Molecular microbiology

Pavel Dřevínek

Where we enter through this door

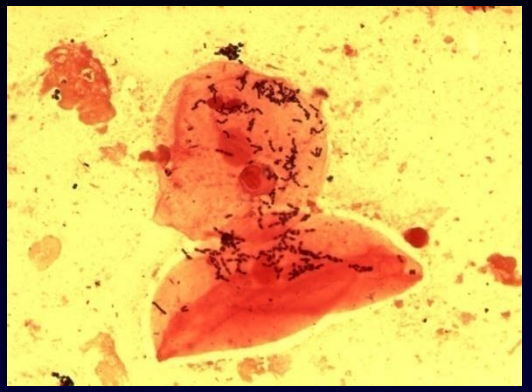
A) Ministry of Health and IHIS

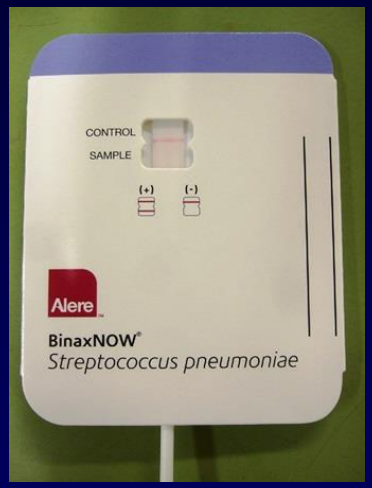
B) Warehouse of civil defense FN Motol

C) Department of Virology, 2nd Medical Faculty, Charles University and Motol University Hospital

Dept. of Medical Microbiology
2. Medical Faculty Charles University and Motol University Hospital

Yesterday

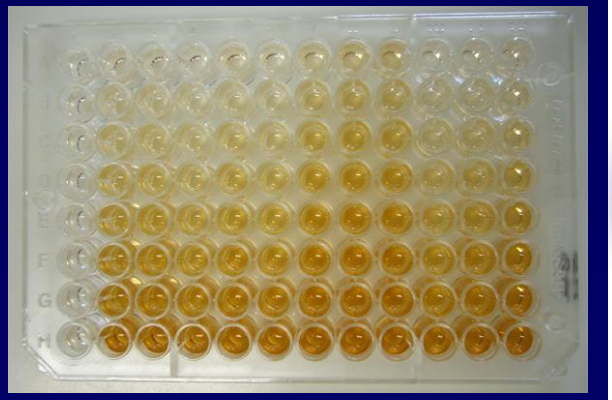


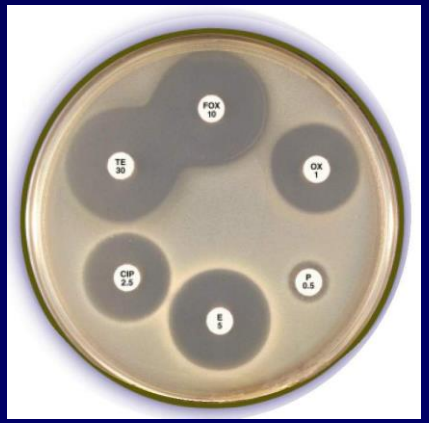


Traditional microbiology

Microscopy
Culture, ID a AST
Antigen detection

Serology and detection of antibodies





Dept. of Medical Microbiology
2. Medical Faculty Charles
University and Motol University
Hospital

Yesterday

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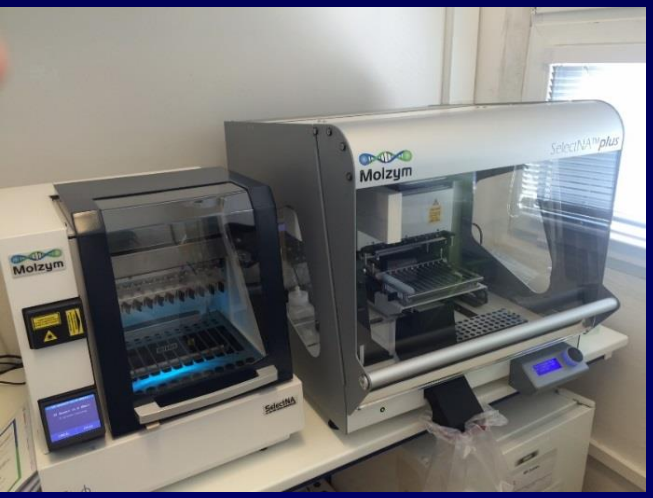
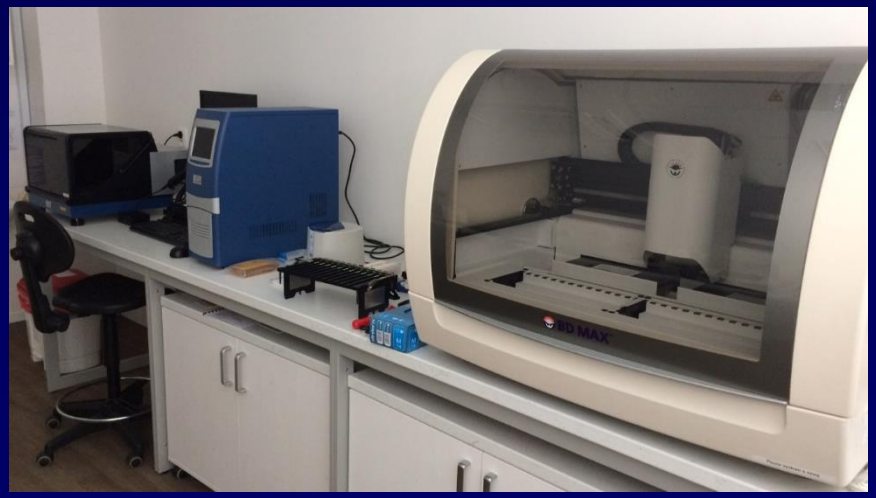
Today

New microbiology

DNA or RNA analysis :

- diagnostics (ID)
- epidemiology
- virulence
- antimicrobial resistance





What is not an ATB resistance gene

A) spA

B) mecA

C) bla

D) vanC

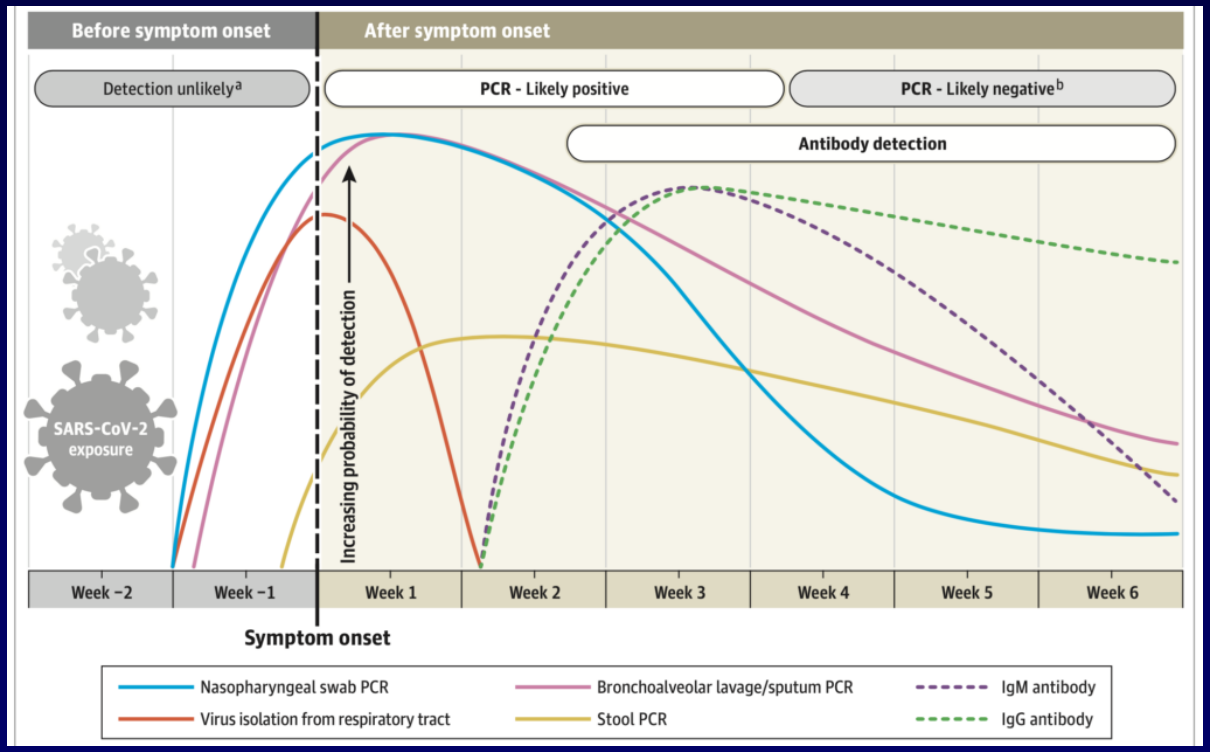
MALDI TOF

Diagnostic reason to detect DNA or RNA

Advantage
• non-culturable agens, slow growing, „fastidious"
• quickly performed
• high sensitivity
• detection even during antibiotic therapy
• quantification

Diagnostic reason to detect DNA or RNA

Limitations
pitfalls in interpretation, invisible at first glance:
• detected DNA originate from a viable agens?
• positive result: artifact, contamination, colonization or patogen?



DNA diagnostics

to a selected pathogen
(patogen-specific)

Examples
Clostridium difficile
Mycobacterium tuberculosis
SARS-CoV-2

DNA diagnostics

selected microorganism
(patogen-specific)

any microorganism
(broad-range)

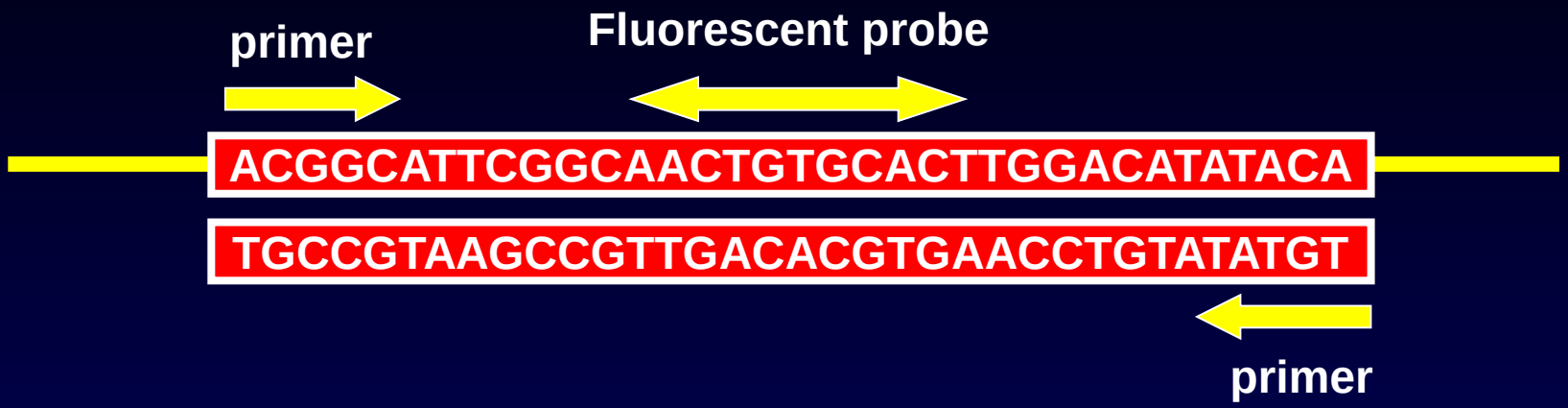
Examples
Clostridium difficile *Mycobacterium tuberculosis* SARS-CoV-2

Examples
infectious endocarditis (heart valves) joint infections (joint puncture, tissue) abscesses, biopsy ...

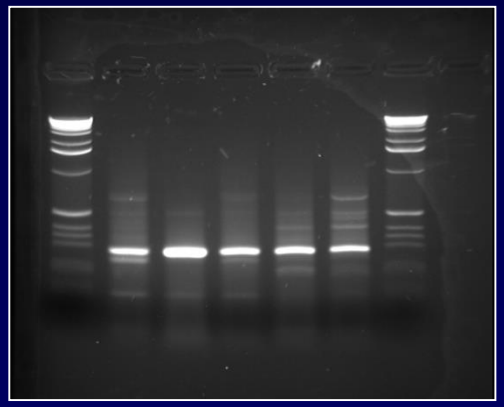
Working cookbook:

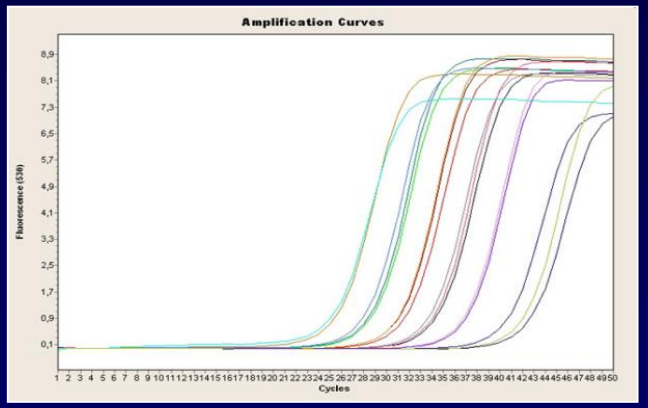
how to actually „design“ PCR to do what we want it to do

detection focused on the nucleotide sequence



PCR result

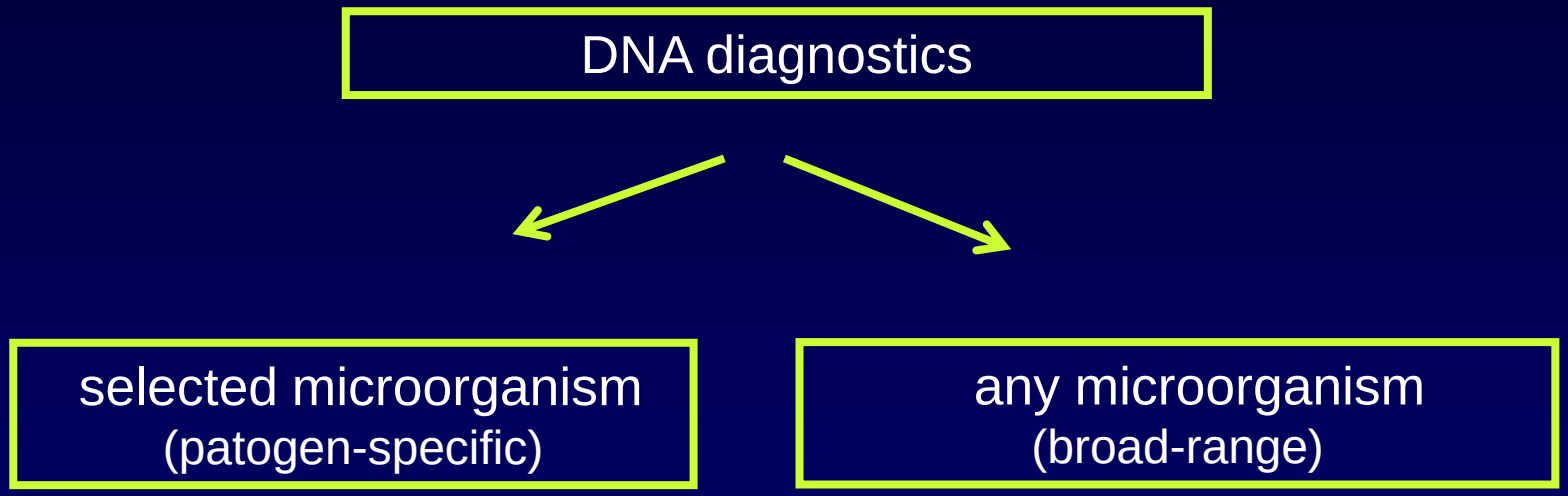




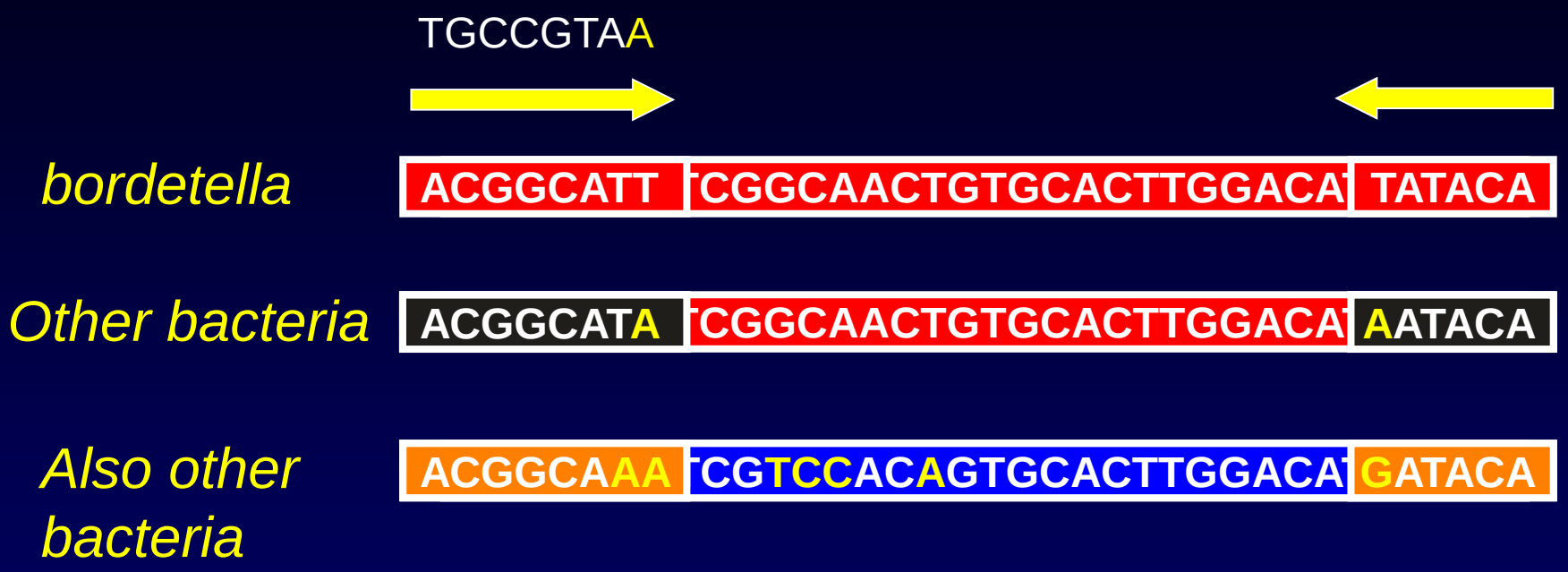
Ct treshold value = PCR cycle,
when the PCR signal starts to grow

the lower the Ct value, the more agent is detected in the sample

Does the patient have a whooping cough?
(need to detect only *Bordetella pertussis*)



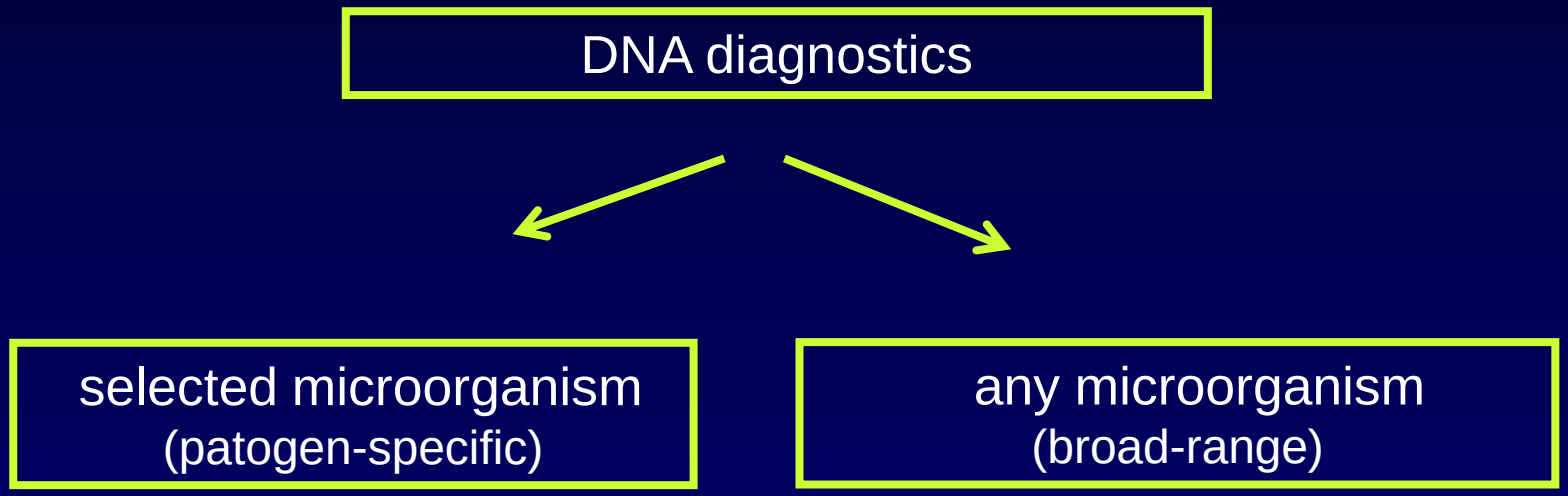
the target sequence for the primers must be unique to bordetella



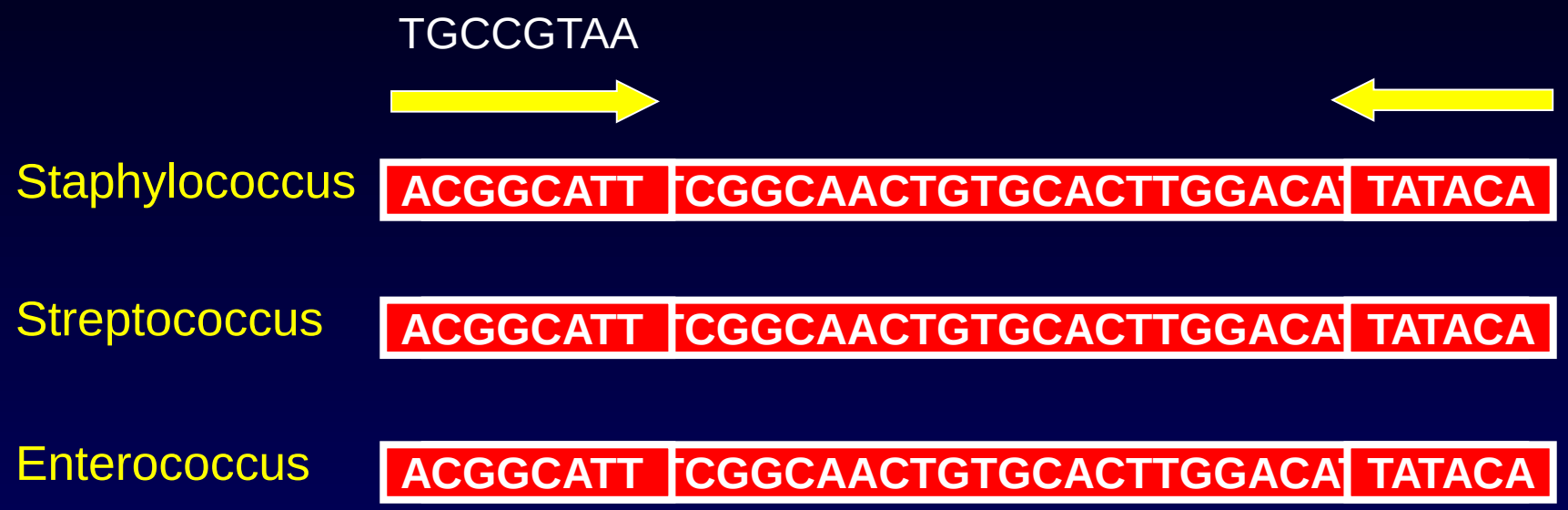
PCR positivity

Bordetella detection

Fever. What is the cause?
(need to detect any bacteria,
because any bacteria can cause sepsis)

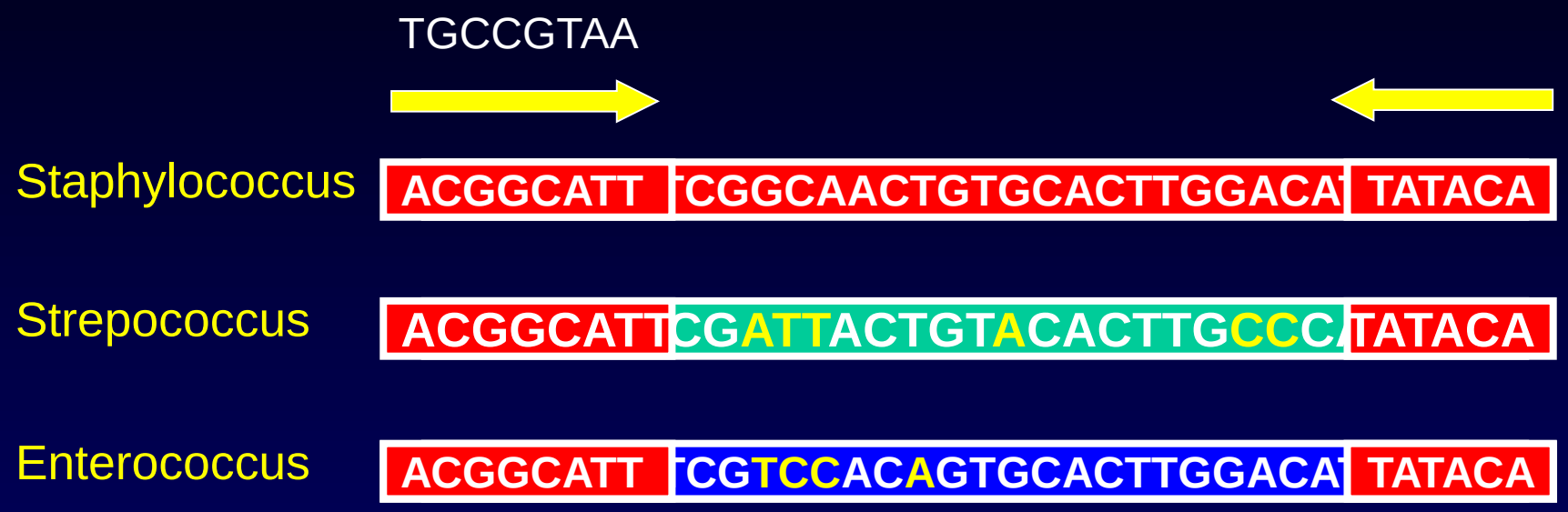


the target sequence for the primers is present in all bacteria



Situation A

the target sequence for the primers is present in all bacteria



Situation B

Which situation is better for diagnosis?

A) Situation A

B) Situation B

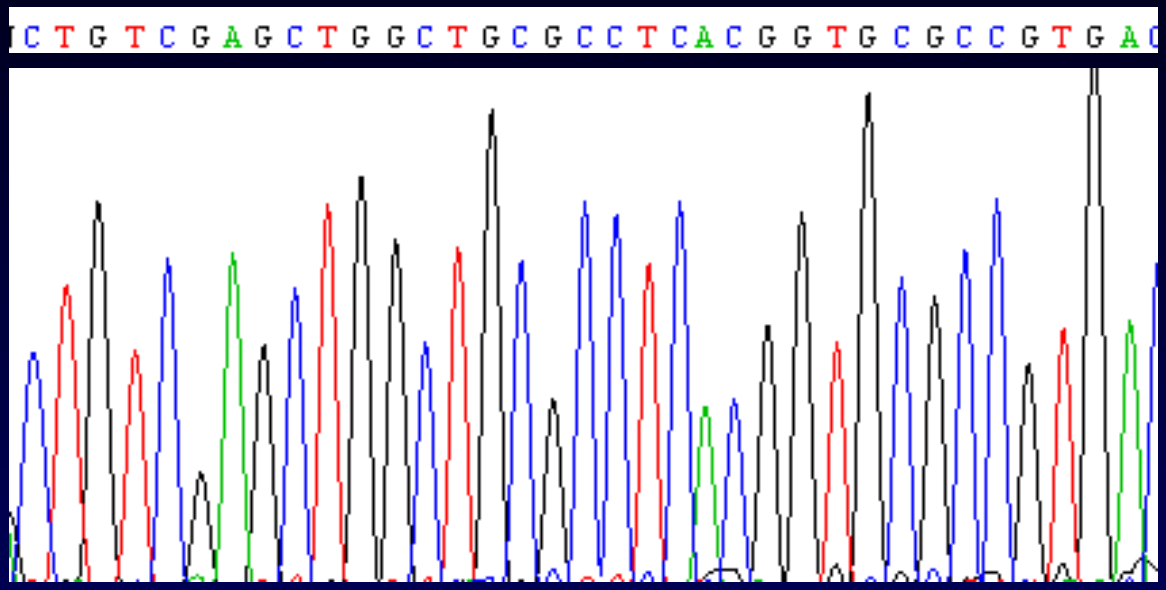
PCR positivity

+ sequencing

DETECTION OF BACTERIA,
but which one?

IDENTIFICATION
on species level

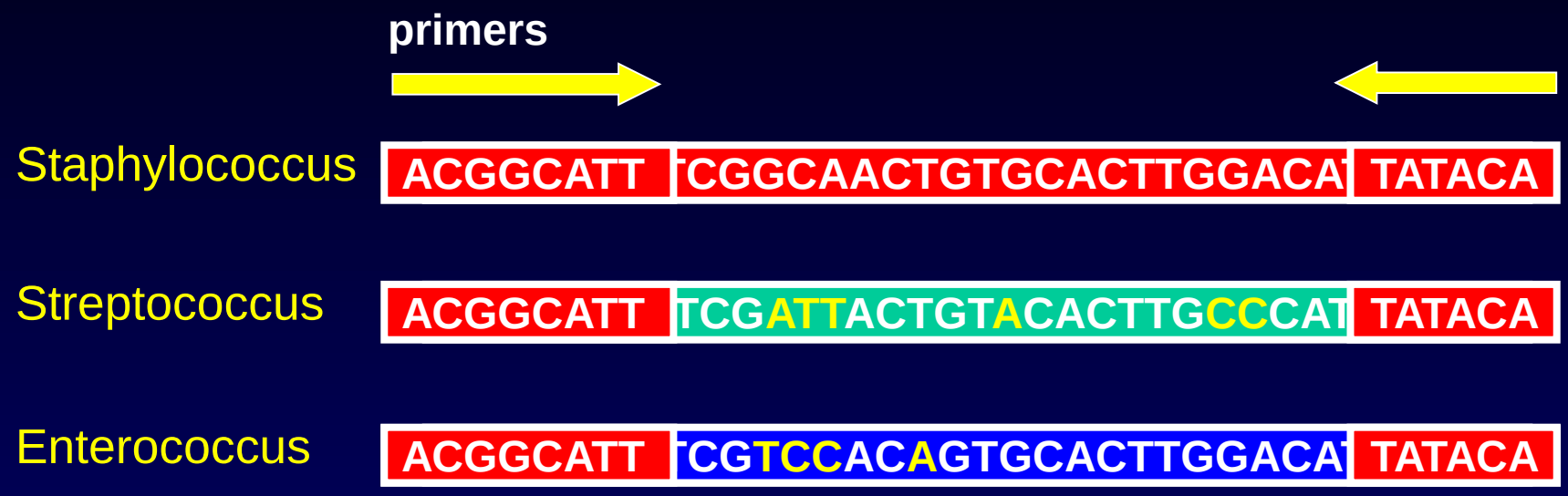
PCR product sequencing



S. aureus

C T G A

the target sequence for the primers is present in all bacteria



situation B !

Which gene meets the criteria for broad-spectrum examination?

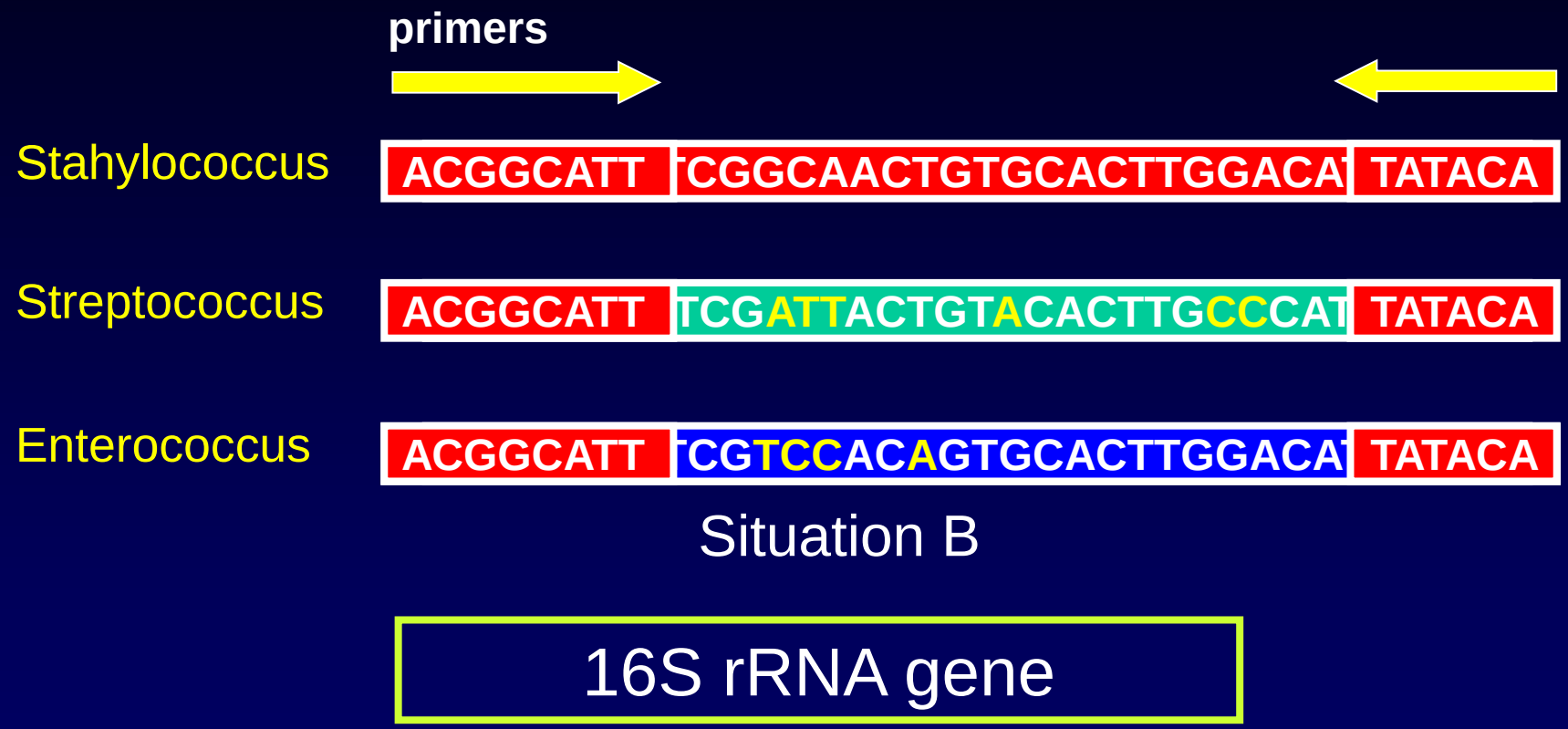
A) Gen for insertion sequence

B) Gen for transpeptidase (PBP)

C) Gen for 16S rRNA

D) Gen for 18S rRNA

the target sequence for the primers is present in all bacteria



Situation B

16S rRNA gene

DNA diagnostics

selected microorganism
(patogen-specific)

Examples
Clostridium difficile *Mycobacterium tuberculosis* SARS-CoV-2
MRSA VRE KPC NDM

DNA diagnostika

selected microorganism
(patogen-specific)

selected microorganism
(patogen-specific using multiplex PCR)

Examples
Clostridium difficile
Mycobacterium tuberculosis
SARS-CoV-2

Examples
respiratory infections
meningitis
GIT infections

MRSA
VRE
KPC
NDM

Which pathogen should not be missing in the meningitis multiplex?

A) N. gonorrhoeae

B) S. pneumoniae

C) Y. pseudotuberculosis

D) S. aureus

Viral respiratoty infections

influenza A including typing
influenza B
RSV
rhinovirus
parainfluenzavirus
adenovirus
metapneumovirus
coronaviruses

Sexually transmitted diseases (STD)

Neisseria gonorrhoeae
Chlamydia trachomatis
Mycoplasma genitalium
Mycoplasma hominis
Ureaplasma parvum
Ureaplasma urealyticum
Trichomonas vaginalis

Atypical pneumoniae

Mycoplasma pneumoniae
Chlamydia pneumoniae
Chlamydia psittaci
Legionella pneumoniae
Pneumocystis jiroveci

GIT infections

Salmonella
Campylobacter
Shigella
enterohemoragická *E. coli*

Meningitis

Streptococcus pneumoniae
Neisseria meningitidis
Haemophilus influenzae
Escherichia coli
Streptococcus agalactiae
Listeria monocytogenes
HSV a VZV
enteroviruses

Patogen-specific analysis and POCT (point-of-care testing)

- ✓ There are many technologies available, commercially
- ✓ offered"two flies with one blow" in the case of a multiplex

Influenza/RSV 20 min
C. difficile 45 min
S. aureus (MRSA) 60 min
M. tuberculosis 80 min

E. coli, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, E. faecium
3,5 h

27 pneumonia agent and 7 resistence markers
60 min

Patogen-specific analysis and POCT (point-of-care testing)

X Price for closed system (and limited capacity)

Influenza/RSV 20 min
C. difficile 45 min
S. aureus (MRSA) 60 min
M. tuberculosis 80 min

cca 1,000 Kč/1 sample

E. coli, S. aureus, K. pneumoniae, A. baumannii, P. aeruginosa, E. faecium
3,5 h

cca 4,000 Kč/1 sample

27 pneumonia agent and 7 resistence markers
60 min

cca 3,500 Kč/1 sample

Patogen-specific analysis and POCT (point-of-care testing)

- X Price for closed system (and limited capacity)
- X Limitation of automatic interpretation

Test Result: SARS-CoV-2 POSITIVE

Analyte Result

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
E	20.2	418	POS	PASS
N2	22.7	216	POS	PASS
SPC	28.6	37	NA	PASS

Test Result: SARS-CoV-2 POSITIVE

Analyte Result

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
E	0.0	3	NEG	PASS
N2	42.1	112	POS	PASS
SPC	28.5	342	NA	PASS

Which result is false positive?

A) The one on the left

B) The one on the right

Test Result: SARS-CoV-2 POSITIVE

Analyte Result

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
E	20.2	418	POS	PASS
N2	22.7	216	POS	PASS
SPC	28.6	37	NA	PASS

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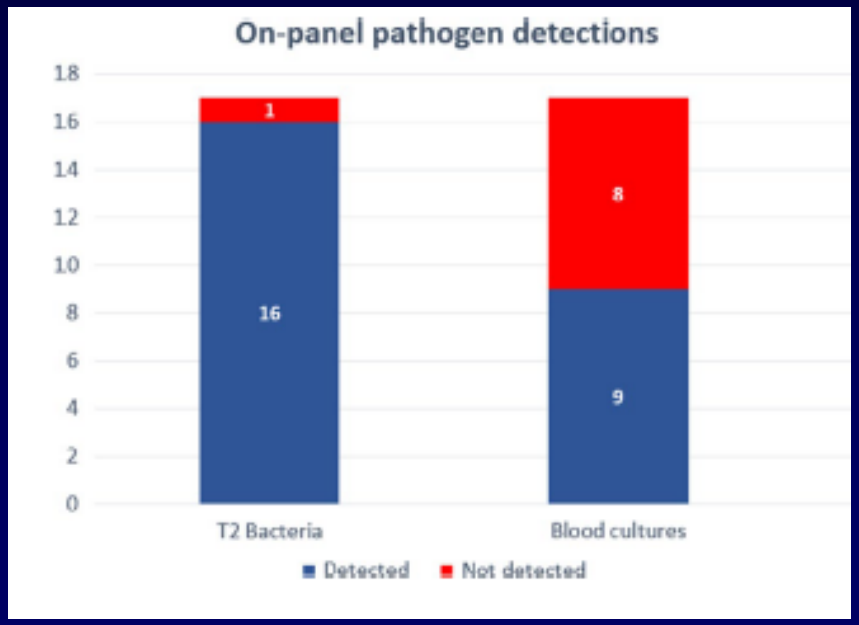
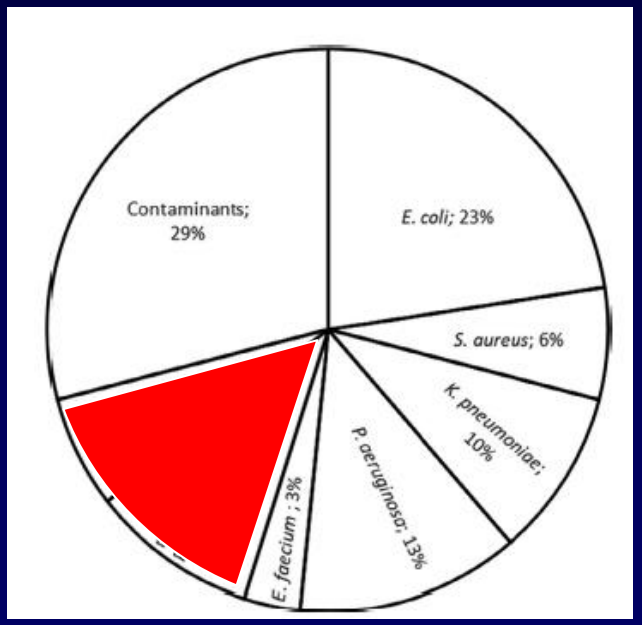
Patogen-specific analysis and POCT (point-of-care testing)

- X Price for closed system (and limited capacity)
- X Limitation of automatic interpretation
- X Missed "off-target" patogens





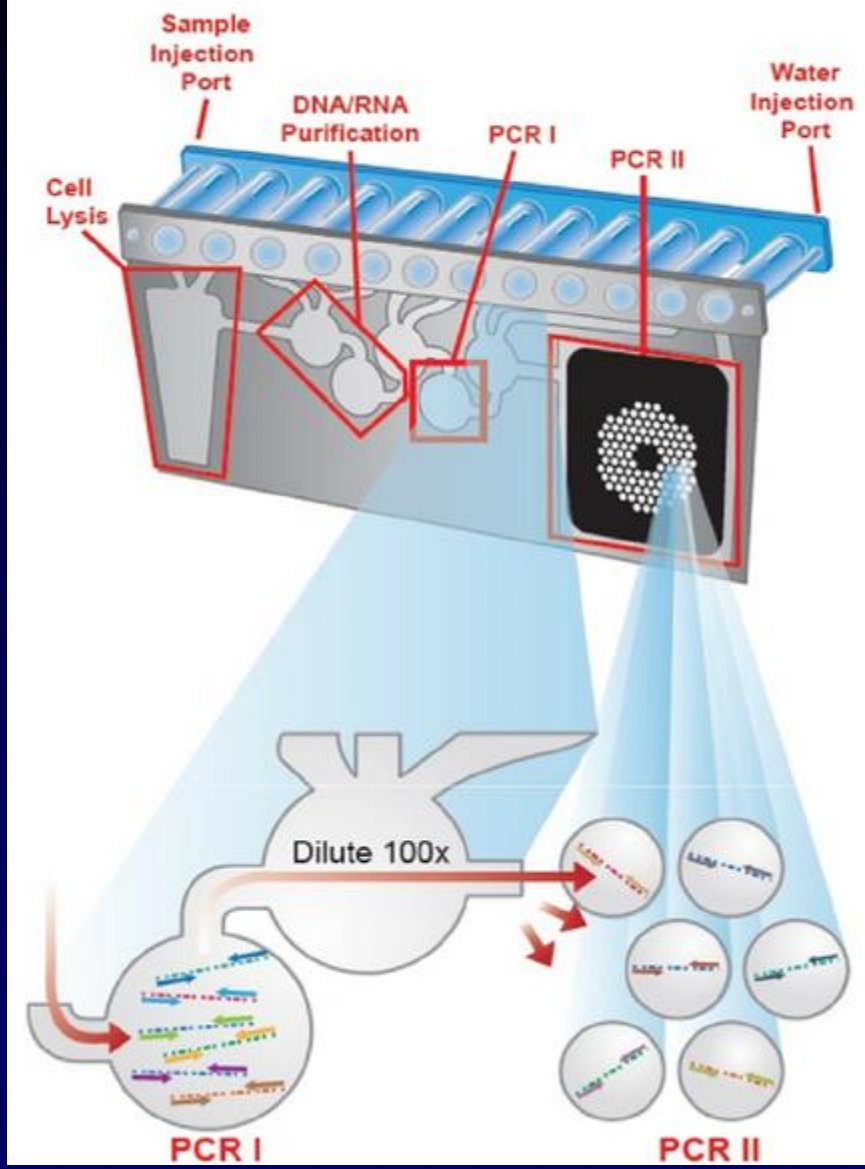
Drevinek et al. Direct Detection of ESKAPEc pathogens from whole blood using T2Bacteria ... MicrobiologyOpen. 2021

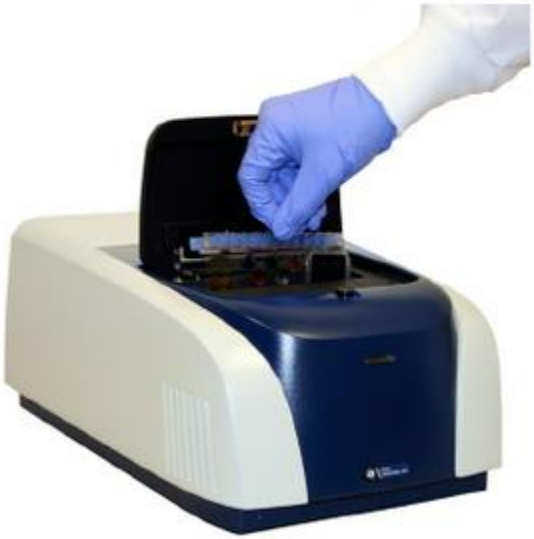
Patogen-specifická vyšetření a POCT (point-of-care testing)

- X Price for closed system (and limited capacity)
- X Limitation of automatic interpretation
- X Missed "off-target" patogens
- X Patogen versus nevinný commensal (infection vs. colonization?)

Bakterie	Viry
Acinetobacter baumanii complex	Adenovirus
Enterobacter cloacae complex	Coronavirus
Escherichia coli	MPV
Haemophilus influenzae	Rhinovirus/Enterovirus
Klebsiella aerogenes	Influenza A
Klebsiella oxytoca	Influenza B
Klebsiella pneumoniae group	MERS-CoV
Moraxella catarrhalis	PIV
Proteus sp.	RSV
Pseudomonas aeruginosa	**Markery rezistence**
Serratia marcescens	CTX-M
Staphylococcus aureus	IMP
Streptococcus agalactiae	KPC
Streptococcus pneumoniae	*mecA/C and MREJ*
Streptococcus pyogenes	NDM
Chlamydophila pneumoniae	OXA-48-like
Legionella pneumophila	VIM
Mycoplasma pneumoniae	

The FilmArray Pouch





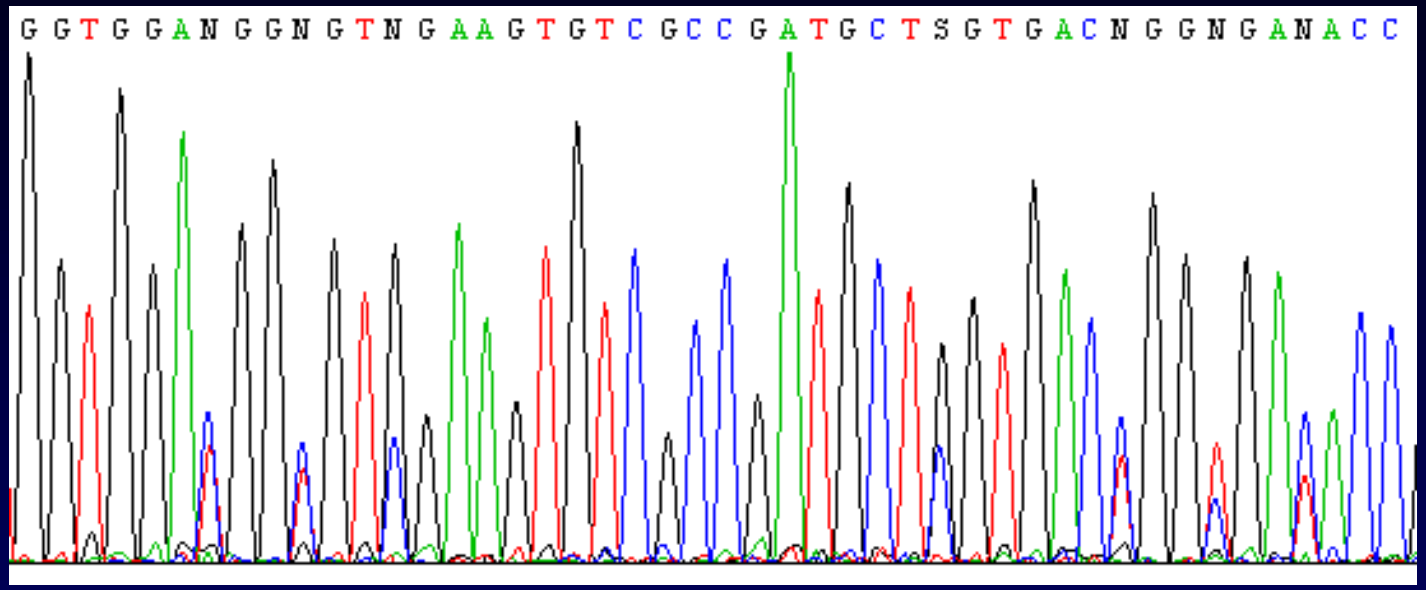
DNA diagnostika

Any microorganism
(broad-range analysis)

Examples
infectious endocarditis (heart valves) joint infections (joint puncture, tissue) abscesses, biopsy ...

- from primary sterile material

.... otherwise this will happen :


G G T G G A N G G N G T N G A A G T G T C G C C G A T G C T S G T G A C N G G N G A N A C C

Broad-range “16S PCR”
– samples from cardiovascular surgery:

ID	clinical material	16S sequence	culture
1	tricuspid valve	***Staphylococcus aureus***	negative
2	aortic valve	***Bartonella quintana***	negative
3	aortic valve	***Enterococcus faecalis***	negative
4	tricuspid valve	***Staphylococcus aureus***	negative
5	mitral valve	***Streptococcus anginosus***	negative
6	mitral valve	***Staphylococcus aureus***	***Staphylococcus aureus***
7	tricuspid valve	negative	negative
8	tricuspid valve	***Staphylococcus aureus***	negative
9	aortic valve	***Bartonella quintana***	negative
10	mitral valve	***Enterococcus faecalis***	***Enterococcus faecalis***
11	aortic valve	***Enterococcus faecium***	negative
12	tricuspid valve	***Enterococcus faecium***	negative
14	aortic valve	***Enterococcus faecalis***	negative
15	mitral valve	***Staphylococcus aureus***	negative
16	valve	***Bartonella quintana***	negative

Why does cultivation fail?

A) Non-culturable material

B) Non-culturable bacteria

C) Low bacterial quantity

D) Antibiotic treatment

Case report

4-year-old boy with meningeal symptoms, in septic condition

leucocytes 26,4 x 10^9/L
CRP 186,9 mg/L
PCT > 100 µg/L

<u>liquor:</u>

Pandy	+	
leukocytes	2432 / 3 µl	(0-12) ↑
PMN	97 %	
erytrocytes	48 / 3 µl	
glukose	0,22 mmol/L	(1,65-5,55) ↓
proteins	984 mg/L	(130-360) ↑
chlorides	123,5 mmol/L	(109-133)
lactate	9,04 mmol/L	(0,9-2,8) ↑

January 24 (Day 1)

Lumbal puncture
Blood culture
Throat swab
Ear swab

liquor: microscopy neg.

January 25 (Day 2)

Liquor, 24. January:

- PCR *N. meningitidis* neg.
- PCR *S. pneumoniae* neg.
- PCR *H. influenzae* neg.

January 26 (Day 3)

Lumbal puncture
Blood culture
Throat swab
Ear swab

Throat swab, 24. January: norma
Ear swab, 24. January: neg.
urine Ag pneumokok: neg.

liquor, 24. january:
PCR herpesviry neg.

January 27 (Day 4)

liquor, 24. January:

- culture neg.
- panbacterial PCR:

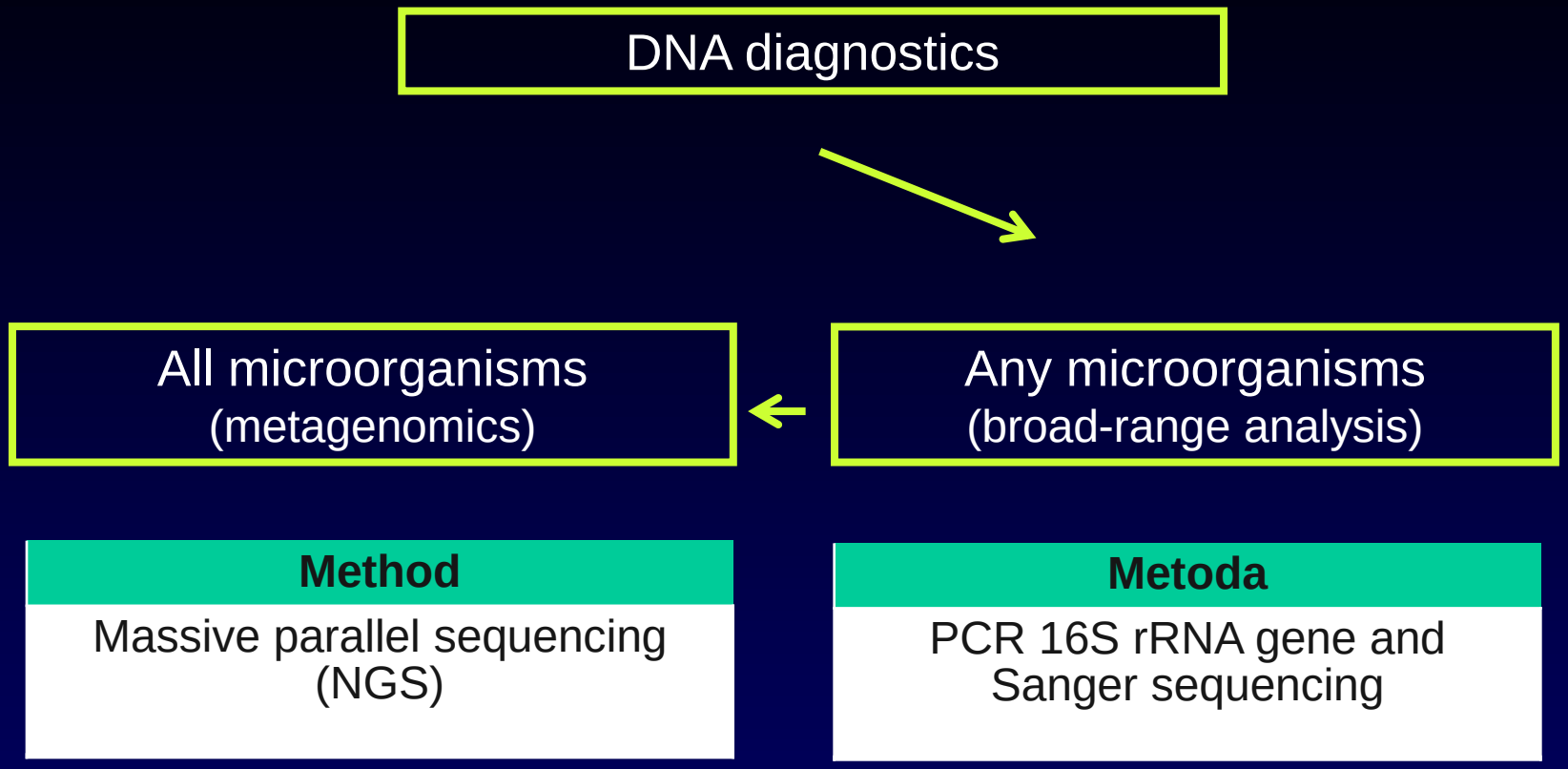
Fusobacterium necrophorum

January 30 (Day 7)

Blood culture, 24. January: neg.

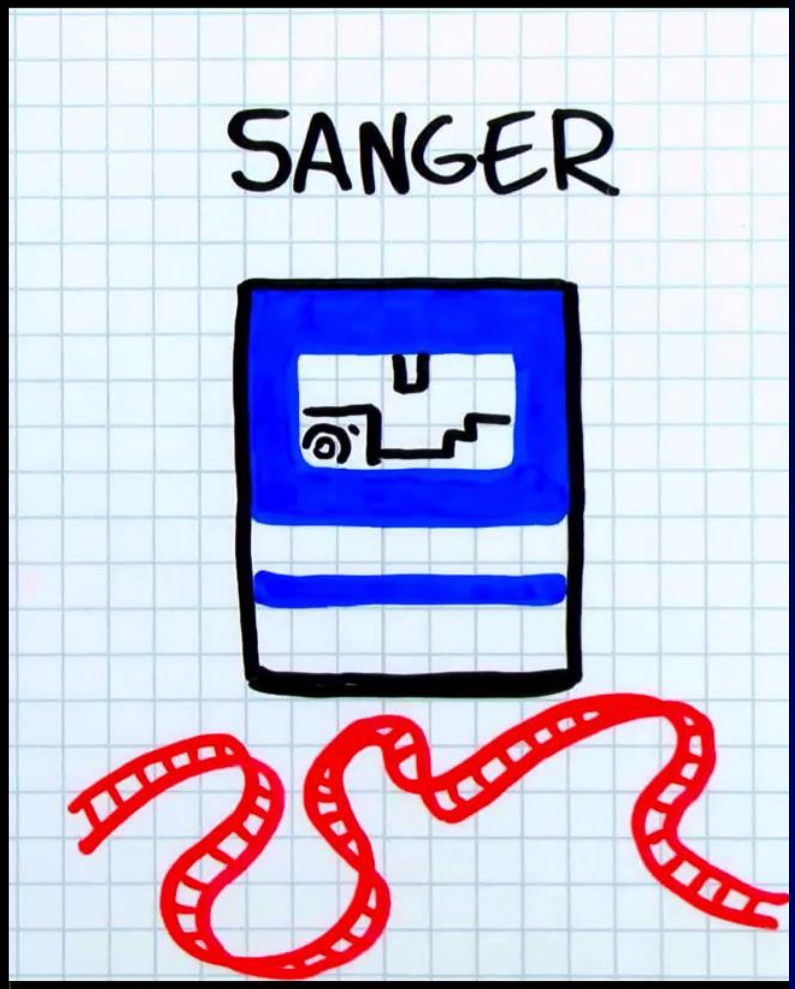
Blood, 26. January:
panbacterial PCR:

Fusobacterium necrophorum


DNA diagnostics
All microorganisms
(metagenomics)
Any microorganisms
(broad-range analysis)
Method
Massive parallel sequencing
(NGS)
Metoda
PCR 16S rRNA gene and
Sanger sequencing

generation SEQ (short)

1st generation SEQ 2nd



DNA diagnostics

Any microorganisms
(broad-range analysis)

All microorganisms
(metagenomics)

Method
Massive parallel sequencing (NGS)

Metoda
PCR 16S rRNA gene and Sanger sequencing

Useful for:

- diagnosis of unexplained diagnosis (directly from clinical material)

diagnosis of unexplained diagnosis and NGS

Sample	Material	Culture	16S-23S rDNA NGS Identification	Bacterial fraction (%)
1	Brain abscess	*Porphyromonas asaccharolytica*	*Fusobacterium spp.*	99,5
		Porphyromonas somerae	*Porphyromonas spp.*	0,2
		Parvimonas micra		
4	Blood culture	Unidentified gram positive rods	*Actinotignum spp.*	100,0
7	Joint tissue	Negative	*Rothia mucilaginosa*	0,2
			Corynebacterium spp.	1,9
			Cutibacterium acnes	0,9
8	Joint punctate	Negative	*Capnocytophaga canimorsus*	99,1
10	Bloodvessel tissue	Negative	*Cutibacterium acnes*	6,7
			Staphylococcus epidermidis	7,2
			Anaerococcus spp	2,5
20	Brain abscess	Negative	*Dialister pneumosintes*	12,3
			Parvimonas micra	5,5
			and 18 additional identifications	
25	Pleural fluid	*Fusobacterium nucleatum*	*Prevotella pleuritidis*	77,5
			Fusobacterium nucleatum	21,1
			Actinomyces meyeri	1,4

What is associated with capnocytophagous infection

A) Tick suction

B) Dog bite

C) Stay in tropical areas

D) MSM

DNA diagnostics

All microorganisms
(metagenomics)

Any microorganisms
(broad-range analysis)

Method
Massive parallel sequencing (NGS)

Metoda
PCR 16S rRNA gene and Sanger sequencing

Useful for:

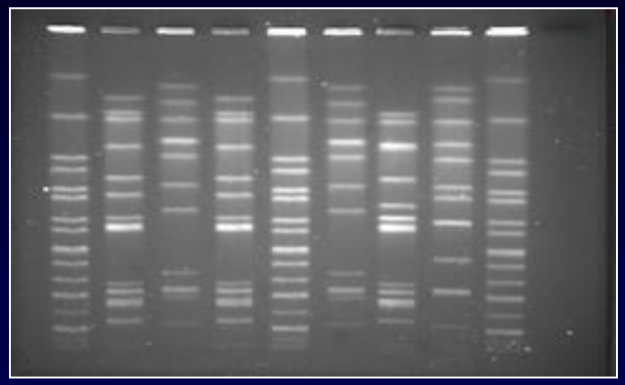
- diagnosis of unexplained diagnosis (directly from clinical material)
- epidemiology (from pure cultures)

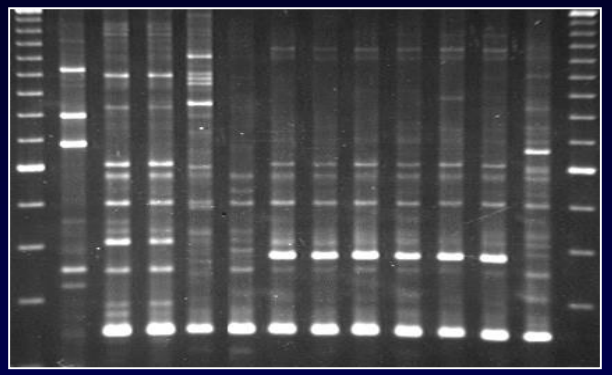
Molecular epidemiology and NGS

Strain identification using genotypic methods

Genotyping

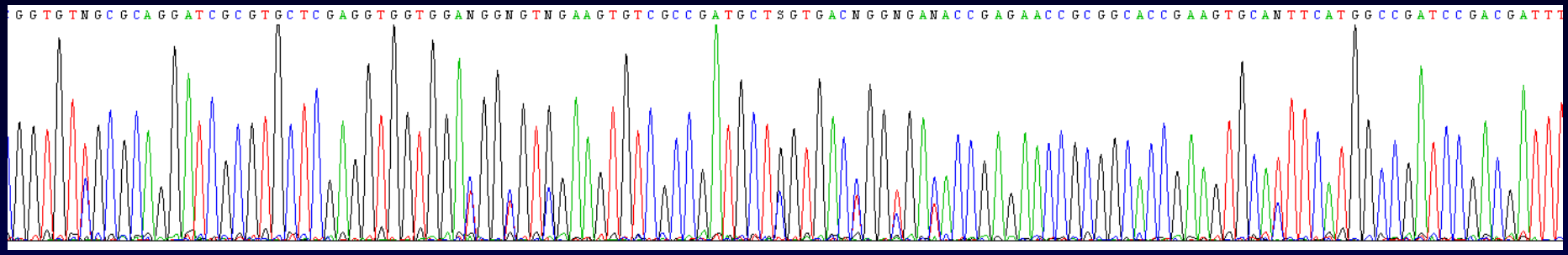
- „Gel (image)-based“



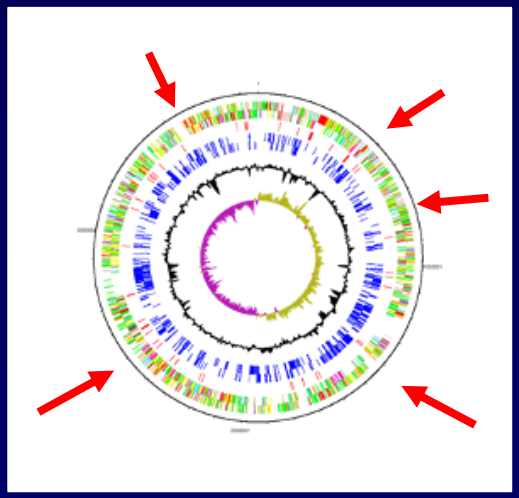


Genotyping

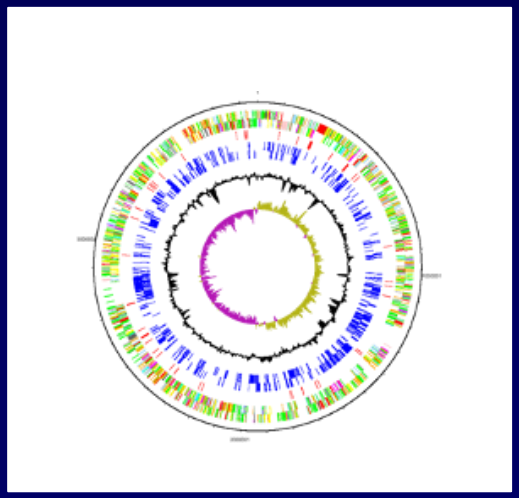
- „Sequence-based“



Selected genes
= multilocus sequence typing (MLST)



All genes
= whole genome sequencing (WGS)



Example 1: "Image-based"

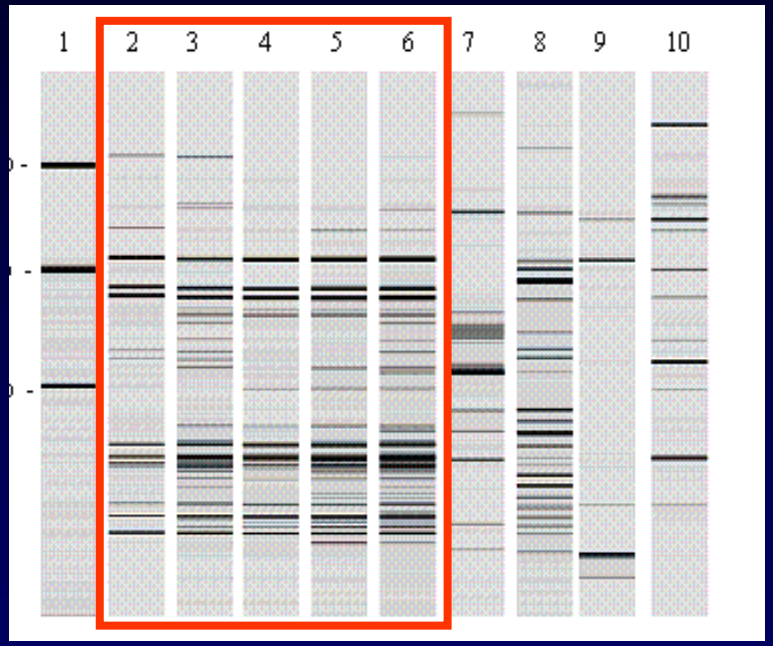
Cystic fibrosis center

(300 pacientů)

1998 - ¨culture diagnostic of *Burkholderia cepacia*
--> 15 % positive patients

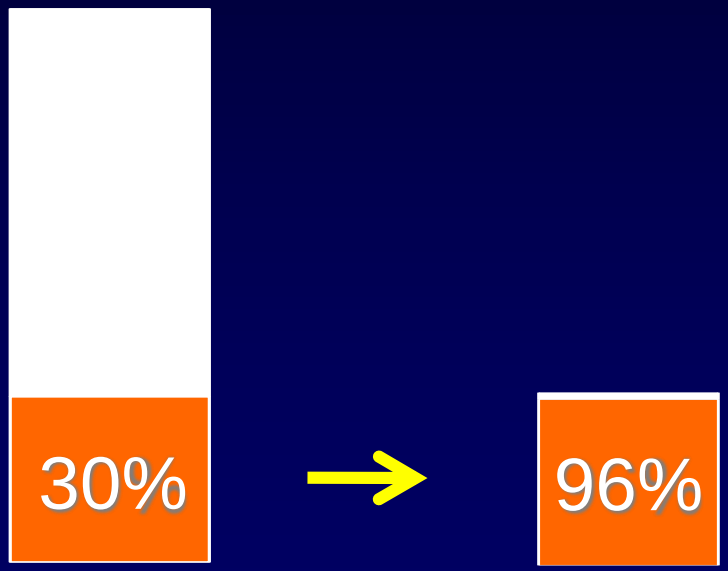
2001 - introduced PCR diagnostics of *Burkholderia cepacia*
--> 30 % positive patients

"Image-based"

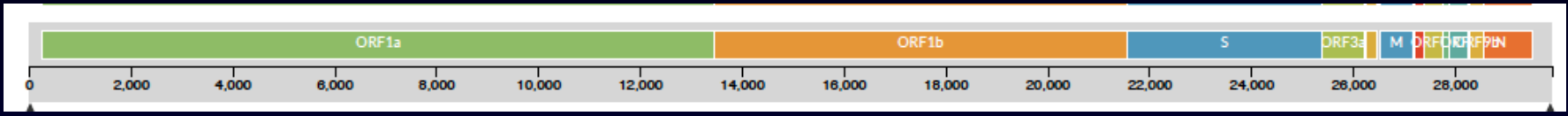


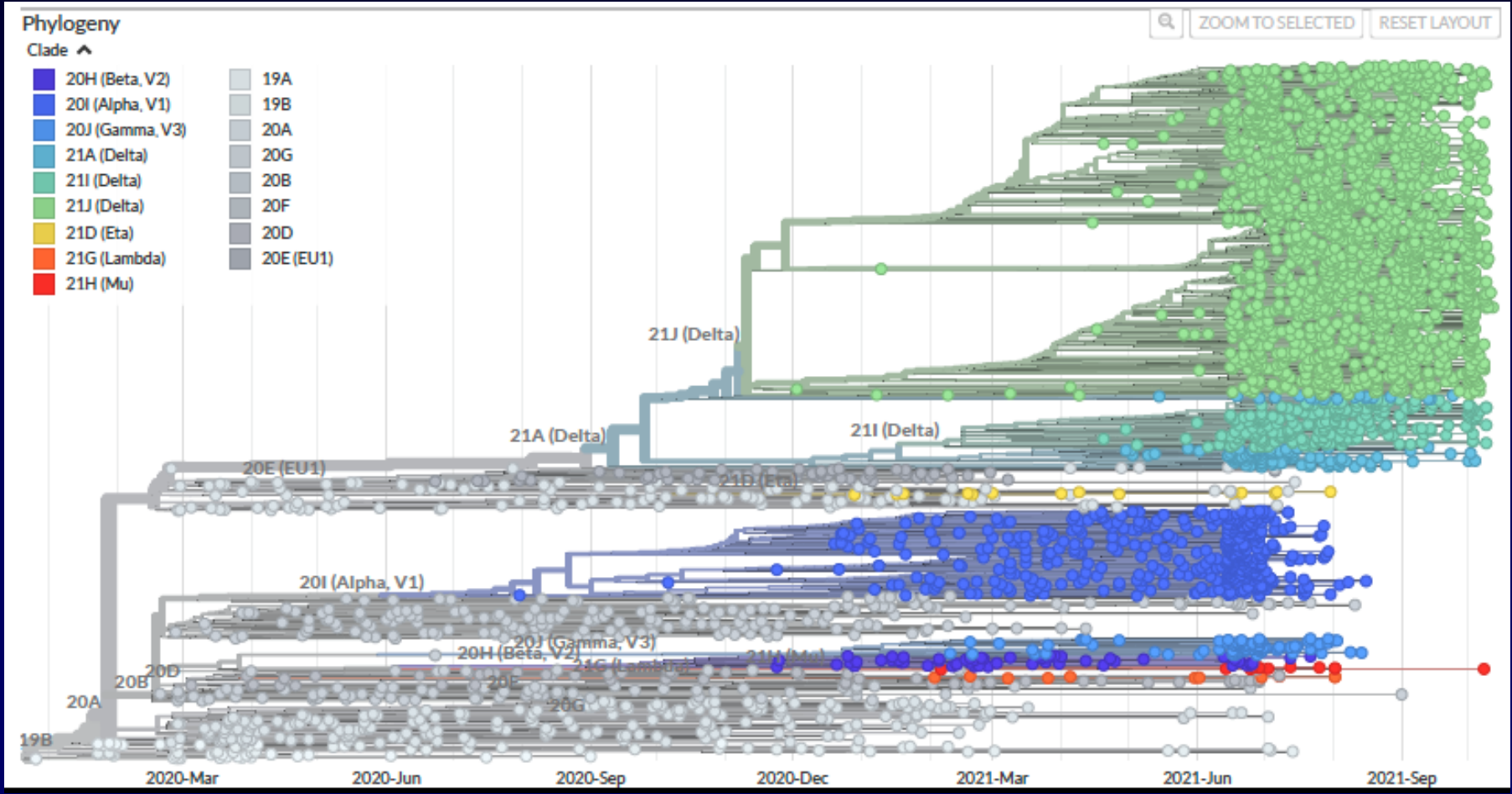
Infected patients

Identical strain



Example 2: "sequence-based" (WGS)





Conclusion

•Main reasons for using molecular microbiology:

- examination speed
- high sensitivity, high specificity
- detection of non-culturable agents
- detection during antibiotic treatment

- The methodology is designed

- Offer a wide range of pathogen-specific PCRs
- sometimes panbacterial PCR

- NGS and metagenomics, molecular epidemiology

- Clinical Microbiology = field with a great future

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