Diagnosis of *Mycoplasma pneumoniae* infections

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Aims of the presentation

- The role of conventional methods for detection of *M. pneumoniae*
  - Non-microbiological diagnosis
  - Cold agglutinins
  - Direct examination and culture
  - Serology
- The role and utility of new diagnostic techniques such as nucleic acid amplification tests to identify *M. pneumoniae*
- Explain the strengths and weaknesses of different diagnostic approaches for implementation in routine setting
- Discuss diagnostic strategies to detect *M. pneumoniae*

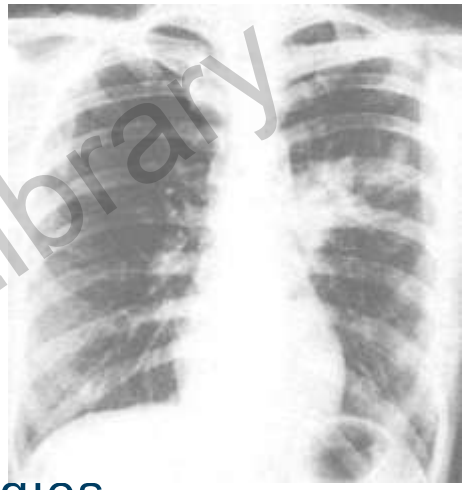
Aetiology of lower respiratory tract infection in the community (%)

Reference	*n*	*S. pn*	*H. infl*	*M. pn*	*C.pn*	Virus
Boldy et al. 1990	42	3.0	3.0	8.0	0	21.0
Creer et al. 2006	80	18.8	6.3	1.2		61.3
Graffelman et al. 2004	145	6.2	9.0	9.0	1.3	39.0
Holm et al. 2007	364	6	4	3	<1	24
Hopstaken et al. 2005	247	2.9	13.8			
Macfarlane et al. 1993	206	30.0	8.0	0.5		8.0
Macfarlane et al. 2001	316	17.1	9.8	7.3	17.4	19.3
GRACE study, 2012	3059	9.1	14.8	2.9	2.2	51.1
Range		**3-30**	**3-15**	**0.5-9**	**0-17**	**8-61**

- ***M. pn* prevalence varies depending on year studied and methods**
- ***M. pn* early data are largely based on serological analysis only**

Woodhead M et al. Clin. Microbiol.Infect. 2011; 17, E1-E59

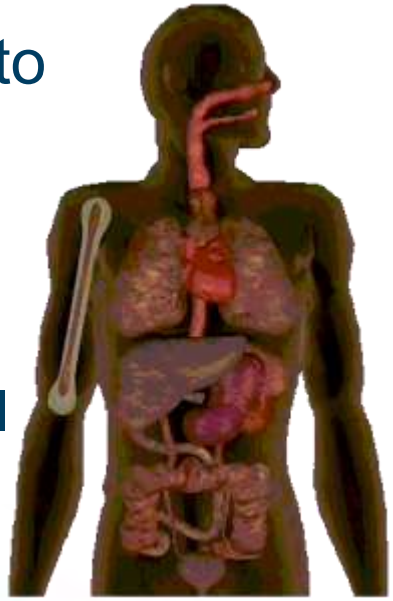
Difficulties with Non-Microbiological Diagnosis



- Clinical signs, symptoms of atypical pneumonia
  - Chest signs on examination minimal
  - “walking pneumonia” frequently confused with virus infection: uniform throughout spectrum of etiologies
  - Prodrome of “flu-like” symptoms
  - Gradual onset, no pathognomonic symptoms

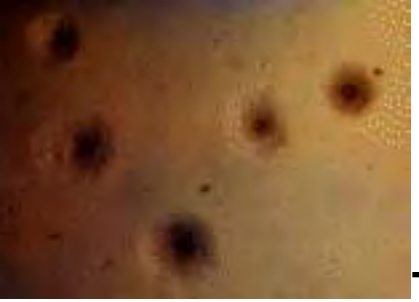
- CXR findings are usually non-specific and difficult to distinguish typical/atypical pneumonia

- Non-microbiological laboratory tests:
  - lack specificity for differentiation bacterial/non bacterial pneumonia

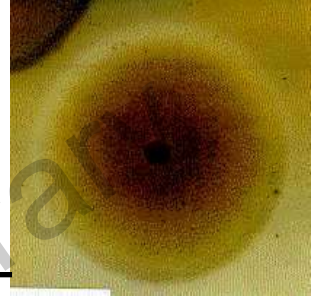


Microscopy for detection of *M. pneumoniae*?

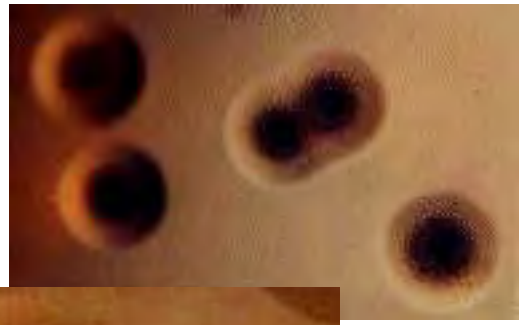
- The smallest among prokaryotic microganisms with circular dsDNA, usually 0.2-0.3μm in size; lack of cell wall; pass through a 0.45 μm filter.
- Because of the absence of cell walls, they do not stain with the Gram stain, and they are more pleomorphic and plastic than eubacteria.
- Pleomorphic, spherical, short rod, filament
- stained hardly, usually use Giemsa stain.
  - they appear as tiny pleomorphic cocci, short rods, short spirals, and sometimes as hollow ring forms.

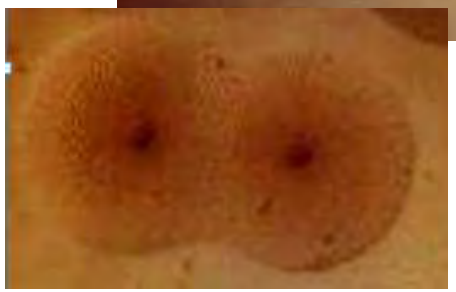


Culture based detection of *M. pneumoniae*

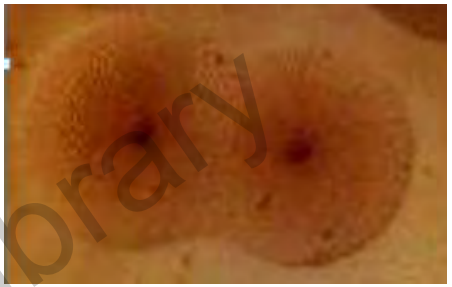


- Most mycoplasmas require a rich medium containing sterols and serum proteins for growth, yeast extract and penicillin to inhibit contaminating bacteria: Technical demanding
- On solid media, transparent fried egg colonies appear
- Slow: may take from two days to 6 weeks to form a colony.





M. pneumoniae : Culture versus PCR



	PCR +	PCR-	
Culture +	7	0	7
Culture -	5	359	364
total	12	359	371

- Culture compared to 2 PCRs: P1 gene and 16S rRNA
- Sensitivity of culture: 7/12 = **58.3%,** specificity: 100%

- In general: sensitivity of culture between 23 to 60%
  - ⇒ sensitivity too low
  - ⇒ Too slow
  - ⇒ Too labour intensive

leven M et al., J Infect Dis 1996; 173:1445-52

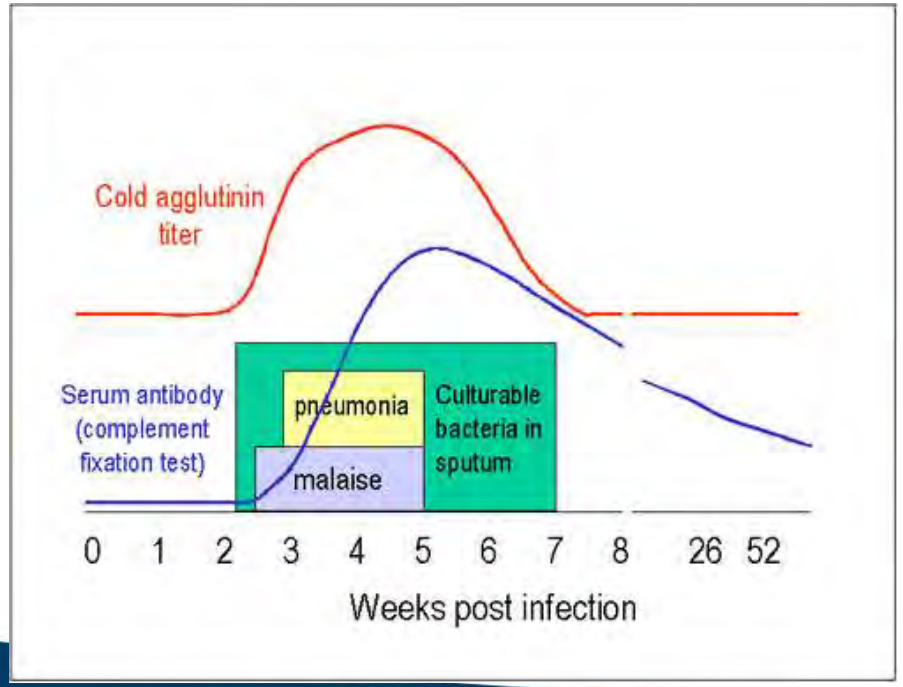
Value of cold agglutinins?

- Cold agglutinins to human O erythrocytes
- The antibodies of cold agglutinins arise before the complement fixing antibodies and they decline faster
- Approximately 34% - 68% of patients with *M. pneumoniae* infection develop cold agglutinins.

⇒ Sensitivity: limited

- Also pos in patients with viral infections:

⇒ Specificity: limited



Serology for diagnosis of *M. pneumoniae* infections

- CFT, ELISAs and EIAs:
  - simple to perform
  - Commercially available
  - Considered objective because of photometrical reading

- However: performance variable due to:
  - Different antigens: whole organisms, protein fractions, …
  - Lack of standardization: moderate concordance between tests
  - sensitivities varying between < 20% and >80%
  - different performances of IgM tests in children and in adults
  - More validation and QC needed

Serology for diagnosis of *M. pneumoniae* infections

Most reliable serological evidence for ongoing infection:

- Fourfold rise in IgG antibodies 3-4 weeks after onset
  - Mostly only one acute phase serum sample available
  - or paired serum samples collected within too short time interval
- Solitary high IgG titers: no diagnostic meaning for acute infection; useful in prevalence studies
- Detection of IgM antibodies in acute phase
  - Often not produced in very young children
  - IgM antibodies may appear late: sensitivity too low in early phase

Impact of serology on diagnosis of acute *M. pneumoniae* infection

- CAP study, Leiden: 11 tests evaluated on ± 100 samples (34 paired sera)

  ⇒ sensitivity of IgM: **7-23%** in first 6 days

  29-86% after more than 16 days

  Beersma et al. J Clin Microbiol 2005;43: 2277-85

- CAP + LRTI: 4 different tests evaluated on 224 pts (205 paired sera)

  ⇒ sensitivity of IgM: **10-31%** in first 6 days

  20-42% after more than 16 days

  Loens et al, ECCMID 2005

- Analysis of only IgM in acute-phase serum:

  only 35% pos in confirmed cases

  Miyashita et al, Eur J Clin Microbiol Infect Dis 2011; 30: 439-46

⇨ **Serology has limited value for early diagnosis of *M. pneumoniae* and impact on antibiotic management**

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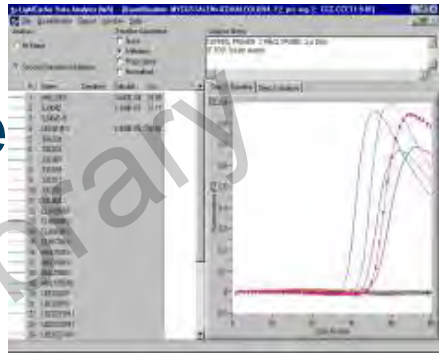
Nucleic acid amplification based tests

Mycoplasma's have the smallest genome of any prokaryote:+/- 20% that of *E. coli*, and the lowest G C content (+/-24%).

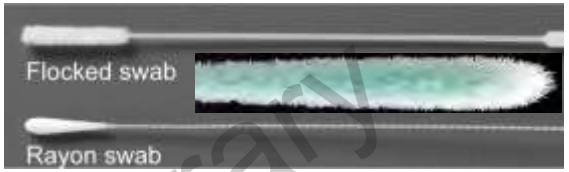
target	Detection procedure
P1 gene	Agarose gel electrophoresis and hybridization, Light Cycler, I-Cycler, Taqman, LAMP
16S rRNA gene, *tuf* gene, ATPase gene	Agarose gel electrophoresis and hybridization
16S rRNA	NASBA and electrochemiluminescence detection, real-time NASBA, Qβ-replicase
CARDS toxin gene	ABI Prism 7500

Conventional and Real-Time Mono in-house NAATs



Ref, year	Assay type	Detection	Gene target
Ieven, 1996	PCR	Agarose gel	P1-gene, 16SrRNA
A. Touati, 2009	PCR	Real-time	P1 gene
Gotoh K, 2012	LAMP	turbidity	P1 operon
Chaudhry R, 2013	Q PCR	Real-time	P1 gene
Schmitt BH, 2013	PCR	Real-time	ptsI
Liu Y, 2014	PCR	Cycleave	23S rDNA+ macrolide R

Comparison of specimens & sampling methods for the detection of *M. pneumoniae*



Sample	Method
Sputum>TW>NPS>OPS	PCR
OPS>NPS, NPA	PCR
OPS>BAL>sputum	PCR
Sputum>NPA	PCR, EIA
Sputum>OPS	PCR, NASBA, Gen-probe test, culture
NPS=OPS	PCR

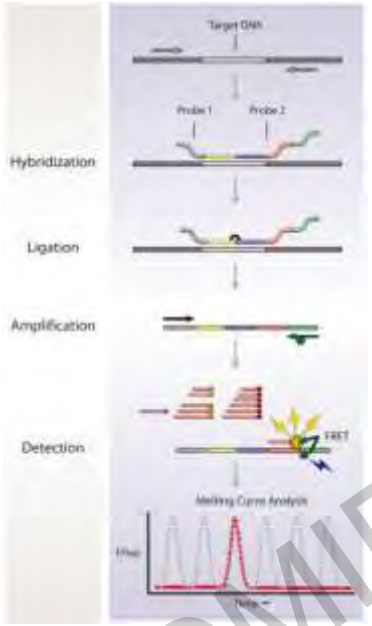
Loens et al., J. Clin. Microb 2008; 47:21-31.

Some amplification and detection kits for *M.pneumoniae* or max 3 atypicals

Kit manufacturer	Assay type	Detection procedure	Nr targets	Pathogens
M. pneumoniae BDProbeTec ET, BD	SDA	Fluorescence	1	*M. pneumoniae*
Illumigene Mycoplasma, MeridianBioScience	LAMP	Turbidity	1	*M. pneumoniae*
ASR MPN, Cepheid	PCR	Real-time	1	*M. pneumoniae*
ProPneumo-1 Hologic	MX-PCR	Real-time	2	*M. pneumoniae, C. pneumoniae*
Chlamylege, Argene	MX-PCR	Hybridization	3	*C. pneumoniae, M. pneumoniae, Legionella spp*
Venor MP	PCR	Agarose GE, RT PCR	1	*M. pneumoniae*

Multiplex tests for >3 pathogens, including *M. pneumoniae*

RespiFinder , Pathofinder



Mx RT amplification
no sample prep
melting curve analysis
Up to 25 targets
TAT 6 hr

RespiFinder SMART 22 Fast

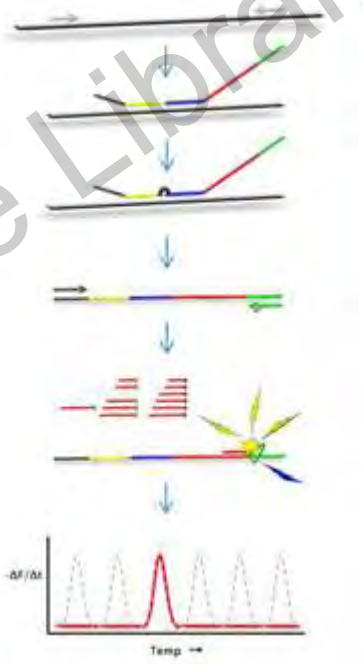
Mx RT amplification
no sample prep
melting curve analysis
22 targets
TAT 4 hr
multiple samples/instrument

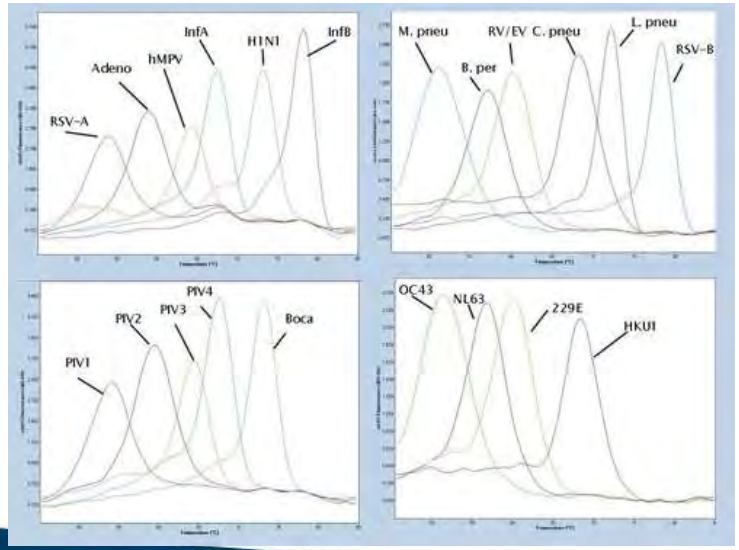
FilmArray RP, BioMérieux

Mx amplification
Sample prep included
Up to 20 targets
TAT VP 1 hr
1 sample/instrument

RespiFinder Mx assay, Pathofinder

- Rapid, conventional & real-time PCR
- Multiplex capabilities expanding till up to 25 targets
- 18 viral + 4 bacterial pathogens in 1 RT assay
- Gene-specific Mx reverse transcription step
- 2 pathogen specific probes hybridized by ligation, amplified and detected by melting curve analysis
- Contains a competitive internal amplification control
- Diagnosis within 6 hours
- Validated on QCMD panels
  - CE-IVD labelled

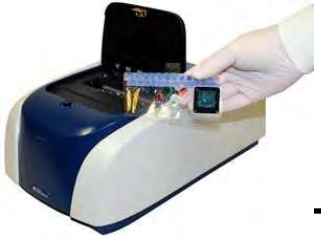




FilmArray RP assay

- System integrates sample preparation, amplification, detection and analysis
- All reagents are freeze dried in 1 pouch
- Closed system prevents cross-contamination
- Internal controls for each step
- Advanced software runs the system and automatically analyzes and reports results
- Multiplexed testing analyzes up to 120 tests per sample
- Rapid results in 1 hr from sample injection

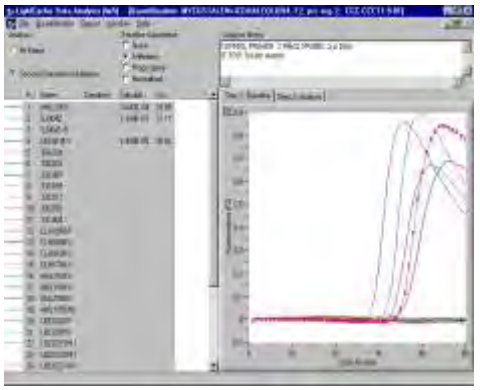
Viral: Adeno, Boca, Corona (229E, HKU1, OC43,NL63), Flu A, Flu A H1, Flu A H1 2009, Flu A H3, Flu B, hMPV, Para (1, 2, 3, 4), RSV, Rhino
Bacterial: *B. pertussis, C. pneumoniae, M. pneumoniae*



Choice of platforms and assays: Commercial versus in-house tests?

"commercial" tests: FDA approved or CE labeled IVD

- More or less extensive validation and standardization
  - More extensive validation for FDA approval required
- positive controls included
- Quantification standards often included
- internal controls often included



"In house tests": developed & used for diagnostic purposes on the premise of the developer / user

- degree of validation and standardization is often not transparent or even lacking; large variability of results within and between laboratories

Accreditation to the requirements of ISO 15189 ***(Medical laboratories-Particular requirements for quality and competence)***

First European multicenter pilot EQA for *M. pneumoniae* and *C.pneumoniae*

Material and methods

- Panel composition: 13 samples in BAL or transport medium
  - 6 *C. pneumoniae* pos samples: high pos ⇨ pos ⇨ low pos
  - 5 *M. pneumoniae* pos samples: high pos ⇨ pos ⇨ low pos
  - 2 negative samples
- Questionnaire for technical information
- Number of participants: 79 labs in 18 countries

Results

- False positives in 2.0% of data sets
- Average % of correct results ranging from 54% for low pos *M.pn* to 90% for pos and >95% for strong pos samples
- Large differences between methods used:
  - Commercial conventional **<<<** in house methods
  - Real-time commercial **>>>** in house methods

First European multicenter pilot EQA for *M. pneumoniae*

PCR

Sample	Sample content	Total datasets n=68	Conventional				Real-time			
			Commercial n=55		In-house n=10		Commercial n=5		In-house n=46	
		n	n	%	n	%	n	%	n	%
CP/MP negative SYM		65	33	60.0	10	100.0	5	100.0	45	97.8
CP/MP negative SYM		65	33	60.0	9	90.0	5	100.0	43	93.5
CP/MP negative SYM		65	33	60.0	9	90.0	5	100.0	42	91.3
CP/MP negative SYM		65	11	20.0	6	60.0	5	100.0	25	54.3
CP/MP negative SYM		65	22	40.0	6	60.0	5	100.0	25	54.3
CP/MP negative SYM		65	44	80.0	10	100.0	5	100.0	45	97.8
CP/MP negative SYM		65	44	80.0	10	100.0	5	100.0	46	100.0
CP/MP negative SYM		65	44	80.0	10	100.0	5	100.0	46	100.0
CP/MP negative SYM		65	44	80.0	10	100.0	5	100.0	45	97.8
CP/MP negative SYM		65	44	80.0	10	100.0	5	100.0	46	100.0
CP/MP negative SYM		65	44	80.0	10	100.0	5	100.0	46	100.0
		65	55	100.0	10	100.0	5	100.0	46	100.0
		65	55	100.0	10	100.0	5	100.0	43	93.5

EQA panel to evaluate the performance of different real-time PCRs in GRACE

- EQA panel: 42 samples spiked various conc of ref strains
  - *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*
  - 13 respiratory viruses
  - 6 negative samples
- Amplification methods:
  - In house developed RT- mono or MX PCRs
  - One commercial assay included

Sample content	Conc. / Dilution	Centre 1	Centre 2	Centre 3	Centre 4
M. pneumoniae	50 CCU/ml	***Mp*** positive	**Negative**	***Mp*** positive	**Negative**
M. pneumoniae	500 CCU/ml	***Mp*** positive	***Mp*** positive	***Mp*** positive	**Negative**
C. pneumoniae	4,9 IFU/ml	***Cp*** positive	***Cp*** positive	***Cp*** positive	**Negative**
L. pneumophila	18 CFU/ml	***Lp*** positive	**Negative**	***Lp*** positive	**ADV positive**
L. pneumophila	60 CFU/ml	***Lp*** positive	**Negative**	***Lp*** positive	**ADV positive**

Clinical sensitivities of five evaluated commercial kits in comparison to in-house RT-PCR for detection of *M. pneumoniae*

Real-time PCR assay	No. of positive specimens	Sensitivity (%)	*P value*
In-house	42/42	100.0	
Nanogen kit	41/42	97.6	0.61
Simplexa kit	37/42	88.1	<0.001
Diagenode kit	36/42	85.7	<0.001
Cepheid kit	35/42	83.3	<0.001
Venor kit	26/42	61.9	<0.001

A. Touati et al, J Clin Microbiol 2009; 47: 2269-71

Limited target detection versus multiplex detection

Limited target detection

- Usually ⇧ analytical sens.
- Lower cost
- Often lower TAT
- In outbreak situations
  - SARS Coronavirus
  - Influenza, H1N1
  - RSV, *L. pn*, *M.pn*
- As first approach
  - in high prevalence periods
  - if therapeutic implications
    - Influenza, *Legionella* spp, *Mycoplasma pn., B. pertussis*

Multiplex detection

- In >90% similar results
- Expensive
- TAT usually > 4-6hours
- For epidemiological studies
  - Prevalence of respiratory etiologies
  - Role of respiratory viruses
- As add-on diagnostic test
  - In severely ill patients
  - In immunocompromised
- For virus discovery studies

Costs and handling and testing times of evaluated real-time PCR commercial kits and in-house assay

Real-time PCR assay	No. of reagents per reaction mixture	No. of tests per series	Thermal cycling time	Range of cost per sample excl. tax. (€)
In-house	7	96	1 h, 49 min	2–4
Nanogen kit	4	96	1 h, 40 min	12–14
Simplexa kit	4	96	1 h, 14 min	10–12
Diagenode kit	5	96	1 h, 32 min	10–12
Cepheid kit	3	16 (independant)	50 min	22–24
Venor kit	6	96	1 h, 3 min	8–10

A. Touati et al, J Clin Microbiol 2009; 47: 2269-71

Impact of molecular detection on the diagnosis of acute *M. pneumoniae* infection

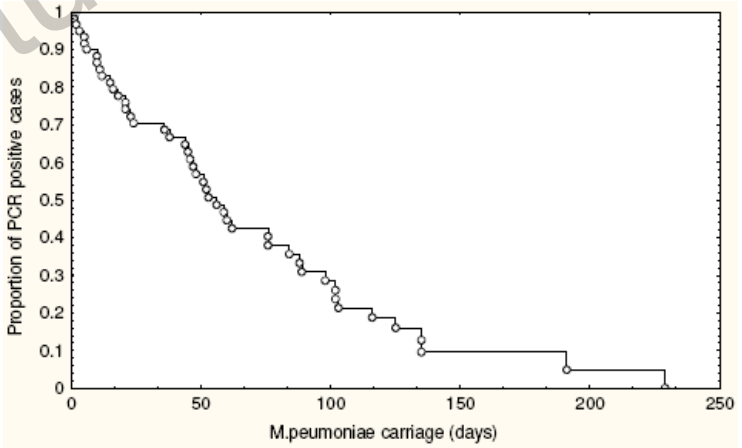
⇒ *M.pneumoniae* carriage among asymptomatic persons is rare

⇒ Persistance of *M.pneumoniae* DNA is common during +/- 7 weeks

- *M. pneumoniae* in 48/164 patients:
- 45/164 (29%): PCR pos in first week
  - 50% of cases still PCR positive after 54 days
  - 44/154 (27%): significant ⇧ in IgG or + IgM

BUT:

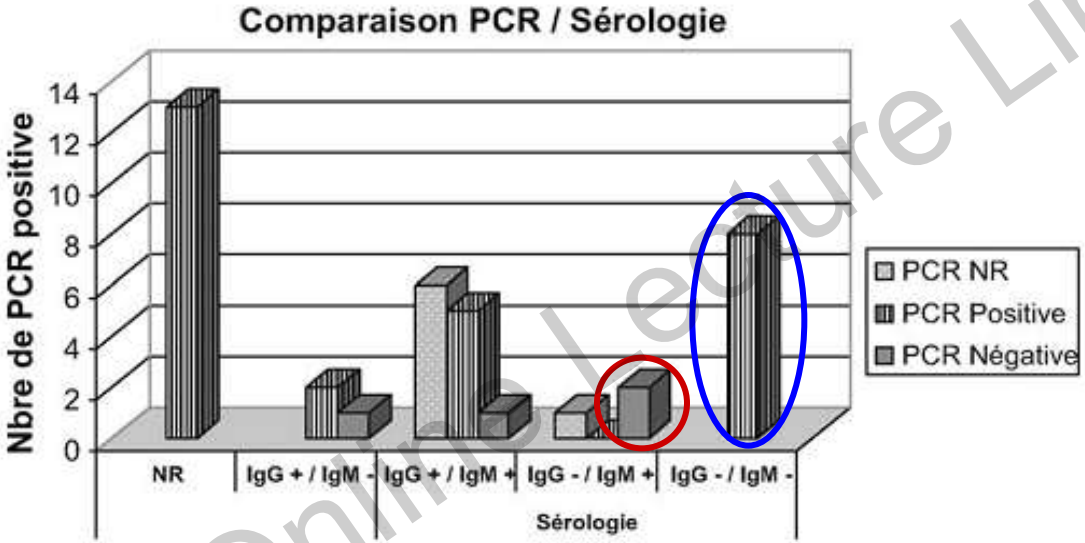
- 21% in 1st week
- 56% during second week
- 100% after 3rd week



⇒ PCR is superior to serology for diagnosis of *M. pneumoniae* during early phase of infection

Nilsson et al., BMC Microbiol 2008; 8: 93-100

Importance of PCR in the diagnosis of *Mycoplasma pneumoniae* infections



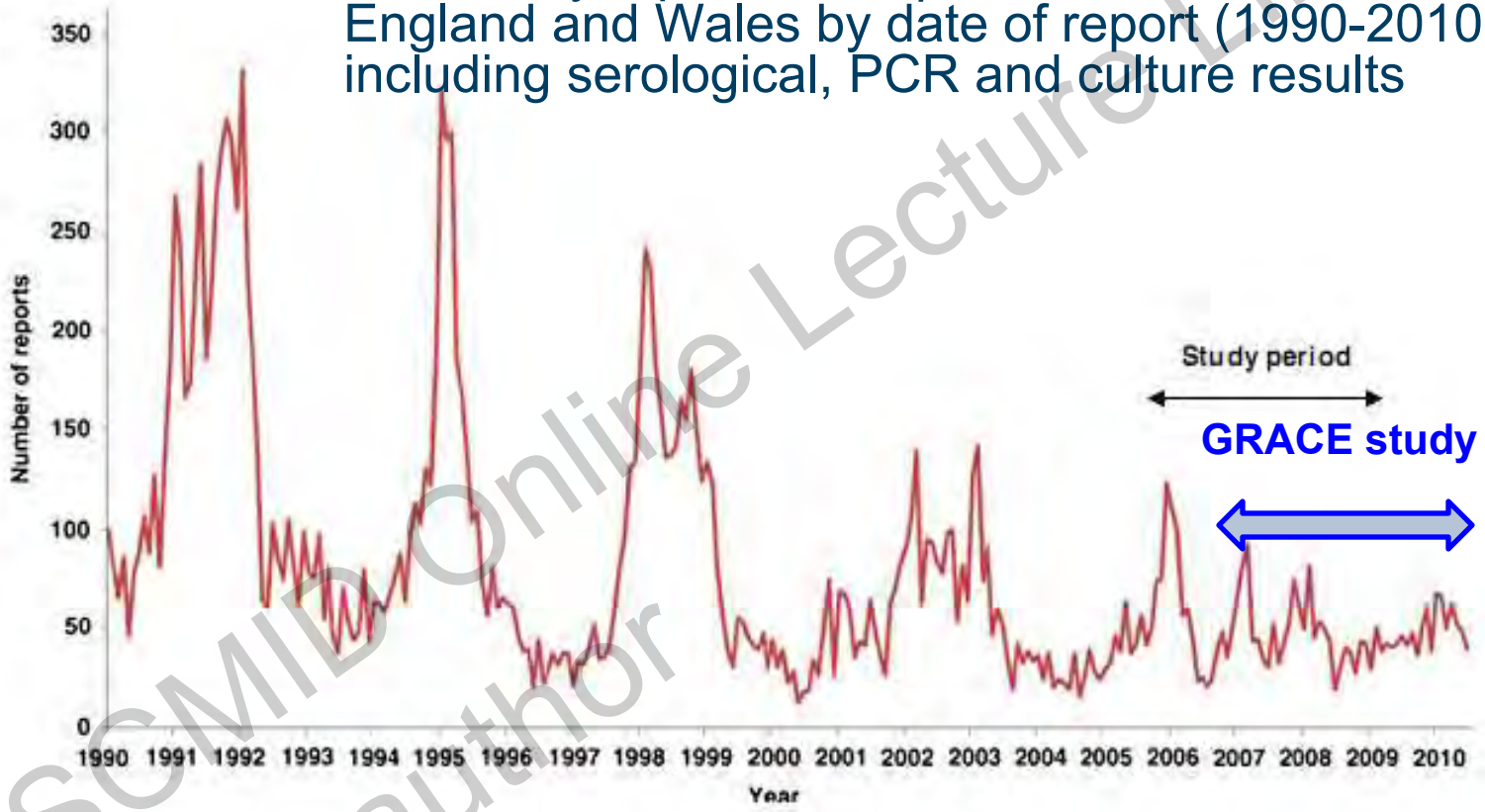
- PCR based detection: most sensitive
  - 28/32 (87%)
- Sensitivity of serology
  - 15/26 (58%)
- 7 patients only diagnosed by serology

- **Combination of PCR and serology detects most cases**

Dekeyser S et al., Pathol Biol 2011; 83-87

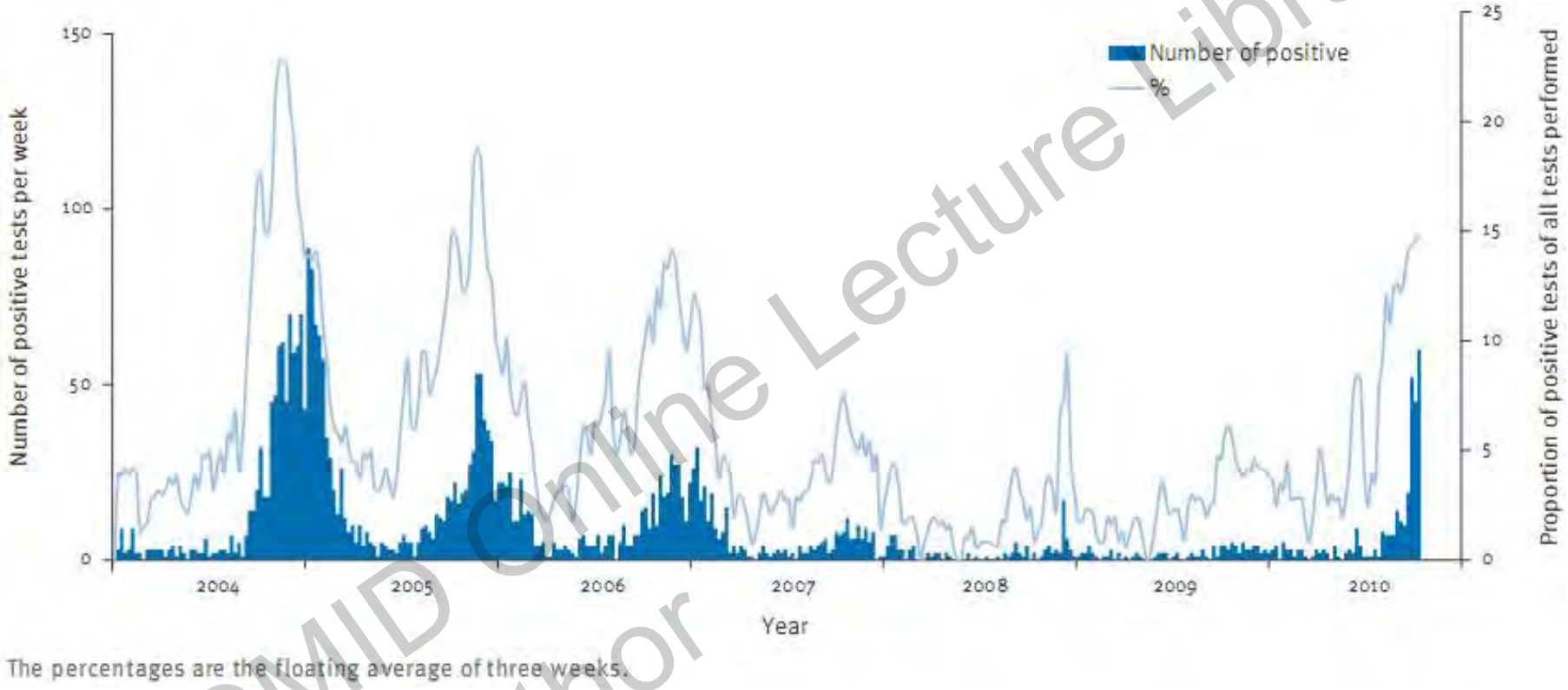
Mycoplasma pneumoniae infection in primary care in England and Wales

- Laboratory reports of *M. pneumoniae* to the HPA in England and Wales by date of report (1990-2010) including serological, PCR and culture results



Chalker VJ et al., Eur J Clin Microbiol Infect Dis 12 February 2011

Incidence of *Mycoplasma pneumoniae* infections in Denmark 2004-2010



- PCR based surveillance system for *M. pneumoniae*
- Denmark From 2007 to 2010: average positivity rate of *M. pneumoniae* in Denmark remained very low
- Increase in positivity rate in late summer 2010

Rasmussen JN et al., Eurosurveillance 11.2010

Limited utility of culture for diagnosis of *Mycoplasma pneumoniae* RTI infections

- Culture for *M. pneumoniae* less sensitive than PCR and serology
  - Only 10/24,677
- Culture for *C. pneumoniae* less sensitive than PCR and serology
  - No culture positives detected in 6,981 specimens submitted
- "Given the extremely low yield of culture and the wide availability of molecular testing and serology, we recommend culture for *M. pneumoniae* and *C. pneumoniae* be discontinued by clinical microbiology laboratories."

Bacterium and method	No. of samples tested	No. positive	% positive	*P* value[a]
M. pneumoniae				
Culture	24,677	10	0.04	NA
PCR	8,509	167	1.9	<0.001
Serology (IgM by ELISA)	92,507	6,049	6.6	<0.001
C. pneumoniae				
Culture	6,981	0	0	NA
PCR	994	6	0.6	<0.001
Serology (IgM by MIF)	58,211	960	1.6	<0.001
Serology (IgM by ELISA)	3,689	143	3.9	<0.001

[a] Compared to culture; determined using Fisher's exact test (one tail). NA, not applicable.

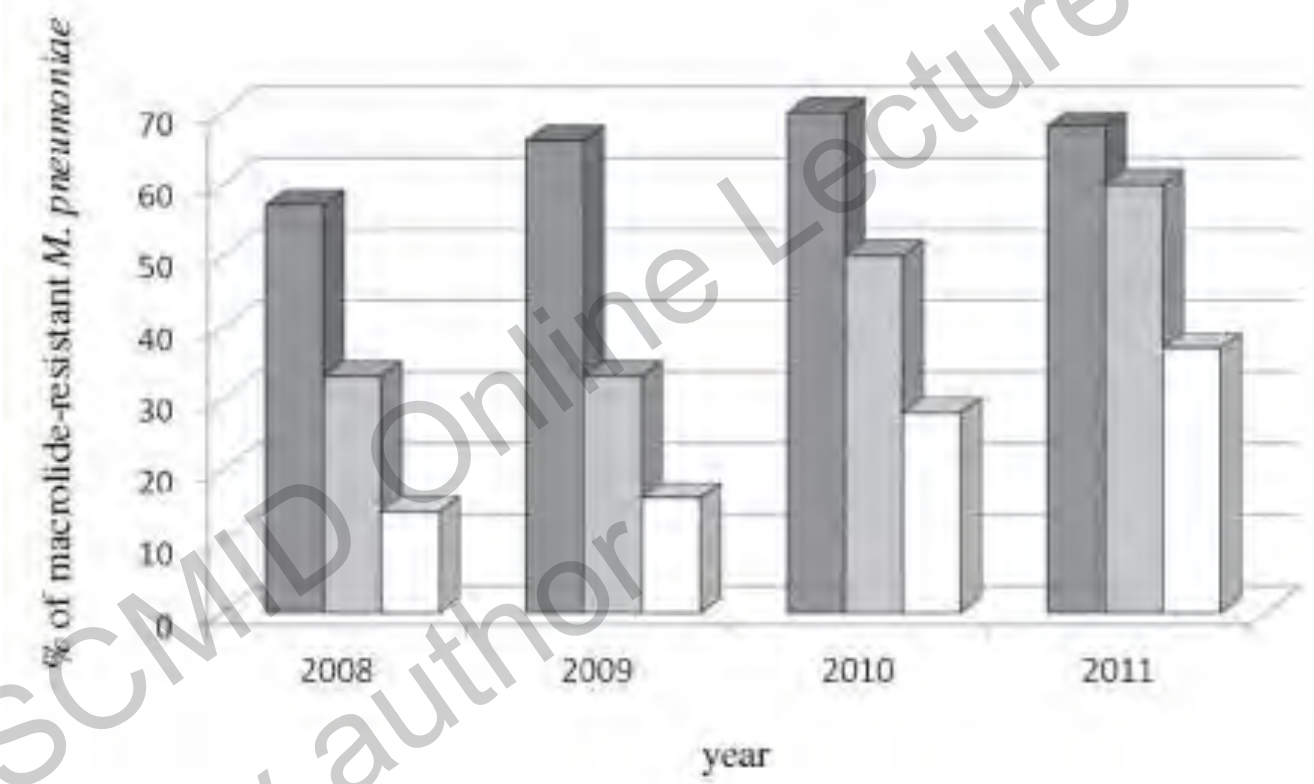
She RC et al., J Clin Microbiol 2010; 48:3380-82

Studies combining PCR and serology to diagnose *M. pneumoniae* infection

Location, year	Nr	Methods	Nr (%) pos	Comments
Belgium, 2008	147	Mono and MX real-time NASBA, real-time PCR, culture, IgM and IgG serology	19 (12.9)	15 PCR positive, 4patients IgG positive of which 1 also IgM positive, 3 pos by IgM only
Sweden, 2010	184	P1-based PCR, IgM and IgG serology	15 (8.%)	8 PCR positive, 7 positive by serology
Israel, 2010	126	PCR, IgM serology	23 (18.3)	23 PCR positive of which 6 IgM positive
UK, 2011	170	P1-based PCR and IgM serology	22 (12.9)	8 PCR and serology positive, 3 PCR positive only, 11 serology positive only

Detection of macrolide-resistant *M. pneumoniae* in different age groups

- New development: PCR based identification of *M. pneumoniae* combined with detection of macrolide resistance for surveillance



Shaded bar: pediatric patients less than 16 years old, hatched bar: 16 to 19-year-old adolescent patients, open bar: adult patients.

Current Diagnosis of *M. pneumoniae* Infections

- Molecular techniques are definitely more sensitive than culture and serology in the early phase of infection.
- Still a great emphasis on serology despite limited added value in diagnosis of acute infections.
  - even IgM may appear late : mean 27 days
- Serology is useful in epidemiological studies.
- Most cases will be detected by a combination of a combination of IgM antibody detection and PCR.