ISTITUTO G. CAPORALE
TERAMO

USDA
FSIS

Non O157 Shiga toxin-producing *Escherichia coli* from meat products

Marina Torresi
Teramo 12 Dicembre 2012

ISTITUTO G. CAPORALE
TERAMO

FSIS Official Methods can be found in the FSIS Microbiology Laboratory Guidebook (MLG). The MLG is updated to incorporate improvements in analytical methods. The latest version can be found at: http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/index.asp

Resources:

- USDA Microbiology Laboratory Guidebook (MLG) Chapter 5B.02: Detection and Isolation of non – O157 Shiga toxin-producing Escherichia coli (STEC) from meat products;
- USDA MLG Chapter 5B Appendix 1;
- USDA MLG Chapter 5B Appendix 2;
- USDA MLG Chapter 5B Appendix 3.

ISTITUTO G. CAPORALE
TERAMO

Background

- Increased awareness of non-O157 STEC in illness and hemolytic uremic syndrome (HUS);
- Estimated 70-84% of human illness caused by non-O157 STEC are caused by 6 major serogroups (O26, O45, O103, O111, O121 and O145);
- Similarly to *E. coli* O157, cattle are a major reservoir for non-O157 STEC.

ISTITUTO G. CAPORALE
TERAMO

Quality controls

1) General

The top six non O157 STEC control strains shall meet the following genetic characteristics: *stx*+ and *eae*+.

2) Sample Enrichment Controls

Positive control: *E. coli* O157:H7 strain 465-97 (*stx*- *eae*+) inoculated into a meat matrix free of the target analyte;
Negative control: Uninoculated media (mTSB)

3) DNA Extraction Control Preparation

E. coli O157:H7 strain 465-97 (*stx*- *eae*+): Positive DNA extraction control.

4) IMS Plating Controls

Streak the serogroup(s) of interest (based on serogroup specific PCR results) onto mRBA and incubate along with the samples that have been treated with the IMS procedure.

Quality controls

5) PCR Controls

a.stx/eae screen PCR

•DNA extraction *eae* positive control: DNA template from bioluminescent *E. coli* O157:H7

•PCR positive control: DNA template from a cocktail of top six STEC cultures analyzed in triplicate

•NTC: PCR certified water

b. Serogroup- specific screen PCR (O antigen gene cluster)

•PCR positive control: DNA template from a cocktail of top six STEC cultures analyzed in triplicate

•NTC: PCR certified water

c. *stx/eae* confirmatory PCR

•PCR positive control: DNA template from a cocktail of top six STEC cultures analyzed in triplicate

•NTC: PCR certified water

d. Serogroup-specific confirmatory PCR (O antigen gene cluster)

•PCR positive control: DNA template from a cocktail of top six STEC cultures analyzed in triplicate

•NTC: PCR certified water

Quality controls

5) PCR Controls

To prepare the controls:

- Streak onto a SBA STEC culture and incubate at 35±2°C for 18-24h
- Create a culture suspension in PCR certified water corresponding to approximately 10^9 CFU/ml.
- In one tube, 1,0ml from each suspension shall be added to 4,0 ml of PCR certified water to create a 10 ml cocktail of all six strains.
- Transfer 100µl of the suspension in PCR tubes and heat at 95-99°C for 10min.
- Centrifuge for 3min at 10.000xg
- Use the supernatant as PCR positive control for all PCR assay.

Note: DNA can be prepared as a batch, aliquoted to smaller volume tubes and stored at ≤20°C for 1 year.

ISTITUTO G. CAPORALE
TERAMO

Method overview

Day 1	Sample Prep and Primary Enrichment 42±1°C for 15-22 hours	**ABI 7500 FAST** **Screen PCR**
Day 2	Multiplex Real time PCR Samples that are PCR + for *eae*, *stx* genes are carried forward to O antigen PCR	
Day 2 cont.	Multiplex O antigen genes Real time PCR Samples that are PCR + for *eae*, *stx* genes and O antigen genes are carried forward to further analysis (IMS bead capture)	
Day 2 cont.	Immunomagnetic Bead Capture & Modified Rainbow agar Plating (mRBA) for specific serogroups	
Day 3	Examine mRBA plates for latex + colonies and streak them onto Sheep Blood agar plates	
Day 4	1. Latex agglutination 2. Multiplex Real time PCR + for *eae*, *stx* genes and O antigen genes 3. Latex + colonies onto VITEK2®	**ABI 7500 FAST** **Confirmatory PCR** **VITEK2®**

ISTITUTO G. CAPORALE
TERAMO

Day 1: Sample arrival and preparation

- Aseptically weigh 325±32.5 g of raw product into sterile filter bag


+

+


- Add 975±19.5 ml of mTSB with 8 mg/L novobiocin to the sample to achieve a 1:4 diluition
- Stomach (homogenize) the sample and incubate the sample enrichment at 42±1°C for 15-22 h.

ISTITUTO G. CAPORALE
TERAMO

Day 2: Screening Step- DNA Extraction Step

Prelabel three sterile 1.5 ml microcentrifuge tubes for each sample and turn on heating block set at 95-99°C

Transfer a 1.4±0.1 ml aliquot of enriched sample (15-22 h incubation) to a microcentrifuge tube

Centrifuge the aliquot at 1500 xg for 1 min.
Transfer the supernatant into the second centrifuge tube

Centrifuge the trasferred supernatant at 10.000 xg for 5 min.
Discard the supernatant

Add 500±50 µl of 0,85% saline to the bacterial pellet and resuspend the pellet with the pipette or by vortexing

Centrifuge the suspension at 10.000 xg for 3 minutes

ISTITUTO G. CAPORALE
TERAMO

Day 2: Screening Step- DNA Extraction Step

Remove the supernatant from the pellet and add 90±9.0µl 1X Tris-EDTA (TE). Resuspend using pipette tip or vortexing

Add water to each of the wells of the heating block for appropriate heat distribution

Heat the suspension for 15 minutes at 95-99°C in a heating block

Centrifuge the cell lysate at 16.000 xg for 4 minutes.
Transfer the supernatant (DNA template) into a sterile prelabeled microcentrifuge tube

If the template is to be used within 24 hours, store at 2-8°C.
For long term storage, store at≤20°C

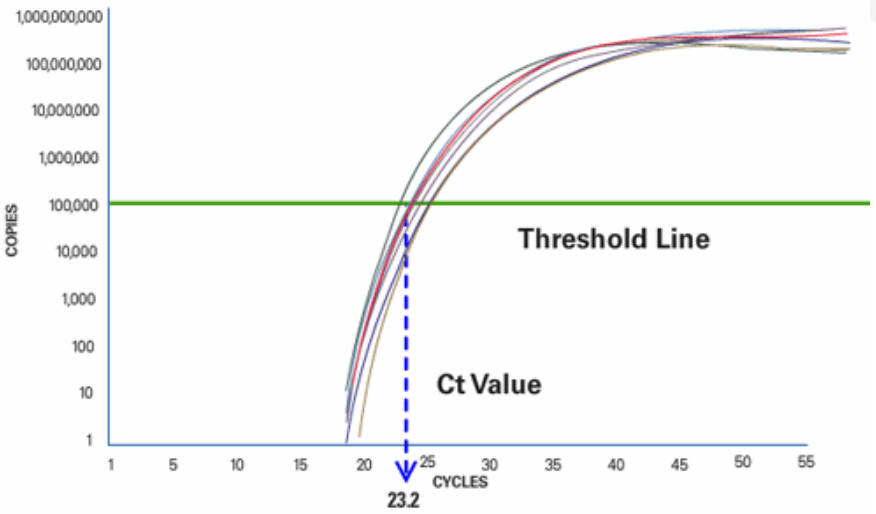
Day 2: Screening Step- real time PCR

- Following DNA extraction from overnight enrichments, Real time PCR will be used as a screen for the presence of *stx* (toxin) and *eae* (intimin)
- PCR reaction components are listed in USDA MLG Chapter 5B Appendix 1
- Assay optimized with ABI Environmental Mastermix
- 16S rDNA gene serves as internal positive control

ISTITUTO G. CAPORALE
TERAMO

Day 2: Screening Step-real time PCR

- Transfer 20µl of master mix to each well on the PCR plate for each reaction
- Add 5µl of DNA template to the appropriate well containing PCR mastermix
- Seal the PCR plate with ABI MicroAmp™ Optical film
- Briefly, centrifuge the plate (2000xg for 30 sec.) to remove any bubbles at bottom of the wells
- PCR cycling parameters are the same for all assays
- Total run time is approximately 1h 45 min



Day 2: Screening Step- real time PCR

Examine each target for each sample:

- Ct value given if sample is (+) for that target
- Undetermined given if sample is (–) for that target

Follow matrix to determine if serogroup PCR is needed

	Stx PCR Negative	*Stx* PCR Positive
eae PCR Negative	Negative **STOP** Report results	Negative **STOP** Report results
eae PCR Positive	Negative **STOP** Report results	Continue with serogroup PCR

Matrix for interpreting *stx* and *eae* PCR screen

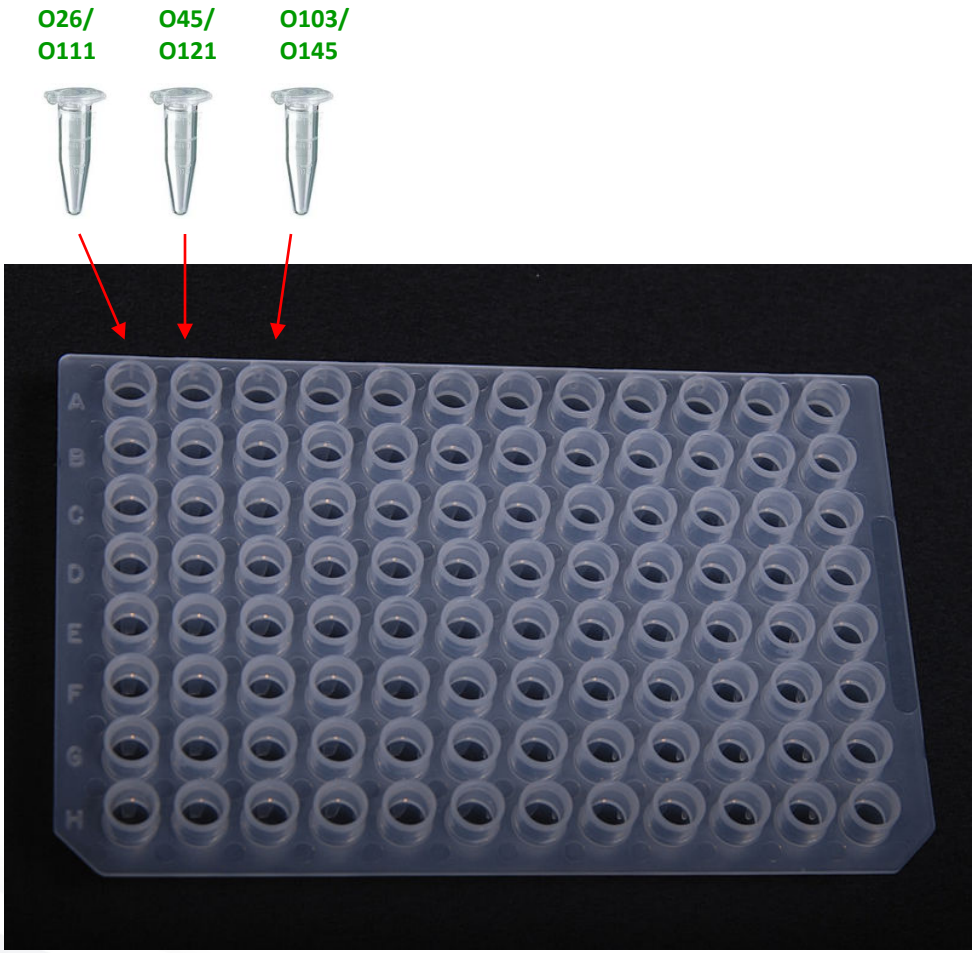
Day 2: Screening Step- real time PCR

- Run the serogroup- specific O antigen PCR assays on *stx* and *eae* screen + samples
- Follow same procedure for the *stx/eae* PCR screen except with different primers and probes
- Serogroups have been grouped into multiplex reactions (O26/O111, O45/O121, O103/O145) with each serogroup having distinct reporter dye

A5B.2.2.1 Serogroup-specific PCR Assay for O26 and O111

	µL	[Final]	Units	[Stock]
ABI® Environmental Master Mix	12.50	1	X	2
Primer 16S rRNA F	0.20	0.16	µM	20
Primer 16S rRNA R	0.20	0.16	µM	20
Primer Wzx O26 F	0.31	0.25	µM	20
Primer Wzx O26 R	0.31	0.25	µM	20
Primer WbdI O111 F	0.31	0.25	µM	20
Primer WbdI O111 R	0.31	0.25	µM	20
Probe 16S rRNA P	0.50	0.1	µM	5
Probe Wzx O26 P	1.88	0.15	µM	2
Probe WbdI O111 P	1.00	0.02	µM	5
dH2O	2.48			

Day 2: Screening Step- real time PCR

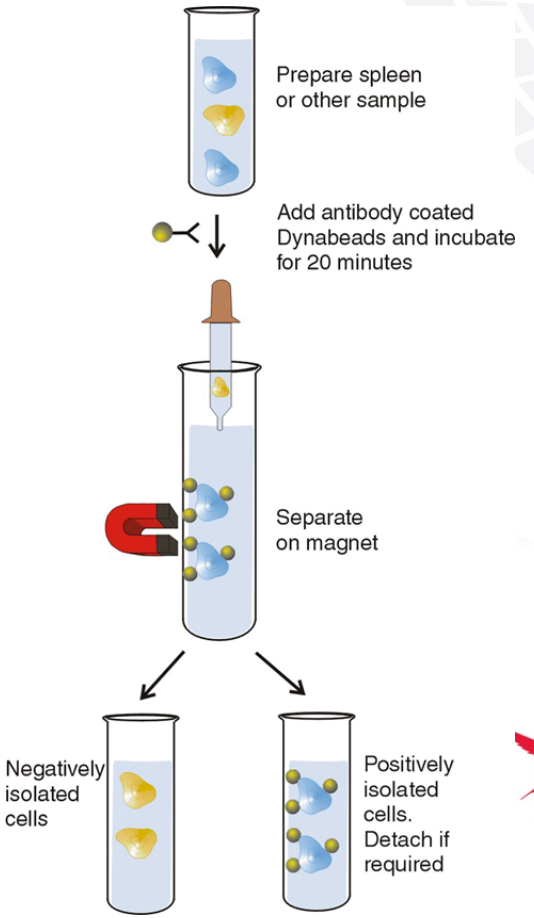


- 3 separate serogroup mastermixes per *stx*+/*eae*+ sample
- Assays for serogroup run simultaneously on the same plate
- Total run time is 1h 45min

Day 2: Screening Step - real time PCR

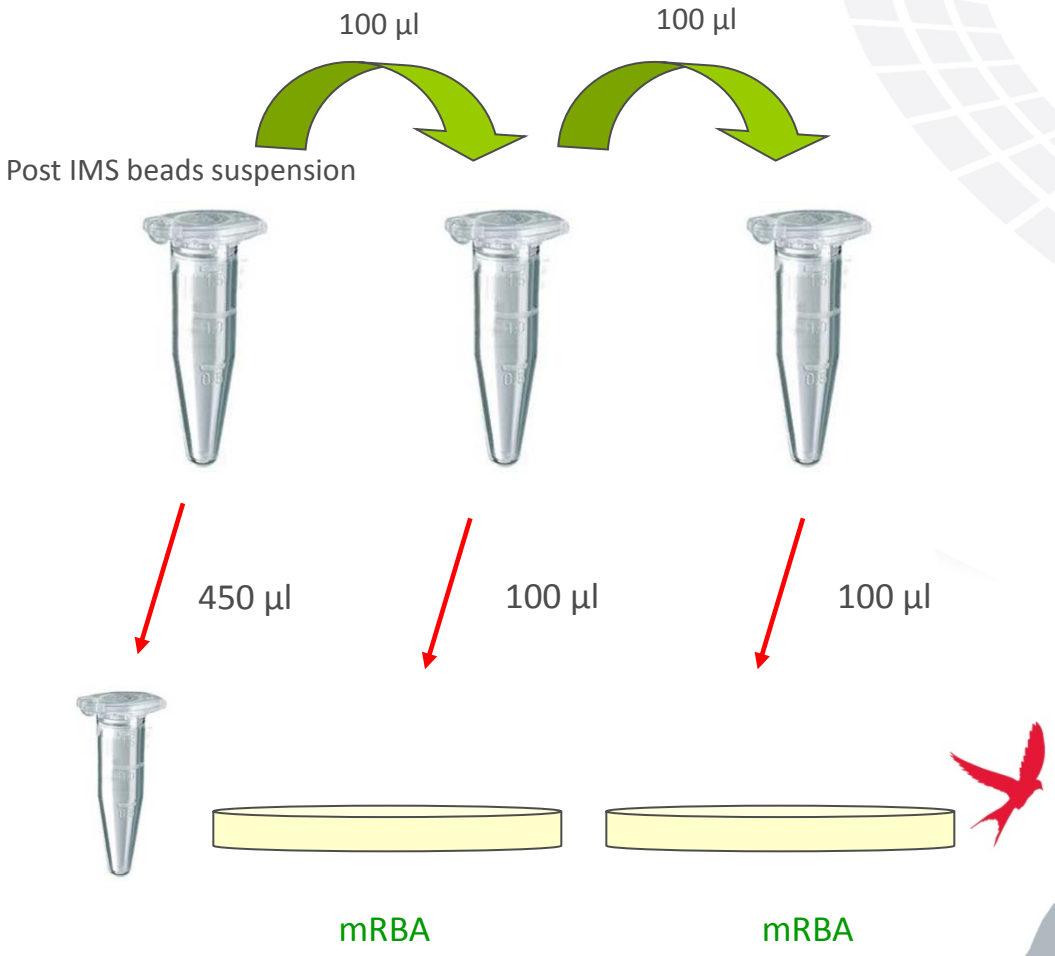
- Examine each target for each sample
- Each serogroup reaction can be distinguished by dye channel
- Samples that are *stx*+, *eae*+ and top six O antigen gene + are carried forward for cultural analyses

- Tranfer 2-5 ml from overnight enrichment through 40µm Cell strainer into a 50ml centrifuge tube.
- 50 µl Serogroup specific immunomagnatic separation (IMS) beads will be used to concentrate target cells (1 ml)
- Place the microcentrifuge tubes on LabQuake® Agitator and rotate tubes for 15min at RT
- IMS beads are washed 4X and eluted with 1 ml E-Buffer



Day 2: Screening Step - Immunomagnetic Separation

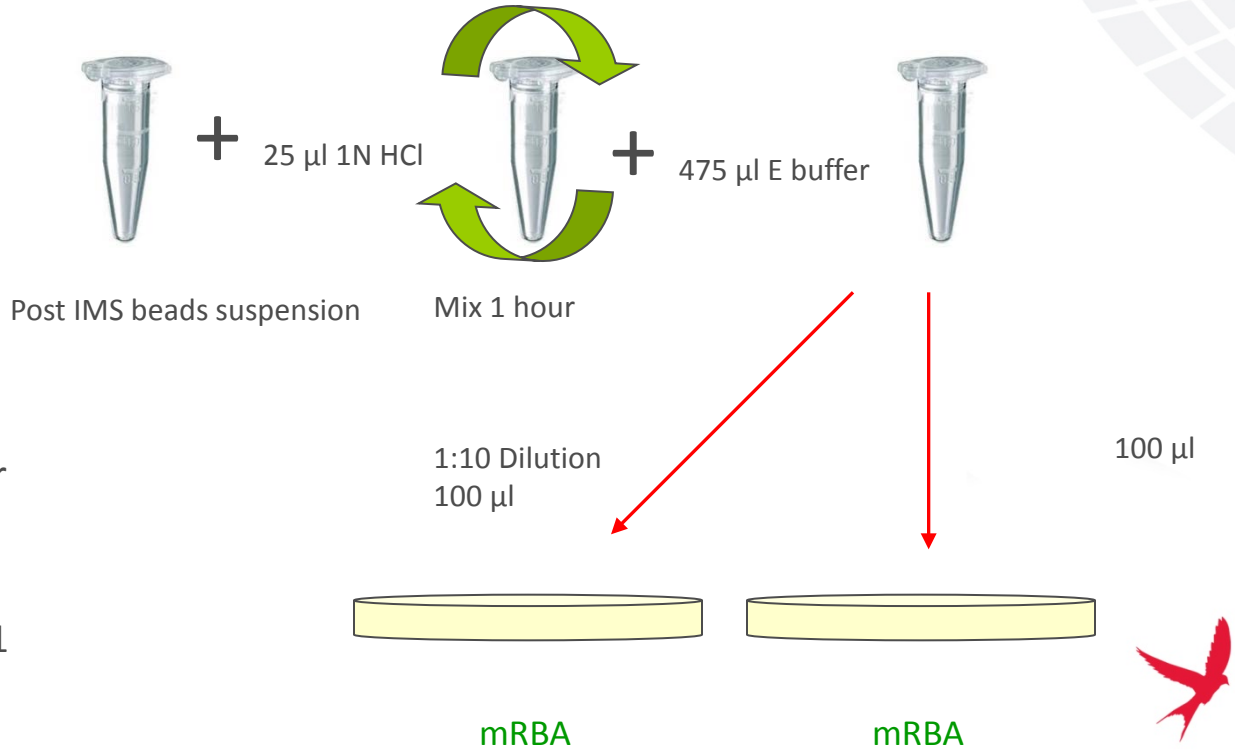
- Make a 1:10 diluition of each post-IMS bead suspension by adding 0.1 ml of the bead suspension to a tube containing 0.9 ml E buffer
- Make a 1:100 diluition by adding 0.1 ml of the 1:10 diluition to a tube containing 0.9 ml E buffer
- Plate 0.1 ml from 1:10 dilution and 1:100 dilution onto a mRBA plate and incubate for 20-24h at 35±2°C
- Transfer 0.45 ml of the undiluted post-IMS bead suspension to an empty 1.5 ml tube



ISTITUTO G. CAPORALE
TERAMO

Day 2: Screening Step- Immunomagnetic Separation

- Add 25µl of 1N HCl to 450 µl post IMS bead suspension and vortex briefly
- Rotate tubes for 1 hour at room temperature
- After 1 hour neutralize the suspension by adding 475 µl of E buffer
- Vortex briefly to maintain beads in suspension and plate 0.1 ml onto a mRBA plate
- Make a 1:10 diluition of the neutralized suspension and plate 0.1 ml onto a mRBA plate



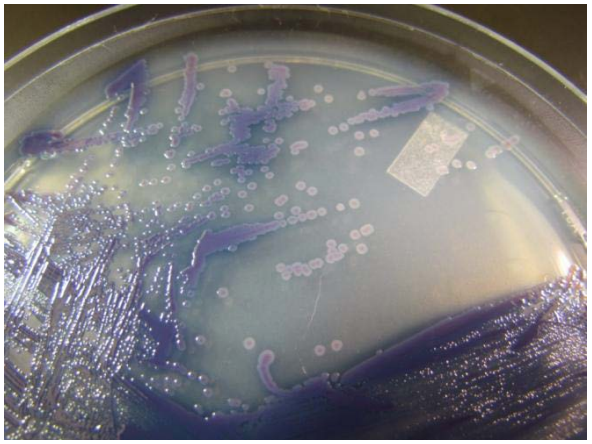
ISTITUTO G. CAPORALE
TERAMO

Day 3: Identification Step - Serological agglutination

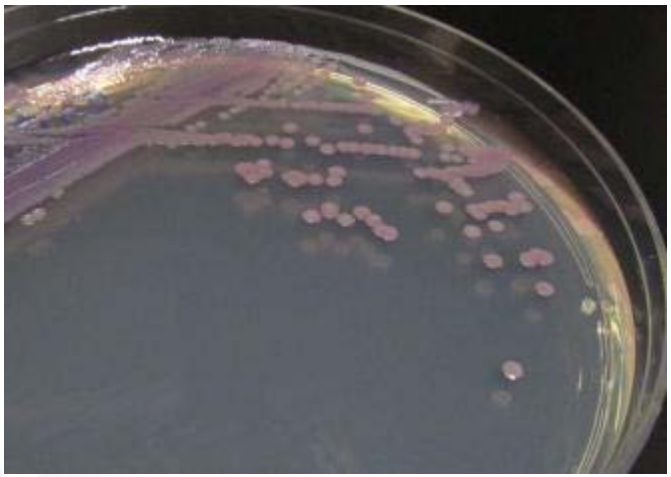
- Remove plates from incubator following 20-24h incubation
- Test representatives colonies of different colors and morfologies present on each plate using the latex agglutination reagents or antisera corresponding to your positive O group PCR result.
- Streak up to five (+) colonies, if available, onto tryptic soy agar with 5% sheep Blood (SBA) and incubate for 18-24 h at 37°C

Day 3: Identification Step - Serological agglutination

O45



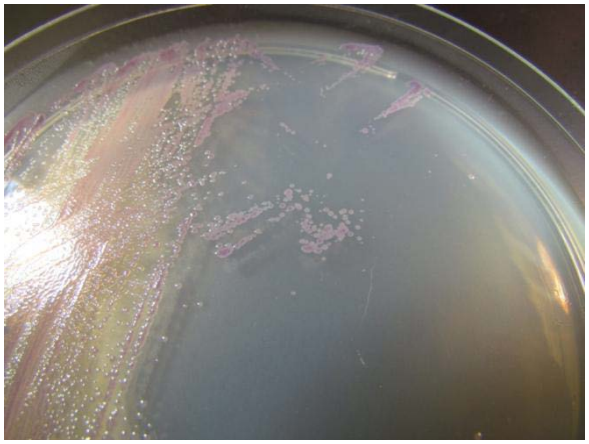
O26



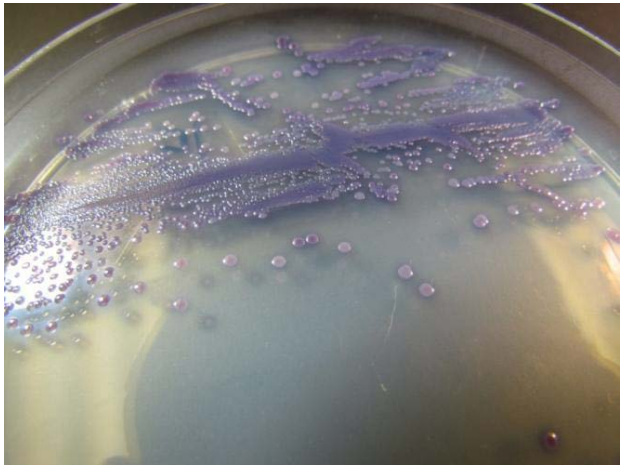
O103

Day 3: Identification Step- Serological agglutination

O111



O121



O145

ISTITUTO G. CAPORALE
TERAMO

Day 4: Confirmation of agglutination positive colonies

- Latex colonies from incubated SBA (18-24h)
  Latex (+) colonies will be confirmed by VITEK2 and PCR
- Extract DNA from isolated colonies on SBA by heating a 50 µl colony suspension in molecular grade water for 10 min at 95-99°C
- Perform *stx* and *eae* PCR assay and serogroup specific PCR assay to confirm the colony
- Confirm the same latex (+) colony by VITEK2 (biochemical identification as *E. coli*)