



Prvé skúsenosti s polymikrobiálnymi realtime PCR panelmi – QIAstat v rutinnom mikrobiologickom laboratoriu

MUDr. Rudolf Botek,
RNDr. Viera Melicháčová, RNDr. Silvia Vašková

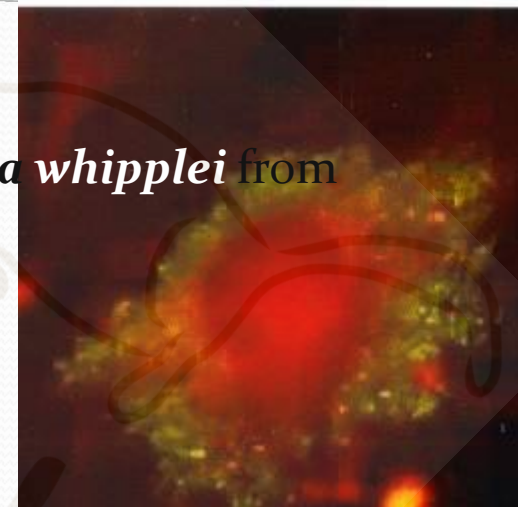
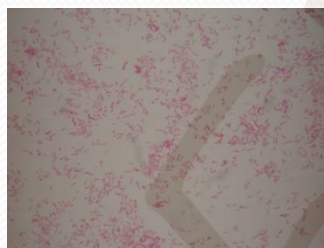
MIKROBIOLOGICKÁ DIAGNOSTIKA

- Priamy dôkaz:
 - **Mikroskopia**
 - **Kultivácia**
 - Dôkaz antigénu
 - Dôkaz špecifickej sekvencie nukleovej kyseliny
- Nepriamy dôkaz:
 - Dôkaz protilátkovej odpovedi
- Report:
 - Vysokoppravdepodobná etiopatogenetická súvislosť**
 - Súčasný nález viacerých relevantných agensov ?
 - Zložitá interpretácia
 - Závislá na triede Ig a mikroorganizme

ZLATÝ ŠTANDARD KULTIVÁCIA



Culture of *Tropheryma whipplei* from Human Sample

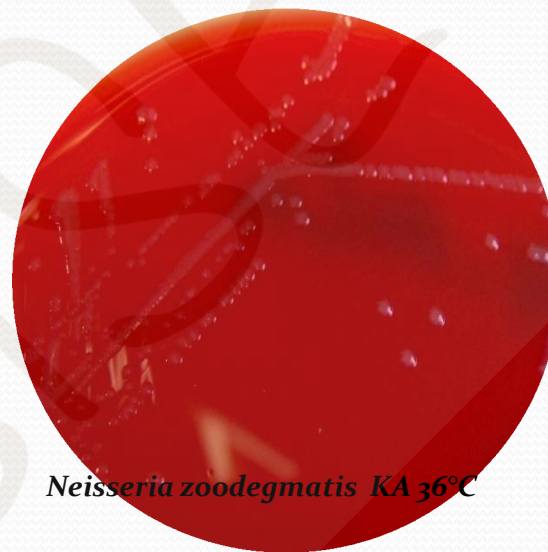
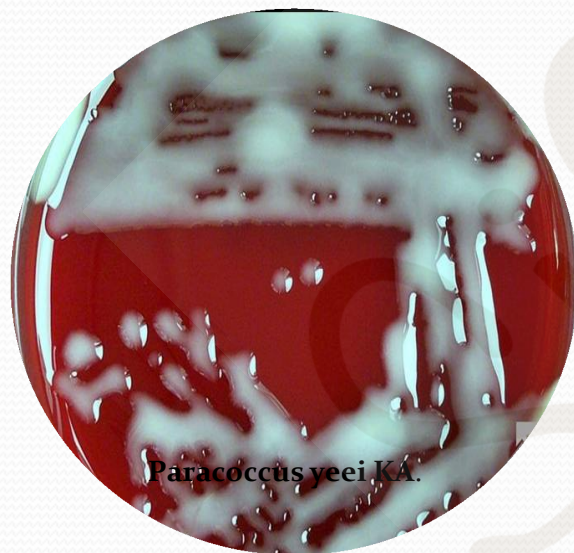
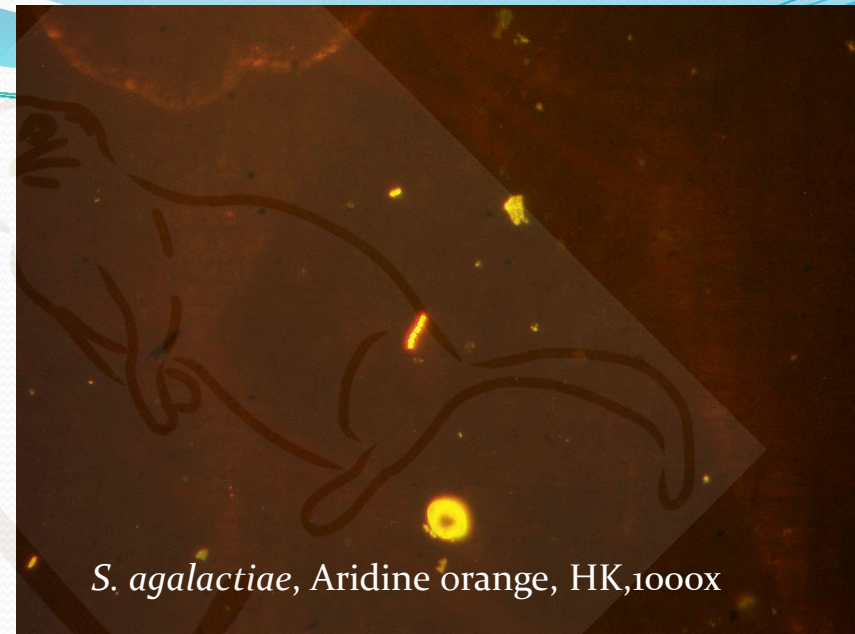
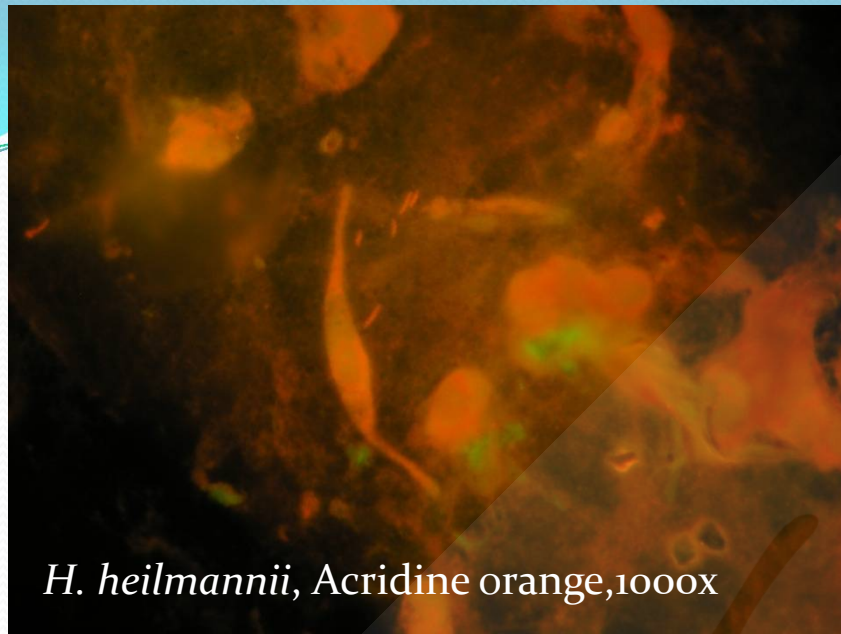


Immunofluorescence staining of the bacteria in infected HEL cells



EUROPEAN CONCERTED ACTION
ON LYME BORRELIOSIS

Culture of spirochetes from patient material is still the gold standard for laboratory diagnosis of LB.



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?

- **Zložitá interpretácia**

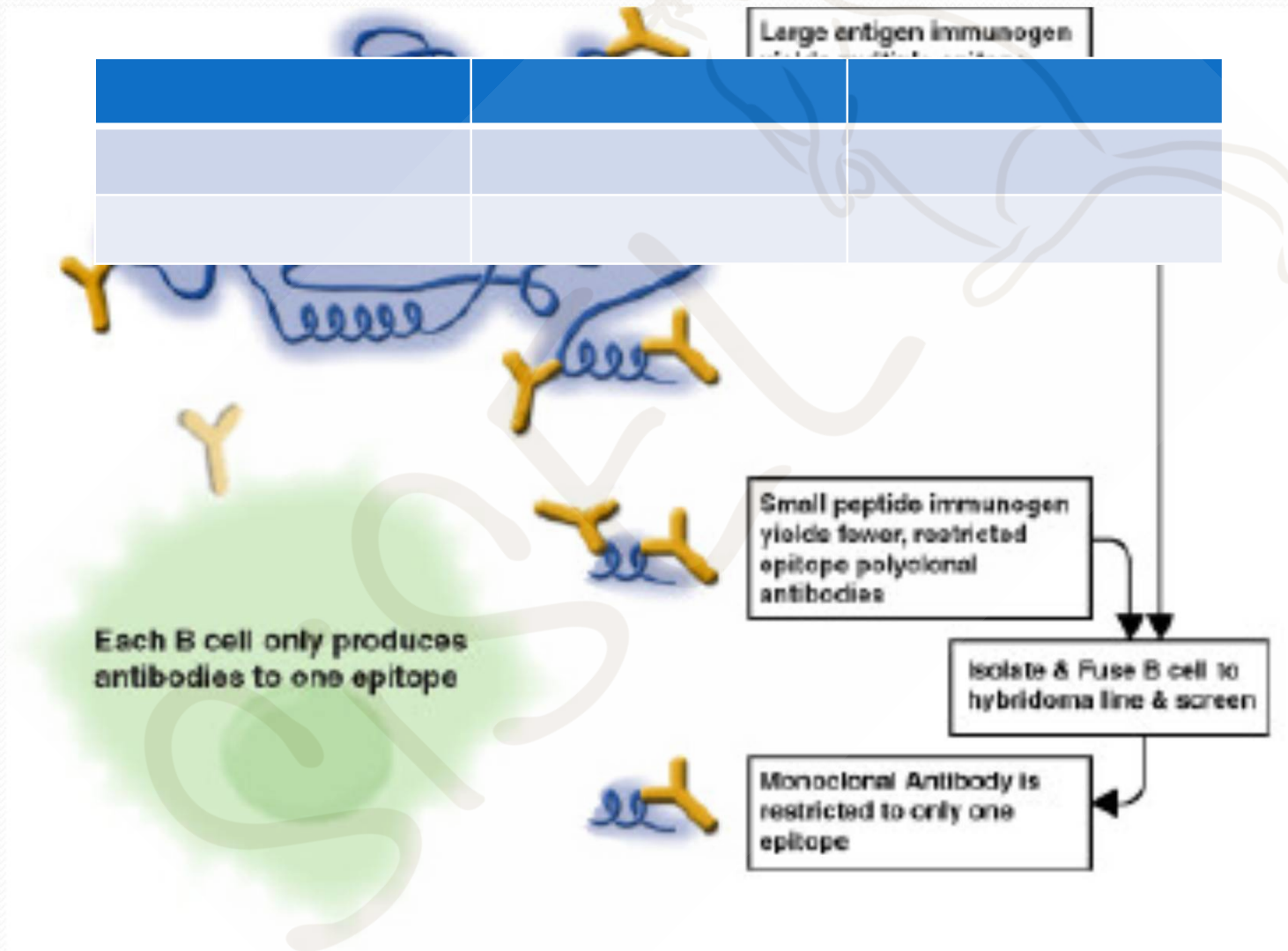
- Závislá na triede Ig a mikroorganizme

SÉROLOGICKÁ DIAGNOSTIKA

Pre klinickú validitu výsledku je rozhodujúca

- ✖ Afinita a avidita jednotlivých tried imunoglobulínov
- ✖ Použitý antigén (*koplexný Ag - skrížená reaktivita*)
- ✖ Proces protilátkovej odpovedi pri rôznych mikrobiálnych agensoch a nozologických jednotkách
 - + Učebnicovú schému dynamiky protilátkovej odpovedi nemožno absolutizovať
 - + Hodnoty vyjadrené v arbitrárnych jednotkách nekorelujú s navyknutým hodnotením „titračných stanovení“ (*napr. KFT*) vzhľadom na nelineárny charakter regresnej krivky
 - + Perzistujúca protilátková odpoveď vo viacerých triedach imunoglobulínov neodráža vždy aktivitu ochorenia

EFFECT OF ANTIGEN SIZE ON HUMORAL IMMUNITY



Materiál	Sérum
Oddelenie	MOD
Diagnóza	A69.2 Lymská choroba
Výšetrenie	B,BWM
Komentár	

Dátum		26.5.2015	11.3.13	25.4.12	29.9.11
Číslo		7938-S	3930-S	6490-S	15148-S
29.5.2015	WRIGM	+ pozitívny	+ pozitívny	+ pozitívny	+ pozitívny
29.5.2015	WBM17	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM19	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM21	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM25	+ pozitívny	+ pozitívny	+ pozitívny	+ pozitívny
29.5.2015	WBM30	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM31	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM39	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM41	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBM83	- negatívny	- negatívny	- negatívny	- negatívny
29.5.2015	WBMVLSE	- negatívny	- negatívny	- negatívny	- negatívny

Konfirmácia opakovane od 29.9.2011 potvrdila špecificitu IgM.

Sérokonverzia neprítomná

St.p. abortívnej infekcii

Protrahovaná prítomnosť špecifických IgM s/bez sérokonverzie nesvedčí pre chronickú boreliózu (ECCMID apríl 2013)

Číslo 14016

Pacient

Rod.čís.

stovňa 25

Odber 19.10.2015

Prech. 2014/15490

Príjem 19.10.2015

Nasl. /

Materiál Sérum

chlamydia trachomatis PCR...

negatívna

Hran. hodnoty

Oddelenie BL

Ureaplasma spp. PCR.....

pozitívna

Diagnóza J41.8 Zmiešaná jednoduchá a hlienovohnisová chronická

Vyšetrenie CP,CPWA,CSWA,CTWA,MP

Komentár

Dátum:		19.10.2015	5.11.14	8.1.14	4.9.13	16.4.12	17.1.12
Číslo		14016-S	15490-S	224-S	12871-S	5861-S	864-S
20.10.2015	WCPAM		- negatívny				- negatívny
20.10.2015	WCPAO2		- negatívny				- negatívny
20.10.2015	WCPAO4		- negatívny				- negatívny
20.10.2015	WCPAO5		- negatívny				- negatívny
20.10.2015	WCPIGA		- negatívny				- negatívny
20.10.2015	WCPSAM		- negatívny				- negatívny
20.10.2015	WCPSIGA		- negatívny				- negatívny
20.10.2015	WCTAHSP		- negatívny				+ pozitívny
20.10.2015	WCTAM		+ pozitívny				+ pozitívny
20.10.2015	WCTAMIP		+ pozitívny				+ pozitívny
20.10.2015	WCPIGA		- negatívny				- negatívny
20.10.2015	WCPSAM		- negatívny				- negatívny
20.10.2015	WCPSIGA		- negatívny				- negatívny
20.10.2015	WCTAHSP		- negatívny				+ pozitívny
20.10.2015	WCTAM		+ pozitívny				+ pozitívny
20.10.2015	WCTAMIP		+ pozitívny				+ pozitívny
20.10.2015	WCTAO2		- negatívny				- negatívny
20.10.2015	WCTIGA		+ pozitívny				+ pozitívny
19.10.2015	CPNIGA		1.48 pozitív	1.164 hranič	1.652 pozitív	1.249 pozitív	1.437 pozitív
19.10.2015	CPNIGG		2.026 pozitív	2.123 pozitív	2.003 pozitív	2.672 pozitív	2.834 pozitív

Findings of microbiological examination

Negative samples	40	14,0%
Chlamydophilla pneumoniae	61	21,0%
Mycoplasma pneumoniae	24	8,0%
Common bacteria	45	15,0%
C. pneumoniae and common bacteria	41	14,0%
C. pn. and common bacteria and M. pn.	82	28,0%
Total patients	293	100%

Tab. 2



ECCMID 2002
Milan / Italy

Chlamydophilla (Clamydia) pneumoniae as a causative agent of respiratory tract infections of children and adolescents, Botek, R. et al., ECCMID MILAN 2002

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The Evolution and Revolution of PCR

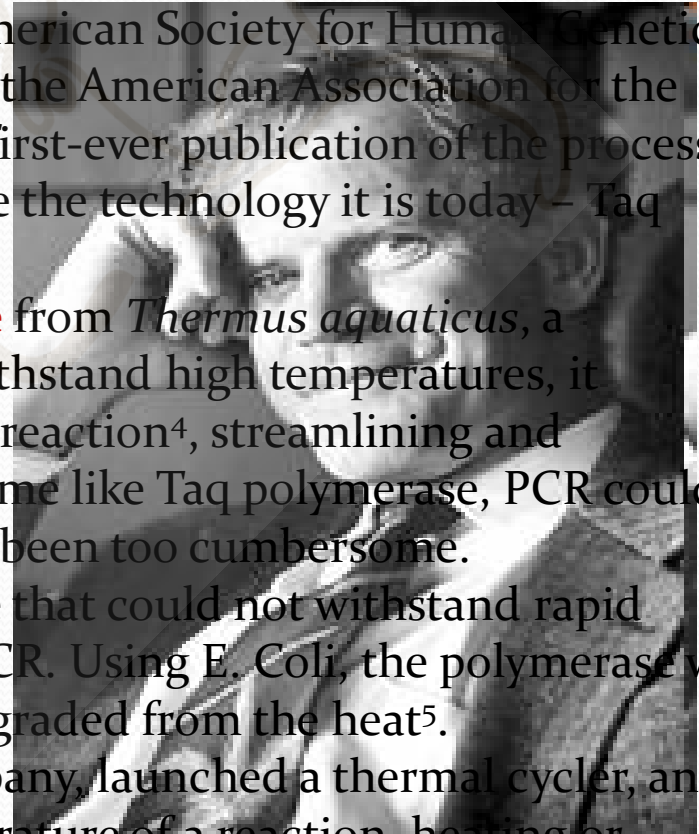
In **1983**, **Kary Mullis**, PhD, a scientist at the Cetus Corporation, conceived of PCR as a method to copy DNA and synthesize large amounts of a specific target DNA. Over the next two years, a team of Cetus scientists that recognized the potential impact PCR could have on molecular biology, researched, refined and made the theoretical process a reality.

The team presented for the first time in 1985 at the American Society for Human Genetics annual meeting.² Later that year, *Science*, a journal of the American Association for the Advancement of Science³, reported the results in the first-ever publication of the process. Two significant advances have enabled PCR to become the technology it is today – Taq polymerase and the thermal cycler.

In **1986**, Cetus scientists isolated the **Taq polymerase** from *Thermus aquaticus*, a bacterium found in hot springs. Because Taq could withstand high temperatures, it removed the need for human intervention during the reaction⁴, streamlining and shortening the process. Without a heat-resistant enzyme like Taq polymerase, PCR could not be used on a large scale as the process would have been too cumbersome.

Prior to Taq, DNA polymerase from *E. coli*, an enzyme that could not withstand rapid heating and cooling, was used in the second step of PCR. Using *E. coli*, the polymerase was manually replaced at each step of the reaction as it degraded from the heat⁵.

In 1987, PerkinElmer, another US-based biotech company, launched a thermal cycler, an instrument that is programmed to regulate the temperature of a reaction, heating or cooling the samples as needed. Once again, this advance minimized human interaction in the reaction, leading to an elegant, efficient and streamlined process.



MOLEKULÁRNA DIAGNOSTIKA - LIMITY

Výhody

1. Jednoduchý odber a transport
2. Rýchla dostupnosť výsledku (relatívne)
3. Vysoká špecificita
4. Vysoká senzitivita
5. Detekcia viac agensov v jednom odbere

Nevýhody

1. NA nevydrží všetko –
 1. Fragmentácia
 2. Exo a endonukleázová deštrukcia
2. Voľba primerov
 1. Reproducibilita
3. Inhibícia PCR

Prečo molekulárna diagnostika

- 1. Detekcia nekultivovateľných agensov**
- 2. Detekcia neobvyklých agensov**
- 3. Detekcia vírusov**
- 4. Vysoká citlivosť a špecificita**

Prístupy k molekulárnej diagnostike

Klasická PCR

Neisseria gonorrhoeae

Chlamydia trachomatis

Ureaplasma spp.

Mycoplasma hominis

Chlamydia pneumoniae

Mycoplasma pneumoniae

Herpes simplex virus I

Herpes simplex virus II

Cytomegalovirus

Dôkaz jedného agensu:

- Presná pracovná diagnóza
- V prípade negatívneho výsledku opakované vyšetrenie a/alebo skúsiť iné agens

=

Strata času/Strata agensu

Table 2. List of pathogens that were considered to be present in the panel of the 88 specimens according the combination of duplex PCR tests (Argene/bioMérieux) used as gold standard.

Pathogens	Single infections	Co-infections		Total
		with another pathogen	with at least 2 other pathogens	
Influenza A viruses	4	3	0	7
Influenza B viruses	0	5	0	5
Respiratory syncytial virus	3	4	6	13
Metapneumovirus	0	2	2	4
Parainfluenza viruses ^a	3	4	3	10
Rhinoviruses/enteroviruses	8	5	10	23
Coronaviruses ^b	7	5	13	25
Adenoviruses	2	9	11	22
Bocaviruses	1	4	7	12
<i>Mycoplasma pneumoniae</i>	2	4	4	10
<i>Chlamydia pneumoniae</i>	1	1	2	4
<i>Bordetella pertussis</i>	2	4	3	9
None	–	–	–	11

^atype 1 (n = 2), type 3 (n = 3) and type 4 (n = 5).

^btype NL63 (n = 6), type OC43 (n = 8), type 229E (n = 1), type HKU1 (n = 6) and untyped (n = 4).

doi:10.1371/journal.pone.0072174.t002

Following testing of the 88 samples with the reference method.....Most pathogens, with the exception of influenza A virus, were frequently associated with at least another pathogen.

Rozdielne mikroorganizmy prevažne spôsobujú rovnakú alebo veľmi podobnú symptomatológiu

Identifikácia etiologického agensu:

- v mnohých prípadoch život zachraňujúca (napr. herpetická encefalitída)
- výrazne ovplyvňuje terapeutickú intervenciu, hlavne pri „banálnych“ vírusových infekciách (napr. respiračného systému)

RESPIRAČNÝ PANEL

- **Influenza A**
- **Influenza B**
- **Parainfluenza 1, 2, 3**
- **Respiratory Syncytial Virus A & B**
- **Adenovirus groups B, C, E, some A, D**
- **Rhinovirus & Enterovirus**
- **Metapneumovirus**
- **Bordetella pertussis**
- **Bordetella parapertussis & B.bronchiseptica**
- **Mycoplasma pneumoniae**
- **Chlamydophila spp.**
- **Legionella pneumophila**
- **Legionella longbeache**

MT-PCR Sample Report - Sample 15

No.	Colour	Gene	Call	Concentration	Corrected Melt	Take-off
A10	Yellow	Influenza A	Check		84.55	12.84
B10	Blue	Influenza B				
C10	Purple	RSV				
D10	Green	RV/EV	Present	15,541	86.43	13.33
E10	Orange	hPIV1				
F10	Brown	hPIV2				
G10	Light Green	hPIV3				
H10	Red	hAdv	Present	219	84.37	19.97
I10	Teal	hMPV				
J10	Magenta	Bordetella				
K10	Pink	B.parapertussis				
L10	Dark Purple	Legionella				
M10	Blue	M.pneumoniae				
N10	Yellow	chlamydiaceae				
U10	Dark Blue	Sample Adequacy	Present	22,821	81.87	12.13
P10	Black	SPIKE	Present	10,000	81.8	14.02

Operator: JV,JB,RB

Sample volume used: 10µl

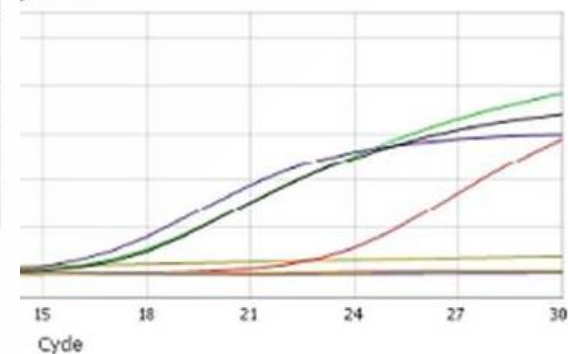
File: C:\Users\AusUx\Documents\177_-_Respiratory_Pathogens_B_(16-well)_2018-01-15_0956.epx

Step 1 Sensitivity: NA extract (15 cycles)

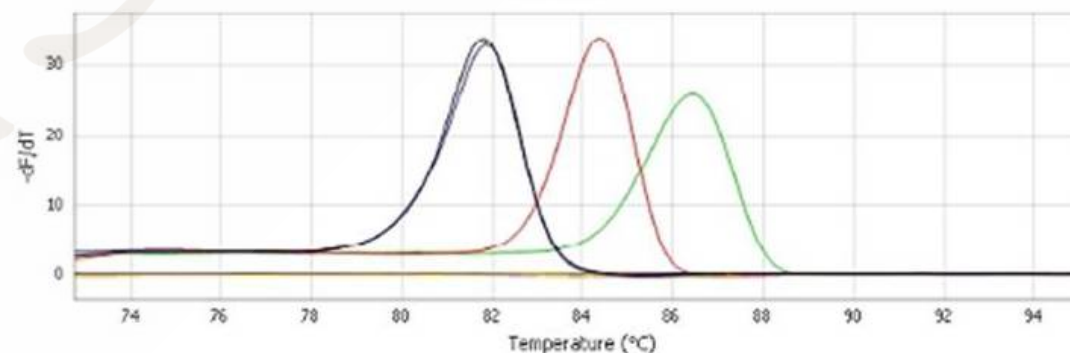
Analysis Sensitivity: Normal

RESPIRAČNÝ PANEL

Amplification Curves



Melt Curves





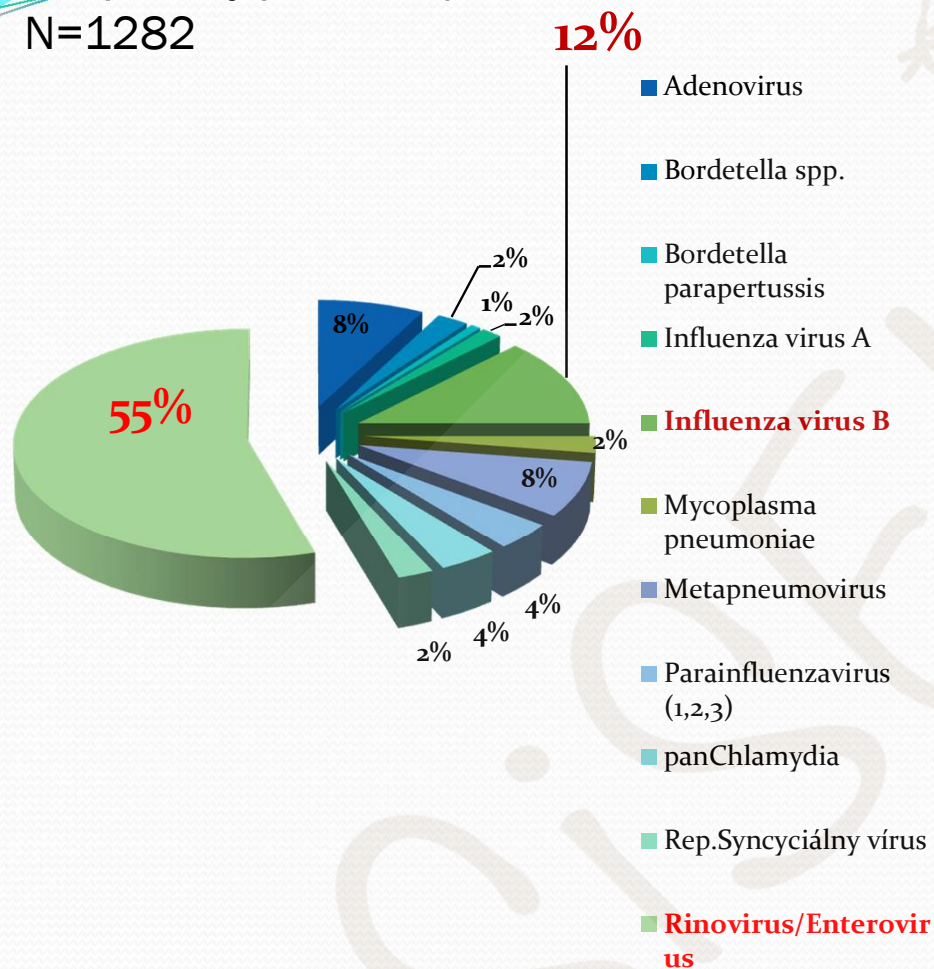
Loyola University Medical Center



<https://www.ncbi.nlm.nih.gov/pubmed/25762770>

Respiračný panel I. 1/2 rok 2018

N=1282



J Clin Microbiol. 2015

Oct;53(10):3110-5. doi:

10.1128/JCM.00382-15. Epub 2015 Mar

11

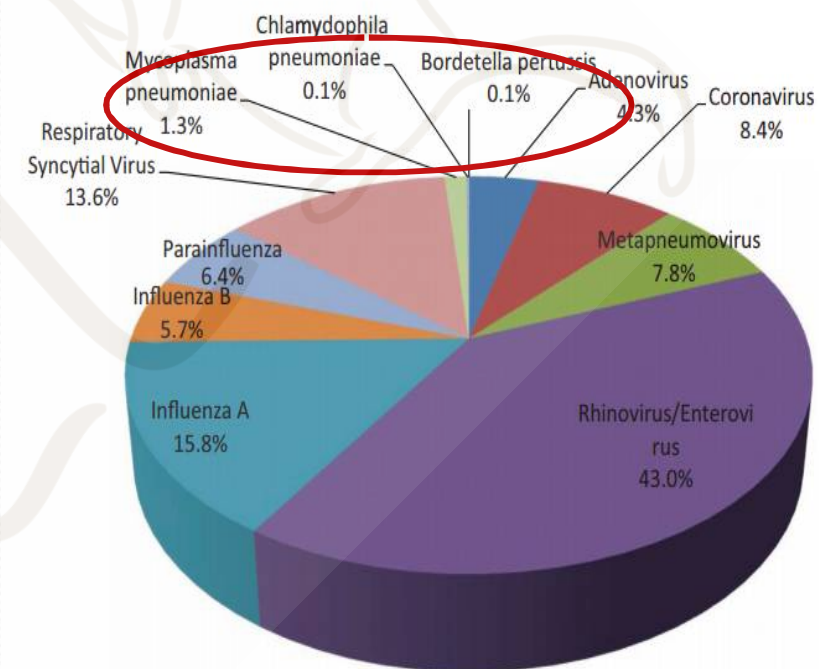


FIG 1 Incidence of viruses present in respiratory specimens at Loyola University Medical Center, 1 October 2013 to 27 September 2014. Note that the percentage total exceeds 100% because some samples contained multiple viruses.

Multiplex PCR panel

COUNTERPOINT

In seeking continued “laboratory improvement” there is a great danger of establishing exhaustive microbiology as an end in itself.... This trend is discouraging to many microbiologists who recognize that the quality of their services will be more effectively improved through careful integration of what is technically feasible with what is clinically important.

Raymond C. Bartlett, Medical Microbiology: Quality, Cost and Clinical Relevance

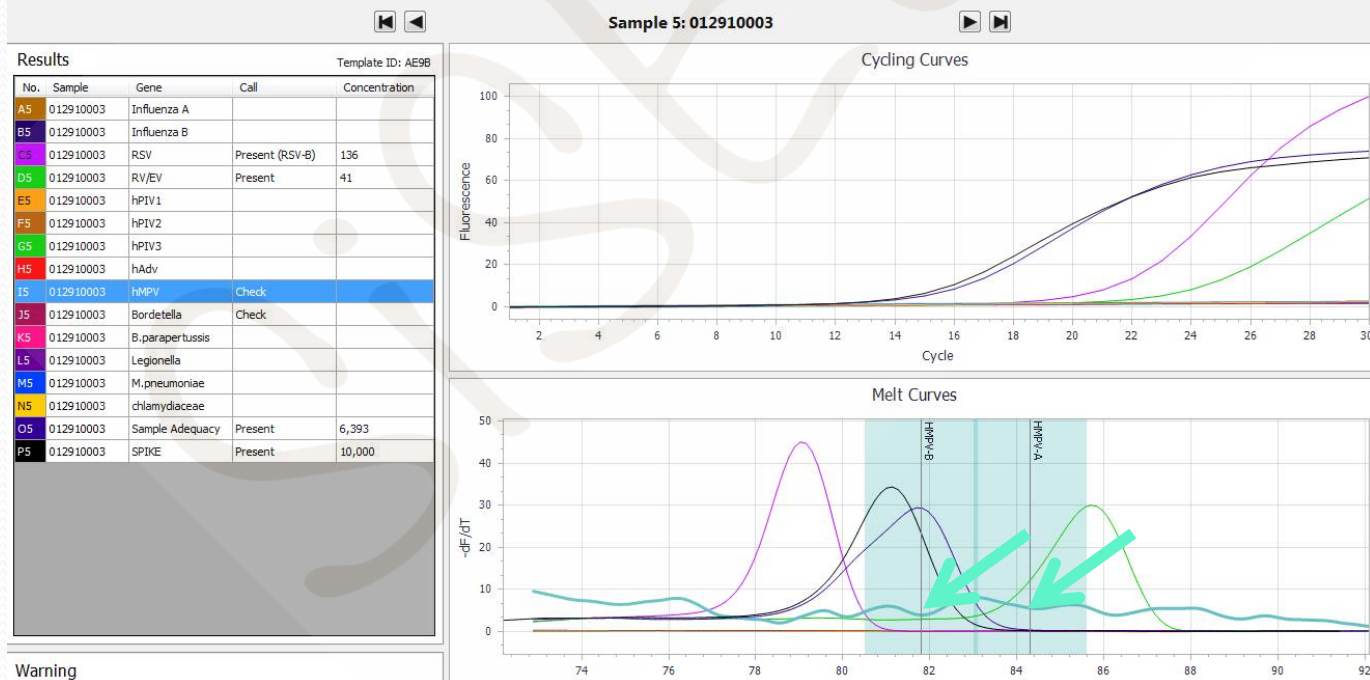
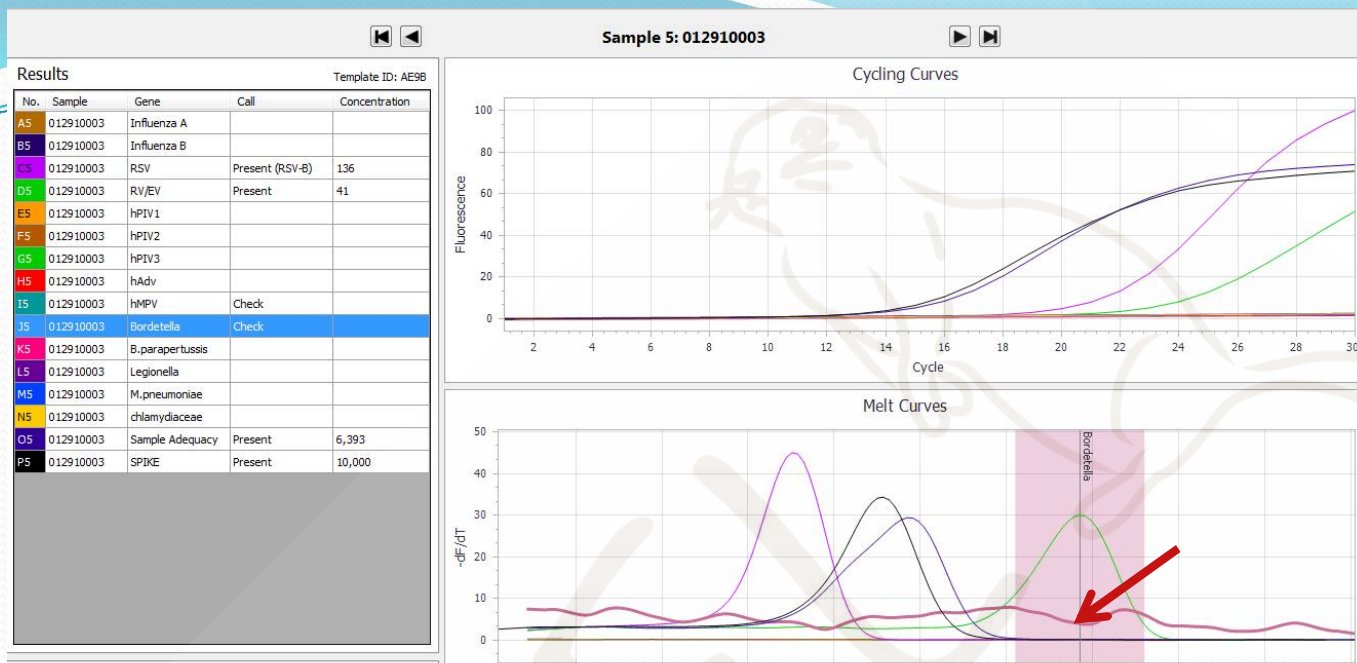
Multiplex PCR panel

Points of agreement

1. Highly multiplexed molecular tests have clinical value; they provide a syndromic approach to diagnostics, which is particularly useful for infections in which it is not possible to determine the etiologic agent based only on symptoms.
2. The use of highly multiplexed tests is more closely aligned with traditional culture methods, where clinicians do not need to identify a specific pathogen for testing but rather think in broad terms about whether there is a bacterial infection in the respiratory tract or the blood.
3. Rapid sensitive diagnostic tests have the potential to transform the medical management of patients with infectious diseases.
4. Multiplex tests should be developed in consultation with clinical microbiologists and clinicians so that the panel members reflect clinical reality.
5. Implementation of panel tests should be done in consultation with clinicians, so there is a clear understanding of the appropriate use and interpretation of test results.

Paul C. Schreckenberger, Alexander J. McAdam

Point-Counterpoint: Large Multiplex PCR Panels Should Be First-Line Tests for Detection of Respiratory and Intestinal Pathogens



Warning

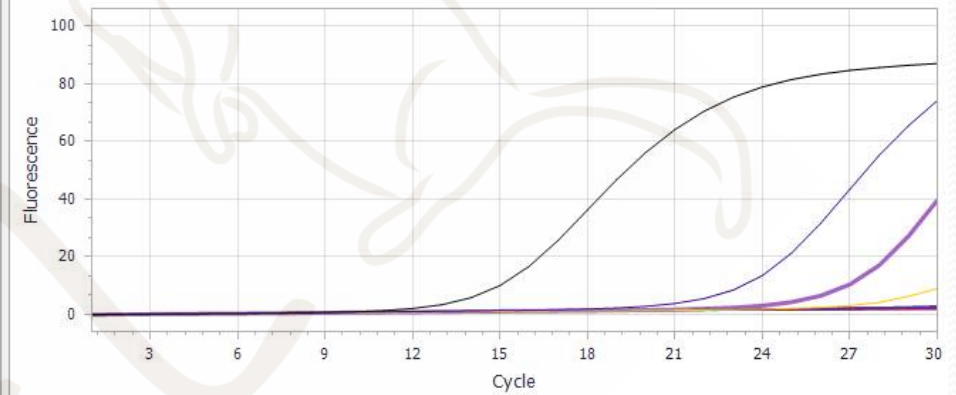
Sample 1: 022010003

Results

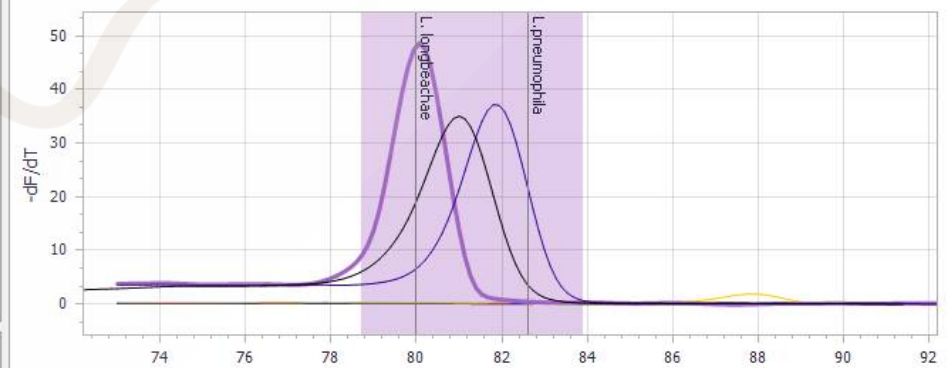
Template ID: AE9B

No.	Sample	Gene	Call	Corrected Melt	Take-Off	Calculated Ct	Concentration
A1	022010003	Influenza A					
B1	022010003	Influenza B					
C1	022010003	RSV					
D1	022010003	RV/EV					
E1	022010003	hPIV1					
F1	022010003	hPIV2					
G1	022010003	hPIV3					
H1	022010003	hAdv					
I1	022010003	hMPV					
J1	022010003	Bordetella					
K1	022010003	B.parapertussis					
L1	022010003	Legionella	Present (L. long...	80.08	24.17	32.69	3
M1	022010003	M.pneumoniae					
N1	022010003	chlamydiaceae					
O1	022010003	Sample Adequacy	Present	81.86	20.41	28.94	35
P1	022010003	SPIKE	Present	81.8	11.6	20.13	10,000

Cycling Curves



Melt Curves



Warning



LABadmin

Amplification Curves

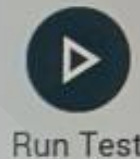
16:57 28-05-2019

1 Available

2 Not installed

3 Not installed

4 Not installed



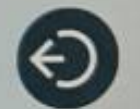
Run Test



View Results



Options



Log Out

PATHOGENS CONTROLS

Detected Pathogens

✓ Respiratory Syncytial ...
CT 33.6 - EP 5,799

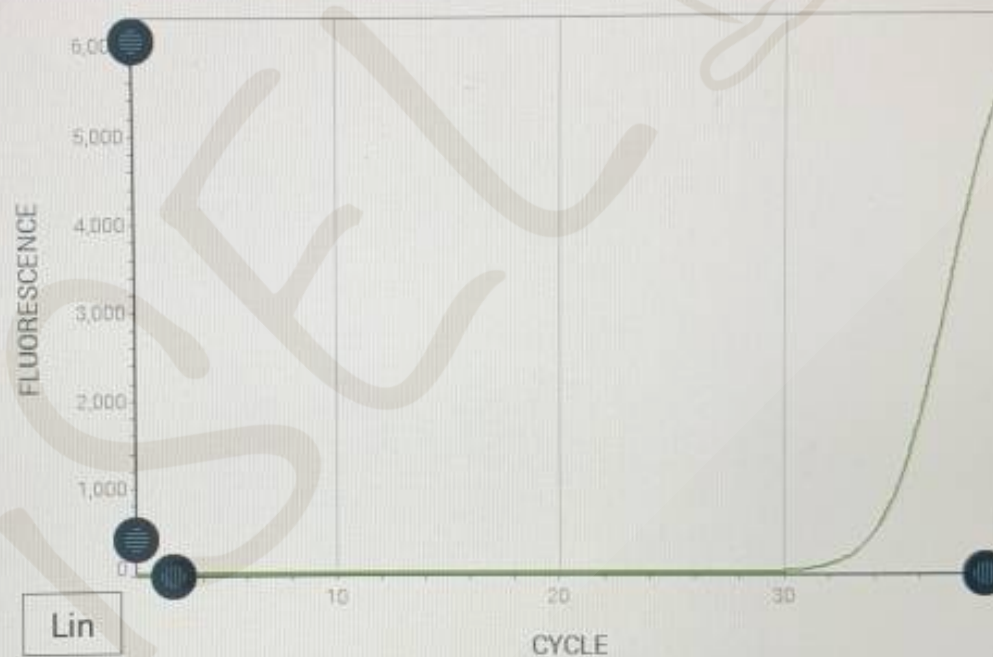
Non detected Pathogens

● Influenza A
CT — - EP —

● Influenza B
CT — - EP —

● Influenza A H1N1 pdm...
CT — - EP —

● Coronavirus 229E
CT — - EP —



Summary

Amplification Curves

Melting Curves

Test Details



Print Report



Save Report

LABadmin

Amplification Curves

17:00 28-05-2019

1

Available

2

Not installed

3

Not installed

4

Not installed

PATHOGENS

CONTROLS

Detected Pathogens



Influenza A

CT 30.7 - EP 110,947



Influenza A H1N1 pdm...

CT 29.6 - EP 15,476



Parainfluenza virus 3

CT 29.0 - EP 81,409



Respiratory Syncytial ...

CT 20.8 - EP 106,041

Non detected Pathogens



Influenza B

CT - EP -

Summary

Amplification Curves

Melting Curves

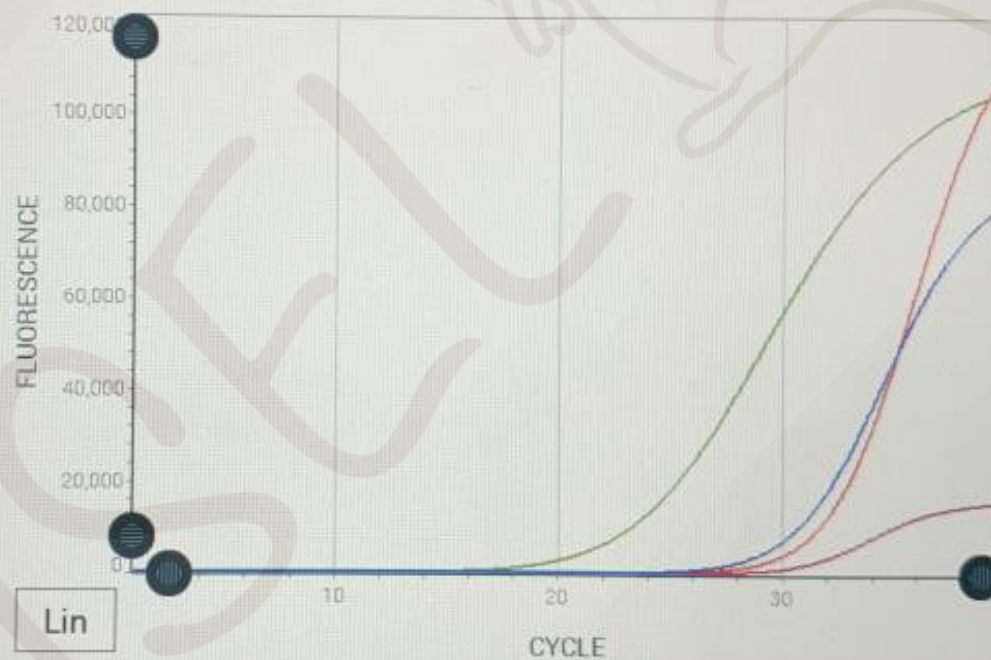
Test Details



Print Report



Save Report



Run Test



View Results



Options



Log Out

AusDiagnostic	AusDiagnostic nález	CT/kópii/* 10 µl	Qiasat nález	Ct/epf
			neg	
Izol. DNA_RNA	RSVINFAPIV ₃		Influenza A	30.7 / 110,947
			Influenza A H ₁ N ₁ pdm09	29.6 / 15,476
			Parainfluenza virus 3	29.0 / 81,409
			Respiratory Syncytial Virus A+B	20.8 / 106,041
011710003	Adenovirus sk. B,C,E	23/1996	Adenovirus	27.3 / 211,805
	Bordetella spp.	24/1122	Bordetella pertussis	29.5 / 184,206
011410005	Rhinovirus a Enterovirus (typy A,B,C)	33/3	neg	
011810001	Human RSV A,B	30/31	Respiratory Syncytial Virus A+B	33.6 / 5,799
011810003			Coronavirus OC ₄₃	35.3 / 42,890
	Human RSV A,B	18/46555	Respiratory Syncytial Virus A+B	23.1 / 255,625
011810004	Human RSV A,B	30/15	Error	
012110001	neg		Influenza A	27.1 / 105,120
			Influenza A H ₃	25.4 / 138,559
013110001	neg		neg	

AusDiagnostic	AusDiagnostic nález	CT/kópií/10 µl	Qiasat nález	Ct/epf
020110018	Len qiasat	CT/kópií/10 µl	Respiratory Syncytial Virus A+B	29.0 / 165,234
021210034	Influenza A (H1, H3, H5, H7)	24/917	Influenza A	31.6 / 79,175
			Influenza A H3	29.6 / 114,789
021910001	Len qiasat		Influenza A	32.3 / 76,268
			Influenza A H1N1 pdm09	30.9 / 9,206
012910001	Adenovirus sk. B,C,E, Human RSV A,B		Adenovirus	34.2 / 19,689
			Respiratory Syncytial Virus A+B	31.8 / 3,285
012910003	Human RSV A,B, Rhinovirus a Enterovirus (typy A,B,C)	27/136_29/41	Rhinovirus/Enterovirus	32.3 / 53,234
	Bordetella spp./HMP V Check	o_3 CT	Respiratory Syncytial Virus A+B	31.2 / 132,960
022010003	Legionella pneumophilla/lonbeachae h raničný	33/3	neg	

AusDiagnosti c	AusDiagnostic nález		CT/kópií/10 μl	Qiasat nález	Ct/epf
022210001	Influenza A (H1, H3, H5, H7)	25/332	Influenza A	29.9 / 188,455	
	Legionella pneumophilla/longbeachae hraničný	33/2	Influenza A H1N1 pdm09	28.9 / 60,226	
022710001	Len qiasat		neg		
030510002	Len qiasat		030510002	Parainfluenza virus 3	19.9 / 309,634
022710008	Influenza A (H1, H3, H5, H7)	33/3	022710008	Influenza A: Positive	30.3 / 104,886
				Influenza A H1N1 pdm09: Positive	28.7 / 15,297
030410005	Human parainfluenzae virus 3	30/16	030410005	Parainfluenza virus 3: Positive	31.0 / 120,093
030510014	Human RSV A, B	34/3	030510014	neg	
022510008	negat		022510008	neg	
031910002	negat		031910002	Error	

Stolice	IMCH	QUIASTAT	CT/epf
Kultúra Cj_SAE	<i>Campylobacter</i> spp.		18.7 / 252,768
	<i>Salmonella</i> spp.		17.8 / 394,710
BREZOVSKA VERONIKA		Enteropathogenic E. coli: Positive	28.8 / 33,891
41110001	Rotavírusy	Rotavirus A: Positive	17.3 / 124,910
		Clostridium difficile toxin A/B: Positive	28.6 / 139,810
41210001	<i>Helicobacter pylori</i>	neg	
41210002	neg	neg	
42410001		Error	
308		neg	
40110001	Norovirus	Norovirus GII: Positive	17.6 / 473,917
313		neg	
40110002	Campylobacter sp. .	neg	
40210001	Clostridium difficile toxin A/B: Positive	Clostridium difficile toxin A/B: Positive	18.3 / 314,279
40210002	Clostridium difficile toxin A/B: Positive	Clostridium difficile toxin A/B: Positive	28.8 / 100,545
41610001		Clostridium difficile toxin A/B: Positive	17.9 / 292,875

1 Available

2 Not installed

3 Not installed

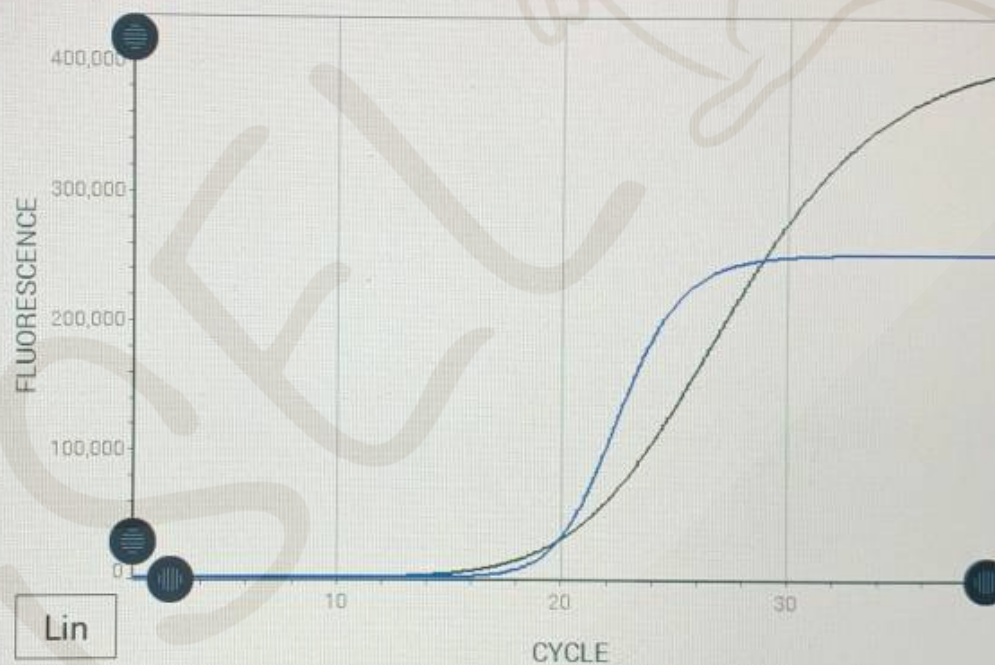
4 Not installed

PATHOGENS CONTROLS

Detected Pathogens

☒ **Campylobacter spp.**
CT 18.7 - EP 252,768☒ **Salmonella spp.**
CT 17.8 - EP 394,710

Non detected Pathogens

☐ **Norovirus GII**
CT — - EP —☐ **Rotavirus A**
CT — - EP —☐ **Astrovirus**
CT — - EP —

Run Test



View Results



Options



Log Out

Summary

Amplification Curves

Melting Curves

Test Details



Print Report



Save Report

Stolice	IMCH	QUIASTAT	CT/epf
40110002	Campylobacter sp. .	neg	
40110001	Norovirus .	Norovirus GII: Positive	17.6 / 473,917
41710003	Clostridium difficile toxin A/B: Positive	Clostridium difficile toxin A/B: Positive	18.9 / 122,272
40310002	Rotavirus	Norovirus GII: Positive	32.9 / 11,100
	Clostridium difficile: Positive	Rotavirus A: Positive	19.4 / 102,059
		Clostridium difficile toxin A/B: Positive	32.1 / 32,960
40310003	neg	neg	
40410003	Clostridium difficile a Salmonella kult. .	Clostridium difficile toxin A/B: Positive	26.9 / 13,919
	Adenovirus, Rotavirus	Salmonella spp.: Positive	29.2 / 15,009
40810002	Clostridium difficile ., toxin negat	Clostridium difficile toxin A/B: Positive	19.2 / 56,770
40410004	negat	Clostridium difficile toxin A/B: Positive	18.4 / 100,392
40510005	Clostridium difficile ., toxin negat	Clostridium difficile toxin A/B: Positive	22.8 / 63,827
41010003	neg	neg	
41010008	neg	neg	

LABadmin

Amplification Curves

17:01 28-05-2019

1 Available

2 Not installed

3 Not installed

4 Not installed



Run Test



View Results



Options



Log Out

PATHOGENS

CONTROLS

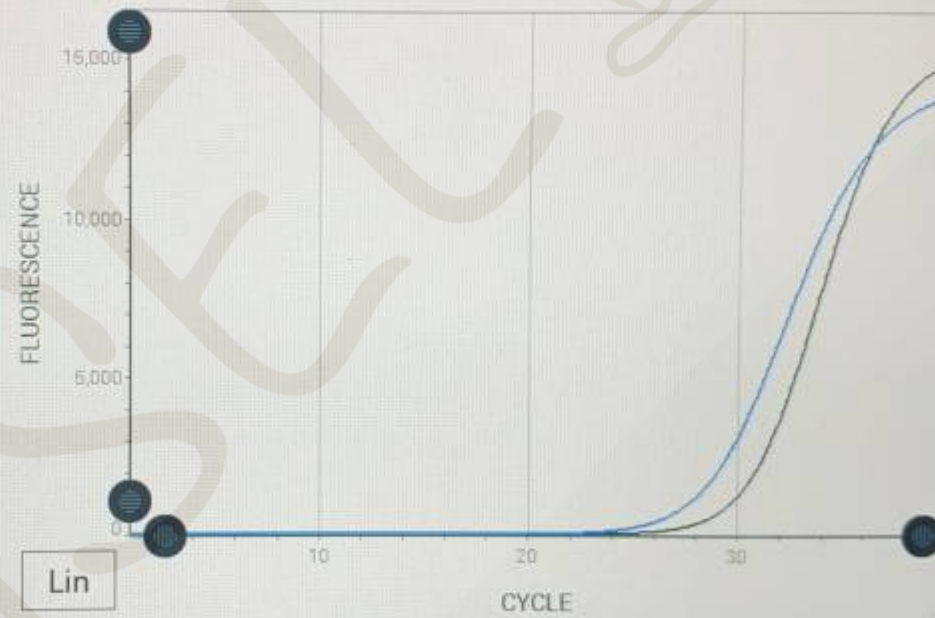
Detected Pathogens

✓ **Clostridium difficile to...**
CT 26.9 - EP 13,919

✓ **Salmonella spp.**
CT 29.2 - EP 15,009

Non detected Pathogens

None



Summary

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Print Report



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LABadmin

Amplification Curves

16:58 28-05-2019

1

Available

2

Not installed

3

Not installed

4

Not installed



Run Test

PATHOGENS

CONTROLS

Detected Pathogens

☒ **Clostridium difficile to...**
CT 17.9 - EP 292,875

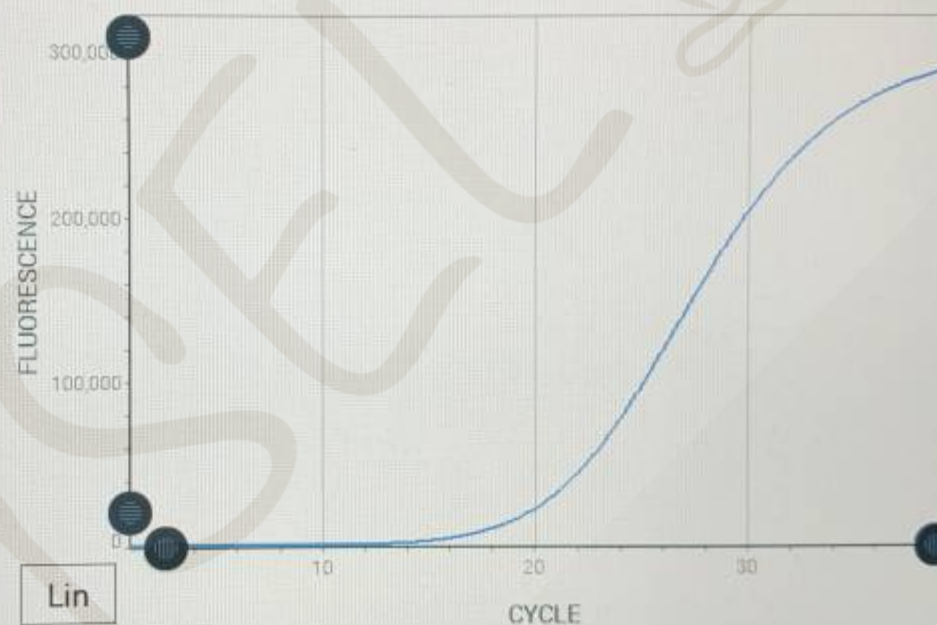
Non detected Pathogens

☐ **Norovirus GII**
CT -- - EP --

☐ **Rotavirus A**
CT -- - EP --

☐ **Astrovirus**
CT -- - EP --

☐ **Norovirus GI**
CT -- - EP --



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ČO NA ZÁVER?

- V našom laboratóriu kombinujeme viaceré molekulárne metódy podľa aktuálnej požiadavky.
- Sme presvedčení, že molekulárne metódy patria do rutinne mikrobiologickej diagnostiky, nesmú byť tabuizované
- Qiasat je „user friendly“ jednoducho obsluhovateľný a spoľahlivý – určite je vhodný aj ako „point of care“
- Automatizácia však neznamená neznamená automatický report výsledkov