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Doctoral Thesis

Title

Molecular characterization by RT-real time PCR and High Resolution Melting Analysis for food safety and veterinary diagnostics

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SUMMARY

ABBREVIATIONS

A/E: Attaching and Effacing

AA: Aggregative Adherence

aEPEC: atypically EPEC

APC: antigen-presenting cell;

APEC: Avian Pathogenic *E. coli*

ATP: adenosine triphosphate;

BQCV: Black Queen Cell Virus

CBPV: Chronic Bee Paralysis Virus

CF: Colonization Factor

CFU: Colony Formation Unit

CGM: Cell Growth Media

CM: Clinical Mastitis

Ct: threshold Cycle

DAEC: Diffusely Adherent *E. coli*

DMEM: Dulbecco's Modiefied Eagle's Medium

DNA: Deoxyribonucleic acid;

dsDNA: DNA double-stranded;

dsRNA: RNA double-stranded;

DWV: Deformed Wing Virus

eae: intimin

EAEC: Enteroaggregative *E. coli*

EHEC: Enterohemorrhagic *E. coli*

EIEC: Enteroinvasive *E. coli*

ELISA: Enzyme Linked Immunosorbent Assay

EPEC: Enteropathogenic *E. coli*

ExPEC: Extraintestinal Pathogenic *E. coli*

FBS: Fetal Bovine Serum

FQ: Fluoroquinolone

FRET: Fluorescence Resonance Energy Transfer

GB3: globotriaosil-ceramide

gDNA: genomic DNA

GFP: green fluorescent protein;

grlA: topoisomerase IV

GTP: guanosine triphosphate;

gyrA: gyrase A

HRMA: High Resolution Melting Analysis

HUS: Hemolytic Uremic Syndrome

IL: Interleukine

IMI: Intramammary Infection

LEE: Locus of enterocyte effacement

LPS: Lipopolysaccharide

LT: Heat Labile

MHC: major histocompatibility complex;

MIC: Minimun Inhibitin Concentration

MNEC: Meningitis *E. coli*

MPEC: Presumed Mammary Pathogenic *E. coli*

mRNA: Messenger RNA;

MRSP: Methicillin Resistence *S. pseudointermedius*

MSA: Manitol Salt Agar

MSSP: Methicillin Susceptible *S. pseudointermedius*

PAMPs: Pathogen-Associated Molecular Patterns

PBPs: Penicillin Binding Protein

PCR: Polymerase Chain Reaction;

pEAF: EPEC adherence factos plasmid

Pet: Plasmid encoded toxin

PIP3: phosphatidylinositol-triphosphate

pMEC: primary Mammary Epithelial Cell

PRR: Pathogen Recognition Receptors

qRT-PCR : Quantitative reverse transcriptase PCR;

RNA: Ribonucleic acid;

RNAi: RNA interference;

RNAse: Ribonuclease;

rRNA: Ribosomal ribonucleic acid;

RT: retrotranscription;

Saa: Shigatoxigenic autoagglutinating adhesion

SCC: Somatic cell Count

SCM: Sub-Clinical Mastitis

SLT: Shiga Like Toxin

SNP: Small Nuclear Poliformism

SPEC: Septicemia Associated *E. coli*

ST: Healt Stable

STEC: Shiga Toxin *E. coli*

STX: Shiga Toxin

Tir: Translocated intimin receptor

TNF: Tumor Necrosis Factor

TTSS: Tir type III secretion system

UPEC: uropathogenic *E. coli*

UTI: Urinary Tract Infection

UTR: UnTraslated Region;

VBA: Visual basic application

VFs: Virulence Factors

Abstract

This PhD thesis is the outcome of a range of activities and experimental results aimed to a better characterization of the risk that *Escherichia coli* and other microrganisms and parasites may pone to the health of animals and finally humans. One of the main activity of the present project was based on the test hypothesis that the virulence profile of *E. coli* strains toward bovine mammary gland can be modulated by the interaction with the host cells. These hypothesis were tested through a gene expression study of some virulence factors of six *E. coli* strains when co-cultured with a bovine mammary cell line, since the *in vitro* models represent both an essential tool to investigate the biological mechanistic of mastitis, and an efficient alternative to animal experiments. Preliminarly, a meta-analysis of existing literature studies on the available bovine mammary cell lines was performed, resulting in the selection of MAC-T as the most responsive cell line to bacteria causing mastitis. The *E. coli* strains used for the coculture experiments with MAC-T cells were isolated from different types of bovine mastitis (acute, chronic and undetermined) and from a VTEC food-borne strain associated to human clinical disease (O157). An upregulation of the virulence factor eae (intimin) in all but one the analyzed mastitis strains following co-culture with MAC-T cell line was detected through RT-reat time PCR, and also the adherence virulence factor ycd and the b12 gene were upregulated in some strains, overall suggesting the possibility that mastitic *E. coli* strains can acquire a more risky molecular profile when exposed to the bovine mammary cells. This finding may have clear implications on the risk assessment related to the *E. coli* strains in bovine mammary tissue and milk. In addition, with the aim to improve the current methodologies for foodborne risk analysis linked to *E. coli*, the project activity provided a preliminary research for the setup and validation of new protocols based on real time PCR-High Resolution Melting Analysis, a widely used technique to target sequence polymorphisms of the same gene in different species without the need to perform DNA sequencing or to use species-specific probes, to help the identification of putative verocytotoxic status in E. coli strains of O26 serogroup, and other serotypes, isolated from bovine milk.

Since the applications of HRMA for the characterization of microorganisms can not be limited to food safety, but can be developed for a large number of issues linked to general veterinary diagnostics, among the objectives of this PhD project some new real-time PCR-HRMA coupled methods were also developed, providing a contribution to the advancement of the existing

molecular tools for sensitive and effective species identification, or variant/mutation screening, applied to different foodborne and veterinary pathogens. Thus, new HRMA-based protocols were designed and tested for the identification of *Pseudomonas* spp responsible for chromatic alterations in mozzarella cheese, for the detection and differentiation of *Dirofilaria repens* and *D. immitis* in canine blood samples, for the detection of the mutation site associated to FQ resistance in *Staphylococcus pseudintermedius* isolated from canine diagnostic samples, and for discrimination of the two most common microsporidial parasites in honeybees, *Nosema apis* and *N. ceranae*. Overall, these new HRMA-based assays could represent additional tools for epidemiological studies, routine disease assessment and therapeutical decisions.

The possibility to identify the presence of risk-predictive SNPs in *E. coli* isolates using these newly established HRMA-based protocols is a novel, and simpler, opportunity with respect to the current, and more complex, surveillance strategies that are based on the amplification of stx genes together with other virulence factors for the evaluation of VTEC status. In the future, a possible way forward of this research is represented, on one side, by the deeper assessment of the reciprocal modulation between *E. coli* mastitis-derived strains and immortalized MAC-T cells using high-throughput RNA sequencing, and on the other side by a large scale validation of the HRMA-based evaluation of risk-predictive SNPs in order to improve the current approaches. And overall, the established HRMA-based protocols when extensively validated would be highly suitable for routine veterinary diagnostics applied to field investigation, as quick and sensitive single step protocols allowing specific and sensitive detection of the targets with shorter analysis time and reduced cost, in parallel or in alternative to the classical approaches.

Chapter 1: Foodborne risk linked to E. coli infection strains in the bovine model: expression analysis of virulence factors and HRMA-based methodology for the detection of predictive SNPs

1.1 Introduction

1.1.1 Generality on Escherichia coli

E. coli is a Gram-negative, non-spore-forming rod, which belongs to the family Enterobacteriaceae and was first isolated by a German paediatrician, Theodore Esherich, in 1884 from faeces of human neonates (Khan and Steiner, 2002). The cell of Gram-negative bacteria is made of three layers, the cytoplasmic membrane and the outer membrane, separated by a peptidoglycan layer. The external cell membrane contains phospholipids, membrane proteins and lipopolysaccharide (LPS). LPS comprises lipid-A, the lipopolysaccharide core and repeated polysaccharide units called O-antigens (Cullor, 1996). Lipid-A is the lipophilic, internal part of LPS. The toxic effects of LPS, also known as endotoxin, are caused by lipid-A (Cullor, 1996). On the outer surface, bacteria may have fimbrias which protrude from the surface. The external of the cell may be covered with a thick polysaccharide layer called capsule. *E. coli* strains can be divided into O:H:K serotypes based on the different antigenic structures of O-antigens, K-antigens (capsular) and H-antigens (flagellar) (Cullor, 1996).

Scientific classification	
Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	Gamma-*Proteobacteria*
Order	*Enterobacteriales*
Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	***E. coli***

Table 1. Scientific classification of *Escherichia coli*.

Escherichia coli is part of the normal intestinal flora of humans and animals. It is the most common facultative anaerobic bacterial species in the intestine and is regularly excreted in the faeces to the environment. Pathogenic *E. coli* bacteria can cause intestinal and extraintestinal infections in human and animals. Infections of the gastrointestinal tract may lead to various kinds of diarrhoeic

diseases, which, in the case of shiga toxin, may even progress to systemic haemolytic ureamic syndrome in humans and oedema disease in pigs. *E. coli* is the predominant cause of urinary tract infection in humans. *E. coli* also causes invasive diseases, such as bacteraemia and meningitis, in humans and animals. In avian species, *E. coli* is an important cause of respiratory and ovarian tract infections (Cullor, 1996).

Although being normally commensal organisms, *E. coli* also exist as pathogenic strains. These are distinguished as causing intestinal or extraintestinal disease and within these two groups they are further divided in different pathotypes. These different pathotypes of *E. coli* have several sets of virulence factors, resulting in a large spectrum of pathologies, including diarrhea, dysentery, septicemia, pneumonia, meningitis, urinary tract and bladder infections (Salyers and Whitt, 2002). *E. coli* is one of the most common causes of diarrhea, particularly in the developing world, where each year over one billion children under the age of five get diarrhea from *E. coli* resulting in over two million deaths (Bryce et al., 2005; Roche et al., 2010). In developed countries, bacteremia is a common cause of death and *E. coli* is responsible for 30% of those cases where a Gram-negative bacterium is implicated. It is one of the most common causes of neonatal meningitis and neonatal sepsis, which can often lead to death. The most common extraintestinal diseases caused by *E. coli* are urinary tract infections (UTIs), including cystitis and pyelonephritis. *E. coli* is the most common cause of UTIs (Johnson and Russo, 2002; Wiles et al., 2008). UTIs are potentially the most common bacterial infections of all those requiring antimicrobial therapy. One half of all women could be infected in their lifetimes. The economic consequences of UTIs in the United States have been estimated at $1.6 billion annually (Foxman, 2003).

Pathogenic *E. coli* bacteria express different, disease-specific and host-specific virulence factors (China and Goffaux, 1999). Bacterial virulence factors are needed to colonize and infect the host and to fight against host defence mechanisms. Major groups of *E. coli* virulence factors include adhesins,toxins, polysaccharide capsules and O-antigens, proteins secreted into host cells and other mechanisms to resist killing by complement or to bind iron ions. Bacteria do not produce virulence factors continually but only when intercepting particular signals from the host or environment (China and Goffaux, 1999). The genes for virulence factors may be present in the bacterial genome or may reside extrachromosomally on plasmids, even though the virulence factor is not produced (Harel and Martin, 1999).

1.1.2 *Escherichia coli* and bovine mastitis

- ***Epidemiology of bovine mastitis***

The inflammation of the mammary gland tissue is defined as bovine mastitis and it induced by microorganisms. Mastitis is caused when the pathogens enter the udder, multiply and produce metabolites or toxins that cause harm to the mammary gland tissue. Damaged tissue allows for increased vascular permeability. This causes alterations in milk composition and properties accompanied by a reduction in milk yield depending on the inflammatory response, mainly influenced by the causative pathogen (Hill, 1994; Kitchen, 2009; Seegers et al., 2003; Pyörälä, 2003; Zadoks et al., 2011). A large spectrum of microrganisms has been identified as potential mastitis pathogens and they are distinguished into either major or minor pathogens. On one hand the main mastitis-causing pathogens are *Escherichia coli*, *Streptococcus uberis* and *Staphylococcus aureus*. These bacteria have been termed major pathogens because of their association to clinical mastitis (CM). On the other hand, other bacteria may be present in the udder and often have an overall beneficial effect by protecting against infection caused by major pathogens. These bacteria produce natural anti-bacterial substances or interfere with the growth of major pathogens and are thus termed minor pathogens. Because of their complex interactions with the mammary gland, these minor pathogens can contribute to increased somatic cell counts (SCCs) and thus to the incidence of sub-clinical mastitis (SCM), but they usually do not cause CM.

- ***Severity and duration***

Mastitis can be acute or chronic, and this definition depends on how long the syndrome lasts. Chronic mastitis usually worsens over months and leads to the development of fibrous tissue in the breast (Gröhn et al., 1990). The most common forms of mastitis are CM or CM and they are usually classified according to the pathogenesis (Gianneechini et al., 2002; Akers, 2002). CM shows visible severe signs, like discoloration of the teat of the infected udder quarter, flakes or clots in the milk, whereas swelling is usually a mild sign. There are other severe signs, but not frequently as watery secretion as well as a hot and swollen udder. More systemic symptoms are fever, rapid pulse, loss of appetite, dehydration, depression and fatal consequences. In case of SCM, there aren't visible signs of infection and both milk and udder appear normal although stagnation in milk has been reported (Mungube et al., 2005). Finally, SCM can be harder to detect although the SCCs

in the milk increase in the same extent as in CM. Currently, the SCC proved to be the most useful diagnostic technique to detect the presence and occurrence of bovine mastitis and especially for bovine subclinical mastitis (Schukken et al., 2011).

The infection Mastitis causing pathogens have been broadly classified into either environmental or contagious pathogens (Radostits et al., 2007). The bacteria could survive in intramammary tissue and are spread from bovine to bovine. Environmental pathogens originate from bedding materials, manure and soil and are considered to be opportunistic invaders with no specialized survival properties. *E. coli* are considered environmental pathogens, are indeed able to persist for prolonged periods within the udder and also cause either chronic or recurrent forms of mastitis (Döpfer et al., 2000; Döpfer et al., 2001; Almeida et al., 2011).

The quarters of a cow include teat cistern, gland cistern, milk ducts and surrounding glandular tissue. The secretory tissue have millions of alveoli and are lined by milk-producing epithelium, which is supplied with nutrients by surrounding blood vessels. Muscle cells encircle each alveolus and squeeze the milk to the milk ducts and further through the teat canal and duct during milking. Milk accumulates in alveolar spaces, milk ducts and gland cistern (Figure 1). Bacteria might either invade the udder through lesions, as teat damage is known to increase susceptibility of mastitis, or more frequently breach the teat canal in several ways (Schroeder, 2010). During milking, the bacteria multiply inside teat duct and are introduced into the gland cistern via the teat canal by their motility properties or pressure placed on the teat end as caused by physical movement of the cow. Another opportunity is that bacteria may be propelled into or through the teat canal during machine milking.

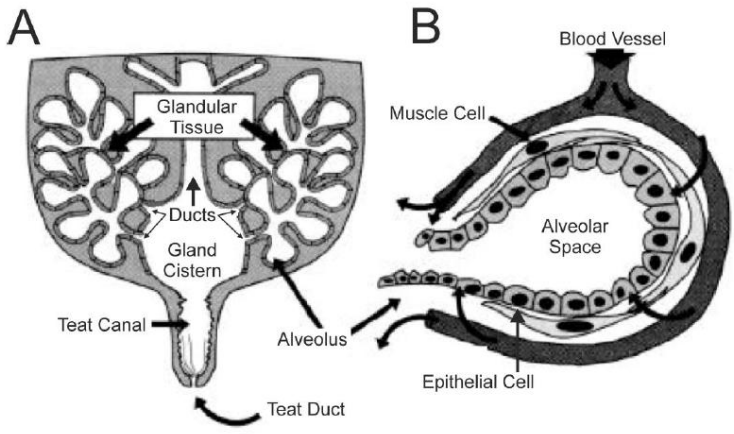


Figure 1: Scheme of the mammary gland modified from Schroeder, 2010. (A) Teat with gland cistern, milk ducts, and secretory glandular tissue. (B) Alveolus encircled by muscle cells and blood vessel.

- **Factors increasing mastitis susceptibility**

Mastitis is defined a multifactorial disease, and it is influenced by many factors ranging from genetics, physiology and finally environmental (Hopster et al, 1998;. Waller, 2000).

An example of an environmental factor is the season, in fact, it is reported an increase in the frequency of CM in the winter months (Olde Riekerink et al, 2007; Steeneveld et al, 2008).

Other factors that are not directly related to infectious pathogens but increase the risk of developing mastitis are poor hygiene, high density of cows per unit, poor ventilation and bad management practices (Barkema et al, 1999 ;Schreiner and Ruegg, 2003, Barnouin et al., 2005; Ericsson et al., 2009 Parker et al, 2007). All these factors generate stress to animals and make them more susceptible to mastitis (Breuer et al., 2003).

The factors related to cattle are genetic background and type of immune response, and other factors can be considered like: age, stage of lactation, nutritional and metabolic status.

Intensive farming is concentrated on increasingly high production of milk. This causes a weakening metabolic status that compromises the resistance of the breast to the pathogenic factors causing mastitis (Waller, 2000; Seegers et al., 2003). It is known that cattle with high milk production are more sensitive to the development of CM rather than cows with less milk production (Gröhn et al., 2004). Pathogens can infect cattle, both during the dry period and during lactation. Lactation has a significant impact on the susceptibility of cows to mastitis (Shafer-Weaver et al., 1996; Mallard et al, 1998; Rinaldi et al, 2008). The risk of developing CM is higher during early lactation (Steeneveld et al., 2008). Conversely, the risk of SCM increases with days of lactation (Busato et al., 2000). Within the periparturient period of the lactation stage, the cow´s defense mechanisms are impaired and therefore the cow is at higher risk of developing mastitis with impact on subsequent intramammary infections (Oliver and Sordillo, 1988; Kehrli et al., 1989; Breen et al., 2009). It is known that multiparous cows show higher incidence of mastitis and there is evidence that increasing parity correlates with increased susceptibility (Rajala-Schultz et al., 1999¸Steeneveld et al., 2008). Both environmental and cow factors are interdependent, whereas the relative impact of each factor is considerably influenced by the causative pathogen (Djabri et al., 2002; Zadoks et al., 2011).

- **Host defense mechanisms and pathogen recognition**

Several defense mechanisms protect the bovine mammary gland at the anatomical and cellular level. The teat end is considered to be the first line of defense against invading bacteria that cause mastitis. At the end of the duct, the sphincter muscles must be securely closed between milkings, this prevents the entry of bacteria into the udder. In addition, a coating keratin layer of the teat canal has a function of physical barrier against the entry of pathogens (Nickerson, 1987). Both, dysfunctional sphincter muscles and diminished keratin lining of the teat canal have been independently shown to increase the risk bacterial invasion and colonization (Capuco et al., 1992). Bacteria that have overcome the anatomical defense mechanisms are then in contact with the bovine immune system. The mammary immune factors have been characterized into cellular and soluble components (Sordillo and Streicher, 2002). More in details, the defense mechanisms the in mammary gland are differentiated into innate immune and specific immune responses. The innate immune response eliminates bacteria before the specific immune system responds. This type of response is activated in the event of abnormalities, such as changes in the composition of milk. The non-specific responses are mediated by the physical barrier and involve macrophages, neutrophils, natural killer cells and particular soluble factors (Sordillo and Streicher, 2002). The innate immune response has soluble factors including defensins, lysozyme, lactoferrin and cytokines. The first line of defense is the complement system, and its function is to maintain homeostasis by recognition and removal of damaged or modified self-material and pathogenic microbes (Zipfel et al., 2013). Moreover, complement is also of importance in the specific immune response. The plasma proteins make up the complement system, these proteins are also present in milk and act by targeting the pathogen, after it has been recognized by the cells of the specific immune system. *E. coli* has been reported to be sensitive to lysis by complement (Korhonen et al., 2000). Additionally, the defensins, also referred to as antimicrobial peptides (AMP), are an evolutionarily conserved component of the innate immune response. Defensins are diverse oligopeptides (<100 amino acids) contributing to the antimicrobial action of granulocytes, mucosal and epithelial host defense (Peschel and Sahl, 2006).

Of the soluble mammary gland defense components, lactoferrin is a predisposing antimicrobial protein reported to increase in concentration when an inflammation occurs (Sordillo et al., 1987). Lactoferrin contributes to neutralization of cytotoxic effects mediated by lipopolysaccharides (Pecorini et al., 2010). Moreover, the main function of lactoferrin is to inhibit the growth of certain

bacteria by binding of iron, an essential factor required for bacterial growth. This growth hindering effect of lactoferrin has already been reported for Gram-negative bacteria such as *E. coli* in ruminants (Chaneton et al., 2008; Rainard, 1986). Cytokines are immunomodulating molecules that account for cell signaling. They are produced after antigen detection by certain cells of the immune system, mainly leukocytes. The different cytokines (e.g. TNFα and IL1β) have a matching cell-surface receptor triggering intracellular signaling cascades and consequently mediating alterations of cellular functions. Figure 2 shows the involvement of the inflammatory cytokines TNFα and IL1β in the immune response against putative mammary pathogenic *E. coli* as possibly induced by lipid A recognition by the TLR4-MD-2 receptor complex (Maeshima and Fernandez, 2013).

If the infecting bacteria evade the innate immune response or are not completely eliminated, the specific or acquired immune response is triggered. In contrast to the innate immune response, the specific immune system needs to recognize the pathogens by specific antigens and therefore it takes time to be mounted. Once a specific response is created, due to the immunological memory, the immunity state is quickly established, intensive and enduring when the same antigen is recognized again. This results in a more effective elimination of the pathogen. Thereby, the most important soluble effectors of the specific immune response are antibodies produced by B lymphocytes after antigen recognition.

Cellular defense factors	Biological function	Immune system
Neutrophils	Phagocytosis and intracellular killing of bacteria; secretion of antibacterial factors	innate
Macrophages	Phagocytosis and intracellular killing of bacteria; antigen presentation in conjunction with MHC	innate & specific
Natural killer cells	Non-immune lymphocytes that secrete antibacterial Proteins upon activation	innate
T lymphocytes		
CD4C (T helper)	Production of immunoregulatory cytokines following antigen recognition with MHC class II molecules; memory cells following antigen recognition	specific
CD8C (T cytotoxic)	Lysis of altered or damaged host cells when complexed with MHC class I molecules; production of cytokines that can down-regulate certain leukocyte functions	specific
γδ T lymphocytes	Biological role in the mammary gland is speculative	specific
B lymphocytes		
Mature B cells	Display membrane-bound antibody molecules to facilitate antigen presentation; memory cells following antigen interactions	specific
Plasma cell	Terminally differentiated B lymphocytes that synthesize and secrete antibody against a specific antigen	specific

Soluble defense factors	Biological function	Immune system
Cytokines	Proinflammatory and immunoregulatory factors	innate
Complement	Bacteriolytic and/or facilitates phagocytosis	innate & specific
Lysozyme	Cleaves carbon bonds and disrupts bacterial cell walls	innate
Lactoferrin	Sequesters iron to prevent bacterial uptake; disrupts bacterial cell wall; regulates mammary leukocyte activity	innate
Antibodies		
IgG1	Selectively transported into mammary secretions; opsonizes bacteria to enhance phagocytosis	innate
IgG_2	Transported into secretions during neutrophil diapedesis; opsonizes bacteria to enhance phagocytosis	innate
IgA	Associated with the fat portion of milk; does not bind complement or opsonize particles; can cause agglutination, prevent bacterial colonization, and neutralize toxin	innate
IgM	Efficient at complement fixation, opsonization, agglutination and toxin neutralization; only opsonic for neutrophils in presence of complement	innate

Table modified from Sordillo and Streicher, 2002

Table 2: Cellular and soluble defenses of the mammary gland

The pathogen recognition receptors (PRRs) allow rapid detection of pathogens and are essential to enable the two types of responses (innate and specific). These receptors can be displayed either on the surface or intracellularly, and recognize the so called pathogen-associated molecular patterns (PAMPs). PAMPs are small molecular motifs, which are conserved within a class of microbes such as lipoteichoic acids in Gram-positive and LPS in Gram-negative bacteria (Kumar et al., 2011). After binding to PAMPs, the PRRs subsequently initiate intracellular signaling cascades or directly promote the attachment, ingestion and destruction of the pathogens. In Table 2 these

pathogen recognition receptors are presented (Sordillo and Streicher, 2002). The extracellular PRRs are called Toll-like receptors (TLR) having an important role in the innate and and specific immune response (Doyle and O 'Neill, 2006).

Factor	Role
Innate Immunity	
CD14	Binds LPS. Membrane version is expressed on several cells including monocytes, macrophages, neutrophils, dendritic cells, and B cells. The soluble version may compete with mCD14 for LPS and is essential in the activation of non-mCD14 expressing cells, including epithelial and endothelial cells, by LPS.
PGRP	Expressed in differentiated, lactating epithelium where it binds and hydrolyzes peptidoglycans.
TLR2	Recognizes peptidoglycan and LTA from Gram-positive bacteria and lipoarabinomannan from mycobacteria. May form a heterodimer with TLR1 to recognize triacylated lipopeptides from Gram-negative bacteria and *Mycoplasma* or with TLR6 to recognize diacylated lipopeptides from Gram-positive bacteria and *Mycoplasma*.
TLR3	Detects double-stranded RNA.
TLR4	Recognizes LPS of Gram - bacteria, heat-shock proteins, fibrinogen, and polypeptides.
TLR5	Recognizes bacterial flagellin.
TLR9	Intracellular recognition of CpG-containing oligodeoxynucleotides (ODNs).
Acquired Immunity	
Fc Receptor	Expressed on macrophages, neutrophils, and natural killer cells and recognize antibodies of infected cells or pathogens.

Table modified from Aitken *et al.*, 2011

Table 3: Pathogen recognition receptors

The interaction of a potential mammary pathogenic *E. coli* (MPEC hereafter) with the host immune cells is reported in Figure 2. TLR2 and TLR4 are the most significant receptors during bacterial mastitis and are primarily activated in response to Gram-positive and Gram-negative infections, respectively (Yang et al., 2008¸Günther et al., 2011). Notably, even though both *S. aureus* and *E. coli* are able to trigger TLR2 and TLR4, only *E. coli* is capable of inducing NF-κB signaling in mammary gland epithelial cells followed by quick induction of TNF-α.

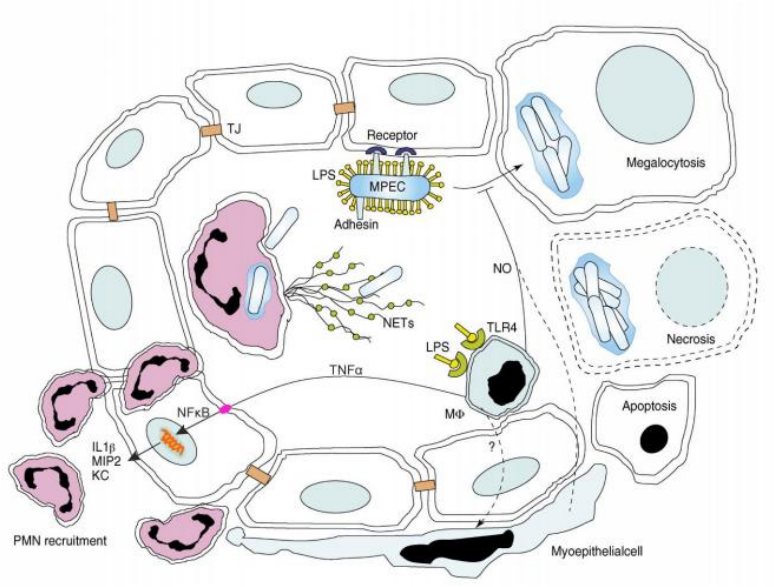


Figure 2: Pathophysiological scheme of presumed mammary pathogenic *E. coli* (MPEC) replicating in the mammary alveolar space, modified from Shpigel et al., 2008. LPS/TLR4 signaling on alveolar macrophages (MΦ) elicits production of inflammatory cytokines (TNFα and IL1β) and chemokines (KC and MIP2), resulting in recruitment of blood neutrophils (PMN) trafficking across the polar alveolar epithelium (TJ, tight junction) into the alveolar space. Recruited neutrophils are killing the bacteria by phagocytosis and neutrophil extracellular traps (NETs). Bacterial epithelial invasion is abrogated by LPS/TLR4 signaling on MΦ possibly mediated by nitric oxide (NO) produced by MΦ and myoepithelial cells. Epithelial invasion by bacteria induce epithelial megalocytosis, necrosis and apoptosis.

Regarding the pathogenic potential for cattle, *E. coli* is considered an environmental pathogen because of its ability to survive in the environment of dairy cows. In particular, *E. coli* represent a group of microorganisms that are frequently associated with bovine mastitis and they are the bacteria most frequently in milk and milk-derivate, and in this last case the are dangerous for humans if associated to VTEC status as serotype 0157. In the past years, several studies have shown an increase in the importance of *E. coli* among all bovine mastitis pathogens. A study from Wisconsin reported an increased prevalence of *E. coli* intrammamary infections (IMI) from 17.7% in 1994 to 24.9% in 2001 of the total mastitis pathogens isolated (Makovec and Ruegg, 2003). In the United Kingdom, *E. coli* was also shown to be the most important pathogen in well managed dairies (Green et al., 2005). The parity number, season of the year, and lactation status affect susceptibility to clinical mastitis caused by *E. coli*. Results from some studies during the last decade indicated that the severity of clinical manifestations of *E. coli* mastitis is mainly determined by cow factors rather than by virulence factors of *E. coli* (Burvenich et al., 2003). On the other hand, recent studies demonstrated that virulence attributes of *E. coli* strains may explain the outcome of *E. coli* IMI (Almeida et al., 2011). There are preliminary evidences that the virulence profile of *E.*

coli strains causing mastitis in cattle can be modulated by the interaction with the mammary cells (Kerro-Dego et al., 2012). Further research is needed in order to define the effect of the host-cell/pathogen interaction on the virulence pattern of *E. coli* and on the possible implications also for human health. With the aim to clarify this issue, in the present project a gene expression study of some virulence factors of *E. coli* strains isolated from bovine mastitis and co-cultured with an immortalized bovine mammary cell line, in comparison with a VTEC food-borne strain associated to human clinical disease, was designed.

The use of in vitro cell-based models for screening and testing has the advantage that several concentrations of mixtures or specific compounds can be assayed at the same time in cells from specific tissues in a controlled environment. Monolayer culture models of mammary cells are easy and convenient in vitro systems, even if they do not recapitulate the glandular structure of epithelium in vivo and therefore cannot provide the optimal system for studying the regulation of proliferation, polarization and differentiation of the glandular epithelium. Furthermore, the use of in vitro models have the advantage that they represent a possible alternative to animal experiments, thereby saving the lives of laboratory animals and reducing costs for expensive animal experimentation (Purup et al., 2000).

A suitable model for the study of mammary immunity needs the selection of a proper cell line. Cell cultures frequently used to understand the mechanisms underlying the function of the mammary gland are the primary lines of bovine epithelium (pMEC) derived from mammary biopsies and two continuous, stabilized cell lines: MAC-T and BME-UV1. Irrespective of the origin, the cells established in culture form a thick monolayer, which in 3 days develops into an epithelial island with a central cell cluster. Four days further, the cell cluster within the epithelial island develops into a dense cellular mass.

Mammary epithelial cells have a strong innate immune defense capability and the capacity to attract circulating immune effector cells such as neutrophils. Primary MECs show a constitutive secretion of IL-1 and IL-6, with different cytokine release pattern compared to MAC-T cells (Okada et al., 1997). Both pMEC and MAC-T cells respond to challenge with LPS through up-regulation of mRNA expression for a range of cytokines, chemokines and β-defensins, including mRNAs coding for several chemotactic proteins likely to promote infiltration of the mammary tissue by neutrophils and monocytes (Strandberg et al., 2005). A comparison of the results obtained with primary cells and MAC-T cells indicate some differences. As an example, the response obtained

with primary cells to exposure to LPS is much higher than the one reported using MAC-T cells regarding β-defensin, LBP (lipopolysaccharide binding protein) and IL-1β expression (Strandberg et al., 2005).

On the other hand, the expression responses of TNF-α, IL-6, MCP-1 and PAI-1 genes in MAC-T cells are similar to primary mammary epithelial cells (Thorn et al., 2008). The reasons for these substantial differences in the responses of the two mammary cell types to LPS are still unclear but may relate to the length of time that cells have been in culture and the immortalization process for the MAC-T cells. Further, there are significant differences in the propensity of each cell type to form globular structure (Stradberg et al., 2005). Also, BME-UV1 cells treated with LPS release a range of cytokines including TNF-α, IL-8 and IL-1β into the serosa compartment of the epithelial monolayer culture model (Fitzgerald et al., 2007).

The exposure of primary mammary cells or established cell lines to physiological exogenous matrices that mimic the composition of the normal basement membrane is also relevant. In a recent study, the growth and transcriptomic profile was studied in BME-UV1 cells cultured in traditional monolayers on a plastic surface, and on extracellular artificial matrix (Matrigel™). It was shown that BME-UV1cells grown on Matrigel™ form polarised acinar structures during 16 days of culture, and the difference in spatial architecture between mammary epithelial cells cultured in monolayer and a three-dimensional structure is reflected by differences in transcriptomic profile. This study shows the importance of developing models with polarized mammary epithelial cells and that mammary cells may be a good model for studying, for example, developmental and even immunological processes of the bovine mammary gland in vitro (Kozlowski et al., 2009).

Co-colture system is a model that in general can help to understand the mechanisms and communication between epithelial cells and white blood cells. This system is suitable also for studies on bovine mammary gland immunology, reflecting that mainly factors of innate immunity are active in mammary gland cells (Didier and Kessel, 2004). Furthermore, a mechanism of co-culture of cells involves the use of supports inducing the cell to make a polarity and then being more comparable to the in vivo situation and could potentially be applied to mammary cells, studying transport or metabolism of blood borne substances across the epithelium (Shin et al., 2006). An interesting applications for mammary cells of the two-compartment system has been described previously regarding polymorphonuclear neutrophil migration, and protein and plasminogen activator production in mammary epithelial cells after extracellular calcium

treatment (Smits et al., 1996; Cheli et al., 2001). Such two-compartment models also provide the possibility of applying epithelial cells in combination with immune cells (adhering or not) and bacterial cells. When microbiota is added to the apical side, this three-component model (epithelial cells, immune cells and microbiota) is close to the in vivo situation.

In literature, there are many studies that investigate on the interaction of the molecular structures of pathogens between with mammary epithelial cells (MECs), which have provided interesting data on mammary immunity.

At present, some researchers have been found in healthy and infected mammary gland a variety of cytokines, such as interleukins (IL-1b, IL-2, IL-6, IL-8, IL-12 and TNF-α) and cathelicidins involved in the mammary immunity response(Sordillo et al., 2002).

For *in vitro* models the primary cell cultures can be used to identify the target organs or a sensitive cell lines for this pathologies. The cell line culture selected are treated using mixtures or specific compounds of different nature, data obtained showed different endpoints inherent to basal or more sophisticated cellular functions such as cell viability and proliferation, cell migration, regulation of gene expression, morphology or metabolic alterations (Purup and Nielsen, 2011).

Primary cultures of bovine mammary epithelium (pbMEC), derived directly from the mammary gland, have also been used as models including both organoids, explants and epithelial cells for studies of effects of growth factors, steroids, lactoferrin, retinoids, cytokines, biogenic amines, mammary extracts, and in such studies endpoint parameters such as mitogenic activity and gene expression have been measured (Matitashvili and Bauman, 1999 and 2001; Weber et al., 1999; Akers et al., 2000; Ellis et al., 2000; Purup et al., 2000 and 2001; Weng et al., 2005; Thorn et al., 2006 and 2008; Ernens et al., 2007; Fusi et al., 2008; Swanson et al., 2009; Wang and Baumrucker, 2010).

Some authors performed studies on bovine mammary mutagenic cell lines, and among these the MAC-T (Huynh et al., 1991) and the BME-UV1 (Zavizion et al., 1996) cell lines, both of which were immortalized by transfection with the SV40 T-antigen. The MAC-T cells have been used for bioactive studies, including fatty acids, growth factors, steroids, retinoids, cytokines and mammary extracts, also for individuate a specific parameters such as viability, proliferation, apoptosis, gene expression, epithelial transport, cell signalling and lipogenesis. Another cell line of the immortalized bovine mammary epithelial, BME-UV1, have been used for elaborating effects of a number of bioactives and parameters studies even more respect the MAC-T sush as mycotoxins,

lactoferrin proteins and retinoids. Until now, despite the numorous studies independently performed using bovine mammary cells, there is not a comprehensive analysis to identify which cell line is more responsive to interaction with bacteria causing mastitis.

1.1.3. Verotoxin-producing Escherichia coli (VTEC)

From the clinical-pathological point of view, *E. coli* pathogens are subdivided into different types associated with different symptoms and modes of pathogenesis (Kaper et al., 2004). These include, but are not limited to, enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroaggregative (EAEC), enteroinvasive (EIEC) and diffusely adherent (DAEC) *E. coli* strains which cause enteric diseases often resulting in diarrhea. Uropathogenic (UPEC) and meningitis associated (MNEC) *E. coli* cause urinary tract infections and meningitis/sepsis respectively, and are classified as extraintestinal pathogenic *E. coli* (ExPEC). EPEC, EHEC and ETEC cause disease in both humans and animals, whereas avian pathogenic *E. coli* (APEC) causes disease in birds.

- Enteropathogenic *E. coli* (EPEC) is linked to many cases of diarrhea and there is a higher incidence in children (Trabulsi et al., 2002). It is the first *E. coli* pathogen described, is one of the most studied and the one most characterized. Its pathology is characterized by the formation of attaching and effacing lesions (A/E positive) on the epithelium of the human intestine and by the consequential bloody, watery diarrhea. The adhesin protein intimin binds to its receptor Tir (translocated intimin receptor). Tir is secreted by a type III secretion system (TTSS) into the cytoplasm of the epithelial cells from where it is inserted into the plasma membrane allowing binding of intimin. The binding causes the formation of actin at the site of adhesion on the intestinal cell membrane, creating a pili effaced pedestal upon which the bacteria perch. Genes responsible for this lesion, including elements of the TTSS, intimin (eae), tir, and other effector proteins are found in the LEE (locus of enterocyte effacement) pathogenicty island (McDaniel et al., 1995). The detection of LEE genes, intimin in particular, has been used to identify EPEC isolates. EPEC may be further divided into typical and atypical varieties. The EPEC adherence factor plasmid (pEAF) is found in typical EPEC isolates. When pEAF is absent, but the LEE is present, the isolate is termed an atypical EPEC (aEPEC). With the recognition that aEPEC isolates are capable of causing disease, it has been discovered that A/E forming isolates come from a

much wider, heterogenous range of serogroups. All serotypes are associated with the disease in developing countries, transmission is direct from person to person, less frequently by water (Hunter, 2003). In developed countries the occurrence of typical EPEC is rare (Ashbolt, 2004), while atypical EPEC does appear to be an emerging pathogen in developed countries (Trabulsi et al., 2002).

aEPEC are very present in animals including cows, rabbits, monkeys, dogs, cats and birds (Trabulsi et al., 2002; Morato et al., 2009). Overall, the environmental distribution of aEPEC is not studied very well and waterborne outbreaks in developed countries have not been reported (Hunter, 2003). Studies in the Great Lakes and connecting channels found that 0-4% of isolates from different sites were aEPEC (Hamelin et al., 2006, 2007).

- Entheroemorrhagic *E. coli* (EHEC) which have the LEE pathogenicity island but do not make shiga-like toxin like EPEC. The secretion of shiga-like toxins (also called as verotoxins) marks an isolate as STEC (or VTEC). *E. coli* that include both the LEE and shiga toxin are enterohemorrhagic *E. coli* (EHEC). This is an example of how a difference of a few genes, in this case the addition of shiga-like toxins, can result in 8 different disease symptoms. In addition to diarrhea, a small number of infected people may develop hemolytic uremic syndrome (HUS), resulting in renal failure and hemolytic anemia. Children and the elderly are most severely affected. Severe, long-term impacts to health and death may follow.
- Enterotoxigenic *E. coli* (ETEC) is the major cause of diarrhea in the world (Turner et al., 2006). it is very frequent in the developing countries, one of the ways of spreading more likely it is the movement of tourists, in fact, the disease is called "traveler's diarrhea". It has been estimated that causes about 800,000 deaths worldwide, mostly children under the age of five years. Death is most frequently the effect of dehydration and loss of electrolytes in those places where sources of clean drinking water are inadequate. In addition to humans, its occurrence has been described in the developed world in pigs, chickens, Canada geese, dogs, sheep, goats and cattle (Hussong et al., 1979; Akashi et al., 1993; Nagy and Fekete, 1999; Khatib et al., 2003).
- Enteroaggregative *E. coli* (EAEC) is another diarrheagenic pathotype that is primarily found in children of the developing world. It is characterized by adherence to epithelial cells in a stacked brick-like aggregation within a biofilm (Kaper et al., 2004). EAEC produce toxins, these contain the plasmid encoded toxin (Pet), EAST1, or ShET1 all of which may be found

in a minority of isolates and the latter two toxins may also be established in other pathotypes. It is uncertain if there are common factors of EAEC that contribute to the phenotype (Kaper et al., 2004). The existence of aggR, the aggregative adherence (AA) probe and aap may detect "typical" EAEC in most cases (Cerna et al., 2003). EAEC strains are present in animal feces from calves, piglets, Canada geese and horses based on their aggregative adherence phenotype or serogroup. Waterborne outbreaks outside of the developing world do not appear to have been reported. (Kullas et al., 2002; Uber et al., 2006).

- Enteroinvasive *E. coli* (EIEC) are responsible for enteritis, toxaemia and septicemic infections; thanks to the presence of different pathogenicity factors (capsule, adhesins, siderophore, α-hemolysin), they are able to adhere to the epithelium of the colon, to penetrate the enterocytes using the mechanism of endocytosis and to destroy them; then they propagate from cell to cell and may reach the lymphatic system; (Kaper et al., 2004). They are almost indistinguishable from Shigella spp. because they share the same essential virulence factors and EIEC infections result in the symptoms of Shigellosis, namely diarrhea and dysentery. Shigellosis is the third most common cause of bacterial gastroenteritis in the United States, with approximately 14 000 cases reported annually (Gupta et al., 2004) Due to the similarity of EIEC and Shigella symptoms and pathology, it is likely that EIEC outbreaks are underreported, having been reported as Shigellosis instead. Although human to human transmission is the major mode of spread for Shigella, food and water transmission also occur (Naimi et al., 2003; Craun et al., 2005). Shigella and EIEC are normally found only in humans and primates.
- DAEC Diffusely adherent *E. coli* (DAEC) are so named for the pattern of diffuse adherence to the surface of epithelial cells. They do not invade cells or produce toxins, but they have been associated with diarrhea in a number of studies (Kaper et al., 2004). DAEC cause the epithelial cells to produce cellular extensions to which the *E. coli* binds. This is the result of binding by Dr fimbriae, however such Dr binding is not limited to DAEC and is also found in UPEC. Overall, what part DAEC play in gastrointestinal disease is not well understood, and unique genetic probes for its identification are still being investigated. DAEC do not appear to have been studied outside of humans and no outbreaks have been documented (Tacket et al., 1990).

- extraintestinal pathogenic *E. coli* (EXPEC). It is therefore expected that they should draw the greatest amount of attention from researchers, the press and the general public. They are guilty for damaging pathologies sometimes linked with high mortality and epidemics. These strains of *E. coli* could cause disease outside of the intestinal system, resulting in sepsis, meningitis, urinary tract infections (UTIs) and infection in a variety of other tissues. They result in a considerable amount of morbidity and expenses. This subgroup is estimated, in the United States, to cause the 11% of UTIs in women aged 18 and over, thus resulting in disease related expenses of over 1 billion dollars annually (George and Manges, 2010). ExPEC strains do, in fact, contain sets of virulence factors (VFs) not found in representative commensal isolates. In animal studies, commensals do not cause disease when introduced extraintestinally as do ExPEC strains. ExPEC strains normally belong to phylogenetic groups B2 or D, rather than A and B1 to which commensals most typically belong. For these reasons, ExPEC strains are now thought of as true pathogens although debate continues as to whether any genetic determinants can distinguish them from commensals (Finlay and Falkow, 1997; Russo and Johnson, 2000). ExPEC isolates contain an assortment of virulence factors including, but not limited to, adhesins that allow colonization of mucosal surfaces, toxins, iron acquisition systems, proteases, invasins and serum resistance proteins (Johnson and Russo, 2002). This particular collection of VFs may optimize an ExPEC strain for colonization of different tissues; uropathogenic *E. coli* (UPEC) colonize the urethra, bladder and kidneys, meningococcal *E. coli* (MNEC) the brain and nervous tissues, and septicemia associated *E. coli* (SPEC) the blood. Despite the existence of these classes of ExPEC, there is great overlap in the variety of VFs expressed by each class, making these designations somewhat artificial (Russo and Johnson, 2000).

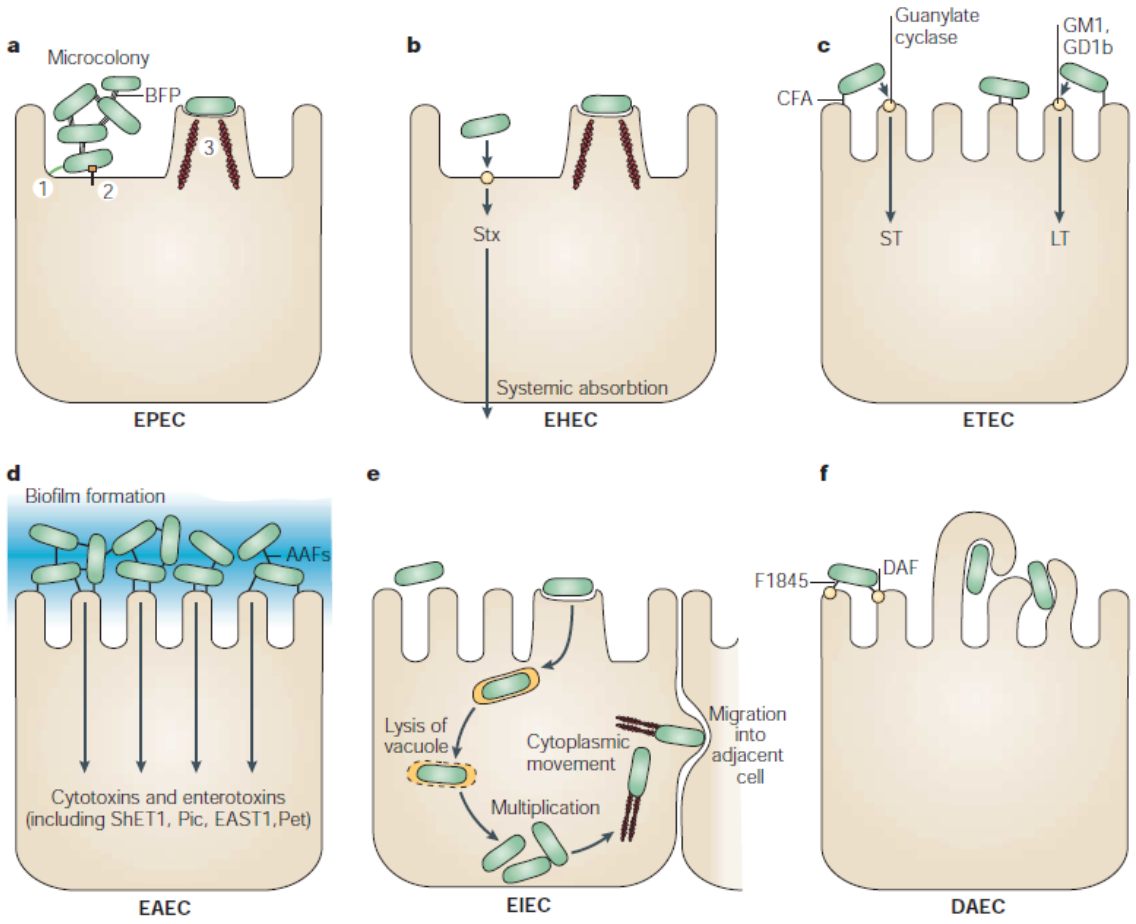


Figure 3 Schematic representation of the pathogenic mechanisms characteristic of the six main histological types of *E. coli*:

a) EPEC: attaching and effacing lesions at the level of the small intestine (1. initial adhesion, 2. injection of Tir by the TTSS, 3. cytoskeletal rearrangement and formation of the "pedestal");

b) EHEC: attaching and effacing lesions at the level of the colon, processing, secretion and systemic absorption of verocytotoxin;

c) ETEC: accession all'enterocita (small intestine), secretion of heat-labile enterotoxin (LT1, LT2) and / or thermostable (STa, STb);

d) CEAA (or EAggEC): adherence to epithelial cells of the small and large intestine, forming a thick biofilm, secretion of enterotoxin and cytotoxin;

e) EIEC: invasion enterocyte (colon), lysis of the phagosome, multiplication inside the cell, migrate from cell to cell through the cell membrane basolateral;

f) DAEC: stimulus to the formation of cellular fingerlike projections that wrap the same adherent bacteria (cells of the small intestine).

AAF: aggregative adherence fimbriae; BFP: bundle-forming pilus; CFA: colonization factor antigen; DAF: Decay-accelerating factor; EAST1: enteroaggregative *E. coli* ST1; LT: heat-labile enterotoxin; ShET1: Shigella enterotoxin 1; ST: heat-stable enterotoxin. (Kaper et al., 2004)

- ***E. coli* and food safety (VTEC/STEC and EHEC)**

Some strains of *Escherichia coli* are characterized by the ability to produce exotoxins called verocytotoxin or verotoxin (verocytotoxins, Vtxs), causing the cytotoxic effect produced in vitro in VERO cell culture (Konowalchuk et al., 1977). Two distinct antigenic groups of these toxins, vtx1 and vtx2, have been described. On the basis of almost identical antigenic structure of toxins belonging to the group vtx1 with Shiga toxin of Shigella dysenteriae, another denomination possible is "Shiga-like toxins (SLTs)" or "Shiga toxins (Shiga toxins, STXs)" (O'Brien et al., 1982). Since not all *E. coli* strains are necessarily pathogenic, the capacity to produce these toxins are referred to as Verocytotoxigenic *E. coli* or Verocytotoxic (Verocytotoxin-producing *E. coli* or Verocytotoxigenic *E. coli*, VTEC) or *E. coli* toxin-producing Shiga- like (Shiga toxins producing *E. coli* STEC).

The EHEC (enterohemorrhagic *E. coli*) are a subgroup of VTEC with strong pathogenicity, thanks to the simultaneous presence in them of the genes encoding the verocytotoxin (stx1, stx2 or both) and the Locus of enterocyte Effacement (LEE), a pathogenetic island of about 33,000 base pairs in the bacterial genome of *E. coli*, in which are encoded the pathogenic factors responsible for attaching and effacing lesions: the intimine (adhesins encoded by the gene eae, responsible for the tenacious bond between EPEC / EHEC and enterocytes), its receptor Tir and TTSS and other proteins involved in the formation of these lesions (EspF-H, EspZ, Map) (Figure 4) (Kaper et al., 2004, Khan et al., 2003).

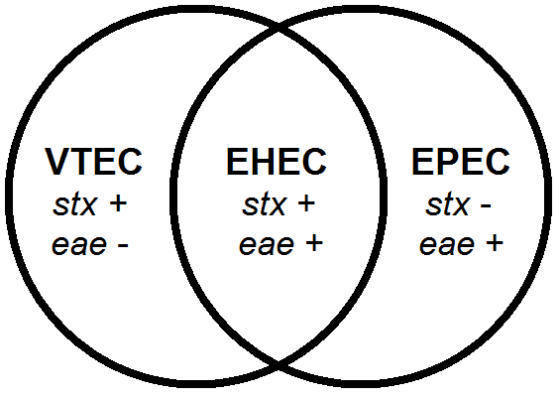


Figura 4: Grafic rappresentation of VTEC, EHEC and EPEC group in base of shigatoxin (stx) and intimin (eae) genes.

The Locus of enterocyte Effacement, characteristic of EHEC is not essential for pathogenicity; Indeed, there are some VTEC LEE-negative can cause serious diseases such as hemolytic uremic syndrome. (Nataro et al., 1998) These strains have other adhesion factors than intimine, as some

fimbriae and adhesion factors without fimbriae (eg Shigatoxigenic Autoagglutinating Adhesin , Saa). (Etcheverria et al., 2013) Some serotypes have also been isolated in cattle strains with LEE which have never been found in humans, suggesting the existence of other relevant factors of pathogenicity. (Karmali et al., 2003).

Other virulence factors are important in the pathogenesis of diseases resulting from infection VTEC: the enteroemolisin, encoded by the gene ehxA, a "poreforming toxin" monomer capable of causing the formation of pores in the cell membrane of enterocytes and correlated to the ability to cause hemorrhagic colitis (Bonardi et al., 2006); the ESPP, a secretory serine protease which cleaves and inactivates pepsin and coagulation factor V (proaxelerin) in humans; catalase-peroxidase, able to inactivate reactive oxygen species (ROS) produced by the host's immune system (Beltz book , 2011).

- **Verocitotoxin**

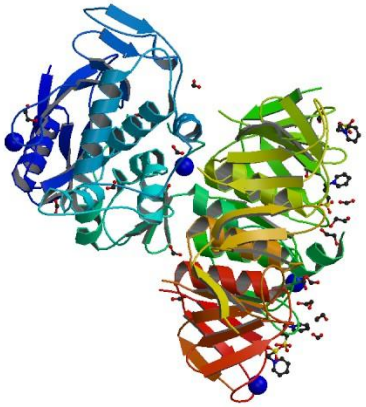


Figure 5: Crystallographic structure verocytotoxin type 2 (Stx2). In blue and blue sub-unit A, in green and red sub-unit B (Fraser et al., 2004).

The verocytotoxin are the main and distinctive virulence factor of VTEC. The toxins of the two groups, Stx1 (vtx1) and Stx2 (vtx2), are encoded, respectively, by genes and genes stx1 stx2, carried by temperate bacteriophage λ which penetrate the bacteria and establish a lysogenic cycle, integrating their DNA with the DNA of the host cell. Several stressful factors, such as exposure to UV light, growth plate or contact with certain antibiotics, can induce the activation of the lytic cycle, with the replication, maturation and the liberation of bacteriophages that, infecting new bacterial cells, can lead to the appearance of new strains of verotoxigenic bacteria with new combinations of virulence factors. Shiga-like toxins are further divided into many variants: three

for the Stx1 subgroup (Stx1a, Stx1c, Stx1d) and seven for Stx2 subgroup (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g), in accordance with the nomenclature established at the 7th International Symposium on Shiga Toxin Producing *Escherichia coli* Infections. The group of clinically more relevant toxins is the Stx2, within which the variants are present more frequently associated with cases of the disease with severe outcome in humans (Stx2a, Stx2c, Stx2d) (Farrokh et al., 2013).

The toxin structure, verocytotoxin AB5 type, consists of a Domain A, in charge of toxic action, and a domain B, responsible for binding with the receptor cell. After cell binding of the holotoxin through its domain B, receptor-binding glycolipid GB3 (globotriaosil-ceramide), the domain A is internalized by endocytosis and enzymatically cleaved into two segments (A1 and A2). The segment A1, with N-glycosilase activity, is released into the cytosol, where acts through the removal of the eukaryotic 28S ribosomal RNA adenine, thus blocking the protein synthesis. The most affected cells are: endothelial cells (mainly enteric and kidney), enterocytes, platelets, red blood cells and B cells. (Doughari et al., 2009)

- **VTEC in bovine**

The most well known VTEC serotype is *E. coli* O157:H7, which has been implicated in many large outbreaks of HC and HUS. However, VTEC strains of other serotypes have increasingly been implicated in sporadic cases and outbreaks of serious illness in humans, e.g., serotypes O26, O111, O103, and O145 (Word Healt Organization, 1998). Among the most important sources of human infection are: direct contact with cattle and other ruminants and contaminated bathing water, beef products, unpasteurized milk, vegetables, fruits, and drinking water (Tozzi et al., 2001).

In particular, the VTEC pathogens for humans have been found in many animal species, but only the gastrointestinal tract of ruminants, primarily cattle but also goats, sheep and wild ruminants can be considered with certainty as a reservoir of these bacteria (EFSA, 2012). Epidemics studied from 1982 to date have shown that ruminants, and in particular bovine, appear almost always involved in the transmission of these bacteria to humans. The intake of foods derived from cattle infected with VTEC is definitely the main route of transmission, also play an important role: the intake of other foods origin (such as horticultural products) or water contaminated with cattle and direct contact with infected animals or their environment (Caprioli et al., 2005).

Studies on the presence of VTEC in cattle have been performed in several parts of the word. In North America, VTEC were isolated in both beef cattle and in dairy cattle (Hancock et al., 1994).

This study reported a prevalence of EHEC O157: H7 equal to 0.71% in beef cattle, and a prevalence of 0.28% in dairy cattle, with, respectively, 16% and 8.3% of infected herds; according to another study, the prevalence of VTEC in the US varies from 10 to 28% of the animals (Karmali et al., 2010). In Brazil, the genes encoding the verocytotoxin (stx) were found in the feces of 53% of beef cattle and 82% of dairy cows, and the strain EHEC O157: H7 was isolated from 1.5% of samples. In Australia, the stx genes were identified in 16.7% of fecal samples from dairy cows; from 1.9% of the samples the serotype O157: H7 was isolated, and from 1.7% the VTEC non-O157 serotype O26: H11. In Japan, fecal samples of 46% of the calves, 66% heifers and 69% of cows were positive for stx genes and EHEC strains belonging to serogroups O26, O111 and O157 were isolated from 9 out of the 78 tested herds (11.5%). In Europe, in the period 2007 - 2011 the VTEC were found in 2.1 to 13.5% of the tested animals and *E. coli* O157 was identified in 0.2 to 2.3% of cases. The prevalence of VTEC in animals varies greatly between different Member States, reaching peaks of 53.8%. The prevalence in a herd ranged from 6.1 to 12.6% for VTEC and 1.5 to 13.7% for VTEC O157. At the slaughterhouse, the 13.0 to 20.2% and from 5.5 to 20.2% of the consignments of slaughter were positive, respectively to VTEC in general, and to O157 VTEC (EFSA, 2013). In Italy, 3.6% of slaughtered are positive for the non-O157 VTEC (Bonardi et al., 2004).

Cattle are asymptomatic excretors of VTEC which are transient or permanent members of the normal intestinal flora (Caprioli et al., 2005) The persistence of VTEC in individual animals is due to the fact that these bacteria colonize the special traits of the gastrointestinal tract. The different possible interactions between the microrganism and its host affect the pattern of fecal elimination in the various subjects: low level ($<10^3$ CFU / g of feces) and short term (<10 days) elimination occurs when the colonization is limited to the rumen; if the colonization is extended to the colon, increasingly low levels of elimination but for prolonged periods (> 30 days) are observed (Duffy 2014). Several authors have shown, for the serotype O157: H7, a particular tropism for a region of the rectum adjacent to the recto-anal junction of cattle. The localization between 3 and 5 cm cranial to the junction recto-anal has been speculated to be cause of eliminating high levels of fecal bacteria (106-8 CFU / g of feces) in animals so-called "super shedders". It is believed that "super shedders" individuals are responsible for the elimination of 95% of the total VTEC issued by a herd and may have, therefore, a very important role in the epidemiology of these microorganisms (Naylor et al., 2010).

Faecal elimination of VTEC in cattle is transitory and is undoubtedly influenced by age. Studies conducted in the US showed a low prevalence of shedding subjects in calves younger than two months (<1.5%), while the highest prevalence (1.8 to 5%) was detected in subjects aged 2 and 4 months; then the proportion of VTEC shedding subjects decreases (Caprioli et al., 2005). Even the studies of experimental infections confirmed the increased elimination in young people than in adults and identify the necessary next step weaning the moment of maximum elimination. The amount of enterohemorrhagic *E. coli* O157 released by calves eliminators is generally between 10^2 and 10^5 cfu / g of faeces.

Excretion of VTEC in cattle was also influences by season. Several studies have reported an increased number of subjects eliminators and quantities of microorganisms emitted during the warmer months and an extension of the elimination period, albeit with lower emission amount of microorganisms, in the cold months (Caprioli et al., 2005).

Some VTEC strains are able to persist in a herd for some time. Studies in the US have shown that certain strains of EHEC O157 have persisted in the dairy industry for more than two years. Furthermore, the introduction of new strains through food, water for the watering and the purchase of new animals, is always possible. The VTEC survive in the feces of animals, preserving their infectivity for periods ranging from a few weeks to a few months. Their survival is mainly influenced by the temperature and water activity (a_w) in the stool, making it higher in conditions of high humidity and low temperatures. Overcrowding and poor housing conditions, such as pavements, litter or soil wet and accumulation of faeces and urine, can promote the persistence of VTEC in the herd of beef cattle. The same factors seem to influence their persistence even in dairy herds (Gautam et al., 2014). At this regard, in the stables where the removal of manure was performed via systems of flushing there was a higher prevalence of bovine excreting EHEC O157 than in the stables where manure was removed by mechanical scraping.

When contaminated feces with 10^8 CFU / g of EHEC O157 are laid on the surface of the pasture, the bacterial count decreases progressively; nevertheless, the micro-organism into the surrounding ground remains detectable for more than 99 days. The persistence of VTEC in the soil favors the infection of the animals, making environmental exposure a major risk factor in animals grazing.

Manure and slurry from cattle farming are frequently used as soil conditioners and fertilizers in conventional farming practices for the production of animal feed and fertilization of meadows and

pastures. Therefore, the presence of VTEC in products intended for cattle represents a potential route of infection and reinfection of herds. The presence of VTEC in foods for cattle has recently been evaluated in the United States: in 504 food samples collected at 54 different fattening farms: *E. coli* O157 was found in 14.9% of cases (75 samples) and there was no correlation between the presence of EHEC O157 and enumerating total coliforms (Farrokh et al., 2013). Fecal contamination of fields, followed by inadequate management silage fodder, is a key risk factor for the persistence of VTEC in the herd. On the contrary, in correct conditions of ensiling, the lactic fermentation is able to eliminate these bacteria and it is therefore wrong to consider silages of good quality a possible source of *E. coli* Verocytotoxic. The inoculation of bacteria producing lactic acid during the ensilage, with the aim to accelerate the descent of the pH, can favor the elimination of VTEC from the product.

Also the composition of the ration is able to influence the presence of VTEC. Several authors have hypothesized that a diet rich in grains may promote, through the lowering of the rumen pH, bacterial adaptation phenomena that can lead to the development of acid-resistance mechanisms able to increase the survival of VTEC and to increase their fecal elimination. However, studies carried out on animals subjected to different diets have often produced conflicting results: in some cases the animals fed with diets high in hay eliminated the EHEC O157 for longer periods than those fed rations high in cereals, while in other studies the subjects fed diets rich in concentrates have shed EHEC O157 for longer than those raised on pasture. Yet other studies have shown no differences between the parties subject to different diets.

The water used for the watering can act both as a vehicle for the introduction of VTEC in breeding, when contaminated in origin, and as a reservoir for the maintenance and circulation of these microorganisms within the herd, due to the contamination of drinking troughs . The VTEC, in fact, are able to survive and multiply in the sediment of water subject to fecal contamination for long periods (LeJeune et al., 2001).

When cattle manure are spread on land in periods of heavy precipitation, verocytotoxic *E. coli* may be transmitted over long distances via the sliding surface of the water or may contaminate groundwater reaching the deep layers of the ground by exploiting the phenomenon of percolation, and spreading infections to other herds and humans through the waters of rivers, lakes, wells and aqueducts.

Cattle farming is undoubtedly a major source of environmental contamination with VTEC; however, other possible sources are sewage contamination of human origin or through the spreading on land of sewage sludge resulting therefrom.

- **VTEC and human trasmission**

The VTEC original source are always the feces of ruminants, and there are many ways through these microorganisms can come to human. The major route of transmission consists of taking food from bovine: meat or meat products contaminated at the slaughterhouse and milk or dairy products made from raw milk, improperly pasteurized or contaminated after the last heat treatment. In addition to beef products, many other foods of various origins are associated with human infection: salami, mayonnaise and fruit or vegetables contaminated with cattle manure, as bean sprouts, lettuce, tomatoes, sprouts and unpasteurized juices (EFSA, 2007). The water contamination is a possible route for human infection, especially in developing countries, but not only these; in this regard, can be cited as an example the "Walkerton tragedy", a source of infection VTEC serotype O157: H7 occurred in 2000 in Walkerton in Ontario (USA), caused by contamination of the municipal water supply, during which were recorded 2,300 cases of the disease, hesitated in seven deaths.

- **VTEC in raw milk**

The possible ways to the entrance of VTEC in milk are fecal contamination and mammary excretion during *E. coli* mastitis. Between the two ways, the first is considered much more frequent in practice, but it can not be excluded that a small part of VTEC found in milk resulting from mammary gland. Indeed, a study conducted in Switzerland found VTEC in the milk of 3% of cows with mastitis from *E. coli* (Stephan and Kuhn, 1999), Besides, a study performed in Brazil found stx genes in 12% of 182 *E. coli* strains isolated from 2,144 samples of milk from cows with clinical and subclinical mastitis (Lira et al., 2004).

Considering the different importanceof the two transmission ways, it is clear that the key point in the control of these pathogens at the farm level is the limitation of the faecal contamination of milk (Hussein et al., 2005). Managerial practices aimed at limiting the presence of VTEC in milk include: the reduction of faecal excretion (through the identification of those "super eliminators", the use of probiotics and vaccines, and minimization of stress for the animals), the limitation of

the spreading of VTEC within the farm (maintenance of hygiene in the housing, the proper management of litter, pest-control, limiting contact between animals of different age groups, limiting contact between raised animals and wild animals, proper management of manure, correct silage of fodder) among different farms (through carrier animals, sewage, water, contaminated food, wild animals and staff), and minimizing faecal contamination of milk during milking (Farrokh et al., 2013).

Even under the best hygienic conditions, the skin of the udder and teats of dairy cattle is always contaminated by a certain amount of fecal material from the environment. The milk from the udder that are dirty and not subjected to cleaning of the teats during milking can reach more than 10^5 CFU / mL. There is a close relationship between the total bacterial count in milk and the hygiene of the udder. The level of contamination of milk by bacteria present on the skin of the udder is influenced by the degree of contamination of the udder itself, the percentage of contaminated udder and by the grade of cleaning of the teats when milking. The contamination of the udder is in turn influenced by many environmental and management factors: housing type (fixed, free with bunks, free with permanent litter), management of litter (frequency of renewal), type of litter (straw, sawdust , sand, rubber mattresses), cleaning of the areas of feeding (mechanical scraping vs. flushing). Regarding milking, pre-dipping is particularly important to reduce the possibility of milk contamination. The cleaning of the teat with a detergent solution and disinfectant, followed by thorough drying, is able to reduce the bacterial load of the skin of 75% and, therefore, contamination of the milk (Galton et al., 1989; Bade et al 2008). Also the wasting of the first jets of milk before the attack of the milking unit (forestripping), eliminating the milk portion closest to the orifice of the teat, allows to reduce the contamination of the milk in the tank. VTEC are also able to persist inside of the milking plant in the case in which the operations of washing and sanitizing are not carried out correctly (Farrokh et al., 2013). The result of the operations of washing of the milking plant is influenced by many factors: temperature, quantity and quality of water used, turbulence and pressure of the washing water, the type of detergent used and its concentration, as well as the duration of the different phases Process (Reinemann et al., 2000).

As described above, in the raw milk is possible to find VTEC. The prevalence of these bacteria in raw milk cattle is generally low and it is usually comprised between 0 and 2%. The latest EFSA report, for the year 2013, shows data from nine member countries (Austria, Belgium, Estonia,

Germany, Greece, Czech Republic, Slovenia, Spain and Hungary); 18 samples out of a total of 860 tested samples were positive for VTEC, equal to 2.09%, and 5 of them (0.58%) were contaminated with O157 serogroup; non-O157 VTEC belonged to serogroups O26, O103, O111 and O145. The prevalence of VTEC was higher in samples of milk taken from breeding (4.52%) and lower in samples taken at retail (1.52%) and in processing plants (1.19%) . In the report for the year 2012 it is not reported any data on VTEC in raw milk. The report showing the data of 2011 collects information from four Member States (Belgium, Germany, Slovenia and Hungary); 499 samples of raw milk were tested, of which 8 (1.6%) were positive for VTEC, and one (0.2%) of these was a serogroup O157 strain. For the year 2010 data were presented from four member countries (Bulgaria, Germany, Slovakia and Hungary), and the VTEC were detected only in Germany, where 17.6% of 318 samples of raw milk destined to processing and none of 117 samples of raw milk destined to direct consumption tested positive for non-O157 VTEC. Italy has provided the data for the years 2009, 2008 and 2007. In 2009, none of 994 samples tested positive for VTEC; in 2008 0.6% of 161 samples of raw milk tested positive for VTEC O157; in 2007, there was no evidence of VTEC in the 436 tested samples.

The reported prevalence of VTEC in raw milk are low, but their presence is always possible, both for O157 and for non-O157 strains, and VTEC can be detected also in raw milk cheese.

Because of the complexity and the enormous variability of the process leading to obtaining the many different cheeses and products all over the world, it is particularly difficult to fully understand the behavior of VTEC during the cheese and predict their survivability during ripening, maturation and commercialization. However, the microbial stability of the cheese is commonly determined by a number of factors that hinder the growth of bacteria; among these, the temperature reached during the production steps, the low pH, the water activity value (a_w), the content of NaCl and the presence of competing flora, are those of greater importance for the achievement of neutralization of VTEC. Several authors have shown how each of these factors, when applied individually, may not be able to achieve the elimination of *E. coli*. Indeed, the survival of VTEC can be compromised only through the simultaneous or sequential application of these stressors. Nevertheless, it is reasonable to assume that in the cheese exists a synergy in the action of these factors and that the combined application of the stress is able to eliminate the VTEC in most cases. All dairy products made with pasteurized milk, since this procedure is effective in the elimination of VTEC, can be virtually considered as VTEC-free, with the exception of

pasteurization failure or contamination of milk in successive steps. It means that the VTEC assume particular relevance only in soft and semi-soft cheese made from raw, unpastorized milk, such as soft cheese. The low prevalence of VTEC in raw milk and effectiveness of most cheese making processes in neutralizing these bacteria, make the presence of VTEC in the cheese and dairy products as quite limited. The EFSA report on animal diseases in 2015, relative to 2013, shows data on VTEC for dairy products other than raw milk from eight Member States (Austria, Belgium, Germany, Greece, Ireland, Italy, Spain and Sweden). Out of 1317 tested samples, 66 (5.01%) were positive for VTEC, and 2 of these were contaminated with O157 (0.15%) and 64 (4.86%) with non-O157 VTEC. Regarding Italy, 796 samples of milk-derived products were tested, among which 24 (3.01%) were positive for VTEC and 2 were O-157 strains.

- **O157 VTEC and non-O157 VTEC**

The strains of *Escherichia coli* are serologically classified based on antigenic differences of antigens O (somatic) and H (flagellar), based on the typing scheme introduced in 1994 by Kauffmann and still used internationally. More than 180 types of O-antigen and more than 60 types of antigen H are currently known (D'Souza et al., 2002). The set of bacterial strains characterized by the same antigen O constitutes a "serogroup" while a specific combination of O antigen and H antigen defines the "serotype" of a strain. Additional antigens taken into consideration in the classification scheme of Kauffmann are K (capsular) and F (fimbrial) antigens.

The membership of a strain of *E. coli* in a given serogroup or serotype may be associated with the presence of certain pathogenic factors and the ability to cause specific clinical symptoms. However, determining only the serotype of a strain can not be useful to predict its genotypic characteristics or its pathogenicity, since the horizontal transfer of genetic material between different bacterial clones may allow strains with identical phenotypic trait (such as serotype) carrying many genotypic differences and different pathogenic factors (Nataro et al., 98, Wu et al 2008).

An extraordinarily important aspect concerning the Shiga-like toxins is their viral origin. In fact, they are encoded by viral genes belonging to prophage of temperate bacteriophage integrated into the bacterial genome by the mechanism of the conversion phage. In this form of genetic transfer the presence of the prophage and the simultaneous expression of viral genes constitute stable genetic alteration in the bacterium (Poli book, 2005). However, following a stressful factors

of different nature (antibiotics, UV rays, etc.), excision from the bacterial chromosome can occur, with the reactivation of the lytic cycle and release of mature viral particles, able to infect new bacterial cells and giving rise, potentially, to new VTEC (Poli book, 2005). The same enterohemorrhagic *E. coli* (EHEC) are, probably, the result of the evolutionary path followed by some enteropathogenic strains (EPEC) after the acquisition of the genes encoding the verocytotoxin (Reid et al., 2000). The most dramatic demonstration of this mechanism, was, probably, the epidemic which occurred in Germany in 2011. On May 26 of that year, Germany signaled a major outbreak of haemolytic uraemic syndrome (HUS) caused by *E. coli* producer verocytotoxin; in one week 470 cases were reported (Askar et al., 2000). The VTEC isolated strain was later characterized by researchers at the laboratory of the National Reference Centre for Salmonella and other Enterobacteriaceae at the Robert Koch Institute in Berlin. This bacteria, belonging to serotype O104: H4, proved capable of producing the verocytotoxin Stx2a but it turned out no characteristic of the classic markers of pathogenicity of EHEC; it was, in fact, LEE enteroemolisin negative; it had, instead, the plasmids encoding the pathogenic factors of enteroaggregative *E. coli* (EAEC), responsible of th autoagglutination and biofilm formation, so-called "stacked-brick" (AATA, AGGR, aap, Agga and aggC) (EFSA, 2011)

This unusual combination of virulence factors associating EAEC status and ability to produce verocytotoxin is extremely rare and it had been previously reported in one case, in a strain with serotype O111:H2 implicated in a small outbreak of hemolytic uremic syndrome in France 1998 (Morabito et al.,1998).

- **EFSA and non-O157 VTEC**

The Panel on Biological Hazards of EFSA scientific opinion on the monitoring and identification of human pathogenic VTEC 2007, concludes that only a small group of serogroups is associated with a high risk to public health (O26, O103 , O91, O145 and O111). EFSA recommends the implementation of monitoring systems aimed at identifying VTEC belonging to these serogroups potentially at risk in food (meat of ruminants, meat products "ready-to-eat" vegetables, milk crude and products derived therefrom) and animal reservoir.

- **Principal methods to detect VTEC status**

The VTEC include 400 different serotypes, and each serotype has its physiological characteristics, its own set of virulence factors and a different potential pathogenic for humans. This diversity is reflected by the complexity and variety of the methods used in their search, especially in the case of non-O157 VTEC.

The large set of VTEC searching methods can be divided into two subsets based on the fundamental concept on which they are built serotyping-independent methods and serotyping-employing methods. The serotyping-independent methods are based on the search of the pathogenicity factors of EHEC or genes coding for them; serotyping-employing methods, otherwise, are intended to identify strains of *E. coli* belonging to the serogroups most frequently implicated in human infections. Obviously, both approaches are not free from errors, in fact, with the first approach strains are frequently labeled as pathogenic VTEC strains but belonging to serotypes not able to cause disease in humans; with the second approach, on the contrary, strains of pathogenic VTEC belonging to serotypes are rare but can cause serious illnesses, such as O113: H21, O174: H21, O104: H4 and many others, could not be detected.

For the identification of VTEC O157: H7, the main cause of pathogenic VTEC recorded in humans, there are several serotyping-employing methods bacteriological, serological and molecular methods; also for the VTEC non-O157, identification methods are available for different serogroups (such as O26, the O103, O111 or the O145) but, because of the greater variability existing between them, they are more difficult to be implemented and less accurate.

The variability of the O antigen that is part of the lipopolysaccharide on the outer membrane of *E. coli* is due to differences in sugar composition and the sequences and linkages of these sugars (Wick et al., 2005). The O-antigen gene cluster consists of genes that are well conserved between O serogroups and also genes that differ across serogroups. These O-serogroup-specific genes are responsible for the synthesis and assembly of the O antigen (Wang et al., 1998). In a recent study (Norman et al., 2012), single nucleotide polymorphisms (SNPs) within the O-antigen gene cluster of non- O157 *E. coli* serogroups O26, O45, O103, O111, O121 and O145, were identified as predictive of VTEC-status within a serogroup. In the study by Norman et al., differences in the genetic sequence of the O-antigen gene cluster corresponded well to the differences in the virulence gene profiles and provided evidence of separate clustering for the majority of VTEC and non-VTEC strains.

1.2 HRMA for polymorphism analysis

1.2.1 Generality on High Resolution Melting Analysis (HRMA)

Amplification product melting analysis is not a novel concept and exploits a fundamental property of DNA, called melting: the separation of the two strands of DNA with heat. PCR product melting analysis in combination with real-time PCR was first introduced with the LightCyclers and the stability of DNA duplexes was detected using intercalating dye, like SYBR Green I. This is able to detect primer-dimers or other non-specific products (Wittwer et al., 1997). This procedure, called Low Resolution Melting has been performed for over a decade. A Low Resolution Melt curve is produced when the temperature increases, normally in steps of 0.5 °C increment per second, thereby gradually denaturing a previously amplified DNA target. Since SYBR Green I is only fluorescent when bound to dsDNA, fluorescence decreases as the double strand of the DNA amplicon is denatured. The melting profile depends on the length, GC content, sequence and heterozygosity of the amplified target. The highest rate of fluorescence decrease is normally at the melting temperature of the DNA sample (Tm). The Tm is defined as the temperature at which 50% of the DNA sample is doublestranded and 50% is single-stranded. The Tm is typically higher for DNA fragments that are longer and/or have a high GC content. The fluorescence data from low resolution melting curves can easily be used to derive the Tm by plotting the derivative of fluorescence vs temperature (-dF/dT against T) as reported in Figure 6 (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

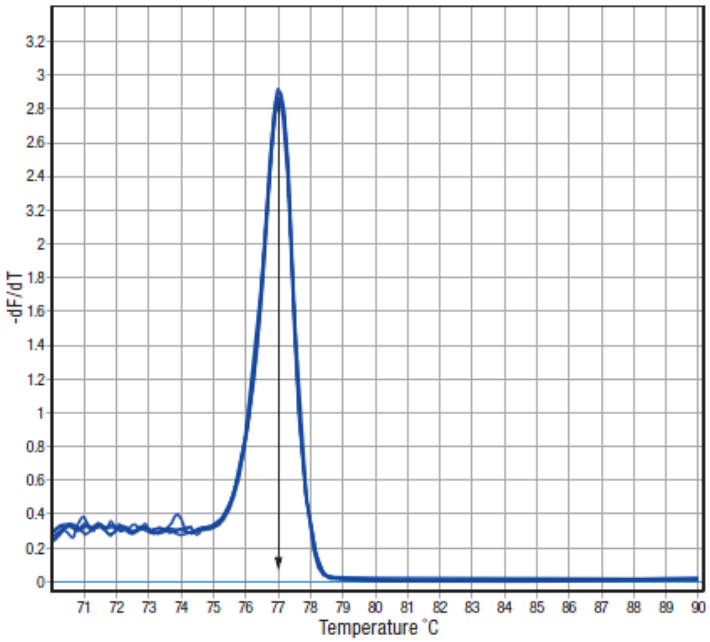


Figure 6: Low Resolution Melt profile derivative plot (-dF/dT against T).

The steepest slope is easily visualized as a melt peak. In this example the Tm of the amplicon is 77 °C (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

The principle of High Resolution Melting Analysis (HRMA) is the same as a Low Resolution Melt, except that the temperature difference between each fluorescence reading is reduced. During a Low Resolution Melt curve analysis, the temperature increases are typically in 0.5 °C steps, but for HRMA this is reduced to 0.008 - 0.2 °C increments, depend on the property of the instrument.

Briefly, the first step of the HRM protocol consists in the amplification of the region of interest (usually, through a real-time PCR protocol), in the presence of a specialized double-stranded DNA (dsDNA) binding dye. This particular dye is highly fluorescent when bound to dsDNA and inadequately fluorescent in the unbound state. This change allows to monitor the DNA amplification during PCR (as in quantitative PCR). After completion of the PCR step, the amplified target is gradually denatured by increasing the temperature in small increments, in order to produce a characteristic melting profile; this is termed melting analysis. The DNA sequence is gradually heat-denatured, the dye is released from the amplicon and therefore resulting in a decrease of fluorescence. If tuned in the correct way, this process allows a much more detailed analysis of the melting behavior. HRMA is thus able to detect a single change in the nucleotide sequence. HRMA uses low-cost dyes and requires less optimization than similar systems based on TaqMan chemistry and fluorescence resonance energy transfer (FRET) probes. Besides, HRMA is a

simpler and more cost-effective way to characterize multiple samples (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).HRMA sensitivity and reliability has been improved with the use of a variety of new dsDNA intercalating dyes and the availability of new instruments and more sophisticated analysis software.

1.2.2 The intercalating dyes

There are a variety of dsDNA intercalating dyes, which have particular different characteristics. The conditions of the dyes used for HRMA are different from dyes typically used for standard quantitative PCR (qPCR) assays. Factors critical in qPCR, such as the signal-to-noise ratio and amplification efficiency, are not essential requirements for HRMA. Instead, the dye must provide detailed information on the melting behavior of an amplified target. Ideally the dye should not bind preferentially to pyrimidines or purines, change the Tm of the amplicon, or inhibit DNA amplification (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). The three main classes of dsDNA binding dyes currently available are:

- **Non-saturating dyes.** SYBR® Green I is the most common non-saturating dsDNA intercalating dye. This type of dye is not very suitable to be used in HRMA. SYBR® Green I stabilizes the dsDNA when used at high concentrations, but it inhibits DNA polymerase. In contrast, low concentrations are not distributed in an appropriate way in the strand of DNA that results in poor discrimination-based difference (Figure 7). For a good performance the dye must be used at the right concentration.
- **Saturating dyes.** These dyes, unlike the previous ones do not inhibit the DNA polymerase and do not alter the Tm of the fragment. These dyes in contrast to other can be added in higher amounts so that the entire sequence is full of dye. Unfortunately this dye is not able to redistribute during melting because the dsDNA is saturated. More accurate examination of the melting performance is therefore possible, as reported in Figure 7. Dyes such as SYTO9® and LCGreen® can be used at the saturating concentrations required for HRMA.
- **Release-on-demand dyes.** The “release-on-demand” class of dyes, which include EvaGreen®, can be added at non-saturating concentrations. This characteristic depends on the new method of fluorescence emission. In fact, the dye signal turns off when the fluorescent is free in solution. After the dye is tied in with the filament dsDNA, when the temperature drops the dye starts to emit the signal. This allows non-saturating concentrations of the dye to be used,

ensuring that there is no PCR inhibition, whilst the unique dye chemistry provides highly sensitive HRMA analysis.

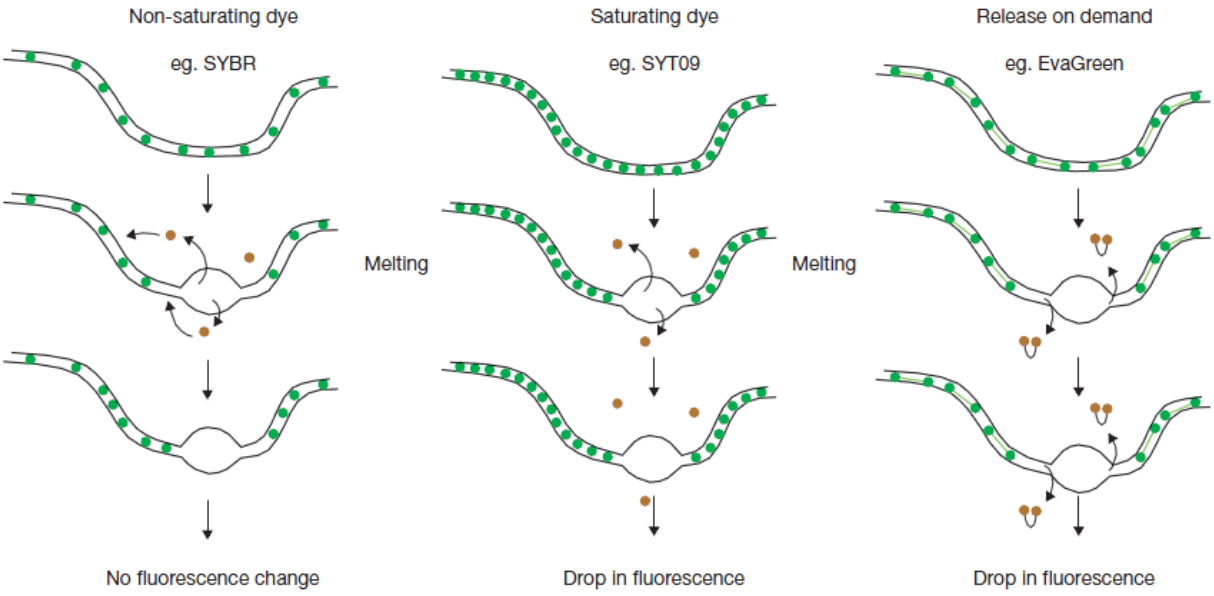


Figure 7: Non-saturating, saturating and 'release-on-demand' dsDNA intercalating dyes (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). Melting of the duplex as the temperature increases releases the intercalated dyes. At non-saturating concentrations the dye rapidly rebinds to regions that remain double stranded; consequently there is no drop in fluorescence. Saturating and 'releaseon-demand' dyes do not redistribute from the melted regions of single-stranded DNA back to dsDNA, resulting in a reduction of fluorescence. This difference gives dyes such as EvaGreenR the high sensitivity required for HRM analysis.

1.2.3 HRM assay and reagent optimization

The HRMA is a very sensitive technique and for this reason the development of the method is crucial. There are several factors that influence the behavior of melting, as genomic DNA (gDNA) quality, the background pattern, the concentration of $MgCl_2$ and polymerase inhibitors. Small differences in the initial configuration can significantly impact the final results. Achieving specific amplification is critical to the success of an assay, since any non-specific amplification will greatly impair the melt analysis. The following most important aspects are listed (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com):

- **DNA quality and quantity:** One of the main factors that can affect the quality of the results is the relationship between DNA samples and PCR preparation. Unsuitable purification techniques can distort the signal. The presence of salts can change the melting behavior of the PCR product, and may result in poor reproducibility, low sensitivity and incorrect genotype calls.

Buffer carryover from the template DNA will not only modify the Tm and melting behavior during the HRM, but can encourage also nonspecific amplification during PCR.

To avoid possible bias in the results, some tricks may be used, as for example: a) to extract the DNA to be analyzed using always the same kit, b) to check the concentration of salts in the kit because any excess can distort the signal, and c) to check that the DNA concentration is equal for all samples. Table 4 shows the recommended concentration of DNA for the different types of targets. At low concentrations of the target, there is a greater possibility of incorporating a mutation early in the PCR that will affect the melting behavior. By contrast, high concentrations of DNA can result in high fluorescence due to an increase in dsDNA intercalation of dye. The positive control(s) for all genotypes should be included if possible, preferably at the same concentration of their corresponding samples. Control DNA should also be eluted and/or diluted in the same buffer of the samples.

Type of DNA	Recommended amount for HRM analysis/reaction
Genomic DNA (gDNA)	10 ng - 100 pg
Plasmid DNA (1 – 10 kb)	1 ng - 10 fg
Amplicon DNA	10 pg - 1 fg

Table 4: Recommended concentration of DNA for different types of template (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com)

➢ **Primer design:** the primers for HRM are not easy to draw, since they should adhere to several requirements. In fact, it should be noted that the HRM analysisis is preceded by a qPCR, then amplicons can not be very long to ensure high sensitivity. Potential primer sets and full-length amplicons can then be tested for specificity using a BLAST search. Factors such as the position of the primer, the need for a GC region on the 3 'end, the presence of any secondary structures in the primers or target, and the specificity of the predicted amplification should be carefully considered. Software as Primer3 or Primer3Plus can be very useful for drawing of primer HRMA, because the user can specify the location, the length and any other characteristics that are needed (Figure 8).

After the primers have been designed, the following factors must be checked:

• The presence of a GC 'clamp' on the 3' end. This is not essential, but usually improves amplification efficiency.

• The specificity of the primers and the presence of known sequence variations. A BLAST search with both primer sets and the expected amplification product should be performed. If possible, sequence variations should also be checked.

• The secondary structure of the primers and the expected amplification product. This should be examined using appropriate softwares, for example, m-fold, http://mfold.rna.albany.edu/?q=mfold/dna-folding-form, or operon, http://www.operon.com/tools/oligo-analysis-tool.aspx. Amplification of any secondary structure within the product may result in unusual melting profiles. Primers may need to be redesigned to avoid areas of secondary structure.

• Amplicon size. This must be optimal for the specific application, typically between 100 - 300 bp.

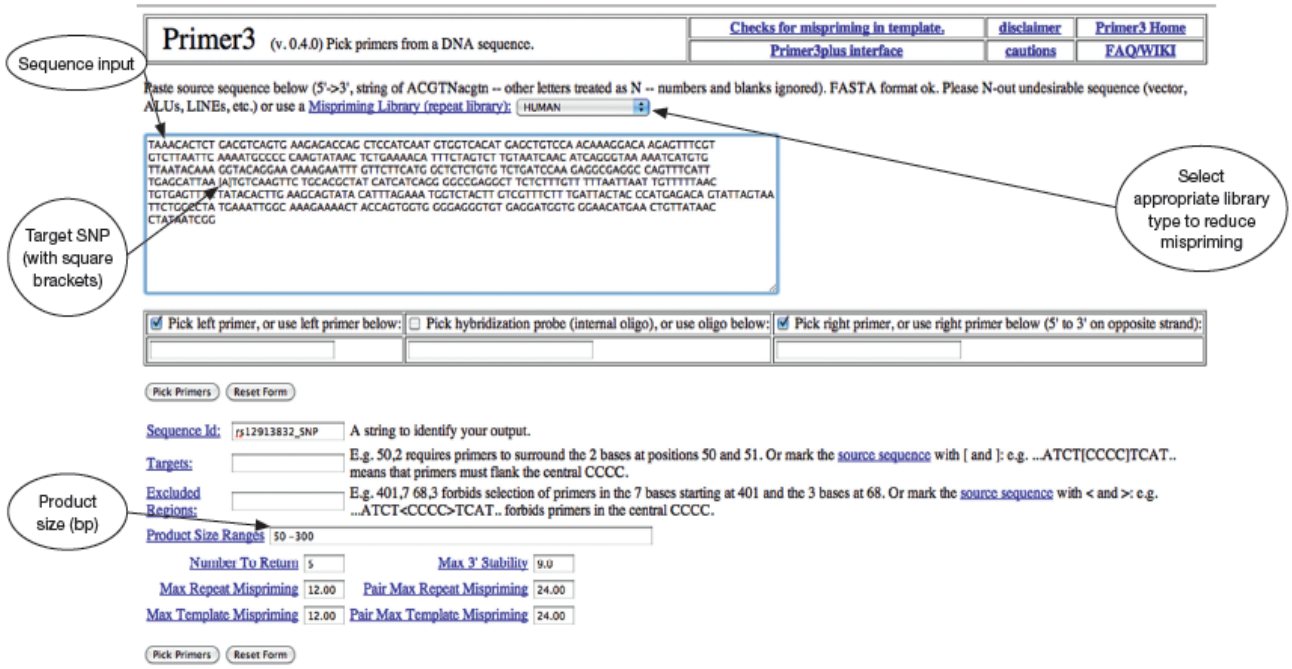


Figure 8. Design of primers for amplification of a human SNP using Primer 3 (http://frodo.wi.mit.edu/primer3/). The target SNP is shown in square brackets (e.g. ... TTAA[A]TGTC...) and the product size is set to 50 – 300 (bp).
Selection of the mispriming library 'HUMAN' helps reduce the chances of non-specific amplification (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

➢ **Primer concentration and purification:** the concentration of primer does not significantly affect the overall result in HRMA, however the recommended primer concentration to avoid nonspecific amplification is between 0.05 and 0.5 µM final. For normal purposes, it can be used the final concentration of 0.3 µM as recommended by the Evagreen's manufacturers.

➢ **Effect of $MgCl_2$ on HRM analysis:** different concentrations of $MgCl_2$ influence the efficiency of amplification, as is the case with conventional PCR. The amount of $MgCl_2$ leads to reduced non-specific amplification, and allows a clearer distinction of sequence variations. It is important to realize that the optimum $MgCl_2$ concentration for amplification efficiency Is not necessarily optimal sensitivity for HRM. The effect of $MgCl_2$ is shown in Figure 9. As the concentration of $MgCl_2$ is increased, the HRM difference graphs change distinctively.

- **Amplicon length:** in HRMA it is difficult to specify the optimal length of the amplicon, since there are many factors to be considered. For example, one factor is the number of nucleotide changes in the sequence, i.e. the less variations there are, the shorter should be the amplicon. Normally the amplicon length can vary from 50 to 300 bp. Clearer discrimination is usually seen with shorter amplicons. However, small amplicons can result in lower fluorescence values, presumably because of decreased incorporation dye. By contrast, very long amplicons are allowed to create complex structures resulting in melting behaviours that can be misinterpreted in the end. When screening for unknown sequence differences, longer amplicons (typically 200-500 bp) can be used. This is useful in gene scanning or determining the variation within at population (e.g., viral) and reduces the number of required primer sets. Furthermore, too short amplicons are difficult to sequence.

- **Effect of PCR enhancers on HRM analysis:** PCR enhancers are commonly added to end-point PCR in order to help reducing non-specific amplification and the number of reaction cycles, and increasing yield. These enhancers function by assisting the melting and annealing of primers and templates, and can affect HRMA performance. For GC-rich amplicons, the addition of DMSO appears not to affect the HRMA significantly, and is recommended if amplification is difficult. Overall, the addition of PCR enhancers is not recommended as they usually detract from HRM performance.

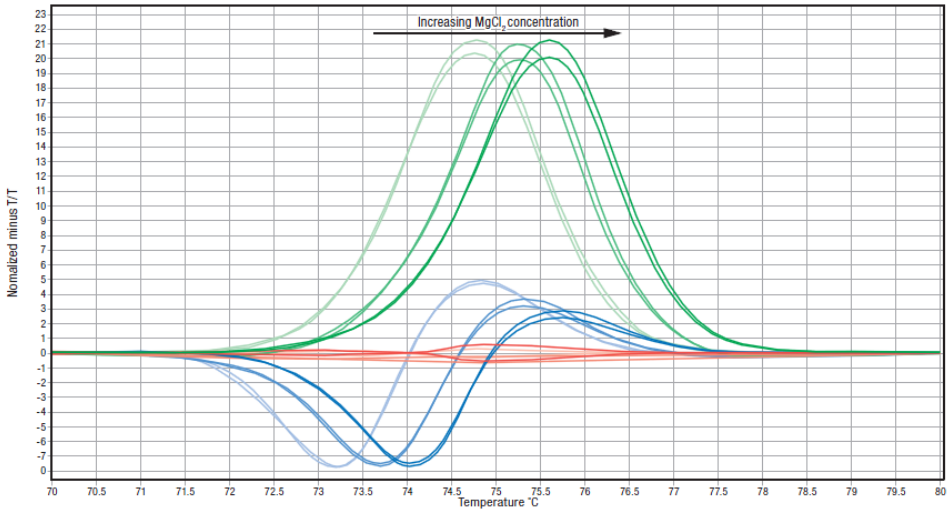


Figure 9. Effect of increasing $MgCl_2$ on melting behavior. Three Difference Graphs are overlayed to demonstrate the dependence of amplicon melting on $MgCl_2$ concentration. The effect of $MgCl_2$ is most pronounced in the heterozygote samples (blue), a result of the magnesium stabilizing the annealing of mispairs. The $MgCl_2$ concentrations are 1.5 mM, 2.5 mM and 3.5 mM final. G/G = green, G/A = blue, and A/A = red (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

1.2.4 Instruments and software

The capacity of HRM analysis depends upon the sensitivity of the instrument and the nature of the PCR reagents used. Certain instruments have been designed specifically for HRM analysis. These can be summarized into two distinct classes:

1. Block based instruments – Samples are placed in a block for cycling and a scanning head or stationary camera is used for detection.

2. Rotary – Samples reside in a single chamber and spin past an optical detector.

There are advantages and disadvantages with each class, and the users should choose the type of instrument that best suits their needs. As a general rule, for high sensitivity and reproducibility a rotary-based instrument should be used, whilst for high-throughput and ease-of-handling, block-type instruments are optimal. The HRM run can vary considerably between instruments; some take considerably longer than others. Every instrument should be calibrated regularly (as recommended by the manufacturer, typically every 6 months), kept in a clean area on a secure and stable benchtop, and checked daily for dust buildup around the optics. The computer hardware and software must be capable of handling the large quantities of data usually generated during a HRM experiment. Analysis of the data is relatively easy with the appropriate software. HRM specific software uses various algorithms to analyze the data and display the results in user-

friendly formats to help discriminate between sequence variants. Additional software packages, designed for more detailed analysis, are available from some instrument manufacturers (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

1.2.5 Analysis of results

Data analysis is typically straightforward, with the correct software, allowing multiple samples to be analyzed at the same time. Low resolution melt plots are often viewed as a derivative plot (−dF/dT against T); however, HRM raw melting curves must be analyzed differently. It is important to know what to look for when interrogating results; unforeseen errors made during setup can be identified at this point.

Amplification plots. For HRMA, the reproducibility of amplification is more important than efficiency amplification, compared with qPCR. As an example, the HRM analysis can still be performed if the product amplifies later than expected (for example due to limitations in the design of primers), provided that all the samples are constants, the no template control (NTC) does not amplify and the product is specific.

Raw data melt curve. The data collected during the analysis HRM has a variety of (pre-melt) initial values of fluorescence. These variations make it difficult to interpret the results, even if different genotype groups can be visible. Figure 10A how to analyze the HRM results and a 124 bp product covering a Type IV SNP is used as an example (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com). The panel A of this figure illustrates how the selection of pre-and post-melt regions is used to align data. It is important to adjust these bars so that the melt region is not selected. If the preor post-melt regions are not clearly defined, it is possible to repeat the HRM run only (without repeating the amplification step), and adjust the temperature range as required. Pre- and post-melt regions must be selected for each primer set, by positioning the parallel double-bars as shown.

Normalization data. If the data is normalized correctly, it will appear as shown in the panel B of the Figure 10. This is termed ‘Normalization Data’. Here the fluorescence variance seen in the Figure has been eliminated, and only the temperature range between the outer bars of the pre- and post-melt regions is shown. The genotypes are now more distinct, but the two homozygote samples (in red and blue) are still difficult to distinguish.

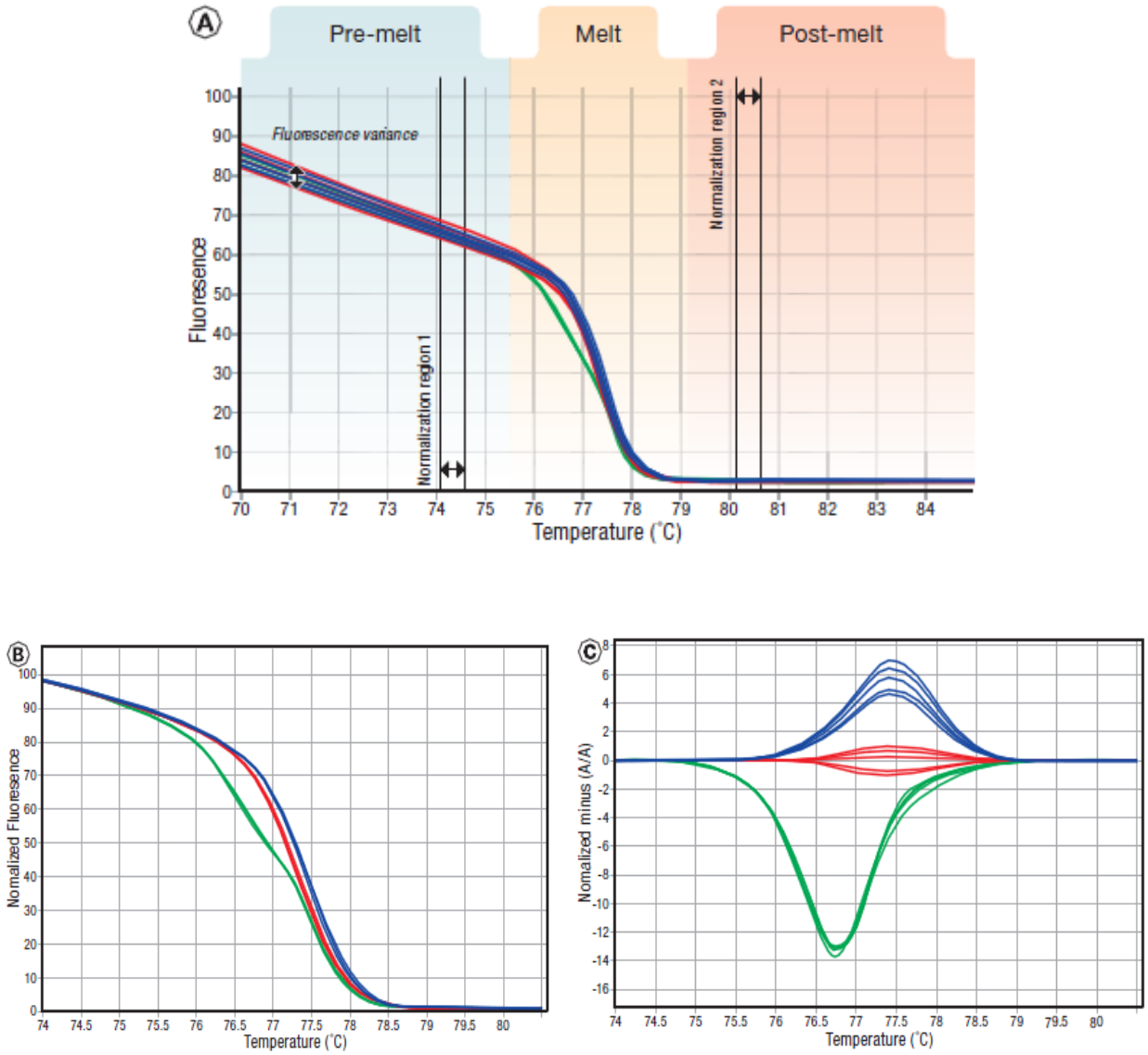


Figure 10. Analysis of HRM data from a type 4 SNP (A/T). Different genotypes are highlighted in different colors. A/A = red, A/T = green, and T/T = blue.

A. Raw data showing the pre-melt, melt and post-melt regions. Notice the fluorescence variance and the positioning of the pre- and post-melt identification bars. **B.** Normalization Data derived from the raw data plots in A. Positioning of the pre-melt, and post-melt bars provides a more detailed view of the melt region. **C.** Difference Graph derived from the Normalization Data (High Resolution Melt Analysis, Application Guide, www.kapabiosystem.com).

Difference graph. Some HRM software applications allow calculation of the difference plot. This is achieved by subtracting the normalized fluorescence data of a user-defined genotype from that of each of the other samples in the HRM analysis. In panel C of Figure 10, the A/A genotype has been selected as a baseline (any genotype can be selected, but usually one of the homozygotes is used). The position of each sample relative to the baseline is plotted against the temperature. This

output of HRM analysis is an aid for visualizing the normalization data. Automatic calling of genotypes of sequence variants can usually be performed with most instrumentation softwares; sometimes confidence ratings are given. However, checking the difference to confirm the genotype is recommended.

1.3 Aim of the work

One of the main activity of the present project was based on the test hypothesis that the virulence profile of *E. coli* strains toward bovine mammary gland is modulated by the interaction with the host cells. Besides, a comparison between mastitis *E. coli* strains and O157 *E. coli* strain might clarify if a similar modulation is induced by the host also on non-bovine strains causing human disease. These hypothesis were tested through a gene expression study of some virulence factors of six *E. coli* strains when co-cultured with a bovine mammary cell line, since the *in vitro* models are an essential tool to investigate the biological mechanistic of mastitis. Furthermore, the use of *in vitro* models have the possible advantage that they represent an efficient alternative to animal experiments, thereby saving the lives of laboratory animals and reducing the expensive costs of animal experimentation. The European Union legislation included an alternatives studies without the use of animals declaring that "authorities will have to assess the animal welfare implications of all scientific experiments involving animals with a view to promoting alternative testing methods where possible" (http://www.euractiv.com/en/science/eu-wantsfewer-animals-used-research-news 497607) (Purup and Nielsen, 2011). For the purpose of the coculture experiment, a meta-analysis of existing literature studies on the available bovine mammary cell lines was preliminarly performed, resulting in the selection of MAC-T as the most responsive cell line to bacteria causing mastitis. The *E. coli* strains used for the coculture experiments with MAC-T cells were isolated from different types of bovine mastitis (acute, chronic and undetermined) and from a VTEC food-borne strain associated to human clinical disease (O157). Among these strains, 727 and McGear strains were reported to be able to induce mastitis in bovine host and were used extensively in the past for experimentally induced *E. coli* intramammary infection and to conduct studies focused on adherence to, internalization into, and trafficking in bovine mammary epithelial cells (Dopfer et al., 2001; Dogan et al., 2006; Oliver et al., 2010; Almeida et al., 2011). The remaining strains were isolated from bovine mastitis (UK *E. coli* ECIMI, UK *E. coli* ECIMI 8 and UK *E. coli* ECIMI EK A) and a foodborne human clinical case (O157:H7). The target genes were selected on the basis of a previous publication preliminary reporting the comparative expression profile of two *E. coli* strains isolated from bovine mastitis, and co-cultured with primary bovine mammary cells (Kerro-Dego et al., 2012).

The experimental design was as follows: 1) *E. coli* strains isolation and growth, 2) DNA screening of strains for virulence genes, 3) MAC-T cell propagation, 4) co-culture assays, 5) bacterial RNA isolation, 6) quantitative RT-real time PCR, 7) data analysis.

The presence of VTEC in cattle and other ruminants calls for further analysis of the pathogenic potential for humans of VTEC strains isolated from non-human sources. An increasing number of putative virulence genes of VTEC have been characterized. To date, a food product found positive to genes VTEC is automatically deleted from production. The tests that are currently being put in place by the EU Reference Laboratory for *E. coli* (Department of Veterinary Public Health and Food Safety Unit of Foodborne Zoonoses) and consequently by the Istituto Superiore di Sanità in Italy, are to verify the presence of stx1 , stx2 and eae . The data of the Istituto Superiore di Sanità in Italy, show that about 60 % of the tested products has a positive test to the above targets but out of these, only a small percentage can actually cause HC and HUS (thus being classifiable as "true" VTEC). In fact, the positivity genes listed above are not automatically characterizing the bacterium as verotoxic. The SNPs discovered in the study by Norman et al., 2012, can in principle be used to develop tests that will specifically identify STEC strains within serogroups O26, O45, O103, O111, O121, and O145. Indeed , unlike the *E. coli* O157: H7 that is always pathogenic, the serotypes O26, O111 , O121 , O145 and O103 may not be pathogenic even if they have the *stx1* , *stx2* and *eae* genes, since also other genes like *ehxA*, *efa1* and *nleB* contribute to virulence (Norman et al. , 2012). One of the targets of this project is to design a new HRMA-based protocol for the evaluation of the risk linked to an isolated *E. coli* strain, based on the detection of virulence-predictive SNPs, as discovered by Norman et al., 2012.

The activity involves the following steps:

1. Serotype-specific primers coding for O-antigen regions of the five major VTEC serogroups available in literature are used to perform conventional multiplex PCR on *E. coli* strains isolated from animals and food products, and preliminarly assessed as stx-positive strains, to assess serotype (Table 7): the wzx, which encodes for a flippase required for O-polysaccharide export, was used to design primers for serogroups O26, O45, O103, O111, and O145 with resulting amplicons ranging from 200 to 900 bps (Paddock et al., 2012). The wbqE gene, which encodes for a putative glycosyl transferase, and wbqF, which encodes for a putative acetyl transferase, were used to design primers for O121. The primer set for rfbE, described in Bai et al. (2010), was included for the O157 serotype identification. The

mPCR conditions used are similar to that of Bai et al. (2010). The amplified DNA is separated on 1% agarose gel and stained with 0.5 μg/ml of ethidium bromide.

2. At this step, the serotype of *E. coli* strain is known. If the analysis finds that it is the strain O157, it is useless to proceed with further analysis, as this serotype is considered the most dangerous between serotypes and the retaled product should always be removed from the market. For the other serotypes, the strains are assessed whether they are truly dangerous or not. The related analysis is carried out on target serotype-specific SNPs, already investigated in a MALDI-TOF pilot study to correspond with differences in virulence gene profiles, as in Norman et al., 2012. This step is carried out through SNP-specific HRMA protocols designed in this project that are currently under test for each representing serotype (Table 8).
3. At this step, if SNP-specific HRMA turns positive, this result predicts that the related strain contains Shiga toxin-encoding genes and other virulence factors predictive of the risk status of the sample. Consequently, the source product should be permanently removed from the market.

1.4. Materials and methods

1.4.1. Selection of the bovine mammary cell line for co-culture experiments

1.4.1.1. Search strategy

The selection strategy for the more suitable bMEC for coculture experiments with E. coli strains was performed following the assumption that the modulation of bacterial gene expression could be more evident when cocultured with a highly responsive cell line. Thus, meta-analysis started to response at one simple question: *Who are the best cell line for the study of immune response in mammary gland?*

The papers was searched using Web of Science and PubMed as electronic databases, whose general settings were "All years" and "All databases". Databases were consulted until December 1st, 2014.

No language restrictions were applied. The reference lists of retrieved articles were searched manually to identify all potential studies so that no articles were missed by the electronic searches.

The first keywords for the selection of the paper were "*bovine mammary epithelial cells*". In this analysis any Boolean operator has been used, but instead a second query: "immune response" was used.

1.4.1.2. Inclusion and exclusion criteria

The papers without quantitative data were excluded from the analysis.

Further selection was based on the following specific criteria: *Data originating from pure mammary lines* (no co-cultures whit other mammary cell lines); *Cultivation on standard substrates, No positive and/or negative control*.

The data of all authors were independently extracted from suitable studies using a standardized form. The information obtained were collected on the basis on surname of first author, year of publication, source of publication, study design, biological stimulus used, time of exposure, quantity of stimulus, method used, cell line response and quantity response (Table 5), for any cell line used. In the cases of conflicting evaluations, different data from the eligible studies were compared for availability of the full text and extensive discussions of the data by the authors.

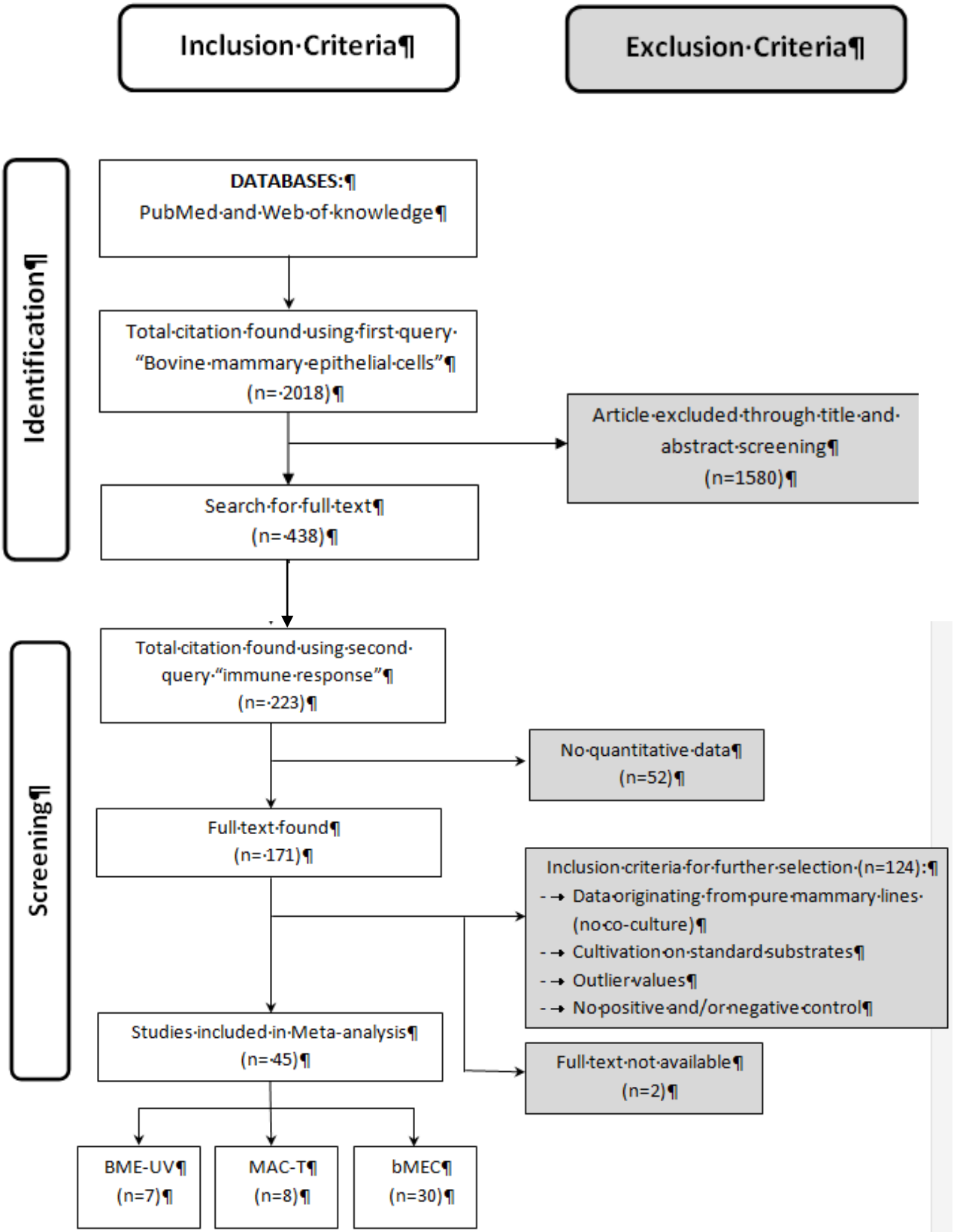


Diagram 1: Grafic rappresentation of paper selection to choise the cell colture for co-colture

1.4.1.3. Statistical analysis.

Data were collected on a database and results were compared when:

- The assay technique was the same (i.e. RT-qPCR).
- There was at least one valid quantitative value for each cell type.

Other factors considered were:

- Stimulating agent concentration and time.
- Time of measurement

In case outlier values were detected based on sufficient sample size, they were withdrawn from the analysis. Data were analyzed by **general linear model** (GLM procedure) on **SPSS 20.0**

The model included as fixed factors: cell line, paper, time of assay and stimulus concentration.

Information collected for any type of cell line		
General information	Author	First author present in the paper
	Year	Year of publication
Information in relationship of stimulus used	Stimulus	Type of stimulus used to induce the immune response
	Time	Time to exposure of the stimulus
	Quantitative	Concentration used of stimulus to induce the immune response
Information in relationship of response analyzed	Response	Immune response considered in the paper (cytokine, interleukins…)
	Method used for the analysis	Technique used to detect the response
	Quantitative data of response	Concentration measured of the response

TABLE 5: Information collected for any type of cell line.

Actually, the studies (papers) were quite different from each other regarding: type of cells, type of stimulus, type of parameters, type of evaluation methods and type of statistical analysis. For this reason, the comparison was at the end limited to a reduced number of studies and targets, and thus the threshold for statistical significance was set at $p \leq 0,10$.

1.4.2. Bacterial strains isolation and growth

For coculture experiments, the *E. coli* strains for were grown in blood-agar plate for 1 day. The bacterial colonies were sub-cultured overnight at 37 °C in Luria Bertani broth (LBB, Becton-Dickinson Company). Before co-culture experiments, the bacterial suspension was diluted at the

density of (~10^7) colony forming units/ml (CFU/ml). in cell growth media (CGM) containing DMEM (Dulbecco's Modified Eagle's Medium - Lonza), FBS (Fetal Bovine Serum - Euroclone), L-Glutammine (Euroclone), Hepes (Euroclone), insuline, cortisone and sodium bicarbonate.

For HRMA experiments, ninety-five *E. coli* strains were isolated from bovine milk samples submitted for microbiological examination to the Laboratory of Malattie infettive of Prof. Alfonso Zecconi, DIVET Department at the University of Milan.

1.4.3. Microbiological isolation

The milk samples were brought to the following dilutions: 10^{-1}, 10^{-2} and 10^{-3}/ml, and as such were sown on plates for the enumeration of coliforms and of *E. coli* (EC 3M ™ Petrifilm ™). The plates were incubated for 24 hours at 37 °C. The reading of the plates was performed according to the directions of the product. About 97% of *E. coli* produce β-glucuronidase which reacts with a color indicator contained in the Petrifilm EC plate, turning blue or red-blue colony; about 95% of *E. coli* ferment lactose, causing the appearance of one or more gas bubbles in correspondence of the colonies. Therefore, on these plates the colonies of *E. coli* appear blue or red-blue and with one or more gas bubbles. Most strains of *E. coli* O157: H7, however, are glucuronidase negative and appear red with gas.

The filters of the milking machine were chopped sterile, introduced in plastic bags with 50 mL of sterile peptone water and processed in Stomacher for 4 minutes. The preparation obtained was brought to the following dilutions: 10^{-1}, 10^{-2} and 10^{-3}/ml and as it were sown on Petrifilm EC plates. The plates were incubated for 24 hours at 37 ° C. The reading of the plates was performed according to the directions of the product. For each sample, up to a maximum of 10 colonies with appearance compatible with *E. coli* and *E. coli* O157: H7 were taken at random. These were plated on nutrient agar and, from the latter terrain, were subjected to biomolecular characterization.

1.4.4. Dna extraction

For HRMA screening, each single colony was tip-sampled and placed in an 0,5 ml Eppendorf containing 50 microliters of molecular grade water. Three alternate cicles of 5’ at 95 °C and 5’ at -80°C were performed to obtain cells lysis and DNA extrusion from bacterial cells, and the lysate were subjected to direct PCR amplification.

1.4.5. DNA screening of strains for virulence genes

For co-culture experiments, the DNA from bacterial strains was extracted using Wizard Genomic DNA Purification kit (Promega) following manufacturer's instruction. To screen for the presence/absence of the virulence factors of interest in the selected strains, a conventional PCR was performed for each target gene on the extracted DNA using the primer pairs reported in Table 6.

Gene	Function
Cdt	Cytolethal distending toxin
Cnf	Cytotoxic necrotizing factor
Eae	Effector protein (Croxen and Finlay, 2010)
iutA	Iron acquisition (Beutin et al., 2005)
Stx1/2	Shigatoxin
Cif	Cell cycle inhibiting factor
Flic	Iron acquisition (Beutin et al., 2005)
tolC	Involved in resistance to fusaric acid (Johnson and Church, 1999)
yciD	Outer membrane protein (Croxen and Finlay, 2010)
Sdfla	Succinate deidrogenase
B12r	Outer membrane protein (Croxen and Finlay, 2010)
16s rRNA	Ribosomal RNA (internal control)
tufA	Elongation factor Tu (housekeeping gene)
MFS1	Protein linked with transportation
SK2	Periplasmatic chaperone 2
Hly	Hemolysin
Ler	Encodes the type III secretion system (Croxen and Finlay, 2010)

Table 6: E. coli genes and relative function

The PCR runs were performed in iCycler thermocycler (Bio-Rad), using GoTaq DNA Polimerase 0.125 U, Buffer 1X, (Promega), dNTPs 0,2mM (Promega), each primer 1µM and water to the final volume (15 µL). The thermal profile was as follows: 2 min at 95 °C for DNA polimerase activation, followed by 35 cycles of DNA denaturation for 20 sec at 95 °C, annealing for 1 min at 55 °C, and elongation for 1 min at 72 °C; final elongation step for 7 min at 72 °C.

1.4.6. MAC-T cell propagation

MAC-T were grown in CGM in T75 tissue culture flasks (Corning Inc. Corning, NY, USA) as described (Calvinho and Oliver, 1998; Almeida et al., 1996). Incubation of MAC-T cells was performed at 37 °C under 5% CO_2/95% air until 90–100% of confluence was achieved.

1.4.7. Co-culture assays

One ml ($\sim 10^7$ CFU) of bacterial suspension in CGM was added to MAC-T cell monolayers in T75 (75 cm^2) tissue culture flasks and incubated at 37 °C under 5% CO_2/95% air for 1 hour.

For each co-culture assay, a T75 flask with ($\sim 10^7$ CFU) of bacterial suspension in CGM but without MAC-T cells was incubated in parallel.

1.4.8. Bacterial RNA isolation

At the end of co-culture step, the supernatants were put into 50 ml Falcons after scraping the cell monolayers where present. The supernatants were centrifuged at 4000g for 5 min and the pellets were resuspended in 2ml of RNALater (Ambion Inc., Austin, TX, USA). The RNA of *E. coli* strains was isolated using RiboPure™-Bacterial RNA purification kit (Ambion Inc., Austin, TX, USA) following manufacturer's instructions. Contaminant DNA was removed from the extracted RNA using turbo DNAse (Ambion) following manufacter's instructions.

1.4.9. Quantitative RT-real time PCR

The expression pattern of the virulence-associated genes whose DNA sequence was detected in all the analyzed *E. coli* strains (see Milestones and deliverables, Table 4) was evaluated by RT-quantitative real time PCR after 1 h co-culture with immortalized MAC-T cells using gene specific primer pairs (Table 7).

Gene	Primer Sequnce	Reference
tufA-qRT-PCR-	F:5'-TGCTCTGAAAGCGCTGGAAG-3' R: 5'-TTGTCAATCGCACGCTCTGG-3'	Kerro-Dego et al., 2012
16S rRNA *E. coli* qRT-PCR-	F-5'-ATAACATCTCCACGCCGTTG-3' R-5'GACCATACGCTTTGCTGTTTC-3'	Kerro-Dego et al., 2012
iutA-qRT-PCR-A-	F: 5'-ATGAGTCGTTGCGGTTCTAC-3' R: 5'ATTTGCCAGCCGTCCATAAG-3'	Kerro-Dego et al., 2012
flic-qRT-PCR-A-	F: 5'-CCGGTGGTGATAACGATGGG-3' R: 5'-CAGGTGTACCGCCTGAAGTG-3'	Kerro-Dego et al., 2012
eae-qRT-PCR-A-	F: 5'-AGGCAGCCGTTACGATCTGG-3' R: 5'-ACCGCCCTGACTGCGTAATG-3'	Kerro-Dego et al., 2012
tolC-qRT-PCR-chr-	F:5'- GGAACTGGCTGCGCTGAATG-3' R: 5'-TCCTGCGCCTGGCGAATTTG-3'	Kerro-Dego et al., 2012
yciD-qRT-PCR-chr-	F: 5'-CAACACTGATGGCGCAGTGG-3' R:5'-CGGAAAGCCCTGCCTCTTTG-3'	Kerro-Dego et al., 2012
sdfla-qRT-PCR-chr-	F: 5'-TTATGCGCCGAACGCCAAAG-3' 5'-TGAGCGAAGGTACGGGAAAG-3'	Kerro-Dego et al., 2012
B12r-qRT-PCR-chr-	F: 5'-GCTGCGCTATAACGGCGAAC-3' R:5'-GCCCACTGGACGGTGTATTG-3'	Kerro-Dego et al., 2012

Table 7: E. coli primer to analyze the differen expression with/without MAC-T.

The amplification was carried out in a total volume of 10µl in 96 well plates (Ambion) using an Quant Studio 6 Flex instrumentation (Ambion). The reactions were carried out coupling retrotranscription and amplification through iTaq Universal SYBR Green One-Step Kit (Bio-Rad) following manufacturer's instructions. The following steps were performed; retrotranscription: 50 °C for 10 min; polymerase activation and DNA denaturation: 95 °C for 1 min; amplification: 40 cycles at 95 °C for 10 sec, 60 °C for 1 min for denaturation, annealing/elongation respectively; melting analysis: 95°C for 15 s, 60 °C for 1 min and 95 °C for 15 s. The samples were run in triplicate.

1.4.10. Data analysis

The cDNAs threshold cycle (Ct) values, obtained by RT-qPCR, were analyzed through the comparative ΔΔCT method (Giulietti et al., 2001). For each gene target, this method compares the Ct value of the test samples with the Ct value of the reference sample, in this case the corresponding strains not co-cultured with MAC-T cells acting as a control. For each target gene, expression Ct values are normalized according to the values of the housekeeping gene(s). In the present study, the Ct values obtained by RT-qPCR were preliminarly analyzed in order to assess the expression stability of the genes in all samples using the software geNorm (Vandesompele et al., 2002), available at http://www.medgen.ugent.be /jvdesomp/genorm/.as described in the corresponding Instructions for Users. The output of geNorm analysis was used to select the most stable gene(s) to be used as housekeeping gene(s) for data normalization. The relative expression of the target genes in the different samples were calculated according to the following formula:

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (housekeeping gene)}$$

where Ct (target gene) indicates the value of the threshold cycle for the gene of interest, and Ct (housekeeping gene) indicates the value of the threshold cycle for the housekeeping gene used as normalizer. Subsequently, the relative expression of all samples in comparison with the Ct values obtained from the corresponding strains not co-cultured with MAC-T cells (control) was calculated as follows:

$$RE = 2^{-\Delta\Delta Ct} \text{[where } \Delta\Delta Ct = \Delta Ct \text{ (sample)} - \Delta Ct \text{ (control)]}$$

where RE indicates the relative expression, ΔCt (sample) indicates the difference between Ct values of the target gene and the housekeeping gene(s) calculated for the test sample, ΔCt (control) indicates the difference between the Ct values of the target gene and the Ct values of the housekeeping gene(s) obtained from the corresponding strains not co-cultured with MAC-T cells (control).

1.4.11. Vtec serotype identification with multiplex-PCR

For the identification of serotypes, a multiplex PCR using primers published by Paddock and co-workers in 2012, as reported in Table 8, was performed. The PCR reactions were performed with an initial DNA denaturation step at 94°C for 5 min, followed by a 30 cycle of denaturation at 94 °C for 30 seconds and annealing at 63 °C for 80 seconds. The procedure ends with a final elongation at 68°C for 8 min and incubation at 5°C for 5 min, and was performed on iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Amplification products were separated in a 2% agarose gel, stained with ethidium bromide and visualized under UV light.

Serogroup	Strain	Primer sequences (5' to 3')	Bps	Reference
O26	TW O1597	F:GGGGGTGGGTACTATATTGG	241	Paddock et al., 2012
		R: AGCGCCTATTTCAGCAAAGA		
O145	KSU 1234-1	F: TGCTCGACTTTTACCATCAAC	374	Paddock et al., 2012
		R: AACCAACACCATACACCTTGTCTT		
O103	KSU 15612-1	F: TAAGTACGGGGGTGCTTTTT	716	Paddock et al., 2012
		R: AAGCTCCCGAGCACGTATAA		
O111	KSU 7726-1	F: CAAGAGTGCTCTGGGCTTCT	451	Paddock et al., 2012
		R: AACGCAAGACAAGGCAAAAC		
O157	ATCC 43894	F: CAGGTGAAGGTGGAATGGTTGTC	296	Bai et al., 2010
		R:TTAGAATTGAGACCATCCAATAAG		

Table 8: Primer sequences and strains used as positive control. Bps, amplicon size in basepairs.

1.4.12. Primers design for the HRMA-based SNPs identification

The design of the primers was performed by aligning all sequences used by Norman and collaborators in 2012 including the SNPs that determine the VTEC status in *E. coli* serotypes linked to food of animal origin, as already shown in Table 8.

Serogroup	Gene	bp position	Polymorphism[a]	AA[b] position	AA change	Association (*P* value)[c]
O26[d]	*rmlA*	30	G→T	10	Synonymous	<0.001
	wzx	953	T→G	318	Phe→Leu	<0.001
	fnl1	88	G→A	30	Ala→Thr	<0.001
O45[e]	*rmlB*	966	T→C	303	Synonymous	<0.001
	wbhQ	721	C→A	241	Leu→Ile	<0.001
	wbhU	241	G→A	80	Val→Ile	<0.001
	wzy	752	T→C	251	Val→Ala	<0.001
	wzy	906	T→C	302	Synonymous	<0.001
	wbhW	21	C→T	7	Synonymous	<0.001
	wbhW	997	T→G	333	Stop codon→Glu	<0.001
	Intergenic	13299[l]	C→A	NA[k]	NA	<0.001
	Intergenic	13340[l]	A deletion[f]	NA	NA	NA
	Intergenic	13534[l]	A→C	NA	NA	<0.001
O103[g]	*wbtD*	937	C→T	313	His→Tyr	<0.001
O111[h]	Intergenic	492[f]	G→T	NA	NA	<0.001
	wbdH	1006	G→A	336	Val→Ile	<0.001
	wbdK	687	C→T	229	Synonymous	<0.001
	wzx	1128	A→T	376	Synonymous	<0.001
O121[i]	*vioA*	313	C→T	105	Pro→Ser	<0.001
	wbqE	437	C→T	146	Ala→Val	<0.001
	wbqI	582	G→A	194	Synonymous	<0.001
O145[j]	*wzy*	37	A→C	13	Ile→Leu	<0.001

[a] Non-STEC→STEC.
[b] AA, amino acid.
[c] Fisher's exact test *P* value testing the association between the STEC-associated allele and O serogoup.
[d] GenBank AY763106 used as a reference.
[e] GenBank AY771223 used as a reference.
[f] Deletion found in the two non-STEC that do not share the other 9 SNPs.
[g] GenBank AY532664 used as a reference.
[h] GenBank AF078736 used as a reference.
[i] GenBank AY208937 used as a reference.
[j] GenBank AY647260 used as a reference.
[k] NA, not applicable.
[l] Position within O-antigen gene cluster.

Table 9: Gene and amino acid data for the single nucleotide polymorphisms identified in the O-antigen gene clusters of non-O157 *Escherichia coli* (From: Norman et al., 2012).

In Table 10 are reported the primers designed for each serotype.

O26		**Fnl1** sequence (5'->3')	**Wzx** sequence (5'->3')
	Forward	ACTCGTTATCACTGGTGGC	CAGTCTATAGCAACCCCG
	Reverse	TGATACAGAAAATCGGCACC	GGATGAACGCGCTTCTAATTTC
	Lengh	219	227
	SNP	**G (no VTEC)→ A (VTEC)**	**T (no VTEC)→ G (VTEC)**
	Annealing T°	56°C	56°C
O111		**WbdK** sequence (5'->3')	**Wzx** sequence (5'->3')
	Forward	TATCATGTATTGTTGTGCCTTCG	GGAATTGGTAAATCTAAGCTTG
	Reverse	AGTGGGCGAACATTGTATCC	CCATAGATATTGCATAAAGGCC
	Lengh	140	109
	SNP	**C (no VTEC)→ T (VTEC)**	**A (no VTEC)→ T (VTEC)**
	Annealing T°	56°C	56°C
O145		**wbtD** sequence (5'->3')	
	Forward	CAAACGGTAAATGGCGCG	
	Reverse	GGTTAATGCATGACTCTAATGTC	
	Lengh	218	
	SNP	**C (no VTEC)→ T (VTEC)**	
	Annealing T°	51°C	
O103		**Wzy** sequence (5'->3')	
	Forward	ATGATCCCGTTATATAAAATTC	
	Reverse	GCATTTAAGTAATATAACGAAG	
	Lengh	123	
	SNP	**A (no VTEC)→ C (VTEC)**	
	Annealing T°	56°C	

Table 10: primer designed for the identification of VTEC status with HRMA.

1.4.13. HRM analysis

In this project, the Eco™ Real-Time PCR System instrument, Illumina Inc. (9885 Towne Centre Drive San Diego, CA 92121 USA), a block based instrument whose technical characteristics are shown in Table 11, was used. The main instrument specifications are:

- Thermal system: silver block with Peltier-based system
- Block format: 48-well block
- Samples volumes: validated for 5-20 µl
- Average ramp rate: 5.5°C/sec
- Temperature range: 40-100°C
- Temperature uniformity : ± 0.1°C
- Optical system: dual LED excitation ($%”-486 nm and 542-582 nm), four emission filters (505-545 nm, 562-596 nm, 604-644 nm, and 665-705 nm) and CCD camera
- Calibrated dyes at shipment: SYBR Green, FAM, HEX, ROX, Cy5.
- Passive reference dyes: use of ROX is supported, but optional
- Data collection: data collection in all four filters for all wells regardless of plate setup; plate setup for data analysis can be altered after run completion.
- Melt curve analysis supports continuous data acquisition in a single filter to provide increased data point collection and reduced run times
- Real-Time PCR run time (40 cycles): less than 40 minutes

Optical	Light Source	Two sets of 48 LEDs (452-486 nm and 542-582 nm)
	Detector	CCD camera (4 filters) (505-545 nm, 562-596 nm, 604-644 nm, and 665-705 nm)
Thermal	Thermal Cycling	Proprietary hollow silver block with Peltier-based system
	Thermal Uniformity	± 0.1°C
Operational	Sample Format	48-well plate
	Reaction Volume	5–20 µl
	Warmup Time	~ 20 minutes
	Typical PCR Run Time	Less than 40 minutes for 40 cycles
	Sensitivity of Detection	1 copy
	High Resolution Melt	Supported resolution to 0.1°C
	Multiplexing	Detection of up to four targets simultaneously (four-plex)
	Passive Reference	Optional (ROX)
Physical	Dimensions	34.5 cm W x 31 cm D x 32 cm H (13.6 in. W x 12.2 in. D x 12.6 in. H)
	Weight	13.6 kg (30 lb) including power supply
Environmental	Electrical	100–240 VAC, 50/60 Hz, 5A
	Temperature Range	Operating: 15°C to 30°C (59° F to 86° F) Storage: 10°C to 100°C (50° F to 212° F)
	Humidity Range	Operating: 15–90% Relative Humidity Storage: 5–95% Relative Humidity

Table 11: Specifications and Environmental Requirements of Eco™ Real-Time PCR System, Illumina.

The analysis was performed in 15 µL reaction mixtures containing 7,5 µL of Supermix SsoFast EvaGreen mix Biorad, 0.3 µM of each primer at, 1 µL and RNase-free distilled water (Invitrogen, Carlsbad, California, USA) to reach a total volume. The real time PCR conditions were as follows: 1 cycle of 95 °C for 2 min followed by 35 cycles at 95 °C for 15 sec and at an appropriate annealing temperature for each primer pair (Table 10) for 30 sec. Reaction mixtures without the DNA template were used as negative controls. All reactions were performed in 48-well optical plates (Euroclone) that were covered with optical adhesive film (Euroclone).

The HRMA step was performed immediately after real time PCR cycling. The amplicons were heated to 95 °C for 15 sec and then were cooled to 55 °C for 15 sec. The melting curves were generated by increasing the temperature from 56° to 95 °C with 15 sec increments and fluorescence was detected each 0.1 s. To make the data uniform, the raw melting curves were normalized by manually adjusting one region before the double-stranded product was melted (pre-melting region) and another region after the amplicon was completely melted (post-melt

region). The normalized melting curve data were analyzed with the Eco Illumina software program (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions.

All non-O157 samples resulted positive in multiplex-PCR were tested by real-time PCR coupled to HRMA in duplicate, to ensure assay reproducibility.

1.5 Results and Discussions

Selection of the bovine mammary cell line for co-culture experiments

In table 12 the cytokines considered by the selected studies (papers) are reported.

A comparison among the 3 cell lines was possible only for the following targets: IL-1, IL-1α, IL-1β, IL-6, IL-8 and TNF-α (Table 12).

Cytokine	BME-UV1	MAC-T	Primary cells
IL-1	●	●	●
IL-1α	●	●	●
IL-1β	●	●	●
IL-2	●	●	
IL-6	●	●	●
IL-8	●	●	●
IL-10	●		●
IL-12			●
IL-15			●
IL-17			●
IL-33			●
TNF-α	●	●	●
TGF-β			●

Table 12: cytokines considered in the papers.

Many (149) other items as, for instance, specific gene transcripts were considered in the papers, and precisely:

- 5 in BME-UV
- 12 in MAC-T
- 137 in Primary Coltures.

But in these cases it was not possible to perform a comparison among the 3 cell lines because there were not common analytical targets among studies.

The stimuli used in the papers were:

- Bacterial origin: LPS, LTA, PGN, whole E. coli, whole S. aureus, PAM_2CSK_4 and PAM_3CSK_4.
- Pro-inflammatory cytokines: ILs, TNF-α, HGF and EGF.

- Others: BMAP-27 and BMAP-28, CD12-iE-DAP, lactoferrin and prolactin, MDP, nonoate, proteinase K, and sodium butyrate
- No stimulus

The stimuli used for all 3 cell lines were: LPS, S. aureus, ILs, TNF-α and no stimulus (used as negative control).

Several analytical methods used in the papers to assess cell line response regarding the abovementioned 6 targets, but only for RT-real time PCR and ELISA it was possible to compare the 3 cell lines and only for TNF-α, IL-8 and IL-1.

TNF-α Expression

The comparison of TNF-α expression was reported in table 13.

TNF-α EXPRESSION	
Stimulus	LPS
Assay	qtPCR
Papers included	3 (1 withdrawn)
Values considered	11

Table 13: parameter considered in TNF-α.

In the table 14 was reported, for any type of cell coltures, mean, standard deviation (StdDev) and range of the analysis (Min and Max).

Cell type	Mean	StdDev	Min	Max
BME-UV	4.80	3.52	2.30	9.85
MAC-T	16.4	2.71	14.00	19.40
Primary cells	0.84	0.77	0.21	1.7

Table 14: statistical parameter in TNF-α.

The datas of final general linear model (GLM) were expressed in tables 15.

	FINAL GLM MODEL	
	MODEL	P= O,O27
	R^2	96.5%
FACTORS	CELL LINE	P=0,013
	TIME	P=0,174
	QUANTITE	*Not Considered*

Table 15: final glm model in TNF-α.

It was statistically significant the difference among the 3 cell lines, in table 16 the difference between type of cells was reported, in particular the difference is linked to MAC-T.

	BME-UV	**MAC-T**	**P.C.**
BME-UV		0.07	
MAC-T	0.07		0.04
P.C.		0.04	

Table 16: p-Value amon cell lines in TNF-α

IL-8

The comparison of IL-8 expression was reported in Table 17.

IL-8 EXPRESSION	
Stimulus	LPS
Assay	qtPCR
Papers included	7 (1 withdrawn)
Values considered	15

Table 17: parameter considered in IL-8.

In the table 18, for any type of cell coltures, mean, standard deviation (StdDev) and variability (Min and Max) were reported.

Cell type	Mean	StdDev	Min	Max
BME-UV	2.78	1.93	1.07	4.59
MAC-T	55.00	31.69	6.00	86.00
Primary cells	10.83	12.04	2.00	32.00

Table 19: statistical parameter in IL-8.

The data of final general linear model (GLM) were expressed in Table 19.

	FINAL GLM MODEL	
	MODEL	P= O,180
	R^2	62,4%
FACTORS	CELL LINE	P=0,099
	TIME	P=0,362
	QUANTITE	*P=0,38*

Table 19: final glm model in IL-8.

It was statistically significant the difference among the 3 cell lines, in table 20 the difference among cell types was reported, in particular the difference is linked to MAC-T.

	BME-UV	MAC-T	P.C.
BME-UV		0.02	
MAC-T	0.02		0.03
P.C.		0.03	

Table 20: p-Value among cell lines in IL-8.

IL-1

The comparison of IL-1 expression was reported in Table 21

IL-1 EXPRESSION	
Stimulus	LPS
Assay	qtPCR
Papers included	5 (1 withdrawn)
Values considered	14

Table 21: parameter considered in IL-1.

In the Table 22, for any type of cell coltures, mean, standard deviation (StdDev) and range of the analysis (Min and Max) were reported

Cell type	Mean	StdDev	Min	Max
BME-UV	1,56	0.31	1,15	1,87
MAC-T	180.5	162,32	6,00	381,00
Primary cells	55.00	84,33	3,00	225,00

Table 22: statistical parameter in IL-1.

The data of final general linear model (GLM) were expressed in Tables 23.

	FINAL GLM MODEL	
	MODEL	P= O,560
	R^2	77,3%
FACTORS	CELL LINE	P=0,418
	TIME	P=0,812
	QUANTITE	*P=0,432*

Table 23: final glm model in IL-1.

The difference among the 3 cell lines wasn't statistically significant, even for MAC-T, and this was probably linked to higher biological variability (Table 22).

Overall, the MAC-T line turned to have a significant higher response than other cell lines regarding TNF-α and IL-8, whereas BME-UV1 and Primary Cultures have similar response in this analysis. Thus, MAC-T were selected as best cell line for the coculture experiments with E. coli strains, assuming tha t more responsiveness of the cells would imply a more effective modulation of bacterial gene expression.

Co-Culture

The achieved milestones and deliverables are the following.

The DNA profile of the strains justified the selection of the targets for gene expression study (Table 24).

GENES	STRAINS					
	Strain 1 727	Strain 2 McG	Strain 3 UK *E. coli* ECIMI	Strain 4 UK *E. coli* ECIMI 8	Strain 5 UK *E. coli* ECIMI EK A	Strain 6 O157:H7
Cdt	-	-	-	-	-	-
Cnf	-	-	-	-	-	-
Eae	+	+	+	+	+	+
iutA	+	+	+	+	+	+
Stx1/2	-	-	-	-	-	+
Cif	-	-	-	-	-	-
Flic	+	-	-	+	-	+
tolC	+	+	+	+	+	+
yciD	+	+	+	+	+	+
Sdfla	+	+	+	+	+	+
B12r	+	+	+	+	+	+
16s rRNA	+	+	+	+	+	+
tufA	+	+	+	+	+	+
MFS1	-	-	-	-	-	+
SK2	-	-	-	-	-	-
Hly	-	-	-	-	-	+
Lir	-	-	-	-	-	+

Table 24: Results after conventional PCR of the gene target to detect the presence of the genes in *E. coli* samples.

After co-culture experiments and RT-qPCR, the Ct values of the genes expressed in all samples were analyzed by geNorm software, thus identifying 16S rRNA and tolC as the most stable gene pair (Figure 11; the genes with the lowest M values are more stable). Thus, the geometric mean of the 16S rRNA and tolC Ct values were used as “Ct (housekeeping gene)” in the calculation of relative expression of the target genes.

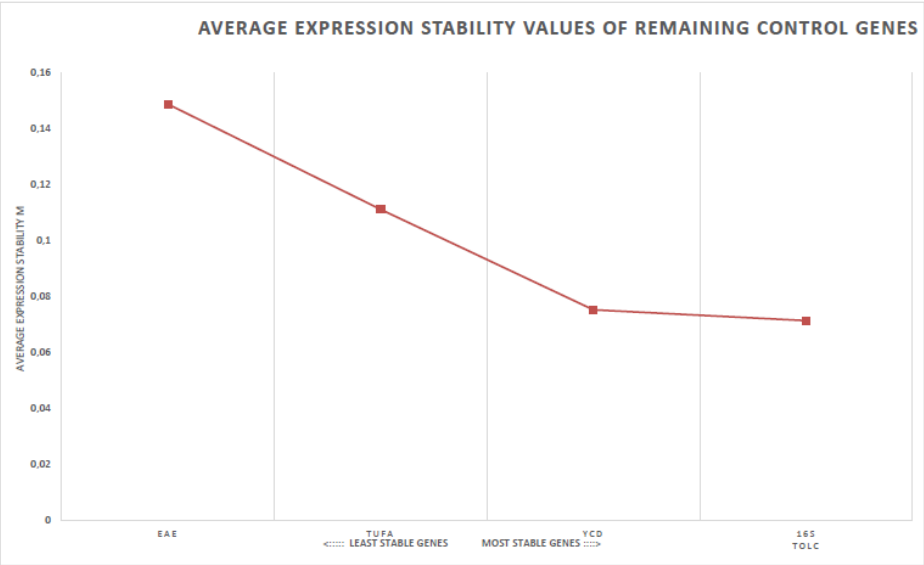


Figure 11: Graphic output of geNorm analysis

The relative expression of each target gene in the different co-cultured strains was obtained with reference to the corresponding strain without co-culture, and using the gene pair 16S rRNA and tolC for normalization. The corresponding values are reported in Table 25.

Strain pair	Sample	tufaA	eae	ycd	b12	iutA	Flic	Sdfla
1S-1M	*E. coli* 727	1,42	0,52	0,54	0,91	2,06	ND	3,00
2S-2M	Mc Gear *E. coli*	0,79	12,76	1,77	1,27	ND	1,15	ND
3S-3M	uk *E. coli* ECIMI	0,57	4,19	1,06	ND	2,08	ND	ND
4S-4M	uk *E. coli* ECIMI 8	3,52	4,13	5,18	4,94	8,76	ND	ND
5S-5M	uk *E.coli* ECIMI ekA	4,09	1,41	2,48	2,15	ND	ND	ND
6S-6M	0157:H7	0,03	1,12	2,78	1,02	ND	ND	0,17

Table 25: Fold-change expression of the target genes in co-cultured strains (letters "M") in comparison with the same strains without co-culture (letters "S"). The sample 6 corresponds to the reference foodborne VTEC strain O157:H7.

A clear upregulation of the virulence factor eae (intimin) in all the analyzed mastitis strains following co-culture with MAC-T cell line can be appreciated, with the exception of the 727 strain that was isolated from an acute mastitis. In literature, the up-regulation of eae in 727 strain when co-cultured with a primary bovine mammary cell line was preliminarly reported (Kerro-Dego et al., 2012). Also the adherence virulence factor ycd resulted to be upregulated in three co-cultured

strains (strain number 2, 4 and 5), and the b12 gene was upregulated in two strains (number 4 and 5). Taken together, these results suggest the possibility that mastitic *E. coli* strains can acquire a more risky molecular profile when exposed to the bovine mammary cells. This finding may have clear implications on the risk assessment related to the *E. coli* strains in bovine mammary tissue and milk.

Vtec serotype identification with multiplex-PCR

The primers amplified correctly at the conditions used, and they were able to correctly discriminate the serotypes of interest. The sequencing confirmed the identity of the VTEC serotypes (figure 12).

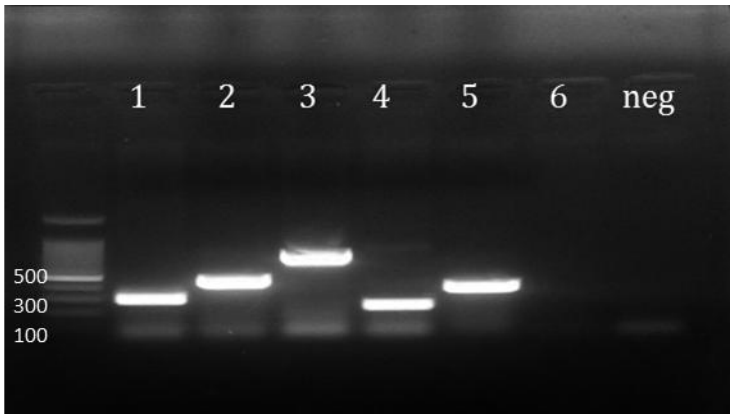


Figure 12: multiplex PCR results. 1) O157 (296bp), 2)O111 (451bp), 3) O103 (716bp), 4)O26 (241bp), 5)O145 (374bp), 6)O104 (N/A)

Until now, 95 bovine isolates of *E. coli* were analyzed, and 3 out of these were positive for O157 and one for O26 serogtype. Sequencing confirmed the obtained serotype.

Identification of VTEC status

As an example, in Figure 13 the result in HRMA serotype O26 is reported, in particular for the gene fnl1.

The amplification profile was regular not showing any aspecific and/or primer dimers, no differences between lysed and DNA extracted samples can be appreciated, and the assay was reproducible as the samples were tested in duplicate with similar results (Figure 13).

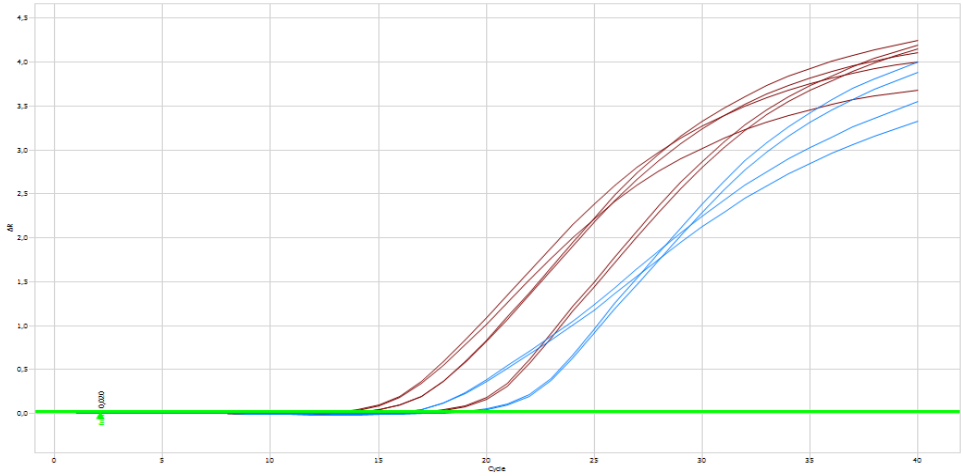


Figure 13: amplification profile in qRT-PCR. In red O26 VTEC and in blue O26 noVTEC**.**

Looking at the profile of melting by HRMA, it is possible to distinguish the isolates of O26 with VTEC status (Figure 14):, in red (on the left) there is the O26 VTEC serotype; in blue (on the right) there is the non-VTEC O26 serotype.

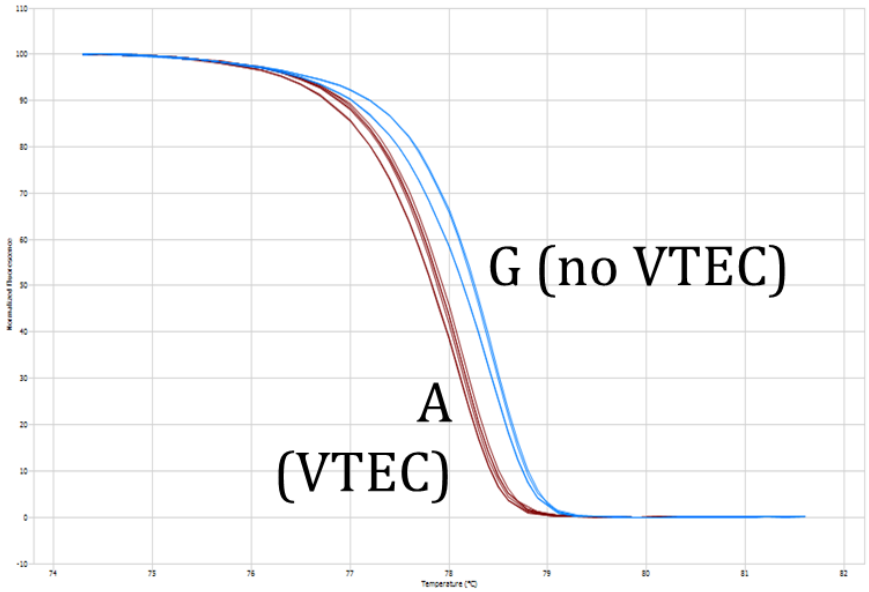


Figura 14: Normalized profile in HRMA of the fnl1 gene in O26 serotype. In red VTEC strain and in blue noVTEC strain.

For a confirmation of the HRM results, the isolated were tested in conventional PCR for positivity to stx genes (both stx-1 and stx-2). For further evidence, the amplified products were sequenced, and the results confirmed that the difference is related to a single SNP (Figure 15).

```
CLUSTAL 2.1 multiple sequence alignment
O26 coli

FNL1D5          TCTAGATACAGATATTACTGAAATACGAATATTTAGCAGGGATGAAAAAAAAACAAGATGA 60
FNL1E1          TCTAGATACAGATATTGCTGAAATACGAATATTTAGCAGGGATGAAAAAAAAACAAGATGA 58
                ********** ****************************************

FNL1D5          TATGCGGAAAAAATATAATAACTCAAAATTAAAATTTTATATAGGTGATGTGCGAGACTA 120
FNL1E1          TATGCGGAAAAAATATAATAACTCAAAATTAAAATTTTATATAGGTGATGTGCGAGACTA 118
                ************************************************************

FNL1D5          TAATTCCGTTCTAAATGCAACGCGTGGTGCCGATTT- 156
FNL1E1          TAATTCCGTTCTAAATGCAACGCGTGGTGCCGATTTT 155
                ************************************
```

Figure 15: alignment of the sequences of the O26 serotype fnl1 gene analyzed and sequenced events. “A” is linked to the verotoxicity SNP and “G” to the no- verotoxicity.

Currently, the collection and analysis of isolated is being increased in order to confirm predictability of the HRM assay also for the other serotypes. Besides, the HRM assay is being improved by including the analysis of predictive SNPs within the O45 and O121 serotypes, as possible VTECs that may be also found in food samples of animal origin.

Chapter 2: High Resolution Melting Analysis for food safety and veterinary diagnostics

2.1. Introduction

Since HRMA is increasingly used in microbiology for species identification and detection of resistance-carrying mutations, the present PhD project aimed, and as additional objectives, to establish some HRMA-based assays to improve the diagnostic tools currently available for different targets, highly relevant to food and animal health research. HRMA offers a low-cost, closed-tube approach to amplicon analysis with the capacity for single-nucleotide discrimination and easy integration with real time PCR, thus very promising for several possible applications in routine diagnostics (Reed et al., 2007). Some examples are reported here below.

First, the phenomenon of pigmented mozzarella cheese is still a topical issue because, even if the initial emergency situation has been redimensioned, it remains a difficult to eradicate problem in Italian dairy farms. Improving identification and tracking methods for the microorganisms that cause chromatic alterations in mozzarella cheese samples in food production and processing systems is of great relevance to understand and reduce the frequency of contamination, as well as to eliminate niche populations in food processing systems such that contamination of food products can be prevented. In particular, bacteria of the genus *Pseudomonas spp.* are the main cause of this type of alteration (Martin et al., 2011).

Second, *Nosema apis* and *N. ceranae* are honeybee parasites widely circulating inside the apiaries around the world (Sprague 1978; Becnel and Andreadis 1999). The infestation of *Apis mellifera* by *N.apis* represents a real danger to the health of the beehive. The type A nosemiasis caused by this parasite is a notifiable disease under the provisions of the Veterinary Police Regulations (Botìas et al. 2013). Only recently *N.ceranae* has been widely admitted as a pathogen of the honey bee. At present, the dynamics of the infection and the pathological consequences it may have on the host are not well defined and give rise to controversial opinions among experts in the sector. The rapid diffusion of the parasite caused emerging concern and for this reason careful monitoring of this phenomenon is required (Martin-Hernández et al. 2007). To take precautionary measures that are efficient and durable it is first necessary to make an accurate assessment of the pathogen at the apiary level. To do this it is important to develop methods of certain identification of the parasite that are able to correctly distinguish the two species of Nosema and to provide information on the degree of infestation of bees.

Third, *Staphylococcus pseudintermedius* is an opportunistic pathogen of dogs and cats, and fluoroquinolone (FQ)-resistant strains are isolated with increasing frequency from infection sites (Bannoehr et al., 2007; Devriese et al., 2009; Penna et al., 2010). Fluoroquinolone resistance in *S. pseudintermedius* and other Staphylococci is predominantly mediated by a mutation located at position 84 of the gyrA subunit of DNA gyrase enzyme (Ser84Leu), together with a mutation located at position 80 of the grlA subunit of topoisomerase IV enzyme (Intorre et al., 2007; Descloux et al., 2008). The standard methods for determining FQ resistance based on the CLSI guidelines (www.clsi.org) are tedious and time consuming. The molecular techniques currently used to characterize FQ resistance usually require sequence-specific probes or post-amplification processing such as gel electrophoresis and DNA sequencing (Kaltenboeck and Wang, 2005). Thus, there is a need for reliable and accurate methods allowing fast identification of gyrA-mediated FQ resistance in *S. pseudintermedius* strains isolated during veterinary clinical routine.

And finally, *Dirofilaria immitis* and *D. repens* are the principal causative agents of zoonotic filariosis and, although the number of dogs subjected to specific prevention is increasing, the prevalence of these parasites remains high in many areas of the world (Genchi et al., 2011). The discrimination between the two *Dirofilaria* species using the classical diagnostic methods can be difficult and may lead to misdiagnosis especially on samples from areas where both *Dirofilaria* are present. Over the last years, several molecular methods with higher sensitivity and specificity compared to classical microscopy and ELISA assays were designed. Nevertheless, a need for simple, rapid and cost-effective molecular protocols to accurately discriminate between *D. immitis* and *D. repens* still remains.

2.1.1. *Pseudomonas* spp

Pseudomonas is a Gram-negative, aerobic gamma-proteobacteria, belonging to the family Pseudomonadaceae containing 191 validly described species.(Euzeby, 1997). The members of this family demonstrate a high metabolic diversity, and consequently are capable to colonize a wide range of biological niches. They are very easy to cultivate in vitro and their presence in different habitats has increased the interest in this bacterium. The most interesting species are: *P. aeruginosa* in his role as opportunistic human pathogen, the plant pathogen *P. syringae*, the soil bacterium *P. putida*, and plant growth promotion of *P. fluorescens* (Madigan and Martinko 2005).

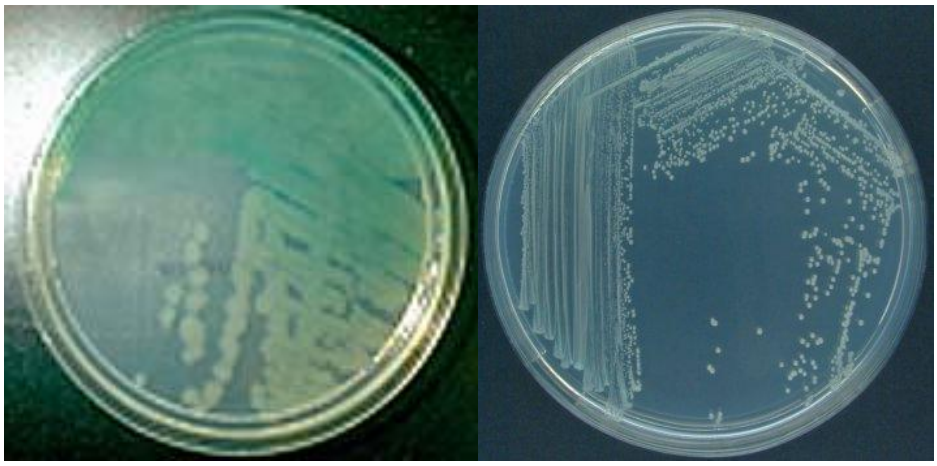


Figure 16: *Pseudomonas aeruginosa(A)* and *Pseudomonas fluorescens* in plate and as Pseudomonas species produces pyoverdin, which fluoresces in ultraviolet light when grown on Kings B agar. This pigment is watersoluble and diffuses into the agar around the colonies. (http://www.uvm.edu/~lwillett/picture3.htm and http://atlas.sund.ku.dk/microatlas/veterinary/pheno_tests/Kings_B_Agar/)

Because of their widespread occurrence in water and plant seeds such as dicots, the pseudomonads were observed early in the history of microbiology. The generic name Pseudomonas created for these organisms was defined in rather vague terms by Walter Migula in 1894 and 1900 as a genus of Gram-negative, rod-shaped and polar-flagellated bacteria with some sporulating species,the latter statement was later proved incorrect and was due to refractive granules of reserve materials. Despite the vague description, the type species, Pseudomonas pyocyanea (basonym of *Pseudomonas aeruginosa*), proved the best descriptor (Madigan and Martinko 2005).

Some species of this bacteria, such as *P. aeruginosa, P. oryzihabitans, and P. plecoglossicida*, are opportunistic infectious pathogens secreting extracellular proteases and adhere to and invade host tissue. Among the most interesting ones, *Pseudomonas aeruginosa* is an opportunistic pathogen and causes disease in immunocompromised individuals, old people and children. In hospitals, Pseudomonas is a leading cause of nosocomial infections via colonization of catheters, skin wounds, ventilator-associated pneumonia and it is also a cause of respiratory infections in individuals with cystic fibrosis (CF) (Pier 1998). The colonization by Pseudomonas spp. It occurs when the host cell fibronectin surrounding coat is destroyed due to trauma or infection (Hosseinidoust et al., 2013). Pets have symptoms identical to that of humans, and even in their incidence is higher in immunocompromised or impaired immune function. The bacteria are typical of the gastrointestinal tract, of the genital regions and upper respiratory tract, but rarely lead to clinical disease because competing with the normal microbial flora typical of these regions. (Hosseinidoust et al., 2013). *Pseudomonas* spp, like other gram-negative bacteria, have an outer membrane plus the inner cytoplasmic membrane and intermediate peptidoglycan layer (Hosseinidoust et al., 2013). This outer membrane is not present in gram-positive bacteria. The polysaccharide capsule (also known as the glycocalyx) sets the outside of the bacteria. Capsule is a virulence factor and acts to protect the outer membrane from attack by the attachment by phagocytic cells (Hosseinidoust et al., 2013). The outer layer of the membrane is composed of LPS, the 'O' antigen is what determines the serogroup of the bacteria. There are approximately 11 variants of this 'O' antigen for Pseudomonas spp. Pseudomonas are involved in more infections range from ear disease to sepsis and its pathogenicity can be influenced by the presence of virulence factors, such as the abovementioned LPS, and also toxins and adhesins (Pier 1998, Klemm et al 2000). In particular, the bacteria, to infect the host, must adhere on epithelium with particular adhesins on the bacterial cell binding to lectins on the host cell surface. Adhesins are structures situated on the bacterial out of the membrane and they are part of the bacterial fimbriae (an appendage on the outer surface of the bacterium). These proteins are very specific and only bind to certain molecules/proteins on the host cell surface (Hosseinidoust et al., 2013), thus activating phosphatidylinositol-3-kinase (PI3K). Protrusions that are enriched with phosphatidylinositol (3,4,5)-triphosphate (PIP3) and actin will accumulate at the apical surface as the site of binding. and at the cell-to-cell junction, to move and to attach on the apical surface.

Pseudomonas spp. live on a different range of substrates and it adapts to change in the environment. Pseudomonas have reduced nutritional requirements and colonize many different environments (Hosseinidoust et al., 2013).The bacteria have a wide range of secretion systems, which export numerous proteins relevant to the pathogenesis of clinical strains (Madigan and Martinko, 2005). A particular characteristic of Pseudomonas bacteria is the capacity to produce pyocyanin, a blue-green pigment. *Pseudomonas fluorescens* group are non pathogenic saprophytes that also produce a pigment, particularly under conditions of low iron availability. This pigment is a soluble, greenish, fluorescent pigment that led to the group's name. These bacteria are generally obligate aerobes; however, some strains can utilize NO_3 instead of O_2 as an electron acceptor. They have multiple polar flagella that assist in the bacteria's movement. Because they have simple nutritional requirements, they "grow well in mineral salts media supplemented with any of a large number of carbon sources". Some researches seek to exploit *P. fluorescens* to partially or completely degrade pollutants such as styrene, TNT, and polycyclic aromatic hydrocarbons. Several strains also have the ability to suppress plant diseases by "protecting the seeds and roots from fungal infection". This ability is due to secondary metabolites produced by these bacteria such as antibiotics, siderophores, and hydrogen cyanide as well as the ability of these bacteria to rapidly colonize the rhizosphere and out-compete some of pathogens (textbookofbacteriology.net/pseudomonas).

In the last years, there is an increase of Pseudomonas spp in food. The estimated minimum infectious dose in food is 10^6 cfu/g, but to raise food-borne risks to humans is necessary that the bacterial load is at least 10^7 cfu/g (Lax et al., 2013). The comsuption of food with such bacterial load is very improbable, since this is usually accompanied by evident modifications of the look and sensory characteristics of the product, sufficient to dissuade the human from eating. Accordingly, these microrganisms are not considered food-pathogenic. At now, they are not considered dangerous for the European and Italian food legislation and so they have not been established any limits of acceptability in foods. The only reference legislation covering this kind of bacteria is contained in the Italian Legislative Decree 31/2001 which, although not considering Pseudomonas spp. among the criteria for potability of water intended for human consumption, it requires the absence of *Pseudomonas aeruginosa* in water offered for sale in bottles and containers (Lax et al., 2013). Overall, foodborne Pseudomonas spp. should be considered more as an issue of quality than of safety.

PSEUDOMONAS IN MOZZARELLA CHEESE

A problem emerged in recent years was concerning the presence of Pseudomonas in products of cheese making, as Mozzarella cheese. The problem seems related to the presence of the bacterium in the process water. Particularly sensitive are the cheeses produced by chemical acidification, as the mozzarella cheese.

Figure 16: esample of blue mozzarella cheese affected of *Pseudomonas fluorescence* and *Pseudomonas putida* (http://www.lucianopignataro.it/wp-content/uploads/2010/07/mozzarella-blu.jpg and http://multimedia.coldiretti.it/Assemblea_Palalottomatica_2_Luglio_2010/Forms/AllItems.aspx)

The water, however, is not the only source of contamination in a dairy company, because there are several environmental microorganisms that can contaminate the raw material or the finished product going to alter the final aspect. Among the most common defects there are blistering or swelling, often associated with the contamination of the milk or the derivate with coliform organisms, that could be resolved through a more accurate hygiene during processing.

2.1.2. *Nosema spp*

Microsporidia are single-cell organisms and are possibly the smallest organisms with a true nucleus (eukaryotes). Mature spores measure 1.5 - 10 µm in length, depending on the species. The honey bee microsporidia, *N. apis* and *N. ceranae*, are about 4 µm in length.

Many pathogens of insects, as some bacteria and fungi, can germinate and reproduce in the environment at least during certain life stages, but microsporidia are obligate pathogens, and can only multiply inside the cells of the host. The only phase that can survive for long period out of environment is the infective mature spore, which is essentially a dormant stage and must be ingested by a susceptible host and invade the tissue cells of the alimentary tract to reproduce once again. Most microsporidian species reproduce only in the cytoplasm of the host cells, but a few species have been known to utilize the cell nucleus. Nuclear invasion, however, rarely occurs in infections caused by *Nosema* species.

The microrganism passes the active reproductive phase of its life cycle within the digestive cells lining the mid-gut of the adult bee. On entering one of these cells, the animal grows and multiplies rapidly, utilising the cell contents for its food supply. Reproduction ceases after several days with the formation of a large number of spores. The cell then reptures, shedding the spores into the mid-gut lumen (Liu, 1990). The spores pass down the gut into the small intestine and accumulate in the rectum from which they are voided at intervals in the bee's faeces. Within the spore, the parasite enters its passive, resting stage until swallowed by another bee. When this happens, the spore "germinates" as it enters the ventriculus from the crop. It enters an epithelial cell and begins to grow (El-Shemy, 1986 and De Grof et al, 1994).

Nosema spores contain simple but important and interesting organelles. These structures, which are only visible using transmission electron microscopy at a magnification of more than 5,000X, are nearly all related to invasion of host cells and reproduction. Figure 17 reports a longitudinal section of a mature spore containing a double nucleus (all true *Nosema* species have double nuclei called diplokarya; other groups may have a single nucleus), layers of membranes at the apical end of the spore deominated polaroplast, a vacuole at the distal end of the spore and, most spectacularly, a polar filament that is attached at the apical end of the spore and winds like a spring around the inside spore wall. The mature spore is covered by an outer exospore formed of protein and an endospore that is composed of a protein-chitin matrix. This thick, tough spore wall protects the spore once it leaves the host cell and is exposed to environmental conditions.

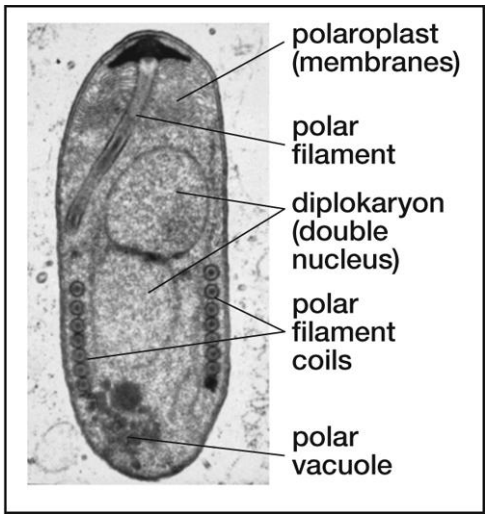


Figure 17: Major organelles inside an infective microsporidian spore. Photo of the Society for Invertebrate Pathology.

There are two forms of the microsporidian (fungus) Nosema associated with clinical signs of disease in honeybees: *Nosema apis* and *Nosema ceranae* (Figure 19). Nosema spp. invade the intestinal cells coating the mid-intestine of the bee, then they multiply fastly and within a few days (3-7 days) the cells are full of spores, the resting phase of the parasite. When the bee intestine cell ruptures, it sheds the spores into the gut where they accumulate in masses, to be later excreted by the bees. If spores from the excreta are picked up and swallowed by another bee, they can germinate and once more become active, starting another round of infection and multiplication. *Nosema apis* is endemic having been first described over 100 years ago, but *N. ceranae* appears to have been a more recent move from the Asian honeybee Apis ceranae being first reported in Europe in 2005, soon being established in many European countries: Denmark, Finland, France, Germany, Greece, Italy, Serbia, Spain, Sweden, Switzerland and United Kindom (Paxton et al 2007; Antunez et al 2009; Botias et al 2012a).

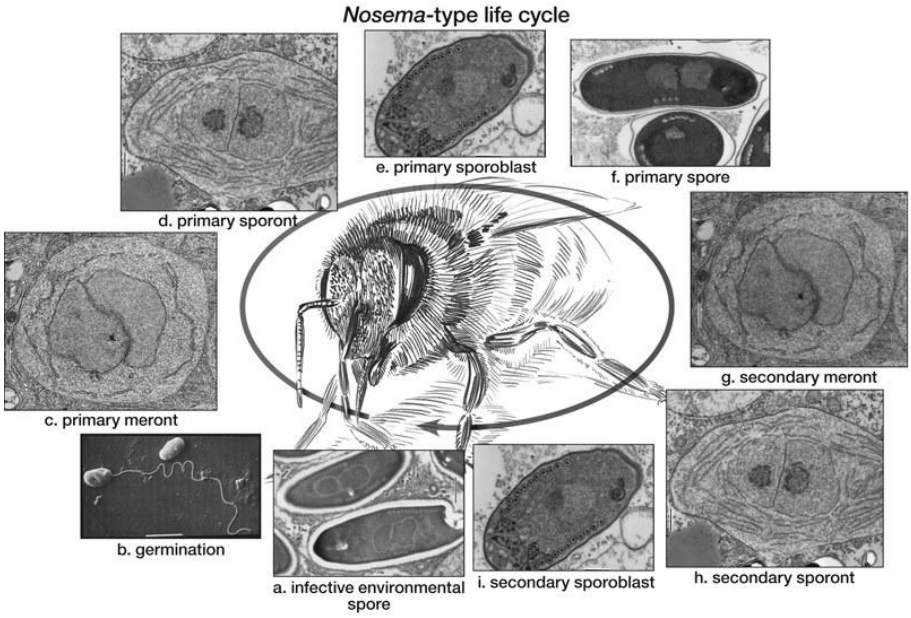


Figure 18: life cycle of Nosema (Scanning electron micrograph of germinating spore courtesy the Society for Invertebrate Pathology; transmission electron micrographs of other stages courtesy Wiley Publishing Co.)

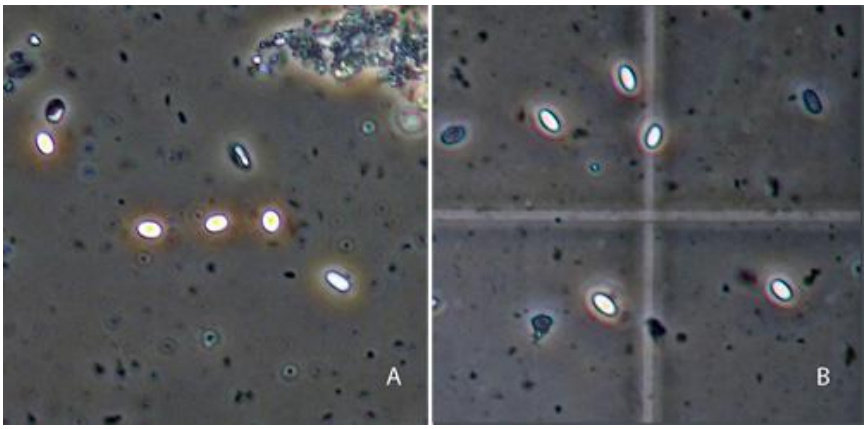


Figure 19: Mature infective spores of *Nosema apis* (a) and *Nosema ceranae* (b), pathogens of the honey bee. Photos by W.-F. Huang

Nosema infection has been associated with impact on pheromone production, immune response, flight behaviour, energetic stress, behavioural fever and hunger mediated conduct (the bees are less inclined to share their food with other bees), effects on brood care, thermoregulation, defence and foraging (Gaulthier et al 2011, Botias et al 2012). All these factors may have an impact not only at the individual but also at the social level and have detrimental effects on colony homeostasis (Botias et al 2012).

N. apis is more prevalent in cooler climates and affects honeybees primarily in the spring and early summer. it has been referred to as “Spring dwindling”, with dysentery and fouling of the outside of hives with faeces and it is known to age bees more rapidly in that they take on the duties of older bees and subsequently die sooner than non-infected bees (Fries 2010). These precocious foragers have been shown to be less effective and resilient than normal foragers (Oskay 2007). Infection with *N. apis* has also been shown to raise Juvenile Hormone III (JHIII) levels in bee hemolymph which is associated with behavioural changes (Ares et al 2012). There is also evidence that colonies infected with *N. apis* collect significantly less pollen than uninfected colonies due to the reduction in their adult bee population (Andersen and Giacon 1992). Although feeding pollen supplements in the laboratory increases the lifespan of bees infected with *N. apis,* feeding field colonies with supplemental pollen has been reported not to counter the reduction in worker lifespan, presumably as it was used primarily for brood rearing (Rinderer and Elliott 1977, Mattila and Otis 2006). In two races of European bee. It was observed that *N. apis* colonizes the entire gut in approximately 2 weeks after infection and an average of 3-5 x 10^6 spores are present at the end of this period, although there was no apparent relationship between bee longevity and spore dose (Malone and Stefanovic 1999, Czekonska 2007).

N. ceranae is more adaptable and less host specific than *N. apis* (Botias et al 2012). There is evidence that *N. ceranae* is far more virulent than *N. apis*, causing colony deaths in warmer drier climates, is more stable at warmer temperatures and that it has a greater effect on longevity (Fenoy et al 2009). The stress may be an important factor contributing to the increased virulence of *N. ceranae* observed. In a recent study, feeding experiments on caged bees showed that both mortality and sugar syrup consumption were higher in *N. ceranae* infected bees than in N. apis-infected and control bees (Martin-Hernandez et al., 2011). The mortality and sugar syrup consumption were also higher in *N. apis*-infected bees than in controls, but less than in N. ceranae-infected bees. With both microsporidia, mortality and sugar syrup consumption increased in function of the increasing spore counts administered for infection, from 10^3 to 10^5 spores/bee. The differences in energetic requirements between Nosema spp. confirms that their metabolic patterns are not the same, which may depend critically on host– parasite interactions and, ultimately, on host pathology. The repercussions of this increased energetic stress may even explain the changes in host behaviour due to starvation, lack of thermoregulatory capacity, or higher rates of trophallaxis *N. c*eranae appears to be more prevalent in the summer than *N. apis*

but seasonal infection peaks appear to differ between geographical zones (Martin-Hernandez et al 2011, Chen et al 2012). Although there is no apparent relationship between bee longevity and spore dose in two races of European bee (Malone and Stefanovic, 1999). There are some suggestions that *N. ceranae* is more adaptable and less host specific than *N. apis* (Botias et al 2012).

Infection with *N. ceranae* has been shown to significantly elevate JHIII titre in infected bees beyond that observed following *N. apis* infection. There are strain differences as *N. ceranae* isolated from Spain had a greater effect on JHIII titres than N. ceranae isolated from the Netherlands (Ares et al 2012). However, there were no differences in virulence of strains isolated from Spain and France (Dussaubat et al 2012) and it was considered that the response of the strain of honeybee to infection may be more critical, e.g. susceptibility of *A. mellifera iberiensis*.

The biological cycle of Nosema spp. in honeybees has been reported to depend on temperature. When expressed as total spore counts per day after infection (2 weeks old bees infected with 10,000 spores), the biotic potentials of *N. apis* and *N. ceranae* at 33°C were similar, but a higher proportion of immature stages of *N. ceranae* than of *N. apis* were seen. At 25 and 37°C, the biotic potential of *N. ceranae* was higher than that of *N. apis* (Figure 20). The better adaptation of *N. ceranae* to complete its endogenous cycle at different temperatures clearly supports the observation of the different epidemiological patterns and supports the increased incidence of problems associated with *N. ceranae* infections in warmer climates (Martin-Hernandez et al 2009). In contrast the viability of *N. ceranae* spores is significantly reduced following one week in a deep freezer, which is not the case for *N. apis* (Fries and Forsgren, 2009).

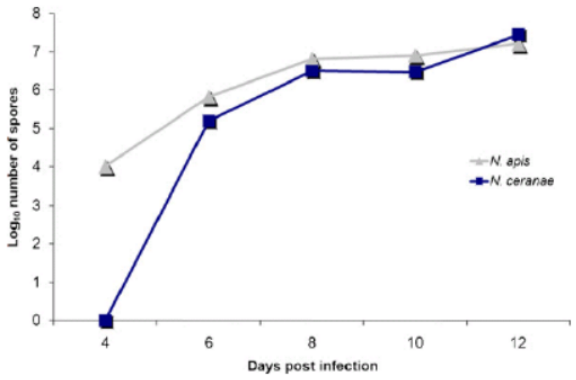


Figure 20: Biotic Potential of N. apis and N. ceranae (Fries and Forsgren, 2009).

N. ceranae has been reported in a number of European countries, including Spain, France, Germany, Switzerland, Denmark, Finland, Greece, Hungary, Holland, United Kingdom, Italy, Serbia, Poland, Slovenia, Bosnia Herzegovina and Sweden (Higes et al 2010). A multiplex PCR assay was used to detect coinfections by the two species of Nosema in a screening approach in Europe. Screening of bee samples from Spain, Switzerland, France, and Germany using the PCR technique revealed a greater presence of *N. ceranae* than of *N. apis* in Europe, although both species are widely distributed. From the year 2000 onward, statistically significant differences have been found in the proportions of Nosema spp. spore-positive samples collected between and within years. In the first period examined (1999 to 2002), the smallest number of samples diagnosed as Nosema positive was found during the summer months, showing clear seasonality in the diagnosis, which is characteristic of *N. apis*. From 2003 onward a change in the tendency resulted in an increase in Nosema-positive samples in all months until 2005, when a total absence of seasonality was detected (Martin-Hernandez et al 2007).

The diagnosis of Nosema disease has been traditionally done by detecting spores of Nosema spp. through microscopic analyses (Shimanuki and Knox, 2000). However, with the recent finding that both Nosema ceranae and Nosema apis affect western honey bees (*Apis mellifera*), molecular techniques are required that can reliably differentiate between these different species of microsporidia, because the spores of the two Nosema spp cannot be reliably distinguished by their morphology (Fries et al., 2010). Moreover, microscopic examination of Nosema spores is costly, laborious and time-consuming. In addition, microscopic analyses are not as sensitive at detecting low levels of Nosema infection as molecular methods, such as PCR, can be. In the last years, several molecular protocols have been published for thes identification of N. ceranae and N. apis, including species-specific PCR assays, or simultaneous detection of the Nosema species using multiplex PCR (Higes et al., 2006; Chen et al., 2008). Until now, published real time PCR-based assays capable of simultaneous identification and quantification of both species in the same reaction mostly use separate primers pairs and species-specific probes (Burgeois et al, 2010).

2.1.3. *Staphylococcus Pseudintermedius* and fluoroquinolone resistance

The genus *Staphylococcus* belongs to the family of *Micrococcaceae*, and is the only of its kind that includes pathogens of medical and veterinary concern. Staphylococci are Gram-positive, spherical in shape (cocci), with a diameter of about 0.5-1,5 µm aerobic and facultative anaerobic, motionless, catalase-positive and oxidase-negative bacteria (Poli G. And Cocilovo A., 2005). The taxonomic classification of staphylococci has changed a lot over the years thanks to new applications of microbiological and enzymatic techniques but especially of molecular methods. Staphylococci are ubiquitary bacteria widely distributed in the environment, and they can be found in water, air, soil, dust, and a wide diversity of inanimate objects (Martino P.A. et al., 2005).
Staphylococci can also be considered the most common saprophytic of humans and animals skin. As well as on the skin, Staphylococci can be isolated from mucous membranes of various apparatuses, including the respiratory, gastrointestinal and urogenital. Many people can be healthy carriers which are then considered a likely source of infection for themselves and for others (Cox H.U. 2006).
Staphylococci are now classified into forty different species on the basis of genotype, habitat, and the related pathological process (Martino P.A. et al., 2005). One of the best criteria to classify staphylococci is the production of coagulase. In fact the various species can be divided in coagulase-positive and coagulase-negative, according to their capacity to coagulate rabbit plasma. Many author considered coagulase a factor closely related to pathogenicity, as it promotes the spread and survival of the bacteria in the body. Coagulase is present in many staphylococci and its main action is to coagulate the plasma resulting in activation of fibrinogen and its subsequent transformation into fibrin. The clot protects the bacteria from the immune system that fails to reach, for which bacteria who develop this enzyme are more resistant to the immune defenses of the host (Cox H.U. 2006). The coagulase is also defined as “free coagulase” to distinguish it from clumping factor, an enzyme that is bound to the bacterial cells and which has the same function.
These bacteria then act as opportunistic pathogens (except *Staphylococcus aureus*, a real pathogen). Each species of staphylococci can therefore be considered as potentially pathogenic, although showing a wide and varied spectrum of virulence as well as host preference. Moreover, given their strong environmental resistance, they can survive the toughest conditions for months,

as they persist for months outdoors (if protected from sunlight), and in the laboratory after transplantation in fresh medium or freezing. They are also relatively resistant to heat (about thirty minutes at 60 °C), drying and, if properly protected from organic material (pus, mucus, milk, serum, debris), also to the common disinfectants (Martino P.A. et al., 2005; Cox H.U. 2006). In addition to being very resistant, these bacteria have developed several factors which allow the colonization and infection of host tissues. They in fact, after a decrease in immune function, invade the epithelium. This colonization can be stimulated by various conditions such as: mechanical insults of the skin (eg., wounds, abrasions, etc.), other infections (eg., demodicosis, dermatophytosis, pyoderma, etc.) or even particular changes (eg., seborrhea, hypothyroidism, Cushing's disease and Addison's disease). In addition, persistently immunosuppressed subjects are more affected and develop more violent and persistent clinical forms. When bacteria invade the skin they may cause from light (acne, impetigo) to severe (furunculosis) local infections or, as a result of colonization of the bloodstream with bacteremia, may develop purulent processes of various kinds (especially abscesses) in different locations: liver, kidneys, lungs, joints. In dogs they can be associated to specific diseases such as otitis, pyoderma and metritis (Cox H.U. 2006).

Staphylococcus pseudintermedius is frequently found on the skin or in the nose or intestinal tract of 50% of more of healthy dogs, and a smaller percentage of healthy cats. Usually this microrganism doesn't cause problems at all, but sometimes it behaves as an opportunistic pathogen. *Staphylococcus pseudintermedius* can infect almost any tissue, but skin and soft tissue infections are more common, particularly when the skin have been damaged by something else (e.g. allergies, scratching, chronic wetness, wounds, surgery). The bacteria easily grow on common growth media, without the need of particular substances or pH indicators, as colonies with color from gray to yellow to orange (Figure 21) (Cox H.U. 2006).

Ear infections are very commonly caused by *S. pseudintermedius.* Infections of other body sites and organs are much less common, but can be very severe. *Staphylococcus pseudintermedius* can also be found in the nose of up to 4% of healthy animal owners. MRSP stands for methicillin-resistant *S. pseudintermedius*, which is a variant that is highly resistant to many antibiotics, including most of the drugs that are commonly used to treat bacterial infections in dogs and cats. Non-MRSP variants of *S. pseudintermedius* are methicillin-susceptible (MSSP). People and animals that carry MRSP without any signs of infection at all are said to be colonized. When infection with

S. pseudintermedius (either MRSP or MSSP) occurs, this causes signs of inflammation (e.g. heat, pain, swelling, discharge, fever) (www.wormsandgermsblog.com).

Figure 21. Colonies of *Staphylococcus aureus* in a growth medium common Agar (ASM Microbel library.org®)

Staphylococci are halophilic, being able to withstand high concentrations of sodium chloride (also 10%), and they can use the ground (MSA-Mannitol Salt Agar-) those contain a large percentage of this salt (approximately 7.5%). As regards instead the condition of incubation and growth, being staphylococci bacteria mesophilic their growth temperature is between 18 and 40 ° C with an optimum at 37 ° C; also being aerobic-anaerobic facultative they can grow both under normal conditions and in modified atmosphere (10% of carbon dioxide) (Martino P.A. et al., 2005; Cox H.U. 2006).

When grown in liquid media, the colonies appear to macroscopical analysis as a uniform turbidity, while in solid media after incubation for 24 hours at 37 ° C, they show up as large colonies roundish of about 2-3 mm in diameter, opaque, smooth and convex; encapsulated strains can form colonies of mucoid appearance (Martino P.A. et al., 2005). The colonies may vary from gray to yellowish, to orange depending on the pigmentation of the cell membrane, and coloring is not associated with pathogenicity. Agar-blood culture can be useful for identification, because some staphylococci, according also to the type of red blood cells used for the ground, show a well clear halo of hemolysis (Figure 22).

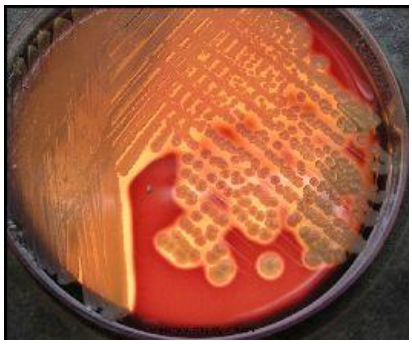


Figure 22. Clear area of hemolysis around the colonies of *S. aureus* on blood-Agar (ASM Microbel library.org®)

There are different cultural and biochemical methods to identify the colony.

The examination of the morphology, to see size, shape, color, odor and presence of hemolysis of the colonies. Staphylococci have a tendency to aggregate in irregular clusters, but occasionally can be found arranged individually, in pairs (diplococci) or in groups of four (tetrads) (Figure 23) (Martino P.A. et al., 2005).

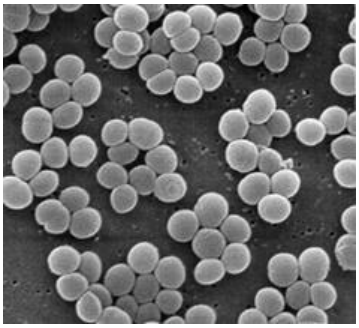


Figure 23. Staphylococci in electron microscopy arranged in small clusters of several subjects. (ASM Microbel library.org®)

The Bacterioscopic examination of stained preparations is another possible application. This test provides further information on the morphological characteristics of the isolated bacteria and also on how they react to certain differentials colors (eg, Gram, Ziehl-Neelsen). In the case of these bacteria, the test with an optical microscope is preceded by Gram dye, which, for Gram-positive bacteria, allows detection of blue-violet microorganisms (Figure 24).

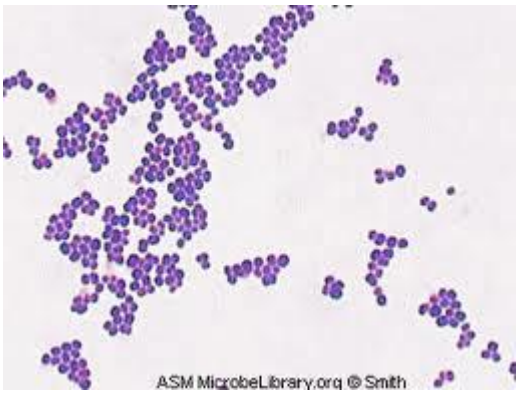


Figure 24. Staphylococci with Gram stain

The catalase proof is based on the detection of the presence of this enzyme. The catalase presence or absence help to distinguish staphylococci catalase-positive and catalase negative. There are several commercial kits but more simply is to dissolve a colony in one drop of water 3% hydrogen peroxide placed on a glass slide (Poli G. and Cocilovo A., 2005).

The oxidase proof is based on the detection of the presence of this enzyme. The procedure prescribes to dissolve the bacterial colony into a drop of saline placed on a glass slide; the suspension is subsequently immersed in a disk of paper, impregnated with a solution of tetramethyl-p-phenylenediamine dihydrochloride (the disks are commercially available). In case of positive reaction, within 10-20 seconds is observed the appearance of an intense purple color. There are also commercial kits, which allow the simultaneous execution, in vitro, of the catalase and oxidase tests. Obviously, in the presence of colonies of staphylococci, this test must be negative (Figure 25) (Poli G. and Cocilovo A., 2005).

Figure 25. Outcome of the catalase test: on the right a positive sample, on the left a negative sample (www.mesacc.edu)

The proof of coagulase is another enzymatic test. This is an easy test to determine the presence of coagulase, as follows. A drop of anticoagulated (using lyophilized citrate or EDTA) rabbit plasma is deposited on a glass slide and a colony is dissolved; the occurrence, within 5 seconds, of microscopic flakes is a sign of positivity (Poli G. and Cocilovo A., 2005).

Biochemical tests and API-System®. API-System ® (bioMérieux) is a miniaturized and standardized system for the identification of microorganisms by biochemical tests. Thanks to this test, it is possible to identify and classify the bacteria according to the final products or intermediates of various biochemical reactions of which they are capable.The testt is used when the first phase of identification has narrowed down the possibilities to a few genera or it only remains to identify the species. It consists of a gallery with 20 wells, each containing a different soil and appropriate chromogenic indicators; therefore it allows the simultaneous execution of 20 biochemical tests. Some reactions are read immediately, according to the spontaneous color changes of the substrates used in the individual well; in other cases, only after the addition of specific reagents. Through this analysis it is possible to simultaneously detect the ability of the microorganism to metabolize various sugars to produce acids or gas, to liquefy the gelatine, to use citrate, to reduce nitrate, etc. There is a version of this identification system, called API-Staph ® specifically targeted to the genera Staphylococcus and Micrococcus.

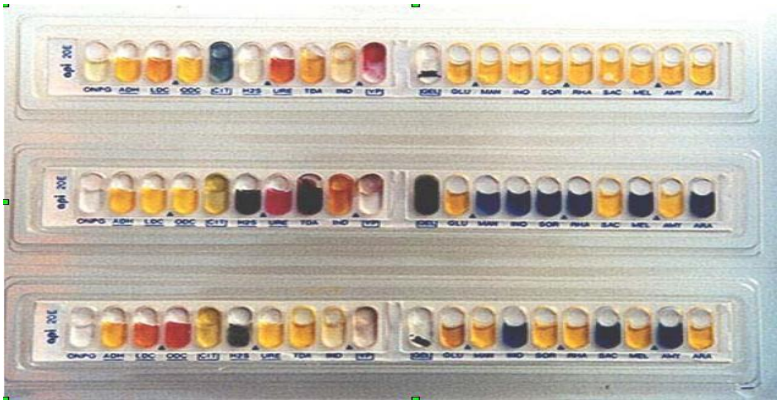


Figure 26: Example of a biochemical test API-System ® (www.jlindquist.net)

Antibiotic resistance is a phenomenon by which a bacterium is immune to the effects of one or more antimicrobial drugs. This phenomenon can be considered as an evolutionary and adaptive biological process that bacteria carry out to escape the selection (Levin B.R. et al., 2000). The selection pressures reach maximum levels when bacteria are exposed to sub-optimal doses of the

drug. The antibiotic resistance may be natural or acquired: the first is determined by the innate characteristics of the microorganism, for example a Gram-negative bacterium is naturally resistant, thanks to the porosity characteristics of their membrane, to glycopeptide antibiotics such as vancomycin. The second appears when occurring genetic modifications that allow to changed subjects the survival and transmission of the modifications (Levin B.R. et al., 2000).

There are two tipe of changes, divided into chromosomal and plasmidic. The chromosomal mutations, also known as endogenous, occur primarily at the level of nuclear DNA by spontaneous phenomena of substitution, insertion and deletion of base pairs (point mutations) or more pairs of bases repeated (mutations for repeated sequences) within the colony to each replication cycle with a frequency of $1:10^3$ - $1:10^9$ for cell division. This type of mutation occurs more slowly and rarely compared to plasmid-mediated mutations but usually gives to the changed population a permanent resistance to antimicrobial agents. Obviously, being this mutation spontaneous and random, it does not mean that, once completed, a mutation affects exactly the portion of DNA that contains sequences related to molecules or structures that are antibiotic targets. The genic modifications mediated by plasmids, however, are caused by fragments of double-stranded circular cytoplasmic DNA. This type of filament is common in bacteria and promotes the exchange of genetic information between phylogenetically related micro-organisms. The plasmids are small DNA fragments of different size: the smaller ones (approximately 10kb) are generally present in large number in the cytoplasm (10-20); those more bulky (100-150kb) are usually present in single or double copy. The plasmids replicate with the aid of specific proteins and are divided among the daughter cells during binary fission. They are able to transmit genetic information that are used to develop new features dangerous for the host: virulence factors, the production of new proteins or enzymes for the survival, detoxification, antibiotic-resistance, etc. (Smillie C. et al., 2010).

There are mainly three other mechanisms that bacteria can use for the exchange of genetic material, with both chromosomal and plasmid nature: a) transformation; in this process the genes are acquired by the recipient cell in the form of naked extracellular DNA. Basic assumption is the death of another microorganism which, after analysis of their structures, releases the DNA into the surrounding environment. The recipient cell adsorbs the free DNA through the cell wall and, as a result of recombination with a homologous segment, it incorporates into its DNA acquiring new characters; b) conjugation; this is a process in which a direct exchange of genetic material takes place from a donor cell to a recipient through the formation of a particular structure called pilo

sexual, which allows the horizontal passage of chromosomal DNA or, more frequently, plasmid DNA; c) transduction, a processes where the gene transfer is mediated by bacteriophages (or phages), as from viruses that infect bacteria. A phage infects a bacterium, basically, and takes control of the genetic processes of the bacteria to replicate. During this process, inadvertently, bacterial DNA (genomic or plasmid) can replace and/or become incorporated into the newly formed phage DNA. Only the phages characterized by dsDNA can be transducing. After bacterial death, which follows the lysis, the new phages are released and go on to infect other bacteria, carrying to them genetic material from previously infected bacteria (an example of transfer by transduction regards the plasmid carrying the gene for β-lactamase in *S. aureus*).

The appearance of antibiotic resistance is related to three main categories of mechanisms (Walsh C. 2000):

1) Alteration of the binding site between the target protein and antibiotic. The alteration of the binding site occurs via chromosomal and/or plasmid-mediated genetic mutations, which alter the affinity for the drug by changing the nucleotide sequence of a certain protein. In this way even if the antibiotic reaches the cytoplasm, it fails to exert its bacteriostatic action or bactericidal activity. Examples of this mechanism are provided by PBPs (Penicillin Binding Proteins), that are target enzymes of β-lactam antibiotics.

2) Production of enzymes that destroy the drug. The production of enzymes that break down the drug is definitely one of the most studied and known mechanisms of antimicrobial resistance. The best known case is undoubtedly that of beta-lactamase enzymes produced by many microorganisms that destroy the antibiotic belonging to the group of β-lactam (penicillins and cephalosporins). These enzymes are encoded both by plasmids, such as the R plasmid which also contains the TEM-lactamase, and by chromosomal sequences such as AmpC, characteristic of many Gram-negative bacteria (Nikaido H. 2009). The beta-lactamases cause hydrolysis and subsequent destruction of antibiotics and have become increasingly refined and perfected by the great use of β-lactam antibiotics. These antibiotics are increasingly improved over the years to escape the enzymes produced by bacteria, but inexorably microorganisms develop resistance by producing and adapting their defense mechanisms.

3) Decrease in the concentration of the antibiotic in the cytoplasm.

The production of pumps for the cytoplasmic efflux is a very refined mechanism of resistance whose transmission to other bacteria is quite difficult because of the complexity of the genes that encode them. Antibiotics enter into the cell through pores of the membrane, but can be extruded from the cytoplasm due to the presence of these pumps which are nothing more than transmembrane proteins that actively expel the drug. This system, developed by both Gram-positive and Gram-negative bacteria, has been particularly studied in E. *coli*, but also in staphylococci, especially *S. aureus*, and is responsible for resistance to multiple antibiotics, especially the fluoroquinolones (NorA), tetracyclines, macrolides and β-lactam antibiotics (Nikaido H. 2009).

All chemoantibiotics compounds are effective only towards certain bacterial species while have no effect on others species. Furthermore, the emergence of resistance makes immune some bacterial strains that commonly fall in the spectrum of action of the antimicrobial. Precisely for this reason when you are in front of a pathogenic micro-organism you should, before embarking on empirical therapy, perform at least one antibiogram that not only gives information about the strain in question but also general information about the antibiotic resistance. The antibiogram, performed according to the Kirby-Bauer technique, is certainly one of the most used techniques in clinical practice and is a standardized method for the amount of bacteria seeded (using a bacterial inoculum equal to Section 0.5 of the McFarland scale, i.e. to 5 x 10^8 CFU / ml) and the type of soil used (Mueller-Hinton, devoid of para-amino acid -benzoic acid). This occurs by the inoculums seeding for inclusion in agar or slither on the surface by swab. Are then deposited, on the surface of the sown soil, a number of cellulose diskettes impregnated with known amounts of antibacterial drugs. After the incubation period of the plates (typically 37 ° C for 24 hours), proceed to the reading of the halos: the chemoantibiotics spread by disks into the surrounding soil and, if effective, inhibit bacterial replication in a much larger area, the greater is their activity. It is observed, thus, the appearance of zones of inhibition of growth around the diskette antibiotic, whose diameter is proportional to the antibacterial activity of the antibiotic content. The absence of a halo is, instead, a sign of ineffectiveness of the drug. With this technique it is therefore possible to classify the bacteria susceptible, intermediate or resistant to several antibiotics tested (Figure 27).

Figure 27. Antibiogram performed according to the Kirby-Bauer technique (www.wikipedia.org)

MIC (Minimum Inhibiting Concentration), is the minimum concentration of antibiotic capable of inhibiting bacterial growth. The most common technique of execution involves dilution in liquid media (or broth): the technique can be performed in a test tube (macromethod) or in 96-well plates (micromethod). It starts from a bacterial inoculum concentration equal to 10^4 CFU/ml, which is mixed at scalar concentrations of antibiotic. The technique performed in a test tube provides the twofold dilution, from 128 micrograms/ml of the antibiotic, in volumes of 1 ml. The micromethod instead provides for the mixing of 100 µl of BHI for growth of the bacterium, 100 µl of bacterial suspension and, in the first tube, 100 µl of stock solution of antibiotic (equal to 1 mg/ml, obtained by dilution of the powder of the principles active in distilled water and filtration), then serial dilutions in base 2. Following incubation at 37 ° C for 18-24-48 hours. The MIC is the highest dilution, i.e. the smallest amount of antibiotic, capable of visibly inhibit bacterial growth.

Quinolones are a class of chemotherapy (i.e. chemical synthesis) antibiotics discovered at the end of the fifties of the last century, whose progenitor may be considered nalidixic acid. Given their spectrum of activity, Gram-negative aerobes and anaerobes, their rapid renal excretion and the achievement of high urinary concentrations were usually used to treat infections of the genito-urinary tract. However, given their narrow spectrum of activity and the emergence of significant resistance phenomena, they have slowly fallen into disuse. In more recent years, starting from quinolones, novel compounds called fluoroquinolones have been synthesized with a broader spectrum of action and smaller phenomena of resistance, due to the presence of a fluorine atom in their structure. These drugs show a good intestinal absorption, good tissue distribution and

prolonged half-life of elimination (Carli et al., 2009). Lately there is a growing series of antibiotic resistance even against this class. Currently, in Italy, enrofloxacin, danafloxacin, difloxacin, flumequine, marbofloxacin and norfloxacin are registered for use in veterinary medicine.

The fluoroquinolones are synthesized starting from the same chinolonic structure, from time to time amended with different chemical substituents (such as fluorine) and with side chains. These modifications, as a rule, do not change the spectrum of action of fluoroquinolones but instead modify the kinetic characteristics, in particular the bioavailability, the ability of tissue distribution and excretion rhythms (Carli et al., 2009). These chemotherapeutics show a bactericidal, very rapid and dose-dependent mechanism of action, mainly targeting two enzymes of the class of topoisomerase: DNA gyrase and topoisomerase IV. The DNA gyrase is an enzyme composed of two GyrA subunit and two GyrB subunits respectively transcribed by gyrA and gyrB genes, and has a crucial role as being the only enzyme able to rotate the DNA windings negative; this ability is important during the step of replication and synthesis of the DNA (Drlica K. and Malik M. 2003). The topoisomerase IV is a type II isomerase being the main enzyme responsible to the separation of DNA strands at the end of the replication process. It consists of two subunits ParC (calls grlA in *Staphylococcus aureus*) and two subunits ParE. The main targets of fluoroquinolones are GyrA and parC, respectively; bonding to these molecules hampers the separation and rejoining of DNA strands and this results in inhibition of bacterial DNA synthesis, poor reparative ability of the genetic material and cell death (Drlica K. and Malik M. 2003). More precisely, the link between drug, enzyme and open DNA, called ternary complex, prevents the progression of replication and transcription, and it is this that causes fragmentation of the chromosome which will lead to cell lysis. The high selective toxicity that these compounds have towards prokaryotic cells is justified by the fact that the eukaryote DNA-gyrase is inhibited at much higher concentrations than those of the bacterial cell (about 100-1000 micrograms/ml against 0.1-10 micrograms/ml) (Carli et al., 2009). For Gram-negative bacteria, the primary target of fluoroquinolones is the DNA gyrase enzyme, while for Gram-positive is the topoisomerase IV enzyme.

The resistance against these drugs is predominantly of chromosomal type, then it is stably transmitted to progeny, and its appearance is directly related to the concentrations of the chemotherapeutic in the site of infection. A repeated exposure to sublethal concentrations of fluoroquinolones can then produce a dangerous and stable resistance; which usually, being of cross-type, makes ineffective the other drugs of the same class (Carli et al., 2009). The onset of

this phenomenon against fluoroquinolones implies the onset of defensive mechanisms by the bacterial cell, which can implement two main strategies: the expression of transmembrane pumps that actively expel the drug outside of the cytoplasm, and the modification of the enzymatic target of the antibiotic. The first mechanism encounters for several efflux systems associated to genes with chromosomal and extra-chromosomal localization coding for transmembrane proteins that are able to reduce the cytoplasmic concentration of the drug (Table 26).

Antibiotic drug	**Working**	**Resistance genes**	**Genes location**	**Bacteria**
Tetracyclines	Outflow	*Tet*	P, T, C	Gram-positive Gram-negative
Macrolides	Outflow	*mefA*	P, T, C	*Salmonella spp* *E.coli*
Chloramphenicol, Fluoroquinolones	Outflow	*blt, norA*	C	*Bacillus spp.* *Staphylococcus spp.*
Chloramphenicol, Fluoroquinolones, β-lactams, Macrolides, Tetracyclines	Outflow	*MexA, MexB AcrA, AcrB, oprM, tolC*	C	*Pseudomonas spp.* *E.coli* *Salmonella spp.*

Table 26: Examples of microbial resistance through development of transmembrane pumps. P= Plasmids, T= Transposons, C= Chromosomes. (Carli et al., 2009).

Over the years, many bacteria have thus developed a tenacious resistance to these chemotherapeutic thanks to the modification of the target enzymes of these antibiotics. As usual, the microorganisms did not implement a single common process but have adapted different strategies according to their features. The resistance usually occurs due to modifications in the chromosomal genome, and its appearance is directly proportional to the concentrations of the drug in the site of infection as mentioned above, especially when the microrganism is exposed to sub-lethal concentrations of the drug for a long time. Typically, the resistance is of cross-type, for which its appearance makes the bacterial strain immune, or at least not very sensitive, to all drugs in the same category.

Fluoroquinolone resistance in *S. pseudintermedius* and other Staphylococci is predominantly mediated by a mutation located at position 84 of the gyrA subunit of DNA gyrase enzyme

(Ser84Leu), together with a mutation located at position 80 of the grlA subunit of topoisomerase IV enzyme (Ser80Ile) (Intorre L. et al., 2007; Descloux S. et al., 2008). All these amino acid substitutions are caused by point mutations (substitution of a single nucleotide) on particular chromosomal genes, gyrA and grlA, which encode for the production of these enzymes. This causes a different reading of the nucleotide triplet and then the change in the protein sequence of the enzyme. The modification of the enzyme structure is the cause of lower affinity which occurs between target enzyme and quinolone that fails to form the ternary complex and thus to cause cell death (Intorre L. et al., 2007; Descloux S. et al., 2008).

2.1.4. *Dirofilaria spp*

The etiological agents of disease defined generally "filariasis" fall into two subfamilies of parasites belonging to the *Onchocercidae* family and to the *Filarioidea* superfamily (Table 27). These subfamilies are represented by: *Dirofilarinae*, which includes *Dirofilaria immitis* and *Dirofilaria repens* (*Nochtiella repens*), and *Onchocercinae*, which includes *Dipetalonema reconditum* (*Acanthocheilonema reconditum*), *Dipetalonema dracunculoides* (*Acanthocheilonema dracunculoides*) and *Dipetalonema grassii* (*Cercopithifilaria grassii*) (Chauve C.M., 1990; Euzeby J., 1990; Urquhart G.M. et al., 1996).

Reign	*Animalia*
Phylum	*Nemathelmintes*
Class	*Nematoda*
Order	*Spirurida*
Suborder	*Spirurina*
Superfamily	*Filarioidea*
Family	*Onchocercidae*
Subfamily	*Dirofilarinae*
Gender	*Dirofilaria*
Species	*Immitis e Repens*

Table 27: Classification of *D. immitis* and *D. repens*. (Urquhart G.M. et al., 1996)

Dirofilaria immitis (Figure 27a) is a filiform parasite that as an adult measures approximately 20-30 cm in length and 1 mm in diameter; the male is distinguished by the female for the smaller size and to the caudal ends wound in a spiral. The mouth is surrounded by eight papillae median and two lateral. The caudal area of the male presents from 4 to 6 pairs of voluminous, pedunculated and ovoid papillae, of which 2-4 are pre-cloacal and 2 post-cloacal and fingerlike, and 3-4 pairs of conical papillae small and located in terminal position. The spicules measure respectively 315 and 200 microns. In the caudal edge of the female, in sub-ventral position, there is a conical structure and papilliforme facing backwards (Euzeby J., 1990).

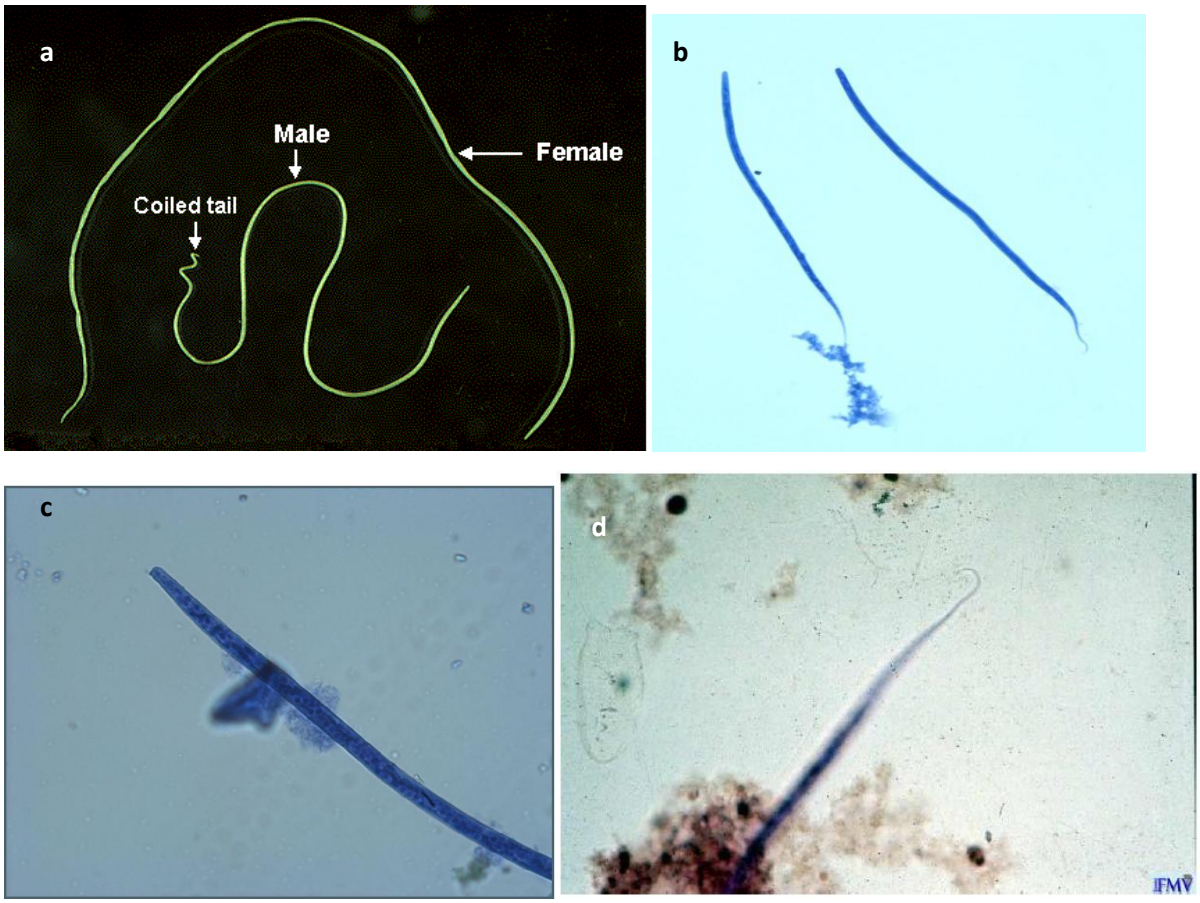


Figure 27: a) Adult specimens of *D. immitis* (cal.vet.upenn.edu/merial/hrtworm/hw_1a.htm). b) Microfilaria of *D. immitis* (left) and *D. repens* (right). (Photo kindly provided by Dr. Marco Genchi). c) Tapered cephalic-end of *D. immitis*. (Photo kindly provided by Dr. Marco Genchi). d) Caudal ends with shape of umbrella handle of D. repens. (http://www.personalweb.unito.it/ezio.ferroglio/atlante/parassiti_cane/dirofilaria/d_repens/foto/coda_rep1.jpg)

Dirofilaria (Nochtiella) repens has smaller dimension than *D. immitis*: in fact the female measures 10-17 cm long and 460-650 microns wide, while the male measures 5-7 cm long and 370-450 microns wide. The male has from 2 to 4 pre-anal papillae on one side (with spicules which measure from 465 to 590 microns) and 5 or 6 on the other (with spicules which measure from 185 to 206 microns). In the female the vulva is distant from 1.15 to 1.60 mm from the front end (Chauve C.M., 1990).

The larval forms of the parasite are represented by the microfilariae (L1): these are found in the circulating blood of the definitive host and, like the adult parasites, their characterization is necessary to reach a proper etiologic diagnosis. The morphological elements that allow the identification of microfilariae are the length and the shape of the cephalic and caudal end.

The microfilariae of ***Dirofilaria immitis*** (Figure 27b) measures 300-330 x 5-7 microns and are not equipped with sheath: the caudal part is straight and sharp while the cephalic end is tapered and blunt-ended (Figure 27c) (Chauve C.M, 1990; Euzeby J., 1990; Manfredi M.T., 1998; Urquhart G.M. et al., 1996).

The microfilariae of ***Dirofilaria (Nochtiella) repens*** (Figure 27b) measures 207-360 microns in length and 5-8 microns in width; does not exhibit sheath, the cephalic end is rounded and the caudal end has a shape similar to an umbrella handle (Figure 27d) (Chauve C.M., 1990; Manfredi M.T., 1998).

The microfilariae are identified, as well as on the basis of morphology, also with histochemical and immunological methods, as described later.

Dirofilaria immitis depends on insect vectors for the completion of the biological cycle, for transmission to the definitive hosts and for its dissemination (Euzeby J., 1990; Urquhart G.M. et al., 1996). It is a hematophagous parasite that can remain for years within the definitive host, continuing to produce microfilariae (Euzeby J., 1990). It is a viviparous parasite: the female lays the microfilariae that circulate in the blood of the host. The microfilariemia has a monthly and seasonal circadian periodicity that is highest in July and August and lowest in winter. To become adult, the larvae make various moults and part of their evolutionary cycle takes place in a hematophagous Diptera (mosquitoes of the genera Aedes, Anopheles, Coquillettidia, Culex, Culiseta and Mansonia) which acts as a carrier and as the intermediate host (Cancrini G., 1998). The first stage larvae (L1, microfilariae) are taken from the Diptera during the blood meal and remain viable in his gut. Subsequently, they migrate towards the Malpighian tubules and are transformed, through two changes, in second stage (L2) and third stage (L3) larvae. These become infesting in two weeks, after reaching the cephalic space and the *labium*. The infesting ability depends on the temperature: if this is too low larval development stops and may then resume in more favorable climatic conditions. The cycle in the final host begins when L3 are inoculated by the mosquito during a blood meal. The L3 remain in the skin for about six days, then turn in the fourth stage larvae (L4) that are localized in the capillaries of the abdominal wall (about 20 days after the bite of the insect) and thoracic (after about 40 days from inoculation of L3). In 60-80 days form the fifth stage larvae (L5) that migrate through the venous system to localize in the pulmonary artery (between 70 and 120 days after infestation). They persist in this location up to a length of 8-11 cm and subsequently perform, after about 110 days infestation, a retrograde

migration into the right ventricle, where they become adult individuals (Euzeby J., 1990; Urquhart G.M. et al., 1996). The prepatent period, i.e. the interval between infection of an individual by a parasitic organism and the first ability to detect from that host a diagnostic stage of the organism, is about 6 months, after which the adult females begin to produce the microfilariae. The infestation of the dog can take place between June and October, and during the first infestation usually L5 reach the pulmonary artery in late August and persist here until December, and then migrate into the right ventricle and start producing microfilariae. In an already parasitized subject, however, the larvae can be found in the blood throughout the year. In the pregnant dog, microfilariae can cross the placenta and reach the fetus, while the larvae infesting (L3) are not able to perform this migration, and for this reason intrauterine transmission of heartworm disease does not occur (in this case microfilariae are not able to evolve because it must spend part of their cycle in the mosquito) (Euzeby J., 1990) (Figure 28).

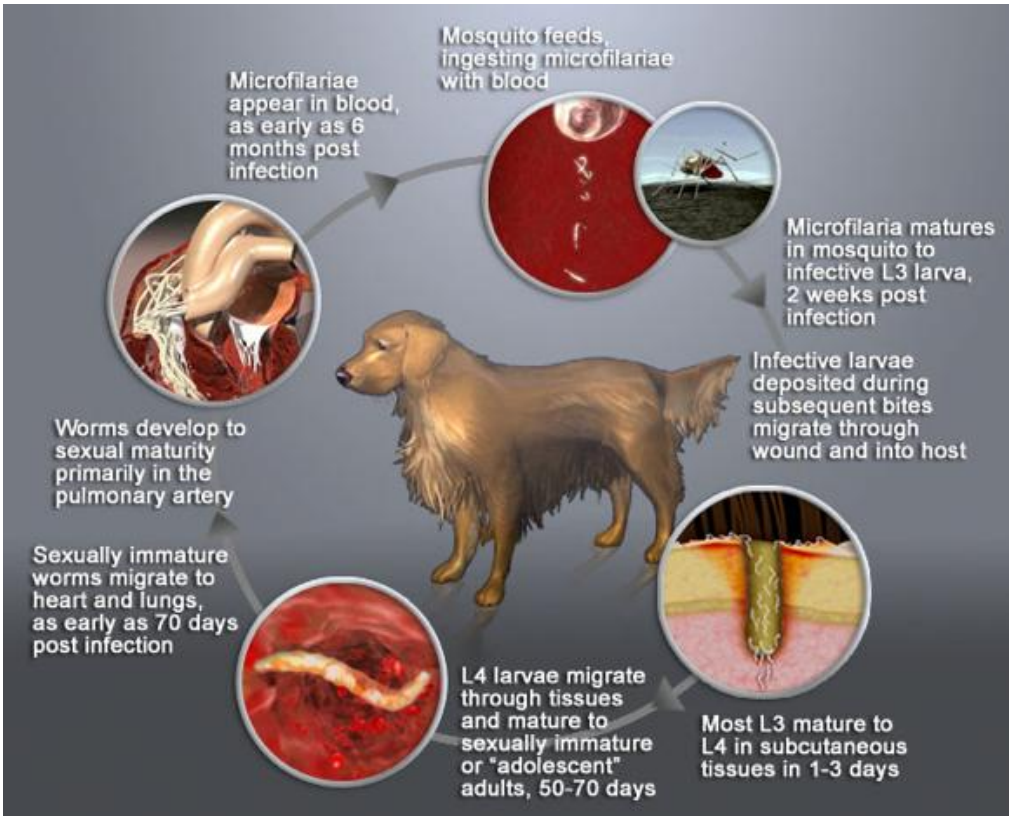


Figure 28: Biological cycle of *D. immitis.* (http://www.capcvet.org/capc-recommendations/canine-heartworm)

Dirofilaria (Nochtiella) repens, like *D. immitis*, to evolve to adult parasite needs a phase of development in an intermediate host (the mosquito) in which processing takes place from L1 to

L3. This is followed by the final inoculation into the host (dog, cat, man, other canids and wild felines) where the parasite is localized in the subcutaneous connective tissue and connective bands of skeletal muscle. In these locations it makes two changes, becoming first L4 and subsequently L5 and reaching then the sexual maturity (Chauve C.M., 1990).

The principal location of *D. immitis* are pulmonary arteries and heartworm must be considered as a pulmonary disease, that during the last stage also affects the heart chambers (Figure 29). A few days after the arrival of the parasite in the caudal pulmonary arteries, the endothelial cells become swollen, the intercellular junctions become dilated in response to trauma and activated neutrophils adhere in the space between endothelial cells. It is also found adhesion and platelet activation. The damaged surface arterial allows to albumins, to the fluid plasma and blood-cells to reach the perivascular space inducing a persistent perivascular inflammatory edema. After the changes endothelial, the tunica intima thickens and leukocytes invade the wall of the smooth muscle cells; these latter multiply within the tunica media and migrate to the surface endovascular as a response to the growth factor released from platelets. The proliferation and migration of smooth muscle cells determines the presence, on the inner surface of the arteries, of villi that are made from smooth muscle cells, collagen and lined by endothelial cells. The surface of the arteries of infected dogs and cats appear to be strongly rough and smooth and the lumen of the pulmonary arteries resulting decreased. Lung disease is secondary to vascular changes; the leakage of liquids and proteins through the wall of the arteries affected produces an edema in the parenchyma. Moreover, the spontaneous death of some filariae is capable of producing pulmonary thromboembolism and severe inflammatory reactions. The reduction of conformity and caliber of the pulmonary arteries, which may also be occluded by a thromboembolism or severe villous proliferation, causes a state of pulmonary hypertension and, consequently, in an increase of the post load in the right ventricle, which can induce the cor pulmonale syndrome and congestive heart failure. The leaking of fluid and protein through the wall of the arteries affected produces further swelling and inflammation in the lung parenchyma.

The clinical evolution of heartworm disease in dogs is usually chronic. Most dogs infected have no symptoms of the disease for a long time, months or years, depending on the amount of heartworms, the individual reactivity and exercise, as it is more serious damage in dogs that perform strenuous exercise in respect dogs at rest. The disease develops gradually and can begin with occasional coughing. The cough may be followed by dyspnea, moderate to severe, weakness,

and sometimes fainting after exercise or excitement. Sound of crackling can be found at lung auscultation. Later, when congestive heart failure is developing occur: swelling of the abdomen and legs, fluid accumulation, anorexia, weight loss and dehydration. At this stage, there is a cardiac murmur on the right side of the chest, because of insufficiency of the tricuspid valve, and the heart rhythm is altered because of atrial fibrillation. Sudden death occurs rarely and usually occurs as a result of respiratory distress or cachexia. In the course of this chronic disease sometimes there is an acute symptomatology due to a severe thromboembolism following the natural death of heartworms, dogs may show acute dyspnea and hemoptysis (Genchi C. et al., 2007). In small dogs, it is also a common event the movement of adult worms from the pulmonary artery to the right chambers of the heart due to pulmonary hypertension and a sudden drop in right cardiac output. In this case, the affected dogs show the so-called *superior vena cava syndrome*. This syndrome shows, as well as dyspnea, cardiac murmur due to the presence of microfilariae in the vicinity of the tricuspid valve. This syndrome is also called *intravascular hemolytic*, because accompanied by hemolytic anemia and hemoglobinuria for mechanical intravascular hemolysis (red blood cell fragmentation occurs for the continuous movement between atrium and ventricle of filarial intertwined). This implies a systemic hemodynamic decompensation and shock with consequent death of the affected animal (Venco L., 1993). Disseminated intravascular coagulopathy often accompanies the superior vena cava syndrome, being secondary to hemolysis and metabolic acidosis. In addition, you may experience liver lesions characterized by hepatomegaly, with expansion venous thrombosis, centrilobular necrosis and fibrosis. Finally, there may be serious kidney disease, resulting from the hemoglobinuria with renal tubular necrosis and cylindruria.

In subcutaneous filariasis, caused by D. repens, the adult parasite is localized in the subcutaneous tissues of the dog and cat. This disease presents with minor clinical signs such as itching, swelling of the skin, subcutaneous nodules or remains asymptomatic (Genchi C. et al., 2007).

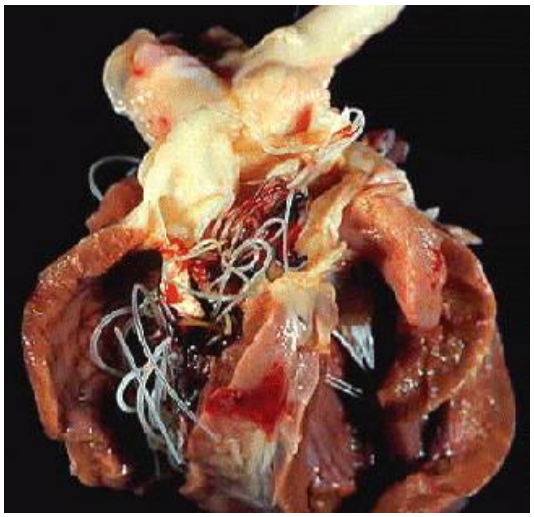


Figure 29: Adult of *Dirofilaria immitis* in dog heart.
(http://www.stanford.edu/group/parasites/ParaSites2001/dirofilariasis/Synonyms.html)

There are several method to detect dirofilaria:

- *Knott test to microfilariae search in the peripheral blood*

The circulating microfilariae can be detected with the Knott test. The microfilariae are detected and identified under the microscope on the basis of morphological criteria, and this is considered as a definitive proof of infection (specificity 100%). However up to 30% of dogs may be oligo-or amicrofilaremic, despite presenting adult worms, due to the presence of worms only of the same sex (quite unusual in dogs), a host immune reactivity towards microfilariae, or administration of microfilaricidal drugs. SThus the sensitivity of the test for microfilariae is not considered sufficient to rule out infection in the event of a negative result. Furthermore, in certain cases the identification based on morphology may be made more difficult by the presence of artifacts and/or intermediate morphologies, especially if the operator is not very experienced or if they are not put in place procedures of sampling and analysis perfectly reproducible (Genchi C. et al., 2007).

- *Blood test for the antigens research of adult females of D. immitis*

Different ELISA kit for detect the presence of circulating antigens of Dirofilaria immitis adult female in serum, plasma or whole blood of a dog are commercially available. Most tests are very specific, sensitive, rapid and easy to perform. The sensitivity is actually very high, but false negatives may occur during the prepatent period, very light infestations or when there are only males (male filariae are not detectable by this test). Most kits are for the clinical diagnosis of a

single sample, but are also available multi-test ELISA plates. Manufacturers claim that for a positive result parasite is sufficient 1 female adult, although many factors may influence the sensitivity of the test as the age of the parasite, the number of parasites female depending on the number of males and the size of the dog. Reliable and reproducible results can be obtained when there are 2-3 or more adult female worms. A detectable antigenemia develops after 5 to 6.5 months from the infestation. After the death of adult parasites, antigens circulating dissolve rather quickly, for this reason, this technique can be used to evaluate the efficacy of a therapy adulticide. To confirm the success of adulticide therapy, dogs must be reassessed at 5 and 9 months after treatment. If the test is negative at 5 months, the test at 9 months can be avoided (Genchi C. et al., 2007).

- *Chest radiographs*

Chest radiographs may show, in an advanced stage, the enlargement of the pulmonary arteries, abnormal lung pattern and, in the worst cases, right cardiomegaly. In the event of congestive heart failure are found right payments and peritoneal effusions. Radiographs are useful for assessing the severity of lung injury, but not for assessing the parasite. The radiographic signs of pulmonary vascular disease in advanced stages may persist long after the infestation has run its course and some of the most severely ill dogs may have a low number of parasites. In contrast, some dogs can host large parasites remained clinically asymptomatic with light or absent radiographic lesions (Genchi C. et al., 2007).

- *Electrocardiography*

Only at the last stage of the disease, when the heart chambers are seriously damaged, the ECG may show a deviation to the right of the axis cardiac and / or atrial fibrillation (Genchi C. et al., 2007).

- *Echocardiography*

Echocardiography allows direct visualization of the cardiac chambers and of communicating vessels. It can also allow the visualization of parasites in the cardiac chambers, the caudal vena cava, the main pulmonary artery and in the proximal portion of both pulmonary caudal arteries. The heartworms are highlighted as floating objects in the right cardiac chambers or in the lumen of the vessels. It is performed mainly in cases where the clinical and radiographic results suggest a severe type of the disease. The cardiac ultrasound can increase the accuracy in the staging of the

disease and in the estimation of filarial influencing the treatment plan and prognosis (Genchi C. et al., 2007).

- *Polymerase chain reaction (PCR*)

The polymerase chain reaction (PCR) is a sensitive and specific technique that allows to detect the presence of DNA belonging to a given organism, and as such can be used to identify the different species of heartworm. During the last years several researchers have developed different molecular protocols with diagnostic and epidemiological purposes. In particular, specie-specific PCR assays for single species identification, and multiplex PCR and PCR-RFLP assays, single and multistep, for simultaneous detection of the different Dirofilaria species either in the vector or in peripheral blood of infected dog have been designed (Favia G. et al., 1996; Mar P.H. et al., 2002; Casiraghi M. et al., 2006; Rishniw M. et al., 2006; Gioia G. et al., 2010). For diagnostic purposes, are particularly useful protocols applicable to the identification of microfilariae in the peripheral blood, in cases where the morphological recognition of microfilariae same proves to be difficult, or even in the case of mixed infections (Genchi C. et al., 2007). In addition, for clinical diagnostics use, is critical to have fast procedures and simple and clear results, including a precise assessment of the level of sensitivity and specificity in the peripheral blood.

2.2. Aim of the work

As stated above, the applications of HRMA for the characterization of microorganisms are obviously not limited to molecular characterization of *E. coli* strains, but can be developed for a much larger number of issues and targets linked to food safety and general veterinary diagnostics. The purpose of this work was to develop some HRMA protocols, fast and relatively inexpensive, able to detect and identify unambiguously a range of foodborne and veterinary pathogens, exploiting the nucleotide differences that exist between the sequences of the different species and/or variants. First, the aim of this work was the development of a novel real time PCR-HRMA method able to identify, in a fast and effective way, the microorganisms that cause chromatic alterations in mozzarella cheese samples. As a second objective, during this PhD project, the research activity was focused on the development of a new real-time PCR- HRM coupled analytical method for the identification and quantification of the *Nosema ceranae* and *Nosema apis* pathogens in honey bees, that could show improved performance for the analysis of *Nosema* spp also in mixed infections when compared to the currently available methods (Martin-Hernandez et al., 2007). As a further activity, two real time PCR-HRMA -based assays were developed for the identification of fluoroquinolone (FQ) resistance in *Staphylococcus pseudintermedius* isolated from dogs, respectively through the analysis of specific mutations of DNA gyrase and topoisomerase IV enzymes as molecular determinant of FQ resistance (Intorre et al., 2007; Descloux et al., 2008). Finally, among the activities of the project, a new real-time PCR- HRMA based method for the identification and discrimination of the filarial pathogens *Dirofilaria immitis* and *D. repens* in dogs was extensively validated.

2.3. Materials and methods

2.3.1. Sampling procedures

2.3.1.1 Pseudomonas

Twenty-seven samples chromatically altered of mozzarella cheese submitted for microbiological examination to the Laboratory of Animal Origin Food Inspection, VESPA Department at the University of Milan were selected for this study.

Briefly, for each mozzarella they were taken 12 g and then laid out in a sterile Stomacher bag. After adding the sterile diluent (1: 5), the samples were placed in 'homogenizer (150 strokes / min for 60 seconds). The compound was put on Petri dishes containing selective medium for *Pseudomonas* spp.: *Pseudomonas* DFD Agar (Oxoid). The plates were subsequently incubated at 30 °C for 48 hours.

After the bacterial growth, characterized by pigmentation (blu-green, brown or with fluorescent), the plates where the growth was not excessive were selected (min 15 colonies and maximum number of 50 colonies/plate). Twenty colonies was tipped from each plate and plated individually on the ground Agar Mascarpone and left to incubate at 30 °C for 48 hours.

2.3.1.2 Nosema

For the methodology's development, control positive samples of *Nosema ceranae* and *Nosema apis* were kindly provided by Istituto Zooprofilattico Sperimentale di Lazio e Toscana (dr. Giovanni Formato). In order to test the specificity of the method, mixed samples were obtained experimentally by mixing different concentrations (1:10, 1:1, 10:1) of *N. ceranae* and *N. apis* samples. Honeybees without *Nosema spp* were included as negative control.

To validate of the new biomolecular method, honeybee samples from 55 field apiaries were used. Forty samples were collected from beehives placed under observation by the monitoring network of Lombardy (Progetto Stranova, Regione Lombardia DG Agricoltura), and 15 samples were collected from apiaries from Lazio. Each sample was consisting of a pool of 5 intestines of foraging bees from 5 different colonies of each apiary, for a total of 25 intestines/pool. Sampling took place in a period between 2011 and 2013. Honey bee crushings underwent preliminary microscopic examination to assess the presence of Nosema spp. spores as described in OIE reference manual (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.04_NOSEMOSIS.pdf).

2.3.1.3 Staphylococcus pseudintermedius

In this study, 30 *S. pseudintermedius* strains were screened for the presence of *gyrA* Ser84Leu mutation and of *grlA* Ser80Ile mutation through specifically designed real time PCR-HRMA protocols. The strains were obtained as the unique or prevalent bacterial isolate from canine skin, external ear and conjunctive clinical samples submitted for microbiological examination to our Department during 2009. Isolation was performed through cultivation on tryptone soy agar plates containing 5% sheep blood (Oxoid S.p.A, Rodano, Italy) for 24 h at 37°C under aerobical conditions. Isolates were identified as *S. pseudintermedius* by morphology, Gram staining, catalase activity, growth in mannitol salt agar selective medium, and API-Staph® analysis.

The strains were tested for FQ susceptibility to enrofloxacin and marbofloxacin by the disc diffusion method on Muller Hinton agar (Oxoid).

For each bacteria was evaluated the profile of antimicrobial susceptibility to enrofloxacin and marbofloxacin. The method used was that of diffusion in agar medium or technique Kirby-Bauer with land Muller-Hinton (Oxoid, Italy).

The bacterial suspension was obtained by drawing a colony pears from cultures grown on blood agar plates and putting in 5 mL of sterile saline to obtain a solution of density equal to the point 0.5 on the McFarland scale. The sowing of land Muller-Hinton has been established through the use of a sterile swab dipped in the suspension, and later affixing to the diskettes containing enrofloxacin and marbofloxacin.

After incubation (37 °C for 18-24 hours) horizontal diffusion of bacterial growth was measured and the results were interpreted on the basis of the framework provided by the manufacturer for:

	enrofloxacina	marbofloxacina
Sensible	≥ 22 mm	≥ 18 mm
Intermediate	18-22 mm	14-18 mm
Resistent	≤17 mm	< 14 mm

Table 28: Interpretation of the data to detect antibiotic resistance in base of horizontal diffusemetry.

2.3.1.4 Dirofilaria spp

D. immitis and *D. repens* microfilariae were collected from anticoagulated canine peripheral blood samples referred to the Faculty of Veterinary Medicine of the University of Milan (Italy). Blood

samples were tested by a modified Knott's test, and circulating microfilariae were identified based on morphology and morphometry (Knott, 1939; Euzeby, 1981; Soulsby, 1982; Genchi 2007), and counted in 20 microliters of blood. In total, 3 *D. immitis* blood samples, 8 *D. repens* positive blood samples and 3 blood samples with natural mixed infection were selected for the HRM analysis. Furthermore, a Knott's test-negative blood sample was included as negative controls and single adults of *D. immitis* and *D. repens* were used as positive controls. Also, positive blood samples from dogs with different filarial loads (i.e., 4 mf/ml and 32250 mf/ml for *D. immitis*; 4 mf/ml and 100000 mf/ml for *D. repens*) were examined to test the range of sensivity of the method.

2.3.2 DNA extraction

2.3.2.1 Pseudomonas

Pigmented colonies resulted positive after plating in Agar Mascarpone were removed and diluted in 50 µl of sterile and demineralized water. The extraction was performed by means of alternating thermal cycles: 10 minutes at 95 ° C and 5 min at -80 ° C for three times. The DNA thus extrapolated is subjected to molecular analysis.

2.3.2.2 Nosema

Genomic DNA was isolated from each pool of 25 honeybee intestins crushing and filtered in 5 ml of double-distilled water. After two centrifugations at 800 g for 6 minutes, the pellets were resuspended in 1 ml of double-distilled water and sonicated for three cycles of 10 seconds each (output=2) (Branson Sonifier 250, Danbury, U.S.A). 1 ml of TNNT lysis buffer (0,5% SDS, 0.5% Tween 20, 0.5% Nonidet P-40, 10mM NaOH, 10mM Tris [pH 7.2]) and 8 µl of 20 mg/ml proteinase K were added in all samples. After incubation at 56 °C overnight, the proteinase K was inactivated at 95 °C for 10 min. DNA was isolated utilizing the standard basic phenol-chloroform extraction followed by ethanol precipitation. The extracted DNA was resuspended in 100 µl of PCR-grade water and then stored at −20 ◦C until tested by real-time PCR. For each sample, 1 µl of resuspended DNA diluited 1:10 was used as template in the real time PCR assay.

2.3.2.3 Staphylococcus pseudintermedius

For each *S. pseudintermedius* isolate was tip-sampled from a single colony and placed in an 0,5 ml Eppendorf containing 50 µl of water. Samples were then subjected to three alternate cicles of 5' at 95 °C and 5' at -80°C to obtain bacterial cell lysis and DNA extrusion.

2.3.2.4 Dirofilaria spp

Genomic DNA was isolated from 300 µl of each mf-positive peripheral blood sample and from isolated *D. immitis* and *D. repens* worms by adding 900 µl of TNNT lysis buffer (0.5% Tween 20, 0.5% Nonidet P-40, 10mM NaOH, 10mM Tris [pH 7.2]) and 50 µl of 20mg/ml proteinase K (Lachaud L. et al., 2002). Samples were then incubated at 56 °C for 18 h. Proteinase K was inactivated by incubation at 95 °C for 10 min. DNA was isolated by standard basic phenol chloroform extraction followed by ethanol precipitation. The extracted DNA was resuspended in 30 µl of PCR-grade water and then stored at −20 ◦C until tested by real-time PCR. For each sample, 2 µl of resuspended DNA was used as template in the PCR assay.

2.3.3. Primer design

Regarding the HRM primers design, for all target was used the same procedure. The target genes sequences were obtained from GenBank (available at http://www.ncbi.nlm.nih.gov/genbank/) and aligned using the online version of ClustalW programme (available at http://www.ebi.ac.uk/Tools/clustalw2/index.htmlref). For each target, the corresponding sequence obtained from GeneBank are reported in Table 29, and the conserved primer sequences designed for the HRMA protocol after sequence alignements are reported in Table 30, respectively.

Target	Gene	GeneBank
Pseudomonas	16S	*Pseudomonas putida*: FN600412.1, AB598738.1, FN652910.1, FN395007.1 Pseudomonas fluorescens: FN666564.1, AJ308306.1, FN666561.1, FN666563.1 *Pseudomonas aeruginosa*: FJ972538.1, FJ972535.1
Nosema	16S	*N. ceranae:* FJ789797.1, FJ789789.1, FJ789785.1, FJ789799.1, FJ789786.1, FJ789788.1, FJ789791.1, DQ374656.2, DQ673615.1, FJ789795.1, EU045844.1, EU025027.1) *N. apis:* FJ789796.1, FJ789790.1
S. pseudointermedius	16S Ribo DNA	From Becker et al 2007
	16S Ribo DNA	From Becker et al 2007
Dirofilaria	cytochrome oxidase sub I	*D. immitis*: AM749226.1, AM749228.1, HQ540424.1, EU169124.1 D. repens (AM749234.1, AM749231.1)

Table 29: resume of gene and relative gene bank tor each target

Target	Gene	primer	Sequence	Annealing T°	lengh
Pseudomonas	16S	16SPseudoF	5' – GCACAAGCGGTGGAGCAT - 3'	60 °C for 30 sec	167 bp
		16SPseudoR	5' – CGGGACTTAACCCAACATCT - 3'		
Nosema	16S	F-NosemHRM	5' –AGGGGCGAAACTTGACCTAT- 3'	60 °C for 30 sec	*N.ceranae* 160 bp *N.apis* 162 bp
		R-NosemHRM	5'–CTCACACATACTACTAACTACG- 3'		
S. pseudointermedius	gyrA	GyrAasmod F	5'-ATGAGTGTTATCGTATCTCGTGC-3	51 °C for 30 sec	262 bp
		GyrAasmod R	5'-CCATCGAACCGAAGTTACCTTG-3'		
	grlA	GrlAsmod F	5'-AATACGTATGATAAACATTTTCG-3'	51 °C for 30 sec	137 bp
		GrlAsmod R	5'-AGCACGTGACGTAATTTCCAGTCTT-3'		
Dirofilaria	cytochrome oxidase sub I	COXdirHRMF	5' –AGTATGTTTGTTTGAACTTC- 3''	52 °C for 1 min	256 bp
		COXdirHRMR	5' –AACGATCCTTATCAGTCAA- 3		

Table 30: Primers, annealing Temperature and fragment lengh for any Target

2.3.4. Conventional PCR

2.3.4.1. Pseudomonas

The new pair of primers was tested in conventional PCR, adding 1 µl of extracted bacterial DNA, 0.2 mM dNTPs, 1X Buffer (Promega Corporation, Madison, WI, USA), 1U Taq (Promega Corporation, Madison, WI, USA), 0.2 mM of each primer and H_2O to obtain a final volume of 20 µl. The thermal profile began with 94°C for 2 minutes, 40 cycles repetitions of: denaturation at 94°C for 10 seconds, annealing at 60°C for 10 sec and elongation at 72°C for 15 sec. The reaction was

performed on Mastercycler® Gradient thermal cycler (Eppendorf AG, Hamburg, Germany). Amplification products were run on 2% agarose gel.

2.3.4.2. Nosema

The new pair of primers was first tested in conventional PCR, adding 1 µl of extracted Nosema DNA, 0.2 mM dNTPs, 1X Buffer (Promega Corporation, Madison, WI, USA), 1U Taq (Promega Corporation, Madison, WI, USA), 0.3 mM of ech primer and H_2O to obtain a final volume of 20 µl. The thermal profile began with 95°C for 2 minutes, 40 cycles repetitions of: denaturation at 95°C for 15 seconds, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. The amplification ended with a final elongation at 72°C for 7 min, and was performed on iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Amplification products were run on 2% agarose gel and purified for DNA sequencing using Qiaquick™ Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The purified amplicons were then sequenced using standard Applied Biosystems technology.

2.3.4.3. Staphylococcus pseudintermedius

The newly designed pairs of primers for gyrA and grlA respectively was first tested in conventional PCR through amplification in a Mastercycler® Gradient thermal cycler (Eppendorf AG, Hamburg, Germany). The PCR reactions were carried out in duplicate in a total volume of 20µl containing 1µl of *S. pseudintermedius* bacterial lysate, 1X Taq buffer containing 1.5mM $MgCl_2$ and 0.2mM dNTPs, 1.25 U Taq Promega and 0.5µM of forward and reverse primers. The thermal profile for the amplification was 94 ◦C for 90 sec; 35 cycles of 94 ◦C for 45sec, 51 ◦C for 30sec, 72 ◦C for 90sec and final elongation step at 72 ◦C for 300sec. Amplification products were run on 2% agarose gel and purified for DNA sequencing using Qiaquick™ Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The purified amplicons were then sequenced using standard Applied Biosystems technology.

2.3.5. Real-time PCR amplification and HRM analysis

The real time PCR-HRMA assays for all the targets were run on an EcoTM Real-Time PCR System (Illumina, Inc., San Diego, CA, USA). The amount of reagents used for real-time PCR were: 7.5 µl Supermix SsoFast EvaGreen mix (BioRad), 0.3 µM of each primer, 1 µl of corresponding target DNA (2 µl for Dirofilaria target) and H_2O to reach a total volume of 15 µl. The thermal protocol started with a denaturation-activation step at 95°C for 8 min, followed by a 40 cycles (45 cycles for

Nosema target) repetitions of: program denaturation at 95°C for 15 s, annealing for 30 sec (1 min for Dirofilaria primers) at temperature depending on the primer pair as reported in Table 30. The melting program comprised 3 steps: denaturation at 95°C for 15 s, renaturation at 55°C for 15 s, and finally melting with continuous fluorescence measurement (ramping 0.1°C/s) to 95°C, each step for 15 s. Eco_v4.0 software was used to analyzed the HRM curve. Raw melting-curve data were normalized by setting the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all samples to uniform values for each target. Pre-melt signals were uniformly set to a relative value of 100%, while post-melt signals were set to a relative value of 0%. Double-distillate water was used as blank in parallel with each experiment. Each sample was tested in two replicates.

2.3.5.1. Comparison between HRMNosem primers and 218MITOC and 321APIS primers

The same Nosema DNA samples analyzed with the real time PCR-HRMA method were tested, in parallel, for *N. ceranae* target with the method based on the primer pair 218MITOC published by Martin-Hernandez et al. in 2007. This method was chosen since being the method currently utilized in conventional and real time PCR for *N. ceranae* detection and reported in COLOSS BEEBOOK (http://www.coloss.org/beebook/II/nosema/2/2/2/3).

2.3.6. Cloning

The efficiency, sensitivity and log range of the methodology for the identification and quantification of *Nosema spp*. was tested against reference curves built with 1:10 diluitions of the corresponding cloned amplicon. Briefly, conventional PCR amplification products from Nosema ceranae and Nosema apis DNA were run on 2% ethidium bromide agarose gel electrophoresis followed by UV-visualization and purified using QiaquickTM Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The concentration of purified amplicons was spectrofotometrically measured using a ND-100 Spectrophotometer. Each quantitated amplicon was ligated the plasmid pGEM T-easy at a concentration of 50 ng/ul (Promega Corporation, Madison, WI, USA) as reported in manifacture's instructions. The corresponding plasmid was included in DH5α competent cells in 3:1 concentration. The colonies used were selected by screening with conventional PCR with T7-SP6 plasmid-specific primers and primer HRMNosem. One positive colony for each target was grown and the corresponding plasmidic clone was purified using Qiaprep^{TM} Spin Miniprep kit

(Qiagen) and spectrofotometrically quantitated. The plasmidic clones were sequenced using T7 and SP6 primes and ABI technology in order to confirm the identity of each ligated target. The amplification was evaluated on ten-fold serial diluition points of the corresponding plasmid. At this regard, the log ranges were chosen encompassing the putative amount of each target sequence that could be expected in DNA prepared from honeybee intestines from 10^6 to 10^0 copies/well.

2.3.7. Sequencing

To confirm the results of HRM analysis DNA sequencing of the amplicons was performed for species identification (for Pseudomonas, Nosema, and Dirofilaria), mutation detection (for S. pseudintermedius) and cloning (for Nosema), as follows: the amplified samples were recovered after agarose gel electrophoresis and purified using QiaquickTM Gel Extraction kit (Qiagen GmbH, Hilden, Germany). The concentration of purified amplicons was spectrofotometrically measured using a ND-100 Spectrophotometer. The purified amplification products were then sequenced using standard Applied Biosystems technology on ABI Prism 310 DNA sequencer (Applied Biosystems). The obtained sequences were aligned to the expected target sequences using ClustalW programme(http://www.ebi.ac.uk/clustalw).

2.4. Results and discussion

2.4.1. P*seudomonas spp*

The alignment performed for the design of the primer pair is reported in Figure 30.

```
CLUSTAL 2.1 multiple sequence alignment


FN600412.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
FN395007.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
AB598738.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
FN652910.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
FN666561.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
FN666563.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
AJ308306.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
FN666564.1      GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTT
                ************************************************************

FN600412.1      GACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAGCTCTGACACAGGTGCT
FN395007.1      GACATGCAGAGAACTTTCCAGAGATGGATTG-TGCCTTCGGGAACTCTGACACAGGTGCT
AB598738.1      GACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCT
FN652910.1      GACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCT
FN666561.1      GACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAGCATTGAGACAGGTGCT
FN666563.1      GACATCCAATGAACTTTCTAGAGATAGATTGGTGCCTTCGGGAACATTGAGACAGGTGCT
AJ308306.1      GACATCCAATGAACTTTCCAGAGATGGATGGGTGCCTTCGGGAACATTGAGACAGGTGCT
FN666564.1      GACATCCAATGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACATTGAGACAGGTGCT
                ***** **  ******** ****** *** * ********** *  *** *********

FN600412.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
FN395007.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
AB598738.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
FN652910.1      GCATGGCTGTGGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
FN666561.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
FN666563.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
AJ308306.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
FN666564.1      GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
                ********** ***********************************
```

Figure 30: Alignment of partial 16S rRNA gene sequences of *Pseudomonas fluorescens* (GenBank accession number: FN666564.1, AJ308306.1, FN666561.1, FN666563.1) and Pseudomonas *putida* (GenBank accession number: FN600412.1, AB598738.1, FN652910.1, FN395007.1). Asterisks indicate identity; gray areas indicate primer sequences.

When preliminarly tested on reference bacterial samples (*P. fluorescens* and *P. putida*), High resolution melting analysis following real time PCR allowed to discriminate the two species of Pseudomonas according to the different melting temperature of the corresponding amplicons (Figure 31A and 31B). All samples showed a robust and reproducible amplification signal after real time PCR, an important prerequisite for optimal HRM analysis. No amplification was obtained from the control negative sample, confirming the specificity of the procedure.

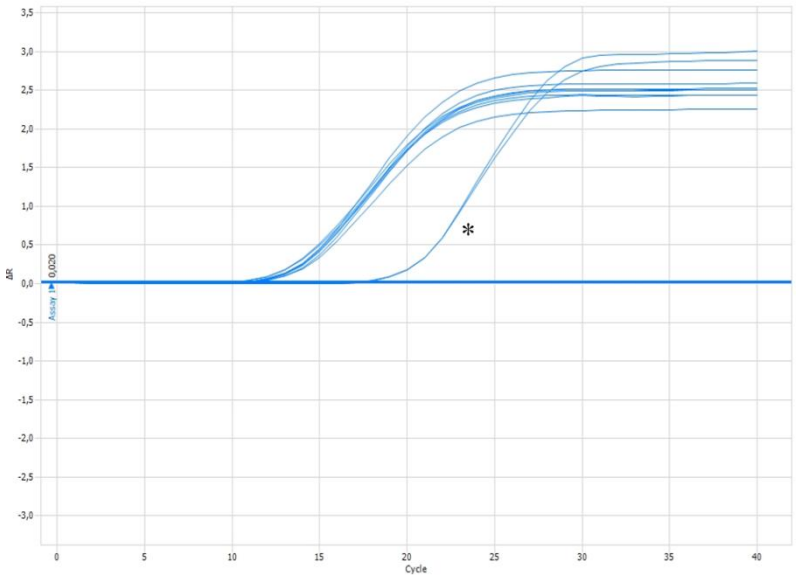


Figure 31A: Setup of the method: amplification curves obtained after real time-PCR of the positive control samples. The bunch of curves on the left indicate *Pseudomonas* spp. samples, asterisk indicates *Staphylococcus* spp. controls.

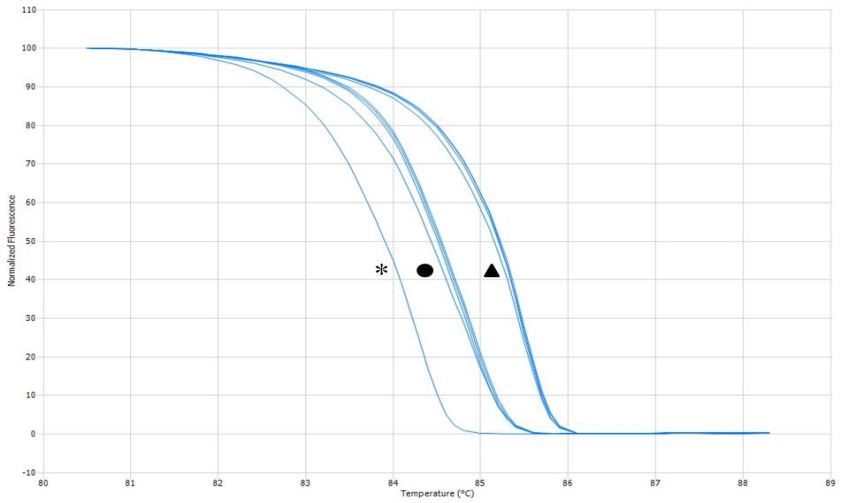


Figure 31B: Setup of the method: normalized graph of the melting curves after HRMA of the positive control samples. The samples of *Pseudomonas putida* (the bunch of curves on the right, indicated by a triangle) are clearly distinguishable from the samples of *Pseudomonas fluorescens* (the bunch of curves in the centre, indicated by a circle) and from the sample of *Staphylococcus* spp. (the curve on the left, indicated by an asterisk).

The agarose gel performed after the amplification and melting reaction confirmed that the amplicons were of the expected size, with undetectable non-specific amplication or primer dimers. (Figure 32).

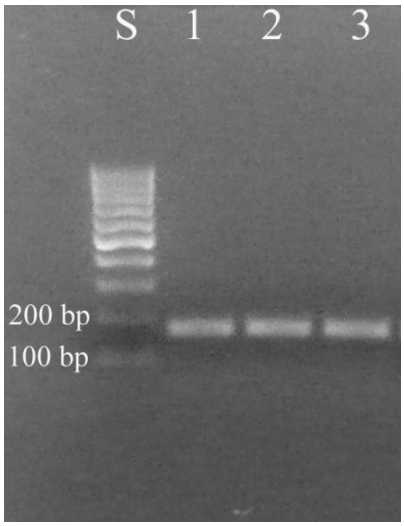


Figure 32: Agarose gel electrophoresis performed after real time PCR-HRMA. S= 100 bp molecular weight marker; line 1= *Pseudomonas putida* positive control sample; line 2= *Pseudomonas fluorescens* positive control sample; line 3= *Staphylococcus* spp. control sample.

When tested on bacterial samples isolated through microbiological analysis and preliminarly assessed as *Pseudomonas spp*, the procedure was able to clearly identify the species of origin. In the normalized plot, the *P. fluorescens* samples were clearly distinguishable from the *P. putida* samples, and the strain of Staphylococcus used as external control (Figure 33A). Also, the differential plot allowed sample classification based on clustering of samples into groups with similar melting curves (Figure 33B). In both plots, an additional, unexpected type of curve was detectable, and the DNA sequencing of the corresponding amplicons led to the identification of *Aeromonas hydrophila* (Figure 33A and Figure 33B).

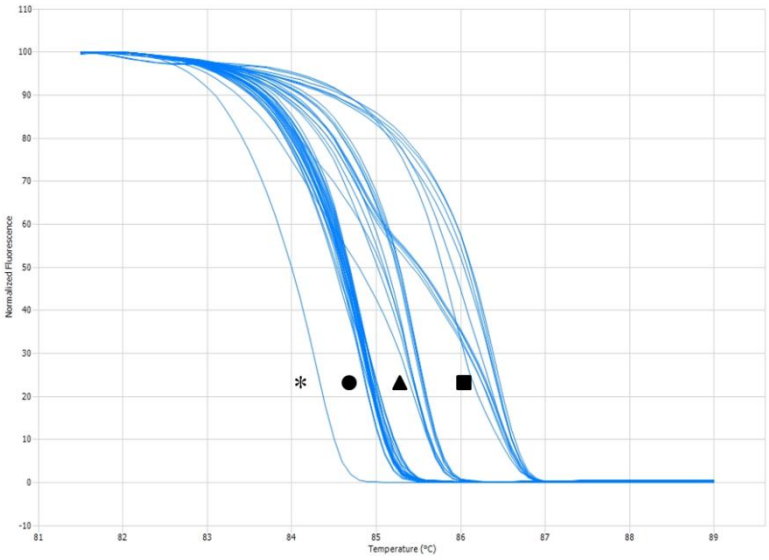


Figure 33A: Normalized graph of the melting curves after HRMA of the positive controls and unknown samples. Black circles, *P. putida¸;* black triangles, *P. fluorescens* The curves on the right (black square) are related to the unexpected amplification of *Aeromonas hydrophila*. Asterisk, *Staphylococcus* spp

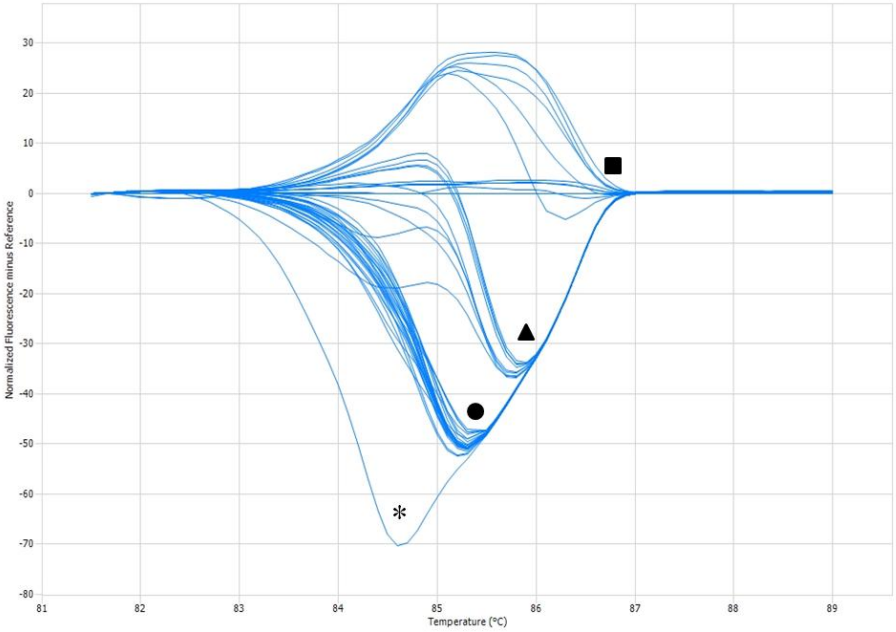


Figure 33B: Differential plot of the melting curves after HRMA. The differential plot allows further analysis of the differences in melting-curve shapes by subtracting the curves from a reference curve (in this case, an *Aeromonas hydrophila* sample). From top to bottom, four different types of sample are distinguishable: the samples of *Aeromonas hydrophila* (indicated by a square), the samples of *Pseudomonas putida* (indicated by a triangle), the samples of *Pseudomonas fluorescens* (indicated by a circle) and the sample of *Staphylococcus spp* (indicated by an asterisk).

2.4.2 *Nosema spp*

The alignment performed for the design of the primer pair is reported in Figure 34.

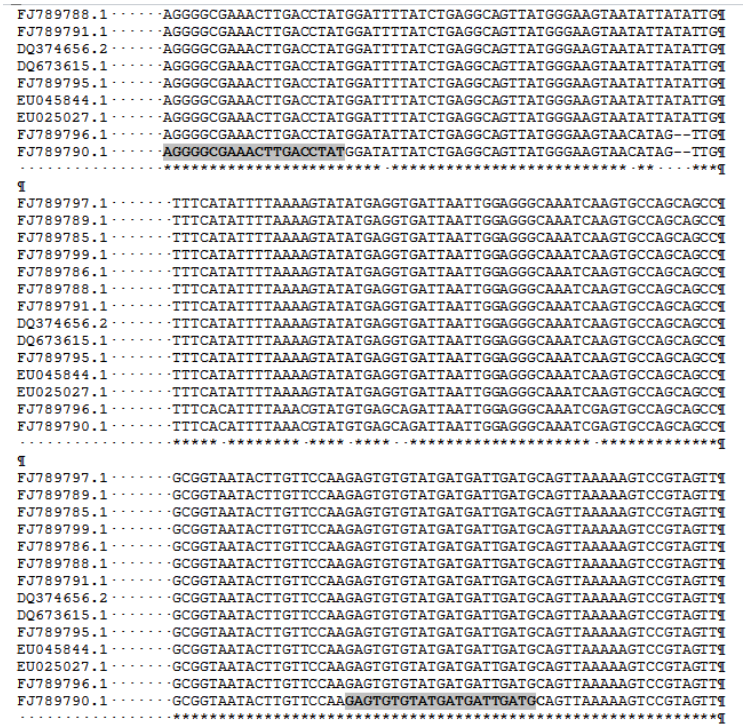


Figure 34: Alignment of partial Nosema ribosomal 16S gene sequences of *N. ceranae* (FJ789797.1, FJ789789.1, FJ789785.1, FJ789799.1, FJ789786.1, FJ789788.1, FJ789791.1, DQ374656.2, DQ673615.1, FJ789795.1, EU045844.1, EU025027.1) and *N. apis* (FJ789796.1, FJ789790.1) Dots indicate identity; gray areas indicate primer sequences. Conserved regions with no intra-species variability and with maximized interspecies variability were chosen as target for the primer design

The new primers designed and reported in this article, have been tested both in conventional and real-time PCR and have demonstrated high specificity and sensitivity for the target sequences. All samples showed a robust and reproducible amplification signal. The amplicons once run on agarose gel electrophoresis were of the expected size, 162 bp for *N. ceranae* and 160 bp for *N. apis*, with undetectable non-specific amplication or primer dimers. No amplification was obtained from Nosema-negative sample, confirming the specificity of the procedure (Figure 2). Eventually, the amplicons' sequencing confirmed the identity of the Nosema sequences.

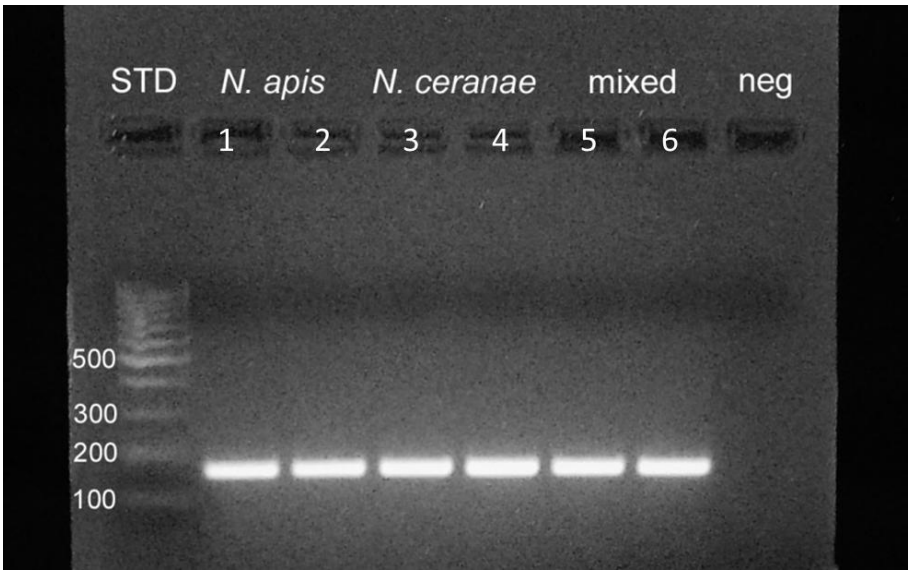


Figure 2: Agarose gel electrophoresis performed after real time PCR-HRMA. STD= 100 bp molecular weight marker; lines 1,2= *N. apis* positive control samples, lines 3,4= *N. ceranae* positive control samples, lines 5,6= 1:1 mixed samples; Neg= negative control.

High resolution melting analysis after real time PCR allowed to discriminate clearly the two species on the basis of the different melting temperature of the two amplicon types. In the normalized plot, the *N. ceranae* samples were clearly distinguishable from the *N. apis* samples and the mixed samples respectively (Figure 35). Also, the differential plot allowed sample classification based on clustering of samples into groups with similar melting curves (Figure 36).

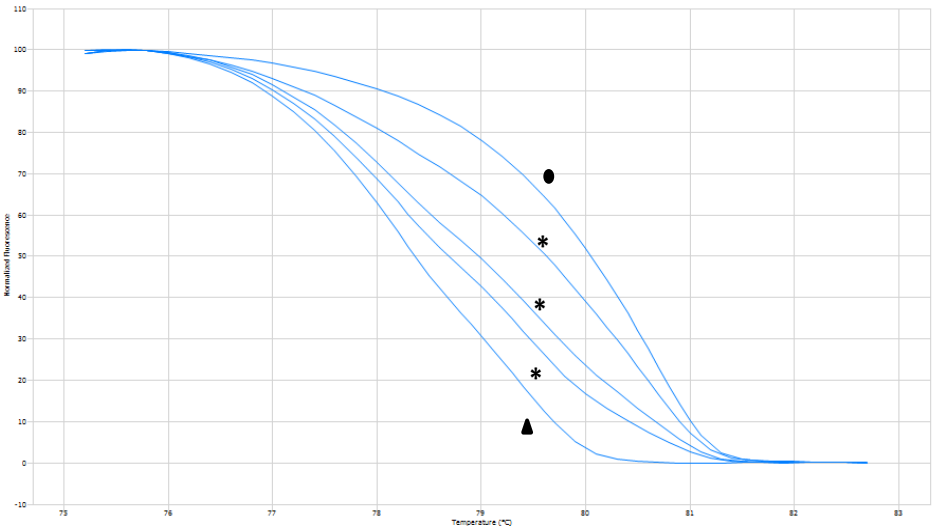


Figure 35: Normalized graph of the melting curves after HRMA. The samples with *N. apis* infection (curve on the right, indicated by dot) are clearly distinguishable from the samples with *N. ceranae* infection (curve on the left, indicated by triangle) and from samples with mixed infestation (the three curves in the center, indicated by asterisks).

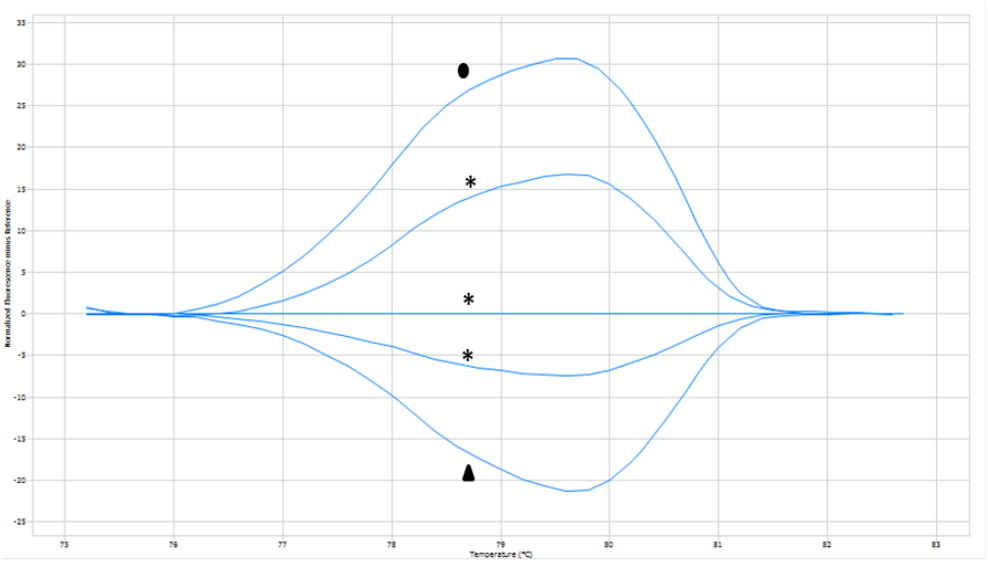
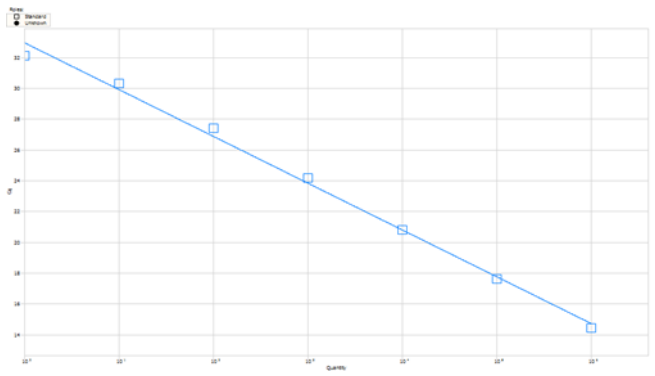


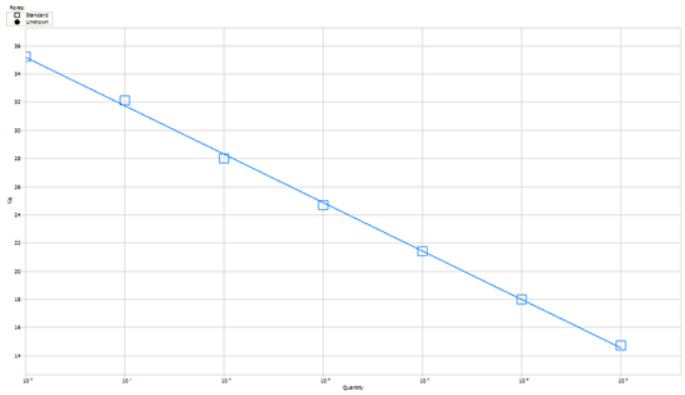
Figure 36: Differential plot of the melting curves after HRMA. The differential plot allows further analysis of the differences in melting-curve shapes by subtracting the curves from a reference curve (in this case, the reconstructed positive mixed 1:1 sample *N. ceranae/N. apis*). From top to bottom, three different types of sample are distinguishable: the sample with *N. apis* infection (indicated by dot), the samples with mixed infection (indicated by asterisks) and the samples with *N. ceranae* infection (indicated by triangle).

The specificity/sensitivity of the method for *N. ceranae* was also tested with referral to a calibration (reference) curve constructed from amplified amplicon (cloned and sequenced) of *N. ceranae* obtained as described. The straight line was built through ten-fold serial dilutions, with a range from 1 million copies to a single copy per sample point (Figure 37). The HRMNosem primers showed an efficiency of 102%. The same procedure was performed for the amplicons obtained using the 218MITOC primers and the calibration curve thus obtained showed an efficiency of 95% (Figure 38).



HRMNosem

Figure 37: *Reference curve for N. ceranae using HRMANosem method*



MITOC218

Figure 38: *Reference curve for N. ceranae using MITOC218 method*

The reference curve for HRMNosem and MITOC218 methods were used to interpolate and calculate the copy number of Nosema ceranae target DNA in the 55 honeybee samples for both methods. The corresponding values obtained with the two methods for the same samples underwent regression analysis in order to check the correlation of the results. The resulting plot showed a very good correlation between the Nosema cerane load calculated with the new HRMA-based method and the reference single target method based on MITOC218 primer pair (Figure 39).

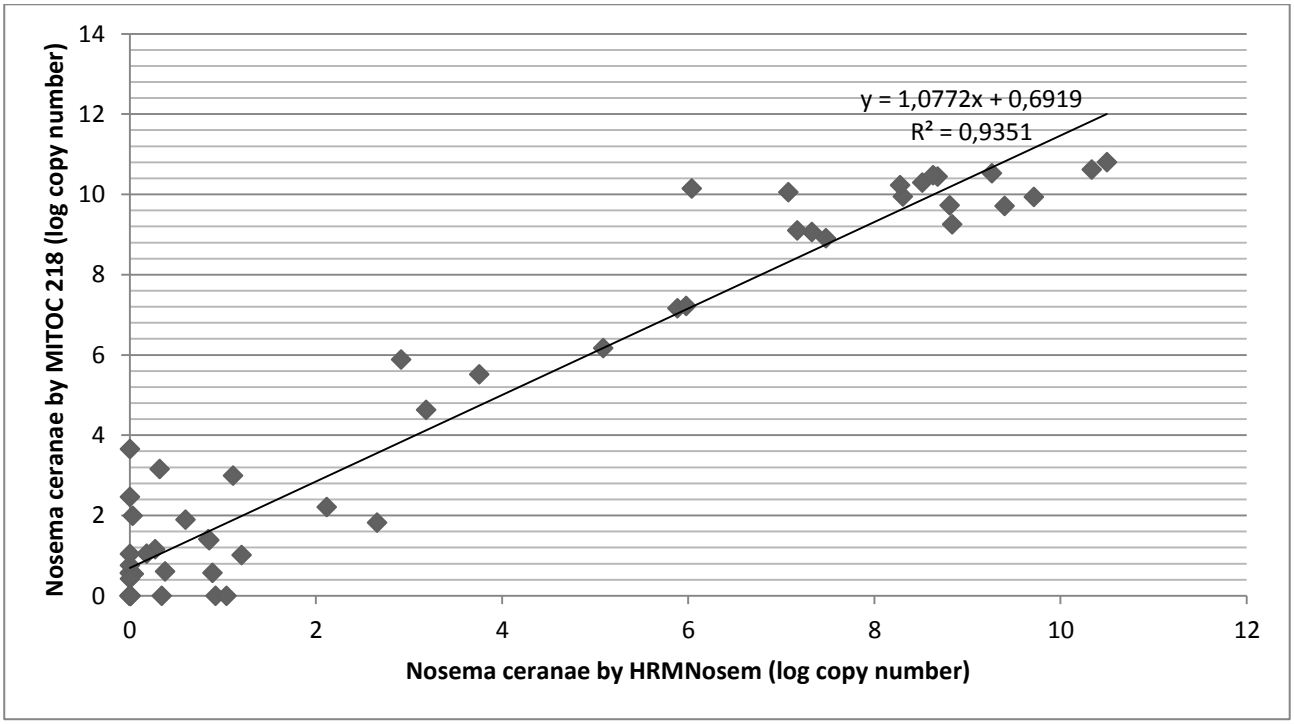


Figure 39: Regression analysis of the *N. ceranae* load evaluated with HRMNosem and MITOC218 methods.

2.4.3. *Staphylococcus Pseudointermedius*

The disc diffusion method on Muller Hinton agar showed that 12 *S. pseudintermedius* isolates were resistant to enrofloxacin and marbofloxacin, while 17 isolates resulted sensitive to both FQs and the one remaining (isolate n. 12) resulted as intermediate resistant (Table 31).

S.pseudointermedius	Enrofloxacina	Marbofloxacina
1	sensitive	sensitive
2	sensitive	sensitive
3	resistant	resistant
4	sensitive	sensitive
5	sensitive	sensitive
6	resistant	resistant
7	sensitive	sensitive
8	resistant	resistant
9	sensitive	sensitive
10	sensitive	sensitive
11	resistant	resistant
12	intermediate	intermediate
13	sensitive	sensitive
14	resistant	resistant
15	sensitive	sensitive
16	sensitive	sensitive
17	resistant	resistant
18	sensitive	sensitive
19	sensitive	sensitive
20	resistant	resistant
21	resistant	resistant
22	resistant	resistant
23	sensitive	sensitive
24	sensitive	sensitive
25	sensitive	sensitive
26	resistant	resistant
27	sensitive	sensitive
28	resistant	resistant
29	sensitive	sensitive
30	resistant	resistant

Table 31: results of the susceptibility testing for enrofloxacin and marbofloxacin for the *S. pseudintermedius* isolates.

When analyzing the melting curve shape obtained through HRM for the gene gyrA, it is appreciable that the respective melting curves have different shapes, as expectable according to the presence of the isolates sensitive (wild type) or resistant (with nucleotide mutation to fluoroquinolones.

As expected based on the the shift in nucleotide sequence, and as appreciable from the respective images of the standardized curves, the amplicons containing the mutations are characterized by a different Tm with respect to the corresponding WT amplicons. Graphically the different melting temperature of the double helix corresponds to a shift of fluorescence, which occurs in a different way between the amplicons based on the nature of the polymorphism: in the specific case, for gyrA it comes to a shift of the temperature towards a lower value linked to the mutation from C (characterized by three hydrogen bonds) to T (characterized by two hydrogen bonds) (Figure 40).

For grlA, it comes to a shift of the temperature again towards a lower value which is linked to the mutation from G to T (Figure 41). An alternative mutation Ser80Arg is also appreciable (Figure 41, grey bunch).

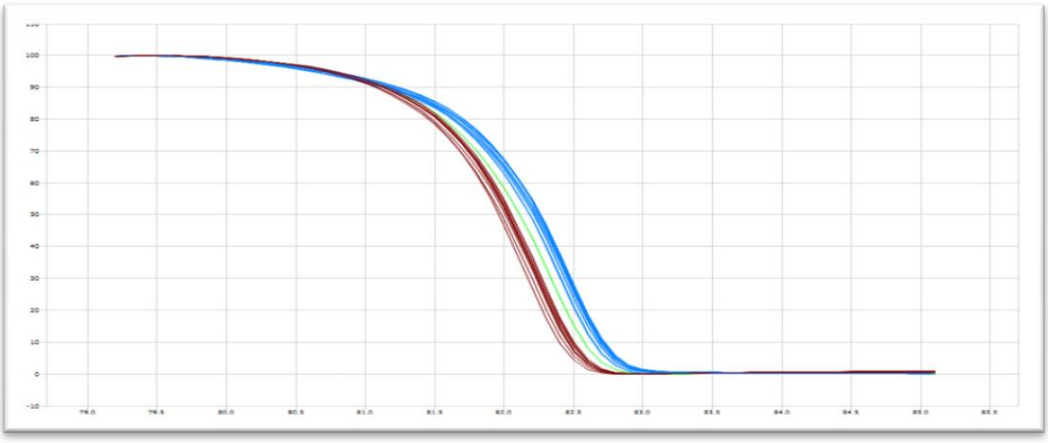


Figure 40: HRMA normalized curve for the gene gyrA for the Ser84Leu mutation: the red samples carry the mutated codon TTG, while the blue samples carry the unmutated codon TCG.

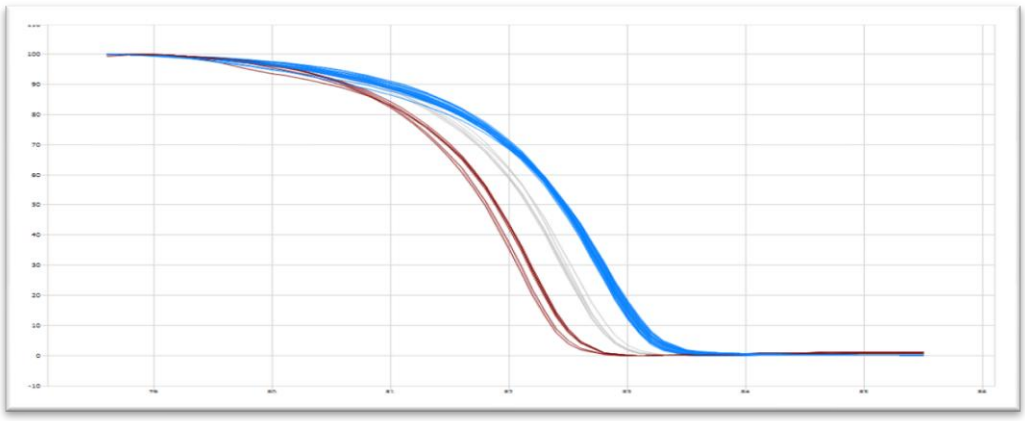


Figure 41: HRMA normalized curve for the gene grlA for the Ser80Ile mutation: the red samples carry the mutated codon ATT, while the blue samples carry the unmutated codon AGT, and the gray samples carry the mutation CGT.

Looking at the differentiated curves, the two different melting patterns due to the presence absence of mutation can be clearly appreciated. At this regard, Figure 42 shows the differentiated plot for the gene gyrA, and Figure 43 shows the differentiated plot for the gene grlA.

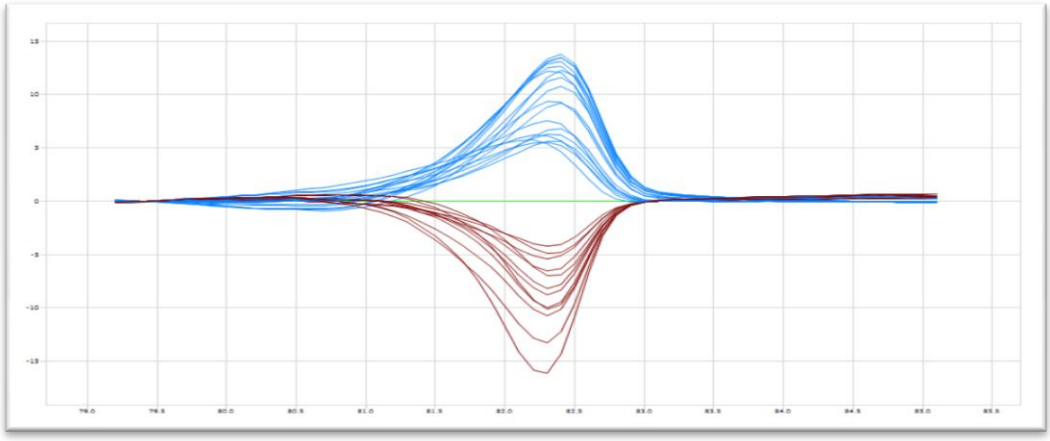


Figure 42: HRMA differentiated plot for gyrA gene: in brown, samples carrying the Ser84Leu mutation; in blue, unmutated samples.

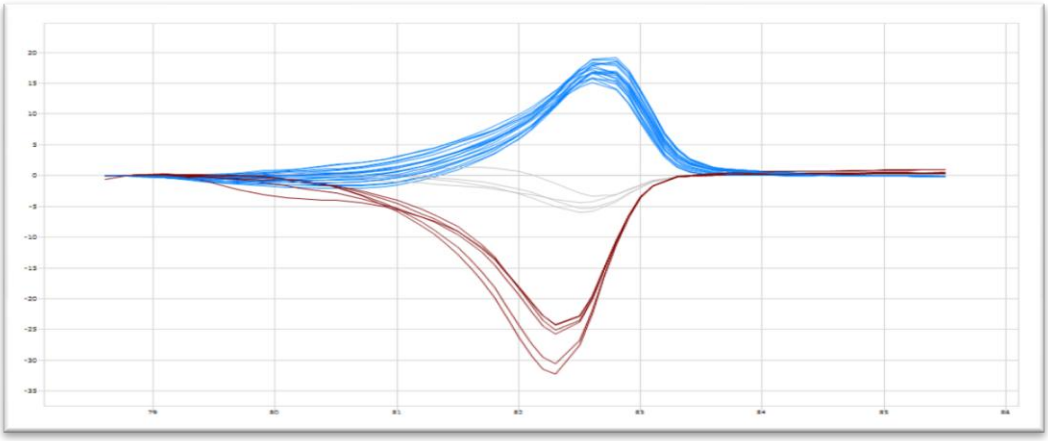


Figure 43: HRMA differentiated plot for grlA gene: in brown, samples carrying the Ser80Ile mutation; in grey, samples carrying the Ser80Arg mutation, in blue, unmutated samples.

When comparing the results of the antibiogram with the results of HRMA screening of both mutation hotspots, an almost full correspondance between sensitivity/resistance and gyrA mutation is showed, as follows: 11 resistant isolated carried also the Ser84Leu mutation of gyrA; 17 sensitive isolated carried the WT codon; 1 intermediate isolate carried only the Ser80Ile mutation of grlA gene; finally, only one resistant isolate carried the WT codon for both gyrA and grlA genes (Table 32).

Thus, eleven out of twelve (92%) resistant isolates assessed by classical sensitivity testing (antibiogram) were also detected as resistant through HRMA method for Ser84Leu mutation of gyrA. Only one resistant isolate was unmutated both for gyrA and grlA codons, and in this case it is conceivable that resistance is mediated by other mutations/mechanisms.

On the other hand, the mutations in grlA gene seemed non correlated to resistance, with the exception of the intermediate strain that carried only the grlA mutation, but not the gyrA mutation, that could explain partial resistance.

S.pseudointermedius	Enrofloxacina	Marbofloxacina	HRMA gyrA	HRMA grlA
1	sensitive	sensitive	unmutated	mutated
2	sensitive	sensitive	unmutated	unmutated
3	resistant	resistant	mutated	mutated
4	sensitive	sensitive	unmutated	mutated
5	sensitive	sensitive	unmutated	unmutated
6	resistant	resistant	mutated	unmutated
7	sensitive	sensitive	unmutated	unmutated
8	resistant	resistant	mutated	unmutated
9	sensitive	sensitive	unmutated	mutated
10	sensitive	sensitive	unmutated	unmutated
11	resistant	resistant	mutated	unmutated
12	intermediate	intermediate	unmutated	mutated
13	sensitive	sensitive	unmutated	mutated
14	resistant	resistant	mutated	unmutated
15	sensitive	sensitive	unmutated	unmutated
16	sensitive	sensitive	unmutated	unmutated
17	resistant	resistant	mutated	mutated
18	sensitive	sensitive	unmutated	unmutated
19	sensitive	sensitive	unmutated	unmutated
20	resistant	resistant	mutated	mutated
21	resistant	resistant	mutated	mutated
22	resistant	resistant	mutated	mutated
23	sensitive	sensitive	unmutated	unmutated
24	sensitive	sensitive	unmutated	unmutated
25	sensitive	sensitive	unmutated	unmutated
26	resistant	resistant	mutated	mutated
27	sensitive	sensitive	unmutated	unmutated
28	resistant	resistant	unmutated	unmutated
29	sensitive	sensitive	unmutated	unmutated
30	resistant	resistant	mutated	mutated

Table 32: comparison between a conventional method of susceptibility testing for enrofloxacin and marbofloxacin with the results obtained in HRMA for gyrA and grlA mutation status.

3.4.4. *Dirofilaria spp*

All samples showed an amplification signal robust and reproducible, this allowing an optimal analysis of the HRMA curves, and besides the amplicons were of the expected size (256 bp) and did not show nonspecific or dimers of primers; no amplification was obtained from Knott's-negative samples, confirming the specificity of the procedure, and sensivity range was confirmed for the two species by successful amplification of the highest (naturally infected blood samples of 32250 mf/ml for *D. immitis* and 100000 mf/ml for *D. repens*) and the lowest (reconstructed blood samples of 4 mf/ml for each single Dirofilaria species) mf load (Figure 44).

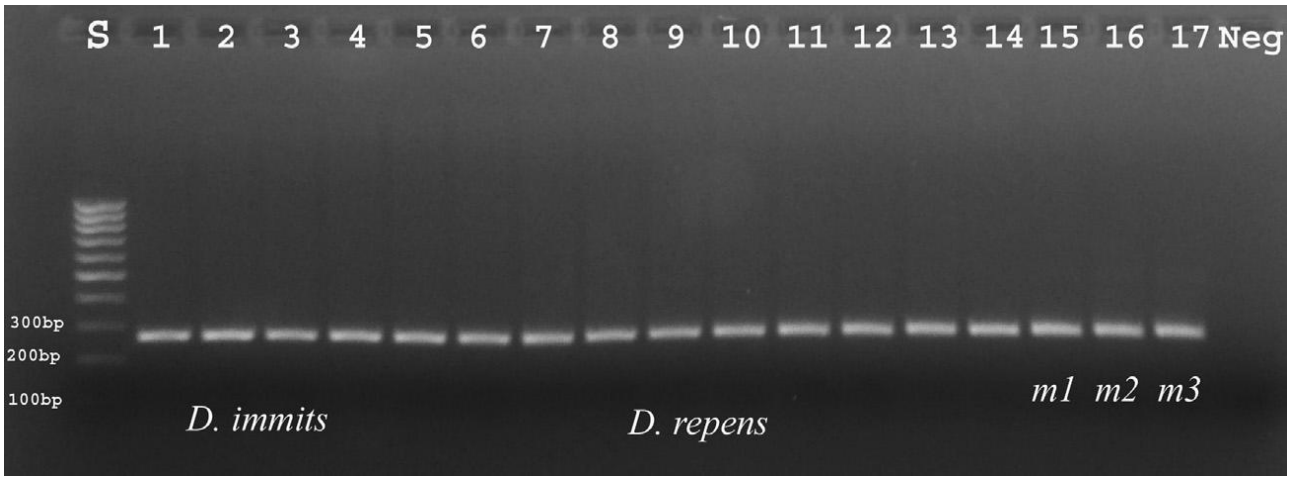


Figure 44: Agarose gel electrophoresis performed after real-time PCR–HRMA. S = 100 bp molecular weight marker; lines 1–17 = Dirofilaria spp samples, includingpositive controls in line 5 (D. immitis) and line 14 (D. repens), and m1–m3 mixed Dirofilaria samples in lines 15–17; Neg = negative control.

After real time PCR amplification , HRM analysis allowed to discriminate clearly the two species according to the different melting temperature of the two sequences, although they have an identical number of bases. Figure 45 shows the normalized graph of *D. immitis* samples (bunch of curves to the right), the *D. repens* samples (bunch of curves to the left) and the three mixed samples in the centre (asterisk). Figure 46 shows the difference plot curve, that allows further analysis of the differences in melting-curve shapes by subtracting the curves from a reference curve (in this case repens 4 mf/ml), which helps to cluster samples into groups that have similar melting curves. Melting profiles showed minimal variability within samples from the same group.

Finally, the specificity of the amplification was confirmed by sequence analysis, thus proving that the clustering pattern presented in the curves was representing two different species, as effectively as DNA sequencing.

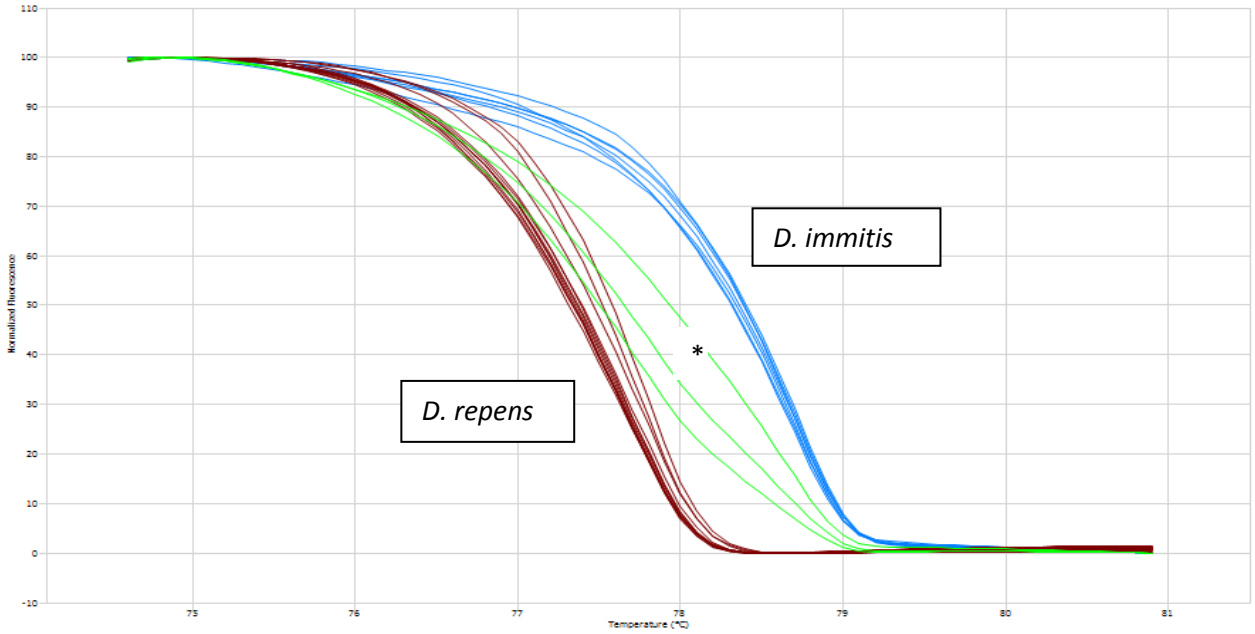


Figure 45: Normalized graph of the melting curves obtained with the HRMA. Are clearly distinguishable the *D. immitis* samples (in blue on the right), from the *D. repens* samples (in red on the left) from samples with mixed infestation (in green in the center, indicated by an asterisk).

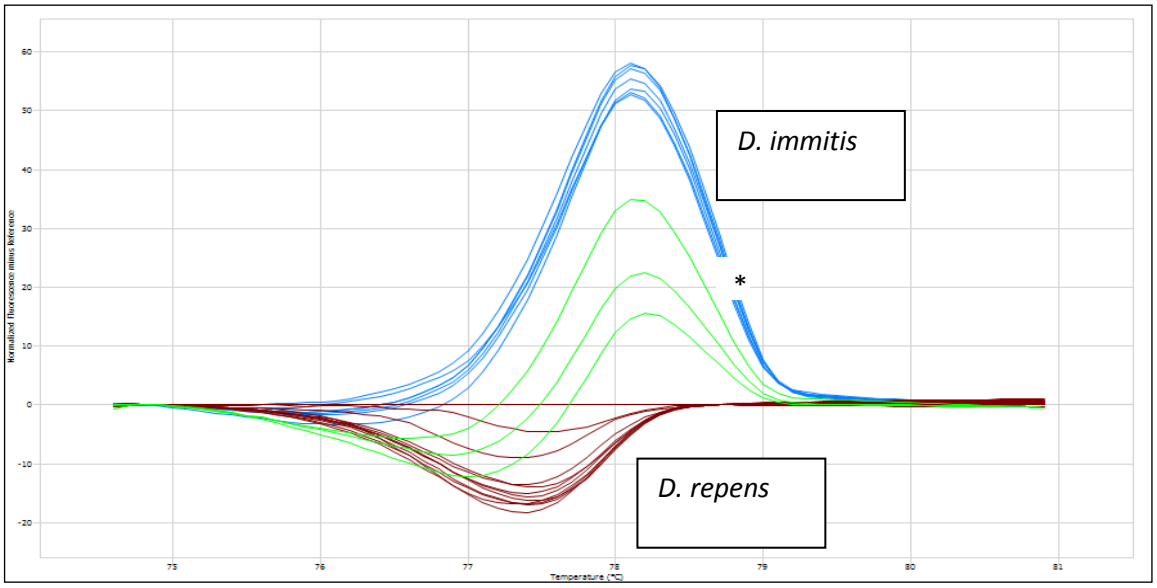


Figure 46: Differential graph of the melting curves obtained with the HRMA. The three different types of sample are easily distinguishable: in blue *D. immitis* samples, in red *D. repens* samples and green (indicated by an asterisk) samples with mixed infestation.

3. Conclusions

The present PhD project was aimed on one side to a better characterization of the foodborne risk linked to E. coli infection strains in the bovine model, and on the other side to the development of new analytical protocols based on HRMA for sensitive and effective species identification, or variant/mutation screening, applied to different foodborne and veterinary pathogens. Regarding the relationship between *E. coli* and the bovine host in the food safety perspective, The experimental hypothesis was linked to a possible expression of virulence factor by *E. coli* strains when exposed to bovine mammary environment, and at this regard a RT-qPCR study in a co-culture model was realized after a comprehensive meta-analysis allowing to select MAC-T cell as the most suitable bovine mammary cell line for this purpose. In addition, with the aim to improve the current methodologies for foodborne risk analysis linked to *E. coli*, the project activity provided a preliminary research for the setup and validation of new real time PCR-High Resolution Melting Analysis (HRMA) protocols helping the identification of putative verocytotoxic status in E. coli strains isolated from bovine milk. The possibility to identify the presence of risk-predictive SNPs using these newly established HRMA-based protocols is a novel, and simpler, opportunity with respect to the current, and more complex, surveillance strategies that are based on the amplification of stx genes together with other virulence factors for the evaluation of VTEC status. In the future, a possible way forward of this research is represented, on one side, by the deeper assessment of the reciprocal modulation between *E. coli* mastitis-derived strains and immortalized MAC-T cells using high-throughput RNA sequencing, and on the other side by a large scale validation of the HRMA-based evaluation of risk-predictive SNPs in order to improve the current approaches. Besides, in the future also the assessment of the effect of co-cultivation with intestinal cell lines would spread more light on the possible virulentation of bovine-derived *E. coli* strains by the host interplay. With the similar aim to develop other novel applications of HRMA-based methodology in food research, a new HRMA-based protocol was designed and tested for the identification of *Pseudomonas spp* responsible for chromatic alterations in mozzarella cheese. More in general, improving identification and tracking methods for these, and other, microorganisms in food production and processing systems through HRMA-based protocols is important in understanding and reducing the frequency of contamination, as well as eliminating niche populations in food processing systems such that contamination of food products can be prevented.

Since the applications of HRMA for the characterization of microorganisms can not be limited to food safety, but can be developed for a large number of issues linked to general veterinary diagnostics, among the objectives of this PhD project some new real-time PCR-HRMA coupled methods were also developed, providing a contribution to the advancement of the existing molecular tools. At this regard, a clear example is provided by the research activity performed regarding the HRMA-based assay for Dirofilaria spp. Currently, most of the existing molecular diagnostic protocols for canine dirofilariosis are based on species specific sets of primers or on additional steps after PCR amplification, thus being quite laborious or not assuring a comparable amplification efficacy between the two targets. Thus, a new molecular method based on real time PCR coupled to HRMA to detect and differentiate simultaneously *D. repens* and *D. immitis* in canine blood samples was designed and validated as part of the activity of the present PhD programme. A single pair of primers was used, as common strategy in HRMA-based assays, thus allowing comparable amplification efficiency between the two different targets and also discrimination of mixed infections. The assay tested positive also on blood samples with a very low mf number, confirming high sensitivity and consequently the suitability of the assay to detect positivity in dogs with a very low parasite load as a common finding in the field. Besides, it was possible to detect both single worm and mixed infections in a repeatable way, another feature that can be very useful in field diagnostics, since the identification of the parasite species using the traditional approaches sometimes can be problematic.

Overall, the cost effectiveness of real time PCR-HRMA assays is comparable to, or cheaper than, conventional PCR and sequencing, with a very rapid post-amplification detection step (Wongkamchai et al., 2013). The availability of a real time PCR-HRMA assay in the epidemiological and clinical setting reduces the reagent cost, the labor time, and the contamination risk. These quick, sensitive and specific single step assays able to discriminate sympatric pathogen species (like for instance Dirofilaria and Nosema targets) or to identify molecular variants that are relevant for clinical management (FQ resistance in *S. pseudintermedius*) represent additional tools for epidemiological studies, routine disease assessment and therapeutical decisions. For instance, rhe results obtained through the HRMA-based analysis confirmed previous reports that in clinical settings, the main mutation site associated to FQ resistance in S. *pseudintermedius* is at position 251 (Ser84Leu) of gyrA as detected in all but one the analyzed resistant isolates of the present PhD project. Besides, a comparable amplification efficiency for the targets of interest, as in HRMA

methods, is especially useful in case of simultaneous infection, as in the case of Nosema spp., where a quick, sensitive and specific single step assay able to discriminate the two most common microsporidial parasites in honeybees represents undoubtedly a valuable new tool for epidemiological studies and routine disease assessment in co-endemic areas for the two species. Furthermore, HRMA-based assays usually prove to be at least as effective as the currently used molecular methods, and a clear demonstration was provided in this PhD project with regard to the HRMNosem in comparison to the MITOC218 method, strictly specific for *N. ceranae*. The PCR-HRMA method here established proved also to be able to detect unexpected species (as *Aeromonas aerophyla* detected by the HRMA assay originally developed for Pseudomonas targets, and previously misinterpreted by the classical micobiological approach) or molecular variants (as the Ser80Arg mutation in grlA gene of *S. pseudintermedius* by the HRMA assay originally developed for the detection of the Ser80Ile mutation), as examples of the potential advantages linked to the introduction of these new assays in the routine diagnostics. Finally, each real time PCR-HRMA method could be easily upgraded to include closely related target species and/or molecular variants.

Overall, the results of this PhD project obtained through a large range of activities and experimental tools, can be considered as an appreciable contribution to a better identification of the risk that E. coli and other microrganisms and parasites may pone to the health of animals and finally humans. In particular, the HRMA approach when extensively validated would be highly suitable for routine diagnostics applied to field investigation, as a quick and sensitive single step protocol allowing specific and sensitive detection with shorter analysis time and reduced cost, in parallel or in alternative to the classical approaches.

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List of publications

Loiacono, M., Mortarino, M., Zecconi, A. Immunological features of bovine mammary epithelial cells: a meta-analysis. Oral presentation, 10th International Veterinary Immunology Symposium, Milan 2013.

Loiacono, M., Albonico, F., Guidi, A., Cersini, A., Formato, G., Corsi, N., Colombo, M., Mortarino, M. (2014). *High Resolution Melting Analysis coupled to Real Time PCR for d*etection and quantification of Nosema ceranae and Nosema apis in honeybees. Atti del XXVIII Congresso Nazionale SOIPA, Roma, 24-27 giugno 2014.

Dell'Orco, F., Loiacono, M., Albonico, F., Minozzi, G., Pagnacco, G., Mortarino, M. (2015). MicroRNA expression correlated with hygienic behaviour in honeybees. International Journal of Health, Animal Science & Food Safety, Vol. 2s, Proceedings of the Veterinary and Animal Science Days, 15-17 July 2015.

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Loiacono, M., Martino, P.A., Albonico, F., Ferretti, M., Zanzani, S., Mortarino, M. High-resolution melting analysis of *gyrA* codon 84 and *grlA* codon 80 mutations conferring resistance to fluoroquinolones in *Staphylococcus pseudintermedius* isolates from canine clinical samples. *Manuscript under submission*.

Loiacono, M., Kerro Dego, O., Zecconi. A., Oliver, S.P., Mortarino, M., Almeida, R. A. Upregulation of virulence factors in *Escherichia coli* strains isolated from bovine mastitis and co-cultured with immortalized bovine mammary cells. *Manuscript in preparation*

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