MICROLAB

MICROGEN BIOPRODUCTS

Microgen Bioproducts Newsletter Nº 24 2006

BACTfish™ Real-Time Microbiology, No Culture and FISH

Background

Infections are responsible for 25% of all deaths world-wide. When expressed in Disability Adjusted Life Years (DALY's, a weighted index taking into account both qualitative and subjective aspects of disease and health), the burden of infectious disease is even worse. Early administration of the appropriate precision antibiotic is vital in such a situation, not only for the treatment of the patient, but also to minimise the risk of the development of antibiotic resistance. Rapid tests which could allow the identification of the causative pathogen and also carry out rapid antimicrobial susceptibility testing are of clear advantage in this situation. Fluorescence *in-situ* Hybridisation (FISH) now offers just such a fast, cost-effective, and rational approach to the detection of pathogens.

The prevalence of nosocomial infections world-wide has been estimated to be between 3.8% and 19.8% (average 11.9%) of all patients, with infants and patients over 64 years of age at greatest risk. The principal conditions arising from nosocomial infections are urinary tract infections, pneumonia, wound infections and septicaemia. Patients suffering from these conditions are invariably pre-treated with antibiotics, rendering the culturing of pathogens difficult, delaying specific treatment and increasing the risk of development of antibiotic resistance. Rapid identification of the causative pathogen and evaluation of its susceptibility to antimicrobial agents can clearly reduce such risks. Fluorescence in-situ Hybridisation (FISH) is a highly sensitive, cost-effective diagnostic procedure that can deliver results in around 1 hour from samples such as smears, body fluids, soft tissue aspirates and formalin-fixed biopsies.

In contrast to other laboratory disciplines, where only precise requests for the determination of already-specified analytes are submitted, the challenge facing a microbiologist is to identify a pathogen out of some 2000 potentially clinically relevant organisms with differing characteristics and growth requirements. The current procedure is to culture and isolate organisms on growth media, to identify the organism from its biochemical characteristics and to
carry out antibiotic susceptibility testing. This procedure can take from two to several days depending on the organism's growth rate.

Fluorescence in-situ Hybridisation (FISH)
The use of FISH in bacteria is dependent on the hybridisation of DNA probes to species-specific regions of cellular protein mechanisms and are themselves synthesised in the cell using highly controlled mechanisms. The reproducible structure of ribosomes means that they are particularly suitable as diagnostic targets. Active ribosomes are large structures consisting of two sub-units and each sub-unit contains both protein and RNA sub-units of various sizes (5S, 16S and 23S). Ribosomal RNA (rRNA) contains functional sequences that are common to all species but also sequences that are very specific for an individual species. The specificity of FISH testing for micro organisms is based on the recognition of specific information stored in the rRNA sequence. It needs only well designed DNA probes to function; no enzymatic techniques are required.

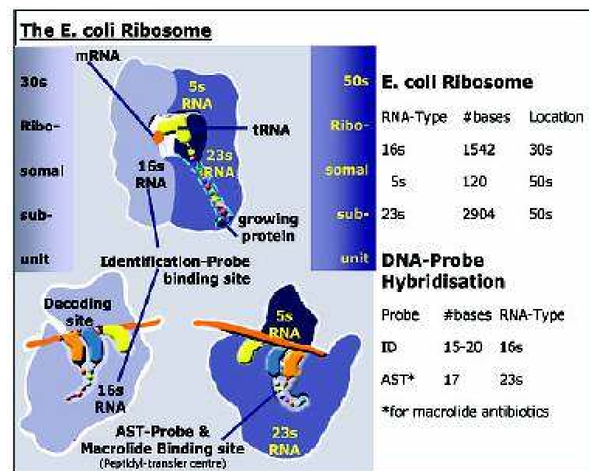


Fig. 1 Representation of the E.coli ribosome and site of probe hybridisation.

In the procedure, holes are generated in the cell wall that are large enough to allow DNA probes to enter the cell but too small for large cellular components like ribosomes to leak out. The species specific regions of rRNA to which the species specific probes bind are predominantly located on the 16S rRNA which is incorporated in the smaller 30S ribosomal sub-unit. Macrolide antibiotics interact at the peptidyl transfer centre, the actual site of de novo protein synthesis, which is situated on the 23S RNA which in turn is incorporated into the large 50S ribosomal sub-unit. The introduction of point mutations into the 23S rRNA renders such antibiotics ineffective. Bacteria

carrying these mutations are thus resistant to Macrolide antibiotics such as clarithromycin so that antibiotic regimens using these antibiotics will fail. FISH tests using probes designed to detect these mutations represent a new, very efficient way of antibiotic sensitivity testing (AST) prior to treatment, not only reducing costs, but also avoiding the administration of unnecessary and inappropriate medication to the patient.

Probe Selection

Ribosomal RNA contains both the highly conserved sequences required for ribosomal functionality as well as sequences of more structural importance that may vary in sequence but not in length. The probes hybridise with a three dimensional protein/RNA structure and not with a linear sequence of RNA alone. Knowledge of the primary sequence alone therefore is not sufficient to allow the design of an efficient probe that will work under normal FISH conditions. In addition since a change in just one base may inhibit or alter significantly the binding of the probe, extensive testing is required to develop probes that hybridise satisfactorily under identical conditions.

Sensitivity

FISH techniques have a sensitivity that is effectively equivalent to those of amplification technologies. Thus, in theory, FISH can detect single cells. This high sensitivity is the result of three factors:

1) High affinity and selectivity of DNA-probes.
   It is well established that DNA probes bind to complimentary DNA/RNA sequences with very high affinity. FISH uses stringent hybridisation conditions where a difference of one nucleotide in a 15 – 20 mer probe is sufficient o discriminate the binding.
2) High Local Concentration of target ribosome.
   The FISH procedure maintains the structural integrity of the cell, confining the reagents in one small vessel. One probe will bind to each of the 1 – 5 x10^4 ribosomes inside. Since the typical cell volume of an average bacterium can be considered to be in the order of one Pico litre, the average molecular probe concentration can be calculated to be approximately 20 – 100 µmolar against a background environment of near zero. This extremely high signal to noise ratio is responsible for the theoretical ability of the technique to achieve single cell sensitivity.
3) Fluorescence.
   Since in fluorescence measurements the wavelength of the emitted signal is higher than that of the exciting light, a low background (noise) signal is generally possible. The greater the wavelength shift between excitation and emission wavelength, the higher the sensitivity, at least as measured by the signal-to-noise ratio. Typically fluorescence techniques are 1000X more sensitive than absorption spectroscopy. In practice however, Fish does not achieve the theoretical sensitivity of being able to detect single cells. For instance if a 10µl sample contains 10 cells, each of which can be detected from their fluorescence, the practical detection level is 10^3 cells per ml. To increase the possibility of getting cells onto the slide, it is possible to spin the cells down or filter the cells out of the body fluid. However the clinical relevance of detecting even lower numbers of specific cells may become debatable. It can be useful to combine a species specific probe with one detecting all bacteria (Eubacteria). This allows the assessment of pathogenic load with respect to the total microbial population present in a sample.

Use of sample source and symptom information to reduce number of probes.

With its increased speed and precision, FISH technology represents a valuable addition to the tools traditionally available to the microbiologist and in particular addresses the needs of intensive care units. As such, it needs to be positioned alongside both culture based and amplification based methods. In culture techniques, samples are inoculated onto several different media to cover as many possible species of potential pathogens as possible, always assuming that they can be coaxed or coerced into growing *in vitro*. For amplification tests, it is necessary to indicate the pathogen(s) to be detected when the test is requested. With specific probe based assays, the detection of all possible clinically relevant microbial pathogens (probably more than 2000 in total) is clearly impractical as well as economically unviable. However, if the sample source and clinical symptoms are known, it is generally possible to narrow down the number of potential pathogens that are likely to be involved. It has been estimated that by using this information, it is possible to selectively identify 95% of the main problem causing pathogens with a reasonable number of probes.

Real-time Microbiology

The term "real-time" (occurring immediately) is generally used to describe computer features such as operating systems that respond immediately to input. In the Polymerase Chain Reaction (PCR), the term is used to describe the continuous monitoring of the PCR reaction. Here, the term real-time microbiology is used to describe the immediate detection of micro-organisms in their environment without culturing or amplification. Real-time microbiology can have a two-fold impact:

1) The significance of individual test results can be greatly enhanced if the bacteriology results are supplied to the clinician at the same time as other laboratory results.
2) Targeted antibiotics may be chosen at a much earlier stage, thus providing clear advantages to the patient, healthcare budgets, as well as reducing the selective pressure for the development of antibiotic resistance.

Additionally, real-time microbiology can allow the early identification of exotoxin-producing bacteria.

This can be vital since such bacteria can continue excreting exotoxins (with a concomitant risk of sepsis) even if the bacteria are no longer proliferating themselves. Rapid identification of such sepsis-causing pathogens is a prerequisite for early appropriate antimicrobial treatment and to inhibit sepsis development before a cytokine "storm" sets in.

Nosocomial infections are a frequent cause of septicaemia, and are usually caused by pathogens such as coagulase-negative *Staphylococci, Staphylococcus aureus*, as well as *Enterobacteria, Pseudomonas aeruginosa* and yeasts. The application of FISH for the identification of pathogens in positively flagged blood culture bottles can achieve a time saving of 26 to 46 hours compared with conventional laboratory methods. Vital, appropriate antimicrobial therapy can thus be initiated in these urgent septicaemia cases one or two days earlier.

Procedure

The FISH procedure does not require the extraction of RNA. In principle, all FISH assays of bacterial pathogens follow the same simple procedure. The sample is smeared or pipetted onto a slide which is then heat fixed. When probes designed for Gram +ve organisms are being used, the cell wall needs to be permeabilised by enzyme pre-treatment. The sample is dehydrated with ethanol and the probes added in a special hybridisation buffer. After incubation the slides are washed and viewed under a fluorescence microscope. The laboratory must have access to a microscope fitted with FITC and Cy3 filters (518 and 570nm emission wavelengths respectively) Up to 16 different probes can be applied to a single slide with eight different fields. Several slides can be run in parallel.

Standard FISH: In the standard procedure, the samples are prepared as above but hybridisation is carried out at 46degC for 90 minutes in a water bath. The slides are then washed for 15 minutes at the same temperature before viewing under the microscope. Hands-on time is around 15 – 20 minutes and the effective turn-around time is 2.5 – 3 hours.

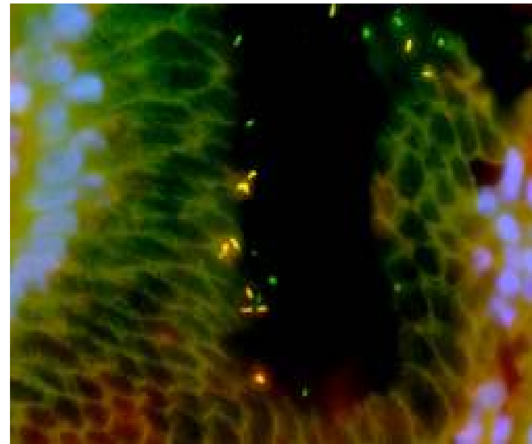
Modified FISH: In this patented procedure, the slides are fixed in a microwave thermal cycler before treatment and dehydration. Hybridisation is carried out in the same unit cycling between 46degC and 48degC. The cycling of the oven induces touch-down annealing which decreases the turn-around time to 60 minutes.

In theory, similar results could also be achieved with PCR, but only with significantly higher reagent and consumable cost not forgetting the use of highly specialised instrumentation.

Probes and probe combinations are available that cover most problem-causing pathogens grouped either by source or disease state. Examples of such groups are septicaemia; necrotising infections; Perinatal Streptococci; meningitis; cystic fibrosis; infections of the middle ear or respiratory tract.

Helicobacter pylori

H. pylori is the cause of both gastric and duodenal peptic ulcers and has also been associated with the development of stomach and duodenal cancer. As such, *H. pylori* is a significant burden to health care systems throughout the world. Isolation, identification and susceptibility testing of H. pylori is technically complex. Isolates may not survive even limited exposure to the atmosphere; regular subculturing is required and older cultures become coccoid with the associated decrease in ability to subculture. Using FISH, it is possible to detect *H. pylori* at the site of infection and to determine susceptibility to Clarithromycin. Resistance to Clarithromycin is a growing problem and currently causes nearly 15% of therapies to fail. Positive identification of *H. pylori* and simultaneous susceptibility testing provides a high predictive value of the probability of successful treatment.

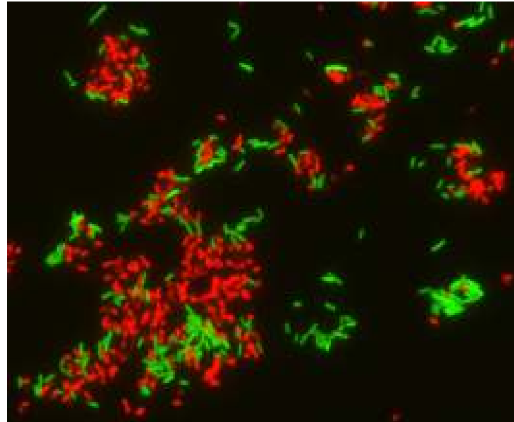


FISH can discriminate between clarithromycin resistant and sensitive H. pylori. Green indicates sensitivity and yellow indicates resistance (viewed under a dual wavelength filter)

Cystic Fibrosis (CF)

Cystic fibrosis is an inherited condition and affects 1 in every 2,500 children born. CF patients are at risk of bacterial chest infections and about half have repeated chest infections and pneumonia. Infection due to *Pseudomonas aeruginosa* has been implicated as a major source of morbidity and mortality in these patients. Nosocomially acquired *P. aeruginosa* isolates tend to be more resistant than community-acquired strains to antimicrobial agents, frequently displaying resistance to multiple classes. This multidrug resistance is of particular concern in

chronically infected CF patients. In recent years the recognition of *Burkholderia cepacia* complex as a significant risk factor in these patients has been recognised. *B. cepacia* (Genomovar I) and other members of the complex (Genomovar II – IX) are some of the most antimicrobial agent-resistant organisms encountered in the clinical laboratory. Strains recovered from CF patients who have received repeated antimicrobial courses are frequently resistant to all known antimicrobial agents.



Mixed population of P. aerogenes (red) and B. cepacia (green).

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This article has been adapted from an article previously published in CLI June 2003 by Dr Ian Thrippleton.

Please contact us for information on the detection and confirmation of:

Pseudomonas aeruginosa
Burkholderia cepacia
Stenotrophomonas maltophilia
Haemophilus influenzae

Helicobacter pylori (including macrolide resistance)

Staphylococcus aureus

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