Laboratory Diagnosis: from Petri Dish to PCR

Anja Siitonen, Research Professor
PhD, MSc, Diploma in Clinical Microbiology
Head of Bacteriology Unit

THL

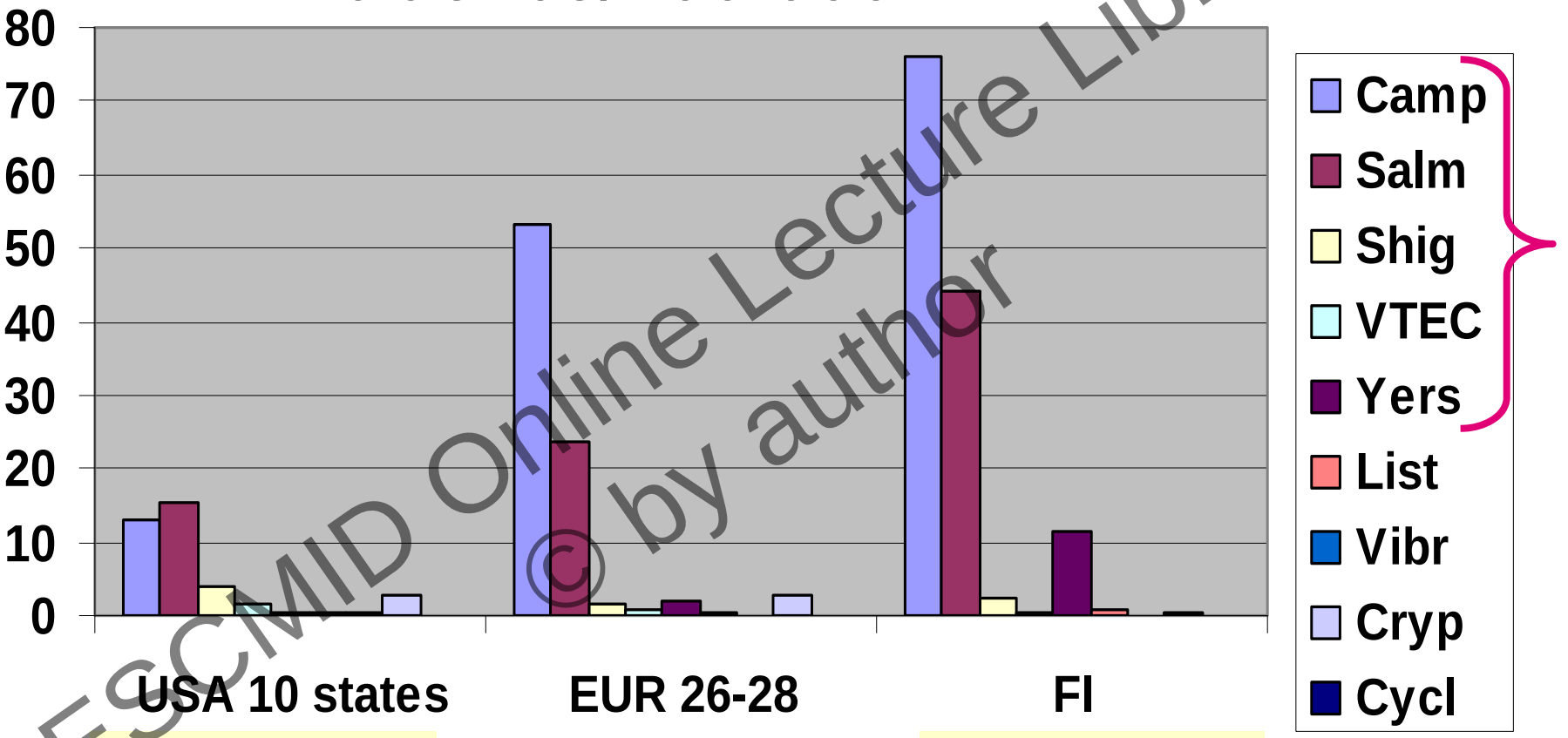
NATIONAL INSTITUTE FOR HEALTH AND WELFARE, FINLAND

ESCMID 16.–18.11.2012

Overview of results by Petri Dish methods and PCR methods

THL
NATIONAL INSTITUTE FOR HEALTH AND WELFARE, FINLAND

Foodborne infections in 2009
Incidence/100 000



USA 10 states
~ 17 500 cases
Bacteria 86.5%

EUR 26-28 states
~ 342 000 cases
Bacteria 97.7%

FI
~ 7 200 cases
Bacteria 99.8%

Refs:
MMWR:59;2010
ECDC; Report 2011
THL; Report 17, 2010

Stool samples from patients / travellers with diarrhoea

Cultivated for

- Salmonella
- Campylobacter
- Shigella
- Yersinia
- VTEC investigated by request

Always routinely in Finland

- Aeromonas
- Plesiomonas

Not requested in Finland
Clinical significance unclear
as causes of diarrhoea

Detection and identification of bacterial pathogens

- Laboratory practices affect patient care and disease surveillance
- Testing practices are changing
  - Conventional culture methods ↓
  - Rapid non-culture methods ↑
    - PCR, antigen testing, …

Non-conventional culture method
MALDI-TOF mass spectrometry

→ Changed practices change epidemiology

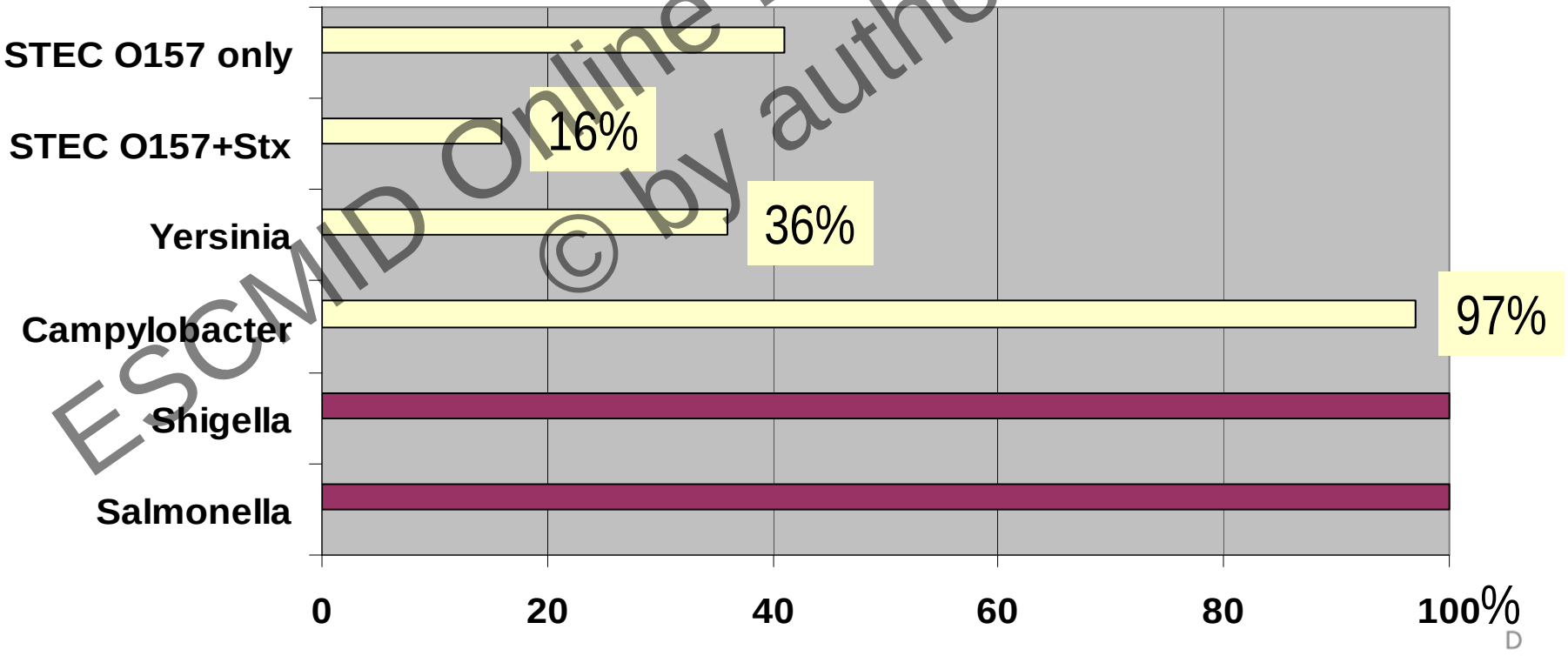
Routine stool cultures practices in Pennsylvania

M'ikanatha NM, *et al*. 2012

- 107 labs

→ only Salm and Shig were searched for in all labs

→ Epidemiology of non-O157 based on finding of Stx and O157 by antigen detection methods

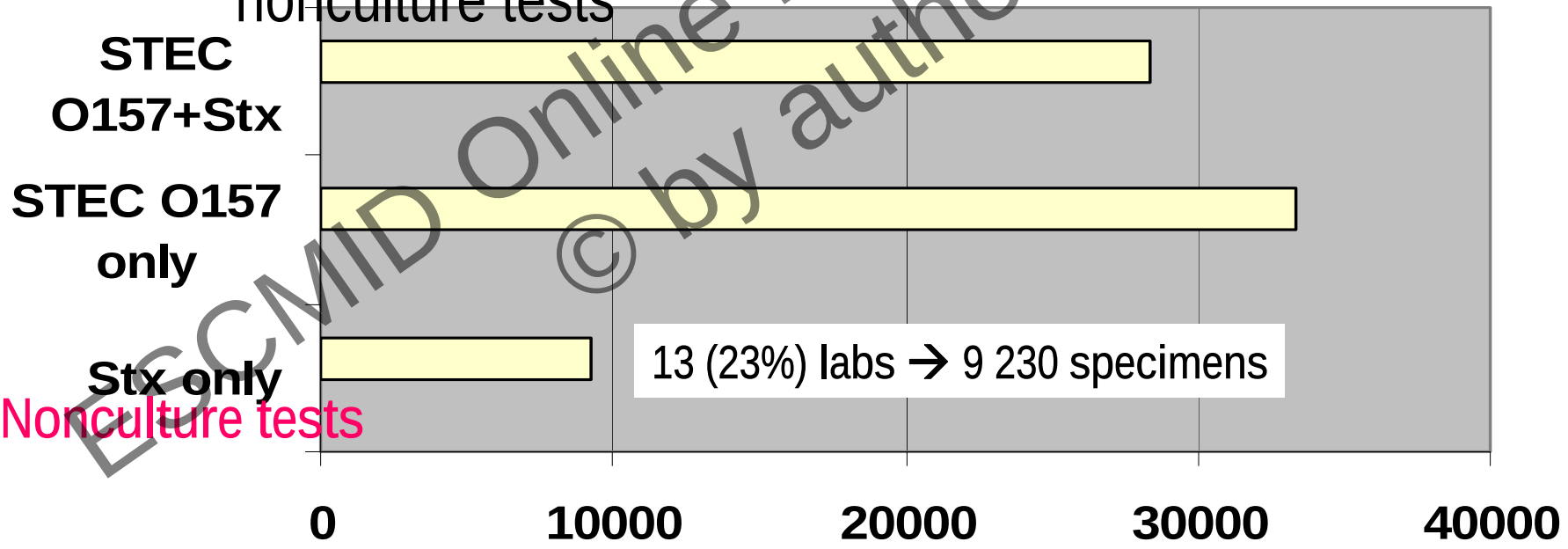


STEC investigations in Washington state

Stigi KA, *et al*. 2012

- 57 labs: investigated 71 000 stool specimens / yr

→ Epidemiology of non-O157 based on detection of Stx by nonculture tests



Nonculture tests

About PCRs very briefly and simply

- Nucleic acid is extracted
  - From stool sample directly, from culture on plate/in broth
  - With commercial kits, boiling, …
- Single PCR
  - Primers targeting one gene
- Multiplex PCR
  - Primers targeting several genes at a time (2- to 16-plex)
- Detection of genes (amplicons) is based on their different sizes in gel-electrophoresis
- Real-time PCR
  - Targets max 5 genes at a time
  - Detection based on use of fluorescent-labelled probes

General aspects on PCR methods

- Pathogens are detected rapidly (outbreaks!)
- Optimization of the method may be difficult
  - inhibitory substances in stool, oligonucleotides may interact with each other
- Working protocols easy to learn – in principle
- Results obtainable from inactivated material
- Increase detection rate (not always)
  - May lead to over-diagnostics – only genes are targeted, not live pathogens
- Can be used to screen for subset of samples for cultivation
- Costs differ, single PCR *vs* real-time PCR
- Isolates not available
  - Subtyping for epidemiological purposes may delay or not done at all
- Detection limit 10^2 – 10^5 CFU/g

General aspects on culture-based methods

- skill, labor, time required
  - Treatment, epidemiological investigations may delay
- Many bacteria inherently difficult to grow → poor yield
  - affected by antibiotics
  - viable but non-culturable state
  - commensals may overgrow pathogens → under-diagnosis
- Lot of infective waste
- Isolates are available for further typing / antimicrobial susceptibility tests
- Present data on diseases, public health epidemiology is based on culture-confirmation
- Detection limit 10^3 – 10^5 CFU/g (usually in diarrheal sample)

Examples of studies where PCRs were done directly from stool samples

Detection rates compared to conventional methods

Single, duplex and real-time PCRs

Amar CFL, *et al*. 2007

- ~ 4 600 stool samples: 2400 from patients, 2200 from controls
- In 1993 – 1996 samples were investigated by conventional methods for 25 foodborne pathogens
  - 53% of cases were +
  - 19% of controls were +
- In 2007 same samples were tested by PCRs for 8 foodborne pathogens
  - 75% of cases were +
  - 42% of controls were +

Single, duplex and real-time PCRs

Amar CFL, *et al*. 2007

- 8 pathogens searched from stools of cases and controls

2400 cases

	Convent %+	and PCR %+
• Salmonella	4	6
• Campylobacter	11	23
• EAEC	5	6
• Cryptosporidium	1	2
• Giardia	1	2
• Sapovirus	1	4
• Rotavirus	6	31
• Norovirus	6	36

Single, duplex and real-time PCRs

Amar CFL, *et al*. 2007

- 8 pathogens searched from stools of cases and controls

	2400 cases		2200 controls	
	Convent %+	and PCR %+	Convent %+	and PCR %+
• Salmonella	4	6	0.3	0.7
• Campylobacter	11	23	0,7	5
• EAEC	5	6	1.7	2
• Cryptosporidium	1	2	<0.1	0.5
• Giardia	1	2	0.5	1
• Sapovirus	1	4	0.2	2
• Rotavirus	6	31	0.4	14
• Norovirus	6	36	0.3	16

For all pathogens percentages were higher by PCR

Real-time PCR, 5 pathogens

de Boer, *et al.* 2010

- 5 pathogens
  - *S. enterica*, *C. jejuni*, STEC, *Shigella*/EIEC, Giardia
- 14 200 samples → cultured → 6.4% +
- 14 000 samples → screened by PCR
  - If PCR+ → cultured → 19.2% +
- Probable reason for high %:
  - when PCR had been+ → cultures on plates were carefully searched for colonies of pathogens

Real-time PCR, 4 pathogens

Cunningham SA, *et al*. 2010

- 3 h-method, including DNA extraction (MagNa Pure kit)
- 392 stool specimens, 104 were culture +
  - PCR from all specimens

PCR compared to culture

	Sensitivity	Specificity
Salmonella	100 %	99 %
Campylobacter	96	99
Shigella	100	100
Yersinia	80	100
Overall	92% (96/104)	98% (283/288)

Real-time PCR, 4 pathogens

Wiemer D, et al. 2011

- 3 h-method, including DNA extraction (QIAmp® DNA Mini Kit)
- 393 diarrheal stool specimens
  - 51% (201 samples) were culture+
- PCR from 201 culture+ and from 192 culture-negative

	PCR+
• *Salmonella*	96% (71/74)
• *C. jejuni*	98% (79/81)
• *Shigella*/EIEC	100% (8/8)
• *Yersinia*	100% (10/10)

→ 3 Salm, 1 Camp not found

8 additional findings from culture-negative samples
- 1 *Salmonella*
- 2 *Shigella*
- 5 *C. jejuni*

6-plex PCR-Luminex Assay

Liu J, *et al*. 2012

- 205 diarrheal specimens
  - DNA extracted with QIAamp kit (Qiagen)
  - Cultured previously (*Campylobacter* by ELISA) for enteric pathogens
- Compared to culture

	Sensitivity	Specificity
Salmonella	96 %	95 %
Campylobacter	89	93
Shigella	94	94
Yersinia	100	100
Vibrio	92	97

PCR: sensitivity less than 100%

Detection of diarrheal *E. coli* pathogroups by PCR

THL
NATIONAL INSTITUTE FOR HEALTH AND WELFARE, FINLAND

Diarrheagenic *E. coli*

- Divided at least to five pathogroups:
  - VTEC verocytotoxic
    - Other names STEC, EHEC
  - EPEC enteropathogenic
  - ETEC enterotoxigenic
  - EIEC enteroinvasive
  - EAEC enteroaggregative
- Without PCR difficult to diagnose
  - all, except O157 sor$^-$, look like normal *E. coli* flora
    - for certain non-O157 groups latex agglutination, immunomagnetic separation are available

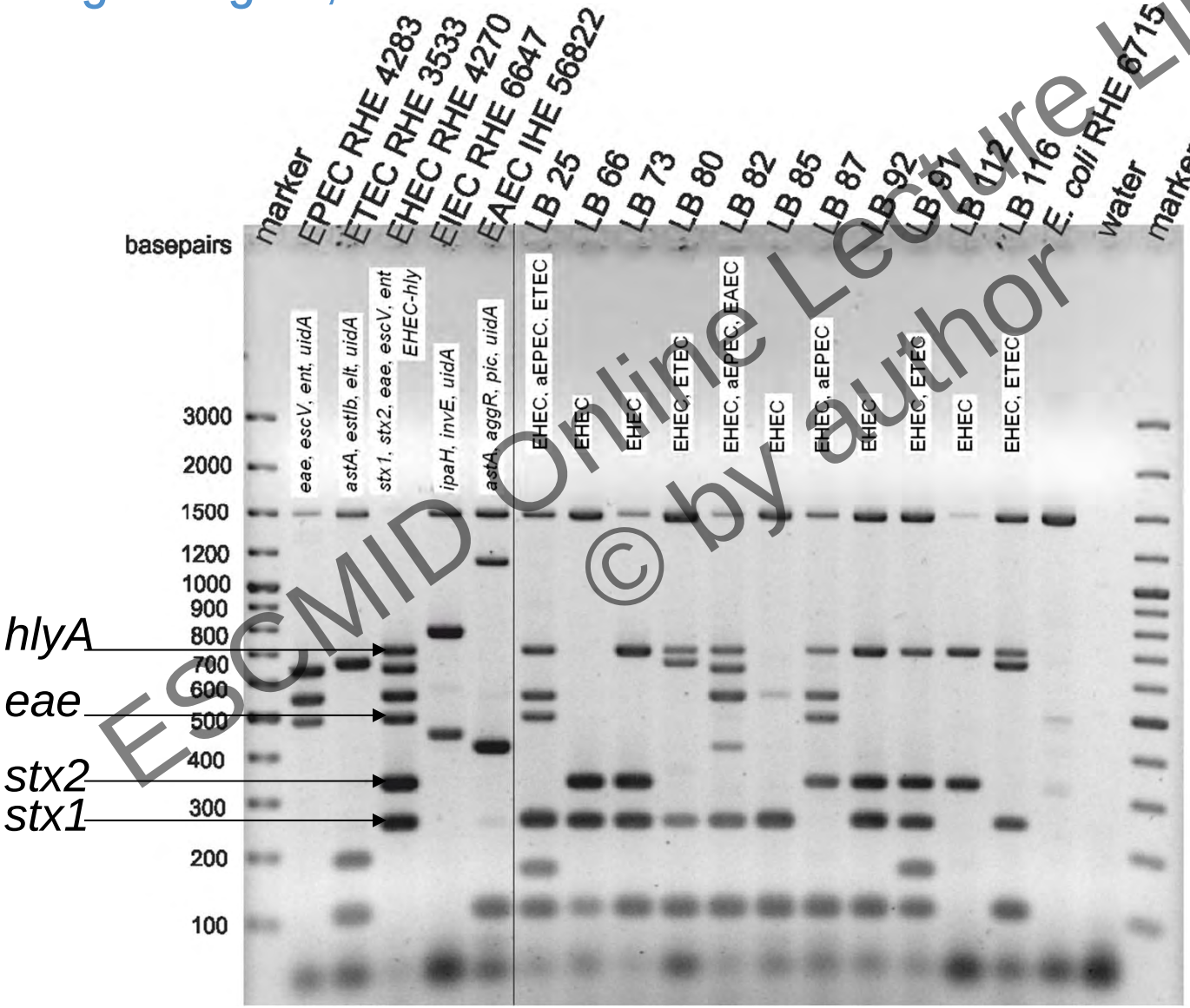
16-plex PCR directly from stool sample or mixed bacterial cultures

Antikainen, *et al*. 2009

- Primary culture on CLED or SMAC, or stool sample
- DNA extraction by boiling (or from stool sample with Genomic DNA purification Kit
- VTEC *stx1*, *stx2*, *eae*, *escV*, *ent*, *EHEC-hly*
- EPEC *eae*, *bfpB*, *escV*, *ent*, *uidA*
- ETEC *elt*, *estIa*, *estIb*, *astA*, *uidA*
- EIEC *invE*, *ipaH*, *uidA*
- EAEC *pic*, *aggR*, *astA*, *uidA*
- *E. coli* *uidA*
- 10 genes in blue define the pathogroup

An example of the 16-plex PCR results after gel-electrophoresis

Kagambèga A, *et al*. 2011



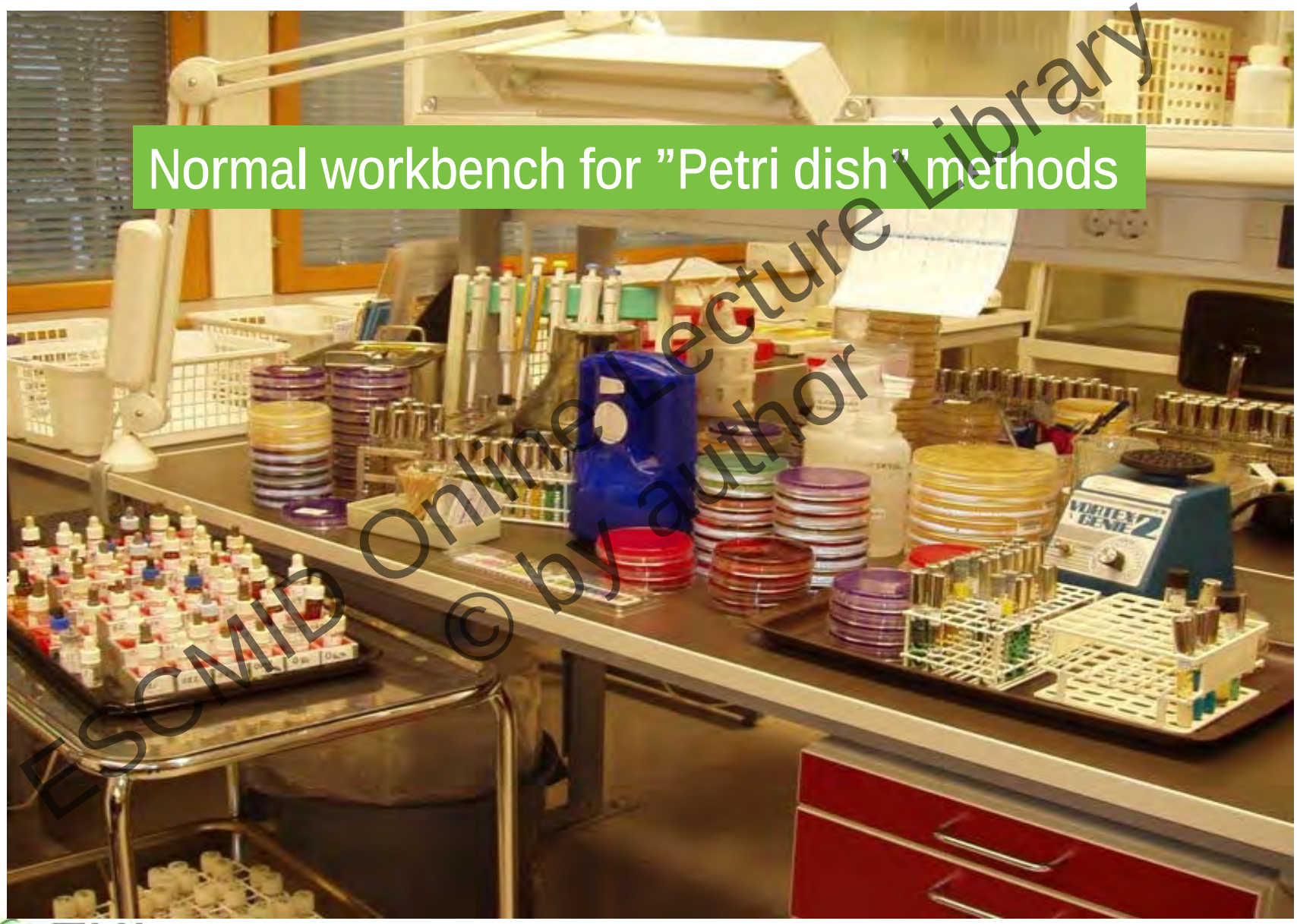
- Isolation of specific strain is challenging
- Hybridization on culture plate

Identification of foodborne bacteria

Conventional methods
vs
PCR methods

A couple of examples

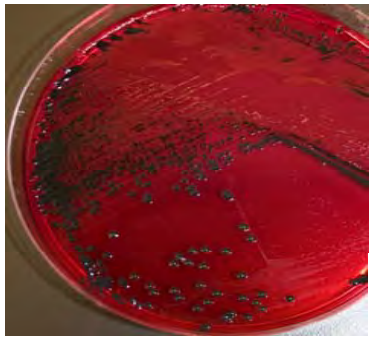
Normal workbench for ”Petri dish” methods



Traditional phenotypic tests for *Salmonella*

CLED

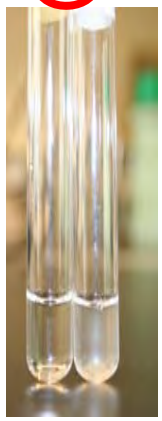
XLD



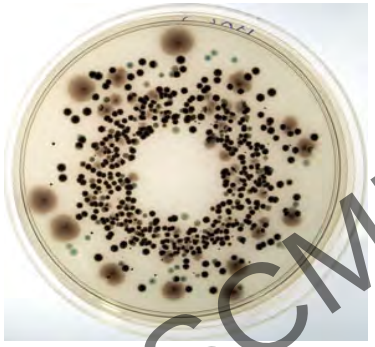
ONPG + -

Lactose + -

KCN -



Harlequin Salmonella Agar (LabM)



+ + + + +

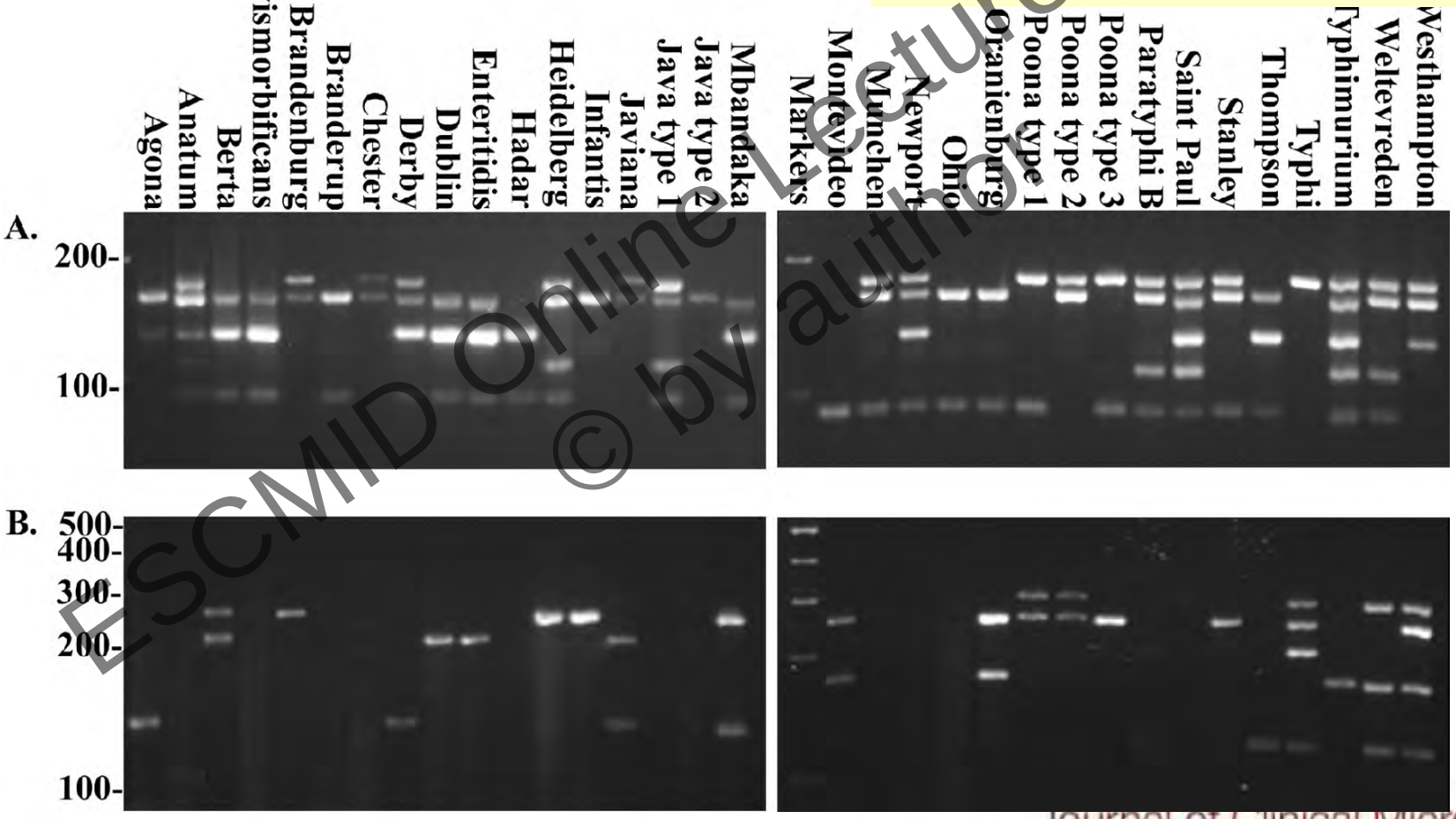
Serotyping by agglutination method with large set of antisera

PCR for serotyping of *Salmonella*

Kim S, *et al*. 2006

- Developed for 30 prevalent serotypes / 2500 serotypes
- 97% of 111 strains were correctly typed

→150 / 5000 strains incorrectly typed



Journal of Clinical Microbiology

PCR for EHEC-EAEC O104:H4 *E. coli*

= combined precise identification and subtyping method

- Detects 4 genes
  - *stx2*
  - *terD*,
  - *wzx*O104,
  - *fliC*H4

Source: Taru Lienemann

NATIONAL INSTITUTE FOR HEALTH AND WELFARE, FINLAND

Identification of *Campylobacter* spp.

- Traditional test is hippurate test: *C. jejuni* + *C. coli* –

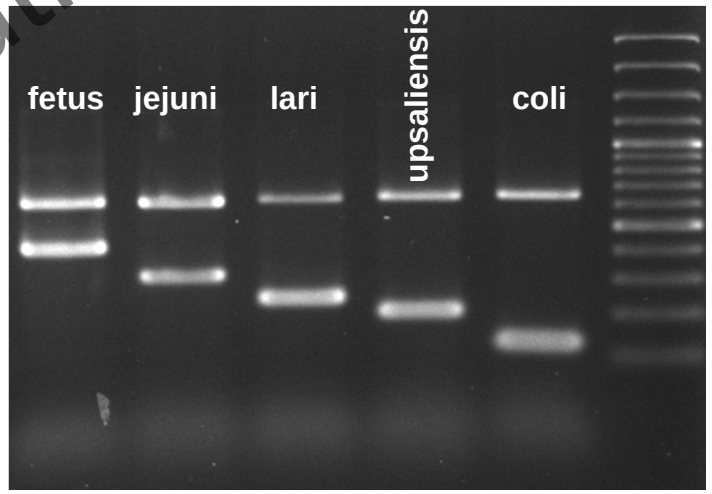
Nakari U-M, *et al*. 2008

- 28% of 145 hip– strains were *C. jejuni*
- → hip– strains should test by PCR

Wang G, *et al*. 2002

- PCR for identification

	Target	
C. jejuni	*hipO*	323 bp
C. coli	*glyA*	126 bp
C. lari	*glyA*	251 bp
C. upsaliensis	*glyA*	204 bp
C. fetus ssp. *fetus*	*sapB2*	435 bp



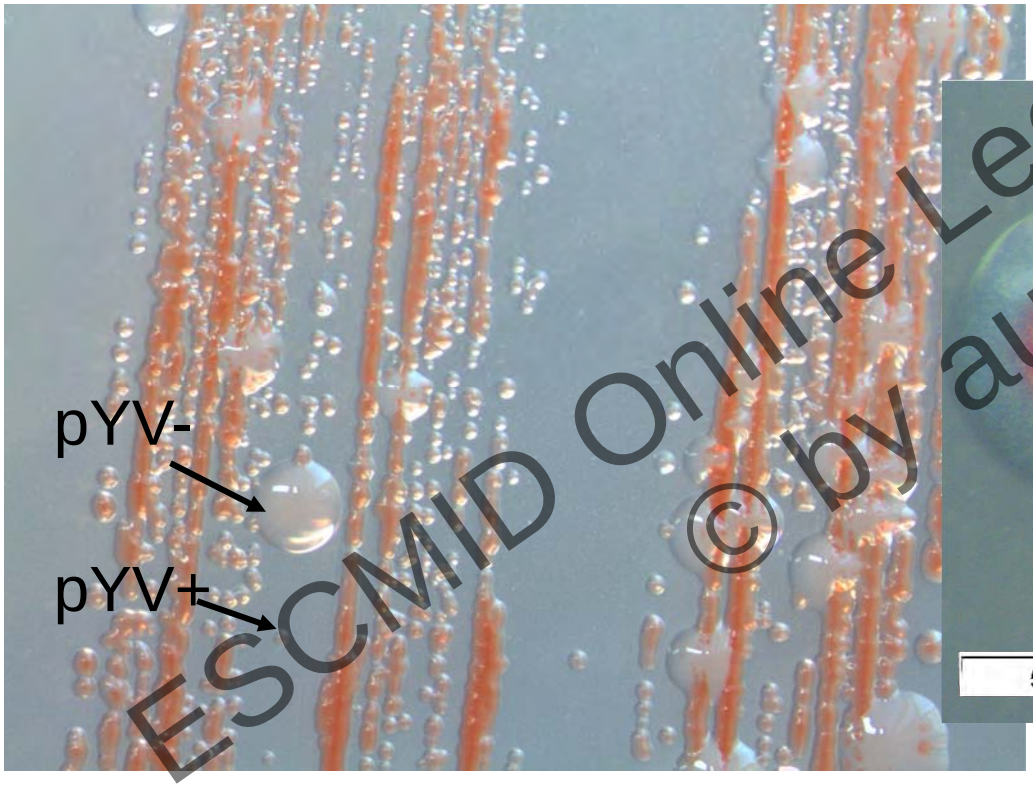
Identification of pathogenic *Yersinia enterocolitica*

- BT1B, BT2, BT3, BT4, BT5 → 5 pathogenic biotypes
  - In Europe most cases BT4/O:3
  - pYV virulence plasmid and chromosomal *ail* gene
    - *lcrE* and *ail* targets in PCR for pathogenic strains
- BT1A regarded as non-pathogenic biotype
  - lack those virulence markers
  - Recently *ail* found in BT1A strains
    - Sihvonen LM, *et al*. 2011
    - Kraushaar B, *et al*. 2011

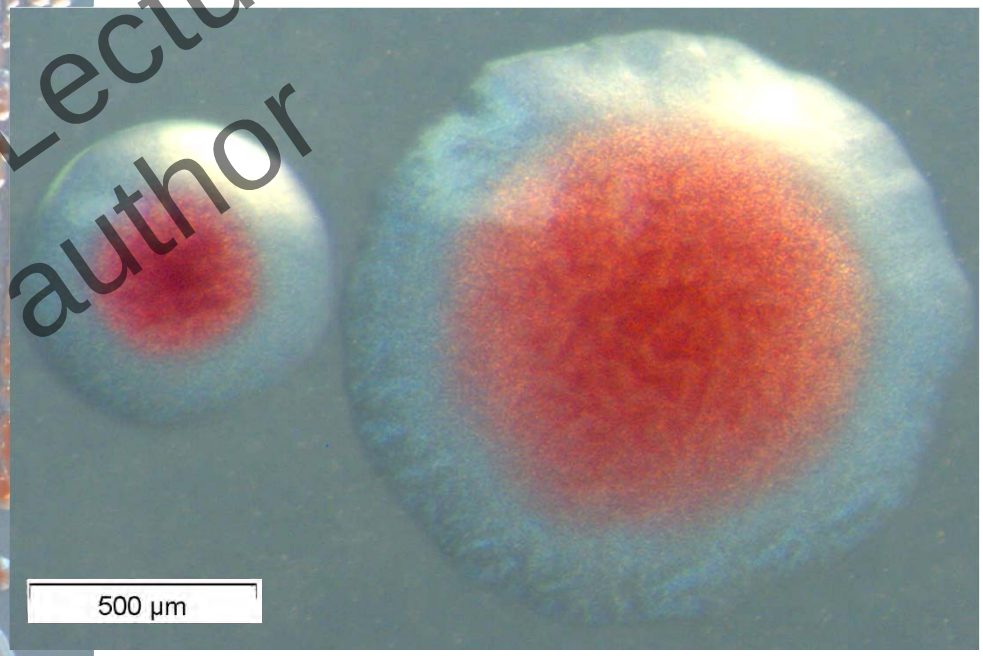
→ Conventional Petri dish methods still usable and reliable

Hallanvuo S, *et al*. 2006. Simplified phenotypic scheme…
Sihvonen LM, *et al* 2009. Identification…

Y. enterocolitica BT4/O:3 on CR-MOX (Congo-red magnesium-oxalate)



Colony morphology of *Y. enterocolitica* BT4/O:3 and BT1A through stereomicroscope on CIN



Photos: Leila Sihvonen

Conclusions

- Foodborne infections are preventable
  - Reliable diagnostic methods are important for surveillance and guiding public health actions
- PCR and other nonculture methods are here to stay
  - Will change the present data on etiology and epidemiology
  - Multiple pathogens in same specimen are found; which one is the likely cause of symptoms?
- Increased sensitivity of PCR may lead to over-diagnostics
  - Many studies showed lower sensitivity than culture
- PCR could be used as high-sensitive screening for a subset of specimens for culture
- Isolation of bacterial pathogens important for further studies

Thank you for your attention

Contact details
Anja Siitonen
National Institute for Health and Welfare (THL)
tel. +358 29 524 6000 (switchboard)
Postal address: P.O. Box 30, FI-00271 Helsinki, Finland
Street address: Mannerheimintie 166, Helsinki
e-mail: forenane.surname@thl.fi website: www.thl.fi

THL

NATIONAL INSTITUTE FOR HEALTH AND WELFARE, FINLAND