

ROUTINE DIAGNOSTIC TESTS FOR FOOD-BORNE PATHOGENS

Authors:

Geraldine Duffy B.Sc. Ph.D

Brendan Kilbride B.Sc. M.Phil.

Justine Fitzmaurice B.Sc.

James J. Sheridan M.A. M.Sc. Ph.D.

The National Food Centre

Teagasc, Dunsinea, Castleknock, Dublin 15

ISBN 1 84170 189 0

January 2001



CONTENTS

Summary	1
Introduction	1
Rapid method for total viable count	2
Rapid method for pathogen detection	4
Enrichment of pathogens	4
Isolation of pathogens	5
DNA detection using PCR	5
Recommendations to Industry	7
Conclusions	8
Publications from this project	9
References	10

SUMMARY

Rapid techniques were developed and applied to the determination of total viable bacteria and to the detection of food borne pathogens (*Listeria monocytogenes*, *Salmonella*, *Campylobacter jejuni* and *E. coli* O157:H7).

The method developed for total viable counts is based on membrane filtration and fluorescent staining and the technique can be performed and a result obtained within 20 min. The results correlate well with the standard plate count and the technique is now being implemented in Irish food factories.

The techniques developed for the detection of food borne pathogens are based on an initial short enrichment of the pathogens in the food sample, isolation of the food pathogen onto a polycarbonate membrane followed by detection using molecular techniques (polymerase chain reaction). The techniques take approximately 28 h for the detection of *Listeria monocytogenes*, *Salmonella* and *E. coli* O157:H7 and 54 h for *Campylobacter jejuni*. The results obtained correlate well with the traditional cultural techniques and validation of the methods is ongoing.

INTRODUCTION

Assessment of the quality and safety of foods requires microbiological analysis. Growth of bacteria can result in organoleptic changes in food including off-colours and off-odours rendering it unacceptable to the consumer. The presence of pathogenic organisms on foods (*Salmonella* spp, *Campylobacter*, *Listeria monocytogenes*, *E. coli* O157:H7 etc.) poses a food poisoning threat and following a number of recent high publicity food-related health scares, consumer concerns regarding the safety of Irish food are now a priority. The production of high quality, pathogen-free food is demanded by consumers and this, coupled with the economic implications of gastroenteritis, is increasing pressure on the agri-food industry to reduce numbers of contaminating organisms in food. To address this problem the industry is implementing quality assurance systems such as HACCP (hazard analysis critical control point).

Traditional methods for the detection of bacteria on foods rely on culturing of the bacteria onto agar plates. These traditional cultural methods are time consuming taking three days to determine a total viable count and five to seven days to detect specific pathogenic bacteria. Currently available rapid methods are often unsuitable for use in industrial laboratories. They lack sensitivity, are expensive and complex to perform, often requiring specialised personnel and significant capital expenditure. The absence of rapid cost-effective methods for bacterial detection poses particular difficulties for food items with a short shelf-life and for the implementation of effective HACCP management systems.

RAPID METHOD FOR TOTAL VIABLE COUNT

The method developed for the determination of total viable counts is outlined in Figure 1. It was based on a membrane filtration epifluorescent technique using pre-treatment of the meat sample by centrifugation, surfactants and a proteolytic enzyme, alcalase 2.4L. The treated sample was filtered through a polycarbonate membrane (0.8 μ m), stained with a fluorescent dye, either acridine orange or *Badlight*, and then viewed using an epifluorescent microscope with a 100W mercury vapour light source and a 100X oil immersion plan objective. Acridine orange stains all micro-organisms while *Badlight* dye is a differential dye, which stains live micro-organisms green and dead micro-organisms orange. Stained bacteria were counted and the number of viable bacteria per gram of meat was determined by counting the fluorescing cells in twenty random fields of vision.

The developed method using the acridine orange (AODC) was applied to fresh meats with correlations (r^2) against the standard plate count of 0.92 for fresh minced beef (Table1).

The *Badlight* direct count method (BLDC) was successfully applied to processed meats. There was a good correlation between the BLDC and the standard plate count for cooked ham, bacon rashers and frozen burgers (Table 1). The *Badlight* technique measures viable cells only. This is an advantage particularly for processed meats which contain a significant proportion of

Table 1 Relationship between the acridine orange direct count or Baclight direct count and the standard plate count for enumerating bacteria in foods.

Rapid technique	Food	r ²	rsd
AODC	Fresh minced beef	0.92	0.23
BLDC	Cooked ham	0.90	0.40
BLDC	Bacon rashers	0.91	0.33
BLDC	Frozen beef burgers	0.93	0.37

Note: r² = correlation coefficient
 rsd = residual standard deviation

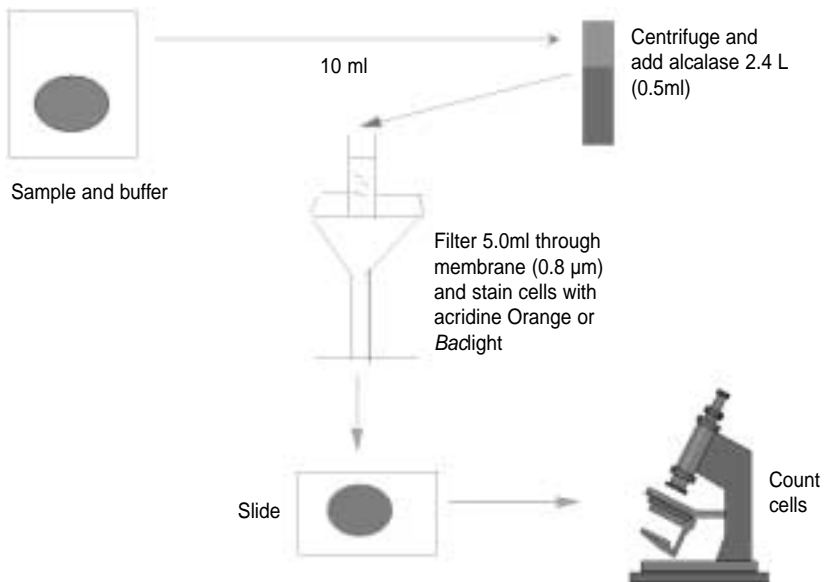


Figure 1 Membrane filtration epifluorescent method for determination of total viable bacterial counts in food products

injured and dead cells. The technique takes approximately 20 min to carry out and is suitable for routine use in a factory laboratory.

RAPID METHOD FOR PATHOGEN DETECTION

Enrichment of pathogens

One of the inherent difficulties in the detection of food pathogens is that they are generally present in very low numbers (< 100 cfu/g) in the midst of up to a million or more other bacteria. The detection of a specific bacterial pathogen is therefore a classic “needle in a haystack” scenario. Traditional techniques for pathogen detection rely on a selective enrichment in liquid broth, which allows the pathogen of choice to grow to detectable levels while limiting the growth of the competing micro-flora. The enriched sample is then plated onto a selective / differential agar and incubated to allow for colony formation. Rapid techniques still rely on an initial enrichment step followed by direct detection of the organism using specific immunological or genetic characteristics of the pathogen. The duration of enrichment depends on the sensitivity of the subsequent detection method.

The food samples (25g) were enriched for selective growth of specific pathogens using the conditions described in Table 2.

Table 2 Enrichment conditions employed for selection of food pathogens in rapid test

Pathogen	Broth	Temperature	Time
<i>Listeria monocytogenes</i>	Buffered peptone water	30°C	20h
<i>Salmonella spp.</i>	Buffered peptone water	30°C	20h
<i>Campylobacter jejuni</i>	Campylobacter enrichment media	37°C	48h
<i>E. coli</i> O157:H7	modified E.coli broth	37°C	20h

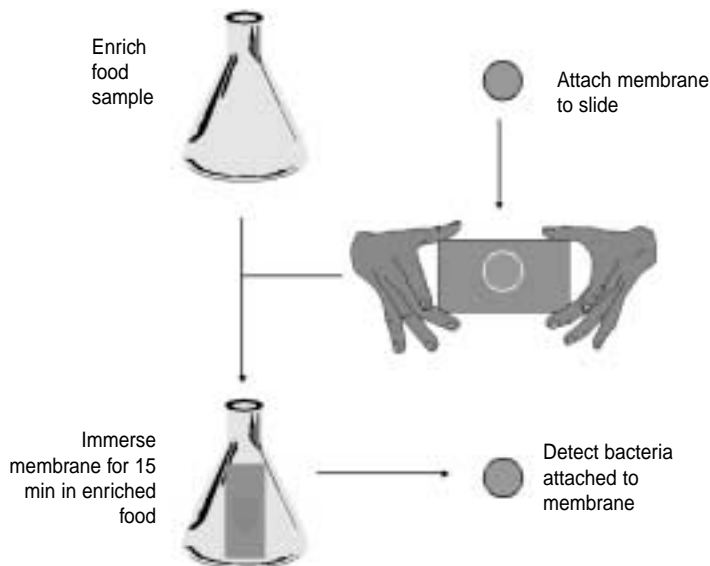


Figure 2 A rapid technique for the detection of pathogens in food products

Isolation of pathogens

The extraction technique required the attachment of a polycarbonate membrane to a glass slide using molten agar. The slide/membrane assembly was immersed for 15 min in the enriched food sample during which time the bacteria attached non-selectively to the membrane. The membrane and attached bacteria were removed from culture and pathogens including *Listeria monocytogenes*, *Salmonella*, *Campylobacter jejuni* or *E. coli* O157:H7 adhered to the membrane were visualised by DNA hybridization (PCR)

DNA detection using the Polymerase Chain Reaction (PCR)

Nucleic acid based methods, which incorporate an amplification step for the target DNA are now widely used. The most popular method of nucleic acid amplification is the polymerase chain reaction (PCR). In this technique, the DNA is extracted from the organism and the double strands are denatured

into single stranded DNA. Short sequence DNA primers are annealed to the complementary DNA target in the organism. The primers are then extended across the target sequence using a heat stable DNA polymerase (usually *Taq* polymerase, a thermostable and thermoactive enzyme from *Thermus aquaticus*) in the presence of free deoxynucleoside triphosphates (dNTPs) resulting in a double replication of the starting target material. Multiple repeats of the denaturation, annealing and extension steps results in an exponential increase in the concentration of target DNA. The PCR product is generally detected by staining with ethidium bromide on an electrophoresis gel.

Bacteria were isolated onto a membrane surface as described earlier and the DNA was extracted from the bacteria using a phenol:chloroform extraction procedure which also degraded the membrane. The DNA was then amplified in a PCR assay with primers specific for the target bacteria and the amplified product subsequently separated and identified by gel electrophoresis. The target primers used for the detection of the specific bacterial pathogens were chosen from the literature (Table 3).

The PCR techniques are currently being validated against traditional cultural methods using a wide range of food sample types with a view to incorporating

Table 3 DNA primers used for the detection of food pathogens by polymerase chain reaction (PCR)

Organism	Target site	PCR product	Reference
<i>Listeria monocytogenes</i>	Listerolysin O	520 bp	Mengaud <i>et al</i> , 1990
<i>Salmonella spp.</i>	1.8 Kb HIND III	1179 bp	Tsen <i>et al</i> , 1994
<i>Campylobacter jejuni</i>	Flagellin A gene	450 bp	Oyofu <i>et al</i> , 1992
<i>E. coli</i> O157:H7	H7, O157, <i>eaeA</i> , <i>ehyIA</i> , <i>vt1</i> and <i>vt2</i> gene	multiplex	Paton & Paton 1998

bp = base pair

them into the commercial microbiological service laboratory at The National Food Centre. In this way, the new rapid techniques will be made available to the Irish food industry.

RECOMMENDATIONS TO INDUSTRY

There are a number of requirements for rapid microbial detection techniques which are suited to use in a food industry laboratory. The technique should be:

- more rapid than the conventional culture technique
- yield comparable results
- suitable for routine sample analysis
- economical
- applicable to a range of sample types.

Current commercial techniques for total viable counts are based on bioluminescence (ATP tests), electrical systems (conductance / impedance systems) and membrane filtration epifluorescent techniques. There are limitations associated with all these test methods. Bioluminescent methods are extremely rapid (5 minutes) but are most suited to hygiene monitoring as opposed to bacterial counts as several factors can interfere with the test reaction including non bacterial ATP, pH, temperature and luciferase inhibitors. Electrical systems are suited to automation and high sample throughput but it can take up to 24 h to obtain a result. Potential interference from the food sample necessitates calibration of the system for every food type analysed. Membrane filtration epifluorescent techniques for total bacterial counts as described in this report conform well with the above criteria for a rapid method. The limitation of the test is that the sample preparation method has to be developed specifically for each food type. To date, membrane filtration epifluorescent techniques have been applied commercially to testing of milk and meat samples. The technique described in this report is currently in use in Irish meat factories. Further development on sample preparation is necessary for other food types.

Most food companies do not test for pathogens “on-site” as this necessitates holding control cultures of pathogens and having specialised facilities available for handling infectious bacteria. In consequence, most companies send samples for pathogen analysis to a commercial microbiology testing laboratory.

Rapid tests for pathogens are based on either immunological or DNA detection. The low number of target bacteria present in foods necessitates an enrichment period of up to 48 h prior to detection which limits the rapidity of the test. Interference from competing bacteria and the food sample matrix can also be problematic. Commercial kits for immunological and DNA testing are available. Immunological are simpler to perform and faster but are in general less specific than DNA tests (PCR). PCR is highly specific, but limitations of the methods relate to the complexity of the technique and potential interference from the food sample and the competitive micro flora. Continued development in this field will fully automate PCR methods and reduce costs encouraging the uptake of rapid tests for routine analysis of food samples.

CONCLUSIONS

- A membrane filtration rapid direct count method was developed for the determination of total bacterial counts on fresh and processed meats. The method takes 20 min to carry out and gives excellent correlation with standard plate count which takes 3 days. This method has been taken up by the food industry.
- A surface adhesion rapid method was developed for the isolation of food borne pathogens (*Listeria monocytogenes*, *Salmonella* and *Yersinia enterocolitica*, *Campylobacter jejuni* and *E .coli* O157:H7) from enriched food samples. The isolated pathogens were detected by DNA hybridisation. The methods have a detection limit of approximately 10,000 cells per ml of enriched broth and were validated with a range of fresh and processed foods.

PUBLICATIONS FROM THIS PROJECT

Cloak, O.M., Duffy, Geraldine, Sheridan, J.J. 1999 Development of a surface adhesion immunofluorescent technique for the rapid detection of *Salmonella* spp. from meat and poultry. *Journal Applied Microbiology* **86**: 583-590.

Cloak, O.M., Duffy, G., Sheridan, J.J. McDowell, D.A. and Blair, I.S. 1999 Simultaneous isolation of three pathogens, *S. enteritidis*, *L. monocytogenes* and *Y. enterocolitica* using a surface adhesion immunofluorescent technique. *Journal Microbiological Methods* **39**: 33-43.

Duffy, Geraldine, Sheridan, J.J. 1998. Viability staining in a direct count rapid method for the determination of total viable counts on processed meats. *Journal Microbiological Methods* **31**: 67-174.

Duffy, Geraldine, Cloak, O.M., Sheridan J.J., Blair, I.S. and McDowell D.A. 1999. The development of a combined surface adhesion and polymerase chain reaction (PCR) technique for the rapid detection of *Listeria monocytogenes* in meat and poultry. *International Journal of Food Microbiology* **49**: 151-159.

Duffy, G., Cloak, O.M., Sheridan, J.J. Blair, I.S. and McDowell, D.A. 1999. The incidence and antibiotic resistance profile of *Salmonella* spp. on Irish retail meat poultry products. *Food Microbiology* **16**: 623-631

Duffy, G. and Sheridan, J.J. 1999. Effect of pH and culture composition on the adhesion of plasmid bearing and plasmid cured *Yersinia enterocolitica* to a polycarbonate membrane in a rapid surface adhesion immunofluorescent technique. *Journal of Applied Microbiology* **86**: 867-873.

Kilbride, B., Sheridan, J.J., McDowell and Blair, I.S. 2000. A rapid membrane immunofluorescent viability staining technique for the detection of *Salmonella* spp. from fresh and processed meat samples. *Journal of Applied Microbiology* **89**: 587-594.

Walsh, D., Duffy, Geraldine, Sheridan, J.J., McDowell, D.A. and Blair, I.S. 1998. Comparison of a selective and non selective broth for the isolation of *Listeria* spp. from retail foods *Journal of Food safety* **18**: 2, 85-101.

Cloak, O.M., Duffy, G., Sheridan, J.J., Blair, I.S. and McDowell, D.A. 2001. The development of a combined surface adhesion and polymerase chain reaction (PCR) technique for the rapid detection of *Salmonella* from food. *Journal of Microbiol. Methods*. (in press).

Cloak, O.M., Duffy, G., Sheridan, J.J., Blair, I.S. and McDowell, D.A. 2001. Incidence of *Campylobacter* in Irish retail meats and a surface adhesion -PCR method for the its detection from food. *International Journal of Food Microbiology* (in press).

REFERENCES

Mengaud, J., Vicente, M.F., Chenevert, J., Pereira, J.M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J.C., and Cossart, P. 1988. Expression in *Escherichia coli* and sequence analysis of listeriolysin O determinant of *Listeria monocytogenes*. *Infection and Immunity* **56**: 4, 766-772.

Oyofe, B.A., Thornton, S.A., Burr, D.H., Trust, T.J., Pavlovskis, O.R. and Guerry, P. 1992. Specific detection of *Campylobacter jejuni* and *Campylobacter coli* by using polymerase chain reaction. *Journal of Clinical Microbiology* **30**, 2613-2619.

Paton, A.W. and Paton, J.C. 1998 Detection and characterisation of shiga toxicogenic *Escherichia coli* by using multiplex PCR assays for *stx1*, *stx2*, *eaeA*, enterohemorrhagic *E. coli hlyA*, *rfbO11*, and *rfbO157*. *Journal of Clinical Microbiology* **36**: 598-602.

Tsen, H.Y., Liou, J.W. and Lin, C.K. 1994. Possible use of a polymerase chain reaction method for the specific of *Salmonella* in beef. *Journal of fermentation and Bioengineering* **77**, 137 - 143.