Wisconsin State
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UNIVERSITY OF WISCONSIN–MADISON

Molecular Diagnostics and Mycobacteria

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Disclosures

- I am an Assistant Director at the State Lab
- This talk is for educational purposes only and is not intended as an advertisement
- I am not paid by any manufacture and will receive no benefits from this talk.

Overview of the Major Molecular Diagnostic Methods Currently Available for Mycobacteria

- Background of methods
- When we use them
- How they work
- Advantages
- Disadvantage

Mycobacterial Culture is Slow

- Slow rate for full lab report
  - TB in culture: 38 days
  - NTM: 28 days
  - NTM rapid growers: 12 days
- Big impact of slow diagnostics
  - Exposes more people to disease
  - Delays start of treatment
  - Delays appropriate therapy (MDR)

We've tried to speed things up

- Concentrating samples
- Media optimization (Broth cultures)
- Automation to detect growth
- Faster ID methods (MALDI)

They only grow so fast-
We need better methods!

Molecular Revolution in Diagnostics

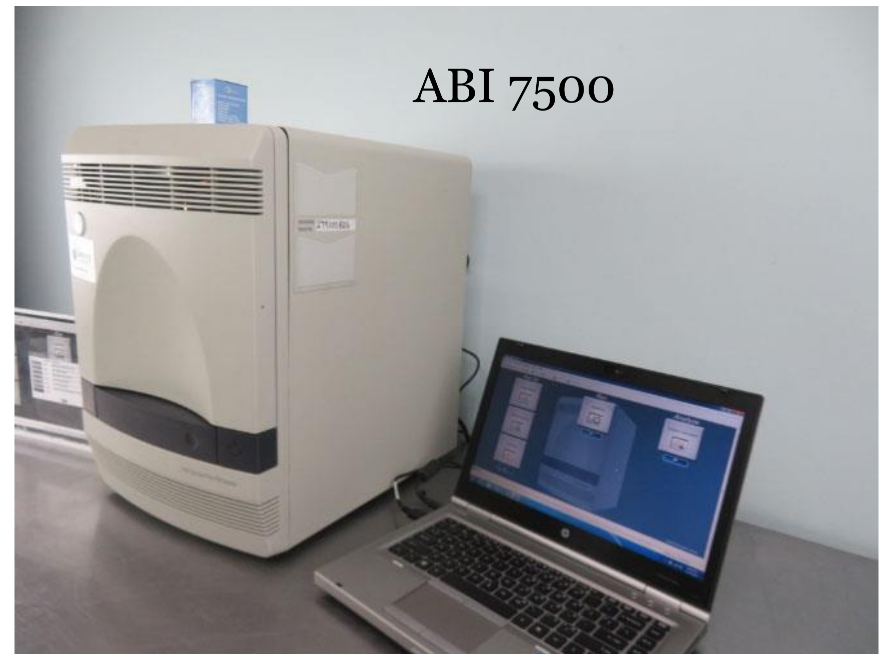
- Rapid advancement in nucleic acid based diagnostics
- Can detect even very small amounts of bacteria
- Does not require growth of the organism
- Huge advantage for Mycobacteriology!

Methods used at WSLH

- PCR
  - TB/MAC PCR
  - GeneXpert MTB/Rif
- Sanger Sequencing
  - 16S
  - RpoB
- NEW! NGS Sequencing

TB/MAC PCR

- Protocol developed at the Wadsworth Center, New York State Public Health Lab
- Real-time PCR
  - Taqman Probe based
- 96 well format



When we use it

- Many sample types: sputum, tracheal aspirates, BALs, tissues, and other fluids.
- Done on sterile site samples and decontaminated samples
- Can be done on isolates and positive broths
- Same day turn around time ~4 hours
- Smear positive samples (due to sensitivity)

How it works

Detects but does not differentiate:

- TB complex members including *M. africanum, M. bovis, M. bovis* BCG, *M. canettii, M. caprae, M. microti*, and *M. tuberculosis*
- *M. avium* complex members including *M. avium, M. avium* subsp. *avium, M. avium* subsp. *paratuberculosis, M. intracellulare, M. chimaera, M. arosiense, M. colombiense, M. marseillense, M. bouchedurhonense*, and *M. timonense*

The Process

- Extraction
  - Bead beat
  - Heat lyse
  - Proteinase K
  - Qiagen spin column extraction

- Amplification
  - 7 controls:
    - MTBC positive, MTBC negative, MTBC NTC
    - MAC positive, MAC negative, MAC NTC
    - RNP positive (human DNA)
  - Pre-made MasterMix stable at -20C of for 31 days

The Targets

- TB PCR:
  - Two primers and a fluorescent dye-labeled probe
    - Targeting a 74-base pair region of insertion sequence 6110 (*IS6110*)
    - Present in TB genomes in multiple copies

- MAC PCR:
  - Six primers and two fluorescent dye-labeled probes
    - Targeting the 16S-23S internal transcribed spacer (ITS) region of MAC

Principle

1b

Forward primer

R TaqMan Probe Q

3' 5'

5' 3'

Reverse primer

Q

3' 5'

5' 3'

40 cycles

$2^{40} \approx$

1000 billion copies

template DNA

1st cycle

2nd cycle

3rd cycle

$2^2 =$ 4 copies

$2^3 =$ 8 copies

$2^4 =$ 16 copies

32 copies

Interpretation

C_t Value

Sample

Fluorescence

No Template

Baseline

Number of Cycles

Disadvantages

- Unable to differentiate members of TB and *M. avium* complexes
- High complexity test requires special training and skill
- Requires special "PCR clean" work-flows and spaces
- Reagent and equipment can be cost prohibitive
- Not FDA approved so requires LDT level validation and oversight
- MAC PCR assay may cross react with *M. nebraskense*

TB/MAC PCR Features

- Culture independent, rapid detection of TB and MAC (same day!)
- Available for many sample types
- As sensitive as AFB stain
- Can be scaled up (cheaper)
- Cheaper than other molecular systems

Cepheid GeneXpert

- Real-time PCR
- For the rapid detection of TB and Rifampin resistance
- Single use, self contained cartridges

https://www.youtube.com/watch?v=mIsBLmjus6Q

Buffer chambers

Cartridge base

Cap

Valve body

Syringe barrel

Gasket

PCR tube

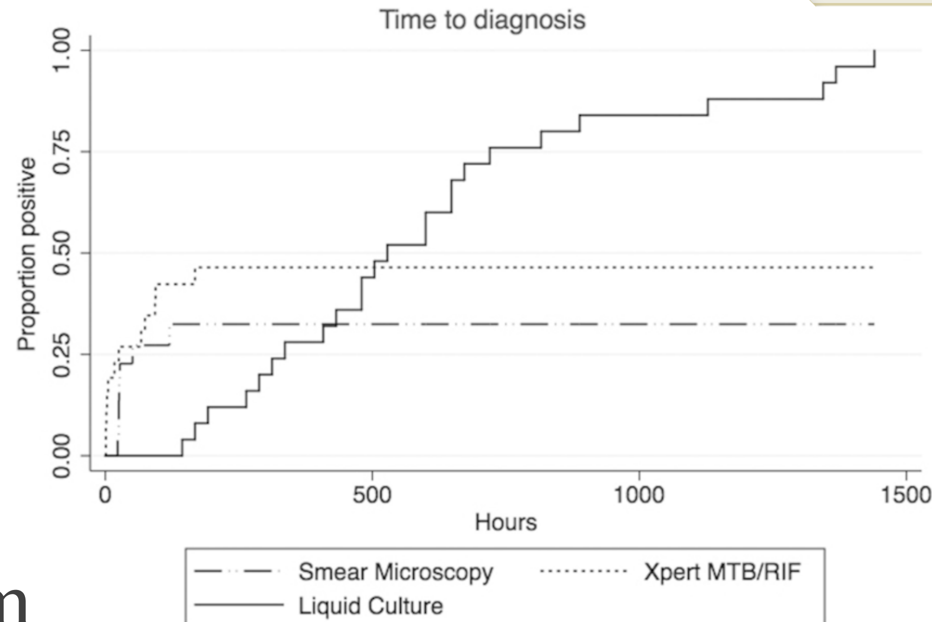
Cartridge body

GeneXpert

1 2 4 16 48

GeneXpert Features

- Random Access
- Drug resistance
- Fast and Easy
  - Set-up ~5 min/sample
  - Results ~2 hours
- FDA cleared for sputum
- Only Moderate complexity
- Does not require molecular workflows
- Platform also can run other diagnostic tests



Sohn, H et al. J Clin. Inf. Dis. 2014

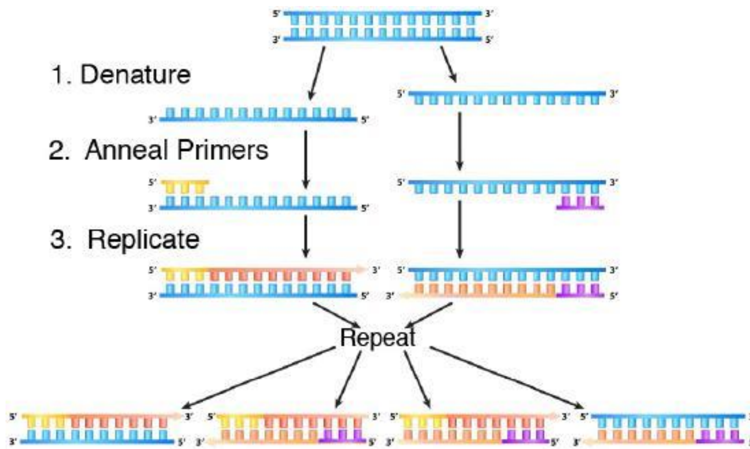
GeneXpert Disadvantages

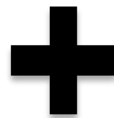
- Only Sputum FDA cleared, BAL is LDT
- Does not detect NTM
- Cannot distinguish strains in the complex
- Expensive platform and reagents
- Cultured still required for negative and positive samples
- Sensitivity (46%) low
  - Improved (86%) for smear positive patients
- Still need drug susceptibility testing
  - Does not detect all Rifampin resistance

Sanger Sequencing

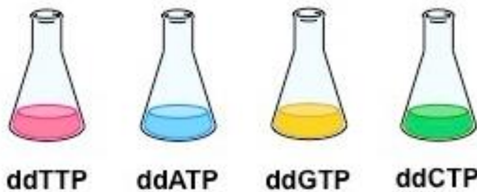
- Sequencing of the 16S and RpoB genes to aid in the identification of mycobacteria

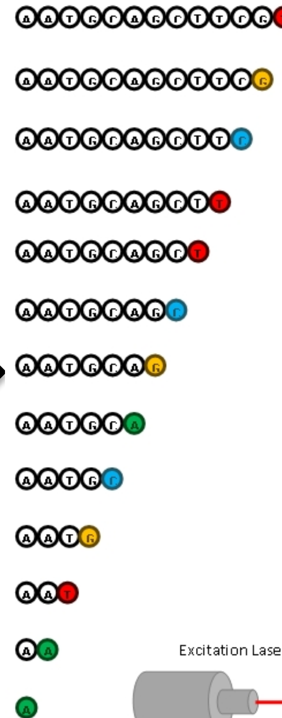
How it works

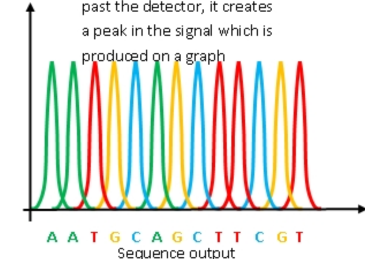




4 × PCR (+ one dideoxynucleotide)







Data Analysis

Strain Query	Genomic island Start position	Genomic island End position	Length (bp)	Total score	Query cover	E.value	Max
Mycobacterium massiliense strain M139	1922873	1992300	69427	1.12E + 005	99 %	0	99 %
Mycobacterium massiliense strain M139	4564885	4576047	11162	8504	74 %	0	80 %
Mycobacterium massiliense strain M139	2800023	2810210	10187	3918	77 %	0	81 %
Mycobacterium massiliense strain M139	254418	263728	9310	1960	38 %	0	84 %
Mycobacterium massiliense strain 23	870688	892899	22212	21406	63 %	0	99 %
Mycobacterium massiliense strain 23	2385439	2401289	15851	10443	56 %	0	89 %
Mycobacterium abscessus strain 137	1922873	1992300	69428	1.07E + 005	88 %	0	99 %
Mycobacterium abscessus strain 137	2172909	2194917	22009	3410	41 %	0	98 %
Mycobacterium abscessus strain 137	4564885	4576047	11163	2879	55 %	0	93 %

[a]First nucleotide blast result with more than 80 % identity, and covering at least Genomic Island
[b]pathogenic mycobacteria

Features

- Quality identification of mycobacteria with a comprehensive library
- Can also be used for other bacteria (16S) and even fungi (ITS genes)
- Cheaper and faster than NGS

Disadvantages

- Similar to TB/MAC PCR
  - High complexity, LDT, PCR clean workflows, special training
- Additional expensive equipment
- Sensitivity poor from primary samples

Molecular Typing

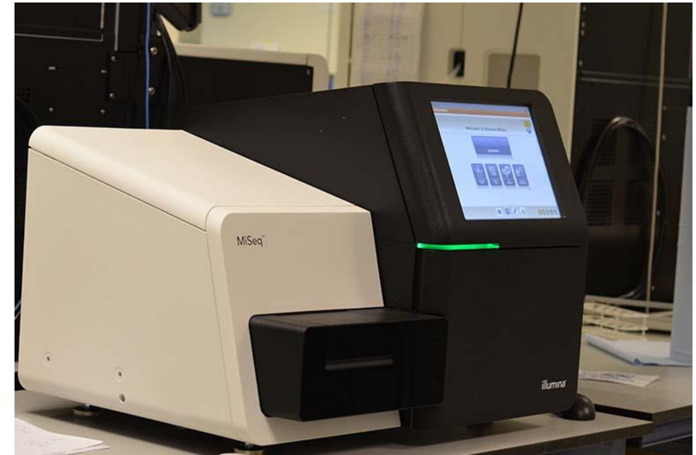
- Spoligotyping
  - Direct Repeat locus
  - 43 unique spacers
  - Numeric code
- MIRU-VNTR
  - Repeats at multiple loci
  - Numeric code
- Next Generation sequencing

Comas, et al. PLoS ONE 4(11):27815 Nov. 2009

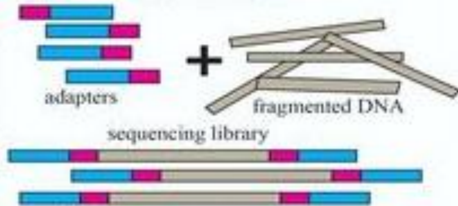
Cost per Genome

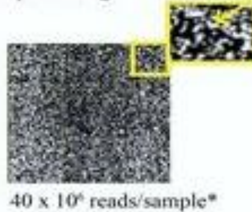
Next Generation Sequencing (NGS)

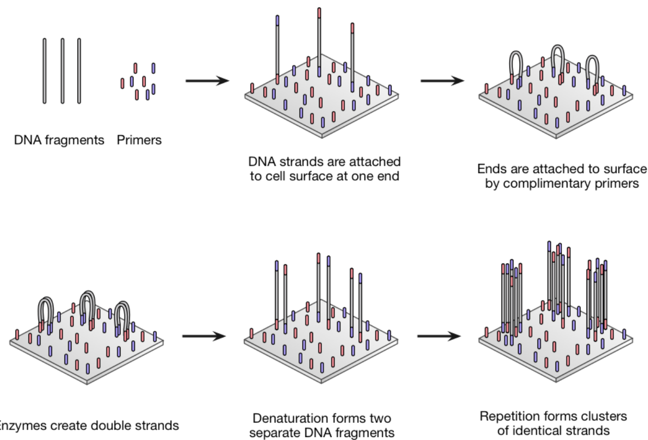
- Analysis of large amounts of DNA
- Whole genome sequencing (WGS)
- Currently being used for surveillance of TB isolates
- WSLH recently funded for a project to evaluate WGS on primary samples

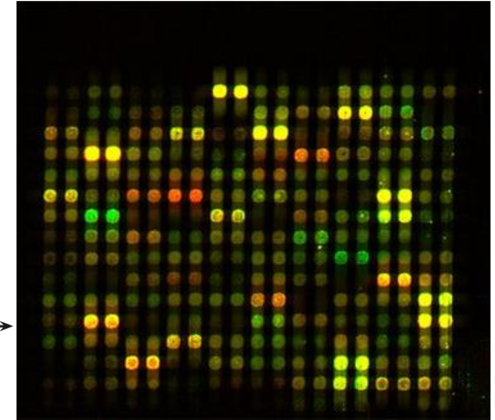


How NGS Works

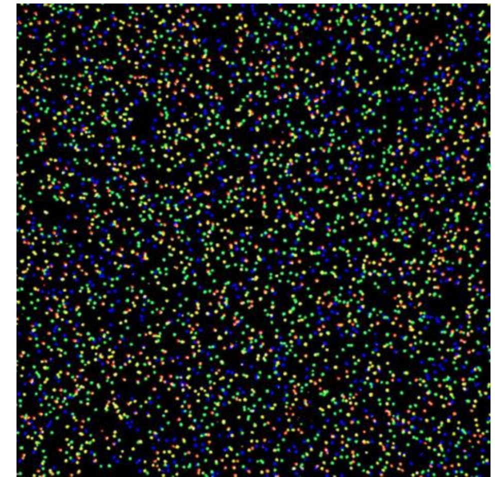








Cost is in the run, so simultaneous sequencing greatly reduces costs



PHASE TWO : INTERPRETATION

I THINK I FOUND A CORNER PIECE.

3 BILLION PIECES

GENOME

Data Analysis- Sequence Assembly

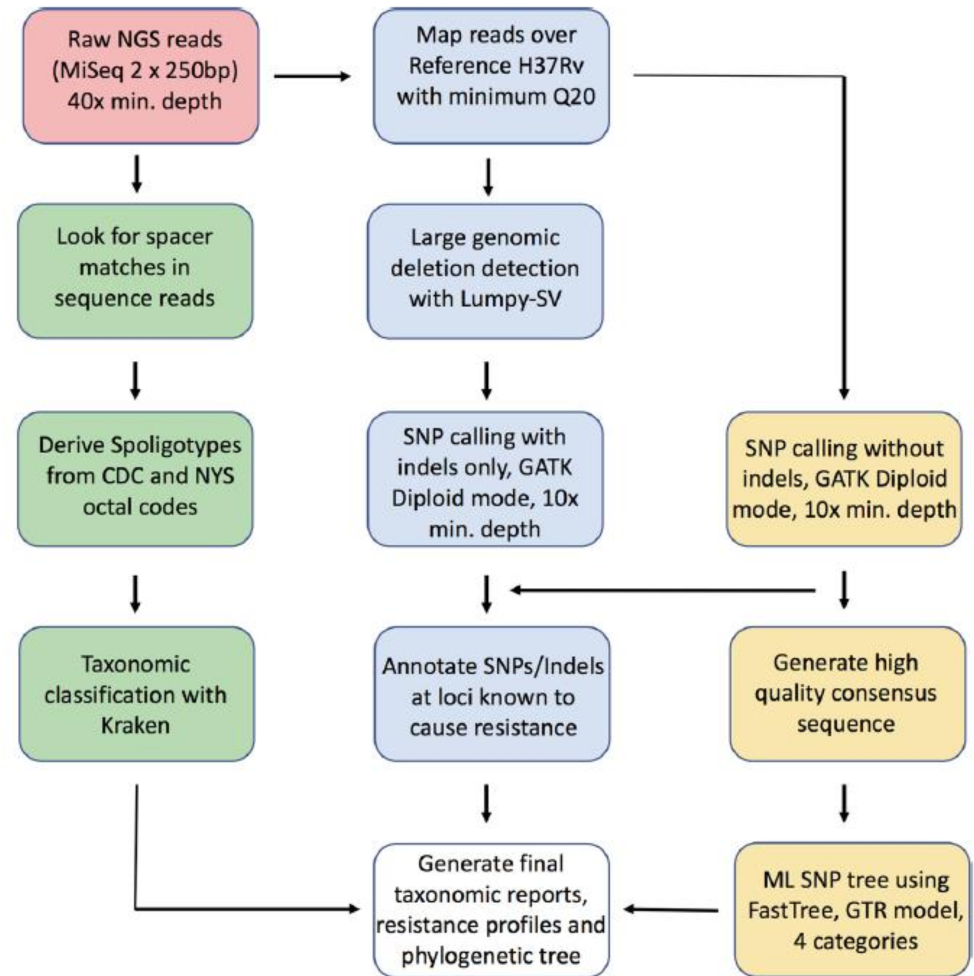
Drowned in next generation sequencing data

HELP!

http://biocomicals.blogspot.com

Data Analysis- Sequence interpretation

- Species identification
- Genotyping
- Drug resistance profiles
- Outbreak analysis



Shea, J et al. J Clin. Micro. 2017

Drug Resistance Genes

TABLE 2 List of high-confidence mutations used to predict resistance by drug/drug class

Antimicrobial (abbreviation)	Locus	Codon/nucleotide position(s)	Function
Rifampin (RIF)	*rpoB*[a]	251, 511, 513, 516, 522, 526, 531, 533, 572[b]	Coding
Isoniazid (INH)	*katG*[a]	279, 315, 525	Coding
	oxyR-ahpC	−81	Noncoding
	mabA-inhA promoter region	−17, −15, −8	Noncoding
	mabA	203	Coding
Pyrazinamide (PZA)	*pncA*[a]	Any nonsynonymous mutation	Coding
	pncA promoter[a]	Any nonsynonymous mutation	Noncoding
Ethambutol (EMB)	*embB*	306, 406, 497	Coding
Streptomycin (SM)	*rrs*	512, 513, 516, 906[c]	Noncoding
	rpsL	43, 88	Coding
Fluoroquinolones (FLQ)	*gyrA*	74, 90, 91, 94	Coding
	gyrB	499	Coding
Ethionamide (ETH)	*mabA-inhA* promoter region	−17, −15, −8	Noncoding
	mabA	203	Coding
Kanamycin (KAN)	*rrs*	1400[d]	Noncoding
	eis promoter	−37, −10	Noncoding

[a]Frameshift deletion/insertions in *rpoB*, *pncA*, and *katG* are considered high-confidence mutations.
[b]*Escherichia coli* numbering system is utilized, which is commonly found in the literature.
[c]Due to the presence of different numbering systems for the *rrs* gene, these may be reported as 513, 514, 517, or 907 in other publications.
[d]Due to of the presence of different numbering systems for the *rrs* gene, this may be reported as 1401 in other publications.

Shea, J et al. J Clin. Micro. 2017

NGS Disadvantages

- Takes longer than other molecular methods
- Requires high complexity testing, LDT standards, PCR clean environments
- Requires extensive computing capabilities
- Needs analysis by specially trained personnel (Bioinformatician)
- Expensive reagents and equipment
- Still in early development

NGS Features

- Thorough identification
  - Can differentiate strains within complexes
  - Genotype and Spoligotype
- Enables outbreak analysis (relatedness)
- Allows for early detection of drug resistance
- All this information is available faster than ever before
- Not limited to TB, already in use for other bacteria and viruses

Molecular Disadvantages

- More expensive than culture
- Requires additional equipment
- Most methods require special workflows and clean spaces
- High complexity testing requires specialized training
- Most tests are not FDA cleared
- Unable to differentiate viable from non-viable organisms
- May miss rare phenotyping differences and new methods of resistance

Advantages of Molecular Diagnostics

1. Reduced time to diagnosis
   - Starts treatment faster
   - Protects others from infection
   - Frees people from isolation quicker
2. Reduced time to drug susceptibility results
   - Gets patients on effective therapy faster
     - Reduces Morbidity and Mortality
     - Shortens time in isolation
3. Reduced time to typing
   - Recognizes outbreaks faster
   - Focuses resources

References/Resources

- CDC. Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. MMWR 2009; 58: 7-10.
- Ngan, G.J.Y., Ng, L.M., Jureen, R., Lin, R.T.P., and Teo, J.W.P. Development of multiplex PCR assays based on the 16S–23S rRNA internal transcribed spacer for the detection of clinically relevant nontuberculous mycobacteria. Letters in Applied Microbiology. 52(5): 546-554. 2011.
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- Shea, J et al. Comprehensive Whole-Genome Sequencing and Reporting of Drug Resistance Profiles on Clinical Cases of *Mycobacterium tuberculosis* in New York State. J Clin. 55(6) Micro. 2017