Development of microbiological molecular diagnostic techniques for the rapid screening and identification of selected human bacterial pathogens and indicators

by

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“A great pleasure in life is doing what people say you cannot do”

Walter Gagehot

“In the field of observation chance favors only the prepared mind”

Louis Pasteur

“Creativity is inventing, experimenting, growing, taking risks, breaking rules, making mistakes and having fun”

Mary Lou Cook

“The greatest mistake you can make in life is to be continually fearing you will make one”

Elbert Hubbard

Academic Dissertation

This thesis will be defended for the degree of Philosophiaea Doctor (Ph.D.) at the faculty of Mathematics and Natural Sciences, University of Oslo, Norway.
The public defence will take place the 19th of February 2009 at the Department of Molecular Biosciences, Blindernveien 31, Oslo.

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Oslo, October 2008

Marc Anglès d'Auriac

Summary

The context directing this project since it was started in 1998, was applied research aiming at industrial applications in the field of microbial diagnostic biotechnology. The first part was the continuation of work started at Colifast ASA, developing and validating an automated solution for monitoring the faecal coliform bacterial indicator group based on measuring the β-galactosidase activity using selective media and conditions to increase specificity. Validation and discussion of this method is presented in paper I, published in 2000.

The project then continued dealing with bacterial indicators but using Nucleic Acid Amplification Tests (NAATs) and in particular PCR-based methods for achieving detection. Definitions and regulations were rapidly evolving, partly due to a better understanding of the ecology of the bacteria and partly due to an evolution in the microbiological techniques used for identification. As a consequence, true faecal indicators among coliforms were restricted to the thermotolerant sub-group even narrowing down to *E. Coli*, as being the only reliable indicator for faecal contamination. On the other hand, the coliform group at large expanded by including more species based on using the presence of the β-galactosidase gene rather than detecting a distant phenotype expression involving the presence of the enzyme. Hence, in 2001 the World Health Organization redefined the total coliform group as a "general microbial indicator". Following this trend, we focussed on identifying reliable and suitable genetic targets to develop PCR based methods for the identification of the Enterobacteriaceae family, to be used as a general microbial indicator. We further extended the search to include relevant pathogenic genetic markers for detecting some of the most relevant aetiological agents of water-borne and food-borne human diseases. Both *stx* coding for the shiga toxin and *eae* coding for intimin rapidly emerged as being the most relevant markers for monitoring Enteropathogenic *Escherichia coli* (EPEC) and Enterohaemorrhagic *Escherichia coli* EHEC as well as other Enterobacteriaceae emerging as new pathogens causing human diarrhoea. In parallel, we evaluated the potential of including another relevant indicator group, the Faecal Streptococci (FS), among which the two most important members are *Enterococcus Faecalis* and *Enterococcus faecium*. These two bacteria have in common with *E. coli* that they are considered reliable indicators of faecal contamination and have been in the past decades identified more often as the aetiological agent of human diseases. We therefore looked for genetic markers specific for these 2 species. This work

was assembled in a patent application filed in 2002 and granted by the European Patent Office in November 2006 (Paper II). The triplex method part for detection of Enterobacteriaceae, *stx* and *eae* has been submitted for publication in September 2008 (Paper III).

An important aspect for the successful implementation of NAAT technology is the NA sample preparation. This becomes an essential element when analysing difficult samples, such as food or clinical samples, containing NAAT inhibitors. Genpoint AS developed a unique magnetic bead based technology for the preparation of NA from such difficult samples. It is unique in that it first non-specifically isolates cells from the matrix followed by NA isolation using the same beads. This technology was first applied to the isolation of Cyanobacteria from environmental samples and further to other bacteria such as *Listeria* in food matrices. In this work we present the validation of an application of this technology developed for the preparation of NA from human urine samples for detection of *Chlamydia trachomatis* (Paper IV, 2007).

A modification of the method allowed the development by serendipity of a shorter and more convenient format, applicable to selected microorganisms such as *Chlamydia trachomatis*. A patent application was filled by Genpoint AS for this new method in 2005, and was published in 2007 which is the last paper presented in this thesis.

Abbreviations

aa:	Amino Acid
AE:	Attaching and Effacing
BSA	Bovine Serum Albumin
CFU:	Colony Forming Unit
DMSO:	Dimethyl Sulfoxide (solvant)
EDTA:	Ethylenediaminetetraacetic acid (chelating agent)
eae:	*E. coli* Attaching and Effacing gene coding for intimin
EAEC:	Enteroaggregative *Escherichia coli*
EHEC:	Enterohaemorrhagic *Escherichia coli*
EIEC:	Enteroinvasive *Escherichia coli*
EPEC:	Enteropathogenic *Escherichia coli*
ETEC:	Enterotoxigenic *Escherichia coli*
ER:	Enzymatic Restriction
FC:	Faecal Coliform
FS:	Faecal Streptococci
HUS:	Hæmolytic Uremic syndrome
LEE:	locus of enterocyte effacement
NA:	Nucleic Acid
NAAT:	Nucleic Acid Amplification Test
P/A:	Presence or Absence
PBP:	Penicillin Binding Protein
PCR:	Polymerase Chain Reaction
SDA:	Strand Diasplacement Amplification
SDS:	Sodium Dodecyl Sulfate (also called Sodium Lauryl Sulfate, it is an anionic surfactant)
STEC:	Shiga Toxin producing *Escherichia coli*
STI	Sexually Transmitted Infections
TC:	Total Coliform
Tn:	Transposon

List of papers

Paper I

Anglès d'Auriac, M. B., H. Roberts, T. Shaw, R. Sirevag, L. F. Hermansen, and J. D. Berg. 2000. Field evaluation of a semiautomated method for rapid and simple analysis of recreational water microbiological quality. Applied & Environmental Microbiology **66**: 4401-4407.

Paper II

Anglès d'Auriac, M. B. and R. Sirevag. 2006. New primers for the detection and identification of bacterial indicator groups and virulence factors, granted European Patent EP1466011, WIPO international publication number WO03052143A2. In the same patent family: US application 2005/0130155.

Paper III

Anglès d'Auriac, M. B. and R. Sirevag. 2008. Multiplex PCR for the simultaneous Detection of the *wecA* gene involved in coding the Enterobacteriaceae Common Antigen (ECA), the Shiga Toxin genes (*stx*) and the Intimin gene (*eaeA*). Submitted

Paper IV

Anglès d'Auriac, M. B., U. H. Refseth, M. Espelund, H. Moi, G. Storvold, and S. Jeansson. 2007. A new automated method for isolation of Chlamydia trachomatis from urine eliminates inhibition and increases robustness for NAAT systems. Journal of Microbiological Methods **70**: 416-423.

Paper V

Anglès d'Auriac, M. B. 2007. Method for detecting the presence or absence of a target cell in a sample. WIPO international publication number WO2007068904A1 and European patent EP 1969138A1 published 17/09/2008.

NOTE: Paper V was written by Genpoint's lawyers based on the DOFI (Disclosure of Invention) delivered by M. Anglès d'Auriac to Genpoint in 2004.

1 Introduction

Water is an indispensable element for all life. For an adult human, it accounts for about 60% of the total body mass and constantly needs to be replenished. Hence, we daily drink water or other aqueous liquids and therefore, the quality of water is of uttermost importance. We further ingest water used to rinse fresh food we eat or during bathing activities. In each case, the possible presence of harmful agents puts at risk several simple basic life activities which are often taken for granted in the western societies. Harmful agents found in water can be categorised as being either chemical or microbial in nature. The latter is reported as causing the most human health disorders and morbidity. The famous findings by John Snow in 1854, that the removal of the handle of a public fountain in London was associated with a reduction of the cholera cases in the neighbourhood was not at the time understood in a microbiological context but was rather the result of statistical observations. He associated cholera deaths to the use of a water fountain and convinced local authorities to disable the pump, therefore stopping water consumption from this spot. The end of the local outbreak was credited to this action although cholera cases were already on the decline when the action was taken. Snow further established a strong statistical correlation between sewage contaminated water consumption and cholera deaths, contributing to the founding of the science of epidemiology. It took another 10 years before the "germ theory" was established by Louis Pasteur and by 1882 Koch had assembled his 4 postulates. The science of microbiology then expanded rapidly associating microbes to the symptoms they produced in their human hosts when the aetiology of the diseases is of microbiological origin. The understanding of the causality of microbes in various human diseases enabled prevention to progress dramatically. The concern for microbial safety especially with regards to products for human consumption, first of all water, lead to the development of microbial "indicator" concept by the end of the 19th century to monitor faecal contamination.

Ever since microbes were recognised and associated with disease, new methods were developed to increase information and speed of analysis. Indeed, microbiological preventive monitoring of water will benefit from increased information and speed of analysis. This will help interpretation, speed up corrective action and therefore reduce exposure duration to

possible microbial hazard and otherwise reduce costs associated to stocks awaiting clearance.
When clinical symptoms of microbial infection sets in, it is essential for the physician to collect as much information as possible on the aetiological agent(s) in order to correctly manage the course of treatment and containment to avoid further spread of the disease.

Microbiology has been traditionally based on the observation and analysis of phenotypic expression such as shape and colour but also metabolic end products, characteristic of the studied microbes. For example, specific media were devised for recognizing production of gas or a decrease of the pH under appropriate and selective conditions. This was later extended to using specific reporter substrates targeting enzymes involved earlier in the metabolic processes of interest. These phenotypic methods have the disadvantage that they depend on the expression of the chosen characters which may vary due to environmental factors or strain variations. Although the use of reporter substrates helped reduce the amount of false negative associated to phenotypic variation, the advent of genotypic microbiology has enabled previously unparalleled discrimination power for microbiological analysis. Indeed, as the nucleic acids (NA) involved in the expression of a character will remain present although environmental factors have suppressed expression, they will be a more reliable material for microbiological analysis. Moreover, the amount of potential targets for microbiological analysis increases dramatically as the choice is no longer reduced to expressed characters requiring appropriate test for detection but rather comprises the whole genome. This includes NA not necessarily involved in any known coding activity but which may have some degree of useful specificity.
The NA diagnostic revolution also brought new challenges to the microbiologist, namely ad hoc sample preparation.

2 Microbiological indicator groups for the assessment of microbial water quality

2.1 Basis for the faecal bacterial indicator groups

The hygienic quality of water is of utmost importance to society, and efficient bacteriological control of water is essential for implementing a good management of this vital resource. Accuracy and speed of diagnosis are essential both for human health protection and for operational cost reduction for the water industry. Cases from the last decades such as the Amazon basin epidemics in 1991 and 1994 (Blake, 1993; Guthmann, 1995; WHO, 1995) and the 1994-95 Ukrainian epidemic (Clark et al., 1998a) where *Vibrio cholerae* was found to be the culprit, shows the actuality of the problem. Even the food industry is more at risk today than earlier due to the development of continuous chain production and world-wide distribution, which enhance pandemic risks (Bell et al., 1994; Cieslak et al., 1997; Eberhart-Phillips et al., 1996; Liddell, 1997; Van Beneden et al., 1999; Watanabe et al., 1999).

Ideally, direct detection of all possible waterborne infectious agents would be a logical strategy to assure microbial quality. In practice it is not feasible as it is too complex, expensive and time consuming. In the early days of microbiology, pragmatism and observation led to building the concept of bacterial indicator. Typically, these organisms would be easier to detect, theoretically be associated to pathogenic bacteria hence indicate its possible presence and not capable of reproducing and sustain itself in the tested material. Although presence of the "indicator" organisms would not necessarily indicate the presence of pathogens, the absence of indicator should on the other hand, assure in theory the acceptable quality of the tested water. Since the most common causative agents of waterborne outbreaks were to be found among enteric bacteria (i.e. Salmonella and Shigella) which spread through the faecal-oral route, the "faecal coliform" indicator group

(FC) emerged to indicate faecal contamination and therefore possible presence of pathogenic organisms. The main representative of the FC group, *Esherichia coli*, was discovered in 1885 by Theodore Escherich who named the bacteria *Bacillus coli.* Although the aetiology of most waterborne diseases were not identified at the time, the association of faeces contamination in water to potential health hazard was first recognised by the Franklands in 1891. They introduced the concept of bacterial indicator by recommending the search of sewage associated microorganism to characterize dangerous pollution. The following year, Schardinger suggested to research "*Bacillus coli*" in water for monitoring microbial quality (Feng et al., 1998; Tallon et al., 2005). This was performed by direct plating on litmus lactose agar using the production of acid as a diagnostic feature, indicated by a colour shift from purple to red when the pH in the media sank bellow 4,5. This test was known as the "Wurtz method" and was later followed by the Durham tube test introduced to demonstrate gas production from lactose catabolism (Ashbolt et al., 2001). As it was soon recognised that other organisms than "*Bacillus coli*" shared these phenotypic properties, the notion of coli-form bacteria was introduced in 1893 by Blachstein to describe bacteria resembling "*Bacillus coli*". New media introducing selective agents such as bile salts were first introduced by MacConkey in 1900 and 1901 to increase selectivity to recover coliforms originating from faeces only (Prescott et al., 1946). In 1919 *Bacillus coli* was renamed *Escherichia coli* and non-pathogenic related species representative of the normal human faecal microbial flora were used to build the core of the faecal coliform indicator group concept based on the ease to detect and their non persistence in the water environments to be monitored. Similarly, the presence in recreational waters of high levels of faecal coliforms (originating from faeces) and more specifically the species *Escherichia coli* has been found to correlate with swimming associated illness (Clinton et al., 1998; Francy et al., 1993; Rice et al., 1998) and with economic loss for shell-fisheries due to coastal shores faecal contamination (Weiskel et al., 1996). Based on such findings, safety guidelines have been developed using the FC group as an indicator for faecal contamination. The emergence of pathogenic *E. coli* types in recreational and drinking water (Francy et al., 1993) illustrates the pertinence of using faecal coliforms as an indicator group. In USA a threshold of 200 cfu / 100 ml for faecal coliforms and 126 cfu / 100 ml for *E. coli*, is recommended for bathing waters (US Environmental Protection Agency, 1986), whereas in Europe the "guideline value" threshold for faecal coliforms has been set at 100 cfu / 100 ml (The council of the European communities, 1976).

The most commonly used quantification reference methods for monitoring microbial quality of water employ membrane filtration (MF) or most probable number (MPN), which both as a rule require 24 to 48 hours for completion (American Public Health Association, 1995). In order to improve public safety, faster microbiological detection is desirable for shortening the time required to implement appropriate measures in the case of an unacceptable level of contamination. To be useful, such methods ought to give results within a working day, be quantitative, sensitive and specific, require less work than the current standard methods, have a high throughput and be non-destructive to the target organisms to allow confirmation work (Sartory et al., 1999).

Traditionally the FC group, which is primarily, but not uniquely, composed of faecal coliform bacteria, is defined as follows: Gram negative, oxidase negative bacteria belonging to the Enterobacteriaceae group, able to ferment lactose with the production of acid and usually gas within 48 h at 44 ^{o}C in the presence of bile salts or other surface active compounds with similar growth-inhibiting properties. This definition is operational rather than taxonomic and in order to better reflect the fact that not all FC are from faecal origin, the denomination thermotolerant coliform has been used instead of FC. The Total Coliform (TC) group is a larger group, comprising the FC, characterised by a less stringent temperature, around 35^{o}C, allowing for the recovery of additional coliform species. These phenotypic characteristics were the basis for the developing of the various coliform selective media as shown in Table 1. In practice, these various methods defined the coliform groups.

Table 1. Selective microbiological media for isolating coliform bacteria

Media	MLSB (+/- agar)	Mac-Conkey Agar	Mac-Conkey Broth	VRBA	m-FC agar	LES Endo agar	Lactose TTC
Peptone	+	+	-	+	+	+	+
Yeast extract	+	-	-	+	+	+	+
Tryptose	-	-	-	-	+	+	+
Casein hydrolysate	-	+	+	-	-	+	-
Sodium chloride	-	+	-	+	+	+	-
Potassium hydrogen phosphate	-	-	-	-	-	+	-
Lactose	+	+	+	+	+	+	+

Media	MLSB (+/- agar)	Mac-Conkey Agar	Mac-Conkey Broth	VRBA	m-FC agar	LES Endo agar	Lactose TTC
Acid indicator	Phenol red	Neutral red	Bromocresol purple	Neutral red	Methyl (aniline) blue Rosolic acid	Sodium sulfite Fuchsin	Bromothymol blue
Selective inhibitor	Sodium Lauryl Sulfate	Bile salts, Crystal violet	Bile salts	Bile salts, Crystal violet	Bile salts	Sodium Lauryl Sulfate, Sodium deoxycholate	TTC + Tergitol 7
Temperature	30.0°C 4h & 37.0°C 14h	35.0°C	35.0°C	30.0°C	44.5°C +/-0.2	35.0°C+/- 0.5 24h	36°C+/- 2 21h +/- 3
Reading	Yellow colonies	Red colonies	Gas and acid	Red colonies	Blue colonies	Red colonies with metallic sheen	
Standard	UK Environment Agency, "The Microbiology of Drinking Water"			U.S.A. "Standard Methods & Bacteriological Analysis Methods"			ISO 9308-1; EU 98/83

2.2 New requirements for the definition of the coliform bacterial indicator group and their implications for detection

Since it has been clearly established that members of the FC group also comprises purely environmental strains, it was globally accepted by 2001 to abandon the FC denomination (Leclerc et al., 2001). For the identification of coliform bacteria, acid, aldehyde and/or gas production from lactose catabolism have been the bases of numerous methods. However, the fact that β-galactosidase positive faecal coliforms, in particular *E. coli*, in some cases do not produce gas, due to the lack or loss of the enzyme formate-hydrogen lyase (Leclerc et al., 1989), has led to the proposition of new definitions. The 1994 edition of Report 71 (Aston et al., 1994) does not any longer mention the requirement for gas-formation and instead requires the presence of β-galactosidase for coliforms and β-glucuronidase specifically for *E. coli*. These changes were further stated in the 2002 version (Barell et al., 2002b) that was published to implement the 1998 EU directive (Anonymous, 98 A.D.). As

shown in Table 2, these changes in definitions had consequences regarding which bacteria were included in the coliform group.

Table 2. Coliform definition evolution according to the various definitions

Bacterial genus	Coliform definition		
	Growth at 37°C, gas and acid production from lactose	Growth at 37°C, acid production from lactose	Growth at 37°C and possess β-galactosidase
Citrobacter	+	+	+
Enterobacter	+	+	+
Escherichia	+	+	+
Klebsiella	+	+	+
Hafnia	-	+	+
Serratia	-	+	+
Yersinia	-	+	+
Kluyvera	-	+	+
Pantoea	-	+	+
Moellerella	-	-	+
Budvicia	-	-	+
Buttiauxella	-	-	+
Cedecea	-	-	+
Ewingella	-	-	+
Leclercia	-	-	+
Rahnella	-	-	+
Yokenella	-	-	+
Morganella	-	-	-
Providencia	-	-	-
Salmonella	-	-	-
Shigella	-	-	-
Proteus	-	-	-
Reference	(Ashbolt et al., 2001; Barell et al., 2002a; Leclerc et al., 1989; Leclerc et al., 2001; Stevens et al., 2003)		

Bold: members found in human faeces

In compliance with these new definitions, various methods based on enzyme-specific substrates have been developed; such as for example the demonstration of β-galactosidase activity, in an appropriate selective medium. These methods include the use of substrates, which will give rise to chromogenic, fluorogenic and luminescent products (Alonso et al., 1996; Berg et al., 1988; Edberg et al., 1988; Manafi, 2000; Robertson et al., 1998; Rompre et al., 2002). The natural substrate for the β-galactosidase enzyme, lactose, is a diholoside composed of β-D-galactose and β-D-glucose bonded through β-1-4 glycosidic linkage. Alternative substrates would typically conserve a β-D-galactoside moiety linked to the

desired reporter molecule. For example, the hydrolysis of 4-methylumbelliferone-β-D-galactoside (MUgal) by β-galactosidase will produce galactose and 4-methylumbelliferone (4-MU), a fluorogenic compound, as shown in Figure 1.

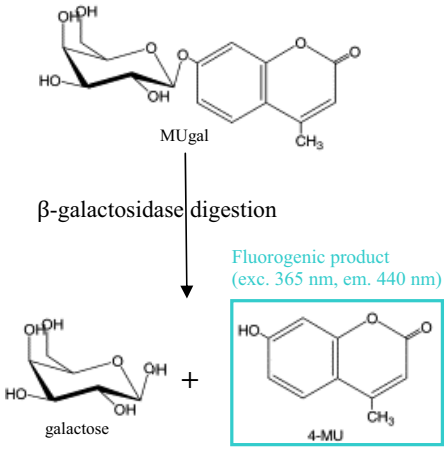


Fig 1. Hydrolysis of 4-methylumbelliferone- β-D-galactoside substrate by β-D-galactosidase

2.3 Chromogenic and fluorogenic substrates used for microbiological analysis

Chromogenic substrate in microbiological media for diagnostic purpose was used as early as 1909 by Harrison and van der Leck for the characterisation of glucosidase activity by hydrolysis of aesculin (Prescott et al., 1946). Hydrolysis of aesculin produces a soluble dark compound in the presence of iron salts, which has been used mainly for the characterisation of Enteroccoci although many other species possess glucosidase. New chromogenic substrates were developed, mainly after the 1990, expanding the range of targeted enzymes as well as the variety of the chromogenic reporters used for detetection. Focus was also given to improve quality of the test by developping non soluble chromogenic products to avoid diffusion in the solid media hence to be able to better differentiate target colonies. One of the main aglycone reporter molecule used for chromogenic detection of glycosidase activity is indoxyl and its derivatives. More recently alizarin, an iron chelator, has been used to develop β-D-glucoside substrates (Perry et al., 2007). Interestingly the authors found that this new

substrate increased the number of known responsive organisms. Typically β-D-glucoside activity would be used to characterise Enterococci and Listeria species, whereas alizarin-glucoside in the presence of iron would also be metabolised by *E. coli* and *Citrobacter freundii*. One explanation suggested by the authors is that this is due to increased uptake by the organism or enzymatic induction.

Fluorogenic substrates followed in the development of new reporters, mainly derivatives of 7-hydroxycoumarin also called umbelliferone. These reporter substrates have been included in either liquid or solid media and appropriate combination of different reporter systems allowed to develop media for the simultaneous detection of two target enzymes i.e. the detection of β-D-galactosidase and β-D-glucuronidase using either different chromogenic substrates or a chromogenic substrate and a fluorogenic substrate. An overview of commercially available and recent developments of chromogenic and fluorogenic media is given in Table 3.

New promissing substrates have been described but do not seem to have been integrated yet in the development of commercial methods. For the detection of β-D-galactosidase, EHC (ethyl-7-hydroxycoumarin-3-caboxylate), a derivative of 7-hydroxycoumarin, seems to avoid the main pitfalls associated to using 4-MU, namely the requirement of a high pH to generate maximum fluorescence, due to a pKa of 4-MU around 8, and toxicity for coliform bacteria (Chilvers et al., 2001). At neutral pH, 4-MU had a fluorescence level only 37% of that produced by EHC and exhibited a higher inhibition level of coliform. Inhibition was observed for concentrations of core coumarin above 0.008 mmol l^{-1}, which is relatively high as levels required for visual detection of 4-MU without alkalinisation is around 0.003 mmol l^{-1} (personal data). However, it is possible that the inhibition differences might affect recovery of injured bacteria so that EHC would allow a higher recovery of target bacteria. Similar data were produced regarding the entire β-D-galactoside substrates, indicating less bacterial toxicity for EHCgal compared to MUgal (Chilvers et al., 2001). When tested after 6h incubation using 12 different coliform bacteria, EHCgal decreased the time needed for detection by 1h compared with MUgal which, within the same time, produced only 27.7% of the fluorescence obtained with EHCgal. This new fluorogenic substrate seems to have the highest potential for rapid assays as maximum sensitivity is obtained without the requirement of hazardous high alkaline solution, hence enabling further microbiological analysis from the same assay if required. This substrate also appears to be metabolised faster by coliform

bacteria than MUgal, therefore shortening the time to detection. Finally, it might favour a better recovery of "Injured" target organisms reducing false negatives.

The fluorogenic substrate 6-chloro-4-methylumbelliferyl- β-D-glucuronide (CMUG) used for detecting β-D-glucuronidase is another example of a new improved fluorogenic substrate reported to have a twofold enhanced sensitivity compared to that of MUG and in addition being non inhibiting to the targeted organisms (Perry et al., 2006).

Enzymatic based methods used for rapid detection are dependant of fluorescence detection limit and non-target enzymatic activity present in the sample. The latter will vary according to the origin of the sample. Environmental samples and particularly sea water samples are known to contain a wide variety of non target β-D-galactosidase activity. Determining the average background level as well as the measurement variations are essential to assess specificity and sensitivity of rapid methods in general. In paper I, we found that the average sample background which had to be taken into account for optimized results corresponded to 2ppb MU and 1ppb for the instrument variation resulting in a pass level of 3ppb. A consequence which we reported in paper I is that the system will perform worse for individual samples without background activity than for samples with background activity. Indeed, when no background activity is present, the target bacteria will then have to produce enough fluorescence to first reach the pass level before it can be scored as a positive sample. Consequently, increased fluorescence sensitivity will not help shorten the required time for detection as the time needed in average to "cover" the pass level will remain the same. This limitation of the system may be overcome by the use of new substrates, i.e. EHC, which are more rapidly metabolized by the target bacteria, hence will help reach quicker the fluorescence threshold. This will in part depend on the non target activity not following a similar increase.

Table 3. Overview of chromogenic and fluorogenic substrate for indicator detection

Target enzyme / organism			Medium or reference
β-D-galactosidase / Coliforms	β-D-glucuronidase / *E. coli*	β-D-glucosidase / Enterococci	
MUgal: 4-methylumbelliferone-β-D-galactoside / fluorescence at 366/455nm	-	-	Colifast (CA 100), Colifast at line (CALM)
EHCgal: ethyl-7-hydroxycoumarin-3-caboxylate-β-D-galactoside fluorescence at			(Chilvers et al., 2001)

Target enzyme / organism			Medium or reference
β-D-galactosidase / Coliforms	β-D-glucuronidase / *E. coli*	β-D-glucosidase / Enterococci	
366/455nm			
DiFMUG: 6,8-difluoro-4-methylumbelliferyl -β-D galactopyranoside	-	-	(Gee et al., 1999), Molecular Probes,
ONPG: o-nitrophenyl-β-D-galactopyranoside / Yellow	**MUG**: 4-methylumbelliferyl-β-D-glucuronide / fluorescence at 365/455nm	-	Colilert & Colilert 3000 (IDEXX, SERES); Coliquick (Hach)
X-GAL: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside / Blue-green	**Salmon-glu**: 6-Chloro-3-indoxyl- β-D-glucuronide / Salmon-red		chromID (Biomérieux); RAPID'*E.coli* 2 (Diagnostic Pasteur)
Salmon-Gal: 6-Chloro-3-indoxyl-β-galactopyranoside / Salmon-red	**X-GLUC**: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide / Blue-green	-	Chromocult® Coliform agar (Merck); CHROMagar™ ECC (Chromagar); Brilliance™ *E. coli* / coliform Agar (formerly Chromogenic E. coli/coliform Agar, Oxoid)
CPRG: Chlorophenol red-β-D-galactopyranoside / Red	**MUG**: 4-methylumbelliferyl-β-D-glucuronide / fluorescence at 365/455nm	-	Colisure (IDEXX)
X-GAL: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside / Blue-green	**MUG**: 4-methylumbelliferyl-β-D-glucuronide / fluorescence at 365/455nm	-	Fluorocult® LMX broth (Merck), Readycult® Coliforms (broth, Merck)
X-GAL: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside / Blue-green	-	-	Fluorocult® ECDC agar (Merck)
	CMUG: 6-chloro-4-methylumbelliferyl- β-D-glucuronide / fluorescence at 365/455nm		(Perry et al., 2006)
	PNPG: p-nitrophenol-β-D-glucuronide / Yellow		(Hansen et al., 1984)
-	**X-GLUC**: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide / Blue-green	-	TBX (Oxoid)
-	**HQG**: 8-hydroxyquinoline-β-D-glucuronide / Black complex	-	Uricult Trio (Orion Diagnostica)
-	Fluorescein-di-β-D-glucuronide	-	ChemScan®RDI (Chemunex)
-	-	**X-GLU**: 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside / Blue-green	Chromocult® Enterococci agar (Merck) Readycult® Enterococci (broth, Merck)
-	-	**Alizarin** (1,2-dihydroxyanthraquinone)-**β-D-glucoside** / purple*; Pink**	(Perry et al., 2007) * with ferric ammonium citrate ; ** with aluminium ions

Most of the methods using chromogenic or fluorogenic substrates are still manually operated (Apte et al., 1994) or a single sample is analysed at one time (Reynolds et al., 1999; Van Poucke et al., 2000), or the total time to obtain a result exceeds a working day (Santurette et al., 1995). More recently an online system, the Colilert 3000, was described (Zuckerman et al., 2008). In an attempt to increase the number of samples processed and reduce the time required to analyse the samples, a semi-automated instrument, CA-100, as well as a specific microbiological culture medium (Berg et al., 1988) were developed by Colifast Systems ASA to assess the presence or absence (P/A) of coliforms. The instrument monitors the activity of β-galactosidase in samples added either directly or after membrane filtration, followed by incubation at the appropriate temperature in the selective Colifast-6 medium. Since the intensity of the fluorescence signal of a sample is proportional to the amount of product formed from the substrate in the medium, information of a semi-quantitative nature is generated.

In a study, performed at the Environment Agency Laboratory, Wales, the CA-100 was used in parallel with reference methods used in the laboratory to assess the microbiological quality of Welsh environmental waters, freshwater as well as seawater. A total of 1011 samples from 206 sites were processed in the period between January and October 1999 (Paper I).

2.4 Molecular microbiological methods for microbial drinking water assessment

As the methods and definitions evolved to include more species within the coliform group, the number of described environmental strains increased such that interpretation of the signification of the redefined group also needed a revision. Even when using the selective temperature of 44^{o}C, the coliform group could no longer be associated exclusively with faecal contamination. As a result, the coliforms are now redefined as a “general (process) microbial indicator” whereas *E. coli* is considered the only true indicator for faecal contamination (Ashbolt et al., 2001) among coliform bacteria. The first methods devised for characterising faecal bacteria were based on detecting end products of lactose catabolism, such as acidification, production of aldehydes or gas. As we have seen, direct characterisation of β-D-galactosidase, the first enzyme involved in lactose catabolism, led to

an increase in the size of the target coliform population. The next step in microbiological method development employs NA for detection and identification. This includes for example targeting the gene(s) involved in the coding and regulation of β-D-galactosidase or β-D-glucuronidase. This change in methods once again has the potential to increase the size of the coliform target group within the Enterobacteriaceae family by detecting MUgal or MUG negative strains which still possesses the corresponding coding sequence (Bej et al., 1991a; Feng et al., 1991). Indeed, the presence of β-D-glucuronidase is used for the characterisation of *E. coli* but it is well known that some *E. coli* strains fail to express the enzyme because of a mutation. This is for example the case for most *E. coli* O157H7 which, in spite of this, can be characterized using PCR for the identification of the mutated gene (Cebula et al., 1995; Feng, 1993). Figure 2 shows the evolution of the coliform definitions and the increase in size it meant within the Enterobacteriaceae.

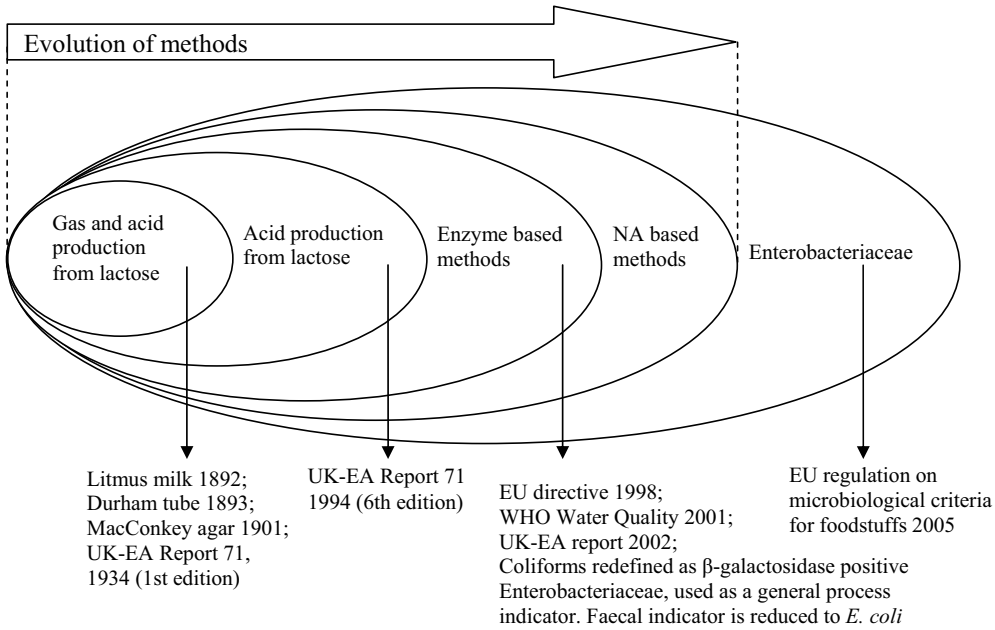


Fig. 2 Evolution of the coliform group definition and extrapolation (Inspired by (Manafi, 2005))

Although the first PCR assays for detecting bacterial indicators in water were developed in the early 1990s (Bej et al., 1990; Bej et al., 1991c; Bej et al., 1991a), Nucleic

Acid Amplification Test (NAAT) does not yet appear to have been established for routine analysis of water samples. Their implementation would necessitate addressing the following points: a) develop the NA assay with the required sensitivity and specificity b) improve sample preparation techniques to allow large volumes (i.e. 100mL) to be processed efficiently in a NA amplification test (typically using 10ul sample) c) get rid of the possible presence of NAAT inhibitors in the sample d) have acceptable cost per analysis.

In the next part of this work, we attempt to explore points a), and c), by defining relevant NA targets, developing robust amplification methods and finally, looking into NA sample preparation.

3 Developing NAATs

3.1 Introduction

As we have seen in chapter 2, the faecal coliform group is no longer considered an indicator for faecal contamination. As the total coliform group expanded within the Enterobacteriaceae and the advent of new NA based methods offered unprecedented possibilities for diagnostic which was first implemented in clinical microbiology (Whelen et al., 1996), we considered the development of NAAT for water microbiological assessment and selected associated diseases to be the next logical step in the present work. The effort would primarily focus on developing specific and integrated assays before addressing the new challenges brought by sample preparation for NAATs. The Enterobacteriaceae family appeared as a logical extension of the enlarged coliform group with the added benefit of being a well defined taxon, and was therefore our primary objective for finding a suitable NA marker and develop a PCR assay. In order to detect some of the emerging Enterobacteriaceae water associated pathogens, specific pathogenic markers were included to characterise mainly Enterohemorrhagic *E. coli* (EHEC) and Enteropathogenic *E. coli* (EPEC). Finally, as an alternative to *E. coli* for faecal contamination indicator we set *Enterococcus faecalis* and *Enterococcus faecium* to be included in the work.

3.2 The Polymerase Chain Reaction (PCR)

After the PCR was invented by Kary Mullis in the mid 80s (Mullis et al., 1986), other alternative amplification systems such as NASBA (nucleic acid sequence-based amplification), TMA (transcription mediated amplification) or SDA (strand displacement amplification) have been described. Although these methods have some appealing features such as using RNA rather than DNA as initial target for amplification, none of them seemed as flexible and accessible as PCR for developing new assays. In the present work PCR was therefore chosen as our primary tool for developing our assays. However, in order to be efficient and practical, it is important that the PCR methods that are used are robust, i.e. that they provide a strong and easily reproducible amplification, with no generation of additional products. Furthermore, when multiple targets are aimed at, the use of multiple primer sets in the same PCR reaction (multiplex PCR; two primer pairs means a duplex PCR, three primer pairs a triplex PCR etc.) is also preferable to the use of a separate PCR protocol for each of the primer sets (simplex PCR). This allows saving time and reagents, and thus lowering the cost of the analysis.

When applying PCR it is possible to use so called universal primers. Universal primers are designed with the aim of working for all variants of a given gene or DNA target. Typically the primers will be chosen in the most conserved areas of the gene in question, which is ideally identical in all variants. When no conserved identical part of the DNA target can be found, two strategies can be employed to accommodate the ambiguous nucleotide positions: silent mismatch and degenerate primers. In the first case, the primers are designed so that the variable nucleotide(s) are positioned in the primers in a way that will allow amplification to proceed in spite of one or more mismatches. Typically, these ambiguous positions will be positioned at the 5' end of the primers. In the case of degenerate primers, all the ambiguous positions are accounted for, and a mix of all possible combination of the variable positions is used. This has the inconvenience of diluting the one full match primer set. However, the advantage is that it will be more efficient for highly variable genes and have more chances of functioning on new unknown variants of the target.

Optimalisation of a PCR reaction aims at improving sensitivity (yield) and specificity (avoid amplification of non target DNA). Typically, the annealing temperature T_a, concentration of primers and MgCl2, will first be optimised. The higher the T_a the more

specific the reaction will be, albeit at the cost of sensitivity beyond a certain limit. Designing primers with a theoretical high T_a can therefore also be advantageous to reduce false priming. Further optimisation can be achieved by using chemical compounds often refered to as "PCR facilitators". They will help alleviate deficience or absence of amplification caused by either difficult target DNA or because of the presence of PCR inhibitors (see Table 4). For example, DMSO, formamide and betaine may be used when the target DNA sequence is GC rich and/or has secondary structures. When PCR inhibitors are suspected to be present, such as with urine or environmental samples, and may be carried through the isolation causing PCR inhibition, BSA or gp32 may be used to facilitate the PCR. Finally, many of the compounds used for chemical lysis have also inhibitory effect to the PCR. In the case they are partly carried over to the eluate due to insufficient NA purification, other facilitators such as Tween 20 or 40 may be used (see Table 4). This kind of PCR inhibition can be avoided by using alternative lysis procedure to avoid chemical lysis (see chapter 4.3).

Table 4. PCR Facilitators

Facilitators	Quantity	Remark	Reference
DMSO (Dimethyl Sulfoxide)	2-10% wt/vol	Reduces secondary structures and is useful for GC rich targets, included in most PCR mastermixes,	(Frackman et al., 1998; Henegariu et al., 1997)
EDTA & then + MgCl2	1mM and 2,5mM	Used to neutralise Fe and other metal cations with stronger complexation constant with EDTA than Mg2+	(Teng et al., 2008)
Formamide	1-5% wt/vol	Reported useful to increase specificity	(Sarkar et al., 1990)
BSA (Bovine Serum Albumne)	0,01-0,06% wt/vol	Useful for samples know to contain PCR inhibitors i.e. blood, faeces, melanin, ancient DNA	(Abu Al-Soud et al., 2000; Giambernardi et al., 1998; Kreader, 1996)
Gp32 (T4 gene 32 protein)	0,01% wt/vol	Useful for samples know to contain PCR inhibitors i.e. blood, faeces, ancient DNA	(Abu Al-Soud et al., 2000; Kreader, 1996)
Glycerol	5% wt/vol		(Abu Al-Soud et al., 2000; Henegariu et al., 1997; Weissensteiner et al., 1996)
PEG (Polyethylene Glycol)	4% wt/vol		(Abu Al-Soud et al., 2000)
Betaine mono hydrate (N.N,N-	1-2,5M	Reduces secondary structures and is	(Frackman et al., 1998; Henke et al., 1997;

Facilitators	Quantity	Remark	Reference
trimethylglycine)		useful for GC rich targets, patents	Weissensteiner et al., 1996)
TMAC (Tetramethylammonium chloride)	15-100mM	To eliminate non-specific priming and reduce potential DNA-RNA mismatch	(Abu Al-Soud et al., 2000; Chevet et al., 1995)
Tween 20-40, Triton X-100	0,5% wt/vol	Useful to neutralise the inhibition effect of SDS carry over during NA sample preparation	(Gelfand, 1989)

Directly using NA genetic material for diagnostic purposes has considerably increased the flexibility and possibilities compared to traditional phenotypical methods. Hence, a large amount of PCR protocols have been developed for detecting higher microbial large taxons, species, and serotypes or for the detection of specific traits such as genes involved in defined aspects of a pathology i.e. coding for toxins. These methods have initially mainly focussed on clinical samples but have increasingly been covering other types of analysis using various sample types such as environmental or food samples. In an effort to gather an overview over existing methods, a list of PCR methods with primer sequences and assay conditions was compiled in appendix 3. Further, a visual summary of relevant PCR multiplex protocols was assembled in appendix 4.

3.3 Enterobacteriaceae

The Enterobacteriaceae is a coherent well-defined taxonomic unit, which is relevant both to clinical diagnostic and to food and water routine microbiological analysis, as it includes important human pathogens and the total coliform group. Traditional microbiological methods used for the identification of this family rely on biochemical properties of isolated re-grown bacterial colonies. Only few faster alternative methods have been developed so far, and they are all based on the identification of a trait or marker specific to the taxon. NA sequences coding for essential biological processes conserved through evolution, commonly referred to as “housekeeping” genes, have been used for phylogenetic and taxonomic analysis as well as for diagnostic applications. For example, sequences of 16S rRNA genes

have been widely used for such purpose including for the detection of Enterobacteriaceae members (Mittelman et al., 1997).

The Enterobacterial Common Antigen (ECA) was first described in 1963 by Kunin et al. (Kunin, 1963) and defined as a cross-reactive antigen that is detectable in all genera of Enterobacteriaceae by indirect hemagglutination and by other methods using antiserum to *E. coli.* It was later found to be strictly family specific with diagnostic as well as prophylactic potential. The only known noticeable reported exceptions are the Enterobacteriacea ECA-negative *Erwinia chrysanthemi* and the non-Enterobacteriacea ECA-positive *Plesiomonas shigelloides,* both of which have disputed taxonomic positions (see review (Kuhn et al., 1988)). The ECA is a glycophospholipid built up by an aminosugar heteropolymer linked to an L-glycerophosphatidyl residue. This surface antigen remained undetected for a long time due to its non-immunogenicity in most Enterobacteriaceae despite its general ability to act as an epitope (hapten). There are seven genes implicated in the synthesis of ECA, *rfe*, *rffD*, *rffE*, *rffC*, *rffA*, *rffT*and *rffM* which are clustered around 85 minutes on the *E. coli* genome and 83 minutes on the *Salmonella typhimurium* genome (Ohta et al., 1991; Rick et al., 1996). Of these seven genes *rfe* is also implicated in the synthesis of many O antigens. The nomenclature of these genes was changed and *rfe-rff* genes are know defined as the *wec* gene cluster (Reeves et al., 1996).

Immunology-based diagnostic tests have been developed to detect the presence of ECA for clinical applications (Malkamaki, 1981) and later to monitor drinking water microbiological quality by detecting bacteria belonging to the Enterobacteriaceae family (Hubner et al., 1992). Such tests rely on the expression of the character being tested for, which might be absent or poorly expressed in mutants, although most of the gene coding sequence may still remain intact. In this connection DNA-based techniques, i.e. PCR, have been successfully used to decrease the amount of false negatives in diagnostic applications, i.e. beta-glucuronidase enzyme and its coding sequence used for the detection and identification of *E. coli* (Feng et al., 1991).

3.4 *Enterococcus faecalis* and *E. faecium*

In the same maner as *E.coli, E. faecalis* as well as *E. faecium* have commonly been considered to be harmless commensal of the human digestive tract of healthy humans, although usually present in amounts up to ten times lesser than *E. coli*, (Leclerc et al., 1989). Members of the *Enterococcus* genus have been used as human probiotics, for ripening and aroma development of various foods and some members produce bacteriocins with anti-*Listeria* activity. However, *E. faecalis* and *E. faecium* have also emerged as important aetiological agents for various nosocomial diseases. Enterococci are responsible for nosocomial bacteremia, surgical wound infection, endocarditis and urinary tract infection. Most infections are caused by *E. faecalis* which, in contrast to *E. faecium*, has virulence genes such as gelatinase, cytolysines, adhesines and invasines (Franz et al., 2003; Jett et al., 1994). Both species also have acquired a steadily increasing pool of resistance determinants to antibiotics such as β-lactams, aminoglycosides and glycopeptides (Arthur et al., 1996; Cetinkaya et al., 2000; Courvalin, 2005; Gholizadeh et al., 2000; Willems et al., 2007) although *E. faecium* is more often resistant to glycopeptides such as Vancomycin and Teicoplanin. These characteristics have turned these species into resilient potentially life threatening pathogenic bacteria.

Thus, in a similar fashion to *E. coli*, they have evolved to cumulate two relevant traits in relation to the present work: they are major members of a traditional indicator group important for water surveillance called the “faecal streptococci” or FS (Ashbolt et al., 2001), and they are potential pathogens important to be identified in the clinical world (Franz et al., 1999a). Detecting and identifying these organisms have therefore become increasingly relevant. Specific phenotypic and genotypic traits have been used for the development of various detection methods. Table 5 summarizes some of the characteristics which have been exploited or could be of interest for diagnostic purpose. When compiling the literature for PCR-based methods (see Annex IV), it seemed few were capable of specifically detecting *E. faecalis* and *E. faecium*, which has emulated the search of new species-specific genetic markers.

Table 5 Selected genetic traits of potential diagnostic interest for *E. faecalis* and *E. faecium*

Selected characteristics	Genetic support	*E. faecalis*	*E. faecium*	Reference
glucosidase	Chromosome	+	+	(Devriese et al., 1993; Jett et al., 1994)
Hyaluronidase[a], gelatinase[b]	[b]*gelE*	variable	variable	
Enterocin A, B & P	Chromosome	-	+	
Enterocin 50	Plasmid PC1Z1	-	+	(Joosten et al., 1997)
Bacteriocin AS48	Pheromone responsive plasmid pMB2	80%	60%	(Franz et al., 1999a; Haas et al., 1999; Herranz et al., 1999)
Bacteriocin 31	Pheromone responsive plasmid pY117	+	-	
Cytolysine (hemolysine)	Pheromone responsive plasmid pAD1	+	-	
Acquired inducible VanA (resistance to high levels of vancomycin and teicoplanin)	Tn*1546* on plasmid or chromosomal mobile elements	(+)	+	(Dutka-Malen et al., 1995; Gholizadeh et al., 2000; Leclercq et al., 1997; Ostrowsky et al., 1999; Perichon et al., 1997)
Acquired inducible VanB (resistance to variable levels of vancomycin)	Tn*1547* associated to pheromone responsive plasmids or mobile elements on the chromosome	+	+	
Acquired constitutive VanD (resistance to moderate levels of vancomycin and teicoplanin)		+	+	
Tetracycline resistance	Pheromone responsive plasmid pCF10 and transposon mediated	+	-	(Dunny et al., 1995) (Murray, 1998)
N-aminoglycoside acetyltransferase	Chromosome	-	+	(Costa et al., 1993)
Enhanced expression of pheromone *eep*	Chromosome	+	-	(An et al., 1999)
Pheromones promoting the conjugative transfer of plasmids	Chromosome	+	-	(Clewell, 1993; Dunny et al., 1997; Hirt et al., 1996; Wirth, 2000)
Agreggation Substance (AS)	Plasmid	+	-	

Enterococci are catalase-negative Gram-positive facultative anaerobic bacteria capable of growing at a temperature range from 10°C to 45°C, in 6,5% NaCl broth or 0,04% sodium azide and bile salts. The presence in these organisms of the enzyme β-glucosidase has been exploited to develop specific growth chromogenic media (Adcock et al., 2001; Rhodes et al., 1997).

The acquired *vanA* phenotype confers high level inducible resistance to both Vancomycin and teicoplanin whereas the VanB phenotype confers variable levels of inducible resistance to vancomycin only. The gene coding for VanA is carried by the transposon Tn*1546* and is mostly found among *E. faecium* isolates. Both *vanA* and *vanB* produce the glycopetide resistant depsipeptide D-alanyl-D-lactate (D-Ala-D-Lac) instead of the glycopeptides sensitive dipeptide D-Ala-D-Ala. The constitutive *vanC* produce D-Ala-D-Ser which has low affinity to glycopeptides antibiotics. Finally, the constitutive *vanD* has a similar mode of action as *vanA* & B but is not transferable by conjugation. An overview of the antibiotic resistance system for *E. faecalis* and *E. faecium* and the *E. faecalis* sex pheromone system is shown in Figures 3 and 4.

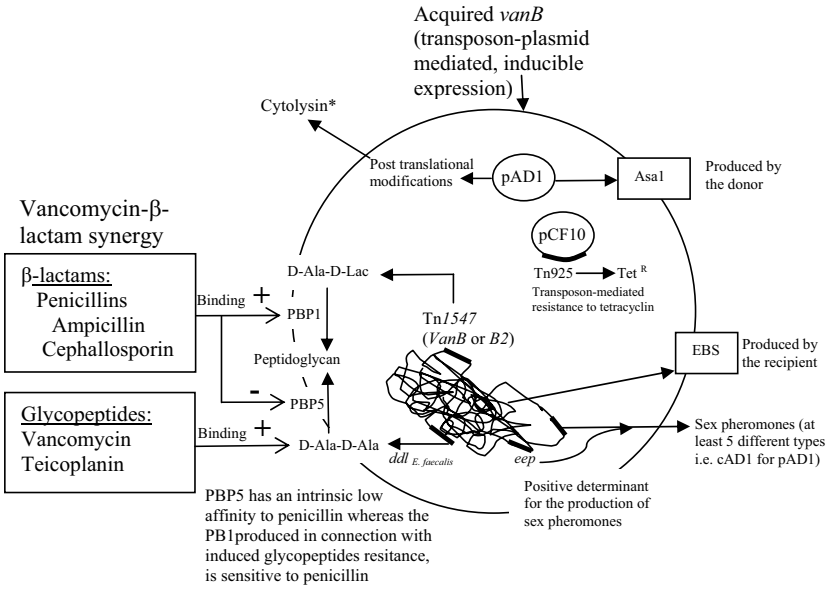


Fig. 3 Overview of antibiotic resistance and pheromone responsive conjugation system of *Enterococcus faecalis*. PBP: Penicillin Binding Protein; Asa1: Aggregation substance; EBS: Enterococcal Binding Substance; *eep* Enhanced expression of pheromones; *ddl*: D-ala, D-ala ligase; * Cytolysin, Bacteriocin 31, & AS48 are Class I bacteriocins (lantibiotics) produced by conjugative pheromone responsive plasmids.

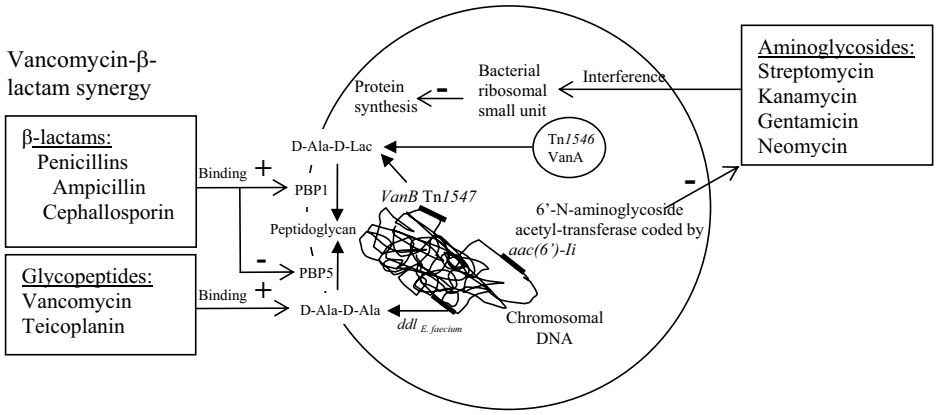


Fig. 4 Overview of antibiotic resistance system of *Enterococcus faecium*.

Several PCR methods have been developed for the detection of the *van* glycopeptides-resistance genes (Dutka-Malen et al., 1995; Modrusan et al., 1999; Petrich et al., 1999) as well as for detecting the species *E. faecium* (Bergeron et al., 1999; Cheng et al., 1997; Dutka-Malen et al., 1995) and *E. faecalis* (Dutka-Malen et al., 1995).

In paper III we present species specific PCR primers for the detection of *E. faecalis* constructed using the *eep* gene and species specific PCR primers for the detection of *E. faecium* constructed using the *aac(6')-Ii* gene. The target genes were selected for their respective species selectivity and for their stability on the host chromosome.

3.5 Shiga toxin gene *stx*

3.5.1 *stx* nomenclature

As indicated by its name, this class of toxin was first discovered in *Shigella dysenteriae*. A similar toxin was later discovered in *E. coli,* characterized as cytotoxic to vero cells and named Vero toxins (VT). The group *of E. coli* producing VT was accordingly named VTEC (Konowalchuk et al., 1977). The toxin was later shown to be related to Shiga toxin, which prompted some authors to rename it Shiga like toxin (SLT) and SLTEC to describe the bacterial group producing it (O'Brien et al., 1983). As it became more evident that all Shiga toxins are related, a new genetic nomenclature was proposed and widely accepted, and is the one we use in the present study (Calderwood et al., 1996; Karmali et al., 1996). Consequently this group of *E. coli* is now referred to as Shiga toxin producing *Escherichia coli* (STEC).

3.5.2 *stx* structure

The Shiga toxin is an A-B toxin type formed of the association of 5 B subunits structured in a ring-like fashion with one A subunit on top of the ring. The ring is responsible for the recognition and attachment to the eukaryotic Gb3 globotriaosylceramide cell receptor of the toxin, whereas the A subunit is the active toxic component that inhibits protein synthesis by removing an adenine from the 28 S rRNA. The two subunits are encoded by two genes organized in an operon in which the B subunit is more frequently transcribed than A, enabling the final molecular ratio of 1/5 for the holo toxin (O'Brien et al., 1992).

The *stxA* gene varies in length from 948 bp for $stxA_1$ to around 960 bp for $stxA_2$ and the “theoretical” maximum length after alignement of all variants is 967 bp, and is the value used as the reference template for numbering the primers (see appendix A1). The gene coding for the B subunit is 267 bp in length. As more *stxA* sequences have been described than *stxB*, and since *stxA* is longer, the latter was chosen here for the development of universal primers to detect the presence of *stx*.

3.5.3 *stx* variability

Many variants of the *stx* gene have been described and new ones are still characterized. They have been classified in 2 main groups according to their sequence similarity. The first, stx_1, is found in STEC and are almost identical to the shiga toxin genes of *S. dysenteriae* type 1, *stx*. The only stx_1 variant, initially called stx_{1ox3} and later renamed stx_{1c}, is primarily found in sheep but is also associated with humans presenting weak diarrhoea symptoms or among asymptomatic carriers (Koch et al., 2001; Zhang et al., 2002b). stx_{1c} can be found alone or together with stx_{2d} mainly in E. coli serotypes O113, O128 O76 or O146 and are characterised by the absence of *eae* (Beutin et al., 2004; Friedrich et al., 2003).

The second group, stx_2 and its variants, is the most divergent and comprises sub-groups which appear to be found in host-adapted strains and other species than *E. coli*. It also encodes for the most potent shiga toxin for humans. Both stx_2 and stx_{2c} are mainly hosted by STEC associated with the aetiology of severe human diarrhea whereas stx_{2d} has been isolated in STEC of both human and cattle origin (Ramachandran et al., 2001). Finaly, stx_{2e} have been found in porcine *E. coli* (Franke et al., 1995) while stx_{2f} are found in *E. coli* hosted by birds (Morabito et al., 2001; Schmidt et al., 2000). Although the toxins Stx2e and Stx2f toxins seem to be adapted to their respective hosts they both have been associated with human disease (Franke et al., 1995; Gannon et al., 1990; Morabito et al., 2001; Muniesa et al., 2000b; Sonntag et al., 2005). Combination of different s*tx* variants can be found in one and the same bacteria as illustrated by the cases of three patients with diarrhoea, each of which had a different STEC serotype carring the genes stx_1, stx_2 and stx_{2c} (Furst et al., 2000). Cattle are considered to be the main reservoir of STEC with 50 to 95% of the animals found to be host (Fegan et al., 1999) although many other domestic animals were also found to host STECs (Beutin et al., 1993). In addition, bacteria carrying *stx* genes have been isolated from marine waters (Miyagi et al., 2001) and are commonly found in rivers (Kurokawa et al., 1999). Although not all Stx-producing bacteria can have phage induced, all *stx* genes, including that of *S. dysenteriae* serotype 1, are considered to be phage borne (Unkmeir et al., 2000). In this connection, Shiga toxin-converting bacteriophages are commonly isolated from sewage (Muniesa et al., 1998) and have been shown to play an important role in the emergence of new STEC variants (Muniesa et al., 2000a; Schmidt, 2001). These findings illustrate how ubiquitous Shiga toxins are in our environment spanning from land to sea and air with the intrinsic potential of horizontally spreading to new bacterial hosts.

3.5.4 STEC diversity

Although the first and main STEC serotype associated with the onset of human disease is O157:H7, more than 200 serotypes have been recognized (Beutin et al., 2004; Coia, 1998; Griffin et al., 1991; Nataro et al., 1998; World Health Organization, 1998) therefore the importance of developing methods for detecting them have been emphasized (Acheson et al., 1996; Johnson et al., 1996; World Health Organization, 1998). The STEC serotype associated with the development of human haemolytic uraemic syndrome might vary from one country to another as shown in a recent Australian survey in which none of the 98 Hæmolytic Uremic syndrome (HUS) cases identified over 4 years were associated to O157:H7 (Elliott et al., 2001). Similarly, in another Australian study, no O157:H7 was detected among the 23 STEC strains isolated from bovine faecal samples (Hornitzky et al., 2001). Non-O157 STEC were possibly previously underestimated because of the use of diagnostic methods targeting typical phenotypic characteristics of the O157:H7 serotype such as delayed sorbitol fermentation and lack of glucuronidase activity rather than toxin production (de Boer et al., 2000; HMSO, 1996; March et al., 1986; Zadik et al., 1993). These tests were developed to enable mass screening by routine laboratory but will obviously miss many STEC including atypical O157 isolates (Karch et al., 2001; Ware et al., 2000). A list of non-O157 STEC is kept updated by (Dr. Bettelheim, National *E. coli* Reference Laboratory, Melbourne, Australia [http://www.microbionet.com.au/vtectable.htm]). The same criticism can be made for serological diagnostic tests, which are specific to the serotype, i.e. O157 detection methods. Other immunological diagnostic methods targeting Shiga toxins have been developed (Kehl et al., 1997; Mackenzie et al., 1998) but rely on toxin expression and lack the analytical flexibility of DNA-based methods.

3.5.5 PCR protocols for detection of *stx*

To circumvent the unreliability of phenotypic expression, various DNA-based methods were developed for detecting *stx*. Due to the diversity of *stx* genes involved with the onset of human disease and the large amount of possible *E. coli* serotype carriers for *stx*, it is clear that a DNA-based method able to detect all variants of the gene encoding Shiga toxin is needed when evaluating human health risk of environmental samples or when identifying aetiological agents of human gastro-enteritis. Although various universal primer pairs for

the detection of *stx* have been described in the literature (see Appendix A3 and A4), few are able to detect all variants or can be used in a multiplex assay comprising a PCR positive control . Only the PCR assay described by Lin et.al. (Lin et al., 1993) was successfully tested so far in order to detect all *stx* variants in a simplex assay (Bastian et al., 1998; Schmidt et al., 2000).

In the present work a "universal degenerate" primer pair, "Ustx" was constructed aiming at the detection of all variants of the *stx* gene (paper II and III). A total of 44 *stx* sequences representating all 7 main *stx* subtypes (including stx_{1c}), have been aligned (see Appendix A1) with the purpose of finding conserved areas suitable for building a universal primer pair. The selected final areas for primer pair design still contained some variable positions. As shown in Fig 5, the Ustx degenerate primer pairs were designed to have the variable positions located away from the terminal 3' end of the primer to enhance priming efficiency. The nomenclature used for ambiguous bases is shown in Appendix 5. As the stx_{2f} gene variant had an extra nucleotide in the chosen reverse primer area, a supplementary primer pair matching stx_{2f}, was added to the degenerate primer pair mix.

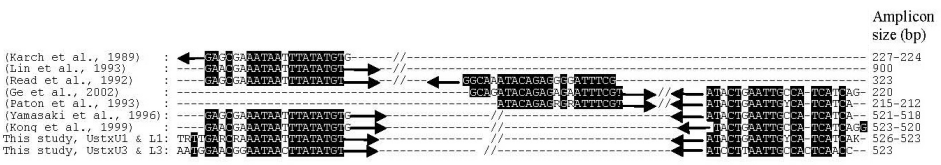


FIG.5 Comparison of selected primers aiming at the detection of all variants of *stxA* gene; arrows are indicating the direction of the primers and complementary sequences are shown for the reverse primers. The conserved bases are shaded in black while the variable positions are unshaded.

As shown in Figure 5, the forward primers used by Lin Z. and Read S.C. (Lin et al., 1993; Read et al., 1992), are overlapping part of the forward Ustx used in the present work but use different reverse primers, thus generating amplicons of a different length. Moreover the overlapping forward primers used by Lin have four mismatched positions, whereas the present work uses degenerate, as well as longer primers. These last two points are important when evaluating the robustness of the method and use of these primers in a multiplex PCR. Furthermore, Lin and Read performed only simplex PCR.

Karch (Karch et al., 1989) developed a simplex PCR which was later included in multiplex PCR protocols (Fratamico et al., 1998; Fratamico et al., 1993; Nagano et al., 1998) in which the reverse primer is complementary to a part of the Ustx forward primer used in the present work. Again, four mismatches are observed with the Karch primer as shown in Figure 5. Paton et al. in the simplex PCR they developed, (Paton et al., 1993b), used an overlapping degenerate perfect match to the reverse primer, although they used a different forward primer. Finally, Yamasaki S. described a simplex PCR (Kobayashi et al., 2001; Yamasaki et al., 1996) in which the forward as well as the reverse primers are overlapping those of Ustx thus generating an amplicon of almost the same size (5 nucleotide less). The aim and the claim of all these protocols are to detect all variants of the *stx* gene using a "single pair" of primers. Few studies have compared and tested extensively such protocols, but two publications (Bastian et al., 1998) among which a study of the latest gene variant stx_{2f} (Schmidt et al., 2000), agree in finding that only Lin (Lin et al., 1993) achieves detection of all variants. A closer study of the reverse primers used by Paton and Yamasaki (Paton et al., 1993b; Yamasaki et al., 1996) indicates they would probably also fail because of a on nucleotide insert in the stx_{2f} variant symbolised by a gap in Figure 5. As indicated above, we have extended the notion of degenerate primers to compensate for this gap by adding an overlapping primer pair with a perfect match to the stx_{2f} variant. Although it appears as an extra pair of primers, it only serves as one more combination to cover all the possible ambiguous positions for the same primer pair location on the *stx* sequence. This was tested on various *stx* types; however, no stx_{2f} was used to verify this particular variant would also be amplified with this protocol.

3.6 Intimin

Intimin is a protein involved in the intimate adherence of a bacterium to the epithelial cell membrane of the host's gut. The *eae* gene (*E. coli* attaching and effacing) encodes intimin of pathogenic *E. coli* producing the typical A/E (attaching-and-effacing) histopathology in infected patients. In an experiment with human volunteers, intimin was proven to be necessary for the full development of diarrhoea caused by EPEC. At least ten different types of intimin have been described: α ((alpha), β (beta), γ (gamma), δ (delta)(Adu-Bobie et al., 1998), ε(epsilon)(Oswald et al., 2000) and more recently the types η(eta), ι(iota), κ(kappa)

(Zhang et al., 2002a), ζ (zeta) (Jores et al., 2003), anḍξ(ksi) (Blanco et al., 2004). These types will often be associated with *E. coli* serotypes and pathotypes. For example, intimin α will be associated witḥ EPEC O56:H6 or O127:H6 wheras intimin γ will be found among EHEC O157:H7 and EPEC O55 (Jores et al., 2003; Ramachandran et al., 2003). The 950 amino acid sequence of intimin can be separated in two regions according to their degree of variability. The 670-aa N-terminal region is highly conserved whereas the 280-aa C-terminal region shows a high degree of diversity. The C-terminal region, also known as Int280, is the exposed immunogenic part of intimin. The *eae* gene is found in the so-called locus of enterocyte effacement (LEE) pathogenecity island of both EPEC and EHEC (Elliott et al., 1998; Yu et al., 1992). The LEE pathogenicity island has preferred insertion sites in the bacterial genome. Thus, for EPEC it is the chromosomal gene *selC* which is disrupted, whereas *pheU* is the preferred site for EHEC.

Plasmid loss during sub-culturing has been reported and demonstrates that pathogenic plasmid borne molecular markers might be unreliable (Hill et al., 1981). Thus, the location of the locus of enterocyte effacement (LEE) on the chromosome rather than on a plasmid, which is often the case for several other virulence factors (Burland et al., 1998; Paton et al., 1998), is of advantage in terms of stability of that DNA segment.

A total of 21 *eae* sequences representative of all 10 main eae subtypes, have been aligned (see Appendix A2) with the purpose of finding conserved areas suitable for building a universal primer pairs which are used in paper II and III.

3.7 Multiplex PCR

After the “universal degenerate” primer pair for detection of *stx* was successfully tested, new primer sets were constructed for the remaining targets of interest (*Wec*A for Enterobacteriaceae and the *eae* gene coding for intimin), checked for compatibility in a multiplex assay and tested (see Papers II and III). Examples of diverse Enterobacteriaceae species or pathotypes carrying stx or eae genes are listed in Table 6.

TABLE 6. Multiplex gene targets and their known hosts[a]

Higher taxon	Gene/marker	GenBank accession no.	Taxonomic unit	Reference
Enterobacteriaceae	Shiga toxin sub-unit A (*stxA*)	X67514	*Citrobacter freundii*	(Schmidt et al., 1993)
		M19473 L04539	*E. coli* stx_1 (EHEC)	(Jackson et al., 1987) (Paton et al., 1993a)
		AF125520 AJ249351 AJ010730	*E. coli* stx_2 (EHEC)	(Plunkett, III et al., 1999) (Muniesa et al., 2000b) (Schmidt et al., 2000)
		Z50754	*Enterobacter clocae*	(Paton et al., 1996)
			Serratia marcessens	(Paton et al., 1997)
		M19437 AJ271153	*Shigella dysenteriaea*	(Strockbine et al., 1988) (Unkmeir et al., 2000)
		AJ132761	*Shigella sonnei*	(Beutin et al., 1999)
	Intimin (*eae*)	Z11541 AF022236 X60439 U60002 AF116899	*E. coli* (EHEC / EPEC / RDEC)	(Yu et al., 1992) (Elliott et al., 1998) (Beebakhee et al., 1992) (Agin et al., 1996) (Oswald et al., 2000)
		L29509	*Escherichia albertii* (formerly *Hafnia alvei*)	(Albert et al., 1991; Albert et al., 1992; Frankel et al., 1994; Ridell et al., 1994)
		L11691 AF311901	*Citrobacter rodentium* (formerly *C. freundii* biotype 4280)	(Schauer et al., 1993) (Deng et al., 2001)
	Enterobacterial common antigen (*WecA* formerly *rfe*)	S75640 AF233324	Enterobacteriaceae	(Ohta et al., 1991)

[a]EHEC, Enterohaemorrhagic *E. coli*; EPEC, Enteropathogenic *Escherichia coli*; RDEC, rabbit EPEC

3.8 Typing for stx

Once the *stx* gene has been identified, it is important to determine which subtype it belongs to in order to gather knowledge on the potential health hazard it represents and help decide on the course of action to be taken (see 3.5.2). In the present work, two complementary approaches were devised based on the sequence of the Ustx amplicon. The first is a seminested PCR protocol to easily differentiate stx_1 from stx_2 whereas the second method uses a restriction enzyme to digest the Ustx amplicon product generating restriction patterns each specific to *stx* subtypes.

3.8.1 Semi-nested PCR

A semi-nested duplex PCR protocol was developed to differentiate stx_1 from stx_2. It is composed of three primers. One reverse primer identical to the one used in the universal *stx* protocol, UstxL, and two forward primers, Nestx1 and Nestx2, complementary to sequences localized within the Ustx amplicon. Succesfull amplification with Nestx1 + UstxL will generate a 210 bp amplicon specific of stx_1 whereas Nestx2 + UstxL will produce a 400 bp amplicon specific of stx_2. The protocol and the results are described in papers II and III.

3.8.2 Enzymatic restriction of the PCR product

To obtain complementary information on the *stx* subtype, an enzymatic restriction (ER) protocol using *Bsr* I for digestion of the Ustx amplicon was devised. The digestion product is subject to a gel electrophoresis, and the obtained fragment pattern will be characteristic of one or several *stx* subtypes as shown in paper II and III. Both the ER and the semi-nested PCR produced the same results for the analysis of the presence or absence of stx1 and stx2 in all 15 tested STEC (paper III).

4 NA sample preparation for NAAT

Although it is possible to some extent to neutralize inhibitors carried over to the eluate after NA extraction (see Ch 3.2) or by using a DNA Polymerase more resistant to the action of the inhibitor (Abu Al-Soud et al., 1998), removal of inhibitors during the extraction process becomes unavoidable at high inhibitory levels. An efficient cleansing might come at the cost of yield or limit the possibilities for automation. Therefore, it is important to consider both the nature of the sample and the purpose of the analysis in order to establish an appropriate NA preparation procedure.

4.1 Methods overview

The nature of the samples to be processed for extracting and purifying NA for NAAT will be determinant for choosing an appropriate methodology and address the two main challenges usually associated with such task: eliminate NAAT inhibitors while securing best possible sensitivity. In order to limit the effect of inhibitors, various NA preparation systems recommend using a small fraction of the prepared eluate, i.e. 1%. However, this will in practice be equivalent to diluting the sample and is a limitation to achieve optimal sensitivity. Diluting and subculturing the sample, when possible and when the time to reach a result is not an issue, may be performed prior to NA extraction, increasing the chances for succesfull NAAT. This is a standard approach used for food matrices when testing for the presence of small number of pathogens by NAAT (PERRY et al., 2007). Solid food samples will also usually require preparation by resuspension in an appropriate buffer, using a stomacher. Methods without enrichment must be considered when subculturing is impossible due to bacteriocidal sampling methods, or because the microorganisms are difficult to culture i.e. obligate intracellular, or when time to reach results is a priority. A range of different techniques can be combined which will involve mechanical, physical and chemical means for separating, concentrating, extracting and purifying NAs (Rudi et al., 2006). An overview of possible preparation combination strategies is shown in Fig. 7.

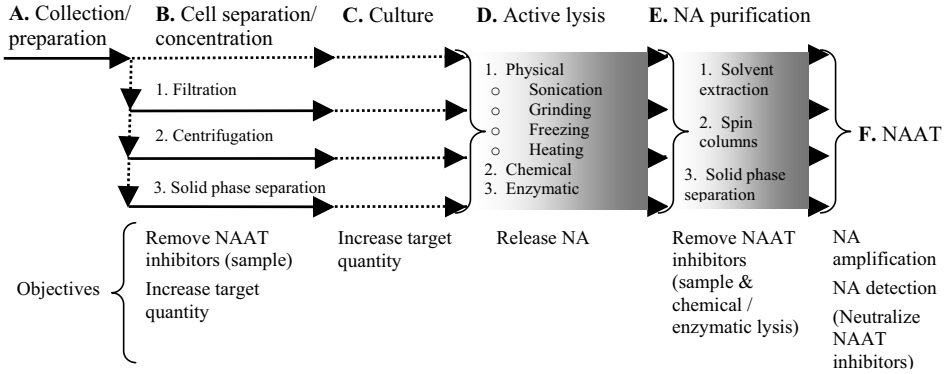


Figure 7. NA sample preparation for NAAT: strategies overview. The doted lines represent possible choices and combinations

Traditional filtration aims at separating and concentrating the target organisms from the matrice prior to starting NA extraction. The FTA filters (Flinders Technology Associates filtrers) will differ in that they have been developed to trap, lyse the target organism and

further recover the NA on the same membrane. The membrane is directly used as PCR template in 100µl final reaction volume or used as archival samples (Lampel et al., 2000). Other methods will use various centrifugation techniques for NA preparation from food samples (Fukushima et al., 2007; Stevens et al., 2004). Antibody-based separation methods are mainly used when a specific bacterial type is the target. As these methods rely on the proper expression of the targeted epitope, they will be sensitive to environmental conditions (Geng et al., 2006; Hahm et al., 2006).

Environmental samples such as fresh waters or sediments will typically be characterized by the presence of humic acids which are known PCR inhibitors. To address this problem, a method was recently developed which uses chemical precipitation to remove humic acids (Persoh et al., 2008).

Clinical samples such as faeces, urine or blood would contain bile salts or hemoglobin as examples of known inhibitors of the PCR. Another problem with such samples is that automation is often highly desirable due to large analysis volumes and the requirement for speed and reproducibility as rapid and accurate diagnosis is essential for optimal treatment of patients.

An important step is the lysis used to release NA from the target organism, which should be adapted to the type of membrane to disrupt. For example, Mycobacterial species are known to be resistant to lysis, requiring enzymatic digestion or various chemicals and mechanical disruption (Aldous et al., 2005; Herthnek et al., 2008), whereas *E. coli* will release its NA content at elevated temperatures (OUYANG et al., 2008). This is a crucial step as many of the chemicals and enzymes used for lysis can have some degree of PCR inhibiting properties. Therefore, mechanical and physical cell disruption should be chosen whenever possible and sufficient.

For NA purification (see Fig. 7), solid phase separation is the most practical for automation and it is non hazardous as opposed to using organic solvents and centrifugation.

4.2 Automated NA preparation from urine samples for detection of *Chlamydia trachomatis*

Genpoint AS, a Norwegian company founded in 1998, launched a new and innovative approach for preparation of NA from complex samples for the purpose of NAAT. As illustrated in Figure 8, the core principle which makes this method different from other beads-based NA methods is that the same beads will be used for cell-isolation and concentration followed by NA purification.

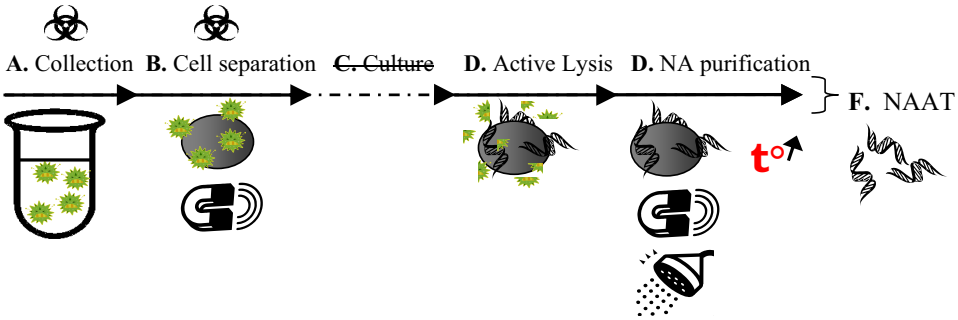


Figure 8. BUGS'n BEADS™ NA sample preparation for NAAT

The bacterial affinity of the beads is general, although variations in the coating of the beads allow targeting various broad groups. The method, called "BUGS'n BEADS™", was first applied to Cyanobacteria in waters (Rudi et al., 1998) and was later successfully applied to other bacteria and matrices (Nogva et al., 2000; Rudi K et al., 2003). Many new applications can be devised as this method is automated, has a broad bacterial target range possibility and is efficient for removing inhibitors from environmental and food matrices. Thus, a clinical application was developed for the isolation of *Chlamydia trachomatis* DNA from urine samples for NAAT diagnostic which was succesfully evaluated in a clinical trial (Paper IV). Extraction of NA from urine for NAAT STI diagnostic is essential for increasing the testing of risk populations, and for the establishing of screening programs (Cook RL et al., 2005; LaMontagne et al., 2004). Due to the non invasive nature of the sampling method and its ease of collection, comfort is increased for the patient and the presence of a medical specialist not required. The main other alternative sample type

employed, urethral swabs, requires a medical specialist and the procedure is invasive. None the less, swabs have been widely used because they require less work to process for the extraction of NA. New preparation methods must take into account that automation of the procedure is highly desirable due to the large volume of samples to be analysed and it must provide a satisfactory removal of inhibitors without affecting the sensitivity.

4.3 New, simplier and faster approach for automated NA preparation

The simplest protocols can be implemented when target concentration is not a limiting factor, and when there is no or little presence of NAAT inhibitors. As some bacteria, i.e. *E. coli* and *Shigella* spp., only require heat for releasing NA, boiling followed by centrifugation is a simple and effective way to extract NA from isolated colonies on agar (Aranda et al., 2004). Further, direct suspension of bacteria in the PCR mastermix is feasible and has been reported efficient with a variety of species including *E. coli* (Hsu et al., 2001), *Bacteroides fragilis* and *Bifidobacterium* (Matsuki et al., 2002), *Pseudomonas aeruginosa* (Song et al., 2000) and *Enteroccocus* species (Patel et al., 1997). This is a practical approach taking advantage of the high temperatures used during the NAAT processes reaching for example up to 95°C for the PCR and 72,5°C for SDA. The limitation of such an approach is the absence of cleansing so that only media or sample matrices compatible with the end NAAT can be used. In order to be able to keep the lysis mainly in the NAAT by heating, and achieve some degree of cleansing, a cell separation must be implemented. Of the various possibilities (see Fig. 7), magnetic beads are particularly attractive as they are easy to implement in an automated solution. In that context, initial bacterial trapping of bacteria provided by the BUGS'n BEADS™ is a well suited method to be combined with simple heat lysis, provided, for example, by downstream NAAT. This process was serendipitously encountered and further organized to develop a new NA method characterized by the absence of "active lysis" as described in Figure 10.

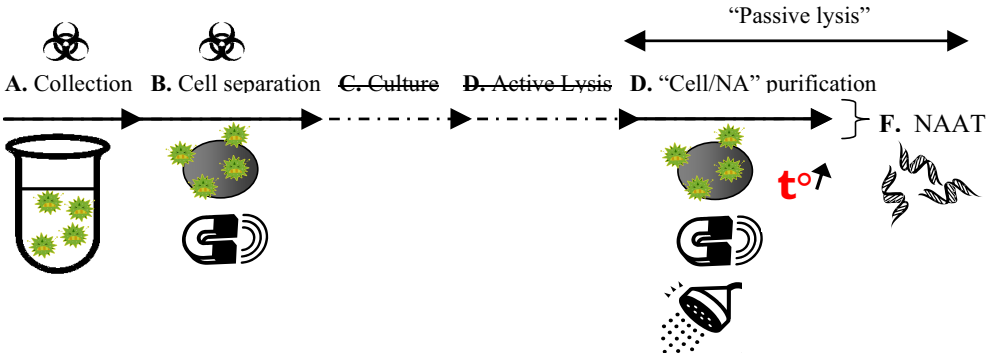
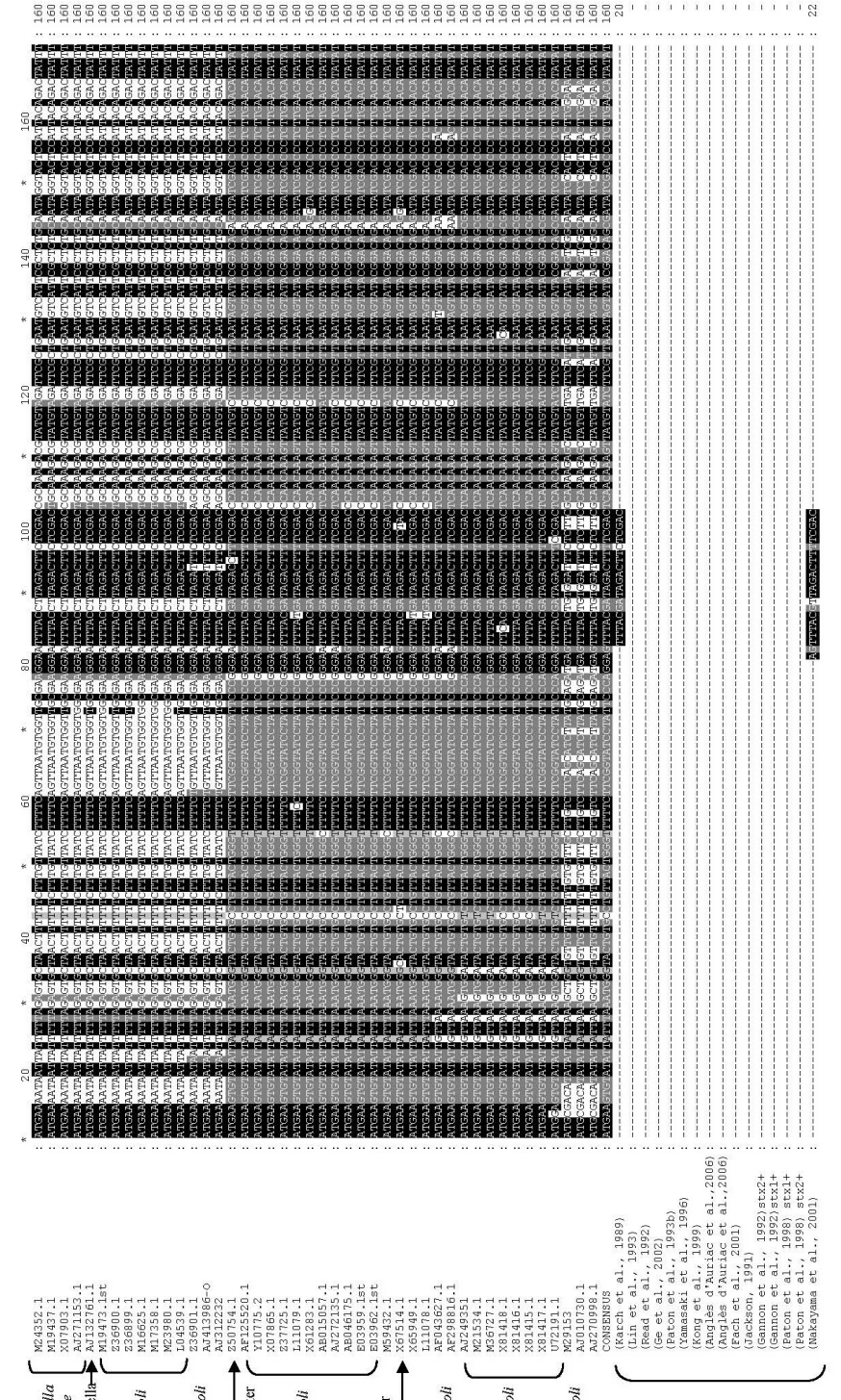


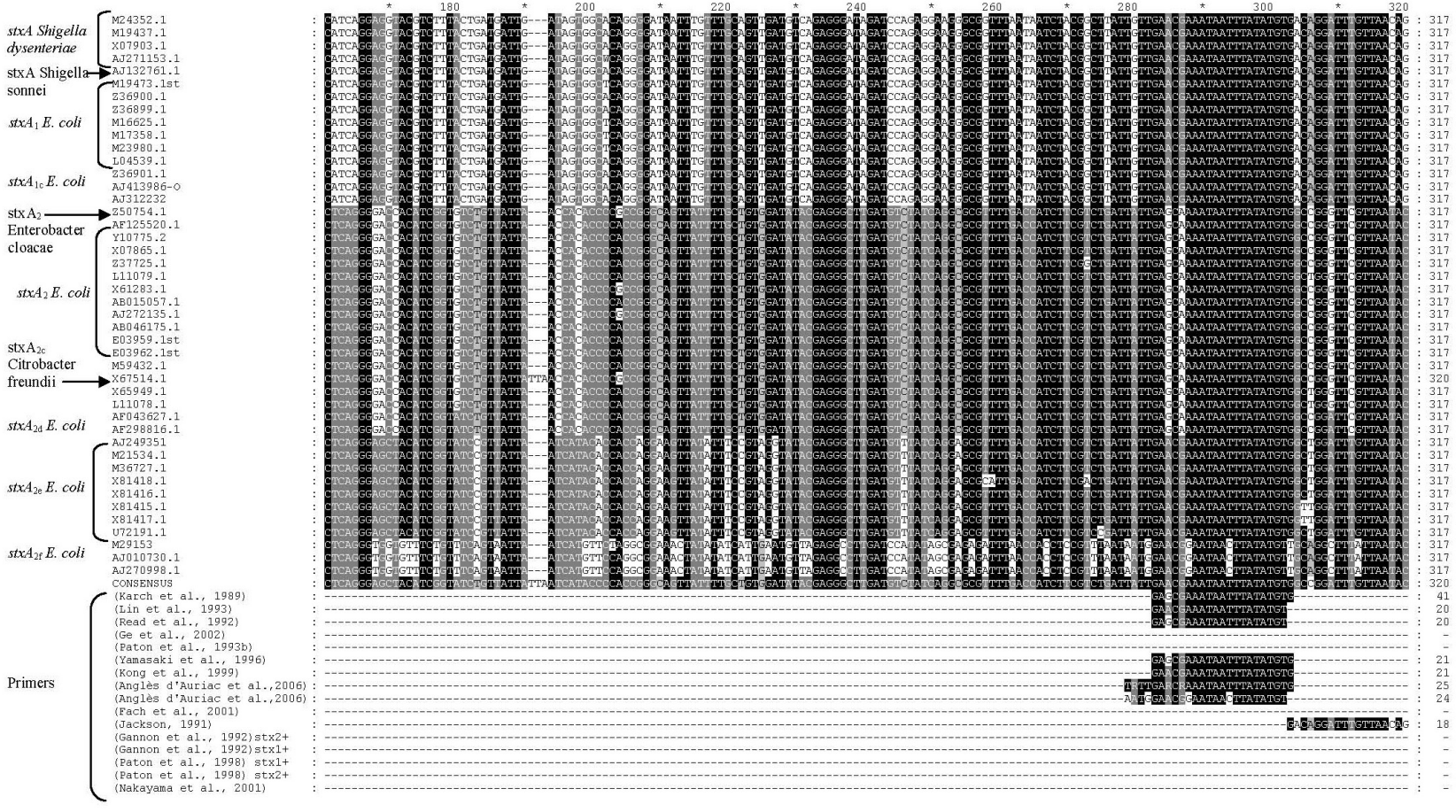
Figure 10. BUGS'n BEADS™ STI-*fast* NA sample preparation for NAAT

This new NA method was first developed for the preparation of *Chlamydia trachomatis* NA from urine samples followed by PCR analysis. It was then succesfully associated to the BDProbeTec™ SDA analysis system and further applied to the detection of other suitable microorganisms (paper V).

Appendix

A1 Alignment of *stx* genes

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stxA Shigella sonnei
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stxA1c E. coli
stxA2
Enterobacter cloacae
stxA2 E. coli
stxA2c
Citrobacter freundii
stxA2d E. coli
stxA2e E. coli
stxA2f E. coli
Primers
M24352.1
M19437.1
X07903.1
AJ271153.1
AJ132761.1
M19473.1st
Z36900.1
Z36899.1
M16625.1
M17358.1
M23980.1
L04539.1
Z36901.1
AJ413986-0
AJ312232
Z50754.1
AF125520.1
Y10775.2
X07865.1
Z37725.1
L11079.1
X61283.1
AB015057.1
AJ272135.1
AB046175.1
E03959.1st
E03962.1st
M59432.1
X67514.1
X65949.1
L11078.1
AF043627.1
AF298816.1
AJ249351
M21534.1
M36727.1
X81418.1
X81416.1
X81415.1
X81417.1
U72191.1
M29153
AJ010730.1
AJ270998.1
CONSENSUS
(Karch et al., 1989)
(Lin et al., 1993)
(Read et al., 1992)
(Ge et al., 2002)
(Paton et al., 1993b)
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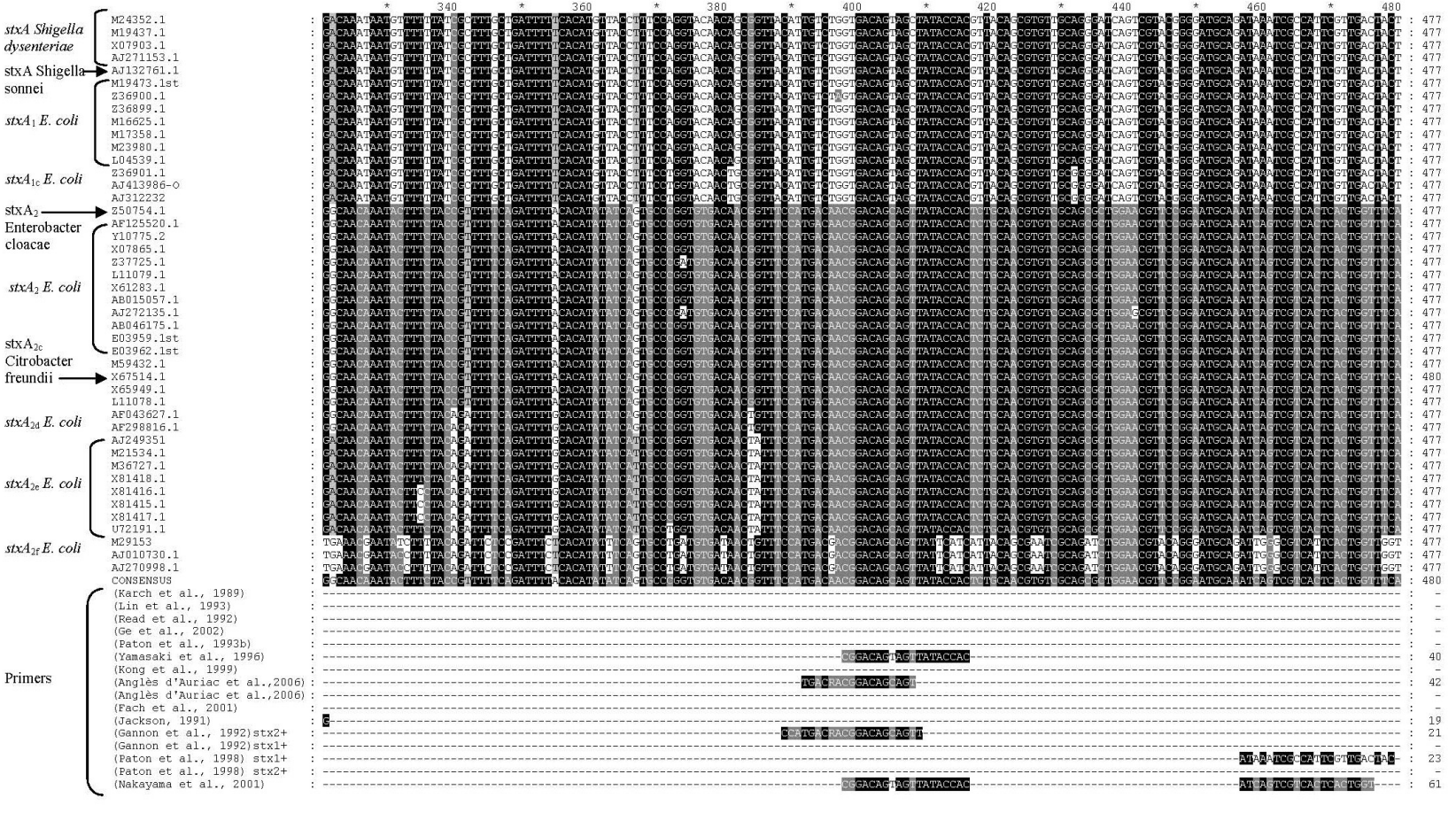
(Nakayama et al., 2001)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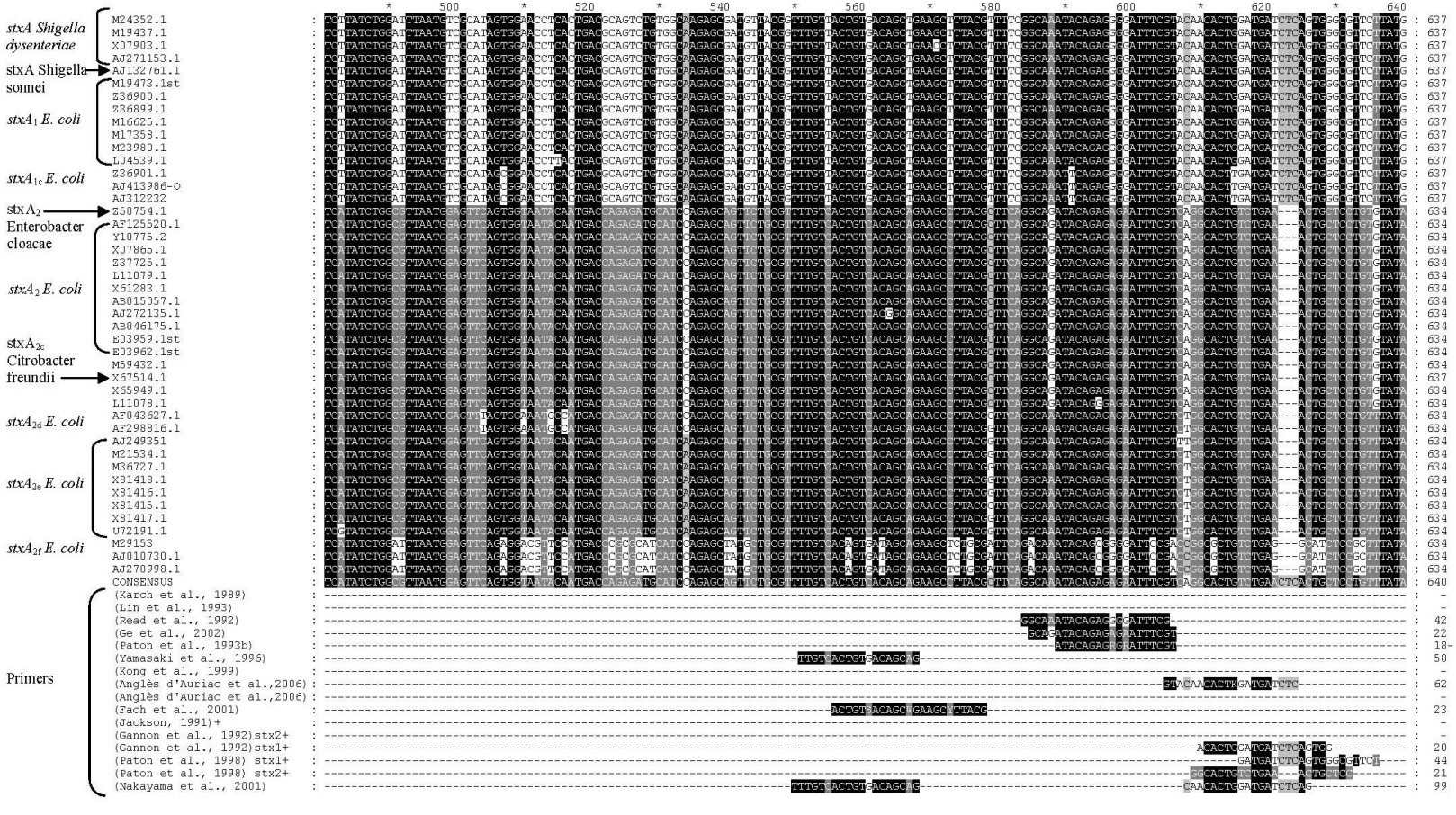
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(Paton et al., 1998) stx1+
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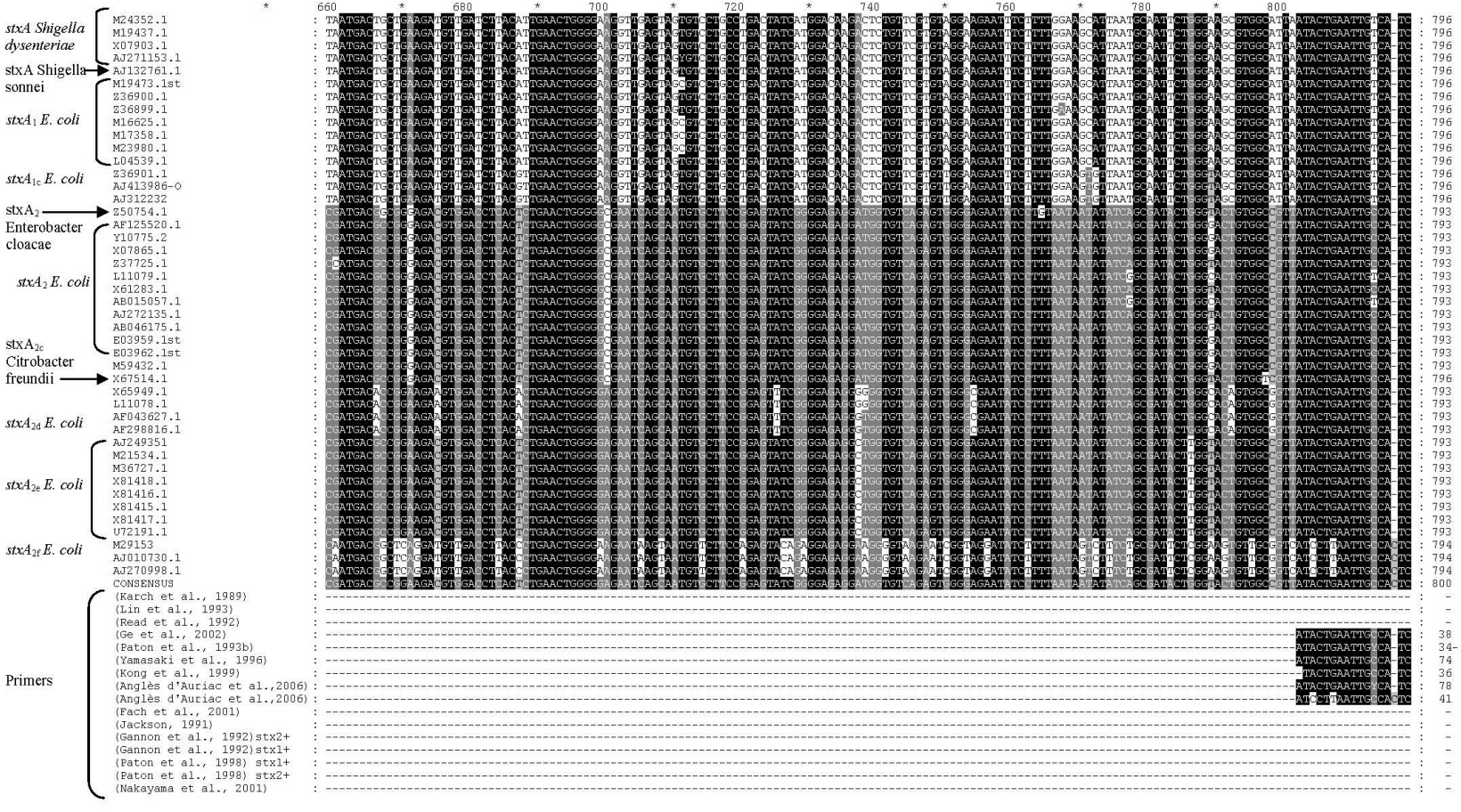
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Z36900.1
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X81417.1
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CONSENSUS
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(Lin et al., 1993)
(Read et al., 1992)
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(Gannon et al., 1992)stx2+
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(Paton et al., 1998) stx1+
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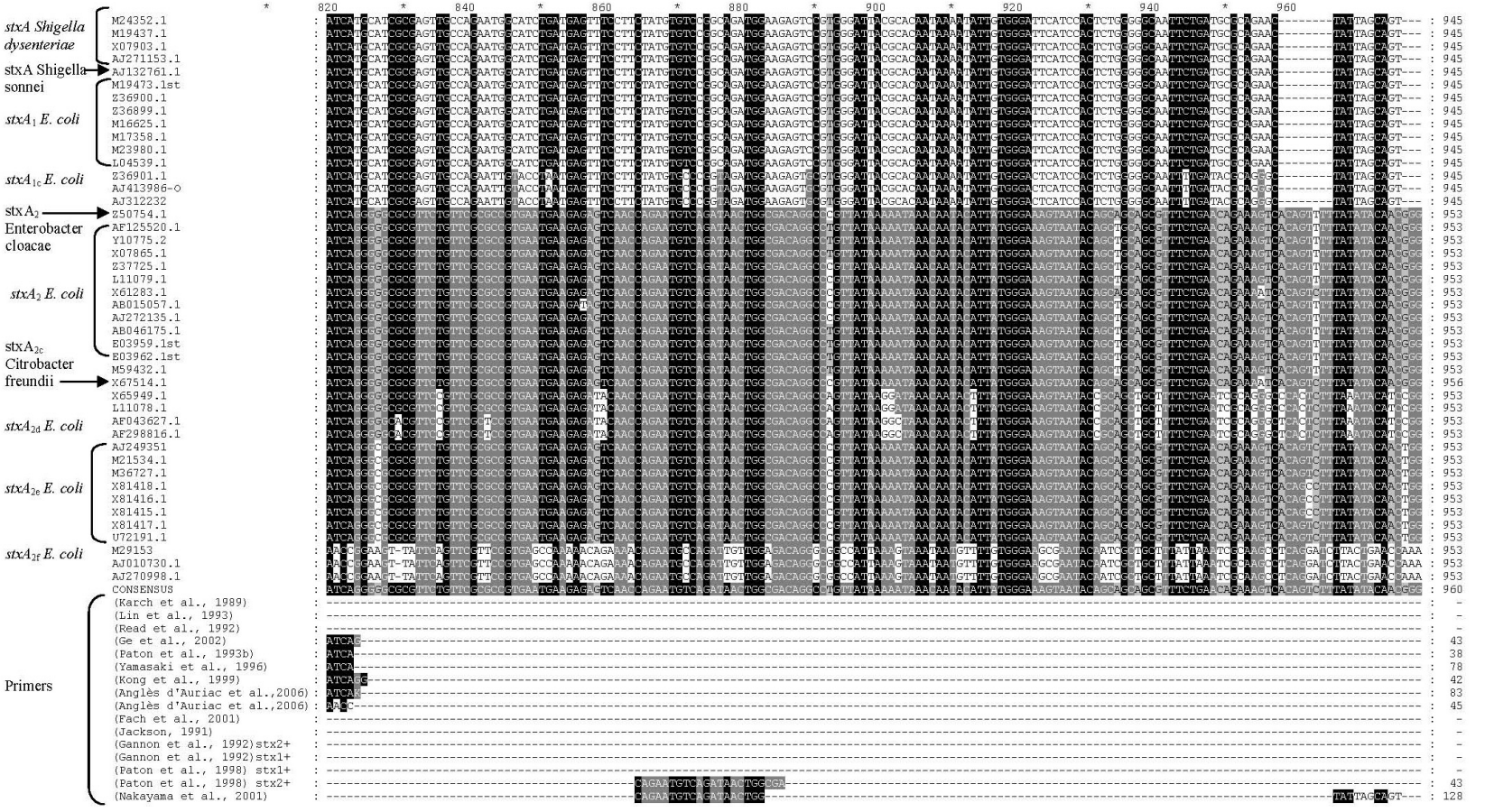
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stxA Shigella sonnei
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stxA2e E. coli
stxA2f E. coli
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(Gannon et al., 1992)stx2+
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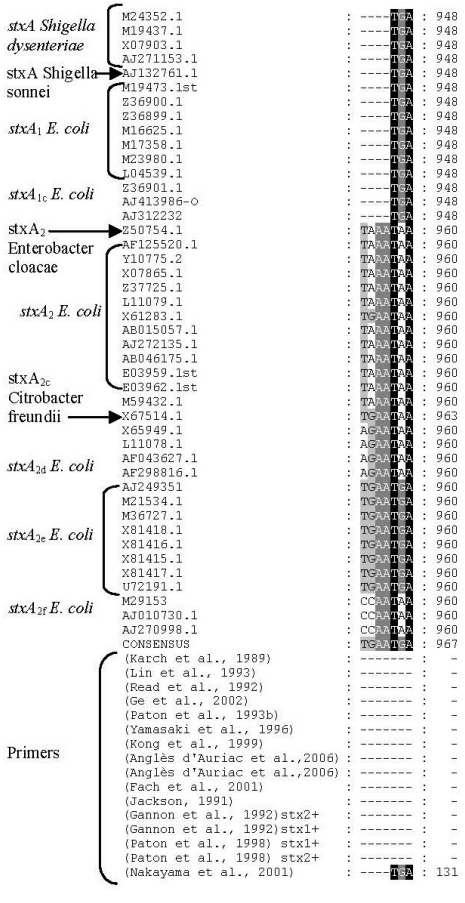
(Paton et al., 1998) stx2+
(Nakayama et al., 2001)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stxA Shigella sonnei
stxA1 E. coli
stxA1c E. coli
stxA2
Enterobacter cloacae
stxA2 E. coli
stxA2c
Citrobacter freundii
stxA2d E. coli
stxA2e E. coli
stxA2f E. coli
Primers
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(Ge et al., 2002) : ------- : -
(Paton et al., 1993b) : ------- : -
(Yamasaki et al., 1996) : ------- : -
(Kong et al., 1999) : ------- : -
(Anglès d'Auriac et al.,2006) : ------- : -
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(Gannon et al., 1992)stx2+ : ------- : -
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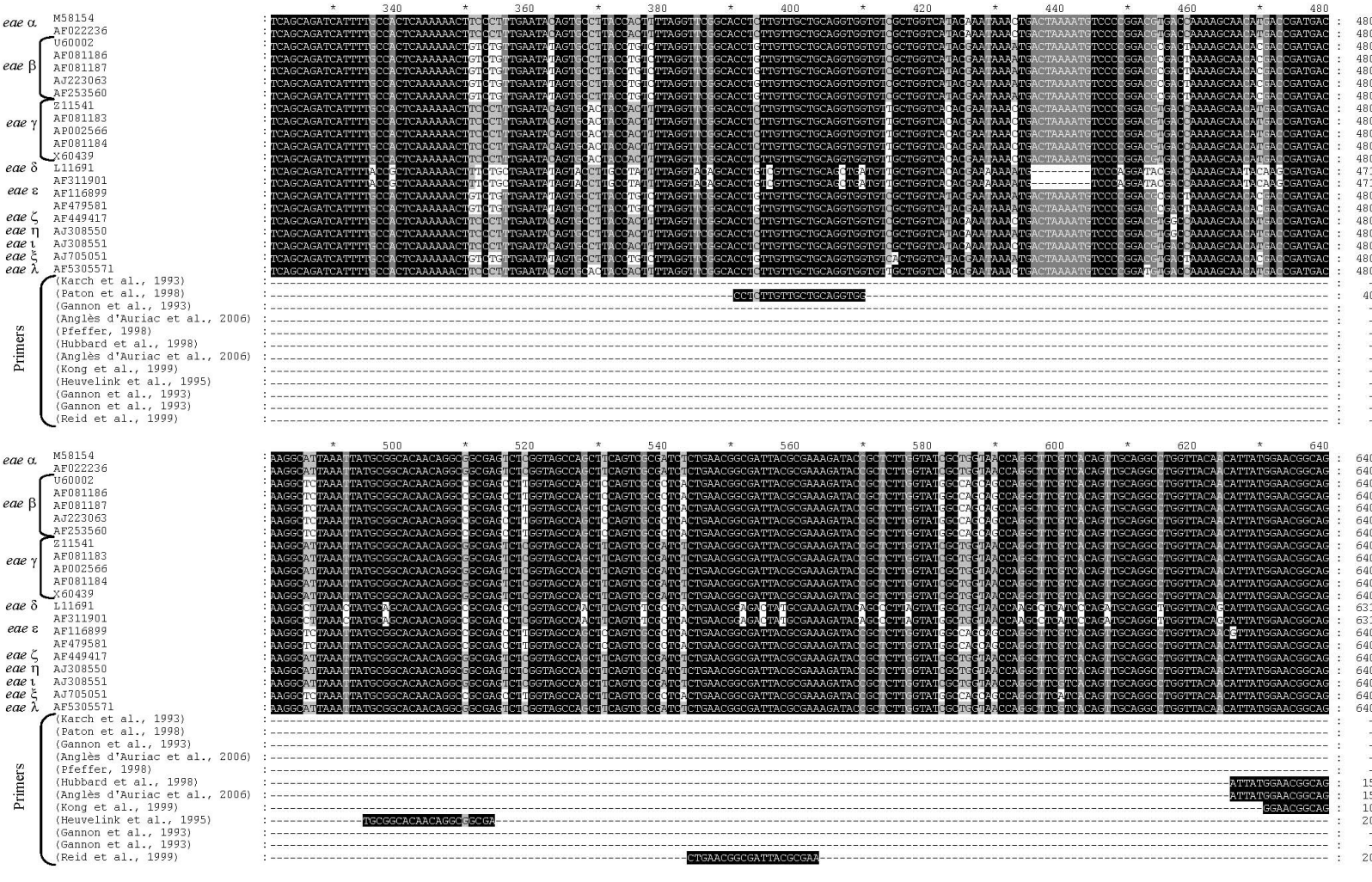
(Paton et al., 1998) stx1+ : ------- : -
(Paton et al., 1998) stx2+ : ------- : -
(Nakayama et al., 2001) : ----TGA : 131

A2 Alignment of *eae* genes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eae λ
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Z11541
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AF081184
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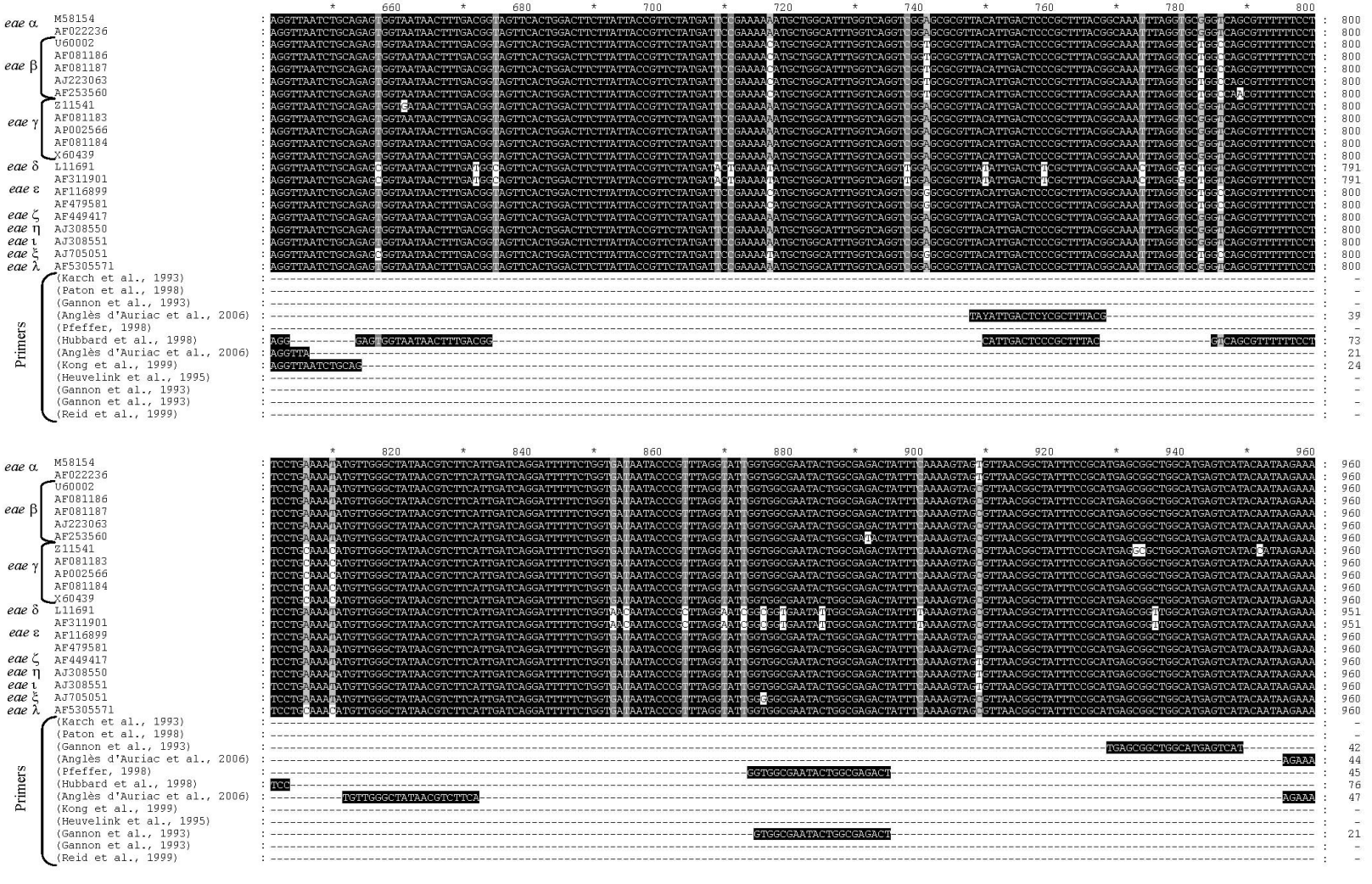
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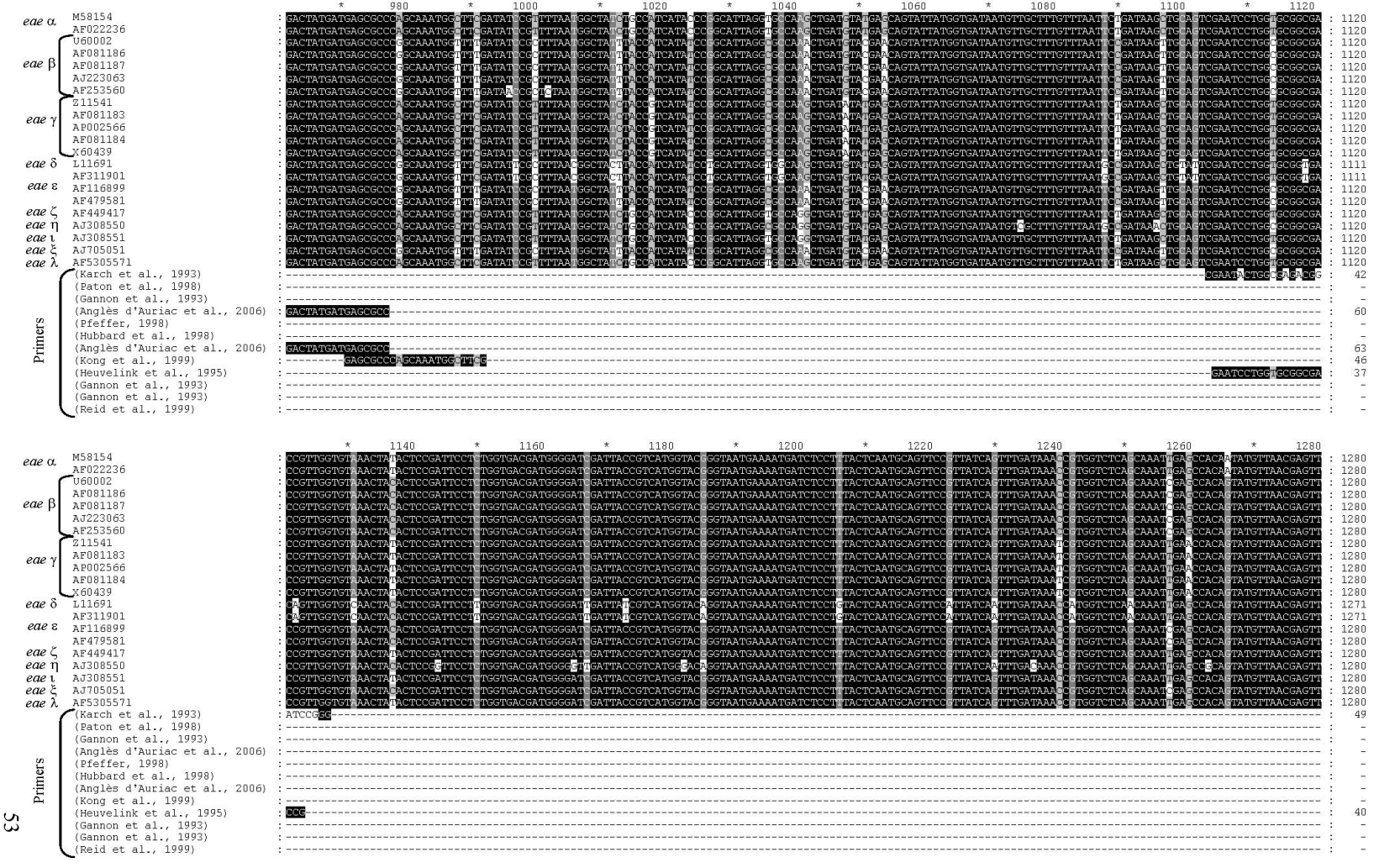
(Reid et al., 1999)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(Paton et al., 1998)
(Gannon et al., 1993)
(Anglès d'Auriac et al., 2006)
(Pfeffer, 1998)
(Hubbard et al., 1998)
(Anglès d'Auriac et al., 2006)
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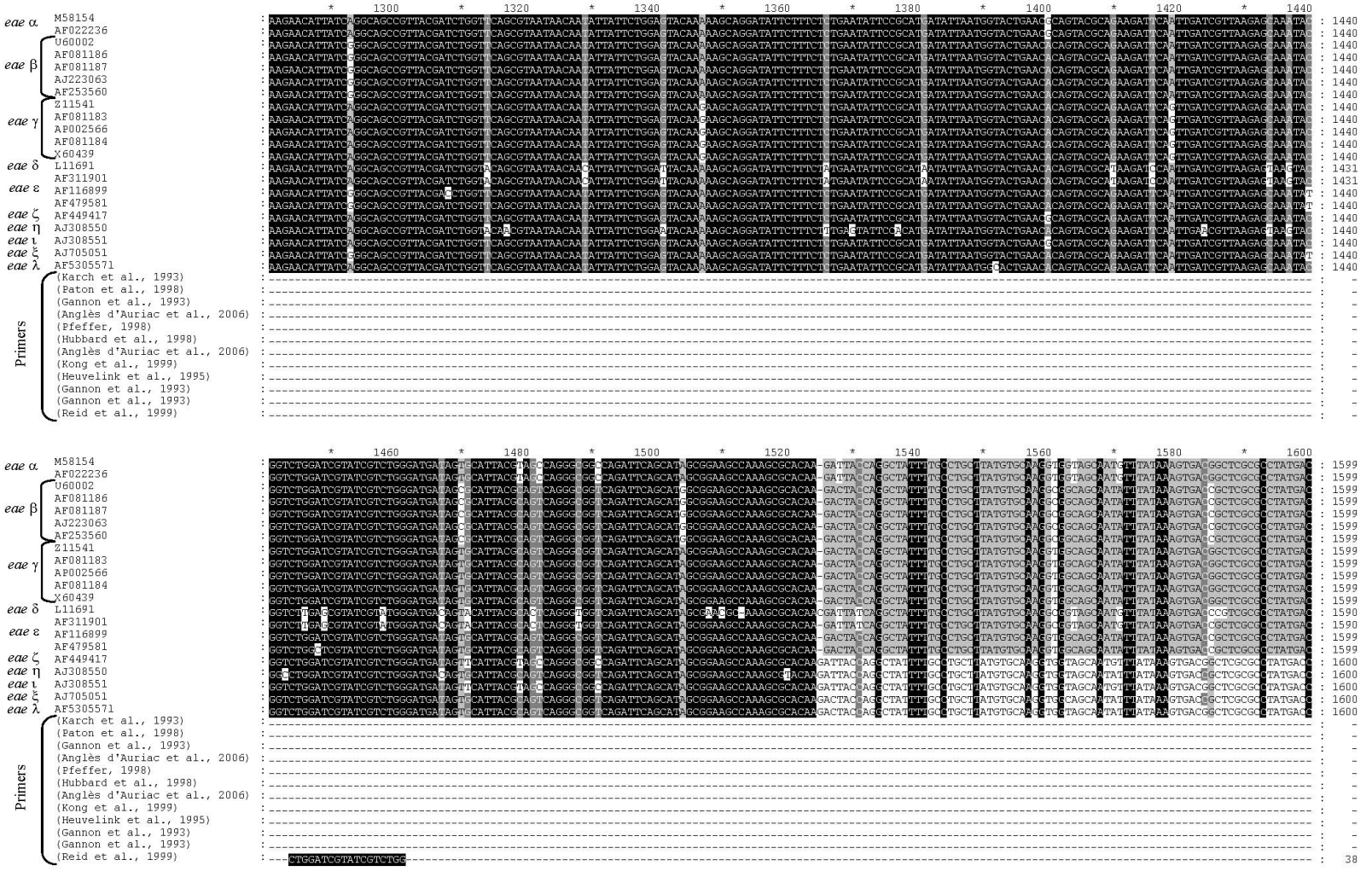
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(Paton et al., 1998)
(Gannon et al., 1993)
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(Heuvelink et al., 1995)
(Gannon et al., 1993)
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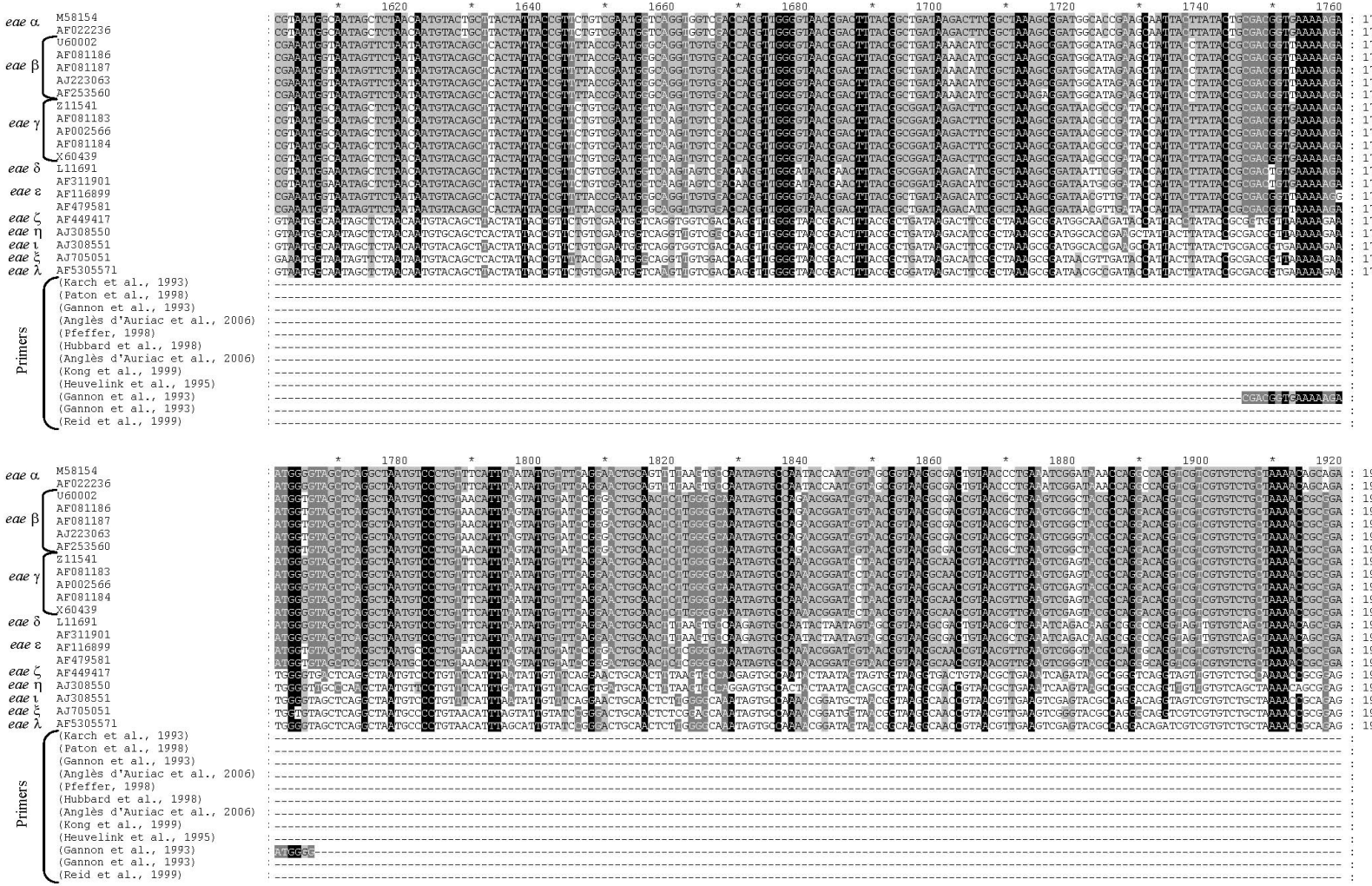
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(Karch et al., 1993)
(Paton et al., 1998)
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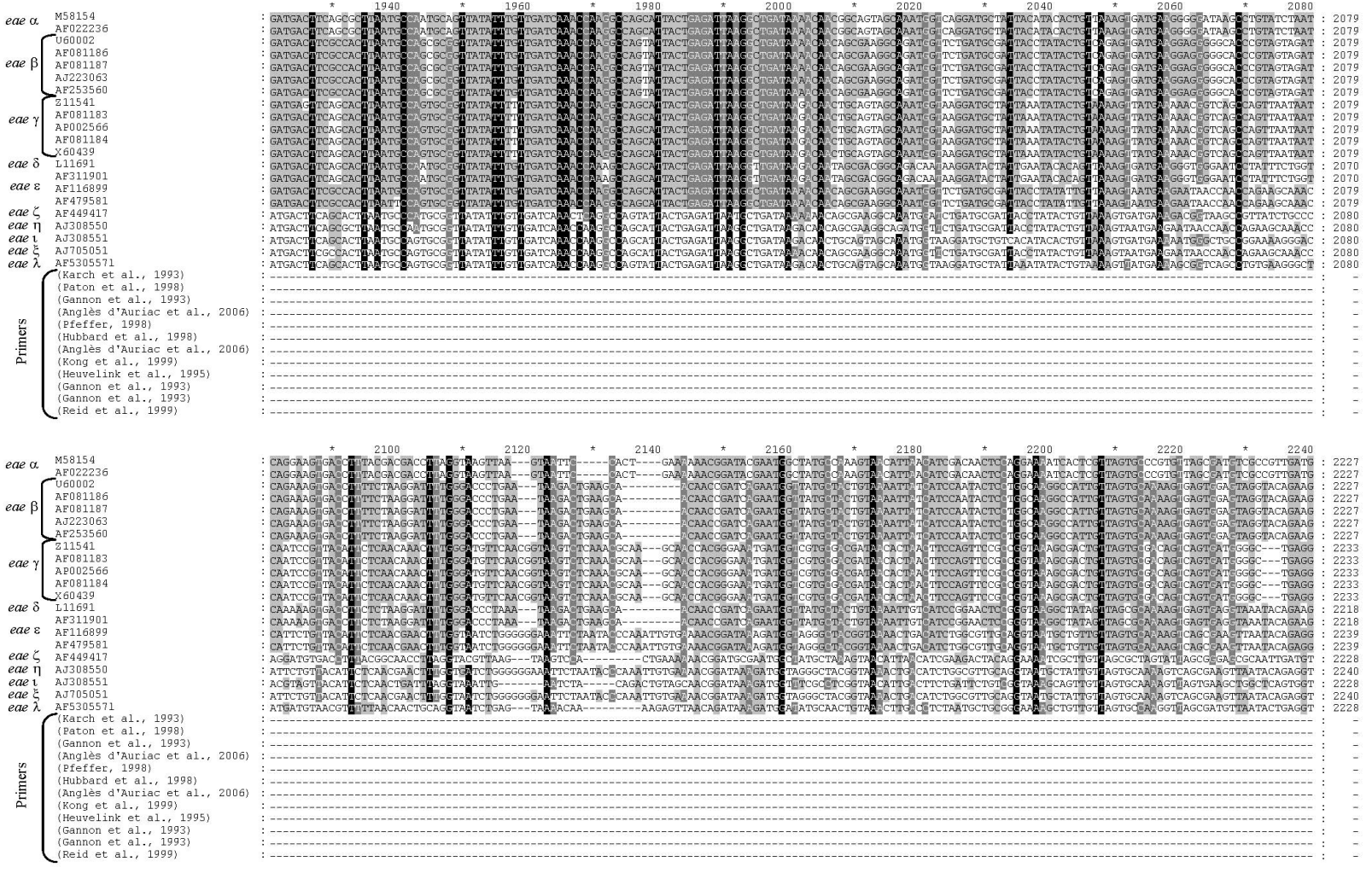
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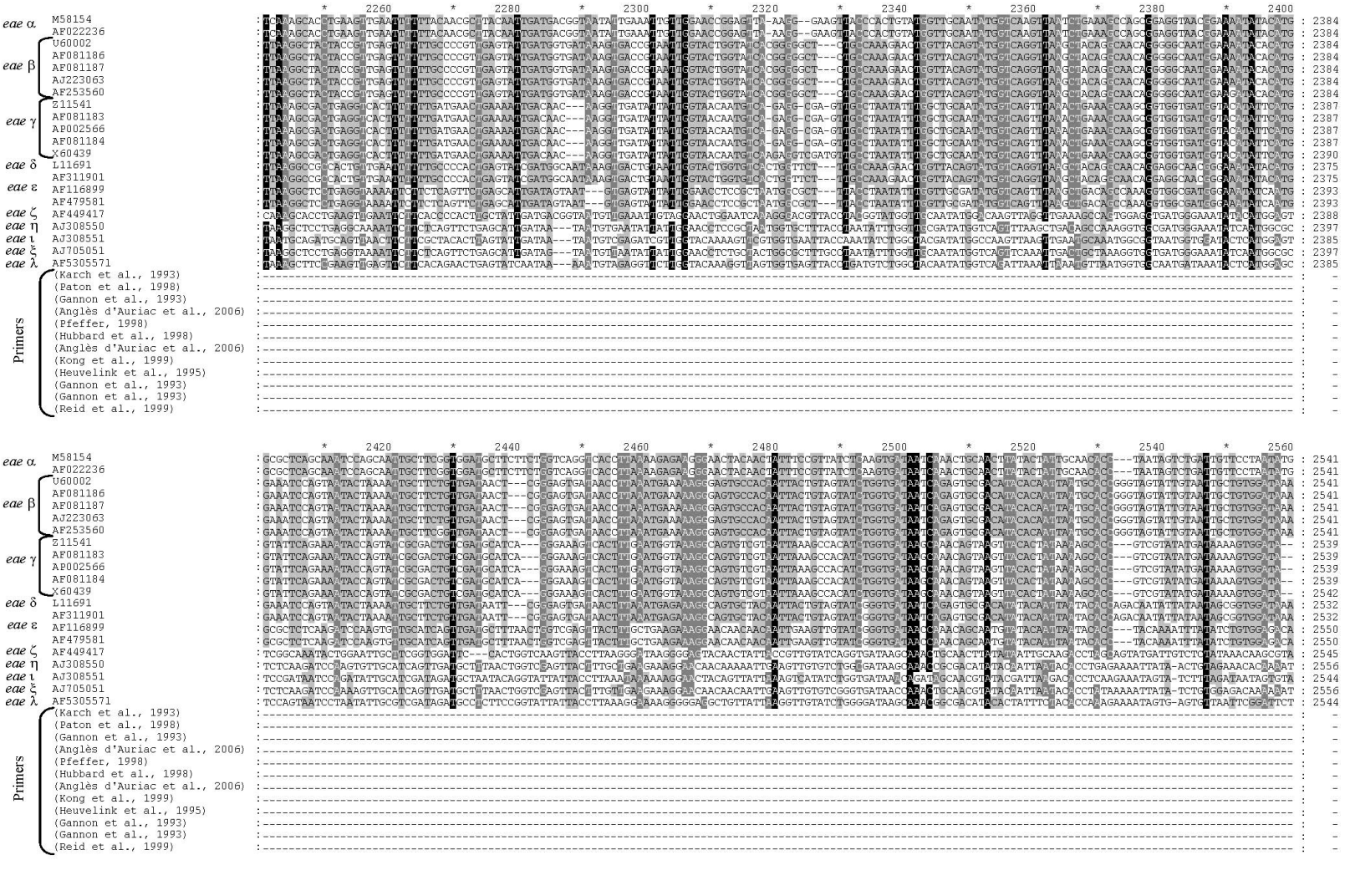
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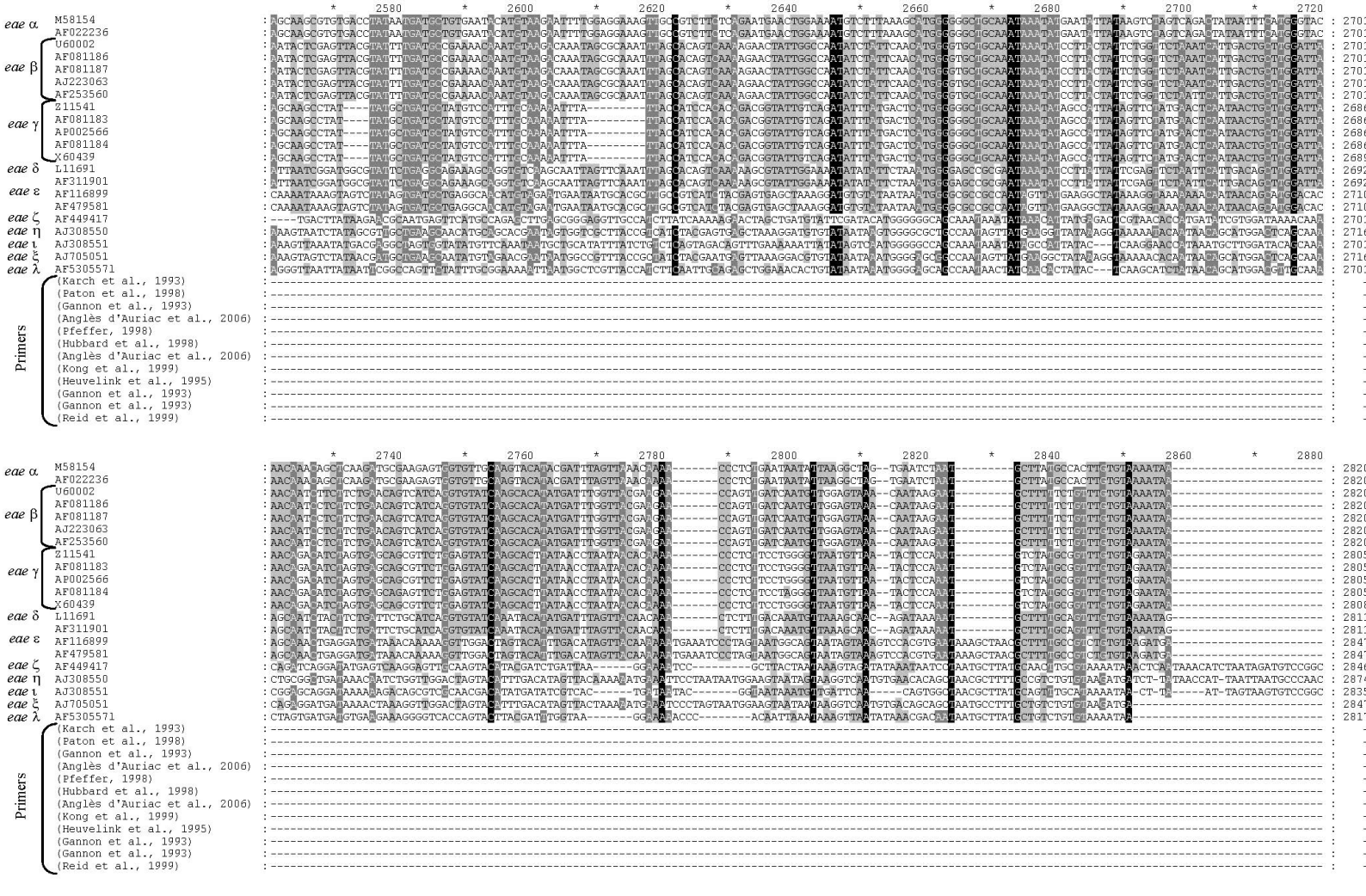
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A3 Pathogenic and indicator multiplex PCR protocol overview

References	Pathogenic markers											Serotyping				Indicator markers											
																Housekeeping genes					Specific genes						
	stx$_1$	*stx*$_2$	All *stx*	*eae*	*bfpA* EAF	*aggR CVD432*	*hlyA*	*elt AB*	*esth estp*	*inv A-E ipaH*	*ast A*	*eae*	*rfb*	*fli*	*uid A*	16-23S rDNA	*uspA*	*alr*	*mdh*	*wecF* (*rff*)	*pho A*	*Uid A/R*	*gad AB*	*lam B*	*lacZ*	*tuf*
(Bellin et al., 2001; Gannon et al., 1992; Olsvik et al., 1991; Pollard et al., 1990)	+	+																								
(Fach et al., 2001; Ge et al., 2002; Karch et al., 1989; Lin et al., 1993; Paton et al., 1993b; Read et al., 1992; Yamasaki et al., 1996)			+																							
(Batchelor et al., 1999; Karch et al., 1993; Meng et al., 1996)				+																						
(Abdulmawjood et al., 2003; Desmarchelier et al., 1998; Maurer et al., 1999; Paton et al., 1998; Paton et al., 1999b)													+													
(Aranda et al., 2004)				+	+																					
(China et al., 1996; Heuvelink et al., 1995; Meng et al., 1997; Pass et al., 2000)	+	+		+																						
(Lang et al., 1994)	+	+						+																		
(Chen et al., 2001)	+	+								+																
(Radu et al., 2001)	+	+												+												
(Cebula et al., 1995)	+	+													+											
(Call et al., 2001; Paton et al., 1998)	+	+		+			+																			
(Osek, 2002)	+	+		+									+													
(Tsen et al., 1998)	+	+						+	+																	
(Aranda et al., 2004)	+	+						+	+	+																
(Vidal et al., 2004)	+	+		+	+			+	+																	
(Fagan et al., 1999)	+	+					+					+														
(Gannon et al., 1997)	+	+										+		+												
(Campbell et al., 2001; Hu et al., 1999)	+	+		+									+	+												
(Feng et al., 2000)	+	+		+			+								+											
(Kimata et al., 2005)	+	+		+	+	+		+	+	+	+															
					+	+			+																	
(Fratamico et al., 1993)			+				+					+														
(Osek et al., 2001)			+				+						+													

References	Pathogenic markers											Serotyping				Indicator markers											
																Housekeeping genes					Specific genes						
	stx$_1$	*stx*$_2$	All *stx*	*eae*	*bfpA* EAF	*aggR CVD432*	*hlyA*	*elt AB*	*esth estp*	*inv A-E ipaH*	*ast A*	*eae*	*rfb*	*fli*	*uid A*	16-23S rDNA	*uspA*	*alr*	*mdh*	*wec*F (*rff*)	*pho A*	*Uid A/R*	*gad AB*	*lam B*	*lacZ*	*tuf*
(Nagano et al., 1998)			+										+	+													
(Fratamico et al., 1998)			+				+			+		+															
(Toma et al., 2003)			+	+		+		+	+	+																	
(Carroll et al., 2000; Greisen et al., 1994; Klausegger et al., 1999; Mittelman et al., 1997)																+											
(Chen et al., 1998a)																	+										
(Daly et al., 2002; Yokoigawa et al., 1999)																		+									
(Hsu et al., 2001)																			+								
(Bej et al., 1991a; McDaniels et al., 1996)																						+					
(McDaniels et al., 1996)																							+				
(Ke et al., 1999)																											+
(Frahm et al., 2003)																+						+					
(Bej et al., 1991b)																						+		+	+		
(Bayardelle et al., 2002)										+			+							+							
(Osek, 2001)								+	+								+										
(Grant et al., 2001)	+	+																					+				
(Heijnen et al., 2006)	+	+											+									+					
(Persson et al., 2007)	+	+		+				+	+	+						+											
(Brandal et al., 2007)	+	+		+	+			+	+	+						+											
(Wang et al., 2002)		+		+												+											
		+																									
(Wang et al., 2002)		+					+						+	+		+											
(Kong et al., 1999)			+	+				+	+												+						

stx: shiga toxin; *eae*: *E. coli* attaching and effacing gene coding for intimin, localized on the Locus of Enterocyte Effacement (LEE) and is required for producing the attaching-and-effacing (A/E) phenotype; *bfpA*: bundle-forming pilus gene encoding for the structural protein BFP located on the EAF 50 to 70-Mda virulence plasmid; EAF: EPEC adherence factor virulence plasmid, responsible for the Localised Adherence (LA) phenotype displayed by EPEC strains; aggR: transcriptional activator of AAF/I and AAF/II; CVD432: part of the virulence plasmid for EAggEC; *hlyA*: hemolysin localized on the 60Mda EHEC plasmid; *eltAB*: coding for heat labile enterotoxin (Enterotoxin LT); *esth* & *estp*: coding for heat stable enterotoxin (Enterotoxin ST); *invA-E*: genes involved with *Salmonella* invasivness; *ipaH*: gene involved with the enteroinvasive mechanism of EIEC and *Shigella* (sequences present at multiple sites on both the large invasive plasmid and the chromosome); *astA* codes for EAST1, an enterotoxin widely distributed in diarrheagenic *E. coli*; rfb: operon involved with the synthesis of the O-antigen of gram-negative bacteria (comprising the *wzx* O-antigen flippase & *wzy* O-antigen polymerase genes); *fli*: coding for flagelin responsible for the H-antigen; *uidA*, β -D-glucuronidase structural gene; *uidR*, upstream regulatory region of β -D-glucuronidase structural gene; 16-23S rDNA: genes coding for the ribosomal RNA 16S and 23S; *uspA*: universal stress protein; *alr*: alanine racemase (provides D-alanine to the peptidoglycan); *mdh*: malic acid dehydrogenase; *wec*F (rff): Involved with the synthesis of the Enterobacterial common antigen (ECA) ; *phoA* alkaline phosphatase; *gadA/B*, glutamate decarboxylase; *lamB*: gene coding for the phage lambda receptor protein, maltose high-affinity uptake system; *lacZ*: β -D-galactosidase structural gene; *tuf*, chromosomal, elongation factor EF-Tu;

A4 Bacterial PCR protocols overview

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
Acinetobacter spp.	(Vaneechoutte et al., 1995)	16S rRNA DNA consensus 5' and 3' ends	TGGCTCAGATTGAACGCTGGCGGC TACCTTGTTACGACTTCACCCCA	50	/ **1500**	PCR, Amplified Ribosomal DNA Restriction Analysis ARDRA, with *Alu*I, *Cfo*I, *Msp*I, *Mbo*I, *Hinf*I 1% agarose EtBr
Aeromonas spp. / M16495; *Shigella* spp. / M76444; *Yersinia enterocolita* / M29945; *Salmonella* spp. / U66877; *Vibrio cholerae* / L13660; *Vibrio parahaemolyticus* / AF429304	(Kong et al., 2002), *Aero*-F & *Aero*-R; *lpaH*-F & *lpaH*-R; *Ail*-F & *Ail*-R, *lpaB*-F & *lpaB*-R; *EpsM*-F & *EpsM*-R; *Vpara*-F & *Vpara*-R	aerA *Aerolysin* *lpaH* Invasion plasmid antigen H *Ail* Attachment invasion locus *lpaB* Invasion plasmid antigen B *EpsM* Enterotoxin extracellular secretion protein 16S-23S rDNA	TGTCGGSGATGACATGGAYGTG CCAGTTCCAGTCCCACCACTTCA CCTTGACCGCCTTTCCGATA CAGCCACCCTCTGAGGTACT CTATTGGTTATGCGCAAAGC TGGAAGTGGGTTGAATTGC GGACTTTTTAAAAGCGGCGG GCCTCTCCCAGAGCCGTCTGG GAATTATTGGCTCCTGTGCAGG ATCGCTTGGCGCATCACTGCCC GCTGACAAAACAACAATTTATTGTT GGAGTTTCGAGTTGATGAAC	62	/ **720** / **606** / **359** / **314** / **248** / **170**	Multiplex (6) PCR and restriction digestion of simplex products, agarose 2% EtBr 0.5µg/ml in TBE / Seawater
Aeromonas trota / AF064068	(Khan et al., 1998; Khan et al., 1999), AL1 & AL2	*aerA* aerolysin (haemolysin*)*	TTGCCGCCCAGGCCGGTGCTG ACCACTGTGTGGACCAGGGTA	69 & 61 / 66	50-71 650-671 / **622**	PCR, agarose 1.5 % EtBr
Campylobacter genus, *C. jejuni, C. coli* & Universal for Eubacteria/ Z29936, AF146727	(O'Sullivan et al., 2000) A1-B (S) B1-B (A) CAMP1F-B (S) CAMP4 CJEJ7 CCOL2	16S-23S rRNA integenic spacer region	Biotin-AGTCGTAACAAGGTAGC Biotin-CYRYTGCCAAGGCATCCACC Biotin-GTTAAGAGTCACAAGCAAGT Amination-GGTAAGCTACTAAGAGCG* Amination-GCTTAGTTGAGACTAAATCA* Amination-GACTTAGTTTAGATATTTTTAG*	57 53	/ 800-950 / 250-500	PCR / DNA probe and reverse hybridization colorimetric assay / Food, poultry
Campylobacter jejuni & *C. coli*	(Mohran et al., 1998; Oyofo et al., 1992), pg50 & pg3	*flaA* & *flaB*, flagellin genes	ATGGGATTTCGTATTAAC GAACTTGAACCGATTTG	37	/ **450**	PCR, agarose gels and DIG labeled probes / Clinical, seeded stools
Chlamidia pneumoniae & *Chlamidia psittaci*	(Tong et al., 1993), CP1 & CP2 CPC CPD	*ompA*, major outer membrane protein for *C. pneumoniae* & *C. psittaci*. Outer primers yield a product for both species but not *C. trachomatis* and inner primer only for *C. pneumoniae*.	TTACAAGCCTTGCCTGTAGG GCGATCCCAAATGTTTAAGGC TTATTAATTGATGGTACAATA ATCTACGGCAGTAGTATAGTT	65-55 / 50	61-80 373-393 / **333** 100-120 286-306 / **207**	PCR nested in 2 phases & "touchdown" Agarose 3% EtBr / Clinical, sputum
Clostridium difficile	(Lou et al., 1997), NK2 & NK3	*C. difficile* toxin A	CCCAATAGAAGATTCAATATTAAGCTT GGAAGAAAAGAACTTCTGGCTCACTCAGGT	55	/ **252**	PCR, polyacrylamide 6% gel / Stool direct
Clostridium difficile	(Lou et al., 1997), YT-17 & YT-18	*C. difficile* toxin B	CGTGGAGCTTCAATTGGAGAG GTGTAACCTACTTTCATAACACCAG	55	/ **399**	PCR, polyacrylamide 6% gel / Stool direct
E. coli	(Chen et al., 1998a; Osek, 2001) Ec1 & Ec2	*uspA*: universal stress protein, specific primers for *E. coli*	CCGATACGCTGCCAATCAGT ACGCAGACCGTAGGCCAGAT	70	4-23 868-887 / **884**	PCR, agarose 1% in 1X TBE EtBr
E. coli	(Hsu et al., 2001), Emdh1 & Emdh2	*mdh* malic acid dehydrogenase	ACTGAAAGGCAAACAGCCAAG CGTTCTGTTCAAATGGCCTCAGG	60	1123-1144 1514-1492 / **392**	PCR, agarose, EtBr / Milk & water
E. coli	(Lin et al., 1999), 16E1 & EV6	16S rRNA gene sequences in the V3 and V6 regions	GGGAGTAAAGTTAATACCTTTGCTC* TCATCTCTGAAAACTTCCG*			*Shigella* F+ no *E. coli* F-, 8h preculture / Tap water & milk
E. coli	(Pollard et al., 1990), (Jerse et al., 1990b)	*eae* on constructed plasmid Pjffeceae / ECEAE-L & ECEAE-R	GGCGAATTCCGCATGAGCGGCTG ATTGAATTCATAGGCGCGAGCCGTCAC		2565-2588 3214-3241 / **680**	PCR, probe
E. coli / / Hoffmann-La Roche Inc US5298392 Claim 8	(Bej et al., 1990), BL-4899 & BR-5452	*lamB*: Phage lambda receptor protein; maltose high-affinity uptake system	GGATATTTCTGGTCCTGGTGCCGC ACTTGGTGCCGTTGTCGTTATCCC	60	4899-4922 5429-5452 / **554**	PCR, agarose 1% EtBr or polyacrylamide 5%
E. coli / / Hoffmann-La Roche Inc US5298392 Claim 9	(Bej et al., 1990; Olsen et al., 1995), BL-4910 & BR-5219	*lamB*: Phage lambda receptor protein; maltose high-affinity uptake system	CTGATCGAATGGCTGCCAGGCTCC CAACCAGACGATAGTTATCACGCA	50	4910-4933 5195-5219 / **309**	PCR, agarose 1% EtBr or polyacrylamide 5%
E. coli EHEC & EPEC	(Batchelor et al., 1999) Int-Fc & Int-Rc	*eae universal*	CCGGAATTCGGGATCGATTACCGTCAT CCCAAGCTTTTATTTATCAGCCTTAATCTC	45	/ **840**	PCR, agarose
E. coli EHEC & EPEC & O157:H7	(Meng et al., 1996) SZ-I / SZ-II (Oberst et al., 1998), SZI-97 (O157:H7)	eae *up-stream of the conserved 5' area used for the construction*	CCATAATCATTTTATTTAGAGGGA GAGAAATAAATTATATTAATAGATCGGA TTGCTGCAGGATGGGCAACTCTTGAp*	62	/ **633**	PCR, agarose 1.2% or probe (Taqman)
E. coli EHEC & EPEC & O157:H7	(Meng et al., 1997) SZ-F & SZ-R SLTI-F & R SLTII-F & R	*eae* up-stream of the conserved 5' area used for the construction *stx*$_1$ *stx*$_2$	CCATAATCATTTTATTTAGAGGGA GAGAAATAAATTATATTAATAGATCGGA TGTAACTGGAAAGGTGGAGTATACA GCTATTCTGAGTCAACGAAAAATAAC GTTTTTCTTCGGTATCCTATTCC GATGCATCTCTGGTCATTGTATTAC		/ **633** / **210** / **484**	Multiplex (3) PCR, agarose
E. coli & coliforms / / Hoffmann-La Roche Inc. US5298392, Claim 6	(Bej et al., 1990) (Olsen et al., 1995), ZL-1675 & ZR-2025	*lacZ*: β -D-galactosidase structural gene	ATGAAAGCTGGCTACAGGAAGGCC GGTTTATGCAGCAACGAGACGTCA	50	1675-1698 2001-2025 / **326**	PCR, agarose 1% EtBr or polyacrylamide 5%
E. coli & coliforms / / Hoffmann-La Roche Inc	(Bej et al., 1990), ZL-1675 &	*lacZ*: β -D-galactosidase structural gene	ATGAAAGCTGGCTACAGGAAGGCC CACCATGCCGTGGGTTTCAATATT	50	1675-1698 2525-2548 / **876**	PCR, agarose 1% EtBr or

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US5298392, Claim 5	ZR-2548					polyacrylamide 5%
E. coli & coliforms / / Hoffmann-La Roche Inc US5298392	(Bej et al., 1990; Bej et al., 1991a; Bej et al., 1991b; Fricker et al., 1994; Gonzalez et al., 1999; Olsen et al., 1995), URL301&URR432 LBL445&LBR790	*uidR*: upstream regulatory region of -D-glucuronidase structural gene	TGTTACGTCCTGTAGAAAGCCC AAAACTGCCTGGCACAGCAATT	60	301-322 432-453 / **154**	Multiplex (3) PCR, agarose 0.8-1.5 %
		lamB: Phage lambda receptor protein; maltose high-affinity uptake system	CTGATCGAATGGCTGCCAGGCTCC CAACCAGACGATAGTTATCACGCA		4910-4933 5195-5219 / **309-346?**	
	LZL389&LZR653	*lacZ*: β-D-galactosidase structural gene	ATGAAAGCTGGCTACAGGAAGGCC GGTTTATGCAGCAACGAGACGTCA		1675-1698 2001-2025 / **264-326?**	
E. coli & *Shigella*	(Yokoigawa et al., 1999) (Daly et al., 2002)	*alr* alanine racemase (provides D-alanine to the peptidoglycan)	Biotin-CTGGAAGAGGCTAGCCTGGACGAG AAAATCGGCACCGGTGGAGCGATC *ATATTCACCGGCTGACGAAC	72	322-345 664-687 / **366**	PCR-ELISA agarose 1%, EtBr / Milk
E. coli diarrheagenic EHEC, EPEC, ETEC, EIEC EAEC	(Brandal et al., 2007); stx_1, (Brian et al., 1992)	*stx1*	AAATCGCCATTCGTTGACTACTTCT TGCCATTCTGGCAACTCGCGATGCA		/ 370	Multiplex (8), fluorescence-based capillary electrophoresis
	stx_2, (Brian et al., 1992)	*stx2*	CAGTCGTCACTCACTGGTTTCATCA GGATATTCTCCCCACTCTGACACC		/ 283	
	eaeA, (Vidal et al., 2004)	*eaeA*	TCAATGCAGTTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG		/ 482	
	(Lopez-Saucedo et al., 2003)	*ST1b*	ATTTTTCTTTCTGTATTGTCTT CACCCGGTACAAGCAGGATT		/ 190	
	(Rappelli et al., 2001)	*LT1*	TCTCTATGTGCATACCGAGC CCATACTGATTGCCGCAAT		/ 322	
	(Toma et al., 2003)	*ipaH*	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGCGAGTAC		/ 619	
	(Toma et al., 2003)	*aggR*	GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC		/ 254	
	(Wang et al., 2002)	*rrs (16SrRNA)*	CCCCCTGGACGAAGACTGAC ACCGCTGGCAACAAAGGATA		/ 401	
E. coli diarrheagenic EHEC, EPEC, ETEC, EIEC EAEC	(Kimata et al., 2005) (Schmidt et al., 1995b) (Gannon et al., 1993)	CVD432 (EAF plasmid) *eaeA*	CTGGCGAAAGACTGTATCAT TAATGTATAGAAATCCGCTCTT CCCGGCACAAGCATAAGCTAA		/ 690	Multiplex (12)
	AE11, EAEk4		TCGCCCTTCAGAGTCGC		/ 526	
	AGGr-3/ AGGrKS-1	*aggR*	CATCTCTTTGATAAGTCCTTCTCG GTATACACAAAAGAAGGAAGG		/ 434	
	EVS-1	*stx2*	Non disclosed (TakaRa Bio, Inc)			
	EVS-2		Non disclosed (TakaRa Bio, Inc)		/ 404	
	EVT-1	*stx1*	Non disclosed (TakaRa Bio, Inc)			
	EVT-2		Non disclosed (TakaRa Bio, Inc)		/ 349	
	INV-1	*invE*	Non disclosed (TakaRa Bio, Inc)			
	INV-2		Non disclosed (TakaRa Bio, Inc)		/ 293	
	ELT-1	*elt*	Non disclosed (TakaRa Bio, Inc)			
	ELT-2		Non disclosed (TakaRa Bio, Inc)		/ 263	
	BFP-3	*bfpA*	GGTCTGTATATTGGGCAGACC GCCGCTTTATCCAACCTGGTA		/ 209	
	BFP-2					
	STH-1	*esth*	TCACCTTTCCCTCAGGATGC ATATTATTAATAGCACCCGG		/ 179	
	STH-2					
	EAF-4	*EAF*	ATTTTAATGAGTATCGAATC TATGGGGACCATGTATTATCA		/ 153	
	EAF-25					
	ESP-1	*Estp*	Non disclosed (TakaRa Bio, Inc)			
	ESP-2		Non disclosed (TakaRa Bio, Inc)		/ 123	
	EAST-1S/EAST-1AS	*astA*	GCCATCAACACAGTATATCC GAGTGACGCCTTTGTAGTC		/ 106	
E. coli diarrheagenic (VTEC, EPEC, ETEC & EIEC)	(Persson et al., 2007), StFh & StRh	Human estA	TTTCGCTCAGGATGCTAAACCAG CAGGATTACAACACAATTCACAGCAGTA		151	Multiplex (7)
	StFp & StRp	Porcine estA	CTTTCCCCTCTTTTAGTCAGTCAACTG CAGGATTACAACAAAGTTCACAGCAG		160	
	PS3 & PS4	vtx1	GTTTGCAGTTGATGTCAGAGGGA CAACGAATGGCGATTTATCTGC		260	
	PS5 & PS6	eae Intimin	GGYCAGCGTTTTTTCCTTCCTG TCGTCACCARAGGAATCGGAG		377	
	PS7 & PS8	vtx2	GCCTGTCGCCAGTTATCTGACA GGAATGCAAATCAGTCGTCACTC		420	
	PS9 & PS10	eltA coding for LT1	AAACCGGCTTTGTCAGATATGATGA TGTGCTCAGATTCTGGGTCTCCT		479	
	PS11 & PS12	ipaH IpaH	TTGACCGCCTTTCCGATACC ATCCGCATCACCGCTCAGAC		647	
	PS13 & PS14	16S rDNA	GGAGGCAGCAGTGGGGAATA TGACGGGCGGTGTGTACAAG		1062	
E. coli diarrheagenic (EHEC, EPEC & ETEC)	(Vidal et al., 2004)	*eaeA*	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG		/ 482	Multiplex (6)
		bfp	GGA AGT CAA ATT CAT GGG GGT AT GGA ATC AGA CGC AGA CTG GTA GT		/ 254	
	((Cebula et al., 1995))	$stxA_1$	CAG TTA ATG TGG TGG CGA AGG CAC CAG ACA ATG TAA CCG CTG		/ 348	
	((Cebula et al., 1995))	$stxA_2$	ATC CTA TTC CCG GGA GTT TAC G GCG TCA TCG TAT ACA CAG GAG C		/ 584	
		lt	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT		/ 218	
		stII	AAA GGA GAG CTT CGT CAC ATT TT AAT GTC CGT CTT GCG TTA GGA C		/ 129	
E. coli EHEC	(Furst et al., 2000), JS1 & JS2	$stxB_2$ and variants	CATGAAGAAGATGTTTATGGCG CTCAGTCATTATTAAACTGCAC			
E. coli EHEC	(Heuvelink et al., 1995)	$stxA_1$-$stxB_1$	GGCAGATGGAAGAGTCCGTGGGATTACGC		1011-1039	Multiplex (3) Agarose 1.5% EtBr 0.1 μg/ml
	(Yu et al., 1992), VT1-1 & -2, VT2-1 & -2	$stxA_2$	CACAATCAGGCGTCGCCAGCGCACTTGCT CCACATCGGTGTCTGTTATTAACCACACC	59	1161-1189 / **179** 408-436	
			GCAGAACTGCTCTGGATGCATCTCTGGTC		751-779 / **372**	
	Eae-1 & -2	eae *conserved 5' area used for the construction*	TGCGGCACAACAGGCGGCGA CGGTCGCCGCACCAGGATTC		2078-2097 2687-2706 / **629**	

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E. coli EHEC	(Russmann et al., 1995) (Beutin et al., 1999; Furst et al., 2000; Schmidt et al., 1999), (Morabito et al., 2001), KS7 & KS8 GK3 & GK4	*stxB*$_1$	CCCGGATCCATGAAAAAAACATTATTAATA GC	52	/ **285**	PCR Simplex
		stxB$_2$	CCCGAATTCAGCTATTCTGAGTCAACG			
			CCCGGATCCATGAAGAAGATGTTTATGGCG		/ **270**	
			CCCGAATTCTCAGTCATTATTAAACTGCAC			
E. coli EHEC	(Schmidt et al., 1995a; Schmidt et al., 1999) (Furst et al., 2000), hlyA1 & hlyA4	*hlyA* 60-Mda plasmid (enterohemolysin)	GGTGCAGCAGAAAAAGTTGTAG			PCR
			TCTCGCCTGATAGTGTTTGGTA	57	/ **1551**	
E. coli EHEC & EPEC	(Aranda et al., 2004; McGraw et al., 1999; Reid et al., 1999) *eae* P1; *eae* P2; Eco*eae*α; Eco*eae*β; Eco*eae*γ	*eae* intimin gene, universal	CTGAACGGCGATTACGCGAA			Multiplex (4) and EtBr gels / Clinical diagnostic on regrown cells
		α EPEC I	CCAGACGATACGATCCAG		544-563	
		β EPEC II + EHEC II (not O111)	CTGGAGTTGTCGATGTT	53	1444-1461 / **917**	
		γ EHEC I	GTAATTGTGGCACTCC		2176-2192 / **1648**	
		δ EPEC O86 *& Citrobacter rodentium*	GCCTCTGACATTGTTAC		2455-2470 / **1926**	
					2298-2314 / **1770**	
E. coli EHEC & EPEC / M58154	(Karch et al., 1993) , (Furst et al., 2000; Schmidt et al., 1994; Schmidt et al., 1999) (Oswald et al., 2000; Schmidt et al., 2000; Toma et al., 2003), SK1 & SK2	*eae* conserved 5′ area used for the construction, universal intimin	CCCGAATTCGGCACAAGCATAAGC	52	26-46	PCR
			CCCGGATCCGTCTCGCCAGTATTCG		879-903 / **863-881**	
E. coli EHEC & EPEC, Citrobaccter rodentium / M58154, Z11541, L11681	(Hubbard et al., 1998), P1 & P2; P3 & P4	*eae* conserved 5′ area used for the construction, universal	ATTATGGAACGGCAGAGG		694-711	PCR, nested primers / Clinical
			GGAAGGAAAAAACGCTGAC	55	852-873 / **179**	
			GAGTGGTAATAACTTTGACGG		702-722	
			GTAAAGCGGGAGTCAATG	50	818-835 / **133**	
E. coli EHEC & O157:H7 & H7	(Bischoff et al., 2005; Gannon et al., 1997) VT1-F & -R, VT2-F & -R (Bischoff et al., 2005; Gannon et al., 1992) EAE-F & -R = AE13 & 14, EAE157-F & -R= AE19 & 20, FLIC-F & -R FLICH7-F & -R,	*stxA*$_1$-*stxB*$_1$	CATTGTCTGGTGACAGTAGCT		639-659	Multiplex (4) with VT1-F & -R + VT2-F & -R + EAE157-F & -R + FLICH7-F & -R
			CCCGTAATTTGCGCACTGAG		1371-1351 / **732**	
		stxA$_2$-*stxB*$_2$ all variants (silent mismatch)	CCATGACAACGGACAGCAGTT	65	624-644	
			CCTGTCAACTGAGCACTTTG		1403-1384 / **779**	
		eae conserved 5′ area used for the construction	GTGGCGAATACTGGCGAGACT		943-963	
			CCCCATTCTTTTTCACCGTCG		1833-1813 / **890**	
		*eae*A variable 3′ (O157:H7 & O55 specific, γ type)	CAGGTCGTCGTGTCTGCTAAA		1959-1970	
		fliC	TCAGCGTGGTTGGATCAACCT		3047-3027/**1087**	
			CCGAATTCATGGCACAAGTCATTAATAC		1-20	
		fliC$_{h7}$	CCGAATTCTTAACCCTGCAGTAGACACA		1736-1755/**1771**	
			GCGCTGTCGAGTTCTATCGAGC		69-91	
			CAACGGTGACTTTATCGCCATTCC		671-694 / **625**	
E. coli EHEC & O157H7	(Call et al., 2001), SZI-97 (*eae*A O157:H7 specific probe) (Oberst et al., 1998),	*stx*$_1$	Biot-TCTTATCTGGATTTAATGTCGC*			Multiplex (4), microaaray detection / Food
			TCAGCTGTCACAGTAACAAACC		/ **89**	
			AACATCGCTCTTGCCACAGACTGCGTCAGT*			
		stx$_2$	Biot-TTATACCACTCTGCAACGTGTC*			
			AACTCCATTAACGCCAGATA	58	/ **96**	
		*eae*A	CCAGTGAGTGACGACTGATTTGCATTCCGG *			
			Biot-CAATTTTTCAGGGAATAACATTG*		/ **104**	
		*hly*A	AAAGTTCAGATCTTGATGACATTG			
			TTGCTGCAGGATGGGCAACTCTTGA*			
			Biot-AAGCCGGAACAGTTCTCTCAGC*		/ **96**	
			CTCCTTCCCGTTGTTTTCTCAG			
			TTCATCAAGAGCCATGCCTGATAAAGCAAT *			
E. coli EHEC / AF043627, X79839, S83460, AF228488 & AB035924	(Wang et al., 2002) Stx2d-a, Stx2d-b HlyA-a HlyA-b RfbE-a RfbE-b FliC-a FliC-b E16S-a E16S-b	*Stx*$_{2d}$	GGTAAAATTGAGTTCTCTAAGTAT		1221-1244	Multiplex (5) PCR, agarose gel
			CAGCAAATCCTGAACCTGACG		1395-1375 / **175**	
		EHEC *hlyA*	AGCTGCAAGTGCGGGTCTG		867-885	
			TACGGGTTATGCCTGCAAGTTCAC		1435-1412 / **569**	
		rfbE$_{O157}$	CTACAGGTGAAGGTGGAATGG		673-693	
			ATTCCTCTCTTTCCTCTGCGG		999-979 / **327**	
		fliC$_{H7}$	TACCATCGCAAAAGCAACTCC		1068-1088	
			GTCGGCAACGTTAGTGATACC		1314-1294 / **247**	
		E. coli 16S rRNA	CCCCCTGGACGAAGACTGAC		1682-1701	
			ACCGCTGGCAACAAAGGATA		2082-2063 / **401**	
E. coli EHEC / M19473 (*stx1*) X07865 (*stx2*)	(Bellin et al., 2001), StxA1 598 & StxA1 1015, StxA2 679 & StxA2 942	*stxA*$_1$	AGTCGTACGGGGATGCAGATAAAT	55	598-621	Multiplex (2) LightCycler TD-PCR, detection with SYBR green and probes / Clinical
			CCGGACACATAGAAGGAAACTCAT		1015-992 / **418**	
		stxA$_2$	TTCCGGAATGCAAATCAGTC		679-698	
			CGATACTCCGGAAGCACATTG		942-922 7 **264**	
E. coli EHEC and EPEC	(Anglès d'Auriac et al., 2006), 7eae213U & 7eae619L 7eae213U & 7eae773L	*eae* conserved 5′ area used for the construction	AACTGTTGCCGATCTTTCTAA			PCR
			TGCCGTTCCATAATGTTGTAA	54	/ **427**	
			AACTGTTGCCGATCTTTCTAA			
			AACGCTGACCCGCACCTAAAT		/ **581**	
E. coli EHEC and EPEC	(Anglès d'Auriac et al., 2006), 7eae626UU & 7eae812LU 7eae626UU-7eae956LU 7eae626UU-7eae1499L	*eae* conserved 5′ area used for the construction	ATTATGGAACGGCAGAGGTTA	67	626-646	PCR
			TGAAGACGTTATAGCCCAACA		812-832 / **207**	
			ATTATGGAACGGCAGAGGTTA	63	626-646	
			GGCGCTCATCATAGTCTTTCT		956-976 / **351**	
			ATTATGGAACGGCAGAGGTTA		626-646	
			CGCTTTGGCTTCCGCTATGCT		1499-1529 / **894**	
E. coli EHEC O157:H7	(Heijnen et al., 2006)	*UidA* (*E. coli*)	ATGGAATTTCGCCGATTTTGC	60		Multiplex (4), Real Time PCR (2 step)
			ATTGTTTGCCTCCCTGCTGC			
		rfbE (*E. coli* O157)	GTAAATATGTGGGAACATTTGG			
			GGCCTTTAAAATGTAAACAACGG			
		stx$_1$	AGTCGTACGGGGATGCAGATAAAT			
			CCGGACACATAGAAGGAAACTCAT			
		stx$_2$	TTCCCGAATGCAAATCAGTC			
			CGATACTCCGGAAGCACATTG			
E. coli EHEC O157:H7	(Cebula et al., 1995), (Feng et al., 2000; Vernozy-Rozand et al., 2000), LP30 & LP31,	*stxA*$_1$	CAGTTAATGTGGTGGCGAAGG			Multiplex (3), agarose EtBr
			CACCAGACAATGTAACCGCTG	64	/ **348**	
		stxA$_2$	ATCCTATTCCCGGGAGTTTACG	or		
			GCGTCATCGTATACACAGGAGC	56	/ **584**	

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	LP43 & LP44, PT-2 & PT-3	*uidA* (MAMA) *β-glucuroniase* mutated O157:H7 (not expressed)	GCGAAAACTGTGGAATTGGG TGATGCTCCATCACTTCCTG		/ **252**	
E. coli EHEC O157:H7	(Hu et al., 1999), (Campbell et al., 2001), RfbF & RfbR, IntF & IntR, SLTI-F & R (Meng et al., 1997), SLTII-F & R (Meng et al., 1997), FLICH7-F&R (Gannon et al., 1997)	*rfb*F (O157 specific) *eae* stx_1 $stxA_2$ $fliC_{h7}$ (H7 specific)	GTGTCCATTTATACGGACATCCATG CCTATAACGTCATGCCAATATTGCC GACTGTCGATGCATCAGGCAAAG TTGGAGTATTAACATTAACCCCAGG TGTAACTGGAAAGGTGGAGTATACA GCTATTCTGAGTCAACGAAAAATAAC GTTTTTCTTCGGTATCCTATTCC GATGCATCTCTGGTCATTGTATTAC GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC		/ **292** / **368** / **210** / **484** 69-91 671-694 / **625**	Multiplex (5), agarose 2%, EtBr
E. coli EHEC O157:H7	(Nagano et al., 1998) H7-F & H7-R O157-F & O157-R MK1 & MK2, (Karch et al., 1989)	*flicC* structural flagellin gene of H7 *rfbE* gene involved in the biosynthesis of the O157 antigen $stxA_1$ & $stxA_2$, A subunit, universal degenerate 1 primer-pair (silent mismatch)	TACCACCAAATCTACTGCTG TACCACCTTTATCATCCACA AACGGTTGCTCTTCATTTAG GAGACCATCCAATAAGTGTG TTTACGATAGACTTCTCGAC (TTTACSWTAGWCTTCTCGAC) CACATATAAATTATTTCGCTC	52	/ **560** / **678** 311-330 515-535 / **227**SLT-I **224**SLT-II	Multiplex (3), agarose 1.5%, EtBr
E. coli EHEC O157:H7	(Radu et al., 2001), LP30 & LP31 Cebula T.A. (Cebula et al., 1995), LP43 & LP44 (Cebula et al., 1995), FLICH7-F & -R (Gannon et al., 1997)	$stxA_1$ $stxA_2$ $fliC_{h7}$	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC	65	50- / **348** 58- / **584** 69-91 671-694 / **625**	Multiplex (3), agarose 1.2% EtBr / Food
E. coli EHEC O157:H7 & EPEC	(Feng et al., 2000), (Fratamico et al., 1998), LP30 & LP31 (Cebula et al., 1995), LP43 & LP44 (Cebula et al., 1995), PT-2 & PT-3 (Cebula et al., 1995), AE22 & 20-2 (Fratamico et al., 1998), MFS1Fb & 1R	$stxA_1$ $stxA_2$ *uidA* (MAMA) *eaeA* *ehxA* on the 60 mDa plasmid (pO157)	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC GCGAAAACTGTGGAATTGGG TGATGCTCCATCACTTCCTG ATTACCATCCACACAGACGGT ACAGCGTGGTTGGATCAACCT GTTTATTCTGGGGCAGGCTC CTTCACGTCACCATACATAT	56	/ **348** / **584** / **252** / **397** / **158**	Multiplex (5), agarose 1% in TBE + EtBr / Clinical
E. coli EHEC O157:H7 & EPEC O55:H7	(Sandhu et al., 1996; Sandhu et al., 1997), C1-C2	eae *conserved 5´ area used for the construction*	TCGTCACAGTTGCAGGCCTCGT CGAAGTCTTATCCGCCGTAAAGT	55	803-824 1912-1890 / **1100**	
E. coli EHEC O157H7	(Maurer et al., 1999), O157PF8 & O157PR8 (Chapman et al., 2001)	*rfb* O157 specific	CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG	55	/ **420**	PCR, agarose 1.5% EtBr
E. coli EIEC & *Shigella*	(Boileau et al., 1984) BAM (Hill et al., 1995)	*220 kb Virulence/invasion plasmid common to both organism*	Probe prepared from EcoRI restriction fragments of recombinant plasmids from the *Shigela* virulence/invasion plasmid		**17000***	Radioactive probe hybridization
E. coli EIEC & Shigellae	(Frankel et al., 1989; Frankel et al., 1990; Luscher et al., 1994) (Rappelli et al., 2001; Svenungsson et al., 2000) *ial*-Pr1 & *ial*-Pr2	*ial* invasiveness associated locus on the large 120-140 Mda plasmid carried by the 4 *Shigella* species and EIEC (frequent false negatives because of loss of plasmid)	CTGGTAGGTATGCTGAGG CC(or GG)AGGCCAACAATTATTTCC	43 or 55	/ **320**	PCR, agarose 1.5 or 2%, EtBr 0.5 μg/ml / Stool direct or after MacCkey agar
E. coli EIEC & Shigellae	(Lampel et al., 1990), (Sunabe et al., 1998), KL1 & KL8	ial *the invasion plasmid locus*	TAATACTCCTGAACGGCG TTAGGTGTCGGCTTTTCTG	55 to 65	149- 897 / **760**	PCR, agarose 1 %, EtBr 0.1μg/ml / Food
E. coli EIEC & Shigellae	(Luscher et al., 1994), (Bischoff et al., 2005)Shig-1 & Shig-2	*ipaH* (invasive plasmid antigen) a multiple copy sequence found on both the chromosome & the invasive plasmid	TGGAAAAACTCAGTGCCTCT CCAGTCCGTAAATTCATTCT	55	/ **422**	PCR, agarose 3%, EtBr / Stool after cultivation on MacConkey agar
E. coli EIEC & *Shigellae*	(Sethabutr et al., 1993) (Sethabutr et al., 1994), (Sunabe et al., 1998) / *ial* primer I & 2 *ipaH* primer III & IV *ial* probe *ipaH* probe	ipaH *(invasive plasmid antigen) a multiple copy sequence found on both the chromosome & the invasive plasmid,* ial *the invasion plasmid locus*	CTGGTAGGTATGGTGAGG CCAGGCCAACAATTATTT GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC CCATCTATTAGAATACCTGTG* AATCTCCGGAAAACCCTCCTGGTC*	50 68 or 60 or 54	/ **320** / **630**	PCR, agarose 1.2% / Clinical / stools
E. coli EIEC & Shigellae	(Theron et al., 2001), H8 & H15 Islam M S (Islam et al., 1993) H10	*ipaH* (invasive plasmid antigen) a multiple copy sequence found on both the chromosome & the invasive plasmid, ial the invasion plasmid locus	GTTCCTTGACCGCCTTTCCGATAC GCCGGTCAGCCACCCTC CATTTCCTTCACGGCAGTGGA	/ 60 / 60	/ **620** / **401**	PCR semi-nested, agarose 2%, EtBr 0.5 μg/ml / Water
E. coli EPEC	(Jerse et al., 1990a), EAF 21	EAF, plasmidal Adherence factor	TATGGGGACCATGTATTATCA*		/ **21**	Colony blot with radiactive labeled probe
E. coli EPEC	(Svenungsson et al., 2000)	*bfpA* bundle forming pili	TTCTTGGTGCTTGCGTGTCTTTT TTTTGTTTGTTGTATCTTTGTAA	55	/ **367**	PCR, agarose 2%, EtBr 0.5 μg/ml / Stool after cultivation on MacConkey agar
E. coli *EPEC & EAEC*	(Aranda et al., 2004), eae1 & eae2 (Reid et al., 1999), BFP1 & BFP2 (Bischoff	*eae* intimin gene, universal *bfpA* bundle-forming pili on the enteroadherence factor	CTGAACGGCGATTACGCGAA CCAGACGATACGATCCAG AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	58	544-563 1444-1461 / **917** **/ 326**	Multiplex (3) and EtBr gels

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
	et al., 2005; Gunzburg et al., 1995) EAEC1 & EAEC2	(EAF) plasmid CVD432	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT		**/ 630**	
E. coli EPEC O55, O111, O119	(Gunzburg et al., 1995) (Sunabe et al., 1998) (Aranda et al., 2004) EP1 & EP2	bfpA *encoding for the bundle-forming pili on the enteroadherence factor (EAF) plasmid*	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	56	/ **326**	PCR, boil prep 10', agarose, EtBr / Clinical
E. coli *EPEC, STEC, ETEC, EIEC & EAEC*	(Toma et al., 2003), SK1 & SK2 ((Karch et al., 1993)), VTcom-u & VTcom-d (Yamasaki (Yamasaki et al., 1996)) AL65 & AL125 LT_L & LT_R ((Tamanai-Shacoori et al., 1993)) IpaIII & IpaIV ((Sethabutr et al., 1993)) aggRks1 aggRkas2 Eaggfp & Eaggbp ((Pass et al., 2000)) aspU-3 & aspU-2	*eae* conserved 5' area used for the construction, universal intimin *$stxA_1$* & *$stxA_2$*, A subunit, universal primer-pair *est* heat stable ST_h-ST_p plasmid-borne toxin genes *elt* heat-labile, LT (B subunit) *ipaH* enteroinvasive mechanism *aggR* CVD432 aspU	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT TTAATAGCACCCGGTACAAGCAGG CCTGACTCTTCAAAAGAGAAAATTAC TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC GTATACACAAAAGAAGGAAGC ACAGAATCGTCAGCATCAGC AGACTCTGGCGAAAGACTGTATC ATGGCTGTCTGTAATAGATGAGAAC GCCTTTGCGGGTGGTAGCGG AACCCATTCGGTTAGAGCAC	52	26-46 879-903 / **863-881** 280-300 778-797 / **518** **/ 147** **/ 322** **/ 619** **/ 254** **/ 194** **/ 282**	Multiplex (6) with the first 6 primer pairs and EtBr gels
E. coli ETEC	(Deng et al., 1996a), LT1-2	*eltB / toxB*, B subunit of the heat-labile enterotoxin (LT)	CAGTCTATTACAGAACTATG B-CCATACTGATTGCCGCAATT AGCGGCGCAACATTTCAGGTCG*	60 ann & ext	70-89 347-366 / **298**	PCR, agarose gels and HRP probes / Food
E. coli ETEC	(Frankel et al., 1989), (Bischoff et al., 2005; Luscher et al., 1994; Olsen et al., 1995) LT-Pr1 & LT-Pr2, ST-Pr1 & ST-Pr2	*elt* heat-labile, LT and *est* heat stable toxin genes	GGCGACAGATTATACCGTGC CCGAATTCTGTTATATATGTC TCTGTATTGTCTTTTTCACC TTAATAGCACCCGGTACAAGC	/ 55	/ **750** / **186**	PCR, agarose 3% EtBr
E. coli ETEC	(Lou et al., 1997), *cfaB*-1 & *cfcB*-p4	*cfaB* colonization factor antigen I operon encoding the colonization factor antigen I fimbriae	GCGGCTCTAATTAGATCT GTGGTCAGACCATTGCACC	55	/ **370**	PCR, polyacrylamide 6% gel / Stool direct
E. coli *ETEC*	(Olive, 1989; Olsen et al., 1995), LTL & LTR	*elt* (heat-labile enterotoxin genes)	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	55	46-65 349-367 / **322**	PCR, agarose
E. coli ETEC	(Olsvik et al., 1993a), (Rappelli et al., 2001) LTIa-1 & LTI-2 LTIb-1 & LTI-2 "mismatch" & LTI-2	*elt* (heat-labile enterotoxin genes)	TCTCTATATGCACACGGAGC (LTIa-1) CCATACTGATTGCCGCAAT (LTI-2) TCTCTATGTGCATACGGAGC (LTIb-1) TCTCTATGTGCACACGGAGC ("mismatch")	60 and 58	46-65 349-367 / **322**	Simplex (1) PCR,
E. coli ETEC	(Osek, 2001) LT3 & LT4 STA1 & STA2 STIIB1 & STIIB2 Ec1 & Ec2 (Chen J. (Chen et al., 1998a))	*elt* I heat-labile enterotoxin gene *est* I heat-stable enterotoxin gene *est* II heat-stable enterotoxin gene *uspA*: universal stress protein, specific primers for *E. coli*	TATCCTCTCTATATGCACAG CTGTAGTGGAAGCTGTTATA TCTTTCCCCTCTTTAGTCAG ACAGGCCGGATTACAACAAAG GCCTATGCATCTACACAATC TGAGAAATGGACAATGTCCG CCGATACGCTGCCAATCAGT ACGCAGACCGTAGGCCAGAT	55	/ **480** / **166** / **278** 4-23 868-887 / **884**	Multiplex (4) PCR, agarose 2% in TAE EtBr staining / Feces swab and regrown colonies on LB agar
E. coli *ETEC*	(Schlor et al., 2000), (O'Meara et al., 1995), ETA1 & ETA2	*eltAB* (heat-labile enterotoxin)	CCGGATTGTCTTCTTGTATGATA GGTCTCGGTCAGATATGTGATTC	60	/ **1100**	PCR, agarose
E. coli ETEC	TW20 (LT-Pr1 (Frankel et al., 1989))-JW11 and JW14-JW7, (Aranda et al., 2004)	*elt* heat-labile, LT heat stable ST_h-ST_p plasmid-borne toxin genes	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT ATTTTTMTTTCTGTATTRTCTT CACCCGGTACARGCAGGATT	/ 50	4-23 443-424 / **450** 22-43 212-193 / **190**	PCR, agarose 3% EtBr
E. coli ETEC	(Tamanai-Shacoori et al., 1993)	*elt* (heat-labile enterotoxin genes) B subunit	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	55	/ **322**	Simplex (1) PCR agarose EtBr
E. coli *ETEC*	(Woodward et al., 1992), Lt-1 & lt-2	*elt* (heat-labile enterotoxin genes)	ATTTACGGCGTTACTATCCTC TTTTGGTCTCGGTCAGATATG	59	27—48 286-307 / **280**	PCR, agarose 1.5%
E. coli *ETEC*	(Woodward et al., 1992), Sta-1 & Sta-2	*estA* (heat-stable enterotoxin genes)	TCTTTCCCCTCTTTTAGTCAG ACAGGCAGGATTACAACAAAG	59	39-60 184-205 / **166**	PCR, agarose 1.5%
E. coli *ETEC & EIEC & Shiga toxin*	(Aranda et al., 2004), LTf & LTr STf & STr ((Stacy-Phipps et al., 1995)) IpaH1 & IpaH2 ((Sethabutr et al., 1993)) Stx1f & Stx1r Stx2f & Stx2r ((Paton et al., 1998))	*elt* heat-labile, LT *est* heat stable ST_h-ST_p plasmid-borne toxin genes *ipaH* enteroinvasive mechanism *$stxA_1$* A subunit coding region *$stxA_2$* A subunit coding region (including *stx* 2 variants)	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT ATTTTTMTTTCTGTATTRTCTT CACCCGGTACARGCAGGATT GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG	50	4-23 443-424 / **450** 22-43 212-193 / **190** **/ 600** 454–477 612-633 / **180** 603–624 857-879 / **255**	Multiplex (5) and EtBr gels
E. coli ETEC-LT & ETEC-ST	(Svenungsson et al., 2000),	*eltB* and/or *estA* (heat-labile enterotoxin and heat stable enterotoxin genes)	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT GCTAAACAAGTARRGGTCTTCAAAA CCCGGTACARRGCAGGATTACAACA	55	/ **322** / **147**	PCR, agarose 2%, EtBr 0.5 µg/ml / Stool after cultivation on MacConkey agar
E. coli O157 / AF061251	(Abdulmawjood et al., 2003), Gi-O157-I & Gi-O157-II	*rfbE* O157:H7	CGAGTACATTGGCATCGTG ATTGCGCTGAAGCCTTTG	60	4596-5079 / **501**	PCR, IAC, agarose 2%, EtBr
E. coli STEC	(Begum et al., 1995; Jackson, 1992)	*$stxA_2$* *$stxA_2$* &	CGCAGCGCTGGAACGTTCC (F) CAGAAGCCTTACGCTT (F)	55	661-679 / **785** 798-813 / **648**	PCR, dig labelled probes

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
		stxB$_2$	CCGGAGCCTGATTCACAGG (R)		1427-1445	
E. coli STEC	(Belanger et al., 2002), 1Slt224 & 1Slt385, 1SltB1FAM, 2Slt537 & 2Slt678b, 2SltB1TET	*stxA*$_1$ (A sub-unit of Shiga toxin-1)	ATGTCAGAGGGATAGATCCA TATAGCTACTGTCACCAGACAAT GCGAGGCGCTTTGCTGATTTTTCACATGTTA CCCCTCGC*FAM	56	224-243 385-407 / **185**	Multiplex (2) molecular beacons with smart cycler
		stxA$_2$ (A sub-unit of Shiga toxin-2)	AGTTCTGCGTTTTGTCACTGTC CGGAAGCACATTGCTGATT GCGAGGCACTGTCTGAAACTGCTCCTGTCC TCGC*TET		537-558 678-696 / **160**	
E. coli STEC	(Chen et al., 1998b) ((Pollard et al., 1990)) ((Pollard et al., 1990))	*stxB*$_1$ (B subunit of the VT1 toxin including detection of *S. dysenteriae* type 1) *stxA*$_2$ (A subunit of the toxin)	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA Biotin-ATTCATCCACTCTGGGGGCA Biotin-TCATTTTACCCCCTCAACTG TTAACCACACCCCACCGGCAGTTTA GCTCTGGATGCATCTCTGGT Biotin-TTGCTGTGGATATACGAGGG Biotin-ACTGCTGTCCGTTGTCATGG	58 & 60	1191-1210 1301-1320 / **130** 1230-1249 1273-1292 / **63*** 426-445 752-771 / **346** 450-469 621-640 / **191***	Multiplex (2) magnetic capture hybridization, agarose 1%, EtBr 1 µg/ml / Food
E. coli STEC	(Chen et al., 1998b; Fratamico et al., 1995; Pollard et al., 1990; Ramotar et al., 1995; Rappelli et al., 2001; Svenungsson et al., 2000) VT1a & b VT2a & b	*stxB*$_1$ (B subunit of the VT1 toxin including detection of *S. dysenteriae* type 1) *stxA*$_2$ (A subunit of the toxin)	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA TTAACCACACCCCACCGGCAGTTTA GCTCTGGATGCATCTCTGGT	55- 60	1191-1210 1301-1320 / **130** 426-445 752-771 / **346**	Multiplex (2) Agarose 2%, EtBr 0.5 µg/ml & dot blot / Stool direct
E. coli STEC	(Cocolin et al., 2000) ECSLT 1a & ECSLT 1b; ECVT 1; ECSLT 2a & ECSLT 2b; ECVT 2	*stxA*$_1$ A subunit coding region *stxA*$_2$ A subunit coding region (including *stx* 2 variants)	GGGCGGTTTAATAATCTACG CCCAGTTGAATGTAAGATCAA GGCAGATACAGAGAGAATTTCG* ATCGGTGTCTGTTATTAACC CGCCAGATATGATGAAACCAG ACGTTCCGGAATGCAAATCAG*	50	405-425 811-831 / **429** 337-356 632-652 / **318**	Multiplex (2)-ELISA agarose 2%, EtBr
E. coli STEC O157	(Desmarchelier et al., 1998), O157AF & AR	*rfbE* O157:H7	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGACAG	66	/ **497**	PCR, agarose 1.5% EtBr
E. coli STEC	(Fach et al., 2001) EH1 & EH5	*stxA-stxB* and variants, universal stx degenerate primers Capture probe Detection probe	ACTGTSACAGCWGAAGCYTTACG ACWGTRAAKGTATCRTYMTCATTATA Biot-GRAGAHGTKGAYCTYACWYTGAACTGGGG* TWATACTGAATTGYCATCATCAKG-dig*	55	/ **789-801**	PCR-ELISA with internal control (IC) / Food (dairy products)
E. coli STEC	(Fagan et al., 1999), (Hornitzky et al., 2001), SLTI-F & -R Gannon V.P. (Gannon et al., 1992), SLTII-F & -R (Gannon et al., 1992), MFS1F & R (Fratamico et al., 1995)	*stxA*$_1$ *stxA*$_2$ all variants (silent mismatch) *eaeA* specific 3' end *hlyA* 60-Mda plasmid (enterohemolysin)	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCACTTTG GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG ACGATGTGGTTTATTCTGGA CTTCACGTGACCATACATAT	58	938-957 1539-1520 / **614** 624-644 1403-1384 / **779** / **890** / **166**	Multiplex (4), agarose 2% EtBr 0.5 µg / ml / Animal feces after pre-incubation
E. coli STEC EHEC O157:H7 US 5,652,102	(Fey et al., 2000; Karch et al., 1989), MK1 & MK2 428-I* 428-II*	*stxA*$_1$ & *stxA*$_2$, A subunit, universal degenerate 1 primer-pair (silent mismatch) SltIA probe SltIIA probe	TTTACGATAGACTTCTCGAC (TTTACSWTAGWCTTCTCGAC) CACATATAAATTATTTCGCTC GATAGTGGCTCAGGGGATAA* AACCACACCCACGGCAGTTA*	43 55-44 1,5'-2'-1'	311-330 515-535 / **227**SLT-I **224**SLT-II	PCR, agarose 2% or polyacrylamide 5%, EtBr
E. coli STEC EHEC O157:H7 specific. US 5,652,102	(Fratamico et al., 1993) (Deng et al., 1995; Deng et al., 1996b; Gannon et al., 1993), MK1 & 2 (Karch et al., 1989), AE19 & 20 (Gannon et al., 1993), MFS1F & R, (Fratamico et al., 1995)	*stxA*$_1$ & *stxA*$_2$, universal primer-pair (silent mismatch) *eaeA* variable 3' area used for construction of specific primers (O157:H7 & O55 specific, γ type) *hlyA* 60-Mda plasmid	TTTACGATAGACTTCTCGAC (TTTACSWTAGWCTTCTCGAC) CACATATAATTATTTCGCTC CAGGTCGTCGTGTCTGCTAAA ACAGCGTGGTTGGATCAACCT ACGATGTGGTTTATTCTGGA CTTCACGTCACCATACATAT	55	311-330 515-535/ **227***stx*$_1$ **224***stx*$_2$ 1959-1970 3047-3027/ **1089** / **166**	Multiplex (3), agarose 1.4% EtBr / Food
E. coli STEC	(Gannon et al., 1992; Gannon et al., 1997; Witham et al., 1996), SLTI-F & -R, SLTII-F & -R	*stxA*$_1$-*stxB*$_1$ *stxA*$_2$-*stxB*$_2$ all variants (silent mismatch)	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCACTTTG	60	938-957 1539-1520 / **614** 624-644 1403-1384 / **779**	Multiplex (2), agarose 1% EtBr 0.25 µg / ml / Ground beef
E. coli STEC EHEC	(Ge et al., 2002) F1 & R1	*stxA*$_1$ & *stxA*$_2$, *A* subunit, universal primer-pair	GCAGATACAGAGAGAATTTCGT CTGATGATGGCAATTCAGTAT	50	/ **220**	PCR-ELISA, agarose 2%, EtBr 0.5 µg/ml / Food
E. coli STEC	(Gilgen et al., 1998), I-1 & I-2 (Gannon et al., 1992), II-1 & II-2 (Gannon et al., 1992), I-3 & II-4, II-3 & II-4	*stxA*$_1$-*stxB*$_1$ *stxA*$_2$-*stxB*$_2$ all variants (silent mismatch) *stx*$_1$ nested primer *stx*$_2$ nested primer	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG CCATGACRACGGACAGCAGTT CCTGTCARCTGAGCACTTTG TTGTCATCATCATGCATCGC AGTTACACAATCAGGCGTCG GTTCTGCGTTTTGTCACTGT AGCTGTATTACTTTCCCATAA	60 55	938-957 1539-1520 / **614** 624-644 1403-1384 / **779** / **247** / **372**	Multiplex (2) + nested PCR, agarose 1.5% EtBr 0.25
E. coli STEC	(Grant et al., 2001; McDaniels et al., 1996) JIB9/4 JIB5/6, (Jinneman et al., 1995)	*gadA* & *gadB* Glutamate decarboxylse: 2 very similar copies per genome. Conserved GAD A/B primers *stx*$_1$ *stxA*$_2$-*stxB*$_2$ amplicon spanning from 3' of A subunit through the 5' of the B subunit	ACCTGCGTTGCGTAAATA GGGCGGGAGAAGTTGATG ? CTGGGGGCGAATCAGCAATG CCGCCGCCATTGCATTAACA	58	307-324 959-976 / **670** **/ 513** 832-855 1195-1176 / **364**	Multiplex (3), agarose gel 1 % EtBr
E. coli STEC	(Kobayashi et al., 2001),	*stxA*$_1$ & *stxA*$_2$, A subunit,	GAGCGAAATAATTTATATGTG		280-300	PCR & nested PCR

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
	VT com-u & VT com-d (Yamasaki (Yamasaki et al., 1996)) (Toma et al., 2003) VT com-nesF & VT com-nesR	universal primer-pair universal nested primer-pair	TGATGATGGCAATTCAGTAT CGGACAGTAGTTATACCAC CTGCTGTCACAGTGACAA	55	778-797 / **518** 395-413 547-565 / **171**	
E. coli STEC	(Osek et al., 2001), MK1 & 2 (Karch et al., 1989), O157PF8 & O157PR8 (Maurer et al., 1999), HlyAF & hlyAR	stxA$_1$ & stxA$_2$, *universal primer-pair (silent mismatch)* rfb *O157 specific* *ehlyA* Enterohemolysin	TTTACGATAGACTTCTCGAC CACATATAAATTATTTCGCTC CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	53	**/ 230** **/ 420** **/ 534**	Multiplex (3) PCR, agarose 2%, EtBr
E. coli STEC	(Osek et al., 2001; Osek, 2002) Int-Fc & Int-Rc (Batchelor et al., 1999), LP30 & LP31 (Cebula et al., 1995), O157PF8 & O157PR8 (Maurer et al., 1999), LP43 & LP44 (Cebula et al., 1995),	eae *universal* *stxA$_2$* *rfb* O157 specific *stxA$_1$*	CCGGAATTCGGGATCGATTACCGTCAT CCCAAGCTTTTATTTATCAGCCTTAATCTC ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC CGTGATGATGTTGAGTTG AGATTGGTTGGCATTACTG CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG	53	/ **840** / **584** / **420** / **348**	Multiplex (4) PCR, agarose 2%, EtBr / Feces
E. coli STEC	(Paton et al., 1993b)	stxA$_1$ & stxA$_2$, A subunit, universal degenerate primer-pair	ATACAGAGRGRATTTCGT TGATGATGRCAATTCAGTAT AGAACGCCCACTGAGATCATC* ATACACAGGGAGCAGTTTCAGA*	47	586 - - 800 / **215**SLT-I 583 - - 794 / **212**SLT-II	PCR, agarose 2% EtBr / Crude fecal culture extract
E. coli STEC	(Paton et al., 1998), (Mansfield et al., 2001) Stx1F & stx1R Stx2F & stx2R eaeAF & eaeAR hlyAF & hlyAR	stxA$_1$ A subunit coding region stxA$_2$ A subunit coding region (including *stx* 2 variants) *eaeA* (this region is conserved between EPEC and STEC) *hlyA* EHEC	ATAAATCGCCATTCGTTGACTAC AGAACGCCCACTGAGATCATC GGCACTGTCTGAAACTGCTCC TCGCCAGTTATCTGACATTCTG GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG GCATCATCAAGCGTACGTTCC AATGAGCCAAGCTGGTTAAGCT	65 & 60	454–477 612-633 / **180** 603–624 857-879 / **255** 27–47 390-410 / **384** 70–91 581-603 / **534**	Multiplex (4) TD PCR agarose 2% EtBr / Stool & food
E. coli STEC	(Pollard et al., 1990; Svenungsson et al., 2000), VT1a-VT1b	stxB$_1$ (B subunit of the VT1 toxin including detection of *S. dysenteriae* type 1) stxA$_2$	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA ACCGTTTTTCAGATTTTRRCACATA TACACAGGAGCAGTTTCAGACAGT	55	1191-1210 1301-1320 / **130** / **298**	Multiplex (2) Agarose 2%, EtBr 0.5 g/ml / Stool after cultivation on MacConkey agar
E. coli STEC EHEC	(Read et al., 1992), ES 151-ES149	stxA$_1$ & stxA$_2$, *A* subunit, universal primer-pair	GARCRRAATAAYTTATATGT CGRAATYCYCKCTGTAYTTKCC GAGCGAAATAATTTATATGT CGAAATCCCCTCTGTATTTGCC	49	611-631 912-933 / **323**	PCR, agarose 2%, EtBr
E. coli STEC	(Thran et al., 2001), VT2a & b (Pollard et al., 1990)	stxA$_1$ stxA$_2$	CAGTTAATGTGGTGGCGAA CACAGACTGCGTCAGTGAGG TTAACCACACCCCACCGGCAGTTTA GCTCTGGATGCATCTCTGGT	55	/ **475** 426-445 752-771 / **346**	Multiplex (2), Agarose 1%, EtBr / Whole cells
E. coli STEC	(Tsen et al., 1998), SLTI-5 & SLT-3 SLTII-5 & SLTII-3 LT 51 & LT 31 STII-FP & STII-RP	stx$_1$ stx$_2$ *elt* heat-labile enterotoxin gene *est* heat-stable enterotoxin gene	AGCTGAAGCTTTACGTTTTCGG TTTGCGCACTGAGAAGAAGAGA TTTCCATGACAACGGACAGCAGTT ATCCTCATTATACTTGGAAAACTCA CCGTATTACAGAAATCTGA GTGCATGATGAATCCAGGGT GCAATAAGGTTGAGGTGAT GCCTGCAGTGAAATGGAC	60	723-744 1312-1291 / **590** 618-641 1311-1287 / **694** 616-635 725-706 / **110** 701-719 1068-1049 / **368**	Multiplex (4) PCR, agarose 2.3% in TAE EtBr staining
E. coli *STEC*	(Woodward et al., 1992), VT1-1 & VT1-2	stx$_1$	TTAGACTTCTCGACTGCAAAG TGTTGTACGAAATCCCCTCTG	59	239-260 748-769 / **530**	PCR, agarose 1.5%
E. coli STEC - EHEC O157:H7 *Salmonella* spp / US 5,652,102	(Fratamico et al., 1998), MK1 & 2 (Karch H. (Karch et al., 1989)), AE22 & AE20-2, MFS1F & R, INVAF & R	stxA$_1$ & stxA$_2$ universal (silent mismatch) *eaeA* (O157:H7 & O55 specific, γ type) *hlyA* 60-Mda plasmid (enterohemolysin) (Fratamico P.M. (Fratamico et al., 1995)) *InvA* , chromosomal, essential for invasion of epithelial cells by *Salmonella*	TTTACGATAGACTTCTCGAC (TTTACSWTAGWCTTCTCGAC) CACATATAATTATTTCGCTC ATTACCATCCACACAGACGGT ACAGCGTGGTTGGATCAACCT ACGATGTGGTTTATTCTGGA CTTCACGTCACCATACATAT CGGTGGTTTTAAGCGTACTCTT CGAATATGCTCCACAAGGTTA	53	 / **228-225** / **397** / **166** / **796**	Multiplex (4) Agarose 1.5%, EtBr / Food
E. coli STEC & EPEC	(Svenungsson et al., 2000)	*eaeA* conserved 5' area used for the construction	CACACGAATAAACTGACTAAAATG AAAAACGCTGACCCGCACCTAAAT	55	/ **376**	PCR, agarose 2%, EtBr 0.5 μg/ml / Stool after cultivation on MacConkey agar
E. coli STEC & ETEC / LT: M35581, M57244, K01995	(Lang et al., 1994), VT1a & b VT2a & b (Pollard D.R. (Pollard et al., 1990),	stxB$_1$ (B subunit of the VT1 toxin including detection of *S. dysenteriae* type 1) *stx* 2 (A subunit of the toxin) LT toxin	GAAGAGTCCGTGGGATTACG AGCGATGCAGCTATTAATAA CCACTCTGGGGGCAATTCTGATGCG* TTAACCACACCCCACCGGCAGTTTA GCTCTGGATGCATCTCTGGT CGTGTCGCAGCGCTGGAACGTTCCG* TGTTTCCACTTCTCTTAG TATTCCCTGTTACGATGT TACAGCCCTCACCCATATGAACA*	45	1191-1210 1301-1320 / **130** 426-445 752-771 / **346** / **258**	Multiplex (3) Agarose 2%, EtBr 0.5 μg/ml / Environment waters
E. coli STEC & O157:H7 / M16625 X07865	(Walford et al., 1999), VS1 & VS2 VS3 VS4 & VS5	stxA$_1$ stxA$_2$	CATAGTGGAACCTCACTGACGCAGT TTTGCCGAAAACGTAAAGCTTCA TGTGGCAAGAGCGATGTTACGGTTTG* GGGCAGTTATTTTGCTGTGGA TGTTGCCGTATTAACGAACCC	60	830-854 917-886 / **80** 442-462 562-542 / **120**	Multiplex (3), TaqMan detection / Food and faeces

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
	VS6		CTATCAGGCGCGTTTTGACCATCTTCG*			
X60439	VS8 & VS9	*eae*A	GGCGGATTAGACTTCGGCTA		1896-1916	
	VS10		CGTTTTGGCACTATTTGCCC		2047-2028 / **150**	
			AACGCCGATACCATTACTTATACCGCGACG*			
E. coli STEC & *Salmonella* spp.	(Chen et al., 2001)	$stxB_1$ (B subunit of the VT1 toxin including detection of *S. dysenteriae* type 1)	GAAGAGTCCGTGGGATTACG		1191-1210	Multiplex (3) magnetic capture hybridization, agarose 1%, EtBr 1 µg/ml / Food
			AGCGATGCAGCTATTAATAA	58	1301-1320 / **130**	
	((Pollard et al., 1990))		Biotin-ATTCATCCACTCTGGGGGCA	&	1230-1249	
	((Pollard et al., 1990))		Biotin-TCATTTTACCCCCTCAACTG	60	1273-1292 / **63***	
		$stxA_2$ (A subunit of the toxin)	TTAACCACACCCCACCGGCAGTTTA		426-445	
			GCTCTGGATGCATCTCTGGT		752-771 / **346**	
	(Stone et al., 1994)		Biotin-TTGCTGTGGATATACGAGGG		450-469	
			Biotin-ACTGCTGTCCGTTGTCATGG		621-640 / **191***	
		invE & *A* , chromosomal, essential for invasion of epithelial cells by *Salmonella*	TGCCTACAAGCATGAAATGG		1219-1238	
			AAACTGGACCACGGTGACAA		278-259 / **457**	
			Biotin-CAGGATACCTATAGTGCTGC		72-91	
			Biotin-CACCAATATCGCCAGTACGA		218-237 / **166***	
E. coli STEC & *Shigella dysenteriae*	(Johnsen et al., 2001; Kobayashi et al., 2001; Olsen et al., 1995; Olsvik et al., 1991; Olsvik et al., 1993a; Vold et al., 1998) SLTI-1 & SLTI-3 SLTII-1 & SLTII-2	$stxA_1$ (A sub-unit of Shiga toxin-1)	CAGTTAATGTGGTGGCGAAG			Multiplex (2) PCR, polyacrylamide 8% or agarose 2% / Isolated colonies
			CTGCTAATAGTTCTGCGCATC	50	/ **894**	
		$stxA_2$ (A sub-unit of Shiga toxin-2)	CTTCGGTATCCTATTCCCGG			
			GGATGCATCTCTGGTCATTG		/ **478**	
E. coli STEC / L04539 and Z36901, L11078, M21534, X60439	(Pass et al., 2000)	$stxA_1$	ACGTTACAGCGTGTTGCRGGGATC			Multiplex (4) direct on cells Agarose Nusieve 3% EtBr 1 µg/ml / Dog feces after pre-incubation
			TTGCCACAGACTGCGTCAGTRAGG	63	/ **121**	
		$stxA_2$	TGTGGCTGGGTTCGTTAATACGGC			
			TCCGTTGTCATGGAAACCGTTGTC		/ **102**	
		$stxA_{2e}$- $stxB_{2e}$	CCAGAATGTCAGATAACTGGCGAC			
			GCTGAGCACTTTGTAACAATGGCTG		/ **322**	
		*eae*A	TGAGCGGCTGGCATGAGTCATAC			
			TCGATCCCCATCGTCACCAGAGG		/ **241**	
E. coli STEC / AJ010730	(Bielaszewska et al., 2000; Morabito et al., 2001; Schmidt et al., 2000), 128-1 & -2	$stxA_{2f}$	AGATTGGGCGTCATTCACTGGTTG	57		PCR
			TACTTTAATGGCCGCCCTGTCTCC		/ **428**	
E. coli STEC / M17358, M29153, X07865 & AB035924	(Wang et al., 2002)	stx_1	TCTCAGTGGGCGTTCTTATG		777-796	Multiplex (4) PCR, *a*garose gel
	Stx1-a, Stx1-b		TACCCCCTCAACTGCTAATA		1114-1095 / **338**	
	Stx2f-a	stx_{2f}	TGTCTTCAGCATCTTATGCAG		300-320	
	Stx2f-b		CATGATTAATTACTGAAACAGAAAC		449-425 / **150**	
	Stx2-a	stx_2	GCGGTTTTATTTGCATTAGC		1228-1247	
	Stx2-b		TCCCGTCAACCTTCACTGTA		1342-1323 / **115**	
	E16S-a	*E. coli* 16S rRNA	CCCCCTGGACGAAGACTGAC		1682-1701	
	E16S-b		ACCGCTGGCAACAAAGGATA		2082-2063 / **401**	
E. coli STEC / M19473, X07865	(Ge et al., 2002)	$stxA_1$ & $stxA_2$, *A* subunit, universal primer-pair, F1 & R1	GCAGATACAGAGAGAATTTCGT	50		PCR, agarose 2% EtBr
			CTGATGATGGCAATTCAGTAT		/ **200**	
E. coli STEC / M59432, M36727, Z11541 & AB035924	(Wang et al., 2002)	stx_{1c}	GCGGTTTTATTTGCATTAGT		1186-1205	Multiplex (4) PCR, *a*garose gel
	Stx2c-a, Stx2c-b		AGTACTCTTTTCCGGCCACT		1309-1290 / **124**	
	Stx2e-a	stx_{2e}	ATGAAGTGTATATTGTTAAAGTGGA		204-228	
	Stx2e-b		AGCCACATATAAATTATTTCGT		506-485 / **303**	
	EAE-a	*eae*A	ATGCTTAGTGCTGGTTTAGG		132-151	
	EAE-b		GCCTTCATCATTTCGCTTTC		379-360 / **248**	
	E16S-a	*E. coli* 16S rRNA	CCCCCTGGACGAAGACTGAC		1682-1701	
	E16S-b		ACCGCTGGCAACAAAGGATA		2082-2063 / **401**	
E. coli STEC and other stx_2 hosts	(Kurokawa et al., 1999; Miyagi et al., 2001), EVS-1 & EVS-2	$stxA_2$ (A subunit of the VT2 toxin)	ATCAGTCGTCACTCACTGGT		454-473	PCR (direct *in situ*) / Environment
			CCAGTTATCTGACATTCTG	55	839-857 / **404**	
E. coli STEC and *Shigella dysenteriae* Type 1	(Jackson, 1991; Olsen et al., 1995)	$stxA_1$ & *stxA*, A subunit of shiga like toxin *slt*-I and shiga toxin	GACAGGATTTGTTAACAGG			PCR, agarose 1% EtBr, southern blot & DIG labeling/detection
			TTCCAGTTACACAATCAGGC	55	/ **680**	
E. coli STEC EHEC O157:H7 ATCC 43895 (EDL933) & SEA 6347 / X07903 & X61283	(Jinneman et al., 1995)	$stxA_1$-$stxB_1$ amplicon spanning from 3' of A subunit through the 5' of the B subunit	GCAATTCTGGGAAGCGTGGC		885-904	PCR agarose 1.5% EtBr 0.8 µg/ml
			CACATTCAGGCGTCGCCAGC	59	1158-1139 / **274**	
	JIB5/6	$stxA_2$-$stxB_2$ amplicon spanning from 3' of A subunit through the 5' of the B subunit	CTGGGGGCGAATCAGCAATG	59	832-855	
			CCGCCGCCATTGCATTAACA		1195-1176 / **364**	
E. coli STEC non-O157:H7	(Louie et al., 1998), SLTI-F & -R (Gannon et al., 1992), SLTII-F & -R (Gannon et al., 1992)	$stxA_1$-$stxB_1$	ACACTGGATGATCTCAGTGG		938-957	Multiplex (3), *a*garose gel 1 % EtBr / Stools
			CTGAATCCCCCTCCATTATG	56	1539-1520 / **614**	
		$stxA_2$-$stxB_2$ all variants (silent mismatch)	CCATGACAACGGACAGCAGTT		624-644	
			CCTGTCAACTGAGCACTTTG		1403-1384 / **779**	
		*eae*A specific 3' end	AAGCGACTGAGGTCACT		2442-	
			ACGCTGCTCACTAGATGT		- 2917 / **450**	
	$eaeA_{O157:H7}$-F & -R (Louie et al., 1994)		ACGTTACTGGTGACTTA		2508 -	
			TATTTATCAGCTTCAGT		- 2917 / **400**	
	$eaeA_{O111:H8}$-F & -R (Louie et al., 1994)		CAGAATGGTTATGCTACTGT			
	$eaeA_{O26:H11}$-F & -R		CTTACATTTGTTTTCGGCATC		/ **450**	
E. coli STEC O113 AF1722324 AF1722324	(DebRoy et al., 2004) O113 *wzx* O113 *wzy*	*wzx* O-antigen flipase gene; *wzy* O-antigen polymerase gene	GGGTTAGATGGAGCGCTATTGAGA			Simplex PCR, agarose 1% EtBr
			AGGTCACCCTCTGAATTATGGCAG	60	/ **771**	
			GCATGTATGATGCATAGCTTCGCC			
			TGATATCGTTCGCTAACCACCCA	60	/ **419**	
E. coli STEC O113	(Paton et al., 1999a; Paton et al., 1999b) O113F & R	*rfb* O-antigen biosynthesis loci; *wzy* O-antigen polymerase gene	AGCGTTTCTGACATATGGAGTG	65 & 60	3690-3712	PCR, agarose 2% EtBr
			GTGTTAGTATCAAAAGAGGCTCC		4259-4282 / **593**	
E. coli STEC O157 & O111	(Paton et al., 1998), O157F & R	$rfbE_{O157:H7}$	CGGACATCCATGTGATATGG	65 &	393–413	Multiplex (2) TD Agarose 2% EtBr / Stool &
			TTGCCTATGTACAGCTAATCC		630-651 / **259**	

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
	O111F & R	*rfb* region ORF 3.4 of *E. coli* O111	TAGAGAAATTATCAAGTTAGTTCC ATAGTTATGAACATCTTGTTTAGC	60	24–48 405-429 / **406**	food
E. coli STEC O157:H7	(Holland et al., 2000), SLTI-F & -R (Gannon et al., 1992),	$stxA_1$	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	56	938-957 1539-1520 / **614**	Multiplex (3), *a*garose 1 % EtBr / Stools direct
	SLTII-F & -R (Gannon et al., 1992),	$stxA_2$ *eaeA* O157:H7-specific 3' end	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCACTTTG AAGCGACTGAGGTCACT		624-644 1403-1384 / **779** 2442-	
	$eaeA_{O157:7}$-F & -R (Louie et al., 1994; Louie et al., 1998; Osek, 2003), 16S-F & -R (Osek, 2003)	16S rDNA not used in the multiplex	ACGCTGCTCACTAGATGT AGAGTTTGATCATGGCTCAG GGACTACCAGGGTATCTAAT		- 2917 / **450** / **798**	
E. coli STEC O26 AF529080 AF529080	(DebRoy et al., 2004) O26 *wzx* O26 *wzy*	*wzx* O-antigen flipase gene; *wzy* O-antigen polymerase gene	GCGCTGCAATTGCTTATGTA TTTCCCCGCAATTTATTCAG TAAATTGCGGGGAAAGAATG GACTTCATGGGTACCGCCTA	54 60	 / **152** / **276**	Simplex PCR, agarose 1% EtBr
E. coli STEC producing Stx1	(Schmidt et al., 1997) (Karch et al., 1999), 356 & 285	$stxA_1$ 5'end of the gene and the adjacent upstream region	GCAGGATGACCCTGTAACGAAG CCTTCGCCACCACATTAACTG	52	 / **400**	PCR, agarose
E. coli STEC producing Stx2	(Karch et al., 1999), 545(F) 422(R) & 457(R)	stx_2 5'end of the gene and the adjacent upstream region	CATGAAGAAGATGTTTATGGGG (F) GGTCTTTTGCATCTGTCTGG (R) CACCCCGTTCTCATCCGTCATG (R)	52	8356-8377 10754-10735 / ? 10177-10096 / ?	PCR, agarose
E. coli STEC, EHEC, EPEC, ETEC	(Kong et al., 1999), VT-F & VT-R, Pho-F & Pho-R LT2-F & LT2-R EAE-F & EAE-R LT1-F & LT1-R ST1-F & ST1-R	$stxA_1$ & $stxA_2$, A subunit, universal primer-pair *phoA* alkaline phosphatase *elt* II heat-labile enterotoxin *eae* intimin *elt* I heat-labile enterotoxin *est* I heat-stable enterotoxin	GAACGAAATAATTTATATGTG CCTGATGATGGCAATTCAGTA GTGACAAAAGCCCGGACACCATAAATGCCT TACACTGTCATTACGTTGCGGATTTGGCGT ATATCATTTTCTGTTTCAGCAAA CAATAAAATCATCTTCGCTCATG GGAACGGCAGAGGTTAATCTGCAG CGAAGCCATTTGCTGGGCGCTC TTACGGCGTTACTATCCTCTCTA GGTCTCGGTCAGATATGTGATTC CTTTCCCCTCTTTTAGTCAG TAACATGGAGCACAGGCAGG		 / **523**-**520** / **903** / **720** / **360** / **275** / **175**	Multiplex (6) Agarose 2%, EtBr / Sea water
E. coli STEC, EPEC & Enterobacteriaceae	(Anglès d'Auriac et al., 2006), Meca202UU20 & Meca633LU21 UstxU1 & UstxL1 UstxU3 UstxL3 eae28UU18 eae748LU21	*rfe* (*wec* A) for the Enterobacterial common antigen (ECA) $stxA_1$, $stxA_2$ and variants, $stxA_{2f}$ universal stx degenerate primers *eae* conserved 5' area used for the construction	GGGTTRTCCWGCGTCTCRTT TATTCTGCCRKYACGCCWAYK TRTTGARCRAAATAATTTATATGT MTGATGATGRCAATTCAGTAT AATGGAACGGAATAACTTATATGT GGTTGAGTGGCAATTAAGGAT ACCCGGCACAAGCATAAG CGTAAAGCGRGAGTCAATRTA	57	202-223 633-653 / **452** / **523**-**526** / **524** 28-45 748-768 / **741**	Multiplex (3) PCR, agarose 1.7% EtBr 1 µg/ml
E. coli STEC/EHEC, EPEC & EAEC	(China et al., 1996), B54 & B55 B56 & B57 B52 & B53	$stxA_1$ (A sub-unit of Shiga toxin-1) $stxA_2$ (A sub-unit of Shiga toxin-2) *eaeA* (intimin)	AGAGCGATGTTACGGTTTG TTGCCCCCAGAGTGGATG TGGGTTTTTCTTCGGTATC GACATTCTGGTTGACTCTCTT AGGCTTCGTCACAGTTG CCATCGTCACCAGAGGA	50	 / **388** / **807** / **570**	Multiplex (3) PCR, *a*garose 2% EtBr
E. coli STEC/EHEC, EPEC, EAEC & ETEC / Z36899, L11078, Z11541, S90827, X14354, M35282, M25607	(Franck et al., 1998)	$stxA_1$ (A sub-unit of Shiga toxin-1) $stxA_2$ (A sub-unit of Shiga toxin-2) *eaeA* (intimin) F41 (fimbriae) K99 (fimbriae) *sta* (heat stable enterotoxin)	TTCGCTCTGCAATAGGTA TTCCCCAGTTCAATGTAAGAT GTGCCTGTTACTGGGTTTTTCTTC AGGGGTCGATATCTCTGTCC ATATCCGTTTTAATGGCTATCT AATCTTCTGCGTACTGTGTTCA GCATCAGCGGCAGTATCT GTCCCTAGCTCAGTATTATCACCT TATTATCTTAGGTGGTATGG GGTATCCTTTAGCAGCAGTATTTC GCTAATGTTGGCAATTTTTATTTCTGTA AGGATTACAACAAAGTTCACAGCAGTAA	50	125-142 659-679 / **555** 30-53 128-147 / **118** 992-1013 1395-1416 / **425** 34-51 390-413 / **380** 21-40 311-334 / **314** 9-36 171-198 / **190**	Multiplex (6) PCR, agarose 3% EtBr
E. coli STEC-EPEC O157:H7 EDL933 O127:H6 E2348/69 / Z11541 M58154 & *M34051* / US 5,652,102	(Gannon et al., 1993) (Sunabe et al., 1998) AE9-AE10 AE11-AE12 AE13-AE14 AE15-AE16 AE17-AE18 AE19-AE20	*eae* conserved 5' area used for the construction *eaeA* variable 3' area used for construction of EHEC specific primers (O157:H7 & O55 specific, γ type)	ACGTTGCAGCATGGGTAACTG GATCGGCAACAGTTTCACCTG CCCGGCACAAGCATAAGCTAA ATGACTCATGCCAGCCGCTCA GTGGCGAATACTGGCGAGACT CCCCATTCTTTTTCACCGTCG TTCTGTCGAATGGTCAGGTGGT ACCAGAAGAAGCATCCACCGA CATGGCGCTCAGCAAATCCAG TGGTACCAGCCTCGGGATTGG CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	55-60	1054-1074 1869-1849 / **815** 1672-1692 2592-2572/ **924**? 2518-2538 3408-3388 / **890** 3286-3307 4073-4053 / **787** 4024-4044 4803-4783 / **779** 3534-3554 / **1087**	PCR, agarose gel 1 to 1.6 %, EtBr 0.25 µg/mL
E. coli UPEC and DAEC	(Le Bouguenec et al., 2001) afa-f & afa-r	*afa* afimbrial adhesive sheats	CGGCTTTTCTGCTGAACTGGCAGGC CCGTCAGCCCCCACGGCAGACC		 / **672**	PCR
E. coli VTEC	(Lin et al., 1993), (Hopkins et al., 2000) (Schmidt et al., 2000), Up & down	$stxA_1$ & $stxA_2$ genes and there variants: universal primer-pair overlaping on the *stxB* subunit	GAACGAAATAATTTATATGT AAATTACCAATGTCAGTA	43	280-299 1184-1167 / **880**-**900**	PCR, polyacrylamide gel 5%, EtBr
E.coli / / Hoffmann-La Roche Inc US5298392 Claim 12	(Bej et al., 1991a), UAL-1939 & UAR-2105	*uidA*: β -D-glucuronidase	TATGGAATTTCGCCGATTTT TGTTTGCCTCCCTGCTGCCG	50	1939-1958 2085-2104 / **166**	PCR, agarose gel EtBr
E.coli / M84024 & M84025	(McDaniels et al., 1996)	*gadA* & *gadB* Glutamate decarboxylse: 2 very similar copies per genome. Conserved GAD A/B primers	ACCTGCGTTGCGTAAATA GGGCGGGAGAAGTTGATG	58	307-324 959-976 / **670**	PCR, agarose gel 1 % EtBr
E.coli / M14641	(McDaniels et al., 1996; Rappelli et al., 2001)	*uidA* : β -D-glucuronidase	CCAAAAGCCAGACAGAGT GCACAGCACATCAAAGAG	58	1066-1083 1671-1689 / **624**	PCR, agarose gel 1 %, EtBr
E.coli Y10412	(Hofinger et al., 1998), colD-1 & colD-2	Colicin D PColD157 activity	GTAAATCTGCCTGTTCGTGGAC CCTTTTTCTCTTCGGTATGTTC	57	 / **587***	PCR, randomly digoxigenin labeled probe & Southern blot

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
E.coli, Hoffmann-La Roche Inc US5298392 Claim 11	(Bej et al., 1991a), UAL-754 & UAR-900	*uidA* : β -D-glucuronidase	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTACAGTCTTGCG	50	754-773 880-900 / **147**	PCR, agarose gel EtBr
EHEC O157:H7 / US 5,652,102	(Fratamico et al., 2000) (Meng et al., 1997), SLTII-F & R(Meng et al., 1997), FLICH7-F&R (Gannon et al., 1997), AE22 & AE20-2 (Fratamico et al., 1998), MFS1F & R (Fratamico et al., 1995)	stx_1 $stxA_2$ $fliC_{h7}$ *eaeA* *hlyA* 60-Mda plasmid (enterohemolysin)	TGTAACTGGAAAGGTGGAGTATACA GCTATTCTGAGTCAACGAAAAATAAC GTTTTTCTTCGGTATCCTATTCC GATGCATCTCTGGTCATTGTATTAC GCGCTGTCGAGTTCTATCGAGC CAACGGTGACTTTATCGCCATTCC ATTACCATCCACACAGACGGT ACAGCGTGGTTGGATCAACCT ACGATGTGGTTTATTCTGGA CTTCACGTCACCATACATAT	57	/ **210** / **484** 69-91 671-694 / **625** / **397** / **166**	Multiplex (5) Agarose 1.5%, EtBr / Food
Enterobacteriaceae	(Loge et al., 1999) ENT1	16S rRNA	CUCGCGAGAGCAAGCGG* (Target)		1273-1289	Insitu probe hybridization / Water
Enterobacteriaceae / AE000455	(Bayardelle et al., 2002) / RFFT7 & RFFT8 O157F & R (Paton A.W. (Paton et al., 1999b)) Shig-1 & Shig-2 ((Vargas et al., 1999),(Bischoff et al., 2005))	*rff* (*wec* F) for the Enterobacterial common antigen (ECA) *rfbE* $_{O157:H7}$ O-antigen biosynthesis loci ipaH Invasion Plasmid Antigen, multiple copies	CGGCTTAACTCCTACAGTCAG GAAAGTAGACCACCAGCATCG CGGACATCCATGTGATATGG TTGCCTATGTACAGCTAATCC TGGAAAAACTCAGTGCCTCT CCAGTCCGTAAATTCATTCT	55	8939-8959 9041-9021 / **103** 393–413 630-651 / **259** 1063-1083 1466-1485 / **423**	Multipplex (3) / Clinical
Enterococcus & Universal, J01690	(Ke et al., 1999), Universal denerate U1 & U2 Ent1 & Ent2	*tuf* , chromosomal, elongation factor EF-Tu	AAYATGATIACIGGIGCIGCICARATGGA AYRTTITCICCIGGCATIACCAT TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	55 for an. & ext	271-300 1051-1073 / **803** 618-639 708-729 / **112**	PCR (nested) agarose 2 % TBE, EtBr 0.5 µg/ml
Enterococcus durans, Enterococcus hirae	(Knijff et al., 2001), DuHiff, DuR & HiR	*ddl*, chromosomal D-Ala D-Ala ligase for peptidoglycan final synthesis step (sensitive to glycopeptides)	TTATGTCCCWGTWTTGAAAAATCAA TGAATCATATTGGTATGCAGTCCG TTTTGTTAGACCTCTTCCGGA		485-510 649-672 / **186** 845-868 / **377**	Multipplex (2), agarose 1.5% in TAE EtBr staining 0.5µg/ml
Enterococcus faecalis	(Betzl et al., 1990), DB8	23S rRNA	TAGGTGTTGTTAGCATTTCG*	42-47 (Hyb-wash)		
Enterococcus faecalis	(Teng et al., 2001)	*groESL* heat shock proteins, signature sequence used for specific detection	GGAATTGTTCTTGCATCCGT ACAATTAAGTATTCTACGCC	52	67-86 251-232 / **185**	PCR, agarose 1.5 % , EtBr
Enterococcus faecalis / AF152237; *Enterobacteriaceae* / M76129 M87049	(Anglès d'Auriac et al., 2006), efam 1U & 1L,	*eep*, Chromosomal gene involved in the production of the peptide sex pheromone cAD1 (An et al., 1999)	AATGCCGTGGGTAATGTGGTT GGCTTTTCGGGGTTCTTCTG		855-875 1329-1348 / **494**	Multiplex (4) PCR, agarose 1.7% EtBr 1 µg/ml
AE000454 S75640	Meca582UU18 Meca826LU21	*rfe* (*wec* A) for the Enterobacterial common antigen (ECA)	TTCCCGYCAGGCRTTTGT CMGGYAWTGGTTGTGTCATCR	55	582-599 826-846 / **265**	
Enterococcus faecium	efuaac1U & 1L	*aac(6')-Ii*, chromosomal aminoglycoside acetyl transferase (Costa et al., 1993)	GGGGGAAGACGTATGATAATC TCGGGAGCTTTCTACAACTAA		191-211 428-448 / **258**	
E. coli	gad259U21 gad402L17	*gadAB*	AAAGAAGAATATCCGCAATCC GCCATTTCATCGCCATC		259-279 402-418 / **160**	
Enterococcus faecium	(Anglès d'Auriac et al. unpublished), efuph 1U & 1L	Putative pheromone (Franz et al., 1999b)	ATAGCCATAGCCCATACCATA AGGAAGTTTTTGACCACGAT		1917-1937 2074-2093 / **177**	PCR, agarose 1.7 %, EtBr 0.5 µg/ml
Enterococcus faecium	(Betzl et al., 1990), DB6	23S rRNA	CACACAATCGTAACATCCTA*	42-47 (Hyb-wash)		
Enterococcus faecium / faecalis -glycopeptide resistance	(Modrusan et al., 1999), van*A*811L-27 Van*B*467-27	*VanA* and *VanB-B2* glycopeptide resistance genes	TTAATAACCC aaa a GGCGGGAGTAGCT* TACATTCTTAC aaa a AATGCGGGCATC*	59		Cycle Probe Technology (CPT), 5' terminal labeled with [γ-^{32}P]-ATP. 20% polyacrylamide, 7M urea denaturating gel
Enterococcus faecium / L78127	(Cheng et al., 1997), EM1A & B	Randomly selected species specific E. faecium DNA sequence, unknown function	TTGAGGCAGACCAGATTGACG TATGACAGCGACTCCGATTCC		310-330 947-967 / **658**	PCR, agarose 1.2 % TBE, EtBr 0.5 µg/ml
Enterococcus faecium / Z49244 / US5,994,066	(Bergeron et al., 1999)	*sod* , superoxide dismutase	ACGCAACAATGGTGGTGGACA TCTTGATTTGCAGTAGAGGTAATAG			PCR
Enterococcus gallinarum	(Clark et al., 1998b) (Angeletti et al., 2001) (Satake et al., 1997)	*vanC-1*, D-Ala D-Ser ligase for peptidoglycan final synthesis step (constitutive low resistance to glycopeptides)	GAAAGACAACAGGAAGACCGC ATCGCATCACAAGCACCAATC	58	/ **796**	Multiplex (3)
Enterococcus casseliflavus	vanC2-1 & -2	*vanC-2*	CGGGGAAGATGGCAGTAT CGCAGGGACGGTGATTTT		/ **484**	
Enterococcus flavescens		*vanC-3*,	GCCTTTACTTATTGTTCC GCTTGTTCTTTGACCTTA		/ **224**	
Enterococcus - glycopeptide resistance	(Dutka-Malen et al., 1995) / A_1 & A_2, (Angeletti et al., 2001; Roger et al., 1999; Satake et al., 1997) (Depardieu et al., 2004)	*vanA* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin & teicoplanin) *vanB* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin)	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA ATGGGAAGCCGATAGTC GATTTCGTTCCTCGACC	54	175-191 907-891 / **732** 173-189 807-791 / **635**	Multiplex (6) PCR, agarose 1.5 %, EtBr 0.5 µg/ml / Clinical isolates
Enterococcus gallinarum	B_1 & B_2 (Roger et al., 1999) (Angeletti et al., 2001) (Satake et al., 1997)	*vanC-1*, D-Ala D-Ser ligase for peptidoglycan final synthesis step (constitutive low resistance to glycopeptides)	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT		246-272 1067-1051 / **822**	
Enterococcus casseliflavus & flavescens	(Dutka-Malen et al., 1995) (Isenberg, 1998)	*vanC-2* & *vanC-3*, D-Ala D-Ser ligase for peptidoglycan final synthesis step (constitutive	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG		455-486 885-869 / **439**	

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	p662, C_1 & C_2	low resistance to glycopeptides)				
Enterococcus faecalis		*ddl*$_{E. faecalis}$, chromosomal D-Ala D-Ala ligase for peptidoglycan final synthesis step (sens. to glycopeptides)	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG		98-116 1038-1021 / **941**	
Enterococcus faecium	(Dutka-Malen et al., 1995) / (Isenberg, 1998)p662, D_1 & D_2	*ddl*$_{E. faecium}$, chromosomal D-Ala D-Ala ligase for peptidoglycan final synthesis step (sens. to glycopeptides)	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC		/ **550**	
	(Dutka-Malen et al., 1995), E_1 & E_2 (Angeletti et al., 2001) (Satake et al., 1997)					
	(Dutka-Malen et al., 1995), F_1 & F_2 (Angeletti et al., 2001) (Satake et al., 1997)					
Enterococcus - glycopeptide resistance	(Patel et al., 1997) vanA-FOR & vanAB-REV	*vanA* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin & teicoplanin)	CATGACGTATCGGTAAAATC ACCGGGCAGRGTATTGAC	56	/ **885**	Multiplex (4) PCR-RFLP (*Msp*I), agarose 1.5 %, EtBr 0.5 µg/ml
	vanB-FOR & vanAB-REV	*vanB* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin)	CATGATGTGTCGGTAAAATC ACCGGGCAGRGTATTGAC		/ **885**	
Enterococcus gallinarum	vanC123-FOR & vanC1-REV	*vanC-1*, D-Ala D-Ser ligase for peptidoglycan final synthesis step (constitutive low resistance to glycopeptides)	GATGGCWGTATCCAAGGA GTGATCGTGGCGCTG		/ **467**	
Enterococcus casseliflavus & flavescens	vanC123-FOR & vanC23-REV	*vanC-2* & *vanC-3*, D-Ala D-Ser ligase for peptidoglycan final synthesis step (constitutive low resistance to glycopeptides)	GATGGCWGTATCCAAGGA ATCGAAAAAGCCGTCTAC		/ **429**	
Enterococcus - glycopeptide resistance	(Petrich et al., 1999), VanA1& VanA2 VanA3	*vanA* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin & teicoplanin)	Biotin-GCTGCGATATTCAAAGCTCA CAGTACAATGCGGCCGTTA	50	/ **545**	Multipplex (2) EIA detection in microtiter plates with anti-FITC HRP conjugate
	(Petrich et al., 1999) (Dutka-Malen et al., 1995), VanB1& VanB3 VanB4	*vanB* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin)	ATTGCGTAGTCCAATTC-Fluorescein* Biotin-ATGGGAAGCCGATAGTC GTTACGCCAAAGGACGAAC GACAATTCAAACAGACC-Fluorescein*		/ **368**	
Enterococcus - glycopeptide resistance / M97297	(Depardieu et al., 2004) / (Dutka-Malen et al., 1995) EA1 & EA2	*vanA* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin & teicoplanin)	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA		176-192 907-891 / **732**	
	(Depardieu et al., 2004) / EB3 & EB4	*vanB* , D-Ala D-Lac ligase for peptidoglycan final synthesis step (aquired high resistance to vancomycin)	ACGGAATGGGAAGCCGA TGCACCCGATTTCGTTC		169-185 815-799 / **647**	
Enterococcus gallinarum, E. casseliflavus & E. flavescens / AF162694/L29638	(Depardieu et al., 2004) / EC5 & EC8	*vanC1 & 2/3*, D-Ala D-Ser ligase for peptidoglycan final synthesis step (constitutive low resistance to glycopeptides)	ATGGATTGGTAYTKGTAT TAGCGGGAGTGMCYMGTAA TGTGGGATGCGATATTCAA		133-150/142-159 & 947-929/ 968-950 **815/827**	
Enterococcus - glycopeptide resistance / AF130997/AY082011 AF430807	(Depardieu et al., 2004) / ED1 & ED2	*vanD* , moderate resistance to vancomycin & teicoplanin	TGCAGCCAAGTATCCGGTAA TGTGGTATCGGAGCTGCAG		357-375 856-837 **/ 500**	
	(Depardieu et al., 2004) / EE1 & EE2	*vanE* , low resistance to vancomycin & teicoplanin	ATAGTTTAGCTGGTAAC CGGCATCCGCTGTTTTTGA	54	364-382 793-777 **/ 430**	Multiplex (10) PCR, agarose 1%, EtBr
AY271782		*vanG* , low resistance to vancomycin & teicoplanin	GAACGATAGACCAATGCCTT CACCTGAAGAAACAGGC		68-86 1008-989 **/ 941**	
Enterococcus faecalis	(Depardieu et al., 2004) / EG1 & EG2	*ddl*$_{E. faecalis}$, chromosomal D-Ala D-Ala ligase for peptidoglycan final synthesis step (sens. to glycopeptides)	ATGGCTACTTCAATTTCACG GAGTAAATCACTGAACGA		206-222 680-661 **/ 475**	
Enterococcus faecium	(Depardieu et al., 2004) / DD13 & ED3-2	*ddl*$_{E. faecium}$, chromosomal D-Ala D-Ala ligase for peptidoglycan final synthesis step (sens. to glycopeptides)	CGCTGATGGTATCGATTCAT GACTATTATTGGTTGATCCACCTG		1-18 1091-1072 **/ 1091**	
Staphylococcus aureus / V01281	(Depardieu et al., 2004) / FAC1-1 & FAC2-1	Thermonuclease *nuc* gene	GCCTTGACGAACTAAAGCTTCG ATCAAAAAGTTGGCGAACCTTTTCA		350-363 567-554 **/ 218**	
Staphylococcus epidermis	/ Tn1 & Tn2 Se705-1 & Se705-2	Specific chromosomal fragment	CAAAAGAGCGTGGAGAAAAGTATCA		21-45 145-121 **/ 125**	
Enterococcus species	(Baele et al., 2000), T5A & T3B*(TET)	tRNA genes flanking conserved edges	AGTCCGGTGCTCTAACCAACTGAG AGGTCGCGGGTTCGAATCC	50	/ **variable**	PCR, tRNA intergenic spacer PCR, capillary electrophoresis of amplicons
Enterococcus species Z49243 E. faecalis Z49244 E. faecium, Srtreptococcus species & Gram positive bacteria	(Poyart et al., 2000) (Poyart et al., 1995), d1 & d2 (Poyart et al., 1998)	*sod* , superoxide dismutase Universl degenerated primers for the amplification of a 480 Nu internal fragment *sodA*$_{int}$	CCITAYICITAYGAYGCIYTIGARCC ARRTARTAIGCRTGYTCCCAIACRTC	37	/ **480**	PCR (broad range) with degenerate primers, sequencing of the product and comparison to a database / Clinical
Enterococcus spp. & *E. coli*	(Frahm et al., 2003)	23S rRNA	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT FAM-	60 for an.		TaqMan PCR / Water samples after filtration and 18h pre-incub.

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		uidA: β -D-glucuronidase	TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA GTGTGATATCTACCCGCTTCGC AGAACGGTTTGTGGTTAATCAGGA FAM-TCGGCATCCGGTCAGTGGCAGT-TAMRA	& ext		
Eubacteria universal	(Corless et al., 2000)	16S rDNA signature	CCATGAAGTCGGAATCGTTAG ACTCCCATGGTGTGACGG FAM-CGGTGAATACGTTCCCGGGCCTTGTAC-TAMRA	60	1320-1341 1413-1431 / **112**	TaqMan real time PCR
Eubacteria universal	(Greisen et al., 1994), DG74 & PLO6 RW01 RDR080	16S rDNA signature	AGGAGGTGATCCAACCGCA GGTTAAGTCCCGCAACGAGCGC AACTGGAGGAAGGTGGGGAT AACTGGAGGAAGGTGGGGAC	60	1522-1540 1088-1109 / **453** 1170-1189 1170-1189	PCR (broad range) & hybridization 2% NuSieve-0.5% Agarose gel
Gram + Gram –	(Carroll et al., 2000) / 16SF 16SR NF NR P2F N6R	16S rRNA	TTGGAGAGTTTGATCCTGGCTC ACGTCATCCCCACCTTCCTC GGCGGCAKGCCTAAYACATGCAAGT GACGACAGCCATGCASCACCTGT GCGRCTCTCTGGTCTGTA GGTGCCTTCGGGAAC	54.2 & 60	4-25 1174-1194 42-66 1044-1067 712-729 1013-1027	PCR nested & Multiplex (2) Universal NF-NR (**1025) +** GRAM+ P2F-NR (**355**) Simplex GRAM- NF-N6R (**985**) Agarose 1% / Ocular pathogens
Gram + Gram –	(Klausegger et al., 1999), DG74 (Greisen K. (Greisen et al., 1994)) & 65ab DG74 & 143 (Gram+) DG74 & 68d (Gram-)	16S rRNA universal signature (DG74) 16S rRNA universal signature (65ab) 16S rRNA gram + signature (143) 16S rRNA gram + signature (68d)	AGGAGGTGATCCAACCGCA AACTGGAGGAAGGTGGGGAY GAYGACGTCAARTCMTCATGC AYGACGTCAAGTCMTCATGG	69 65 69	1522-1540	PCR (broad range), identification by sequencing of the product
Listeria monocytogenes / *AF067409*	(Deneer et al., 1991) / Lis-1 & Lis-2	*hlyA* Listeriolysin O,	GCATCTGCATTCAATAAAGA TGTCACTGCATCTCCGTGGT	60	130-149 284-303 / **174**	PCR,agarose 1.4 % EtBr
Listeria monocytogenes / *AF067409*	(Winters et al., 1999), SK6 & AP4	aminopeptidase	GGTCGGTGCATTAATAAG CAAGAGTTACAAATTACACC	52	542-559 612-631 / **90**	PCR, NuSieve agarose gel 4 %, EtBr
Mycobacterium bovis / X61270	(Wards et al., 1995), Bw6 Bw7 Bw8 Bw9	IS*1081* chromosomal Insertion sequence (6 copies)	CGACACCGAGCAGCTTCTGGCTG GTCGCCACCACGCTGGCTAGTG ACAGGCGAGCCCGGATCTGCTG GTTCAGCTCGCTTGCGGCGCTG	68 68	405-427 710-689 / **306** 438-459 685-664 / **248**	PCR followed by nested PCR Agarose 1.4 %, EtBr.
Mycobacterium tuberculosis	(Ahmed et al., 1998), Bw6 Bw7	IS*1081* chromosomal Insertion sequence (6 copies)	CGACACCGAGCAGCTTCTGGCTG GTCGCCACCACGCTGGCTAGTG	68	405-427 710-689 / **306***	PCR, agarose 1.4 %, EtBr, followed with Southern blot with 306 $_{32}$P-amplicon
Mycoplasma pneumoniae & M. genitalium	(Cadieux et al., 1993; Tong et al., 1999), P4A & B G3A & B H6A & B	P1 adhesin gene (*M. pneumoniae*) P1 adhesin gene (*M. genitalium*) Human mitochondrial cytochrome oxidase subunit 3	AGGCTCAGGTCAATCTGGCGTGGA GGATCAAACAGATCGGTGACTGGGT GCTTTAAACCTGGTAACCAGATTGACT GAGCGTTAGAGATCCCTGTTCTGTTA ATGACCCACCAATCACATGCCTATCA ACTAGTTAATTGGAAGTTAACGGTACTA	65	3947- -4291 / **345** 3754- -4260 / **507** 9207- -10034 / **828**	Multiplex (3), Agarose 1.5 %, EtBr, / Clinical / sputum
Mycoplasma pneumoniae, J01690 J01691(*E. Coli*)	(Luneberg et al., 1993), Mpn38 Mpn39 Mpn46*	*tuf* , chromosomal single copy, elongation factor EF-Tu	TACTCGTTACGACCAAATCGATAAG-Biotin GTTCAACTGTAATCGAGGTATTG TCCACGTGAGCGGAGTTAA-Dig*	60	132-156 1065-1087 / **950** 188-206	PCR, agarose, EtBr, Biotin / Streptavidin, Dig/AP microtiter plates
Pseudomonas aeruginosa	(Anglès d'Auriac et al. unpublished), PapU & PapL	Gene coding for the siderophore pyoverdin	GCAGCATGACTGAGCGCAAA GGCGAACCAGGCGGAGAC			PCR
Pseudomonas aeruginosa	(Khan et al., 1994), ETA1 & ETA2 ETA3 ETA7 ETA8	*eta* Gene coding for the exotoxin A, protein biosynthesis inhibition by ADP-ribosylation of the eukaryotic elongation factor II	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT AGCCACATGTCGCCGATCTACACC* TTCCGCTCCCCGCCAGCCTC AGTAGTGCAGCACGCCCTGG	68	1001-1024 1373-1396 / **396** 1199-1122 618-637 937-956 / **339**	PCR, agarose 1.5% / Water / clinical
Pseudomonas aeruginosa	(Song et al., 2000), ETA1 & ETA2 (Khan et al., 1994)	*eta* gene coding for the exotoxin A, protein biosynthesis inhibition by ADP-ribosylation of the eukaryotic elongation factor II	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	54	/ **367**	PCR, direct cell (95 °C 18' pre-heat), agarose gel, EtBr / Clinical (ocular infection)
Salmonella spp.	(Rahn et al., 1992) 139 141	*invA* , chromosomal, essential for invasion of epithelial cells by *Salmonella*	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC	53	287-312 571-550 / **284**	PCR, agarose 2%, EtBr (some F-)
Salmonella spp.	(Stone et al., 1994) (Feder et al., 2001) /	*invE* & *A* , chromosomal, essential for invasion of epithelial cells by *Salmonella*	TGCCTACAAGCATGAAATGG AAACTGGACCACGGTGACAA CTGGTTGATTTCCTGATCGC*	52	1219-1238 278-259 / **457** 106-125	PCR-hybridization, agarose 1.5% EtBr (*Y. pseudotuberculis* & *E. tarda* F+)
Salmonella spp., *Shigella* spp. & *E. coli* EIEC	(Vantarakis et al., 2000), 139 & 141 (Rahn et al., 1992), (Villalobo et al., 1998),	*invA* , chromosomal, essential for invasion of epithelial cells by *Salmonella VirA*	GTGAAATTATCGCCACGTTCGGGCAA TCATCGCACCGTCAAAGGAACC CTGCATTCTGGCAATCTCTTCACATC TGATGAGCTAACTTCGTAAGCCCTCC	50	287-312 571-550 / **284-275?** / **215**	Multiplex (2), agarose 3 %, EtBr 0.5 mg/ml
Salmonella spp./	(Chiu et al., 1996) SPVC-1 SPVC-2 INVA-1 & 2	*invA* , chromosomal, essential for invasion of epithelial cells by *Salmonella SpvC*, on a virulence plasmid	ACTCCTTGCACAACCAAATGCGGA TGTCTTCTGCATTTCGCCACCATCA ACAGTGCTCGTTTACGACCTGAAT AGACGACTGGTACTGATCGATAAT	56	505-528 1052-1075 / **571** 104-127 324-347 / **244**	Multiplex (2), Agarose 2 %, EtBr
Salmonella spp./ U43271, U43272,	(Ferretti et al., 2001; Manzano et al., 1998),	*invA* , chromosomal, essential for invasion of	GCTGCGCGCGAACGGCGAAG TCCCGGCAGAGTTCCCATT	58	586-605 954-972 / **389**	1) PCR, agarose 1.5%, EtBr, + Southern blot with

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
U43273 & M90846 / Patent pending	Salm 3 Salm 4 Salm 5*	epithelial cells by *Salmonella*	TTTGTGAACTTTATTGGCGG* (Probe labeled with Biotin / Fluorescein for Southern blot analysis with an antifluorescein-HRP)		697-716 for *S. thyphimurium* gene	fluorescein probe / antifluorescein-HRP 2) microtiter plates with DIG PCR products + Biotin labeled probes on streptavidin plates
Shigella *spp Universal primers + specific Ab for concentration*	(Peng et al., 2002)	16S rRNA DNA Universal consensus	AAACTCAAAGGAATTGAC GACGGGCGGTGTGTACAA	51	/ **500**	Beads + specific antibody to isolate prior to PCR
Shigella spp.	(Rafii et al., 1995),	*spa* virulence region (Orf 3)	AGCGATCTTACGTCTTG CGAGATGTGGAGGCAT		/ **118**	PCR, agarose 3% EtBr
Shigella spp. & EIEC / AF010147	(Villalobo et al., 1998)	*virA* *16S rDNA* bacteria signature	CTGCATTCTGGCAATCTCTTCACATC TGATGAGCTAACTTCGTAAGCCCTCC AGACTGCTACGGGAGGCAGCAGT GTTGCGCTCGTTGCGGGACTTAA	65	/ **215** / **755**	Multiplex (2) Agarose 1 %, EtBr 0.8 mg/ml / Mayonnaise
Shigella spp. and *Campylobacter* spp. and *E. coli* ETEC	(Oyofo et al., 1996) and (Oyofo et al., 1992), E-1 E-2, Pg50 pg3, LT1 LT2	*ipaH* (invasive plasmid antigen) a multiple copy sequence found on both the chromosome & the invasive plasmid of *Shigella* spp, *flaA* of *Campylobacter* and *elt* heat-labile enterotoxin (LT) of ETEC	GCTGGAAAAACTCAGTGCCT CCAGTCCGTAAATTCATTCT ATGGGATTTCGTATTAAC GAACTTGAACCGATTTG CCATACTGACCGCAATTG CCAGTCTATTACAGAACTATG	40 + 1.25% formamide	/ **424** / **450** / **300**	Multiplex (3), garose 2%, EtBr 0.5 µg/ml / Stool with chaotropic glass matrix DNA extraction
Staphylococci MRSA	(Killgore et al., 2000; Vannuffel et al., 1995), M1 M2, 16SRRI 16SRRII	*mecA* antimicrobial resistance gene producing PBP 2A with reduced affinity for β–lactam for *Staphylococci* *rRNA* universal used as a control	TGGCTATCGTGTCACAATCG CTGGAACTTGTTGAGCAGAG CAGCAGCCGCGGTAATAC CCGTCAATTCCTTTGAGTTT TGCTAAAGTTCAAAAGAGTATTTATAACAA CA TGTGCTTACAAGTGCTAATAATTCACC ATTATGGCTCAGGTACTGCTATCCACCCTCA AA*	 56 60	/ **310** / **409** (Universal)	PCR,TaqMan 5'nuclease PCR
Staphylococcus aureus	(Vannuffel et al., 1995), F1 & F2	*femA* gene required for the correct expression of methycillin resistance but is specifically present in all *S. aureus*	CTTACTTACTGGCTGTACCTG ATGTCGCTTGTTATGTGC	56	217-237 884-902 / **686**	PCR, agarose 2 %, EtBr 0.5 µg/ml
Staphylococcus aureus / J01859 (U) AFO33191 (Sa)	(Martineau et al., 1998), Sa442-1 & Sa442-2	Randomly selected species specific chromosomal DNA fragment + conserved 16S rRNA for universal bacterial amplification (control)	AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA (Sa) GGAGGAAGGTGGGGATGACG ATGGTGTGACGGGCGGTGTG (U)	 55 ann -ext	5-34 83-112 / **108** 1244-1263 1469-1489 / **241**	Multiplex, direct cell-PCR from re-grown isolates Agarose 2 % EtBr 0.5 µg/ml / Clinical
Staphylococcus aureus + *Staphylococcus epidermis* J01859 (U) AFO33191 (Sa) M13771 (*aac*(6')-*ph*(2'))	(Martineau et al., 2000), Se705-1 & Se705-2 Sa442-1 & Sa442-2	Randomly selected species specific chromosomal DNA fragment + conserved 16S rRNA for universal bacterial amplification (control) + antibiotic resistance locus	ATCAAAAAGTTGGCGAACCTTTTCA CAAAAGAGCGTGGAGAAAAGTATC (Se) AATCTTTGTCGGTACACGATATTCTTCACG CGTAATGAGATTTCAGTAGATAATACAACA (Sa) GGAGGAAGGTGGGGATGACG ATGGTGTGACGGGCGGTGTG (U)	55 ann -ext	21-45 145-121 / **125** 5-34 83-112 / **108** 1244-1263 1469-1489 / **241**	Multiplex (Se + Sa + U + one of the antibio. res. Primer set), direct cell-PCR from re-grown isolates PCR, agarose 2 % EtBr 0.5 µg/ml / Clinical
M60253 (*blaZ*)			TTGGGAAGATGAAGTTTTTAGA CCTTTACTCCAATAATTTGGC (*aac*(6')-*aph*(2'))		159-180 311-332 / **174** 511-531	
K02987 (*ermA*)			ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC (*blaZ*)		663-683 / **173** 370-392 487-508 / **139**	
U35228 (*ermB*)			TATCTTATCGTTGAGAAGGGATT CTACACTTGGCTTAGGATGAAA (*ermA*)		366-389 484-507 / **142**	
M17990 (*ermC*)			CTATCTGATTGTTGAAGAAGGATT GTTTACTCTTGGTTTAGGATGAAA (*ermB*)		214-235 382-403 / **190**	
X52594 (*mecA*)			CTTGTTGATCACGATAATTTCC ATCTTTTAGCAAACCCGTATTC (*ermC*)		1059-1085 1206-1232 / **174**	
X52593 (*mecA*)			AACAGGTGAATTATTAGCACTTGTAAG ATTGCTGTTAATATTTTTTGAGTTGAA (*mecA*)		891-910 1034-1053 / **163**	
X52085 (*msrA*)			TCCAATCATTGCACAAAATC AATTCCCTCTATTTGGTGGT (*msrA*)			
Staphylococcus epidermis / J01859 (U)	(Martineau et al., 1996), Se705-1 & Se705-2 Se705-3 & Se705-4	Randomly selected species specific chromosomal DNA fragment + conserved 16S rRNA for universal bacterial amplification (control)	ATCAAAAAGTTGGCGAACCTTTTCA CAAAAGAGCGTGGAGAAAAGTATC (Se) TCTCTTTTAATTTCATCTTCAATTCCATAG AAACACAATTACAGTCTGGTTATCCATATC (Se) GGAGGAAGGTGGGGATGACG ATGGTGTGACGGGCGGTGTG (U)	55 ann -ext	21-45 145-121 / **125** 448-477 622-593 / **175** 1244-1263 1469-1489 / **241**	Multiplex, direct cell-PCR from re-grown isolates Agarose 2 % EtBr 0.5 µg/ml / Clinical
Staphylococcus spp. & *S. aureus* MRSA	(Vannuffel et al., 1995), M1 M2, F1 F2, C1 C2	*mecA* antimicrobial resistance gene producing PBP 2A with reduced affinity for β-lactam for *Staphylococci* *femA* regulator gene essential for the expression of methicillin and specific to *S. aureus* IS*431* used for positive control	TGGCTATCGTGTCACAATCG CTGGAACTTGTTGAGCAGAG CTTACTTACTGGCTGTACCTG ATGTCGCTTGTTATGTGC AGGATGTTATCACTGTAGCC GATGTACAATGACAGTCAGG	56	885-904 1194-1175 / **310** (Staph. spp.) 217-237 902-884 / **686** (S. aureus) 32-51 476-457 / **444**	Multiplex, Agarose 2%, EtBr 0.5 µg/ml / Clinical in blood Sensitivity = 5x10^5 CFU /ml after 1st PCR and 50 CFU / ml after 2nd PCR
Streptococcus pneumoniae	(Rintamaki et al., 2002), WO506 & WO507	*pneumolysin*	Biot-CCCCACTCTTCTTGCGGTTGA TGAGCCGTTATTTTTTCATACTG Eu-CGAGAAAGCTATCGCTACT*	55	/ **209**	PCR simplex, Europium/TRF & agarose 2%, EtBr
Universal & *Mycobacteria* genus /	(Zolg et al., 1994), Z205 & Z212	*sod* , superoxide dismutase	AGCTTCACCACAGCAAGCACCA TCGKCCCAGTTCACGACRTTCCA	37	188-209 683-661 / **489**	PCR simplex, agarose

Target strains / GenBank ID/ Patent	Reference & primer name	Target NA	Primers, forward & reverse and/or Probe*	Ta °C	Position / product in bp	Methods/Application
X52861						
	Z261 & Z212		CCAARCTCGAAGAGGCGCGSGCCAA TCGKCCCAGTTCACGACRTTCCA	 60	245-268 683-661 / **434**	
Universal + *Staphylococcus* species* / U13618 & D14711	(Goh et al., 1996), H279 & H280	HSP60 (Cpn60), chromosomal single copy, 60-kDa heat shock protein (Chaperonin 60)	GAATTCGAIIIIGCIGGIGAYGGIACIACIAC CGCGGGATCCYKIYKITCICCRAAICCICGIGCY TT	37	/ **600*-Dig**	PCR agarose 2 %, EtBr. Specific dig-amplicons to identify by probing *Staphilococcus* species
Vibrio cholerae	(Nandi et al., 2000), 1 & 2 3 & 4, 5 & 6, 7 & 8	*ompW* outer membrane protein (detect all *V. cholerae*) *toxR* *ctxA* phage encoded cholera toxin	CACCAAGAAGGTGACTTTATTGTG GAACTTATAACCACCCGCG CCACCTACCTTTATGGTCC GGTTTGTCGAATTAGCTTCACC ATGTTCGGATTAGGACAC TACTCACACACTTTGATGGC CTCAGACGGGATTTGTTAGGCACG TCTATCTCTGTAGCCCCTATTACG	 64	 / **588** 1-18 865-884 / **884** / **301**	Multiplex (2) with primers 1 & 2 (*ompW*), 7 & 8 (*ctxA*), agarose 1.5 % EtBr / Laboratory isolated colonies
Vibrio cholerae	(Theron et al., 2000), CTX2 (PCR 1 & 2) CTX3 (PCR 1) CTX15 (PCR 2)	*ctxA*	CGCGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC GAGTATGGAATCCCACCTAAAGC		/ **564** / **347**	PCR semi-nested, centrifugation + pre-enrichment + boil, agarose 2 % EtBr 0.5 µg/ml / Environmental waters
Vibrio cholerae O1 (toxigenic)	(Fields et al., 1992), CTX2 & 3	*ctxA* phage encoded cholera toxin A subunit	CGGGCAGATTCTAGACCTCCTG CGATGATCTTGGAGCATTCCCAC	60 or 50 or 55	73-94 614-636 / **564**	PCR, garose 0.8 % EtBr
Vibrio cholerae O1 (toxigenic)	(Olsvik et al., 1993b), CTX9B & CTX7	*ctxB* phage encoded cholera toxin B subunit	BIOTIN-GATACACATAATAGAATTAAGGAT GGTTGCTTCTCATCATCGAACCAC	 55	 / **460**	PCR, polyacrylamide 6% and automated laser sequencing
Vibrio cholerae O1 and O139	(Chow et al., 2001), *rtxA-F* & *rtxA-R*; *rtxC-F* & *rtxC-R;* *ctxB*$_2$ & *ctxB*$_3$ = CTX9B & CTX7 (Olsvik et al., 1993b)	*rtxA* repeat toxin gene *rtxC* repeat toxin gene *ctxB* phage encoded cholera toxin B subunit	CTGAATATGAGTGGGTGACTTACG GTGTATTGTTCGATATCCGCTACG CGACGAAGATCATTGACGAC CATCGTCGTTATGTGGTTGC GATACACATAATAGAATTAAGGAT GGTTGCTTCTCATCATCGAACCAC	 55	 / **417** / **263** / **460**	PCR, agarose gel 2 % EtBr
Vibrio cholerae O1 and O139	(Hoshino et al., 1998), O1F2-1 & R2-2, O139F2 & R2, VCT1 & 2	*rfb* O antigen biosynthesis (O1) *rfb* O antigen biosynthesis (O139) *ctxA* phage encoded cholera toxin A subunit	GTTTCACTGAACAGATGGG GGTCATCTGTAAGTACAAC AGCCTCTTTATTACGGGTGG GTCAAACCCGATCGTAAAGG ACAGAGTGAGTACTTTGACC ATACCATCCATATATTTGGGAG	55	 / **192** / **449** / **308**	Multiplex (3) / Stools
Vibrio cholerae O139 Bengal	(Falklind et al., 1996) (Albert et al., 1997), O139-1 & O139-2	*rfb* locus, genes which replaced the O1 El Tor *rfb* complex	GCGTTATAGGTATCATCAAGAGA GTCATTATTAAAACTGCTCCATT	 50	 / **417**	PCR, agarose 1 % EtBr / Stool
Vibrio parahaemolyticus	(Bej et al., 1999), L-tl & R-tl, L-tdh & R-tdh, L-trh & R-trh	*tl* thermolabile hemolysin (Universal for all *V. para*) *tdh* thermostable direct hemolysin *trh* Thermostable direct hemolysin-related	AAAGCGGATTATGCAGAAGCACTG GCTACTTTCTAGCATTTTCTCTGC GTAAAGGTCTCTGACTTTTGGAC TGGAATAGAACCTTCATCTTCACC TTGGCTTCGATATTTTCAGTATCT CATAACAAACATATGCCCATTTCCG	 58	 / **450** / **269** / **500**	Multiplex (3) 8h pre enrichment agarose 1% , EtBr / Oyster
Vibrio parahaemolyticus	(Kim et al., 1999)	*toxR*	GTCTTCTGACGCAATCGTTG ATACGAGTGGTTGCTGTCATG	63	609-629 956-958 / **368**	PCR, agarose 2 % EtBr
Vibrio vulnificus	(Lee et al., 1998) P1-P2 & P3-P4	*vvh*: cytolysin/hemolysin Nested PCR *vvh* = 2237 bp	GACTATCGCATCAACAACCG AGGTAGCGAGTATTACTGCC GCTATTTCACCGCCGCTCAC CCGCAGAGCCGTAAACCGAA	57 59	1360-1390 2043-2063 / **704** 1460-1480 1650-1680 / **222**	PCR, agarose 1.5% & 2.5%, EtBr 0.2 µg/mL
Yersinia enterocolitica	(Feng et al., 1992) PF-17 & PF-18	*virF* gene of the plasmid pYV	CATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	 57	 / **590**	PCR, agarose 1% EtBr 0.2 µg/ml
Yersinia enterocolitica	(Feng et al., 1992), PF-23 & PF-24	*ail* gene sequence on the chromosome that encodes for cellular attachment & invasion	TTAATGTGTACGCTGCGAGTG GGAGTATTCATATGAAGCGTC	 57	 / **425**	PCR, agarose 1% EtBr 0.2 µg/ml
Yersinia enterocolitica	(Feng, 1992), BAM (Hill et al., 1995), PF-13	*ail* gene sequence on the chromosome that encodes for cellular attachment & invasion	GACGCTTCATATGAATAC*			Colony blot with radiactive labeled probe
Yersinia enterocolitica & *Aeromonas hydrophila*	(Ozbas et al., 2000) Y1 & Y2, Y1b (semi-nested) 1a & 1b Aero 2a (semi-nested)	*yst*, *Yersinia* heat-stable enterotoxin present in pathogenic serotypes *aer*, aerolysin gene present in haemolytic strains of *A. hydrophila*	AATGCTGTCTTCATTTGGAGC ATCCCAATCACTACTGACTTC AAGGCAGTTCAGTGATGCATT CCAAGGGGTCTGTGGCGACA TTTCACCGGTAACAGGATTG AAGCAATATTGTCGGCATGA	 56	1117-1139 1242-1262 / **145** 1160-1180 / **110** 645-664 834-853 / **209** 705-724 / **150**	Multiplex (2) & semi-nested PCR, agarose 1.5% EtBr 0.5 µg/ml / Raw milk
Yersinia enterocolitica / M29945	(Wannet et al., 2001) A1 & A2 (same as Feng P. (Feng et al., 1992)), Y1 & Y2	*ail* gene sequence on the chromosome that encodes for cellular attachment & invasion. 16S rRNA *Yersinia enterolitica* species specific	TTAATGTGTACGCTGGGAGTG GGAGTATTCATATGAAGCGTC AATACCGCATAACGTCTTCG CTTCTTCTGCGAGTAACGTC	 62	 / **425** / **330**	Multiplex (2) 1.5% agarose, EtBr
Yersinia pseudotuberculosis / (gb) M17448 (gi) 155439	(Feng, 1992) Isberg R.R. (Isberg et al., 1987) BAM (Hill et al., 1995), INV-3	*inv* gene of *Yersinia pseudotuberculosis*	GGTCCAGCCTTATTCTGTCTC*			Colony blot with radiactive labeled probe

A5 Ambiguous bases nomenclature

Symbol	Meaning
B	Not A (C or G or T or U)
D	Not C (A or G or T or U)
H	Not G (A or C or T or U)
V	Not T (A or C or G)
K	**K**eto (G or T)
M	a**M**ino (A or C)
R	pu**R**ine (A or G)
Y	p**Y**rimidine (C or T or U)
S	"**S**trong" (C or G)
W	"**W**eak" (A or T)
I	**I**nosine
N	Unknown (A or C or G or T)

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