Phylogenetic Diversity of Coliform Isolates in USA

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Phylogenetic Classification

- group organisms together based on probable evolutionary relationships
- usually based on direct comparison of genetic material and gene products

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Constructing a "phylogenetic tree" for mickey mouse

Finding differences in their appearance (phenotype) or genetic code (genotype)

- Define characters: wearing shoes or not (1,0), color of skin (1,0), nose tilt (1,4)…

- Establish matrix:

```
M1  0 0 0 0 0 1
M2  1 0 1 0 1 1
M3  1 1 3 1 ? 1
M4  1 1 3 ? ? 1
M5  1 1 4 1 2 1
MX  1 1 2 1 2 1
```

- Calculate distance within the matrix
- Construct phylogenetic tree

From Professor Gustavo Caetano-Anolles

Constructing a phylogenetic tree based on phenotype
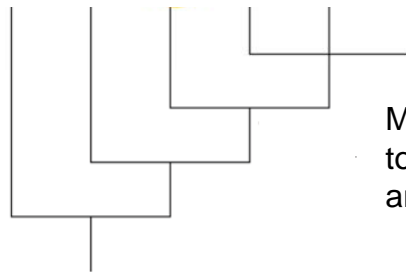
Mickey mouse X is more closer to M3, M4, and M5 than to M1 and M2

From Professor Gustavo Caetano-Anolles

Constructing a phylogenetic tree based on genotype

```
1.   TTGACATGCCGGGGAACCG
2.   TTCGACATGCCGGTGGTAACGCCG
3.   TTGACATCCTAGGAACGCCG
4.   TTCGACATGCTAGGGAACACCG
```
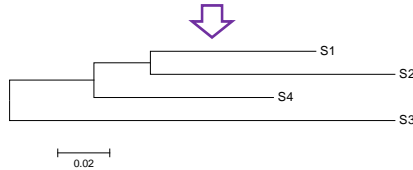
Characters: genetic code

```
1.   TT-GACATGCCGG--GG-AA--CCG
2.   TTCGACATGCCGGT-GGTAACGCCG
3.   TT-GACAT-CC--TAGG-AACGCCG
4.   TTCGACATG-C—TAGGGAACACCG
```

Establish matrix: alignment

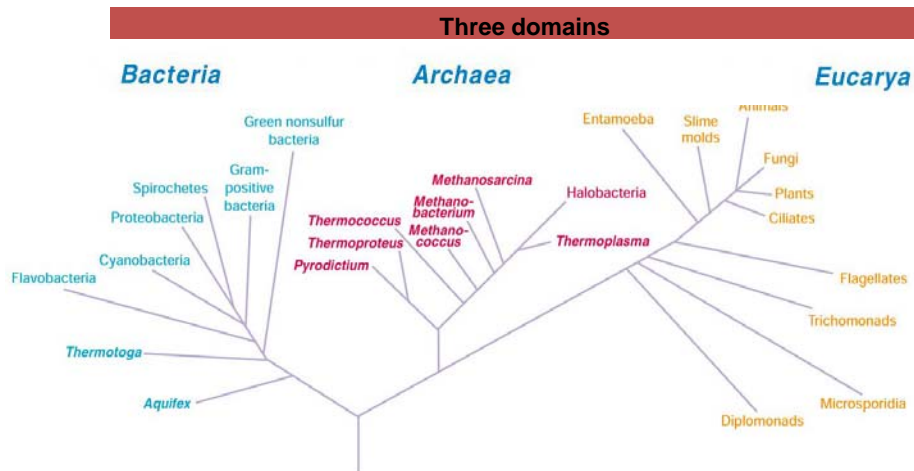|	S1	S2	S3	S4
S1	0.00			
S2	0.16	0.00		
S3	0.26	0.30	0.00	
S4	0.16	0.18	0.25	0.00

Calculate distance within the matrix
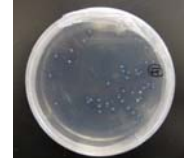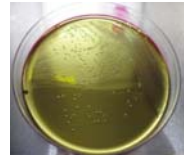
Construct phylogenetic tree

0.02

Phylogenetic systems

1. Direct comparison of genetic material and gene products such as **rRNA** and proteins
2. Three domains (Carl Woese 1970s)
3. Most accepted by microbiologists

Three domains

Bacteria

Green nonsulfur bacteria
Spirochetes
Gram-positive bacteria
Proteobacteria
Cyanobacteria
Flavobacteria
Thermotoga
Aquifex

Archaea

Methanosarcina
Methano-bacterium
Thermococcus
Methano-coccus
Thermoproteus
Pyrodictium
Halobacteria
Thermoplasma

Eucarya

Entamoeba
Slime molds
Animals
Fungi
Plants
Ciliates
Flagellates
Trichomonads
Microsporidia
Diplomonads

EPA approved coliform tests

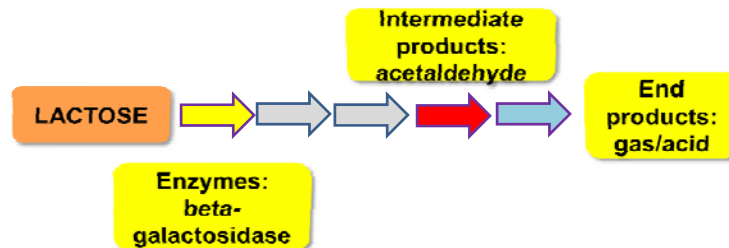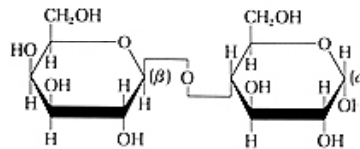| Technique	Medium	Working basis
MTF	LTB-BGLB broth	Lactose degradation and acid and gas production
	P-A broth-BGLB broth	Lactose degradation and acid and gas production
MF technique	m-Endo or LES-Endo-LTB, BGLB	Lactose degradation and metallic (golden) sheen production
	MI medium	MUGal-IBDG
	M-coliBlue 24®	Fermentation to produce color change. Use of X-Gluc to detect E. coli
	Chromocult®	Salmon-Gal and X-Gluc
	Coliscan® C MF	Salmon-Gal and X-Gluc
P/A tests	Colilert® Colilert-18®	ONPG-MUG
	Colisure®	CPRG-MUG
	Readycult® Coliforms 100	X-Gal-MUG
	E*Colite®	X-Gal-MUG
	Colitag™	ONPG-MUG
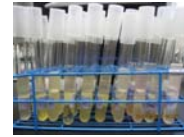
Use of lactose fermentation to detect coliforms

Lactose

Lactose is a disaccharide composed of D-galactose and D-glucose.

LACTOSE → → → → End products: gas/acid

Enzymes: beta-galactosidase

Intermediate products: acetaldehyde

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Leclerc, H. *et al.* 2001. Annu. Rev. Microbiol. 55:201-234.

EPA approved analytical methods

Medium	Working basis
MI medium M-coliBlue 24® ★ Chromocult® Coliscan® C MF Colilert® Colilert-18® Colisure® Readycult® Coliforms 100 E*Colite® Colitag™	Enzymes: beta-galactosidase (total coliform) & beta-glucuronidase (*E. coli*)
M-Endo	Intermediate product (acetaldehyde)
Lauryl Tryptose Broth (LTB) Presence-Absence (P/A) broth	End product: acid and gas production

★ For detection of total coliform, it targets on end product

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What are coliforms?

Definition of Total Coliform

- **Original:**
  - "Gram-negative, facultative anaerobic rod-shaped bacteria that ferment lactose to produce acid and gas within 48 h at 35-37° C".
- **Lactose Tryptose Broth**:
  - "all facultative anaerobic, Gram negative, non-spore-forming, rod shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35°C"
- **Membrane test**:
  - "… that develop red colonies with a metallic (golden) sheen within 24h at 35°C on an Endo-type medium containing lactose."
- **P/A (Beta-galatosidase) test**:
  - "A member of the *Enterobacteriaceae which produces the enzyme* β-D-galactosidase"
  - "a facultative, Gram-negative organism which grows at 35°C in the presence of bile salts, is cytochrome oxidase negative and produces the enzyme β-D-galactosidase"
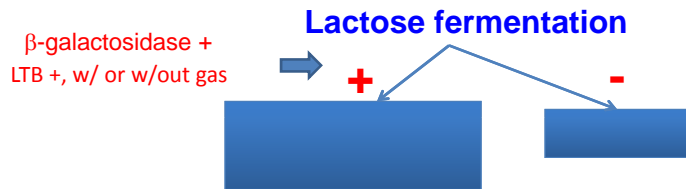 - *"Escherichia coli: bacteria giving a positive coliform response and possessing the enzyme β-D-glucuronidase"*.

WaterRF report 4024

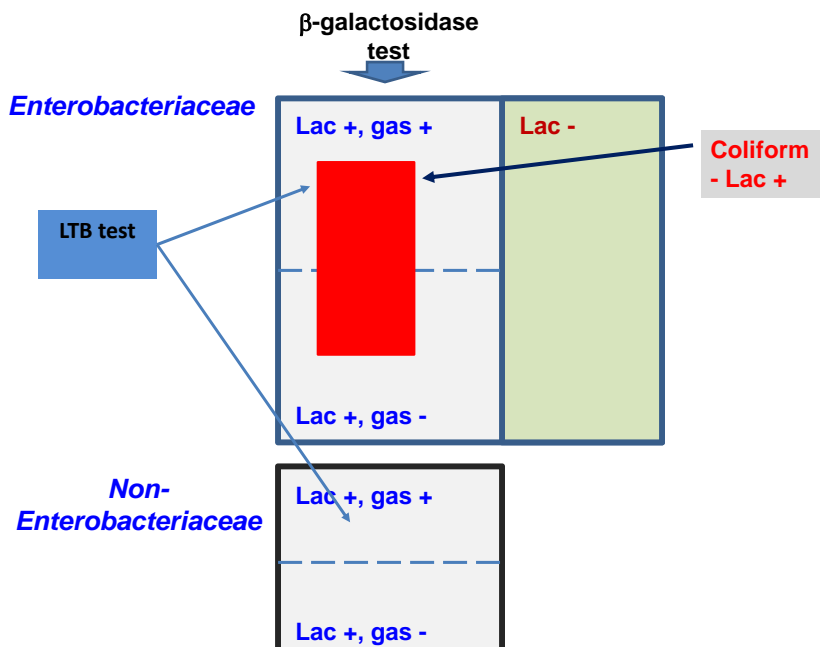# Classification by Bergey's Manual of Systematic Bacteriology, 2nd Edition
Facultatively Anaerobic Gram-Negative Rods

- Family:
  - *Enterobacteriaceae* (44 genera and 176 names species → 60 genera and 160 species in the next edition?)
    - *Arsenophonus, Budvicia, Buttiauxella, Cedecea, Citrobacter, Edwardsiella, Enterobacter, Erwinia, Escherichia, Ewingella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Moellerella, Morganella, Obesumbacterium, Pantoea, Pragia, Proteus, Providencia, Rahnella, Salmonella, Serratia, Shigella, Tatumella, Xenorhabdus, Yersinia, Yokenella*
  - Classification
    - Morphological traits
    - Phenotypic traits
      - Biochemical tests including substrate utilization, antibiotics resistant, etc…
    - Genetic traits
      - 16S rRNA gene analysis (species cutoff, 97% similar or higher)

Family *Enterobacteriaceae* – Phenotypic classification

β-galactosidase +
LTB +, w/ or w/out gas

Lactose fermentation

+   **-**

Combining phylogeny with phenotypes in TC testing

β-galactosidase test

Enterobacteriaceae

Lac +, gas +   **Lac -**

Coliform - Lac +

LTB test

Lac +, gas -

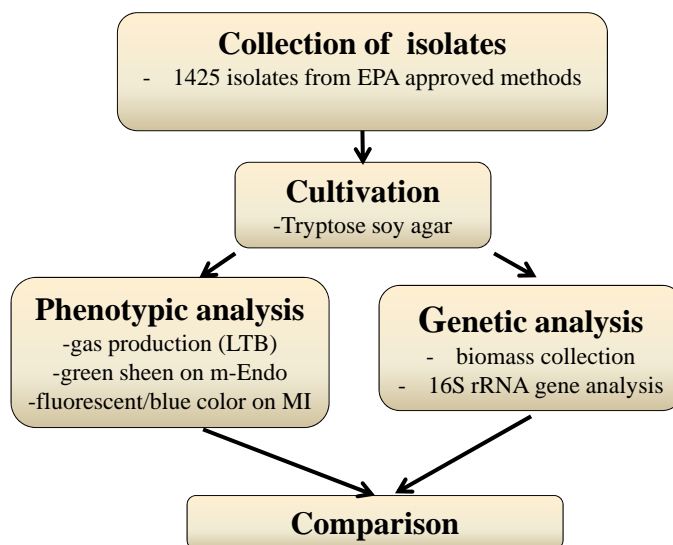*Non-Enterobacteriaceae*

Lac +, gas +

Lac +, gas -

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Experiment objectives

- What are coliforms from the view of phylogeny?

- How can phylogeny information of coliforms be related to current coliform tests?
  - True coliforms
  - False '+' and false '-'

Experimental procedure

Collection of isolates
- 1425 isolates from EPA approved methods

↓

Cultivation
-Tryptose soy agar

Phenotypic analysis
-gas production (LTB)
-green sheen on m-Endo
-fluorescent/blue color on MI

Genetic analysis
- biomass collection
- 16S rRNA gene analysis

Comparison

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Responses on different media

M-Endo   **LTB**   **MI**

+   **-**

Typical

Atypical

Non-coliforms

Colilert

Growth only   TC+

EC+   TC+ ₁₇ -
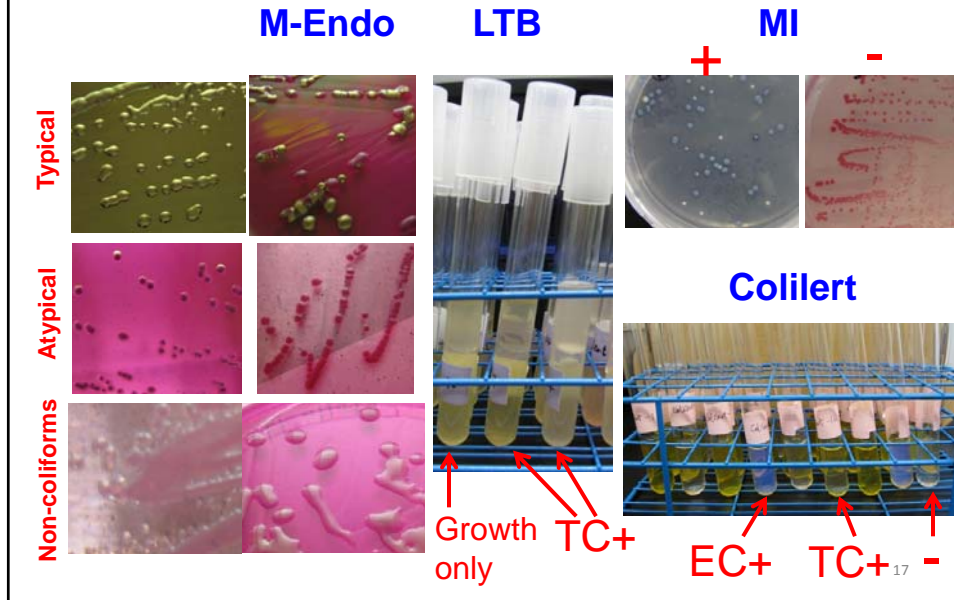
1425 bacterial isolates obtained from 12 different coliform tests

■ POS   ■ NEG

Test	Value
LTB	105
M-Endo	137
MI	123
M1	205
M2	109
M3	91
M4	91
M5	119
M6	112
M7	121
M8	94
M9	102

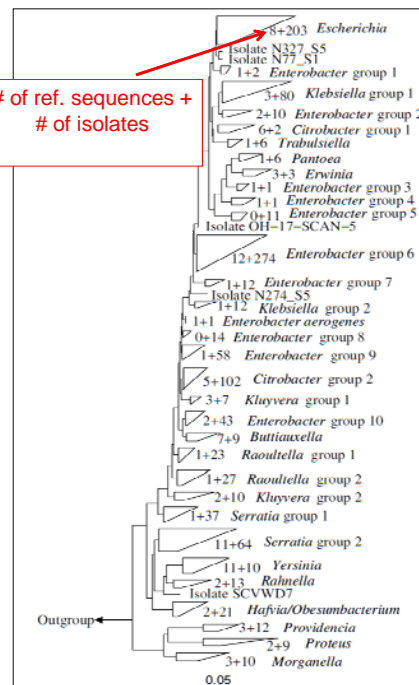*Number of isolates* (y-axis: 0, 50, 100, 150, 200, 250)
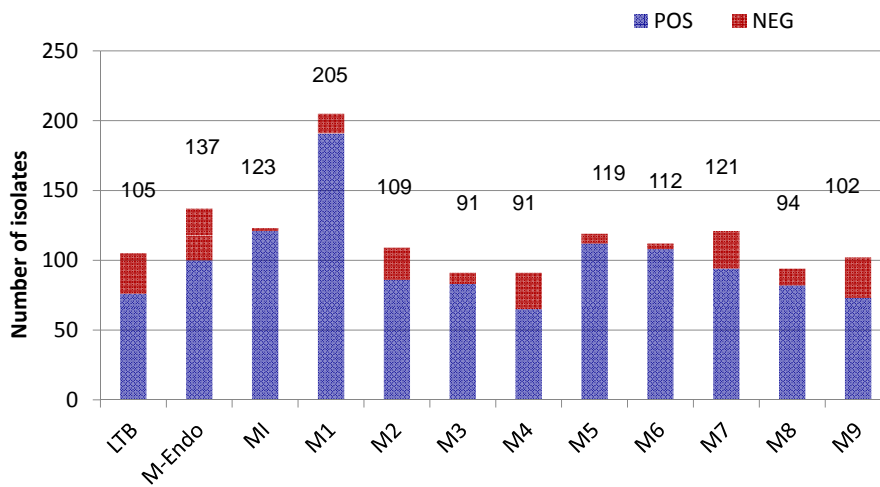
Criteria used for forming phylogenetic clusters

① 16S rRNA gene sequences that can form a cohesive cluster with known coliform bacteria at the **species** level within the family *Enterobacteriaceae*

② To group sequences that form a cohesive cluster with reference sequences at a known **genus** level.
- *Escherichia* cluster
- *Buttiauxella* cluster.

③ For sequences from a same genus that could not form a cohesive group, **more than one sub-cluster** was formed within each genus.
- *Enterobacter, Citrobacter, Klebsiella, Raoultella, Serratia,* and *Kluyvera*
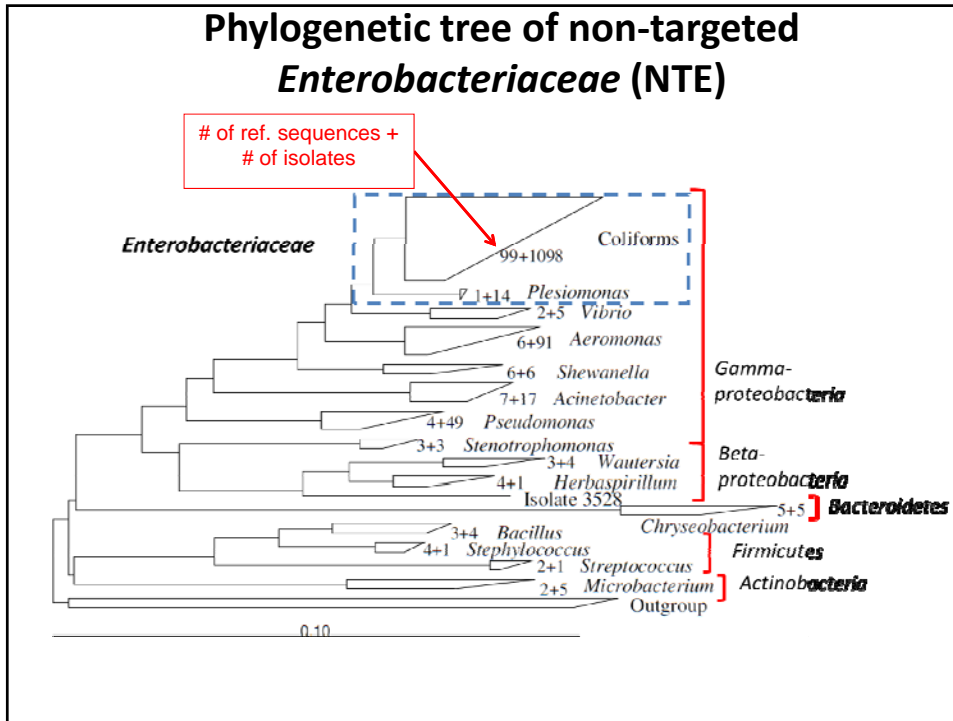- sequence similarity: 98.1% to 99.5%, higher than 'species' definition in microbial taxonomy.

Phylogenetic tree of targeted *Enterobacteriaceae* (TE)

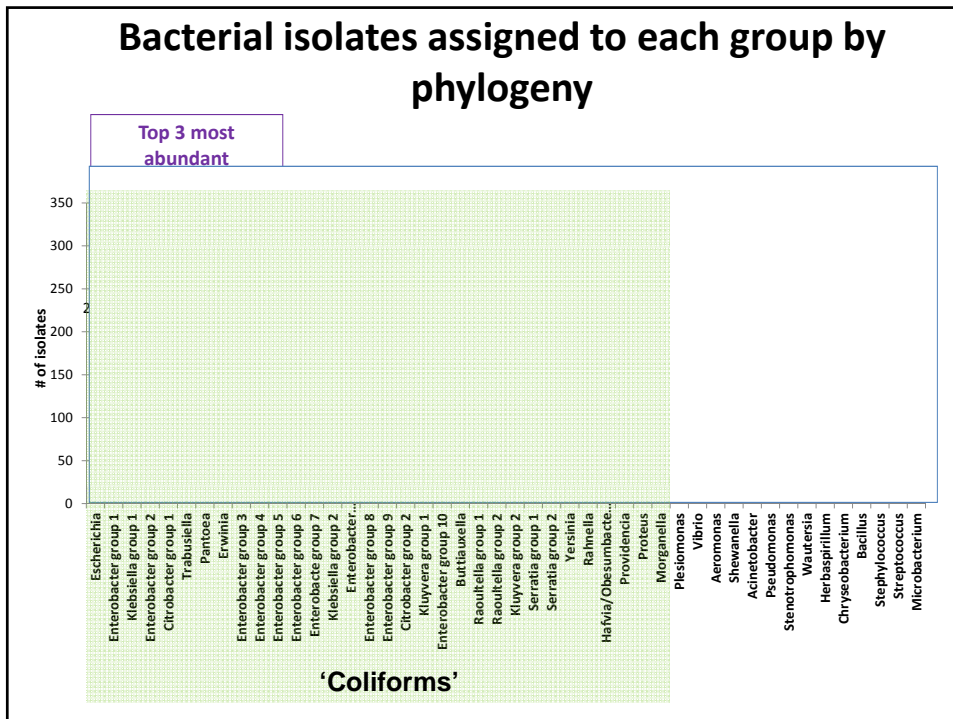- **32** groups are formed.
- Some genera contain more than 1 sub-cluster.

Genera	Group #
Enterobacter	10
Klebsiella	2
Citrobacter	2
Kluyvera	2
Raoultella	2
Serratia	2

# of ref. sequences + # of isolates

8+203 *Escherichia*
Isolate N327_S5
Isolate N77_S1
1+2 *Enterobacter* group 1
3+80 *Klebsiella* group 1
2+10 *Enterobacter* group 2
6+2 *Citrobacter* group 1
1+6 *Trabulsiella*
1+6 *Pantoea*
3+3 *Erwinia*
1+1 *Enterobacter* group 3
1+1 *Enterobacter* group 4
0+11 *Enterobacter* group 5
Isolate OH–17–SCAN–5
12+274 *Enterobacter* group 6
1+12 *Enterobacter* group 7
Isolate N274_S5
1+12 *Klebsiella* group 2
1+1 *Enterobacter aerogenes*
0+14 *Enterobacter* group 8
1+58 *Enterobacter* group 9
5+102 *Citrobacter* group 2
3+7 *Kluyvera* group 1
2+43 *Enterobacter* group 10
7+9 *Buttiauxella*
1+23 *Raoultella* group 1
1+27 *Raoultella* group 2
2+10 *Kluyvera* group 2
1+37 *Serratia* group 1
11+64 *Serratia* group 2
11+10 *Yersinia*
2+13 *Rahnella*
Isolate SCVWD7
2+21 *Hafvia/Obesumbacterium*
Outgroup
3+12 *Providencia*
2+9 *Proteus*
3+10 *Morganella*
0.05

Phylogenetic tree of non-targeted *Enterobacteriaceae* (NTE)

Bacterial isolates assigned to each group by phylogeny

Principles	Methods	POS (%)		NEG (%)	
		TE	**NTE**	**TE**	NTE
Enzymes	MI				
	M4				
	M5				
	M1				
	M2				
	M3				
	M7				
	M8				
	M9				
Intermediate product	m-Endo				
End product	LTB				
	M6				

(Membrane-based: MI, M4, M5; Liquid-based: M1, M2, M3, M7, M8, M9)

Summary

- 'coliforms':
  - at least 32 phylogenetic clusters.
  - Dominant phylogenetic clusters include for example *Enterobacter, Klebsiella, Pantoa, and Serratia*.
- 'non-coliforms':
  - at least 14 phylogenetic groups within and outside of the *Enterobacteriaceae*.
- Phylogenetic clustering together with coliform tests provides an effective way to identify coliform isolates that cause false-positive or false-negative results with different coliform testing methods.
  - False '+' rate: 0 – 67%
  - false '-' rate: 12 – 100%

Acknowledgement

- Water samples and coliform isolates provided by anonymous water utilities and testing laboratories

- WaterRF #4300

- WaterRF #4371