IZSAM G.CAPORALE
TERAMO

Messa a punto di un test diagnostico per *Campylobacter* basato su tecnologia LAMP. *IZS AM 01/13 RC.* –

Studio dei fattori di rischio nell'allevamento del broiler che concorrono alla contaminazione da *Campylobacter* e messa a punto di un test diagnostico per *Campylobacter* «on site» basato su tecnologia LAMP.

Alessandra Alessiani, Francesca Marotta

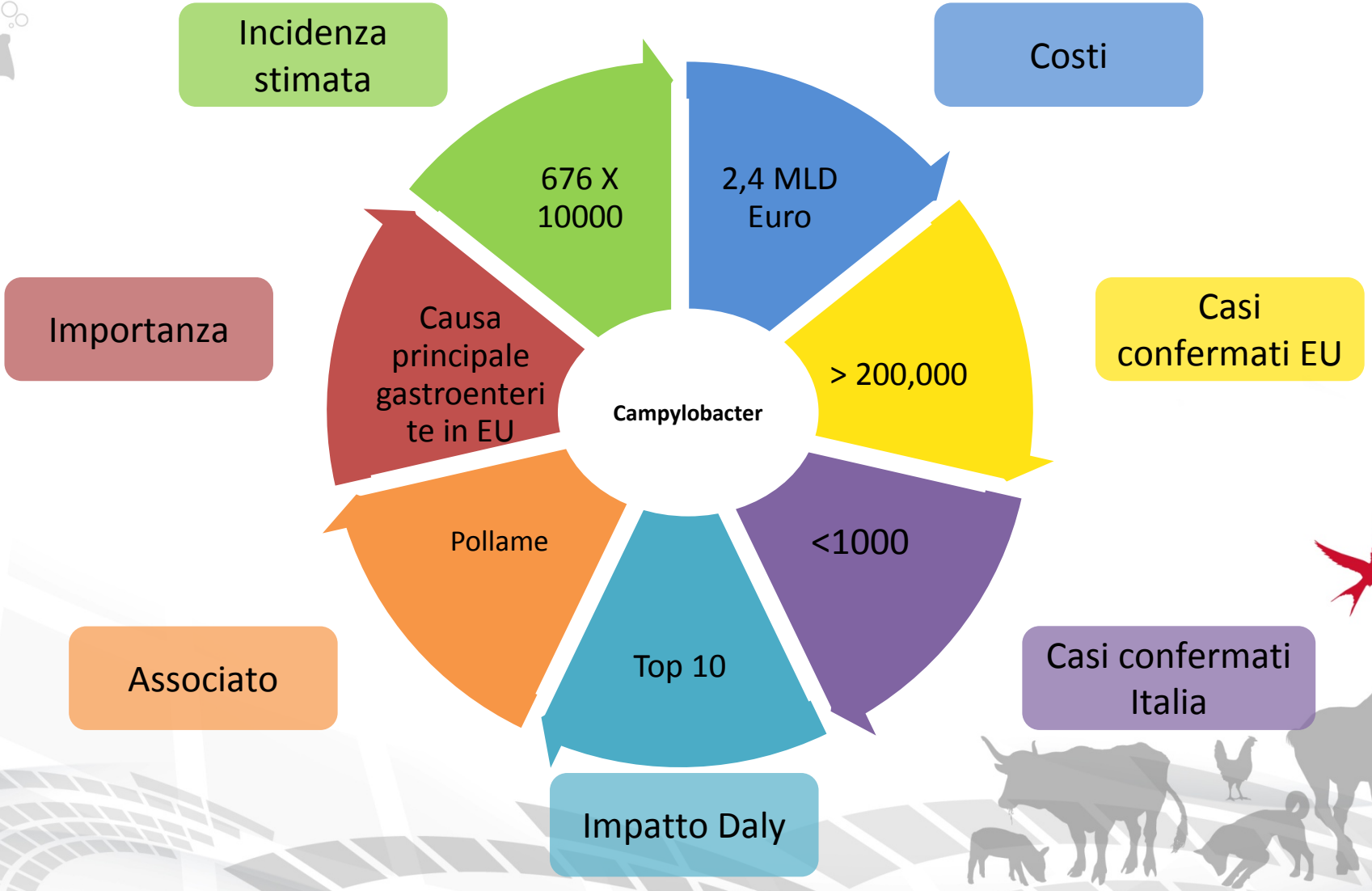
Teramo, 13 Giugno 2017

Centro Internazionale per la Formazione e l'Informazione Veterinaria "Francesco Gramenzi"

IL PROGETTO

- Studio dei fattori di rischio da *Campylobacter* associato alla filiera del pollo da ingrasso
- Sviluppo e validazione di protocolli per la ricerca tramite metodi molecolari di *Campylobacter* da campioni ambientali

CAMPYLOBACTERIOSI



ITALIA

63,3%
allevamenti
avicoli italiani
positivi

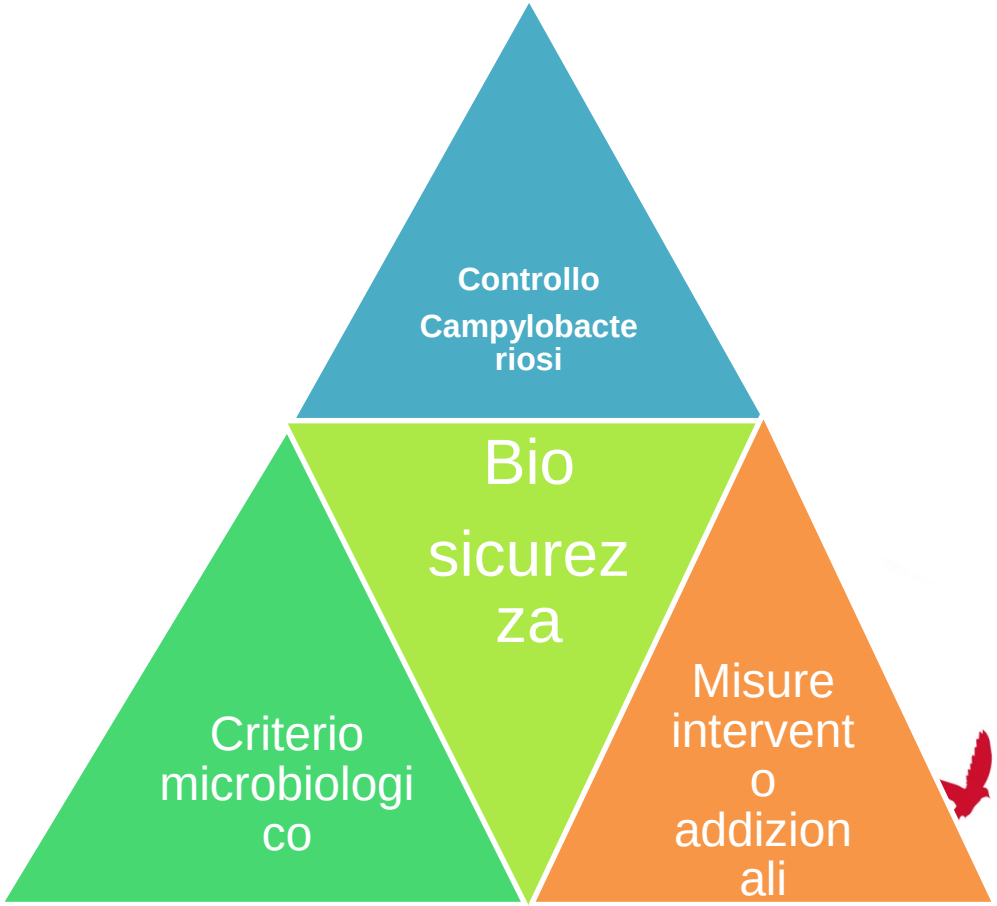
ITALIA

Criterio microbiologico 1000 UFC riduzione rischio 50%

- 15% di produzione fuori dal criterio in EU

Criterio microbiologico 500 UFC riduzione rischio 90%

- una produzione fuori dai limite per il 45%



RIDUZIONE PREVALENZA NEL POLLAME DEL 50%

Table 8.3 Summary costs of *Campylobacter* control strategy CS1a

Control name and (efficacy %)	Reduction in EU incidence (%)	Annual cost per DALY avoided		Availability	Industry impact	Consumer impact
		Lower estimate	Upper estimate			
F1 Enhanced Biosecurity (40%)						
T1 On-farm Testing						
S2 Chemical Decontamination (40%)						
T2 Post slaughter Testing	59%	€141				

91-136 milioni di euro

ON FARM TESTING!

Table 8.4 Summary costs of *Campylobacter* co

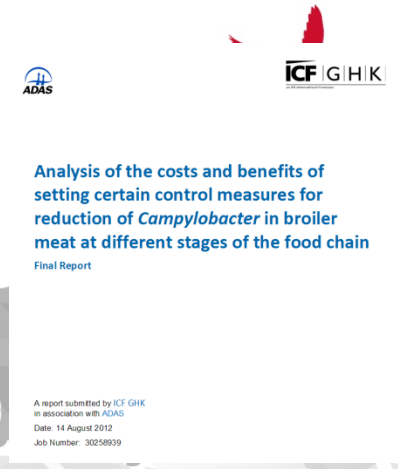
Control name and (efficacy %)	Reduction in EU incidence (%)	Lower estimate				
F1 Enhanced Biosecurity (70%)						
T1 On-farm Testing						
T2 Post slaughter Testing	56%	€581	€872			

...ni di euro

Table 8.6 Summary costs of *Campylobacter* control strategy CS1d

Control name and (efficacy %)	Reduction in EU incidence (%)	Annual cost per DALY avoided		Availability	Industry impact	Consumer impact
		Lower estimate	Upper estimate			
F1 Enhanced Biosecurity (55%)						
T1 On-farm Testing						
S1 Best Practice Hygiene (25%)						
T2 Post slaughter Testing	57%	€866	€1298			

54-80 milioni di euro



RIDUZIONE PREVALENZA NEL POLLAME DEL 90%

Table 8.7 Summary costs of *Campylobacter* control strategy CS2a

Control name and (efficacy %)	Reduction in EU incidence (%)	Annual cost per DALY avoided		Availability	Industry impact	Consumer impact
		Lower estimate	Upper estimate			
F1 Enhanced Biosecurity (40%)						
T1 On-farm Testing						
S3 Freezing (2-3 weeks) (90%)						
T2 Post slaughter Testing	93%					

239 milioni di euro

ON FARM TESTING!

Table 8.8 Summary costs of *Campylobacte*

Control name and (efficacy %)	Reduction in EU incidence (%)					
		Lower estimate	estim			
F1 Enhanced Biosecurity (40%)	90%	€1146	€1720			
T1 On-farm Testing						
S1 Best Practice Hygiene (30%)						
S2 Chemical Decontamination (80%)						
T2 Post slaughter Testing						

167 milioni di euro

Table 8.9 Summary costs of *Campylobacter* control strategy CS2c

Control name and (efficacy %)	Reduction in EU incidence (%)	Annual cost per DALY avoided		Availability	Industry impact	Consumer impact
		Lower estimate	Upper estimate			
F1 Enhanced Biosecurity (55%)	92%	€2739	€4108			
F4 Vaccination (90%) OR F5 Bacteriocins (90%)						
T1 On-farm Testing						
S1 Best Practice Hygiene (25%)						
T2 Post slaughter Testing						

273-410 milioni di euro

MACELLAZIONE PROGRAMMATA

EFSA Journal 2011

DIAGNOSI IN CAMPO

Liljander et al., JCM, 2015

Procedure

PMA-LAMP for rapid detection of *Escherichia coli* and shiga toxins from viable but non-culturable state

Muxia Yan [a], Ling Xu [a], Hua Jiang [a], Zhenwen Zhou [b], Shishui Zhou [c, **], Li Zhang [a, *]

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[b] Clinical Microbiogy, Guangzhou Women and Children's Medical Centre, Guangzhou Medical University, Guangzhou 510180, PR China
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Keywords:
LAMP
PMA
VBNC
Enterohemorrh
Shiga toxins

ABSTRACT

In exposure to outer pressure, microorganisms are capable of entry into the Viable But Non-Culturable (VBNC) state, and thus survive under various elimination processing. The survival microorganisms may yield negative results on culturing, and cause false negative for this golden standard methodology. In this study, a novel PMA-LAMP assay on the detection of *Enterohemorrhage E. coli* and shiga toxins has been developed and evaluated, with further application on a number of food borne *E. coli* strains. LAMP primers were designed on the target of *rfbe* for *Enterohemorrhaze E. coli* and *stx1*with *stx2* for shiga

J Food Prot. 2014 Sep;77(9):1593-8. doi: 10.4315/0362-028X.JFP-14-056.

Development of a loop-mediated isothermal amplification assay for rapid, sensitive detection of Campylobacter jejuni in cattle farm samples.

Dong HJ[1], Cho AR[1], Hahn TW[2], Cho S[3].

Author information

Abstract

Campylobacter jejuni is a leading cause of bacterial foodborne disease worldwide. The detection of this organism in cattle and their environment is important for the control of C. jejuni transmission and the prevention of campylobacteriosis. Here, we describe the development of a rapid and sensitive method for the detection of C. jejuni in naturally contaminated cattle farm samples, based on real-time loop-mediated isothermal amplification (LAMP) of the hipO gene. The LAMP assay was specific (100% inclusivity and exclusivity for 84 C. jejuni and 41 non-C. jejuni strains, respectively), sensitive (detection limit of 100 fg/μl), and quantifiable (R(2) = 0.9133). The sensitivity of the LAMP assay was then evaluated for its application to the naturally contaminated cattle farm samples. C. jejuni strains were isolated from 51 (20.7%) of 246 cattle farm samples, and the presence of the hipO gene was tested using the LAMP ass
~10.8 min) in all C. jejuni isolates (n = 51) demonstrated its rapidity and accu
enrichment broth cultures of cattle farm samples either by boiling or using a c
compared between the LAMP and PCR assays. In DNA samples prepared b
compared with the PCR assay (35.5%) indicates that it is less susceptible to
prepared using a commercial kit, both the LAMP and PCR assays showed 1
and simple LAMP assay, which is the first of its kind for the identification and
role in the prevention of C. jejuni contamination in the food chain, thereby red

Accepted 16 March 2017
Available online 18 March 20

Keywords:
Loop-mediated isothermal a
Foodborne pathogens
Application

A novel rapid

ity, Guangzhou 510623, PR China

Front Microbiol. 2016; 7: 1582.
Published online 2016 Sep 30. doi: 10.3389/fmicb.2016.01582

PMCID: PMC5013100

Use of Direct LAMP Screening of Broiler Fecal Samples for *Campylobacter jejuni* and *Campylobacter coli* in the Positive Flock Identification Strategy

Islam I. Sabike,[1,2] Ryoko Uemura,[1,3] Yumi Kirino,[3] Hirohisa Mekata,[3,4] Satoshi Sekiguchi,[1,3] Tamaki Okabayashi,[1,3] Yoshitaka Goto,[1,3] and Wataru Yamazaki[1,3,*]

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diagnosis as well as surveillance of infection diseases. Moreover, the main purpose of this paper is to provide theoretical basis for the clinical application of LAMP technology.

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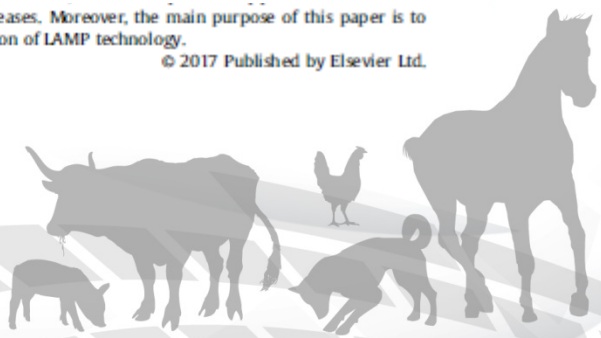
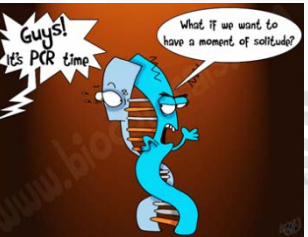


IL METODO

FOCUS ON!

LAMP

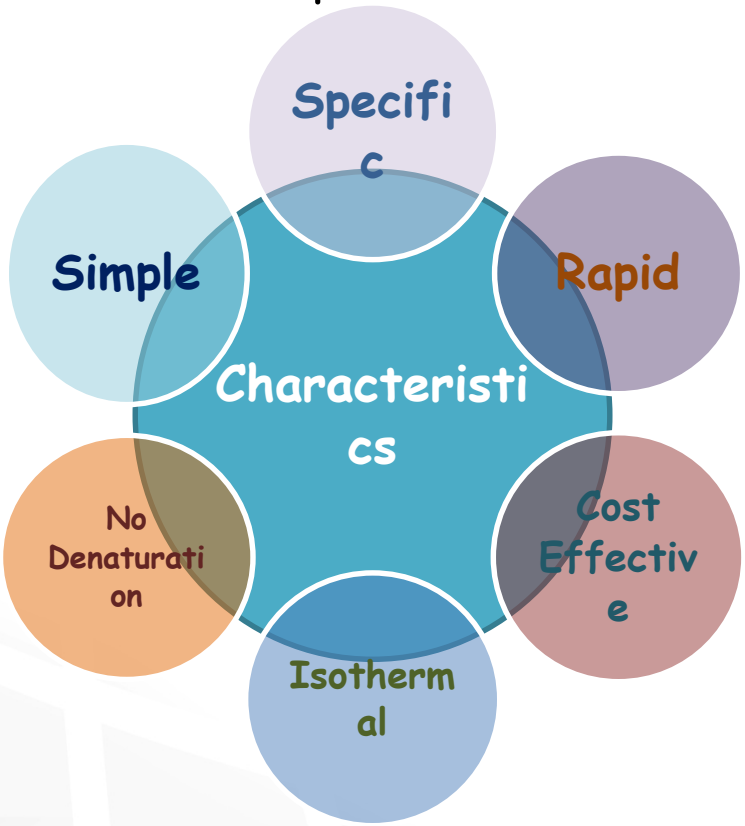
Loop – Mediated Isothermal Amplification: è una tecnica di amplificazione del DNA isotermica. Si contrappone alla classica PCR per il tipo di polimerasi e soprattutto per la necessità di un'unica temperatura di reazione.



IL METODO

Solely developed by **Eiken Chemical Co., Ltd.**
First reported by **Notomi *et al*** in **2000** of EIKEN Chemical Co. Ltd., Japan.

Eiken Chemical Co., Ltd.
Eiken GENOME SITE
(http://www.eiken.co.jp/en/)



Amplification of DNA via non-cyclic and cyclic amplification step with the use of 4 different primers specifically designed to recognize 6 distinct regions on the target gene while the reaction process proceeds at a constant temperature using strand displacement reaction.

DISEGNO DEI PRIMERS

In generale si hanno 4 tipi di primers basati su 6 distinte regioni dei geni target: F3c, F2c ed F1c al 3' e B1, B2 e B3 al 5'.

Le coppie di primer indicate come **FIP** o **BIP**, che stanno per forward inner primer e backward inner primer, sono costruite in modo da contenere al loro interno due regioni consecutive separate da uno «spacer» fatto da timine TTTT.

I primer per F3 e B3 sono semplicemente complementari alla regione 3, la più esterna, sono più corti e meno concentrati nella mix di reazione rispetto a FIP e BIP

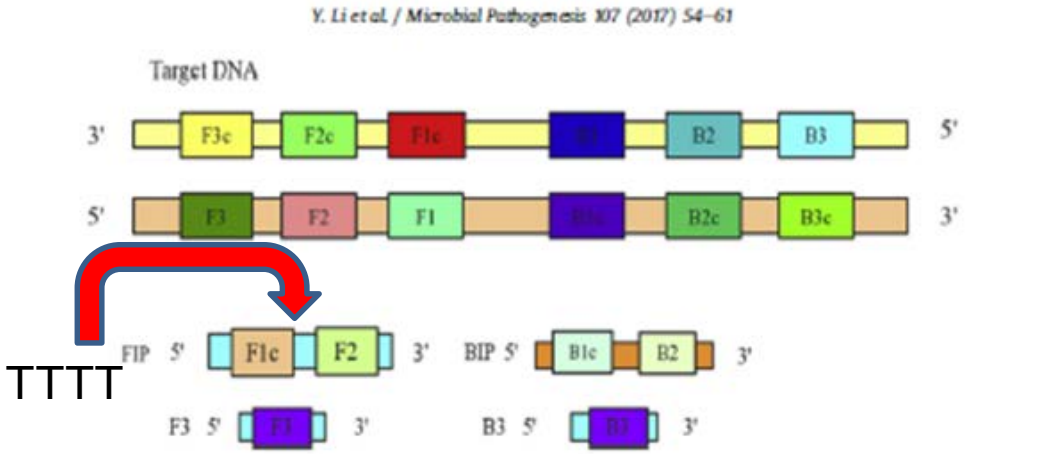


Fig. 1. Schematic representation of primer design for LAMP assay showing the position of the four primers spanning the target.

DISEGNO DE[illegible]ERS

Oligo Name	Sequence (5'->3
h1_F3	CTTGCGGTCATGATGGACAT
h1_B3	AAATTTCTTATCTCTACCAA
h1_FIP	CCTCTTCAGCAGGTTGAA[illegible]TTATTGCT
h1_BIP	GCTTGGGTGGTGCTAAG[illegible]AAAAACAT
h2_F3	GGTCATGATGGACATAC
h2_FIP	CCTCTTCAGCAGGTTGAAA[illegible]GCTGCAAAGTATTTAGC
h3_F3	ATG[illegible]
h2*_B3	[illegible]TATT
m1_F3	
m1_B3	
m1_FIP	[illegible]CTATA
m1_BIP	[illegible]GTA
m2_B3	[illegible]GA
m2_FIP	[illegible]TTTAAATGCTACC
m2_BIP	GCAA[illegible]GTGTCTAAAGTGCGTTTAA
	GCAGTTCTTGTGAAAGTCCTG
	AGCAATACCAGTGTCTAAAGTGCGAAAC
	[illegible]TCAAGCACAACTATTCCTCTAGC
	[illegible]TGCGGTCATGATGGACAT
	[illegible]TTTCTTATCTCTACCAAAAGGCA
	[illegible]TCTTCAGCAGGTTGAAAATAAAGATT
	[illegible]CTTGGGTGGTGCTAAGGCAGTGCCATC
	GGTCATGATGGACATACTACTTCT
	CCTCTTCAGCAGGTTGAAAATAAAGATGCTGCAAAGTATTTAGCAAGTC
h3_F3	ATGCATGCTTGCGGTCAT
h2*_B3	TTCTTATCTCTACCAAAAGGCATATT
[illegible]	AGTTGCTAACGGTTCGGC
	ACTAGCGATTCCGGCTTCA
	TGGGCATAAGGGCCATGATGACACTGCCTTCGTAAGGAGGAG
	AATGAGACGCAATACCGCGAGGGCTCTCGAGTTGCAGCAGAAC
	GCCTTCGTAAGGAGGAGGAA
	GGCTTCATGCTCTCGAGTTG
	TGCCATTGTAGCACGTGTGTCGGGTGTGGACGACGTCAAG
	AATGAGACGCAATACCGCGAGGGAACAATCCGAACTGGGACA

Tm

Hip0
mapA,16s
rDNA

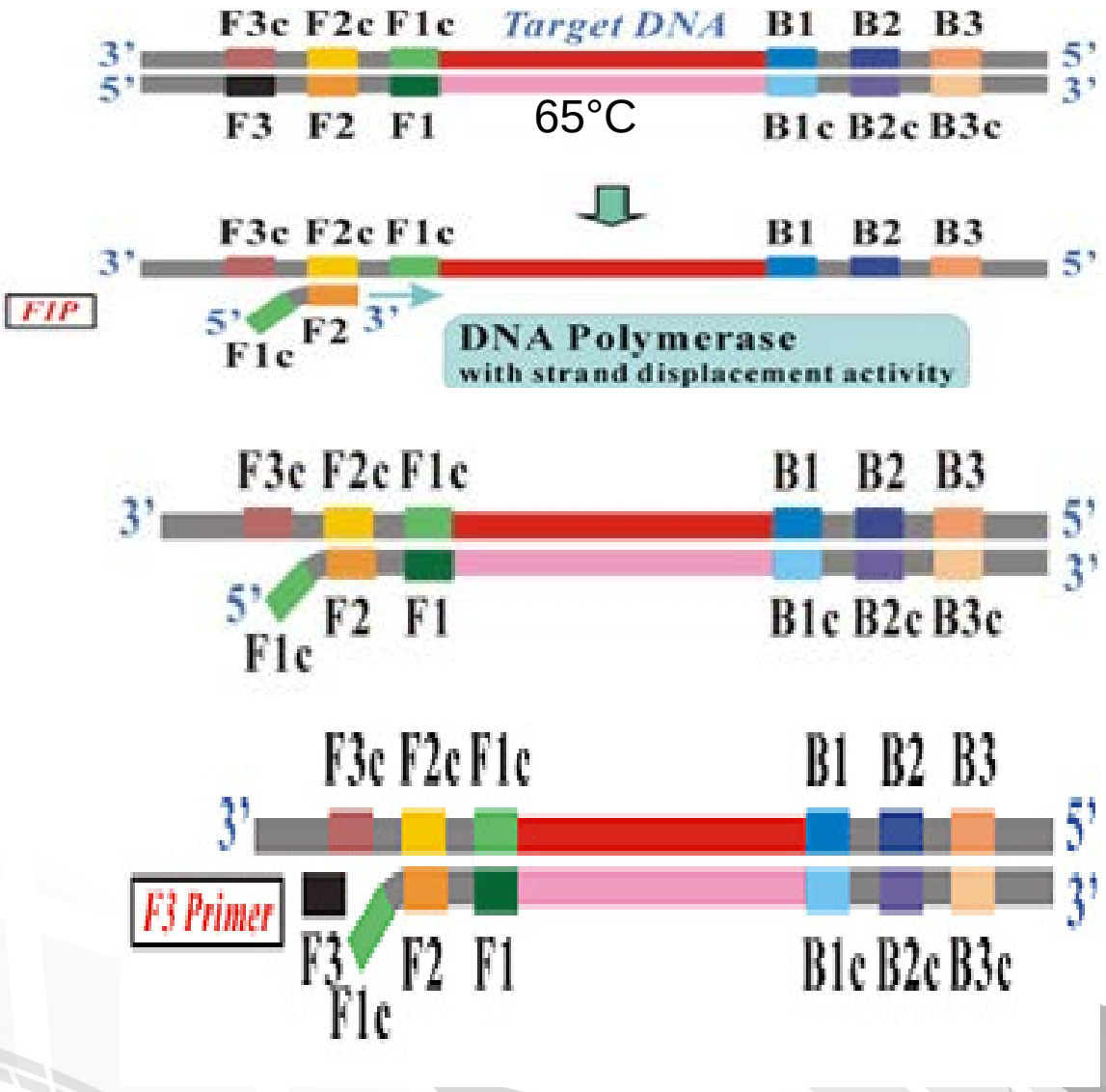
GC 50-60%,
40-50% di AT
nel primer…

Concentrazione
dei primers

PrimerExplorerV4
http://primerexplor
er.jp

17 coppie di
primers!!!

AMPLIFICAZIONE



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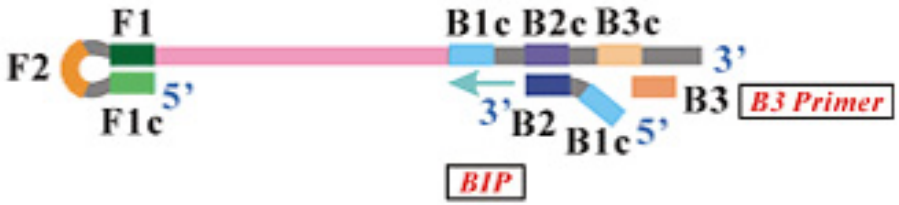
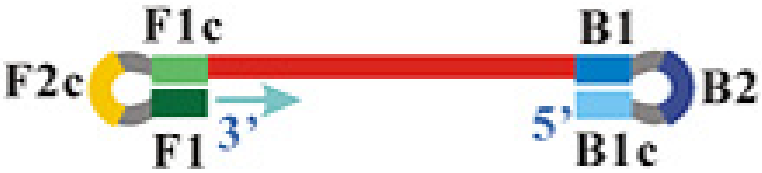
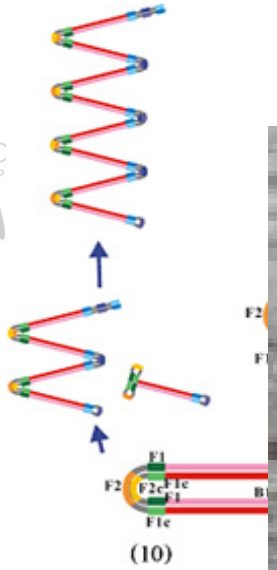
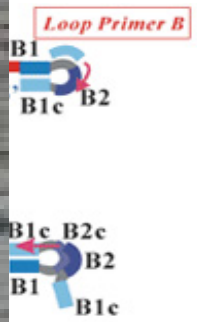
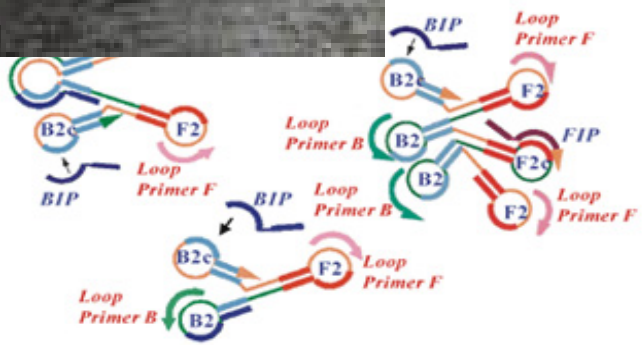




AMPLIFICAZIONE





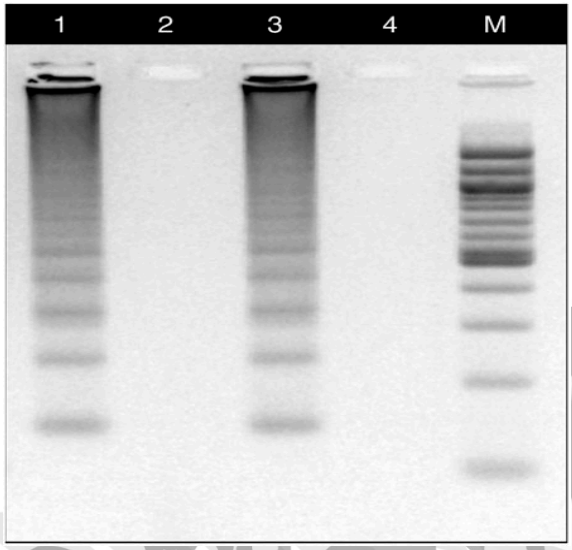


RILEVAZIONE

- Torbidità
- Fluorescenza
- <u>Colorazione</u>
- <u>Gel Electrophoresis</u>

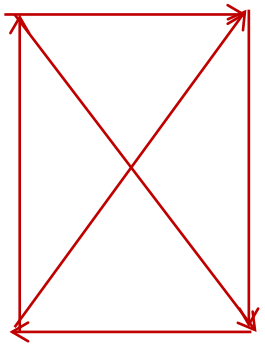
– +
template
(SARS-CoV)

– +
template
(*Salmonella* spp.)



MATERIALI E METODI

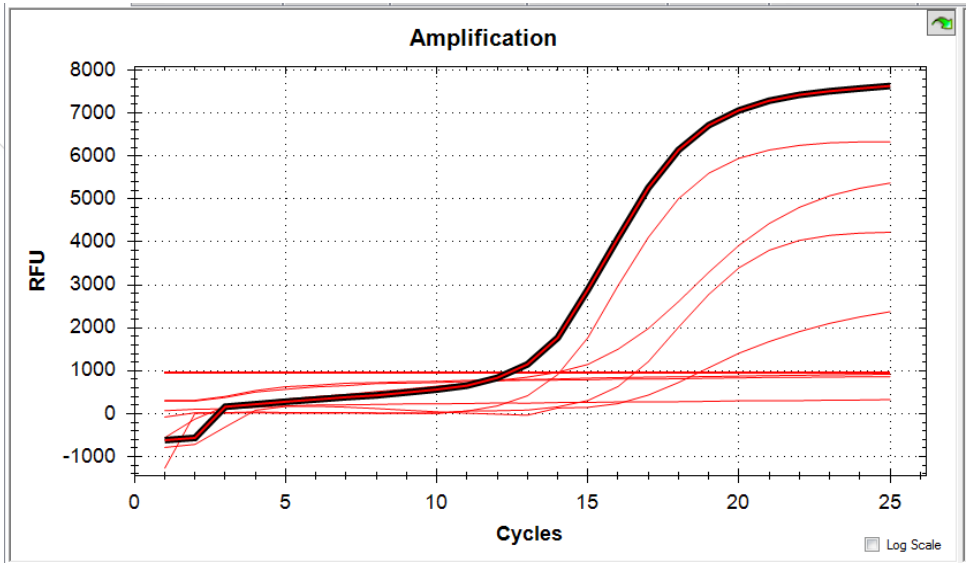
5 allevamenti di riproduttori e 38 di broiler, su differenti capannoni (49), 5 giorni prima della macellazione.



Tamponi cloacali, boot socks, pollina, mangime, acqua di lago, insetti.

REAL TIME PCR, LAMP, ISO, SEMINA DIRETTA, PCR, ANTIBIOGRAMMA, PFGE, MLST

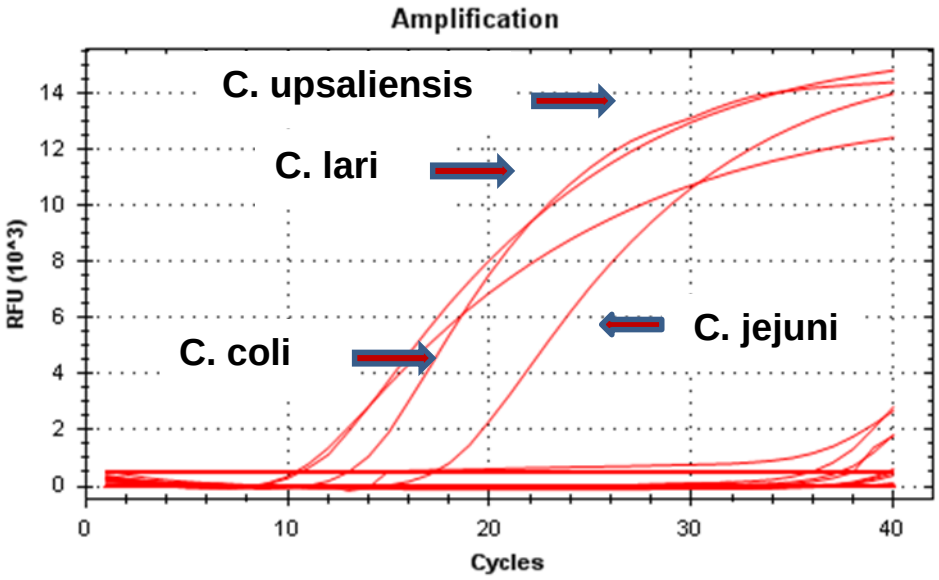
VALUTAZIONE ANALITICA - LAMP



Campylobacter jejuni ATCC 33291
Campylobacter coli NCTC 11353
Campylobacter lari NCTC 11552
Campylobacter upsaliensis NCTC 11541
Campylobacter fetus subsp. fetus ATCC 27374
Arcobacter butzleri NCTC 12481
Helicobacter pylori NCTC 11637
Campylobacter jejuni 20686_14
Campylobacter jejuni 1962_4
Campylobacter fetus subsp. venerealis
Campylobacter sputorum 11367
Campylobacter jejuni 22185-3
Campylobacter jejuni 1962-4
Campylobacter jejuni 22185-8
Campylobacter coli 22748
Campylobacter coli 27987
Campylobacter coli 27988
Campylobacter jejuni 206846_4
Campylobacter jejuni 20670_6

	SAGGIO Lamp 16S I				
		Campioni positivi (83)		Campioni negativi (19)	
Risultati del test	Positivi	83	TP	FP	2
	Negativi	0	FN	TN	17
		Sensibilità TP/(TP+FN) 100%		Specificità TN/(TN+FP) 89.47%	

VALUTAZIONE ANALITICA – REAL TIME PCR



Campylobacter jejuni ATCC 33291
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Campylobacter jejuni 22185-8
Campylobacter coli 22748
Campylobacter coli 27987
Campylobacter coli 27988
Campylobacter jejuni 206846_4
Campylobacter jejuni 20670_6

Gene target: 16s rDNA

Sonda TaqMan

95°C – 5 min
95°C – 15 sec
58°C – 30 sec
72°C – 30 sec
72°C – 5 min

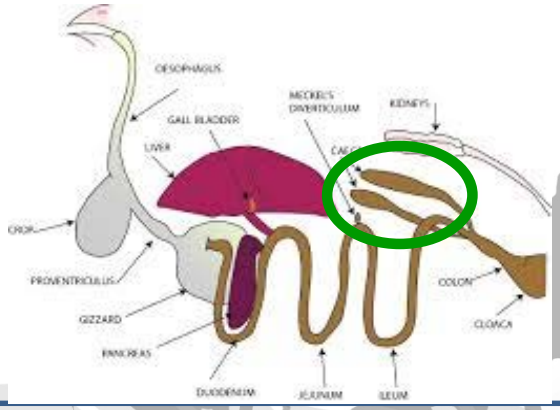
VALUTAZIONE DIAGNOSTICA REAL TIME PCR

Risultati Real-time PCR	Risultati semina diretta		
	+	-	Totale
+	43	0	43
-	0	6	6
Totale	43	6	**49**

Conferma dei risultati al mattatoio

Ricerca Campylobacter

+



VALUTAZIONE DIAGNOSTICA LAMP

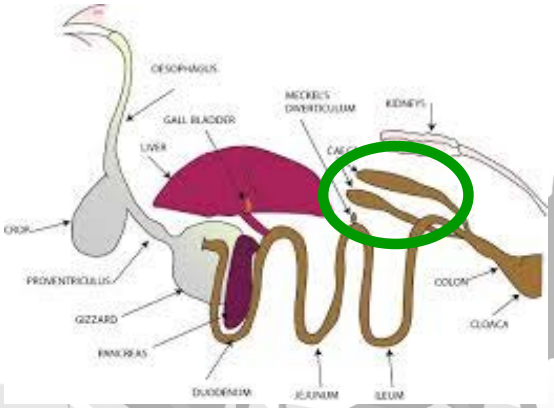
Lamp	Risultati semina diretta		
	+	-	Totale
+	33	3	36
-	10	3	13
Totale	43	6	**49**

Conferma dei risultati al mattatoio

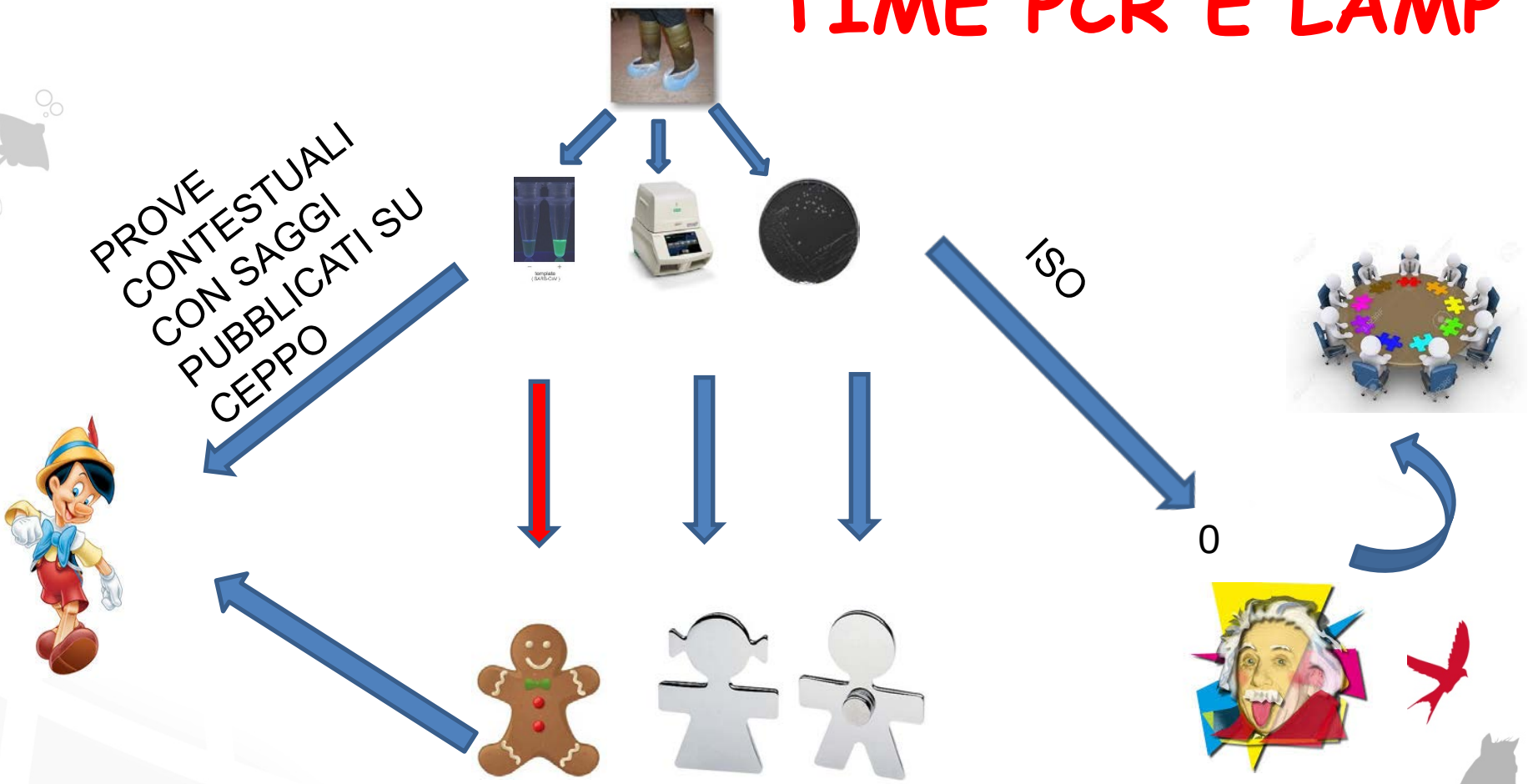
Ricerca Campylobacter

+

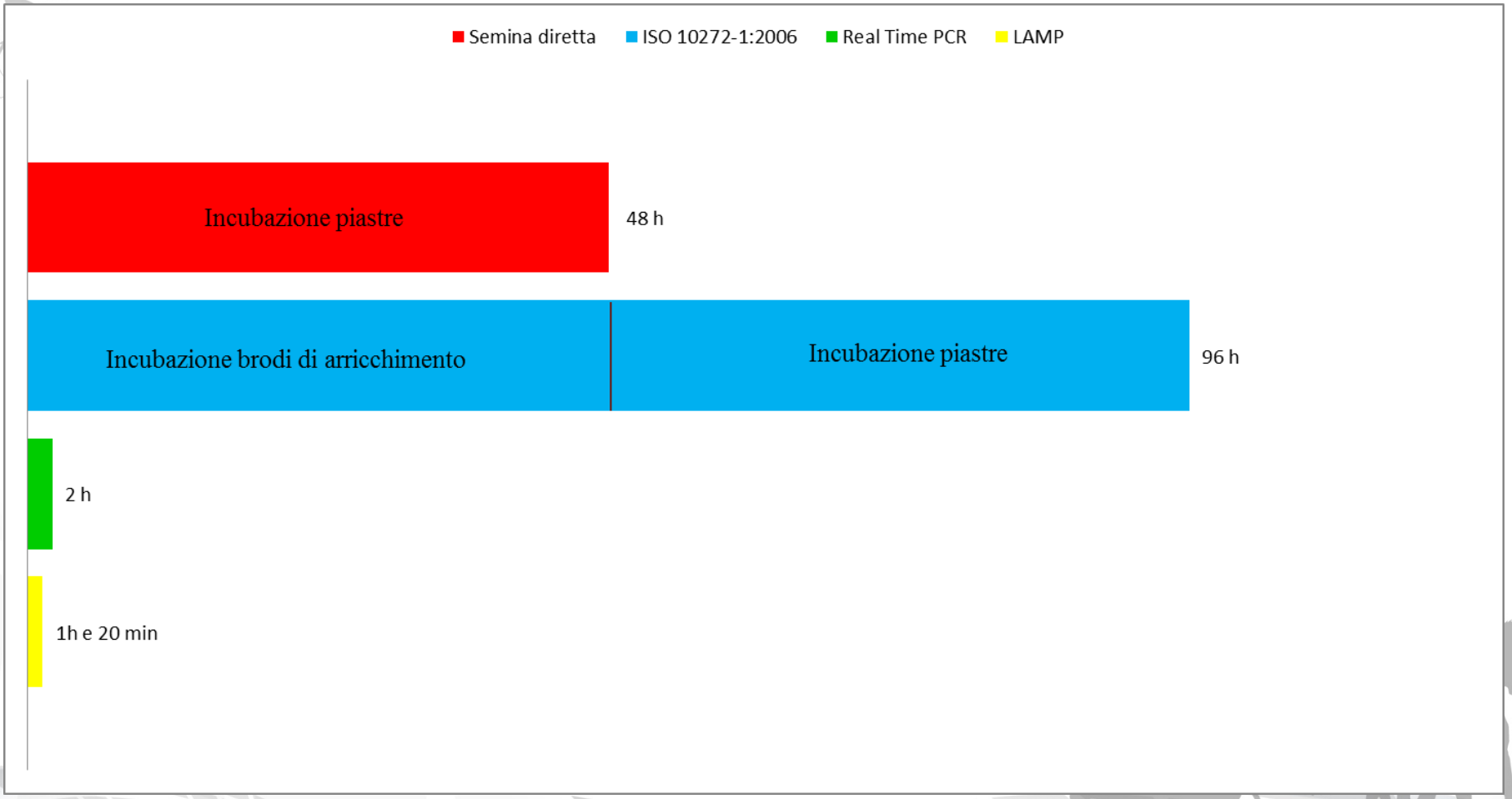
Specificità 76%
Sensibilità 50%



VALUTAZIONE DIAGNOSTICA REAL TIME PCR E LAMP



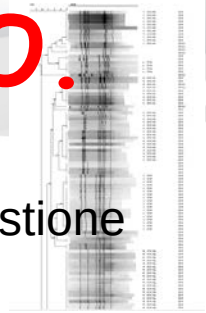
VALUTAZIONE TEMPI DI DIAGNOSI



RISULTATI

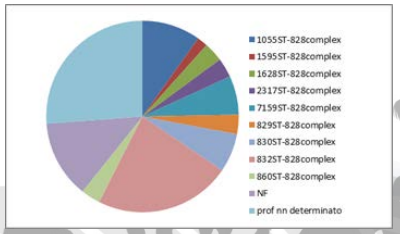
La Real Time PCR si è dimostrata come la tecnica d elezione per l'applicazione diagnostica in campo, mostrando perfetta concordanza con il metodo microbiologico a fronte di un tempo di diagnosi di sole due ore.

ALTRI RISULTATI DEL PROGETTO.



- Individuazione statisticamente significativa di alcune fonti di infezione: gestione del capannone, tinning, acqua di abbeveraggio, etc…
- Sono stati identificati complessivamente 550 ceppi: 300 di *C.jejuni* e 250 di *C.coli.*
- Due campioni di acqua di lago sono risultati positivi per *Campylobacter coli*.
- I mangimi sono risultati sempre negativi.
- I tamponi cloacali effettuati sugli animali negli allevamenti dei riproduttori, sono risultati positivi con una prevalenza dal 53% all'87%
- Le popolazioni evidenziate con la PFGE si dividono tra «stanziali» e «stagionali», particolare risalto ha la stabilità della popolazione dei riproduttori.
- La MLST ha evidenziato la presenza dei più comuni Clonal Complex 828 e 353, inoltre siamo in attesa dell'assegnazione di 10 nuovi profili.
- L'antibiotico resistenza ha fornito valori preoccupantemente in linea col trend degli ultimi anni, in netto aumento purtroppo, su Ciprofloxacin, Acido Nalidixico, Tetraciclina, etc…

Antimicrobial	MIC Breakpoints (µg/ml)		N° Resistant isolates (%)
	S	R	
Chloramphenicol	≤ 8	≥ 32	0
Ciprofloxacin	≤ 1	≥ 4	77,27%
Erythromycin	≤ 0,5	≥ 8	36,36%
Gentamicin	≤ 4	≥ 16	0
Nalidixic Acid	≤ 16	≥ 32	77,27%
Streptomycin	≤ 2	≥ 8	9,09%
Tetracycline	≤ 4	≥ 16	63,63%



CONCLUSIONI

- La buona affidabilità, i brevi tempi di analisi e la possibilità di avere una quantificazione, rendono possibile l'utilizzo del metodo di **Real Time PCR** in un piano di programmazione delle macellazioni in relazione alla positività del gruppo di animali
- Le tecniche andrebbero migliorate superandone i punti critici fra cui la gestione dei campioni e dei reattivi in loco e la valutazione di ripetibilità e riproducibilità, completandone la validazione.
- Le aziende dovrebbero prevedere comunque una gestione corretta dei capannoni per migliorarne la biosicurezza.

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