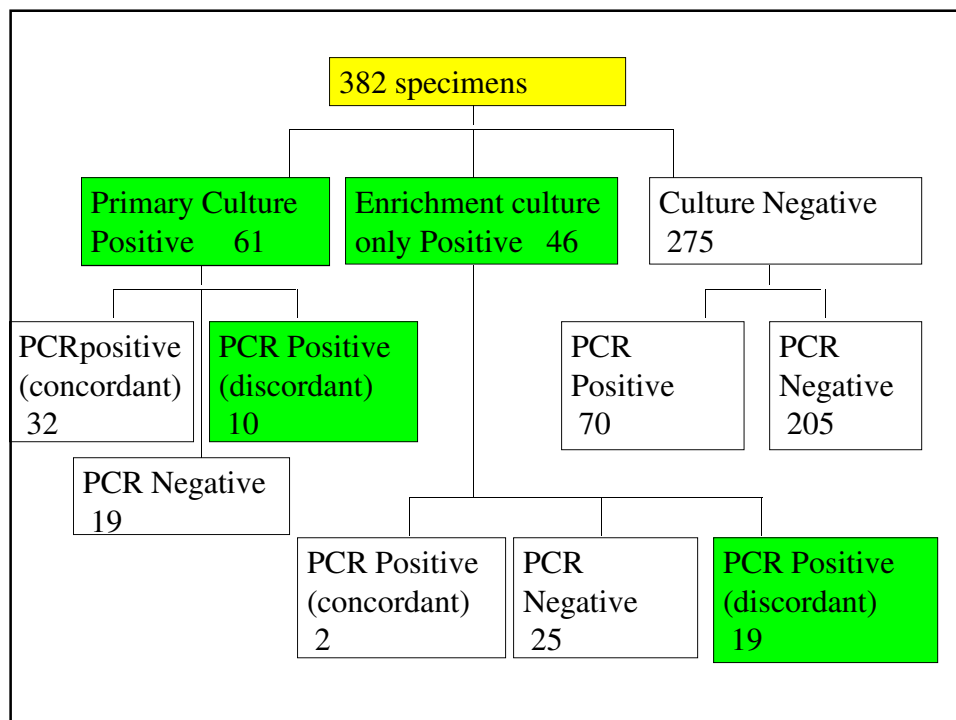
### Culture negative/PCR positive

Organism	No. of patients
<i>Streptococcus pneumoniae</i> *	20
<i>Staphylococcus epidermidis</i>	4
<i>Haemophilus influenzae</i> *	4
<i>Streptococcus pyogenes</i> *	3
<i>Staphylococcus aureus</i> *	3
<i>Propionibacterium acnes</i>	3
<i>Neisseria meningitidis</i> *	3
<i>Streptococcus agalactiae</i> *	2
<i>Corynebacterium</i> sp.	2
<i>Acidovorax</i> sp.	2
<i>Mycoplasma orale</i> *	2

## Culture negative/PCR positive

One detection each of

Streptococcus sp. (mitis group)*	Streptococcus intermedius*
Streptococcus anginosus*	Stenotrophomonas maltophilia*
Helicobacter sp.*	Staphylococcus capitis
Pseudomonas lanceolata	Pseudomonas aeruginosa*
Proteus mirabilis*	Propionibacterium granulosum
Neisseria cinerea*	Moraxella catarrhalis*
Helicobacter sp.*	Haemophilus paraphrophilus*
<b>Prevotella sp.*</b>	<b>Peptostreptococcus micros*</b>
<b>Fusobacterium necrophorum*</b>	<b>Fusobacterium naviforme*</b>
Eubacterium sp.	Enterococcus faecalis
Comomonas sp.	Acinetobacter sp.
Variovorax paradoxus	Mixed



Organism detected by PCR (and not by culture)	Number of samples (each from a different patient)
Streptococcus pneumoniae	4
Staphylococcus aureus	2
Peptostreptococcus sp.	2
Streptococcus pyogenes	2
Streptococcus intermedius	1
Ureaplasma urealyticum	1
Fusobacterium necrophorum	1

Culture  
positive/PCR  
positive  
(discordant)

Potential human pathogens, determined clinically significant by clinical review, detected by 16S rDNA PCR in samples that grew only CNS.

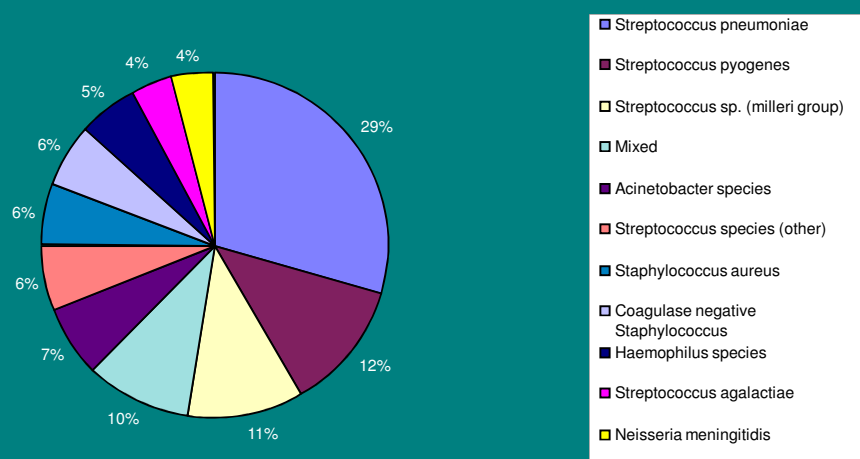
## 16S rDNA in the routine lab

- After this validation period we stopped looking at culture positive samples.
- To date we have tested a further 4000 samples by 16S rDNA PCR
- This includes a considerable number of referred specimens from adults

## What have we found?

- Majority of samples are culture negative, some may have grown other (non-significant) organisms. Sometimes an organism will be isolated later.
- Around 20% of samples positive by 16S rDNA PCR
- Still finding mostly common organisms, presumably rendered unculturable by empirical antibiotics.
- Impressive range of species and many “one-off”, including some fastidious organisms. This demonstrates the power and utility of a broad-range PCR.

## Most common organisms detected by 16S rDNA PCR (% of total number of positives)



### Other organisms found in >1 patient Nov 2001 – May 2009

Peptostreptococcus species  
 Fusobacterium sp.  
 Streptococcus sp. (mitis group)  
 Prevotella sp  
 Propionibacterium acnes  
 Kingella kingae  
 Enterococcus sp.  
 Enterobacteriaceae  
 Finegoldia magna  
 Tropheryma whippelii  
 Pseudomonas aeruginosa  
 Neisseria species  
 Mycoplasma sp.  
 Mixed (anaerobes)  
 E. coli  
 Corynebacterium species  
 Bartonella henselae  
 Salmonella species  
 Peptoniphilus species  
 Mycobacterium species (non-tb)  
 Klebsiella pneumoniae

### Organisms found in a single patient November 2001 – May 2009

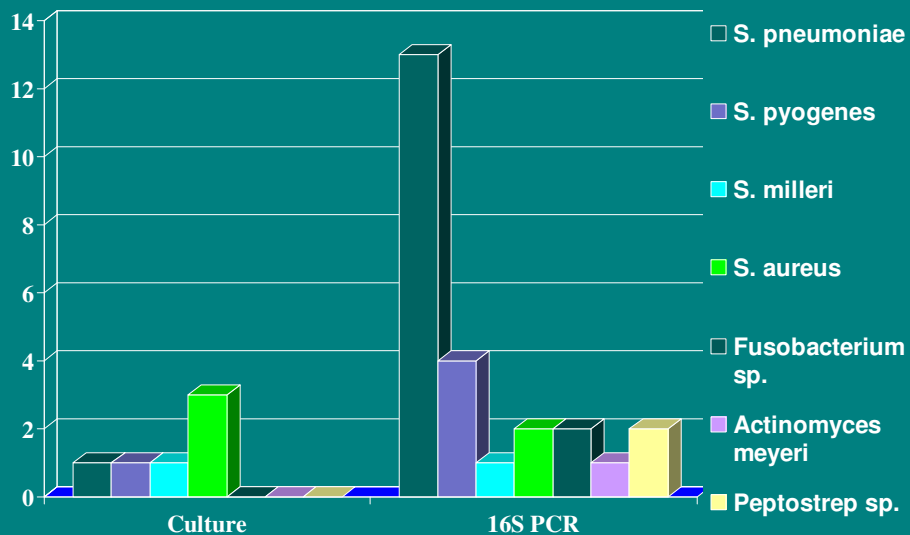
Ureaplasma urealyticum	Helicobacter pylori
Serratia marcescens	Halomonas variabilis
Ralstonia species	Granulicatella adiacens
Pseudomonas species	Gordonia terrae
Proteus mirabilis	Gemella species
Paaracoccus species	Eikenella corrodens
Paenibacillus sp.	Dialister microaerophilis
Mycobacterium tuberculosis complex	Devosia sp.
Morganella sp.	Coxiella burnetii
Moraxella species	Comamonas sp.
Micrococcus luteus	Clostridium indolis
Methylophilus sp.	Citrobacter sp.
M. tuberculosis complex	Burkholderia cepacia
Listeria monocytogenes	Bacteroides sp.
Legionella pneumophila	Arthrobacter species
Lactococcus lactis	Aggregatibacter actinomycetemcomitans
Klebsiella species	Actinomyces neuii

Sample type	% positive	Most common organisms
Abscess	50	<i>S. pneumoniae</i> <i>S. intermedius</i> Anaerobes
Heart valve	44	Viridans Streptococci Then a wide range including: <i>T. whipplei</i> <i>B. henselae</i> <i>C. burnetii</i>
Pleural fluid / empyema	21	<i>S. pneumoniae</i> <i>S. pyogenes</i> <i>S. aureus</i>
Joints	15	<i>K. kingae</i> <i>S. aureus</i> <i>S. pyogenes</i>
CSF	12	<i>N. meningitidis</i> <i>S. pneumoniae</i> Enterobacteriaceae
Blood	3	<i>Acinetobacter</i> spp <i>S. pyogenes</i> <i>Haemophilus influenzae</i>

## Empyema

- Majority now culture negative in children
- S. Saglani et. al. 2004 Archives of Disease in Childhood* – Looked at 32 paediatric pleural fluids that had culture and 16S rDNA PCR – 22 / 32 (69%) were PCR positive (only 18% culture positive).

## Organisms Identified in the 32 patients having culture and 16S PCR



## Pleural fluid

- 54 / 255 (21%) pleural fluid or Empyema Nov 2001 – May 2009. Includes a lot of referred adult samples.
- Range of organisms detected very similar to 2004 study – dominated by *S. pneumoniae* followed by *S. pyogenes*, and *Streptococcus sp.* (milleri group).
- We find more *S. aureus* now that we look for it with specific real-time assay

## Heart valves

- 41 / 94 (44%) positive
- Wide range of organisms – 16S rDNA PCR is the best assay we have for these samples.

Streptococcus species (other)	7
Tropheryma whippelii	4
Haemophilus species	4
Staphylococcus aureus	3
Staphylococcus epidermidis	3
Streptococcus sp. (mitis gp)	3
Bartonella henselae	2
Propionibacterium acnes	2
Streptococcus pneumoniae	2
Acinetobacter sp.	1
Coxiella burnetii	1
Enterococcus faecalis	1
Finnegoldia magna	1
Legionella pneumophila	1
Micrococcus luteus	1
Neisseria meningitidis	1
Peptostreptococcus micros	1
Prevotella sp.	1
Mixed- Staphylococcus aureus; Acinetobacter sp.	1

## Most frequently detected species from bones and joints

Kingella kingae	5	Acinetobacter sp.	1
Streptococcus agalactiae	4	Actinomyces neuui	1
Streptococcus pyogenes	4	Aeromonas species	1
Staphylococcus epidermidis	3	Burkholderia cepacia	1
Staphylococcus aureus	2	Gordonia terrae	1
Streptococcus dysgalactiae	2	Helicobacter species	1
Corynebacterium species	2	Mycobacterium kansasii	1
Klebsiella pneumoniae	2	Peptoniphilus asaccharolyticus	1
Fusobacterium necrophorum	2	Prevotella species	1
Streptococcus pneumoniae	1	Propionibacterium acnes	1
		Salmonella species	1
		Streptococcus bovis/equinus group	1
		Streptococcus canis	1
		Neisseria species	1

[Pediatr Infect Dis J.](#) 2007 May;26(5):377-81.

**Specific real-time polymerase chain reaction places *Kingella kingae* as the most common cause of osteoarticular infections in young children**

CONCLUSION: The *K. kingae*-specific real-time PCR places *K. kingae* as the leading cause of OAI in children at our hospital.

[J Pediatr Orthop.](#) 2009 Mar;29(2):182-8.

**The use of polymerase chain reaction for the detection and speciation of bacterial bone and joint infection in children.**

.....We conclude that current PCR methods are not superior to standard bacterial culture methods when applied to children with presumed bone or joint infections, but that PCR may complement existing microbiologic cultures for detection of bone and joint infections in children.

## In Summary

- This data shows us that 16S rDNA PCR often finds “the usual suspects”. We are building up a panel of organisms that we can detect by specific real-time (taqman) PCR
- We need more sensitive assays for some organisms or sample types.
- The range of organisms detected demonstrates the continued value of broad-range 16S rDNA PCR

## Where next for broad-range bacterial PCR?

- Inherent problems with sensitivity are hard to fix, but 16S rDNA is still the perfect technique in many scenarios (eg abscess, empyema).
- We know that our primers are not so good at some anaerobes but we are bringing in an additional set of broad-range primers to overcome this.
- Mixtures of different species are a problem because of primer competition, but you are still more likely to detect polymicrobial infections with a broad-range method.
- Analysis of our data to show value of 16S rDNA PCR in certain samples types eg Heart valves, CSF, Joints.

## Specific real-time PCR

- Organism specific real-time PCRs can be ~100 times more sensitive than the broad-range 16S rDNA PCR
- Testing algorithm that uses panel of real-time PCR assays prior to performing broad-range 16S PCR:
  - Reduces time and cost
  - Improves pick-up rate – particularly for low-yield samples types e.g. blood

## Organism specific real-time PCR

- *S. pneumoniae* PCR was introduced in 2006 and method published in 2008:

Harris K A et al (2008). Duplex real-time PCR assay for detection of *S. pneumoniae* in clinical samples and determination of penicillin susceptibility. *J Clin Microbiol* 46: 2751 – 275.

- We also have real-time assays in routine use for the following organisms:
  - *Staphylococcus aureus*
  - *Streptococcus pyogenes*
  - *Streptococcus agalactiae*
  - *Neisseria meningitidis*
  - *Tropheryma whipplei*
  - *Kingella kingae*
  - *Mycobacterium tuberculosis*
  - Atypical *Mycobacteria*

## Organism specific real-time PCR (2)

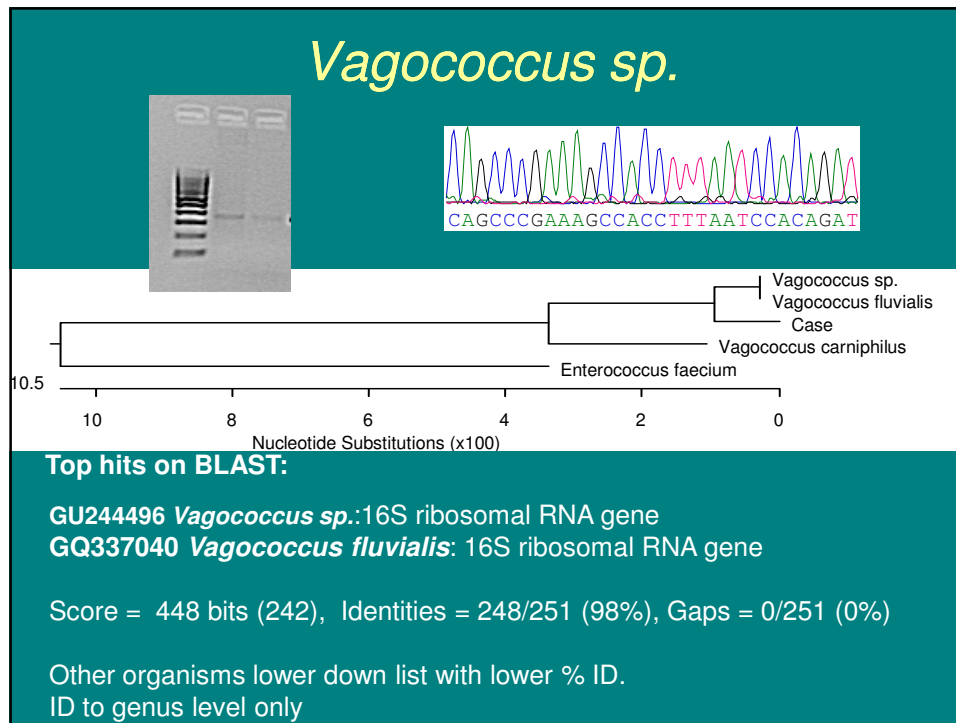
- And the following that are still undergoing validation or are part of a research study:
  - *Fusobacterium* spps
  - *Peptostreptococcus* spps
  - *Mycoplasma* / *Ureaplasma*

## Problematic sites for interpretation

- Non sterile sites (eg BAL)
- From possibly infected Prosthetic joints/material

## A tricky result

- 54 year old man
- THR some years ago
- Felt to have loosening and instability
- Possible ALVAL
- ESR was 80
- Underwent 1st stage revision – an unexpected amount of pus like material was encountered
- 5 samples
- Culture negative
- 3 sent for broad range 16S - *Vagococcus* sp in one of three.



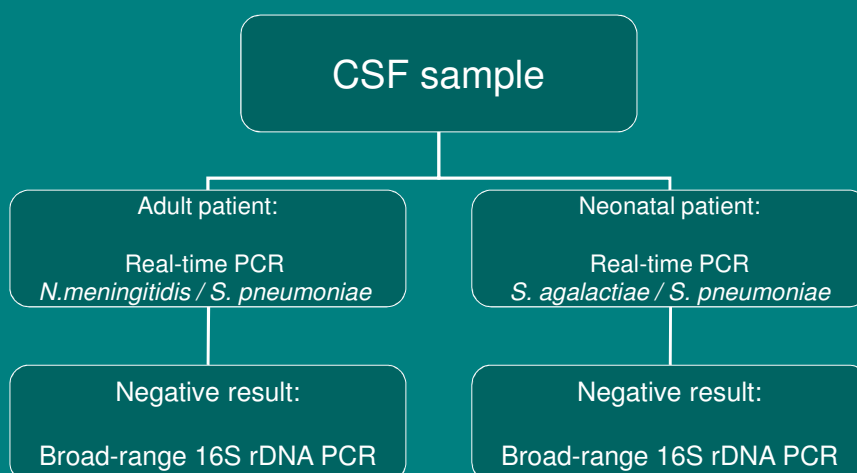
## Acknowledgements

- John Hartley
- Katie Layton
- Judith Phillips
- Lesley Gould
- Yabom Kamara

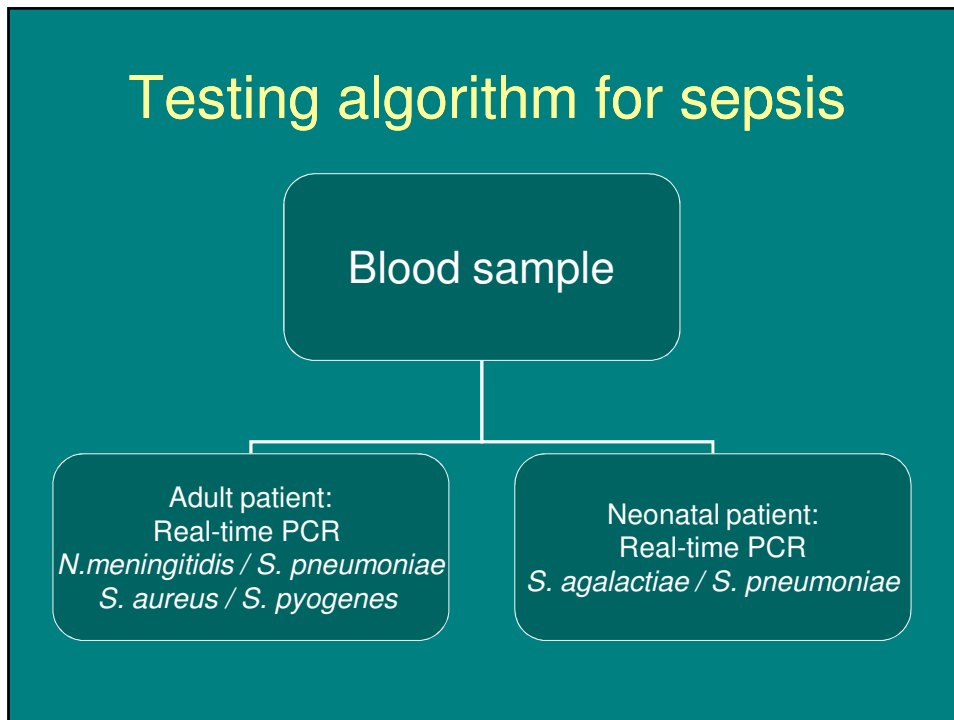
## Testing algorithm

- Abscess and heart-valve tissue – broad-range 16S rDNA PCR only.
- Most other samples are subjected to one or more (multiplexed) real-time PCR assays.

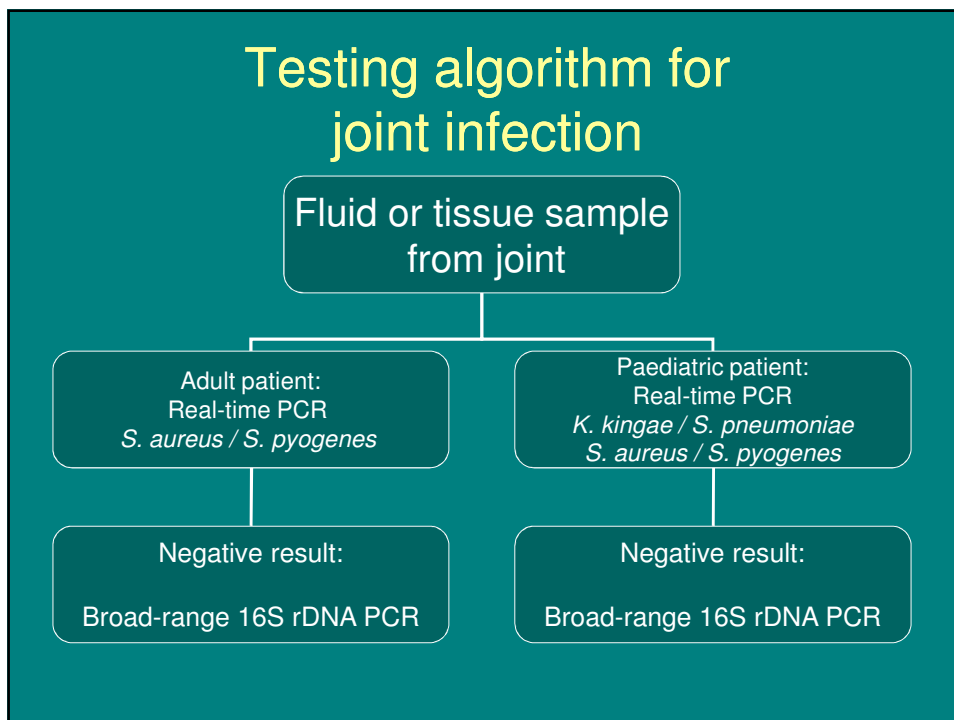
## Testing algorithm for meningitis



## Testing algorithm for sepsis



## Testing algorithm for joint infection



## Testing algorithm for respiratory infection and empyema

Pleural fluid / empyema sample

Real-time PCR  
*S. pneumoniae*

Negative result:  
Real-time PCR *S. aureus* / *S. pyogenes*

Negative result:  
Broad-range 16S rDNA PCR



## Molecular service at GOSH

- CPA accredited
- Validation and quality control of assays is crucial
- Positives and negatives in every run and internal positive control (IPC) in every sample, added at DNA extraction stage.

## Test selection

- Currently users request “16S rDNA PCR” or “bacterial PCR” and we select organism specific real-time assays where appropriate (from clinical details provided on request form).
- A “bacterial PCR screen” is £75 and includes a 16S rDNA PCR plus up to 4 real-time assays.
- Additional charge for sequencing 16S rDNA PCR positives
- Can also request specific assay eg *S. aureus* PCR, 16S rDNA PCR only.

## Future directions

- 16S rDNA deep sequencing for analysing complex ecologies
- Improving diagnosis of Sepsis / SIRS
- Improving diagnosis of **fungal** infections