Molecular Epidemiology, Inc.





CONTRACT MICROBIOLOGICAL SERVICES



"Providing clear solutions to microbiological challenges""

Polyphasic Microbial Identification & DNA Fingerprinting

Microbial Contamination Tracking & Trending

cGMP/ISO-17025-2005 CLIA





What is in your Vial? The Requirement for Polyphasic Microbial Identification and Strain Characterization of Escherichia coli (E. coli) ATCC® 8739™

Jaspreet Sidhu, Ph.D. Molecular Epidemiology, Inc

15300 Bothell Way NE, Lake Forest Park, WA 98155 | P 206.306.8882 | F 206.306.8883 | sidhuj@molecularepi.com | www.molecularepi.com MEI © 2010



Overview

- Regulatory, Guidance and Economic Issues
- Microbial Identification in the Pharmaceutical Industry
- Methodologies and Tools for Microbial Identification
- Case Study linking Microbial ID, DNA Fingerprinting to demonstrate specific needs for Polyphasic Approach to Microbial Identification of a critical Compendia QC organism: *Escherichia coli (E. coli)* ATCC® 8739™



Microbial ID in the Pharmaceutical Industry

- The identification of microbes recovered from the pharmaceutical (manufacturing) environment is of critical concern
 - Regulatory requirement
 - Process requirement
 - Implications for product safety and efficacy



Concerns regarding Mis-identified Microbe

- Product Recall
- Economic Loss
- Regulatory Issues
- Patient Safety
- Public Relations



Level of Microbial Characterization and ID

- Gram Stain and cell morphology: EM in ISO 7/8, excipient derived isolates, finished product, below alert level EM
- ID to Genus: EM in ISO 5/6 with number below alert level
- <u>ID to Species</u>: EM in ISO 5 areas; alert and/or action level isolates from all excipient, finished product, EM and water monitoring
- <u>Strain Typing</u>: Significant product failures, e.g. media fill, sterility test and microbial limit test. Significant adverse trends in EM and water monitoring



Comparison of Phenotypic and Genotypic Approaches to Microbial ID

- Genotypic
 - DNA sequence based
 - Stable
 - Found in all organisms
 - Independent of environmental factors
 - Independent of protein expression
 - *Footnote-case study

- Phenotypic
 - Protein/enzyme based
 - Expression variability
 - Absent in some organisms
 - Environment and growth dependent
 - Lack of functional expression



How far has microbial ID evolved....

- Classification of bacteria based on:
 - Cellular morphology
 - Staining reactions (Gram, spore etc.)
 - Physiological requirements e.g. oxygen, pH, salt tolerance etc.
 - Biochemical (substrate utilization, metabolic profiles)
- Bergey's Manual of Determinative Bacteriology Ed. 9 now an in depth phylogenetic understanding of bacterial taxonomy: Bergey's Manual of Systematic Bacteriology, Ed. 2. "Critical role for DNA Sequencing"



Genotypic ID-Benefits

 "Genotypic methods have been shown to be more accurate and precise than traditional biochemical and phenotypic techniques. These methods are especially valuable for investigations into failures (e.g., sterility test; media fill contamination). However, appropriate biochemical and phenotypic methods can be used for routine identification of isolates"

FDA Guidance for Industry - September 2004 Sterile Drug Products Produced by Aseptic Processing Current Good Manufacturing Practice

This reflects general acceptance that changes in Microbial Taxonomy have been made as a result of advances in Phylogenetics and therefore genetic speciation is now considered the "Gold Standard"

MEI ©2010



Genotypic Methodologies

- DNA Sequencing-ID (16S/23S/28S highly conserved across organisms but also divergent amongst species)
- Pulsed Field Gel Electrophoresis of whole chromosomal DNA
- Southern blotting and Restriction Fragment Length Polymorphism (RFLP)
- PCR-based locus-specific RFLP
- Random Amplified Polymorphic DNA
- Rep-PCR
- Amplified Fragment Length Polymorphism



"Microbial Contaminant Mapping"

- Goal is to link effective Microbial Identification with Microbial Tracking and Trending
- Complete picture of potential contamination sources by matching genetic fingerprints to process flow throughout production (area, operators, raw materials, supply chain and inventory), packaging, QC testing and post-market
- Using a "risk based approach" to problem solving before problem escalates and production is impacted or worse still, actual finished product is discarded



MEI Polyphasic Approach to ID

- Using a Polyphasic approach that combines Phenotypic and Genetic ID (rRNA Gene Sequence) together with DNA Fingerprinting (Genetic Subtyping)
- Reduce the burden of mis-Identification as well as incomplete ID
- Complete the analysis to "Identify and Authenticate" fidelity of QC Microorganism or confirm source of potential contamination (e.g. Sterility Test Failure Investigation)



Aerobic/Anaerobic



MEI ©2010



MEI ©2010



Polyphasic Identification





Polyphasic approach is critical to provide differentiation between very closely related species of B. cereus group

Test		and the set of the	Test Results	-			9
Gram Stain Re Micro O C: Parasporal o Beta-hen	eaction scopic vidase atalase Spore Motility Crystal nolysis	Positive Rods Positive Subterminal, ellip Positive Negative Positive	tical, non-swollen		N.	N N	1 And
Genetic ID Comparisons to g	genetical	ly similar microorg	anisms	Notes: Phenotypic of	data support Ba	cillus cereu	s.
Genetic Distant	ce	Genus	Species				
0.0001 0.0003 0.0003	Bad Bad Bad	cillus cillus cillus	cereus anthracis thuringiensis				
Deviations	None						
Microbial ID Conclusion	Bacille	us cereus					





B. cereus







MEI ©2010



The Requirement for Polyphasic Microbial Identification and Strain Characterization of *Escherichia coli* (*E. coli*) ATCC® 8739™

"Identify and Authenticate™"



Escherichia coli (E. coli) ATCC® 8739™

Isolated pure colonies Subjected to: Genetic (16s), Phenotypic and PFGE Analysis

MEI ©2010



Escherichia coli (E. coli) ATCC® 8739™



Raw Electropherogram



16S SSU rRNA Sequence: Escherichia coli (E. coli) ATCC® 8739™

GGCTTTTCTGCGGGTACGTCATGAGCAAAGGTATAACTTTACTCCCTTCC TCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGC ATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCT CCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTC TCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCCCACCTACTA GCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCCCGAAGGTCCCCCT CTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTCCAGTAGTTAT CCCCCTCCATCAGGCAGTTTCCCAGACATTACTCACCGTCCGCCACTCG TCAGCAAAGAAGCAAGCTGCTTCCTGTTACCGTTCGACTTGCATGTGTA GGCCTGCCGCCAGCGTTCAATCTGAGCAGGATCAAAACTCAAA



16S ID is only possible to "Family Level"

Genetic Distance	Genus	Species
0.0000	Shigella	sonnei
0.0000	Shigella	flexneri
0.0000	Escherichia	coli
0.0000	Shigella	boydii
0.0000	Escherichia	sp.
0.0007	Enterobacter	hormaechei
0.0019	Shigella	dysenteriae
0.0022	Enterobacter	sp.
0.0031	Cronobacter	sakazakii
0.0081	Citrobacter	freundii
0.0090	Enterobacter	dissolvens
0.0100	Klebsiella	oxytoca



Genetic Similarity Comparison of select Enterobacteriaceae





Phenotypic Characterization of select Enterobacteriaceae

Sample Description	ı	Cultural and Phenotypic Data			
Sample Name	Sample Source	Colony Morphology	MaC (Lactose)	Indol	Vitek GNI Number
Escherichia coli ATCC 8739	ATCC Submission	Flat	+	+	GNI 6204724631
Escherichia coli ATCC 8739	MEI Frozen Stock	Flat	.	+	GNI 6204724631
Escherichia coli ATCC 8739	Customer QC Strain	Flat	+	+	GNI 6204724631
Klebsiella pneumoniae ATCC 10031	ATCC Submission	Raised (inhibited)	+	-	GNI 6634773631
Klebsiella oxytoca ATCC 43863	ATCC Submission	Raised	÷	t	GNI 6674773631
Shigella sonnei ATCC 25931	ATCC Submission	Flat	-	[]	GNI 6000300632
Escherichia coli ATCC 25922	MEI Frozen Stock	Raised B -Hemolytic	+	+	GNI 6004720633
Escherichia coli O157:H7 ATCC 35150	MEI Frozen Stock	Raised	+	+	GNI 6004754633
Escherichia coli O157:H7	MEI Lab Isolate	Raised	+	+	GNI 6004754633
Escherichia coli	Environmental Isolate	Flat	Weak	+	GNI 6004724631



Polyphasic ID MEI 35065 ATCC® 8739[™] Source: ATCC

Test			Test Results	6 m 20 11 12 1
Test Ba Gram Stain Re Micros Ox Ca Indologe Acid from X	asic ID action scopic xidase talase enesis Kylose	Bacterium Negative Rods Negative Positive Positive Positive	Test Results	
Acid from Su Acid from La Acid from So Genetic ID	actose orbitol	Negative Positive Positive	poleme	Notes:
Genetic Distanc	e	Genus	Species	Lysine decarboxylase: Positive
Ocenetic DistanceGenus0.0000Shigella0.0000Shigella0.0000Escherichia0.0000Shigella0.0000Escherichie0.0007Enterobacter0.0019Shigella		sonnei Malonate Utilization: Negative flexneri Acid from Adonitol: Negative coli Citrate Utilization: Negative boydii Acid from Raffinose: Negative sp. Phenotypic data support Escherichia coll. hormaechei dysenteriae		
Deviations	None			
Microbial ID Conclusion	Escher	richia coli		



Polyphasic ID MEI 35068 ATCC® 25931[™] Source: ATCC

Test	Test Results			
Ba	asic ID	Bacterium		· · · · · · · · · · · · · · · · · · ·
Gram Stain Re	action	Negative		1 - 6 6 V. F 14 -11
Micros	scopic	Rods		Call of the state of the state of the
0:	xidase	Negative		1
Ca	Italase	Positive		1 27 4 1 3
Indologe	enesis	Negative		1 + - + - + + + - + - + - + - + - +
Acid from Xylose Acid from Sucrose		Negative		1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1. 1
		Negative		L'al manager and a state
Acid from La	actose	Negative		The start of the
Acid from Se	orbitol	Negative		and the set of the
Genetic ID Comparisons to g	jenetical	ly similar microorg	anisms	Notes: Additional Tests and Results:
Genetic Distanc	:e	Genus	Species	Lysine decarboxylase: Negative
0.0000	Shi	gella	sonnel	O-nitro-phenyl b-galactosidase: Positive
0.0000	Shi	gella	dysenteriae	Ornithine decarboxylase: Positive
0.0000	Shi	gella	flexneri	Phenotypic data support Shigella sonnei.
0.0000	Esc	herichia	coli	
0.0000	Shi	galla	boydii	
0.0000	Esc	herichia	sp.	
0.0014	Klei	bsiella	oxytoca	
Deviations	None			
Microbial ID Conclusion	Shigel	lla sonnei		



Polyphasic ID MEI 35066 ATCC® 10031[™] Source: ATCC

Test			Test Results	4 . 1
Ba Gram Stain Rea Micros Ox Cat Indologe Urea Hydro Citrate Utilia	sic ID action copic tidase talase mesis olysis zation	Bacterium Negative Negative Positive Negative Positive Positive		
Genetic ID Comparisons to ge Genetic Distance	enetical	ly similar microorg Genus	anisms Species	Notes: Phenotypic data support Klebsiella pneumoniae.
0.0000 0.0000 0.0028 0.0058 0.0110 0.0110 0.0112	Klei Klei Klei Sen Sen Rad	osiella osiella osiella ratia ratia outtella	pneumoniae granulomatis singaporensis variicola nematodiphila marcescens ornithinolytica	
Deviations	None			
Microbial ID Conclusion	Klebsi	ella pneumonia	e	



Polyphasic ID MEI 35067 ATCC® 43863[™] Source: ATCC

Test			Test Results	
B	asic ID	Bacterium		2 A 8 6 A 1 5
Gram Stain Re	action	Negative		n 74 - 348
Micro	scopic	Rods		· · · · · · · · ·
0	xidase	Negative		
Ci	atalase	Positive		at with a start way of
Indolog	enesis	Positive		9 . 1 . 18 . 6
Acid from L	actose	Positive		- 12 . 3".
Urea Hyd	rolysis	Positive		1 m 1 1 1 1 1
Citrate Util	ization	Positive		1 - 1 - 5 - 7 - 4
Acid from A	donitol	Positive		1 - + + - of - one
Comparisons to g	genetical	ly similar microorg	anisms	Notes: Phenotypic data support Klebsiella oxytoca.
Genetic Distant	ce	Genus	Species	
0.0000	Kle	bsiella	oxytoca	
0.0027	Kle	bsiella	pneumoniae	
0.0030	Ent	erobacter	cancerogenus	
0.0057	Ent	erobacter	cloacae	
0.0078	Par	Noea	aggiomerans	
0.0078	Ent	erobacter	dissolvens	
0.0095	Ent	erobacter	aerogenes	
Deviations	None			
Microbial ID Conclusion	Klebsi	iella oxytoca		



The Requirement for Polyphasic Microbial Identification and Strain Characterization of Escherichia coli (E. coli) ATCC® 8739™

"What is it and is it the right one?"



PFGE Analysis for Select Enterobacteriaceae















Note
ATCC [®] 8739 [™] ◀
ATCC [®] 43863 [™]
ATCC [®] 25931 [™]
ATCC [®] 10031 [™]





Polyphasic ID MEI 35069 ATCC® 25922™ Source: MEI

Test			Test Results	·
E Gram Stain R Micro C Indolo Acid from Acid from S Acid from I	Basic ID leaction oscopic Oxidase Catalase genesis Xylose Sucrose Lactose	Bacterium Negative Rods Negative Positive Positive Positive Negative Positive		A Star top 200
Genetic ID Comparisons to	genetica	lly similar microorg	anisms	Notes: Additional Tests and Results:
Genetic Distance Genus 0.0020 Shigalla 0.0020 Escherichia 0.0020 Escherichia 0.0078 Shigella 0.0079 Shigella 0.0079 Shigella 0.0079 Shigella 0.0079 Shigella 0.0079 Shigella 0.0086 Escherichia 0.0098 Enterobacter		Species dysentariae coli sonnei flexneri boydii sp. hormaechei	Acid from Adonitol: Negative Acid from Adonitol: Negative Citrate Utilization: Negative Acid from Raffinose: Negative Phenotypic data support Escherichia coli.	
Deviations	None			
Microbial ID Conclusion	Escherichia coli			



Polyphasic ID MEI 35065 ATCC® 8739[™] Source: ATCC

Test		Test Results	4 - 12	11 5 F2
Basi	c ID Bacterium		12 1.3	* 5 TTE
Gram Stain Reac	tion Negative			1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1
Microsco	opic Rods		6 18 43 . 37	
Oxid	lase Negative		3	2.30
Cata	lase Positive			and a state
Indologen	esis Positive		4 23 4 2 4	P
Acid from Xylose Positive			· · · · · · · · · · · · · · · · · · ·	Sec. 1. 3.
Acid from Sucr	ose Negative		Sec. 2 5	2.42.34
Acid from Lact	tose Positive		P 9. 200 4 4	2
Acid from Sort	bitol Positive		11 5 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4 4	· · · ·
Genetic ID Comparisons to gen	etically similar microorg	anisms	Notes: Additional Tests and Results:	
Genetic Distance	Genus	Species	Lysine decarboxylase: Positive	
0.0000	Shigella	sonnei	Malonate Utilization: Negative	
0.0000	Shigella	flexneri	Acid from Adonitol: Negative	
0.0000	Escherichia	coli	Acid from Raffinose: Negative	
0.0000	Shigella	boydii	Phenotypic data support Escherichia c	oli.
0.0000	Escherichia	sp.		
0.0007	Enterobacter	hormaechei		
0.0019	Shigella	dysenteriae		
Deviations N	one			
Microbial ID Conclusion E	scherichia coli			



Polyphasic ID MEI 35075 Source: Environmental Isolate

Test		Test Results	This and have the second
Gram Stain Rea Microso Oxi Cat	sic ID Bacteriu ction Negativ copic Rods idase Negativ alase Positive	e	
Acid from Xy Acid from Sud Acid from Lac Acid from Lac Acid from So	viose Positive crose Negative ctose Positive rbitol Positive	6	
Genetic ID Comparisons to ge	netically similar	microorganisms	Notes: Additional Tests and Results:
Genetic Distance	Ge	Genus Species	Lysine decarboxylase: Positive
0.0032Escherichiacoli0.0054Shigelladysen0.0055Shigellasonne0.0056Shigellafiexne0.0056Shigellaboydi0.0057Escherichiasp.0.0172Escherichiaalbert		coli dysenteriae sonnei flexneri boydii sp. albertii	Malonate Utilization: Negative Acid from Adonitol: Negative Citrate Utilization: Negative Acid from Raffinose: Negative Phenotypic data support <i>Escherichia coli</i> .
Deviations /	Vone		
Microbial ID Conclusion	Escherichia c	oli	



Polyphasic ID MEI 35071 ATCC® 35150[™] Source: MEI Note: O157:H7

Test	Test Test Results			1	1.4			
Ba	asic ID	Bacterium			1. W. J.M			
Gram Stain Re	action	Negative			and the second			
Micro	scopic	Rods			" A A A 2			
0	xidase	Negative			1. 1. 1. 1.			
Ca	Italase	Positive		the same has a				
Indologenesis Positive Acid from Xylose Positive Acid from Sucrose Positive Acid from Lactose Positive		Positive Positive			1 10 - 1 - 2 5 T			
					1 1 1 1 1 1 1			
		Positive						
				1 2 3.4				
Acid from S	orbitol	Negative			1 " ~ " !			
Genetic ID Comparisons to g	genetical	ly similar microorg	anisms	Notes: Additional Te	sts and Results:			
Genetic Distance	e	Genus	Species	Lysine decarboxylase: Positive				
0.0000	Esc	herichia	coli	Malonate Util	ization: Negative			
0.0063	Shi	gella	dysenteriae	Citrate Utiliza	tion: Negative			
0.0063	Ent	erobacter	hormaechei	Acid from Ad	sitol: Negative			
0.0077	Cro	onobacter sakazakii iterobacter sp.		Phenotypic data support Escherichia coli.				
0.0080	Ent			*Serotyping necessitated by Sucrose and Sorbito	ecessitated by Sucrose and Sorbitol rxn.			
0.0111 Sh		igella flexneri		50 ST	120			
0.0111	Shi	gella	boydii					
Deviations	None							
Microbial ID Conclusion	Esche	richia coli*						



Polyphasic ID for MEI 35073 Source: MEI Lab Isolate Note: O157:H7

Test		Test Results				
Ba	sic ID	Bacterium			5	
Gram Stain Rea	nction	Negative				
Micros	copic	Rods			2	
Ox	idase	Negative			1	
Cat	talase	Positive				
Indologenesis Acid from Xylose		Positive			F	
		Positive			- 0 4" 1-	
Acid from Sucrose		Positive				
Acid from Lactose		Positive				
Acid from Sorbitol		Negative			· · · ·	
Comparisons to genetically similar microorganisms				Notes: Additional Tests and Results:		
Genetic Distance		Genus	Species	Lysine dec	Lysine decarboxylase: Positive	
0.0000	Esc	herichia	richia coli	Malonate Utilization: Negative		
0.0054	Enterobacter		sp.	Citrate Utilization: Negative		
0.0057	Ent	erobacter	hormaechei	Acid from Inopitol: Negative		
0.0067 Shi 0.0094 Cro 0.0096 Shi 0.0110 Shi		gella	dysenteriae sakazakii	Phenotypic data support Escherichia coli. Serotyping necessitated by Sucrose and Sorbitol rxn.		
		nobacter				
		gella	boydii		5	
		gella	flexneri			
Deviations	None					
Microbial ID Conclusion	Escherichia coli*					



Multiplex PCR Analysis of Pathogenicity markers for Unknown E. coli





PFGE Analysis for Select Enterobacteriaceae





PFGE Comparison for Select Enterobacteriaceae





Conclusion

- Study demonstrates the limitations of genetic based ID (16S rRNA) in ID of *Escherichia coli* ATCC® 8739[™] relative to other select closely related Enterobacteriaceae as well as other *Escherichia* and potential pathogenic forms of *E. coli* such as serotypes of O157, EHEC, ETEC, EPEC, EIEC
- Use of a Polyphasic approach that combines genetic derived information with key biochemical and phenotypic characteristics to correctly "Identify" *Escherichia coli* ATCC® 8739[™] and exclude potential laboratory and or process related cross contaminants
- Use of PFGE DNA Fingerprint to "Authenticate" and "Verify" ATCC derived QC strain of *Escherichia coli* ATCC® 8739™