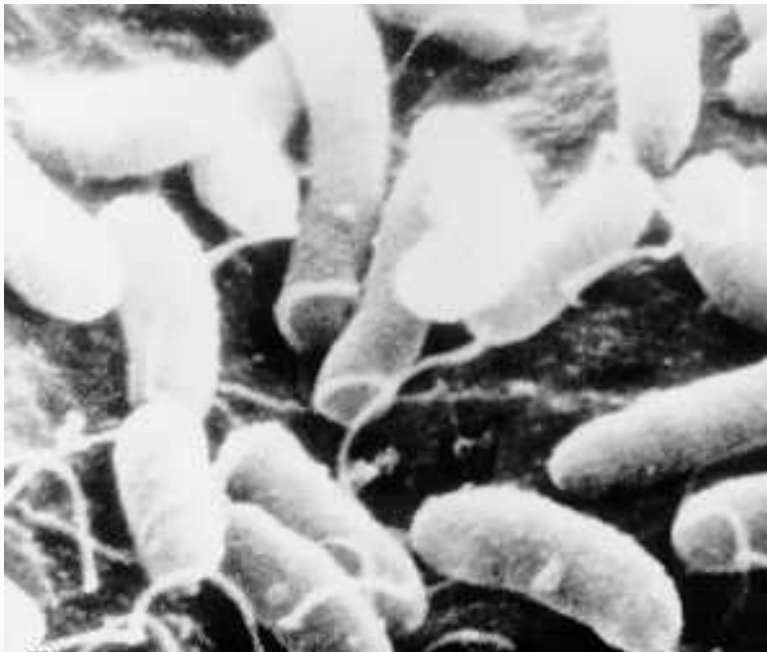


# Rapid methods for detection of *Vibrio* spp. in European seafood



[www.seafoodplus.org](http://www.seafoodplus.org)

# Introduction

- Vibrios are a significant cause of seafood-associated illness worldwide
- Three main species of interest are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*
- A number of other species may also cause seafood-associated infection



# Vibrios and the environment

- Widespread in coastal and estuarine environments
- Absolute requirement for sodium
- Location of individual species reflects salinity (and temperature) range
- Pathogenic species usually multiply in the environment when temperature in the range 15-30°C
- No association between faecal pollution indicators and occurrence of pathogenic vibrios

# Occurrence in human clinical specimens

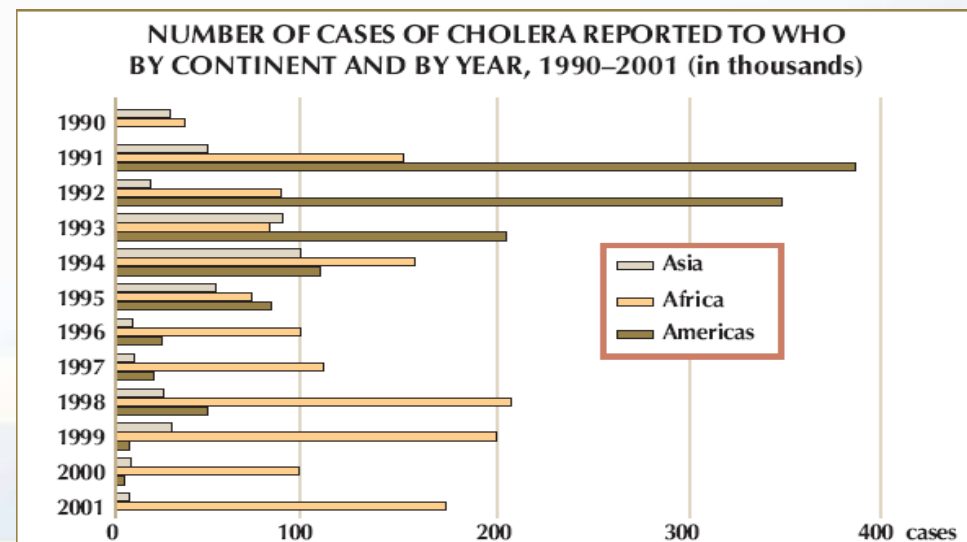
	Intestinal	Non-intestinal
<i>V. cholerae</i> O1	++++	+
<i>V. cholerae</i> non-O1	++	++
<i>V. parahaemolyticus</i>	++++	+
<i>V. fluvialis</i>	++	-
<i>V. furnissii</i>	++	-
<i>V. hollisae</i>	++	-
<i>V. mimicus</i>	++	+
<i>V. metschnikovii</i>	+	+
<i>V. vulnificus</i>	+	+++
<i>V. alginolyticus</i>	-	++
<i>V. carchariae</i>	-	+
<i>V. cincinnatiensis</i>	-	+
<i>V. damsela</i>	-	+

# **Foodborne vibrio infections**

- **Primarily associated with consumption of raw shellfish**
- **May also be associated with consumption of undercooked or recontaminated shellfish**
- **A proportion of cases also associated with consumption of crustacea (shrimps, crab, crayfish) or finfish**

## *V. cholerae* O1 (and O139)

- Cause of epidemic and pandemic cholera
- Cholera toxin the principle cause of the symptoms



2004 first half report: 133000 cases; 1270 deaths



## ***V. parahaemolyticus***

- **Causes gastro-enteritis**
- **Widely present in coastal and estuarine environments**
- **Ability to produce illness mainly associated with ability to produce certain haemolysins (Thermostable Direct Haemolysin (TDH) & TDH-Related Haemolysin(TRH))**
  - **e.g. in the US, only 0.2-3.2% of environmental isolates are TDH +ve**

## *V. vulnificus*

- Causes primary septicaemia or wound infections
- Infection via GI tract (raw oysters) or wounds
- Death may occur within 2 days of start of symptoms
- Predisposition by certain illnesses



*V. vulnificus*  
cellulitis



# Vibrio infections in the EU

- **Most cases are imported**
  - Mainly as a result of people having been infected abroad
- **Outbreaks of *V. parahaemolyticus* have been reported in Spain (oysters and crabs), France (shellfish) and some other EU countries**
- ***V. vulnificus* wound infections and/or septicemia reported from Belgium, Netherlands and Denmark**

# Occurrence in harvested shellfish

- **Britain**
  - 14% positive for *V. parahaemolyticus*
- **Spain**
  - 5% *V. cholerae* non-O1,
  - 30% *V. parahaemolyticus*, 16% *V. fluvialis*
- **Denmark**
  - 41% positive for *V. vulnificus* at one site
- **However, it is important to know the concentrations and pathogenic potential**
  - Little information available within Europe

# Causes of rejection/detention of seafood imported into the EU

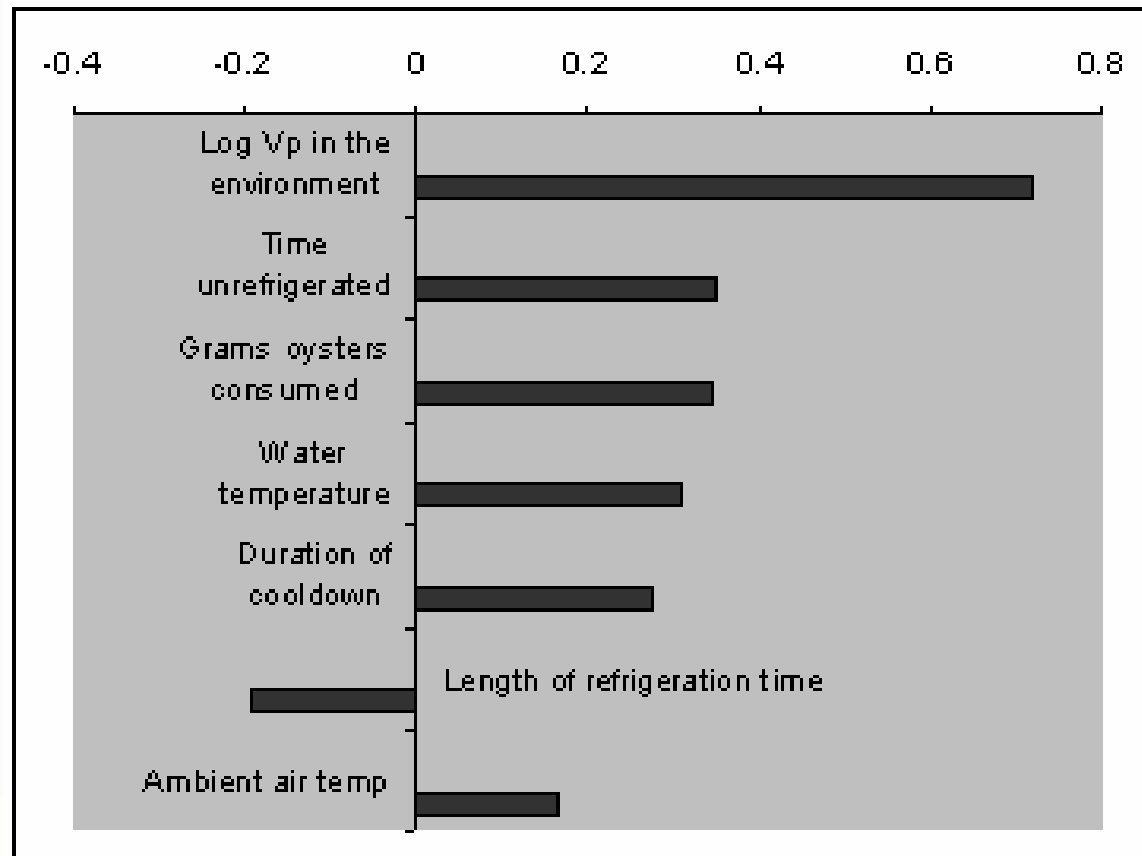
Cause of detention/rejection	No. of rejections / detentions			
	1999	2000	2001	2002
Microbial	59	53	49	47
<i>V. parahaemolyticus</i>	13	10	19	14
<i>V. vulnificus</i>		2	1	3
<i>V. cholerae</i>	9	8	9	5
Other vibrios		1		
Enterobacteria	6	2	4	6
<i>S. aureus</i>	7	0		
<i>Listeria</i>		0		
<i>Salmonella</i>	20	18	10	12
Hepatitis	1	1		
Total plate count	1	8	4	7
Molds		1	1	
<i>Clostridium</i>		2	1	

(Ababouch and Gandini, unpublished)

# Removal from bivalves

- Depuration - only about 40-50% of pathogenic vibrios removed at 48 hours
- Pasteurization - heat shucked oysters at 50°C for 10 minutes followed by rapid cooling in ice-water for 5 minutes
  - 6 log reduction of *V. vulnificus*
  - 3-4 log reduction of *V. parahaemolyticus*
  - Only reduces *V. cholerae* by approximately 1 log
- Freezing –40C followed by 3 weeks storage results in 4-5 log reduction
- Ultra-High Pressure treatment results in up to 6 log reduction

# Influences on risk from *V. parahaemolyticus*



- The relative importance of parameters that influence risk of *V. parahaemolyticus* (Vp) illness in the non-Louisiana Gulf Coast summer harvest

# **Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs**

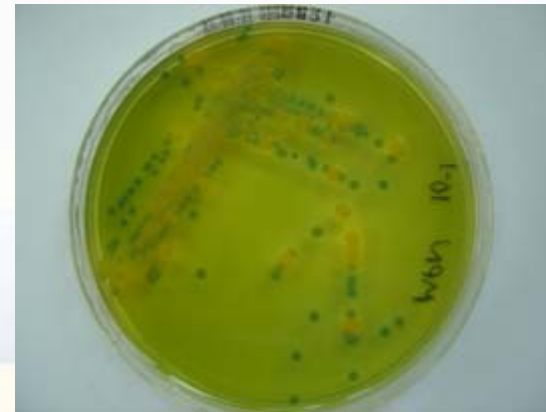


**There is a need for development of  
reliable methods for other microbial  
hazards too, e.g.**

***Vibrio parahaemolyticus***

# Conventional enumeration methods

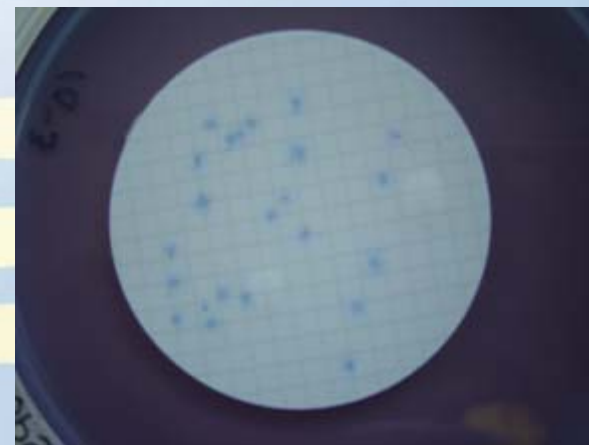
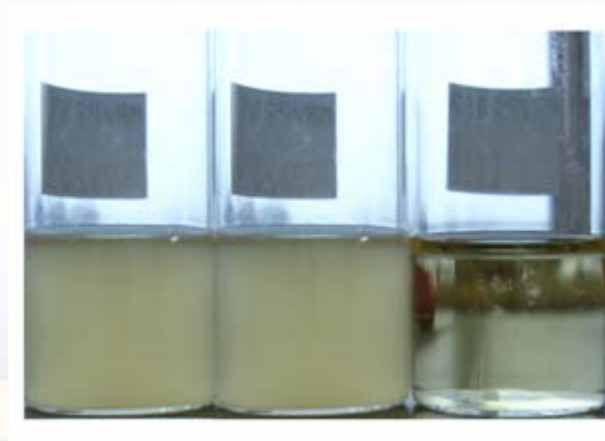
- **Direct plating**
  - only has a recovery efficiency of about 50% (and variable with TCBS)
  - Overgrowth by non-pathogenic vibrios
- **Most Probable Number (MPN)**
  - Resource intensive
  - Subject to prozone effect





# Alternative conventional enumeration methods

- **Semi-quantitative method**
  - Lower resource requirements than MPN
  - Lower accuracy and precision than MPN
- **Hydrophobic Grid Membrane Filtration (HGMF) method**
  - Appears to perform better than MPN
  - Only evaluated to date for *V. parahaemolyticus*



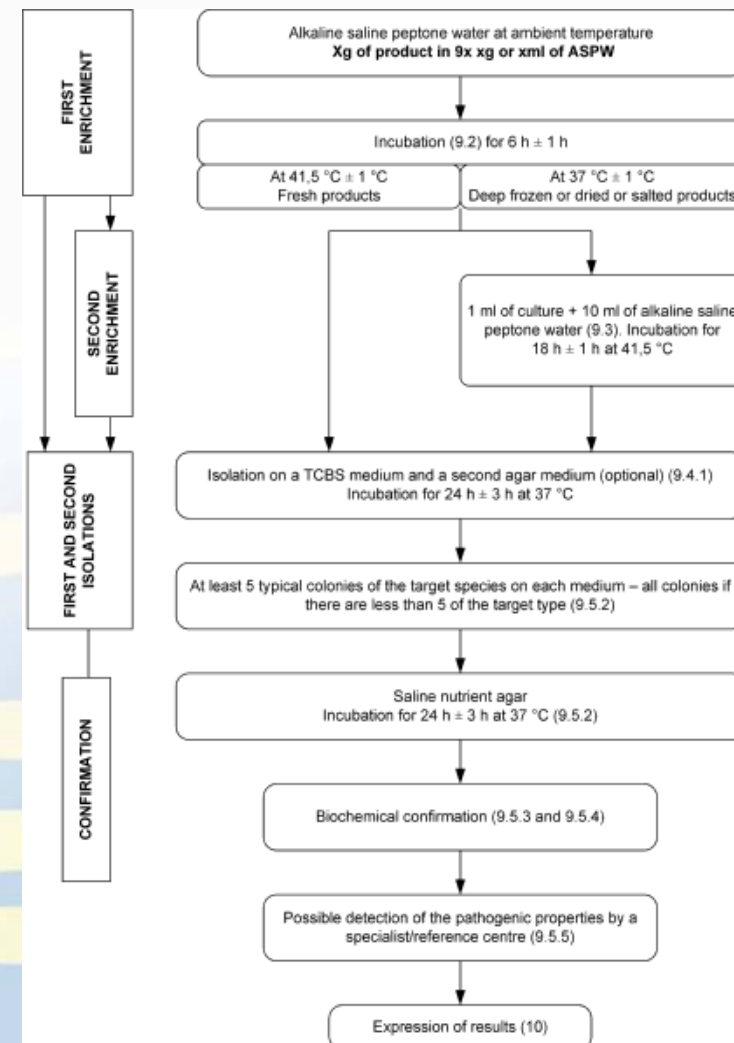
# Identification methods

- Significant problems of misidentification with commercially available identification galleries
- None appears to be suitable on its own for the identification of *Vibrio* spp from seafoods



# ISO 21872 parts 1 and 2

- Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio cholerae*
- Part 2: Detection of species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*

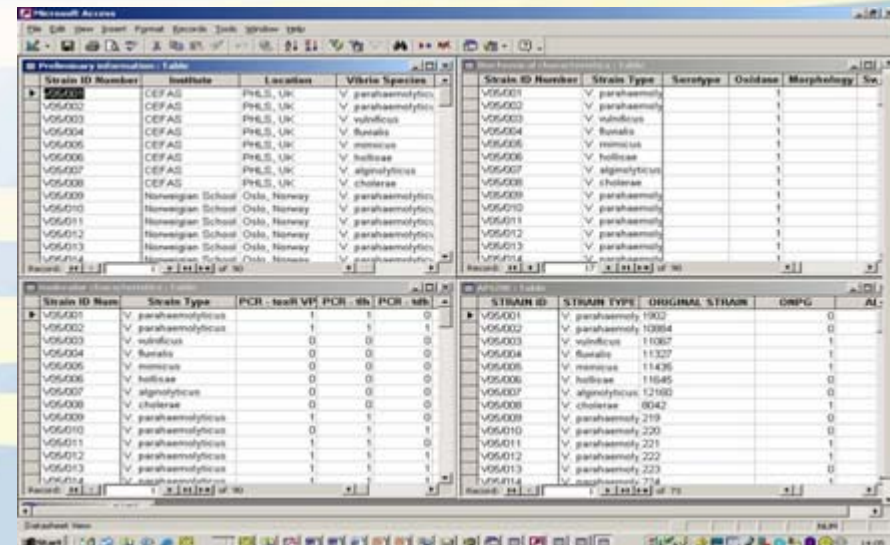


# SEABAC PROJECT

- **Project components**
  - Development of simple molecular methods for detection and enumeration
  - Development of real-time PCR procedures
  - Depuration and survival studies
  - Characterisation of strains
- **Partners**
  - Cefas, UK
  - Ifremer, France
  - ISS, Italy
  - IFL, Iceland
  - IPIMAR, Portugal
  - University of Santiago, Spain

# Establishment of strain bank

- Difficult to differentiate between *Vibrio* spp
- Even culture collection strains poorly characterised
- Need well-defined target and non-target strains for development and validation work

The screenshot shows a Microsoft Access database with two main tables: 'Preliminary Information' and 'Strain Information'. The 'Preliminary Information' table lists strain IDs, institutes, locations, and Vibrio species. The 'Strain Information' table provides detailed data on strain types, serotypes, oxidase, and morphology.

Strain ID Number	Institute	Location	Vibrio Species
V05001	CEFAS	PHLS, UK	V. parahaemolyticus
V05002	CEFAS	PHLS, UK	V. parahaemolyticus
V05003	CEFAS	PHLS, UK	V. vulnificus
V05004	CEFAS	PHLS, UK	V. vulnificus
V05005	CEFAS	PHLS, UK	V. cholerae
V05006	CEFAS	PHLS, UK	V. cholerae
V05007	CEFAS	PHLS, UK	V. alginolyticus
V05008	CEFAS	PHLS, UK	V. cholerae
V05009	Norwegian School	Oslo, Norway	V. parahaemolyticus
V05010	Norwegian School	Oslo, Norway	V. parahaemolyticus
V05011	Norwegian School	Oslo, Norway	V. parahaemolyticus
V05012	Norwegian School	Oslo, Norway	V. parahaemolyticus
V05013	Norwegian School	Oslo, Norway	V. parahaemolyticus
V05014	Norwegian School	Oslo, Norway	V. parahaemolyticus

Strain ID Number	Strain Type	Serotype	Oxidase	Morphology
V05001	V. parahaemolyticus	1902	1	1
V05002	V. parahaemolyticus	1902	1	1
V05003	V. vulnificus	11087	1	1
V05004	V. vulnificus	11327	1	1
V05005	V. cholerae	11436	1	1
V05006	V. cholerae	11645	1	1
V05007	V. alginolyticus	12182	1	1
V05008	V. cholerae	3042	1	1
V05009	V. parahaemolyticus	219	0	0
V05010	V. parahaemolyticus	220	0	0
V05011	V. parahaemolyticus	221	0	0
V05012	V. parahaemolyticus	222	0	0
V05013	V. parahaemolyticus	223	0	0
V05014	V. parahaemolyticus	224	0	0

# Simple molecular methods

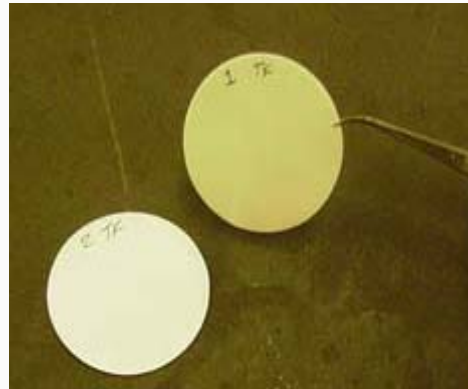
- Direct plating/hybridisation method
- Suitable for use by routine labs
- Total time is 2.5 to 3 days
- Looking at the following genes:
  - *ctx* for *V. cholerae*
  - *toxR*, *tdh* and *trh* for *V. parahemolyticus*
  - *vvh* for *V. vulnificus*



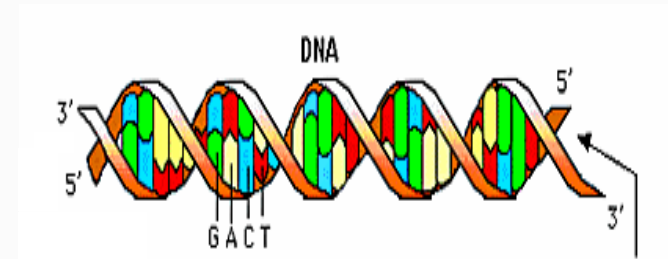
# Nucleic Acid Hybridisation: Preparation of Colony Lifts



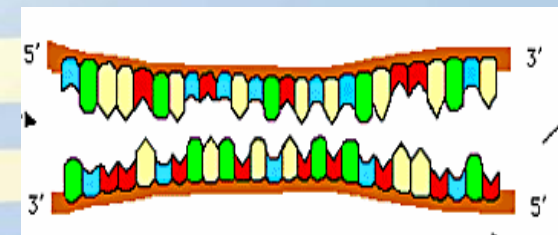
T<sub>1</sub>N<sub>5</sub> Plate with colonies to be probed



Lift colonies by  
transferring onto  
Whatman filter paper and  
denature DNA by lysis



Denatured DNA  
strand



Immobilise denatured strand onto  
filter by heating in microwave

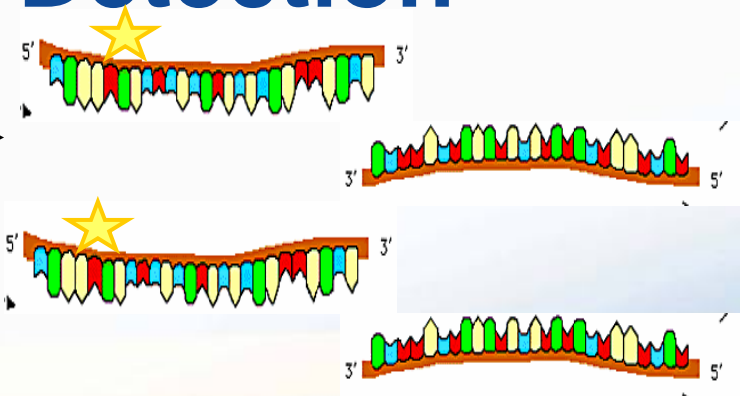


# Nucleic Acid Hybridisation: Hybridisation and Detection



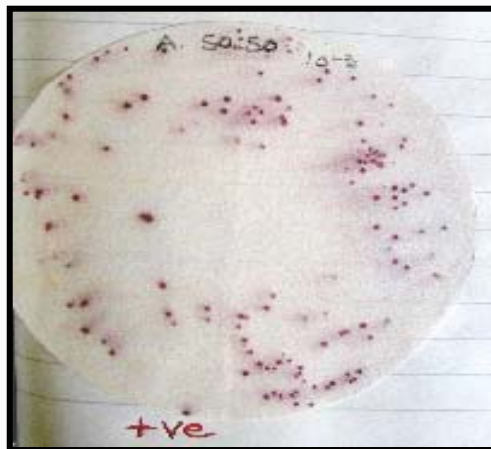
Label DNA with  
biochemical probe

Hybridisation



Complementary target DNA  
hybridises to labelled DNA probe

Labelled DNA hybrid is detected using  
a biochemical substrate



Labelled Probe is bound to DNA hybrid



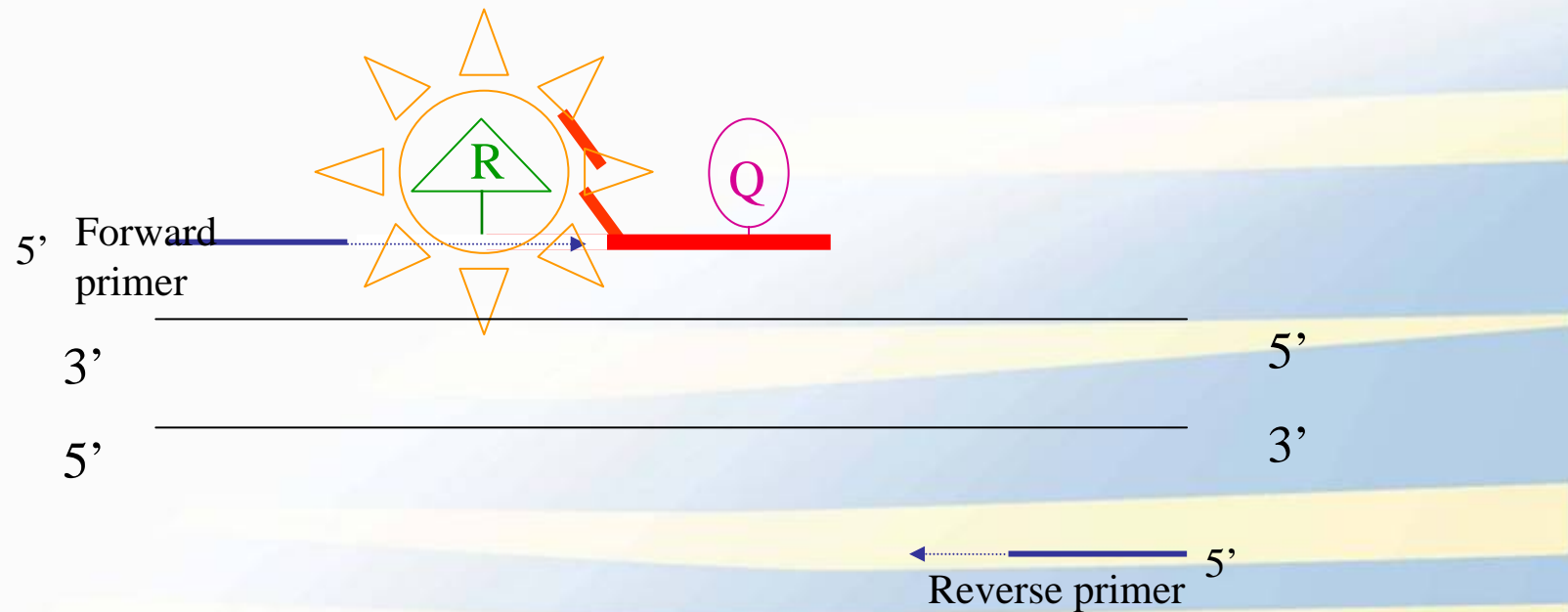
# Real-time PCR

- **Seen as appropriate for specialised labs in the medium term**
- **Can be undertaken on 6 hour enrichment broths**
- **Total analytical time less than 3 hours**
- **Offers the possibility of direct detection and quantification in food extracts**

# How Is Exponential Amplification Detected?



## ...The 5' Fluorogenic Nuclease Assay (TaqMan™)

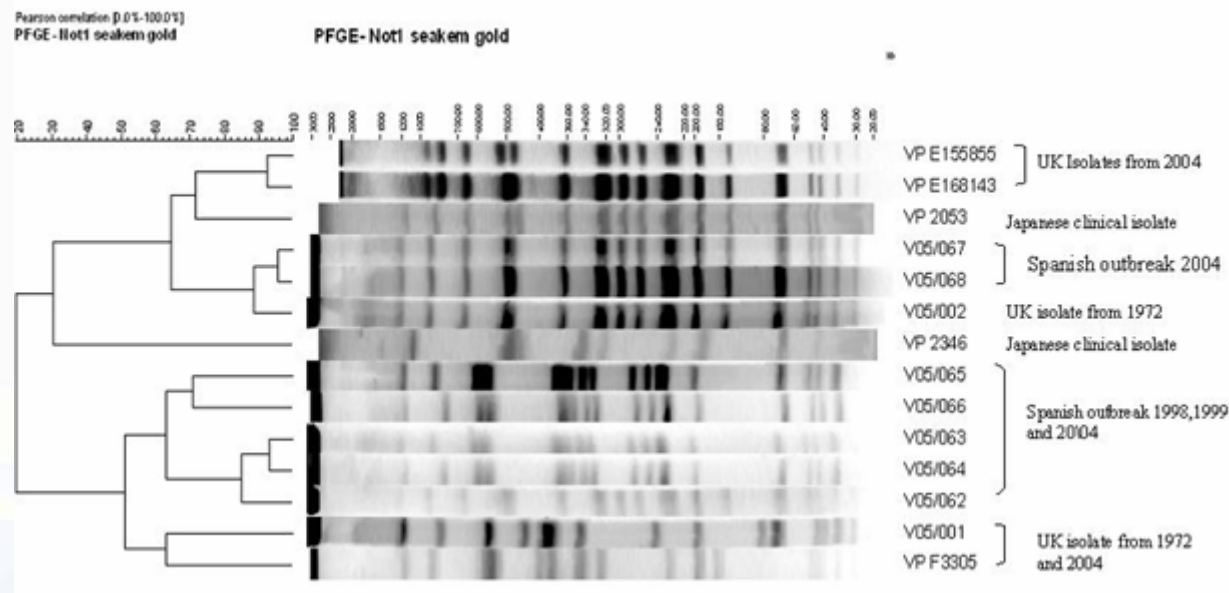


R = 5' Reporter dye

Q = 3' Quencher dye

# Characterisation

- To compare
  - different environmental isolates
  - environmental and clinical isolates



# Progress at ISO/CEN

- **New work item proposed to ISO/CEN on molecular approaches to detection and enumeration of pathogenic vibrios**
  - To cover both direct plating/probe hybridisation and real-time PCR
- **Time to develop a Technical Specification would be at least 2 years**
  - Fits in with the completion of the SEABAC development work

# Summary

- **Current methods not appropriate for setting microbiological criteria in the EU**
- **Molecular methods offer a way forward**
  - Direct plating/probe hybridisation for routine labs
  - Real-time PCR for specialist labs
- **SEABAC progressing development of standard specific and rapid methods through ISO**

# Thanks to

- |                          |         |
|--------------------------|---------|
| • Rachel Rangdale        | Cefas   |
| • Luciana Croci          | ISS     |
| • Dominique Hervio Heath | Ifremer |
| • Sónia Pedro            | IPIMAR  |
| • Sigrun Gudmundsdottir  | IFL     |
| • Jaime Martinez Urtaza  | UoS     |

**...and other co-workers in SEABAC**



# A better life with seafood...



[www.seafoodplus.org](http://www.seafoodplus.org)