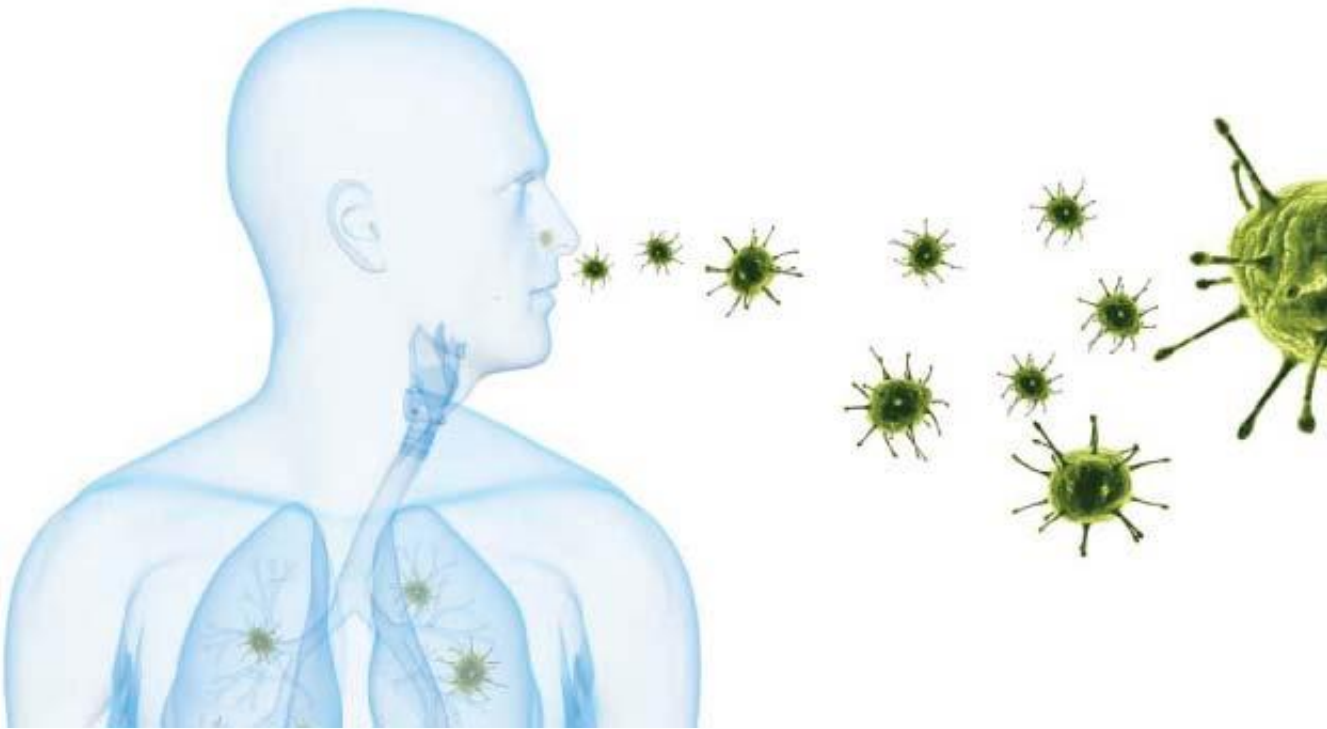
DIAGNOSIS OF RESPIRATORY TRACT INFECTIONS



Laboratory diagnosis of infectious diseases

Direct method

Detection of:

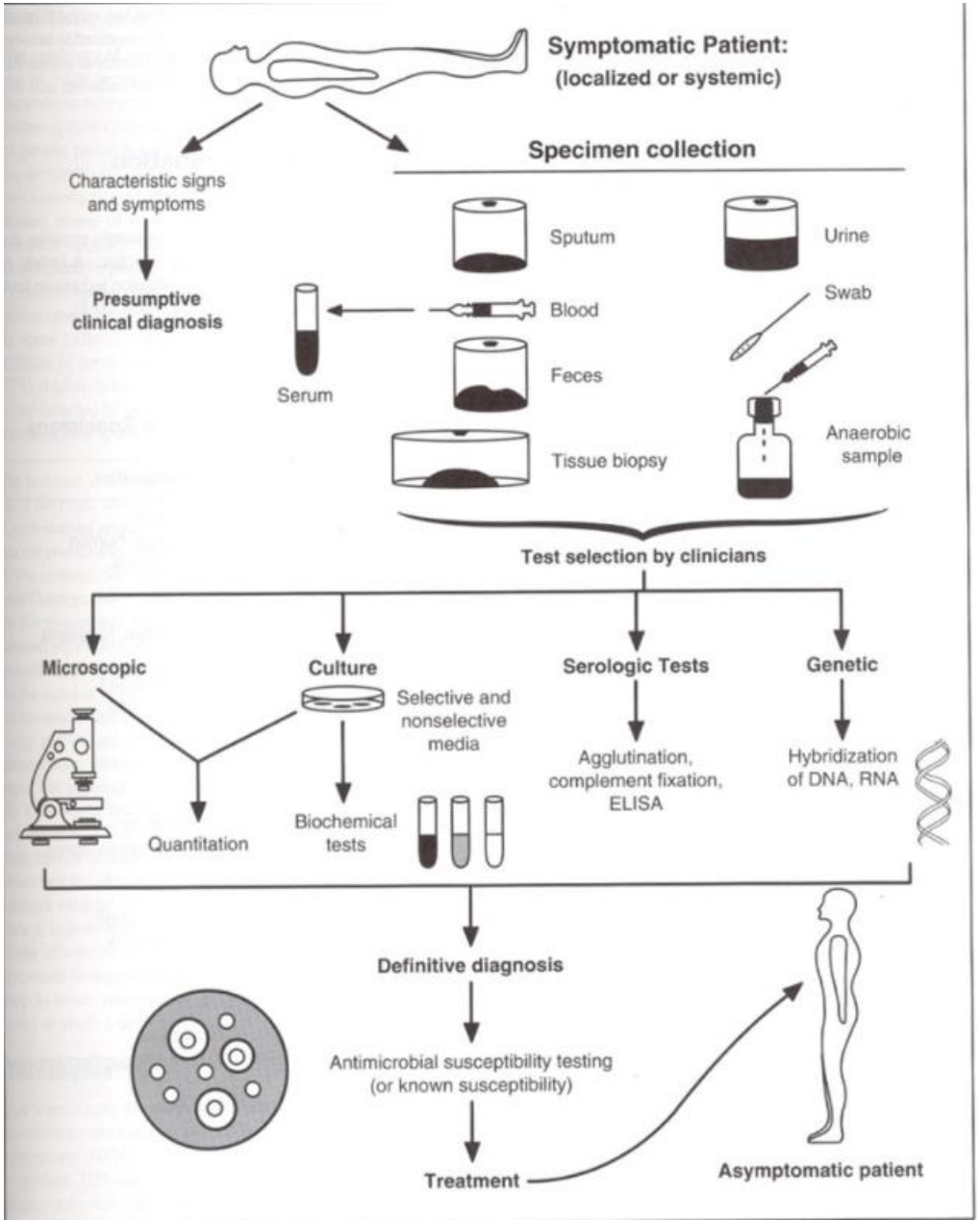
- **microorganisms**,
- their **structural components**
- their **products**

in **specimens** collected from the patient (e.g. urine, blood, sputum, CSF……etc).

Indirect (serologic) method

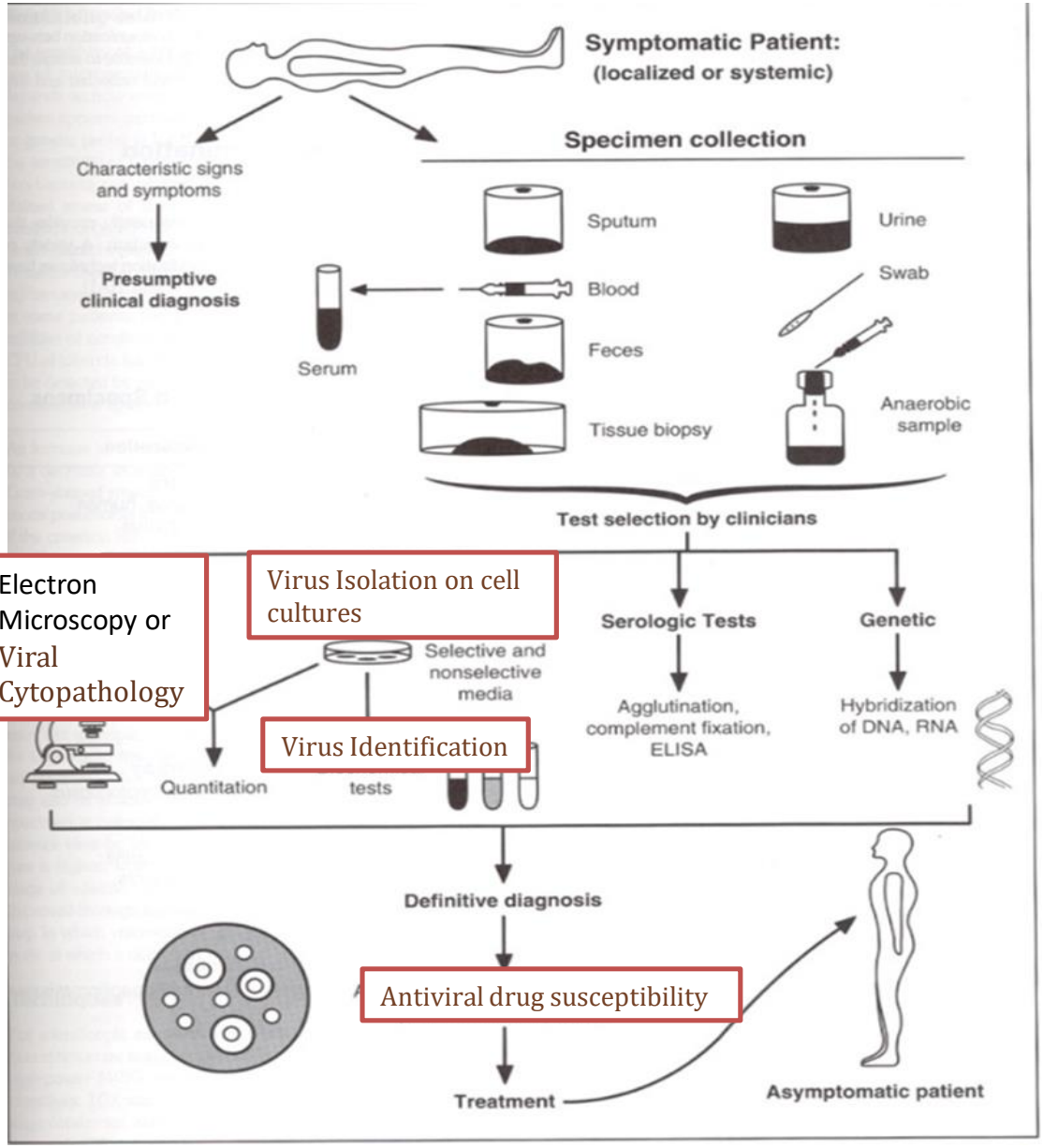
Detection of **antibodies** against the microorganism in the **patient's serum.**

Principles of Bacterial Diagnostics



From: Medical Microbiology. 4th edition.

Principles of Viral Diagnostics



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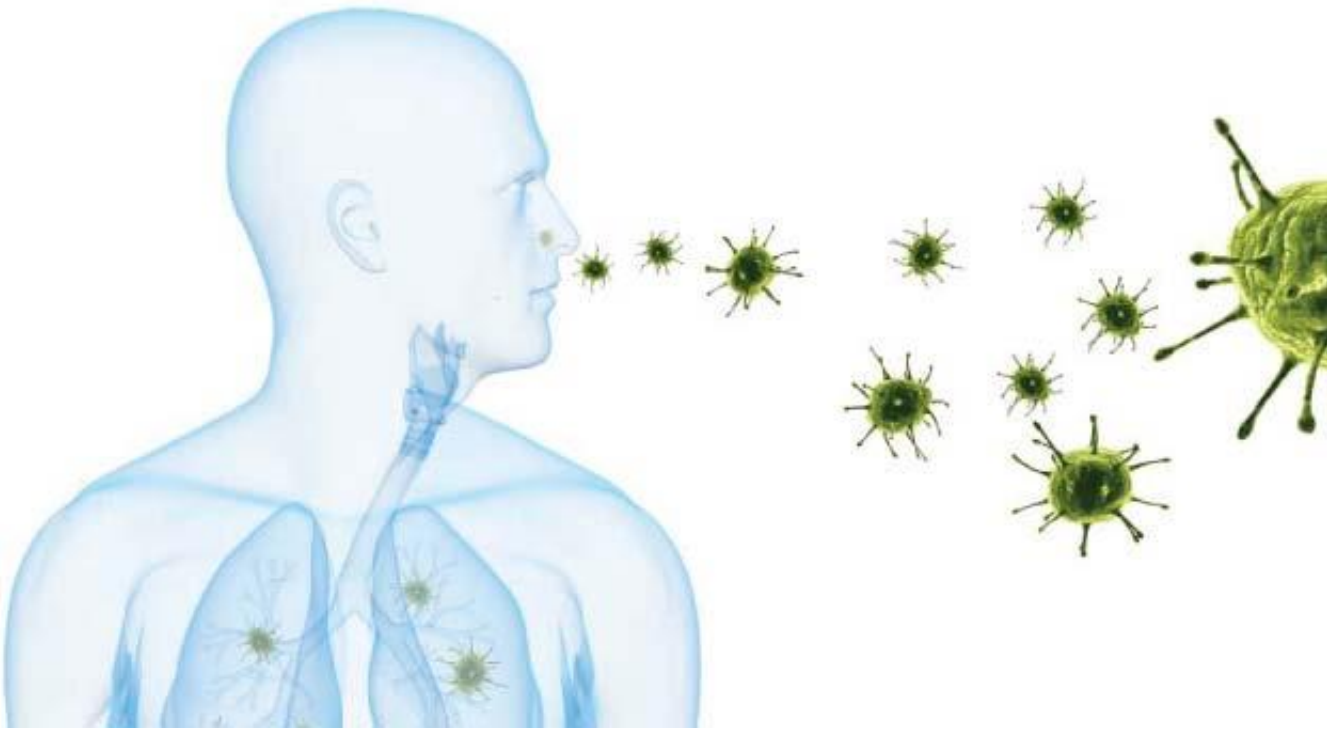
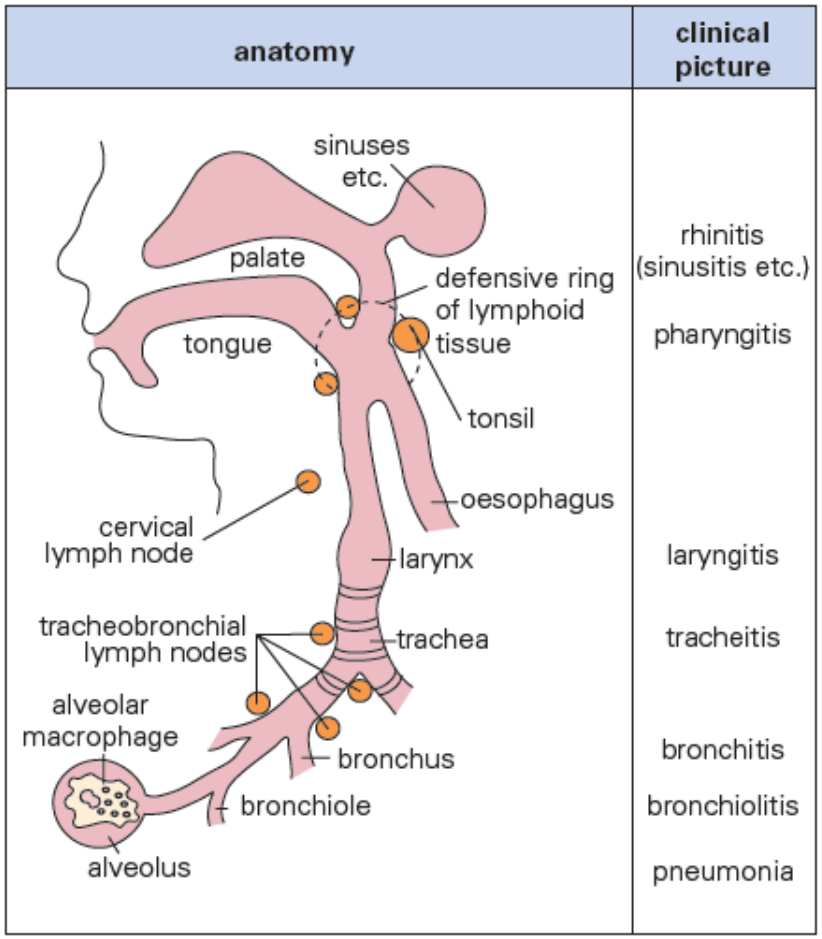
DIAGNOSIS OF RESPIRATORY TRACT INFECTIONS



RESPIRATORY TRACT

- Lower respiratory tract infection is the most common infectious cause of death in the world, 3.5 million deaths yearly (The top 10 causes of death. WHO 2013)
- The respiratory tract is a main site of entry for infections
- The respiratory tract is a continuum as far as infectious agents are concerned



Upper respiratory tract infections

Lower respiratory tract infections

Normal flora of the respiratory tract

Type of resident[a]	Microorganism
Common residents (>50% of normal people)	Oral streptococci *Neisseria* spp. *Moraxella* Corynebacteria *Bacteroides* Anaerobic cocci (*Veillonella*) Fusiform bacteria[b] *Candida albicans*[b] *Streptococcus mutans* *Haemophilus influenzae*
Occasional residents (<10% of normal people)	*Streptococcus pyogenes* *Streptococcus pneumoniae* *Neisseria meningitidis*

- Commensal organisms

(the oropharyngeal microbiota)

- Mainly present in the upper tract
- Mainly Gram+

- Occasional residents, Commensal Symbionts, Pathobionts

Type	Examples	Consequences
Restricted to surface	Rhinoviruses Influenza *Streptococci* in throat *Chlamydia* (conjunctivitis) Diphtheria Pertussis *Candida albicans* (thrush)	Local spread Local (mucosal) defences important Adaptive (immune) response sometimes too late to be important in recovery Short incubation period (days) PATHOGENS of the respiratory tract
Spread through body	Measles, mumps, rubella EBV, CMV *Chlamydophila psittaci*[a] Q fever *Cryptococcosis*	Little or no lesion at entry site Pathogen spreads through body, returns to surface for final multiplication and shedding, e.g. salivary gland (mumps, CMV, EBV), respiratory tract (measles) Adaptive immune response important in recovery Longer incubation period (weeks)

PATHOGENS of the respiratory tract

- **«PROFESSIONAL» INVADERS**: successfully infect the normally healthy respiratory tract and generally possess specific properties (such as capsule, toxins, enzymes for bacteria, mechanisms to evade local host defences …)
- **«SECONDARY» INVADERS** : cause disease only when host defences are impaired

Type	Requirement	Examples
Professional invaders (infect healthy respiratory tract)	Adhesion to normal mucosa (in spite of mucociliary system)	Respiratory viruses (influenza, rhinoviruses) *Streptococcus pyogenes* (throat) *Strep. pneumoniae* *Chlamydia* (psittacosis, chlamydial conjunctivitis and pneumonia, trachoma)
	Ability to interfere with cilia	*Bordetella pertussis* *Mycoplasma pneumoniae* *Strep. pneumoniae* (pneumolysin)
	Ability to resist destruction in alveolar macrophage	*Legionella* *Mycobacterium tuberculosis*
	Ability to damage local (mucosal, submucosal) tissues	*Corynebacterium diphtheriae* (toxin) *Strep. pneumoniae* (pneumolysin)
Secondary invaders (infect when host defences impaired)	Initial infection and damage by respiratory virus (e.g. influenza virus)	*Staphylococcus aureus* *Strep. pneumoniae,* pneumonia-complicating influenza
	Local defences impaired (e.g. cystic fibrosis)	*Staph. aureus* *Pseudomonas*
	Chronic bronchitis, local foreign body or tumour	*Haemophilus influenzae* *Strep. pneumoniae*
	Depressed immune responses (e.g. AIDS, neoplastic disease)	*Pneumocystis jirovecii* Cytomegalovirus *M. tuberculosis*
	Depressed resistance (e.g. elderly, alcoholism, renal or hepatic disease)	*Strep. pneumonia* *Staph. aureus* *H. influenzae*

Upper respiratory tract infections

The symptoms of an upper respiratory tract infection include fever, rhinitis and pharyngitis or sore throat

- **RHINITIS**
- **SINUSITIS**
- **PHARYNGITIS**
- **TONSILLITIS**
- **EPIGLOTTITIS**
- **OTITIS**

RHINITIS or common cold

- Etiology:
  - Generally **VIRAL INFECTIONS**
  - Possibility of secondary bacterial infections
- Transmission: by aerosol, direct contact or fomitis
- No vaccines
- Treatment is symptomatic
- Diagnosis: unnecessary

VIRAL INFECTIONS

RHINOVIRUSES (3 species: -A, -B, -C Around 160 genotypes)

CORONAVIRUSES (Low pathogenic species: OC43, 229E, HKU1, NL63)

ADENOVIRUS (Around 40 genotypes)

PARAINFLUENZA VIRUS 1-4

...

PHARYNGITIS and TONSILLITIS

- **About 70% of acute sore throats are caused by viruses**
- A laboratory diagnosis is not generally necessary but **it is important to diagnose *Streptococcus pyogenes*** infection because of the possible complications

Microorganisms causing acute pharyngitis

Organisms	Examples	Comments
Viruses	Rhinoviruses, coronaviruses	A mild symptom in the common cold
	Adenoviruses (types 3, 4, 7, 14, 21)	Pharyngoconjunctival fever
	Parainfluenza viruses	More severe than common cold
	Influenza viruses, CMV, EBV	Not always present
	Coxsackie A and other enteroviruses	Small vesicles (herpangina)
	Epstein–Barr virus	Occurs in 70–90% of glandular fever patients
	Herpes simplex virus type 1	Can be severe, with palatal vesicles or ulcers
Bacteria	*Streptococcus pyogenes*	Causes 10–20% of cases of acute pharyngitis; sudden onset; mostly in 5- to 10-year-old children
	Neisseria gonorrhoeae	Often asymptomatic; usually via orogenital contact
	Corynebacterium diphtheriae	Pharyngitis often mild, but toxic illness can be severe
	Haemophilus influenzae	Epiglottis
	Borrelia vincentii plus fusiform bacilli	Vincent's angina; commonest in adolescents and adults

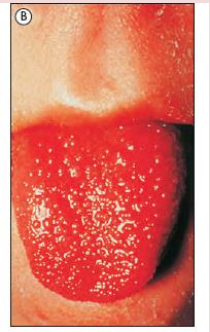
CMV, cytomegalovirus; EBV, Epstein–Barr virus.

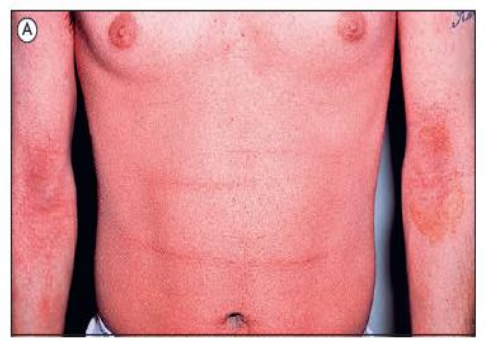
Streptococcus pyogenes

COMPLICATIONS:

- **Quinsy**: Peritonsillar abscess, uncommon complication of untreated streptococcal sore throat
- **Otitis media, sinusitis and mastoiditis**
- **SCARLET FEVER**
  - from strains of *S. pyogenes* producing **an erythrogenic toxin** coded for by a lysogenic phage.
  - Highly contagious
- **Impetigo, erysipelas and cellulitis**
- **PNEUMONIA**
- **RHEUMATIC FEVER**

(Immune-mediated disease)



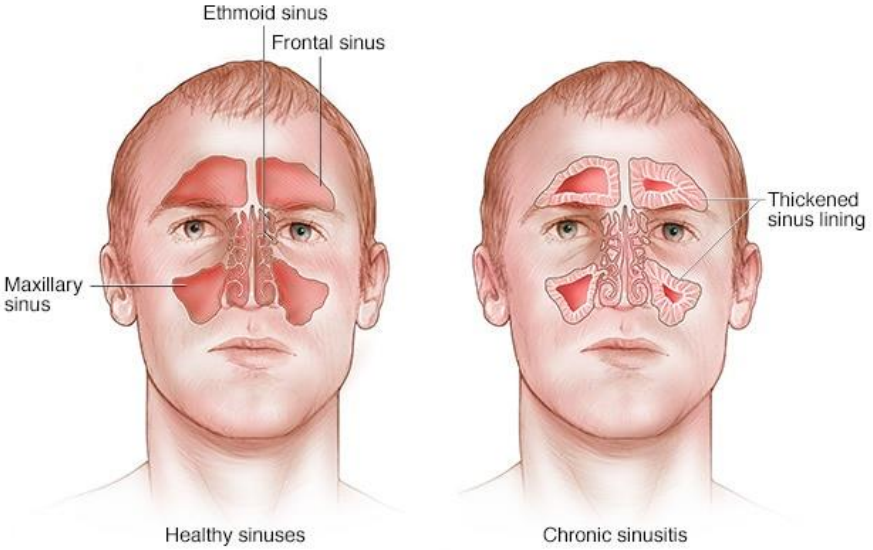


- **Symptoms**:
  - Rash
  - Sore throat
  - Red cheeks
  - Swollen tongue.

Punctate erythema

- Begins as facial erythema, then spreads to involve most of the body
- Rash fades over the course of 1 week
- Followed by peeling for 2–3 weeks

OTITIS and SINUSITIS



- Invasion of the air spaces associated with the upper respiratory tract (sinuses, middle ear, mastoid)

- **ETIOLOGY:**
- **Many viruses (**Rhinovirus, AdenoV, parainfluenzaV)
- **Secondary bacterial invaders** (i.e. *Strep. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis* and sometimes anaerobes, such as *Bacteroides fragilis*)

- Brain abscess is a major complication

Otitis externa

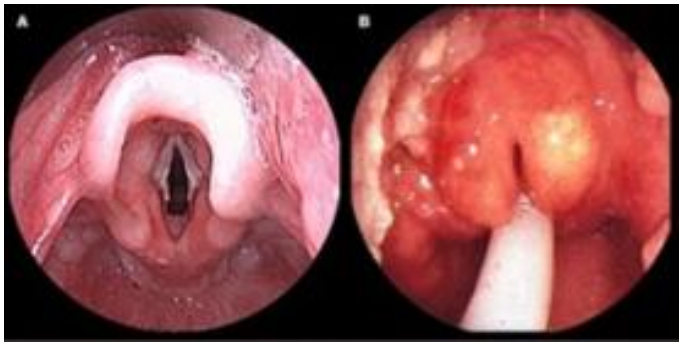
The warm moist environment (swimmers) favours ***Staph. aureus, C. albicans*** and Gram-negative opportunists such as ***Proteus*** and ***Pseudomonas aeruginosa***

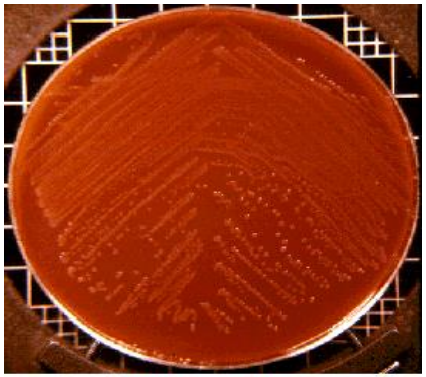
CHRONIC SUPPURATIVE OTITIS MEDIA

ACUTE EPIGLOTTITIS

- In young children, the responsible is ***H. influenzae*** **capsular type B** in 85% of cases
- Usually bacteraemia is present
- Severe inflammation and oedema → difficulty in breathing due to respiratory obstruction

Acute epiglottitis is an emergency and necessitates intubation and treatment with antibiotics





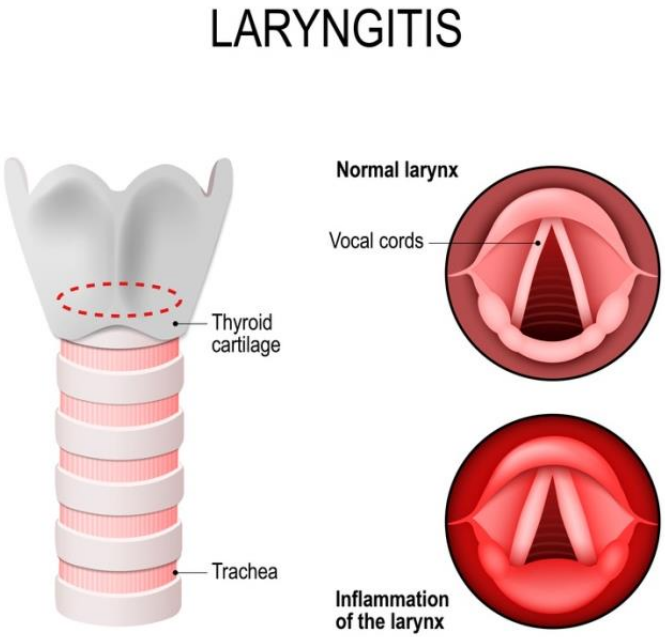
DIAGNOSIS

- Clinical diagnosis
- Confirmation by isolating bacteria from the blood

→ **a pharyngeal swab is strongly discouraged** in cases of suspected epiglottiditis because it can aggravate the obstruction

LARYNGITIS and TRACHEITIS

- Easily obstructed in children, due to their narrow passages, leading to hospital admission
- Swelling may lead to a dry cough and inspiratory stridor (‘crowing’) known as **croup**



ETIOLOGY

VIRUS

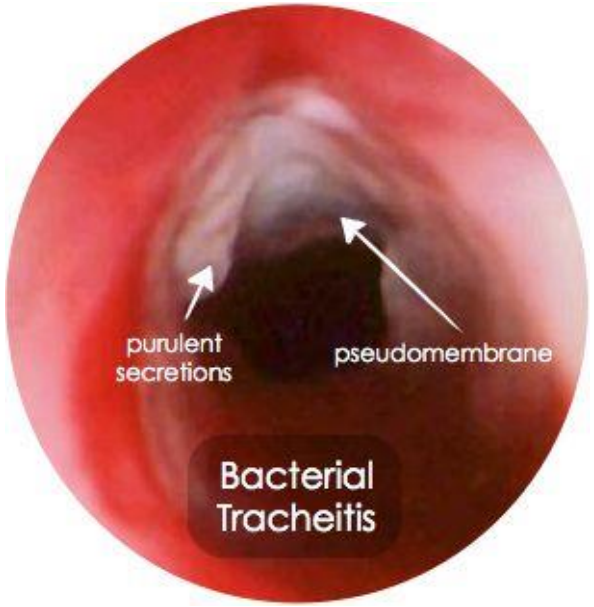
Viral infections of the upper respiratory tract may spread downwards

Broad range: **rhinovirus, parainfluenza virus, influenza virus, adenovirus, respiratory syncytial virus (RSV)**

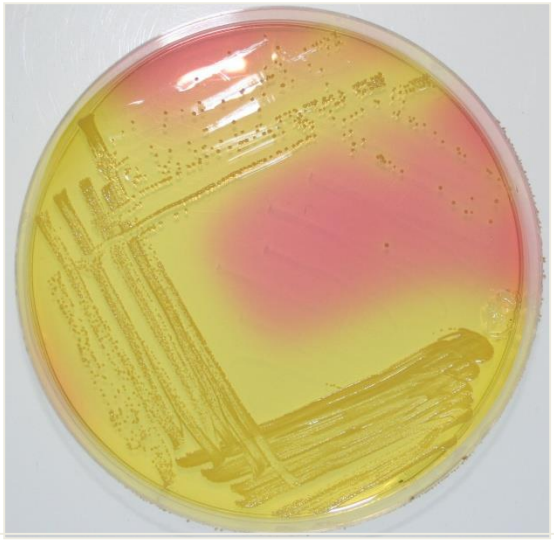
BACTERIA

Less common

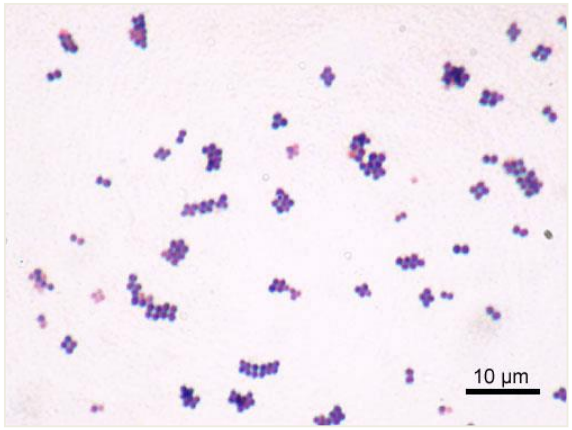
Group A streptococci, *Haemophilus influenzae* and *Staphylococcus aureus*



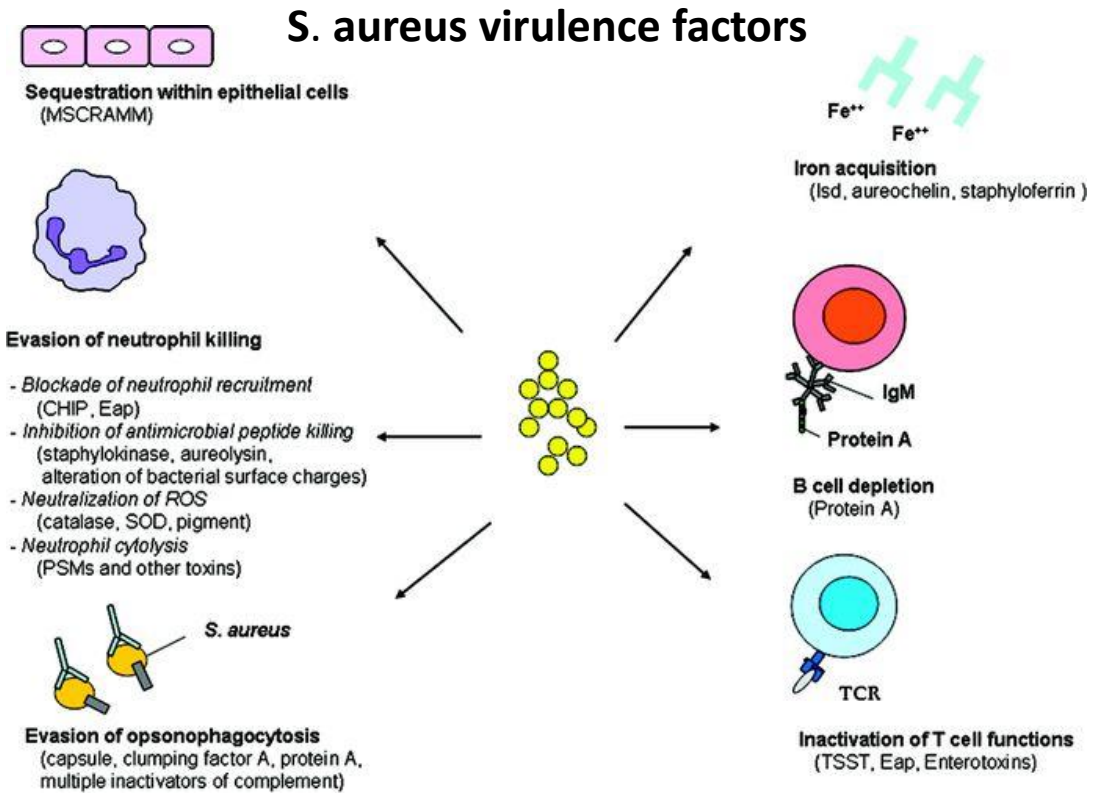
STAPHYLOCOCCUS AUREUS



S.aureus colonies in MSA (Mannitol Salt Agar)



- **S. aureus** can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, scalded skin syndrome, and abscesses, to life-threatening diseases such as **pneumonia**, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis.

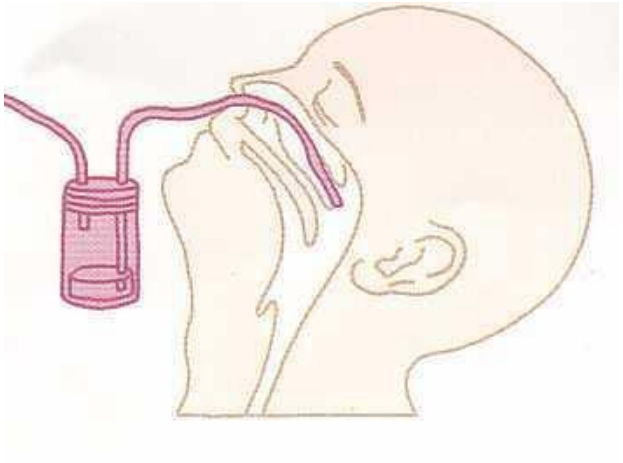


WHOOPING COUGH

Caused by ***Bordetella pertussis*** and ***B. parapertussis***

Infants, if not **immunized**, are at risk of severe complications

DIAGNOSIS



NASOPHARYNGEAL ASPIRATE: recommended sample

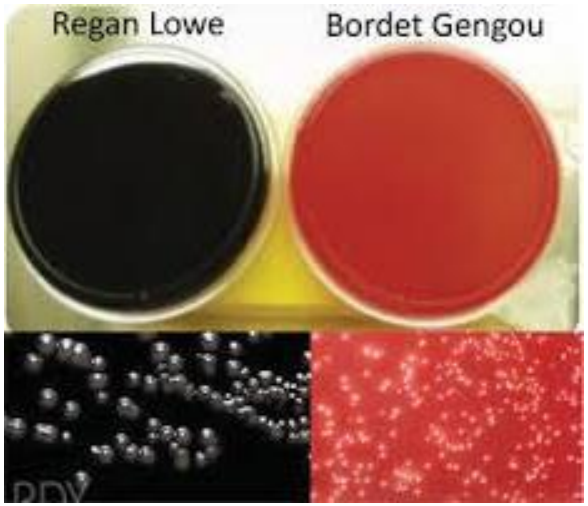
Nasopharyngeal swab

Nasal swab

Culture on specific growth media

- Fails to grow on routine blood agar
- **Enriched medium** are required (e.g. Bordet–Gengou or blood charcoal agar)
- Requires **3–7 days' incubation** in moist atmosphere

→ Iridescent bisected pearl colony type characteristic on Bordet–Gengou



Identification by reaction with specific antisera (agglutination assay)

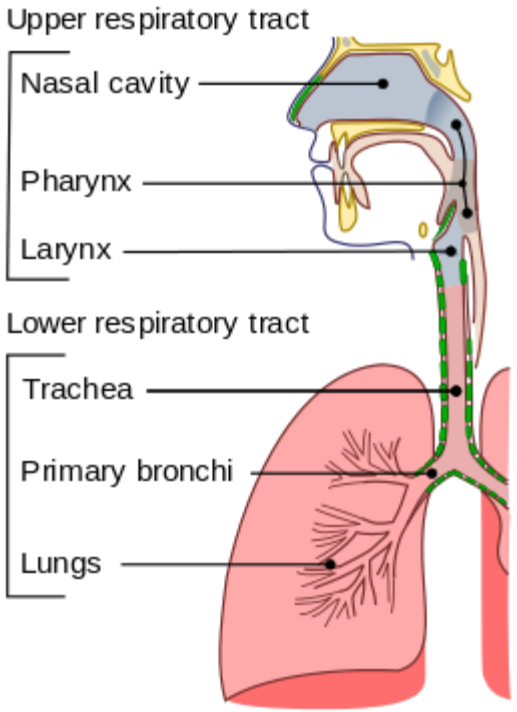
Lower respiratory tract infections

- Infections are spread by the airborne route (except parasites)
- Acute or chronic
- Tend to be more severe than infections of the upper respiratory tract
- May be fatal without correct treatment.

They are caused by a wide range of organisms – usually bacteria or viruses, but also fungi and parasites

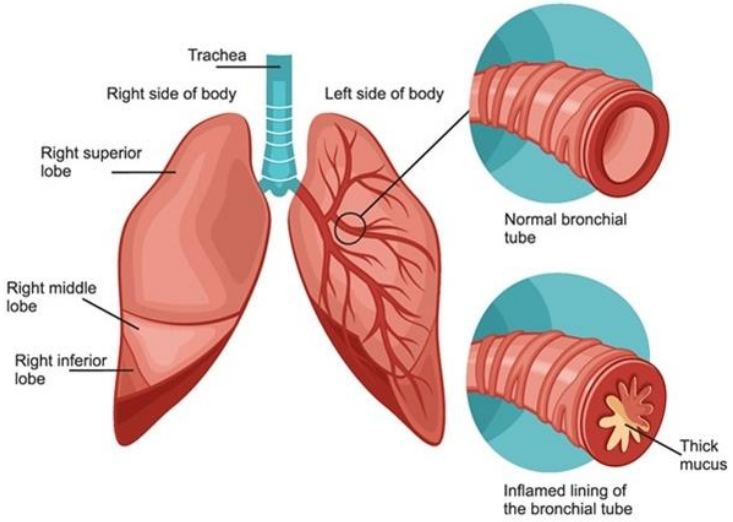
- **BRONCHITIS**
- **BRONCHIOLITIS**
- **PNEUMONIA**

- **TUBERCULOSIS**



BRONCHITIS

- Inflammatory condition of the tracheobronchial tree
- Characterized by cough and excessive mucus production
- The diagnosis is clinical



ACUTE BRONCHITIS

- Rhinoviruses
- Coronaviruses
- Influenza viruses
- Adenoviruses

- *Mycoplasma pneumoniae*

Secondary bacterial infection:

- *Streptococcus pneumoniae*
- *H. influenzae*

CHRONIC BRONCHITIS

(cigarette smoking, inhalation of dust or fumes) have infection-associated acute exacerbations

Bacteria most frequently isolated:

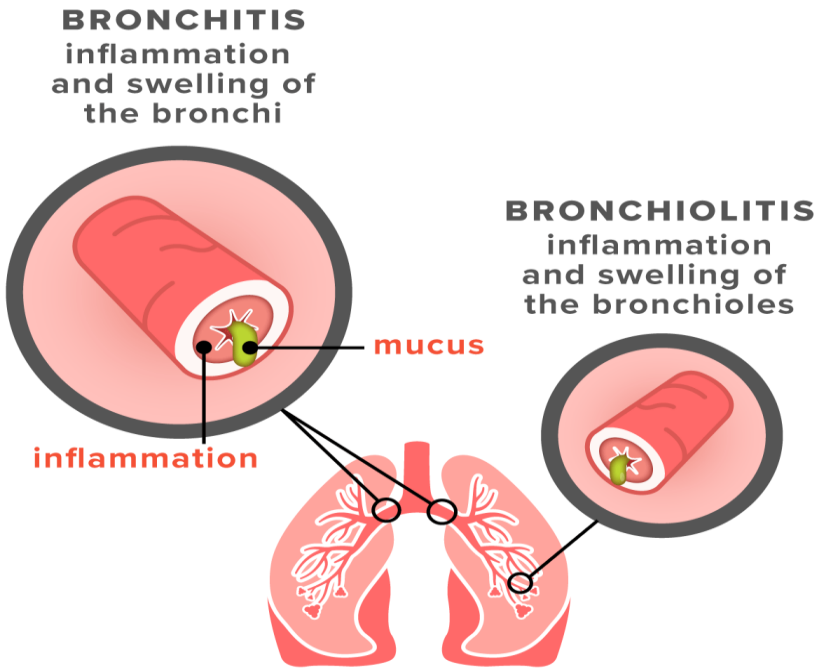
- *S. pneumoniae*
- uncapsulated strains of *H. influenzae*

Less commonly associated:

- *Staph. aureus*
- *M. pneumoniae*

Viruses

BRONCHIOLITIS

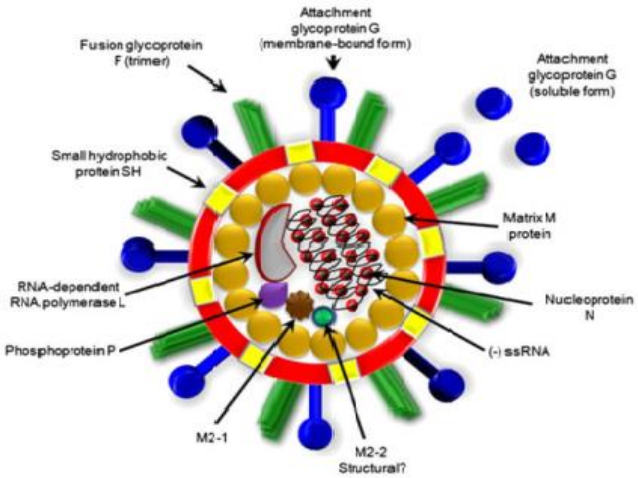


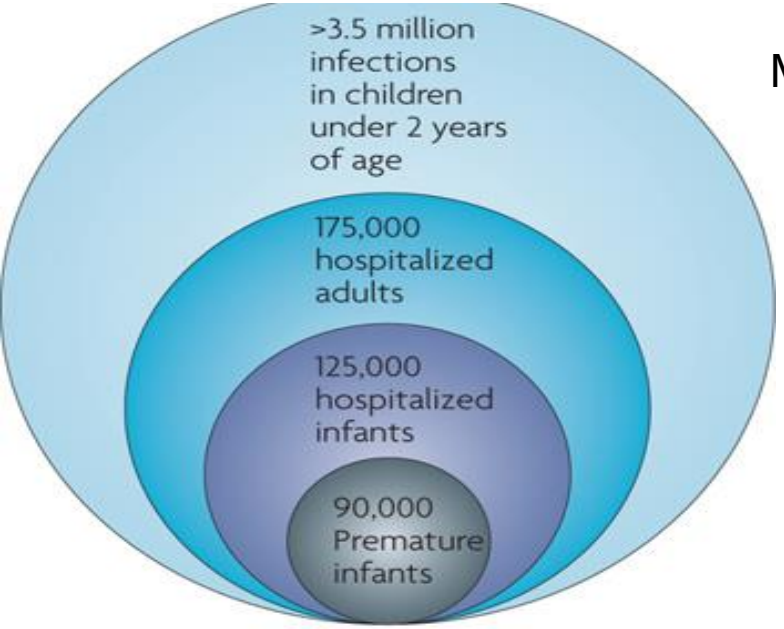
- Children less than 1 years of age

Around 75% of bronchiolitis are caused by RSV

The remaining are also of viral aetiology (rhinoV, parainfluenza viruses, human metapneumovirus and influenza viruses)

Respiratory Syncytial Virus (RSV)





Nature Reviews | Drug Discovery

RSV--
The first cause of infants' hospitalization

Risk factors for severe bronchiolitis include prematurity, immunodeficiency, cardiovascular, pulmonary and chronic diseases, but most hospitalisations occur in previously healthy infants aged 3 to 9 months.
Monoclonal Ab are available for prevention of severe disease in infants

But is also responsible for 7-10% ILI and 20-30% pneumonia in the older

Two vaccines against RSV have recently been approved

Tuberculosis

Species	Clinical disease
Slow growers[a]	
M. tuberculosis	Tuberculosis
M. bovis	Bovine tuberculosis
M. leprae	Leprosy
M. avium[b] *M. intracellulare*[b]	Disseminated infection in AIDS patients M. avium complex (MAC)
M. kansasii	Lung infections
M. marinum	Skin infections and deeper infections (e.g. arthritis, osteomyelitis) associated with aquatic activity
M. scrofulaceum	Cervical adenitis in children
M. simiae	Lung, bone and kidney infections
M. szulgai	Lung, skin and bone infections
M. ulcerans	Skin infections
M. xenopi	Lung infections
M. paratuberculosis	? Association with Crohn's disease
Rapid growers[a]	
M. fortuitum *M. chelonae*	Opportunist infections with introduction of organisms into deep subcutaneous tissues; usually associated with trauma or invasive procedures

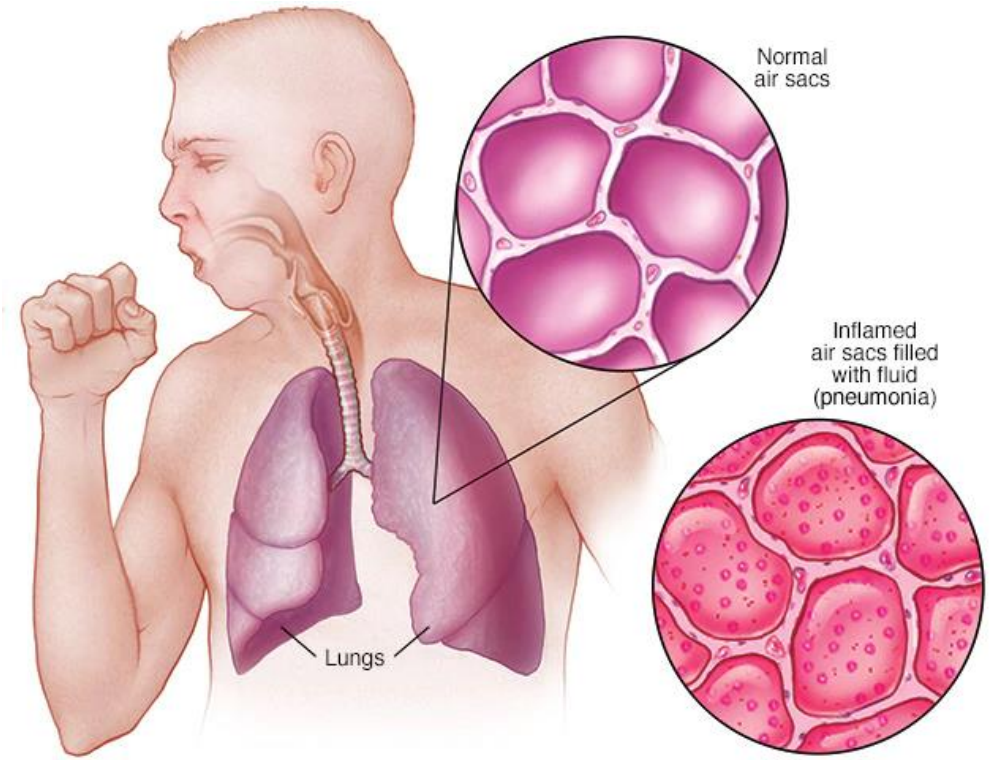
TB (one of the top 10 causes of death globally) is caused by ***Mycobacterium tuberculosis***

Non-tuberculous mycobacteria (NTM) also cause infection in the lungs

TB is primarily a disease of the lungs, but may spread to other sites or proceed to a generalized infection

PNEUMONIA

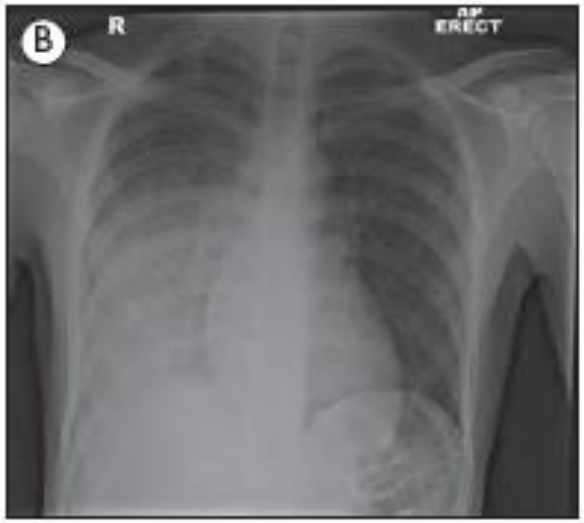
- The most common cause of infection-related death in the USA and Europe
- It is caused by a wide range of microorganisms
- **Simple clinical diagnosis**, but **difficult laboratory identification** of the microbial cause



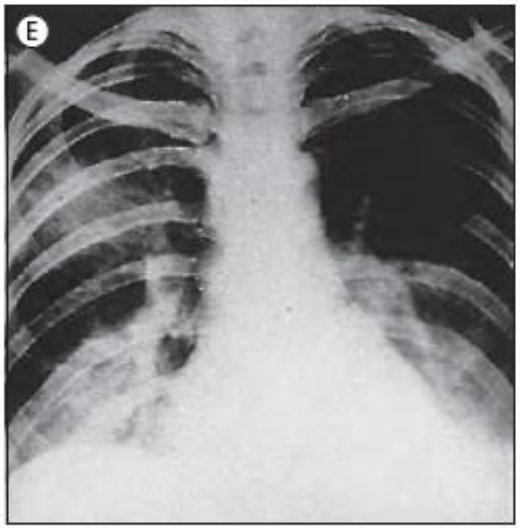
The host's response can be defined by the pathological and radiological findings:

- **Lobar pneumonia**
- **Bronchopneumonia**
- **Interstitial pneumonia or pneumonitis** particularly characteristic of viral infections and in atypical bacterial and P*neumocystis* infection
- **Lung abscess**, or **necrotizing pneumonia**, is a cavitation and destruction of the lung parenchyma

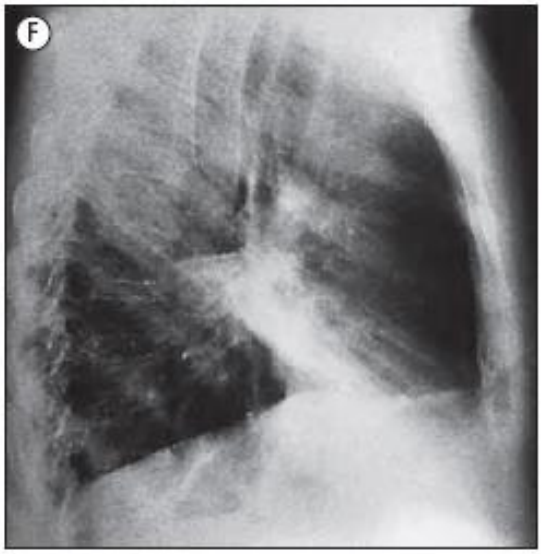
PNEUMONIA



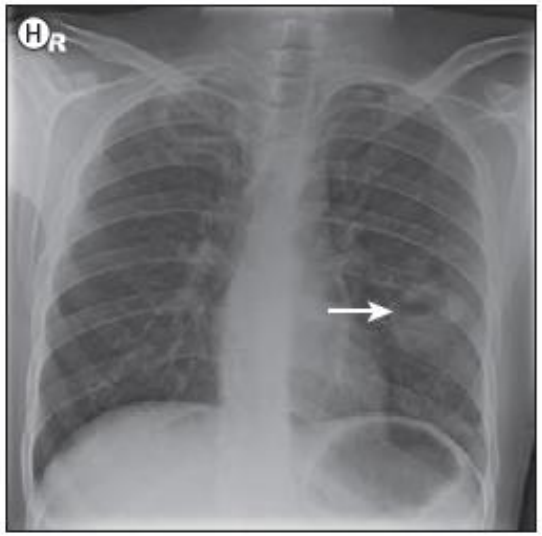
Right lower lobe pneumonia



Mycoplasma bronchopneumonia



Interstitial pneumonia due to viruses



Lung abscess, showing an abscess cavity

PNEUMONIA

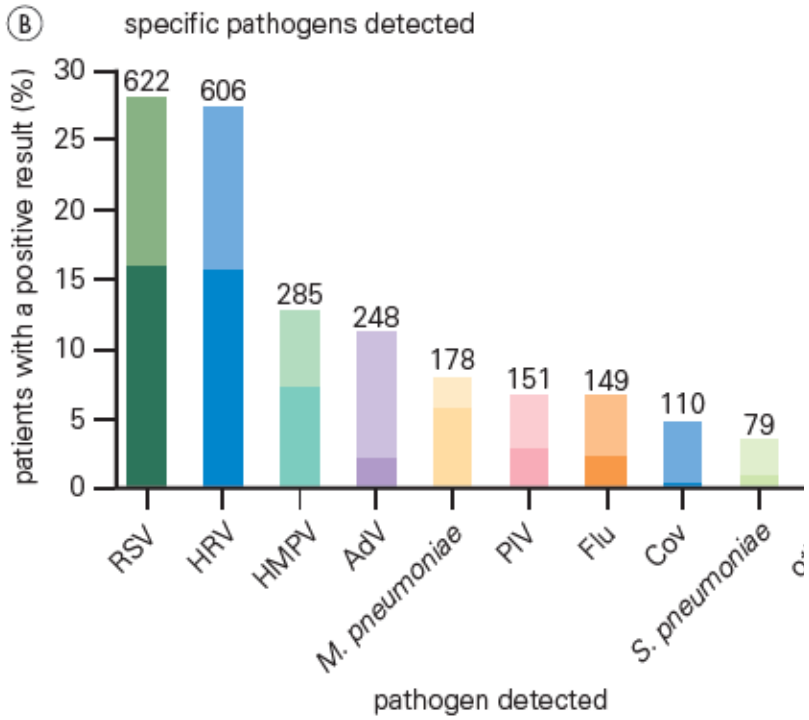
the **range of microorganisms** causing pneumonia differ by **age**

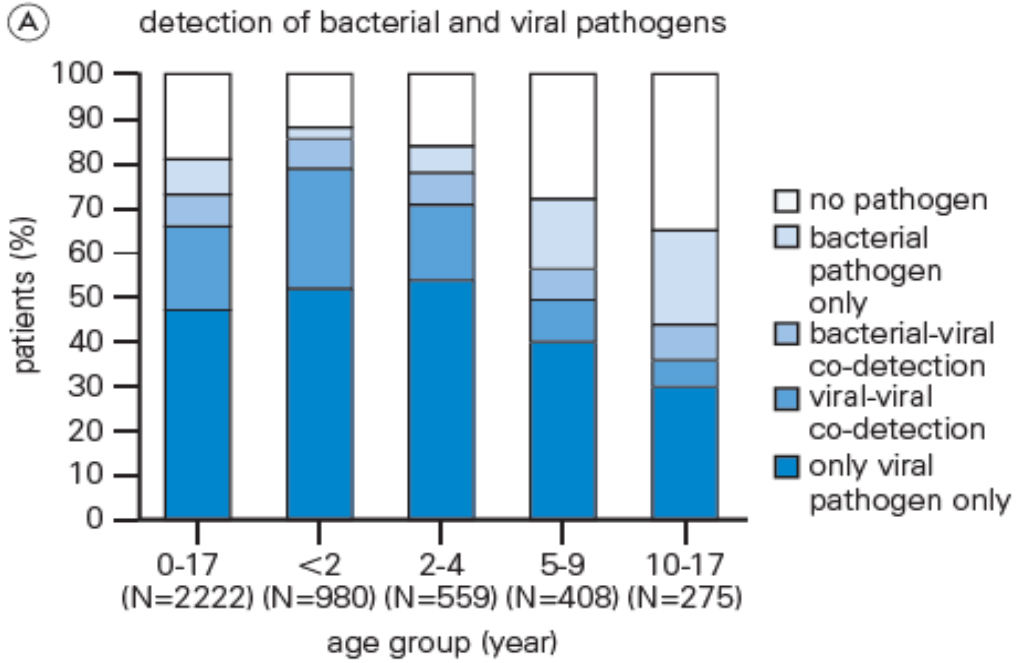
Children	Adults
Mainly viral (e.g. respiratory syncytial virus, parainfluenza) or bacterial secondary to viral respiratory infection (e.g. after influenza, measles)	Bacterial causes more common than viral
Neonates may develop interstitial pneumonitis caused by *Chlamydia trachomatis* acquired from the mother at birth	Aetiology varies with age, underlying disease, occupational and geographic risk factors

PNEUMONIA

the **range of microorganisms** causing pneumonia differ by **age**

CHILDREN



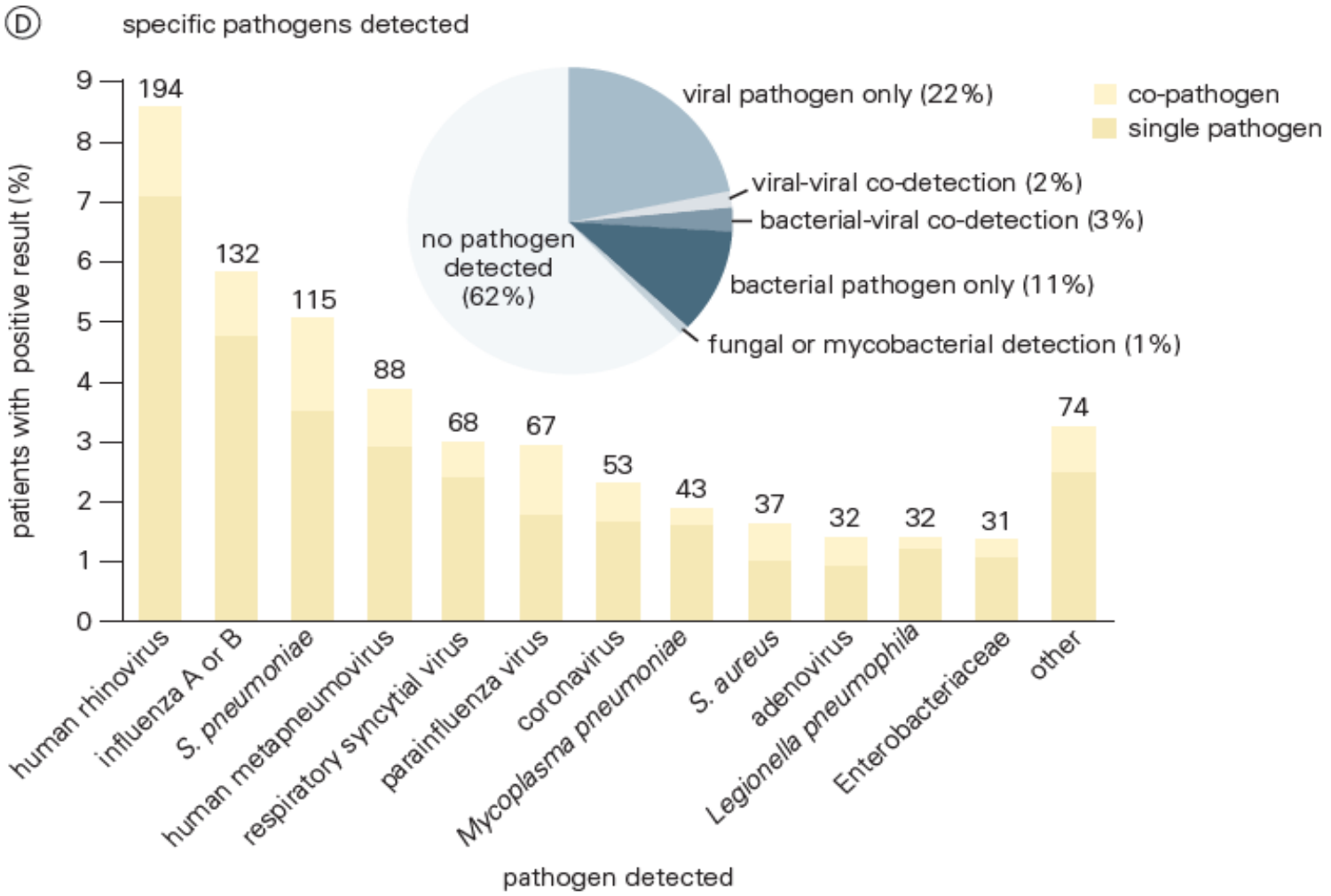


Pathogens detected in US children with community-acquired pneumonia requiring hospitalization

PNEUMONIA

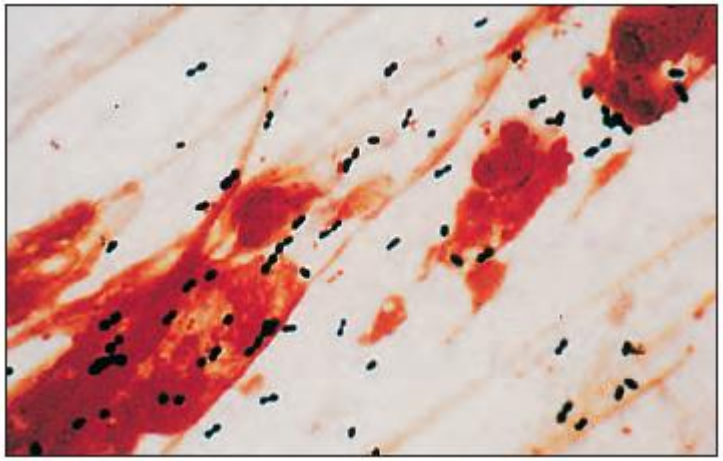
the **range of microorganisms** causing pneumonia differ by **age**

ADULTS



Pathogens detected in US adults with community-acquired pneumonia requiring hospitalization (NEJM 2015)

BACTERIAL pneumonia

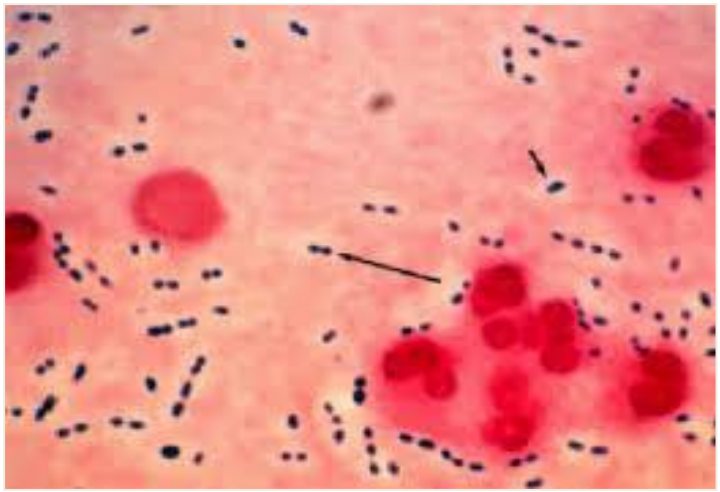


- ***Streptococcus pneumoniae*** is the classic bacterial cause of acute community-acquired pneumonia
- ***H. influenzae*** is the second most common cause

Other bacteria:

- *M. pneumoniae*
- *Chlamydophila pneumoniae*
- *C. psittaci*
- *Legionella pneumophila*
- *Coxiella burnetii*

Streptoccus pneumoniae



- ***Diplococcus pneumoniae*** or ***Streptococcus pneumoniae or*** **PNEUMOCOCCUS,** is a Gram-positive, **facultative anaerobic,** alpha-hemolytic **capsulated**, **asporigens**

- typically colonize the respiratory tract, sinuses, and nasal cavity (healthy carriers)
- Pneumococcus is one of the most common causes of severe pneumonia.

- **Pneumococcal bacteria are resistant to one or more antibiotics in 3 out of every 10 cases**

- Can also cause **invasive pneumococcal diseases**:
  meningitis, sepsis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, and brain abscess
  The introduction of **pneumococcal conjugate vaccines** has universally resulted in a decline in vaccine-serotype pneumococcal meningitis incidence throughout Europe and northern America.

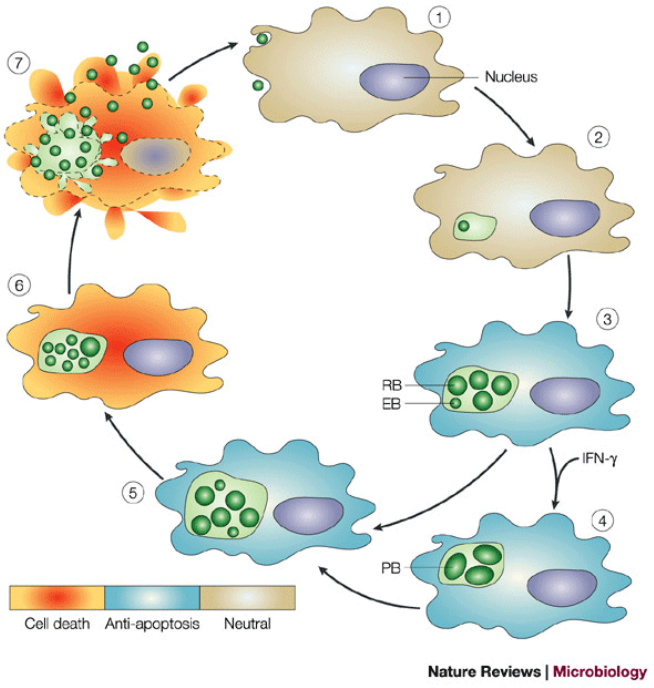
Atypical pneumonia
Mycoplasma pneumoniae

- Mycoplasma, one of the smallest bacteria (0.2 to 0.3 µm) with the smallest genome (between 0.6 and 1.35 Mbp)
- Mycoplasma lacks the cell wall structure: insensitive to beta-lactam anti microbial agents, no gram's staining

- *M. pneumoniae* is an important cause of respiratory tract infections and atypical pneumonia called “walking pneumonia” because of benign nature in young adults.
- The overall mortality is low, but up to 30% among the elderly
- Macrolides are the primary drugs of choice (Macrolide resistance rates reported to be 26% in Italy)
- Responsible for non-pulmonary manifestations including neurological, hepatic, cardiac diseases, hemolytic anemia, polyarthritis and erythema multiforme.

Atypical pneumonia
Chlamydia pneumoniae

- *C. pneumoniae* unique developmental cycle consists of two alternating forms: elementary and reticulate bodies.
- **Elementary bodies,** metabolically inactive, can infect the host cell
- C. pneumoniae growth takes place within host cells where it differentiate into **reticulate bodies**, which are metabolically active and divide by binary fission
- After 48 to 72 hours, the reticulate bodies reorganize themselves and condense to form new elementary bodies then leave the host cell and start a new infectious cycle
- The incubation period is generally between 3 to 4 weeks
- **Culture requires growth within eukaryotic cells** rather than on cell-free culture media.



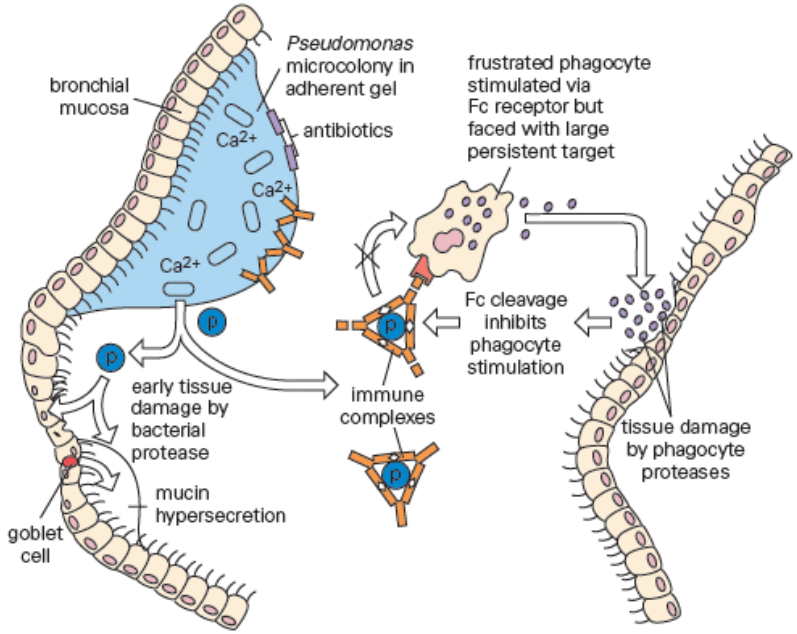
Hospital-acquired **pneumonia**

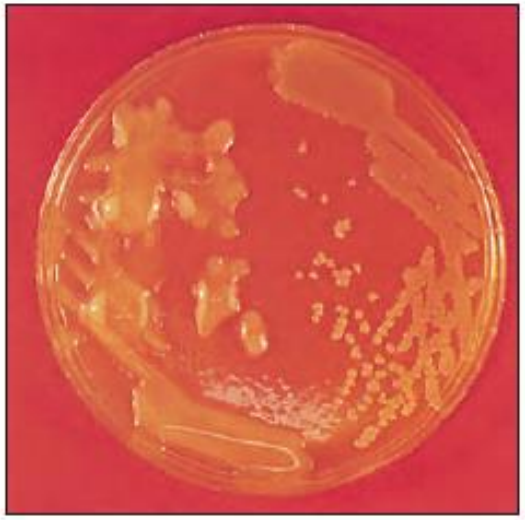
- Methicillin-resistant Staphylococcus aureus (MRSA) is a common causative organism in **pneumonia**, particularly health care associated **pneumonia** (HCAP) and hospital-acquired **pneumonia** (HAP)

- ventilator-associated pneumonia (VAP) develops in intensive care: high mortality rate in cases caused by *Pseudomonas aeruginosa* and *Acinetobacter* spp.

Individuals with cystic fibrosis are predisposed to develop lower respiratory tract infections

- ***P. aeruginosa*,** the main pathogen in **cystic fibrosis**
- *Staph. aureus*
- *Burkholderia cepacia*
- *H. influenzae*, found in association with *Staph. aureus* and *P. aeruginosa*
- *Aspergillus fumigatus*
- Non-tuberculous mycobacteria



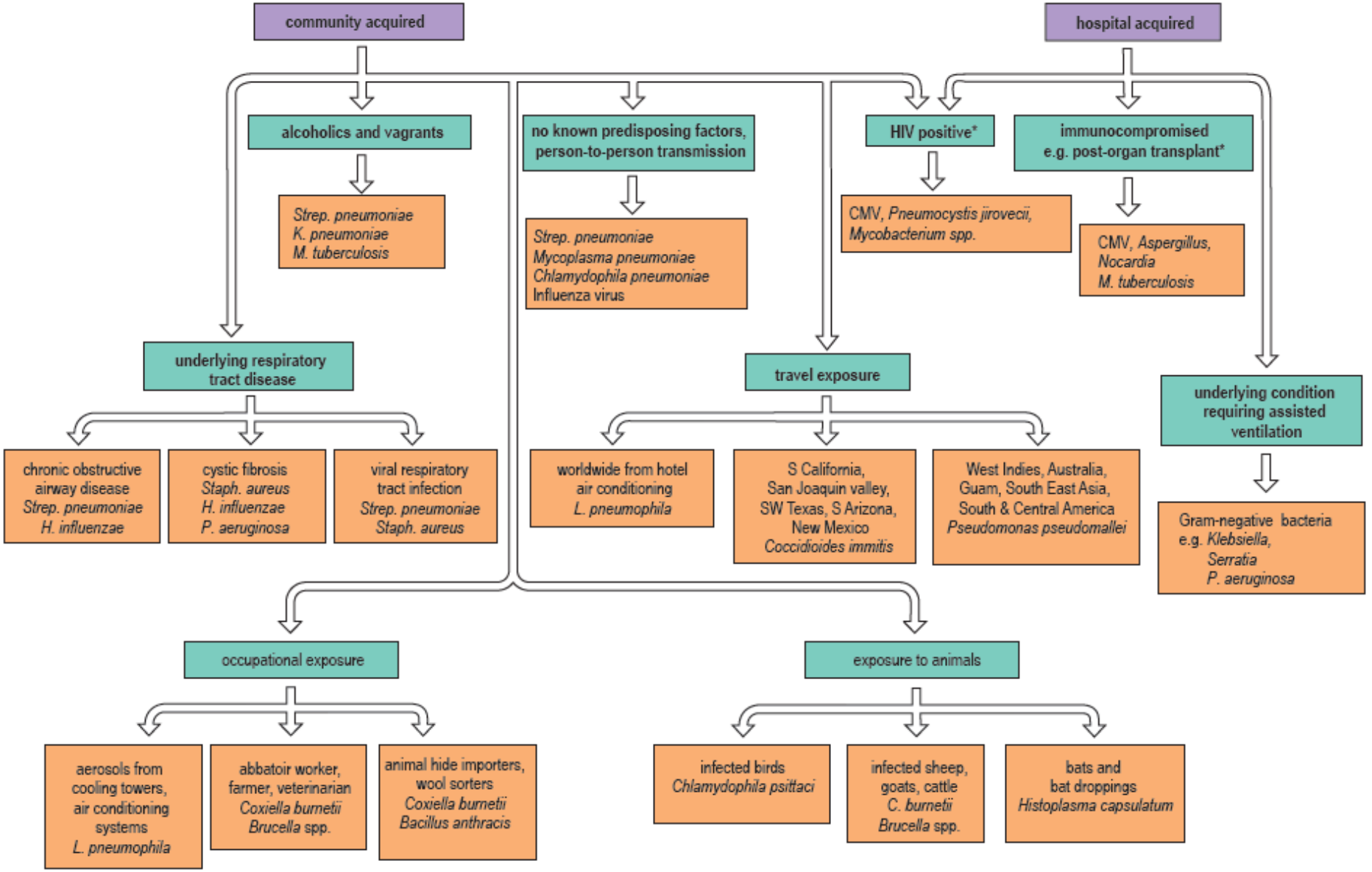


P. aeruginosa infection is uncommon in cystic fibrosis patients under 5 years of age, but colonizes the lungs of almost all aged 15–20 years

As infection progresses, *P. aeruginosa* changes to a highly mucoid form

BACTERIAL pneumonia

COMMUNITY acquired and **HOSPITAL** acquired pneumonia caused by different microrganisms



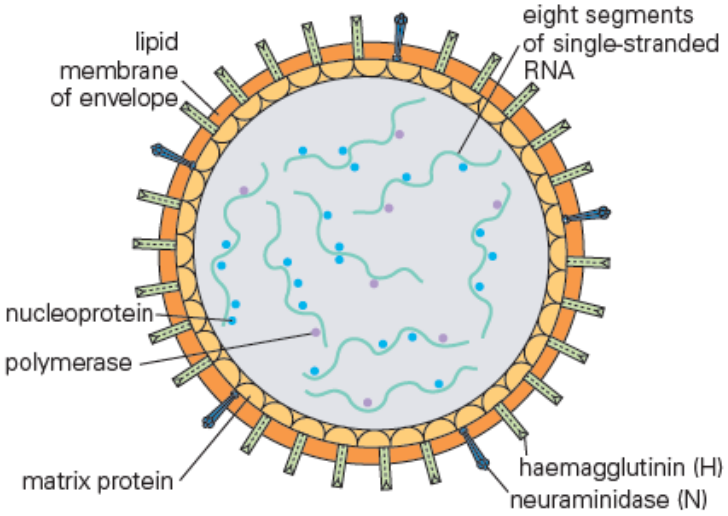
VIRAL pneumonia

- Many viruses cause pneumonia in the face of normal host defences
- Even when viruses do not themselves cause pneumonia, they may damage respiratory defences, laying the ground for secondary bacterial pneumonia

Virus	Clinical condition	Comments
Influenza A or B	Primary viral pneumonia or pneumonia associated with secondary bacterial infection	Pandemics (type A) and epidemics (type A or B); increased susceptibility in elderly or in certain chronic diseases; antivirals and vaccine available
Parainfluenza (types 1–4)	Croup, pneumonia in children <5 years of age; upper respiratory illness (often subclinical) in older children and adults	No treatment available (no published evidence of ribavirin being effective), supportive care, vaccines not available
Measles	Secondary bacterial pneumonia common; primary viral (giant cell) pneumonia in those with immunodeficiency	Adult infection rare but severe; ribavirin may be used as treatment, the King and Queen of Hawaii both died of measles when they visited London in 1824; vaccine available
Respiratory syncytial virus	Bronchiolitis (infants); common cold syndrome (adults)	Peak mortality in 3- to 4-month-old infants; ribavirin treatment available, palivizumab prophylaxis if at high risk
Adenovirus	Pharyngoconjunctival fever, pharyngitis, atypical pneumonia (military recruits)	Cidofovir or ribavirin could be used in specific clinical settings, vaccine available for military
Cytomegalovirus	Interstitial pneumonitis	In immunocompromised patients (e.g. bone marrow transplant recipients); antivirals (e.g. ganciclovir, valganciclovir, foscarnet, cidofovir) and immunoglobulin available
Herpes simplex	Interstitial pneumonitis	In immunocompromised patients; antivirals (e.g. aciclovir, valaciclovir, foscarnet)
Varicella-zoster virus	Pneumonia in young adults with chickenpox	Uncommon; recognized 1–6 days after rash; lung lesions may eventually calcify; antivirals (e.g. aciclovir, valaciclovir, foscarnet) and vaccine available

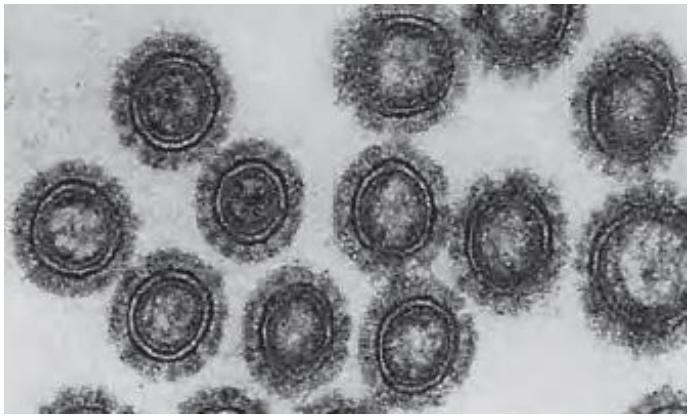
VIRAL pneumonia

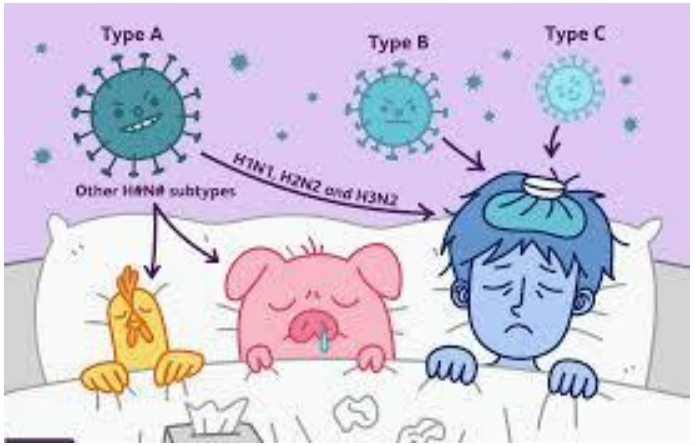
INFLUENZA VIRUS



Antigenic differences between the nucleocapsid and matrix proteins distinguishes 4 types of viruses:

- **A**: causing epidemics, occasionally pandemics
  animal reservoir, notably in birds
- **B**: causing only epidemics
  no animal hosts involved
- **C**: causing no epidemics
  only minor respiratory illness
- **D**: mostly affecting cattle





INFLUENZA VIRUS INFECTION

TRANSMISSION

- By droplet inhalation
- Ubiquitous infections mostly in the coldest months of the year
- Different disease prevalence by years depending on **antigenic drift**

PATHOGENESIS

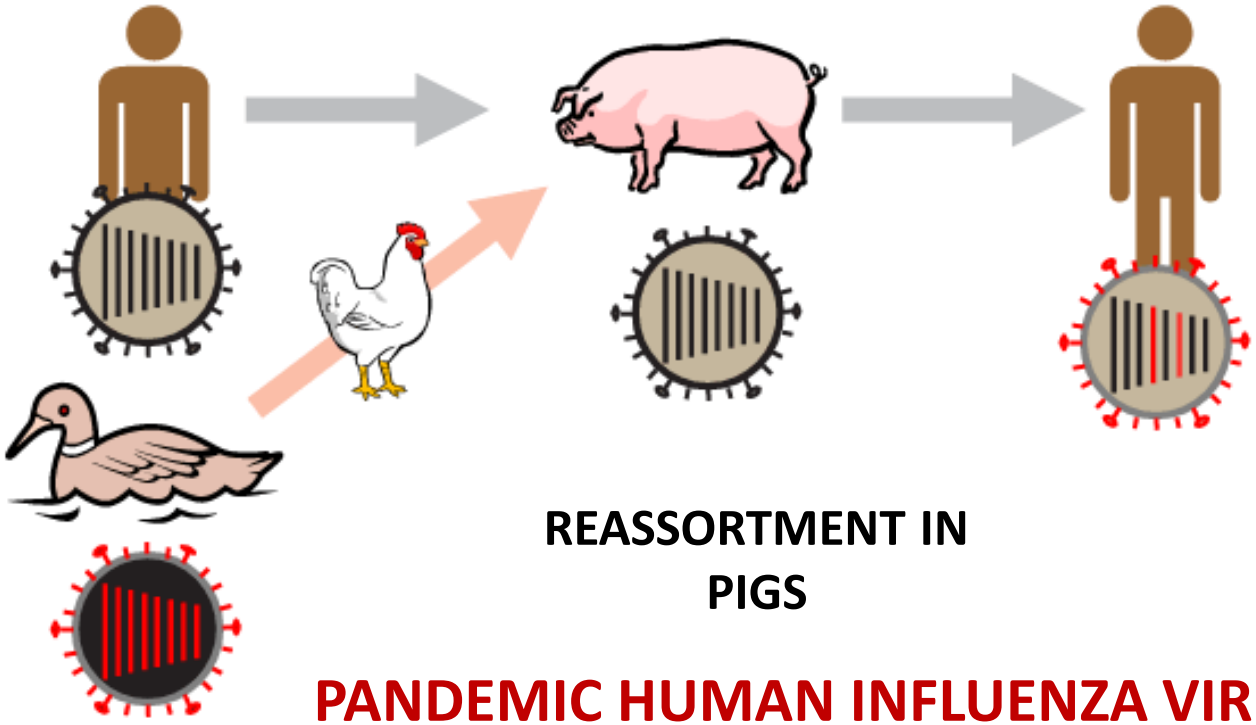
Direct viral damage + associated **inflammatory responses**

Secondary bacterial invaders: staphylococci, pneumococci, *H. influenzae*

→ Mortality due to secondary bacterial pneumonia is higher in apparently healthy individuals over 60 years of age and in those with impaired resistance

→ Pregnant women are also vulnerable

INFLUENZA VIRUS SHIFT MAY CAUSE PANDEMICS



REASSORTMENT IN PIGS

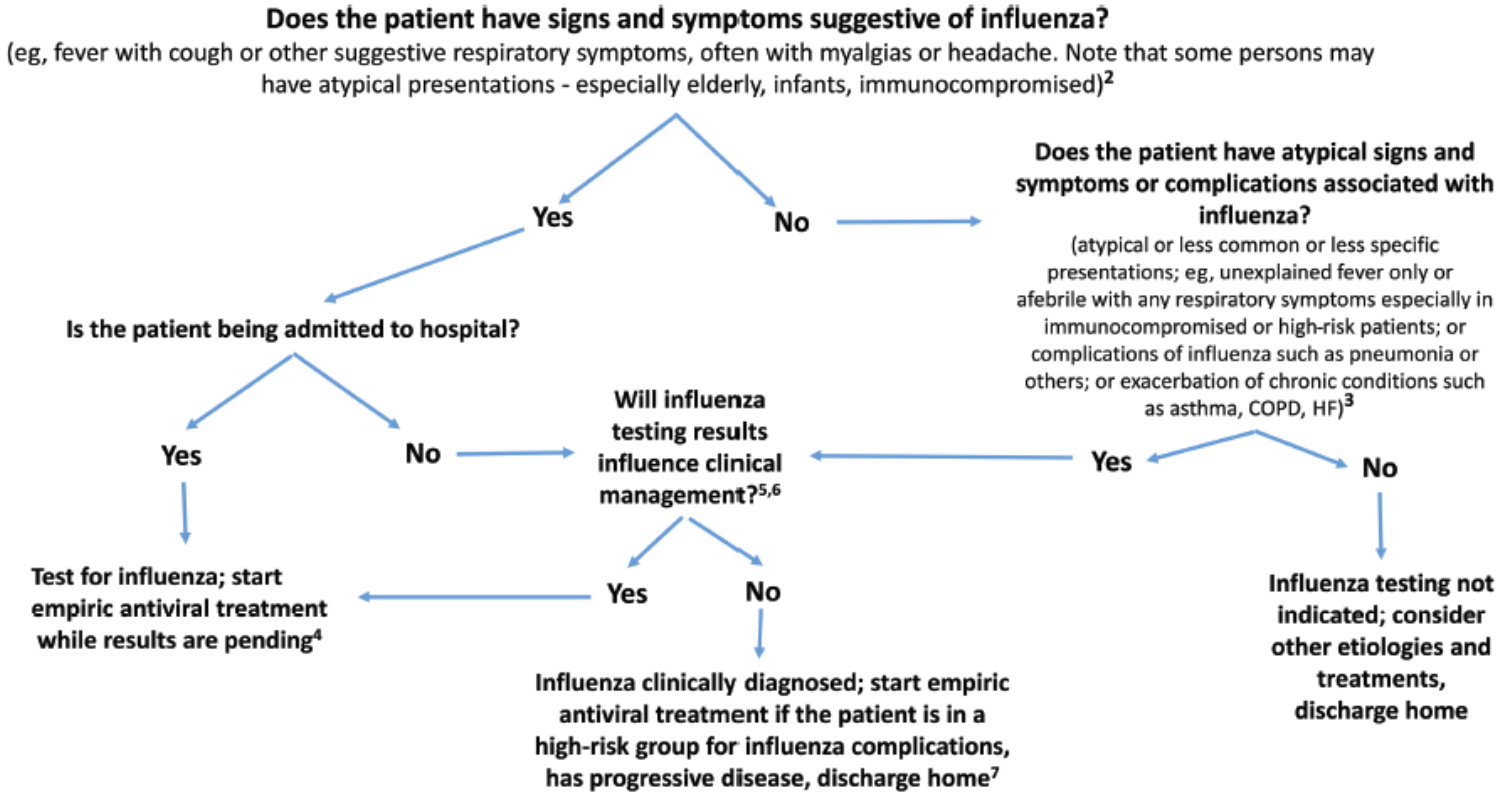
PANDEMIC HUMAN INFLUENZA VIRUSES

Type	Subtype[a]	Year	Clinical severity	Prototype virus
A	H3N2 (?)	1889	Moderate	Designation based on serological studies
	H1N1 (avian)[b]	1918	Severe	H1N1 virus sequenced retrospectively
	H2N2 (Asian)	1957	Severe	A/Japan/57/H2N2
	H3N2 (Hong Kong)[c]	1968	Moderate	A/Hong Kong/68/H3N2
	H1N1	1977	Mild	A/USSR/77
	H1N1pdm09	2009	Mild	H1N1 virus sequenced

Influenza diagnosis guidelines

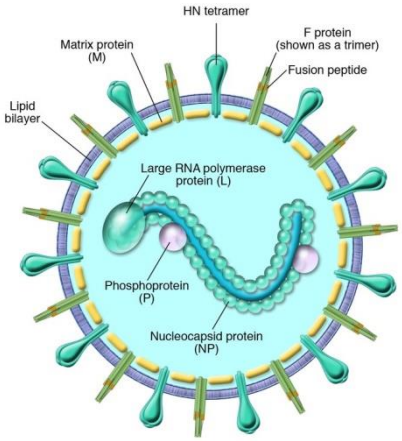
Clinical Infectious Diseases
IDSA GUIDELINE

Clinical Practice Guidelines by the Infectious Diseases Society of America: 2018 Update on Diagnosis, Treatment, Chemoprophylaxis, and Institutional Outbreak Management of Seasonal Influenza[a]



VIRAL pneumonia

PARAINFLUENZA VIRUS

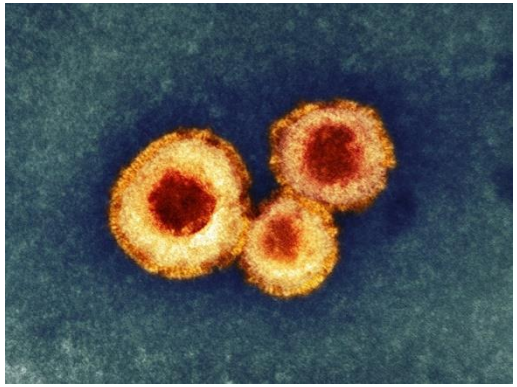


Parainfluenza viruses 1–3

- Pharyngitis
- Croup (in children less than 5 years of age)
- Otitis media
- Bronchiolitis
- Pneumonia

Parainfluenza virus 4

- Less common
- Common-cold-type illness



HUMAN METAPNEUMOVIRUS

HUMAN BOCAVIRUS

RHINOVIRUSES

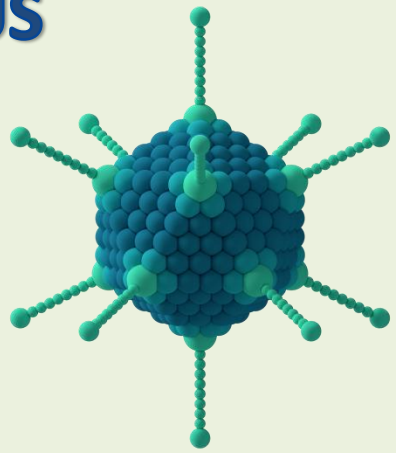
ENTEROVIRUS-D68

RESPIRATORY ENTEROVIRUSES

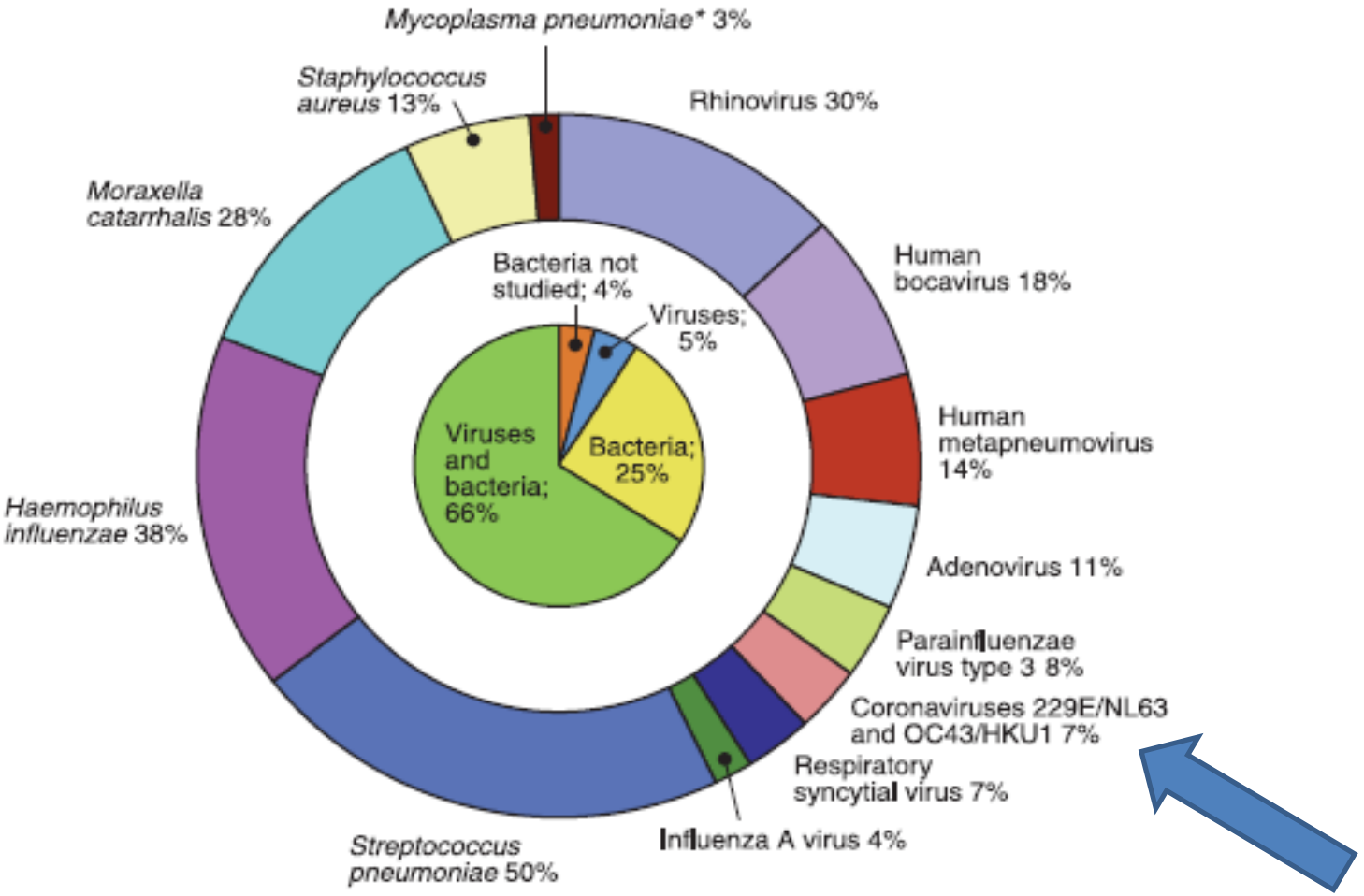
ADENOVIRUS

Types 3, 4 and 7 may cause outbreaks ranging from pharyngitis to atypical pneumonia

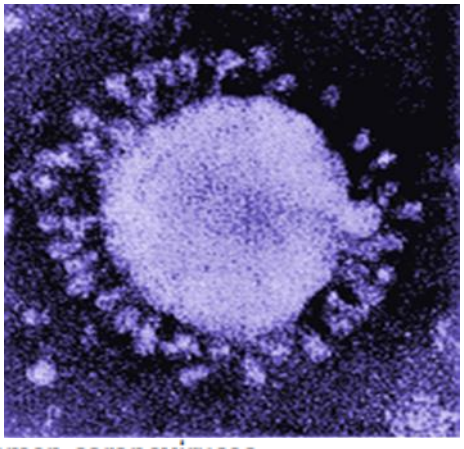
An **emerging variant**, -**14p1**, in United States caused outbreaks of acute respiratory disease with high rates of illness and death



Virus in paediatric pneumonia



From: Honkinen et al Viruses and bacteria in sputum samples of children with community-acquired pneumonia. CMI 2011

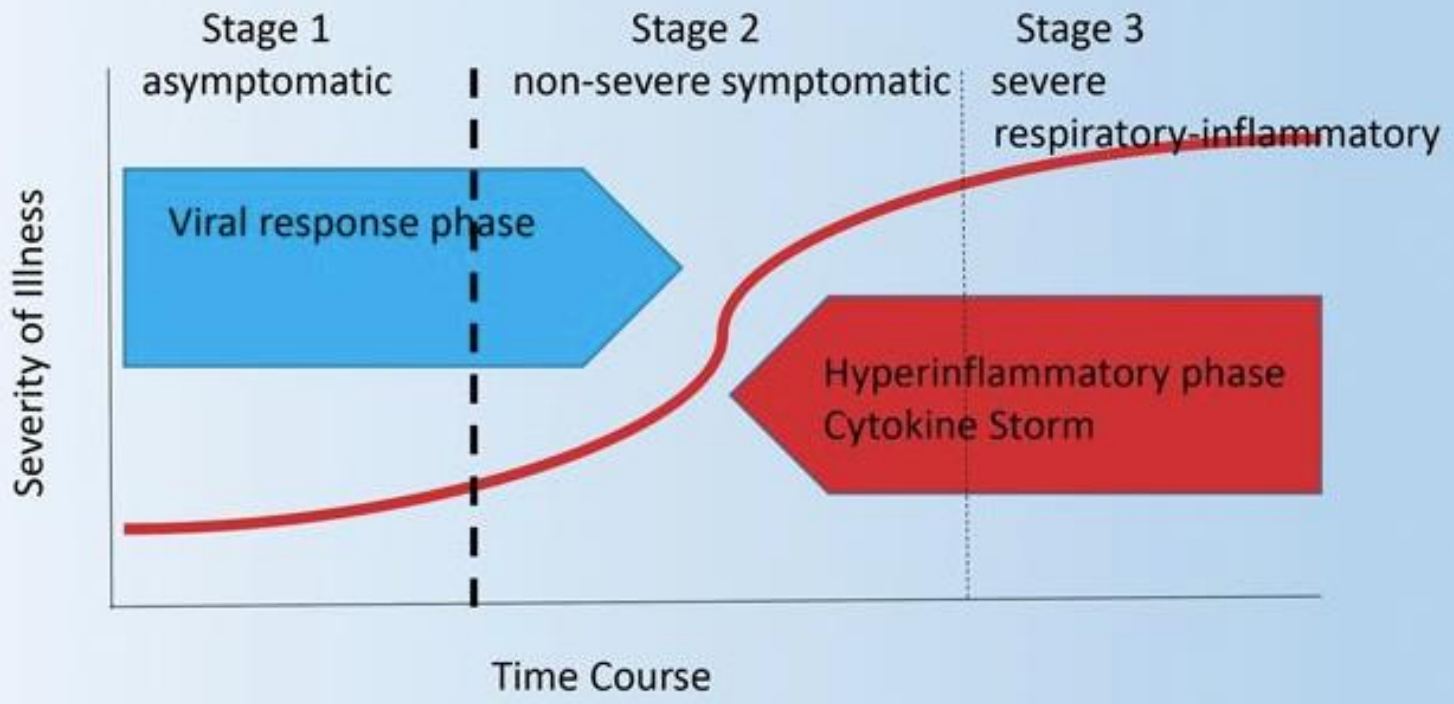


CORONAVIRIDAE

Human coronaviruses.

Coronavirus	Transmission	Disease outcome	Human receptor	Classifica
HCoV-229E	Bats to humans through alpacas, camelids	Mild respiratory symptoms in immuno-compromised patients	Amino-peptidase N	α-CoV
HCoV-NL63	Bats to humans through an unknown intermediate	Mild respiratory symptoms in immuno-compromised patients	Angiotensin- converting enzyme 2, Heparan sulfate	α-CoV
HCoV-OC43	Rodents to humans through cattle	Mild respiratory symptoms in immuno-compromised patients	9-*O*-acetylsialic acids	β-CoV
SARS-CoV	Bats to humans through wild animals, palm civets	Acute pneumonia and respiratory disease	Angiotensin- converting enzyme 2, C-type lectin, Pulmonary surfactant protein D	β-CoV
MERS-CoV	Bats to humans through dromedary camels	Acute pneumonia and respiratory disease	Dipeptidyl-peptidase 4, Sialic acid	β-CoV
HCoV-HKU1	Rodents to humans through an unknown intermediate	Mild respiratory symptoms in immunocompromised patients	9-*O*-acetylsialic acid	β-CoV
SARS-CoV-2	Bats to humans possibly through pangolins	Acute pneumonia and respiratory disease	Angiotensin- converting enzyme 2	β-CoV

Course of COVID-19 Infection

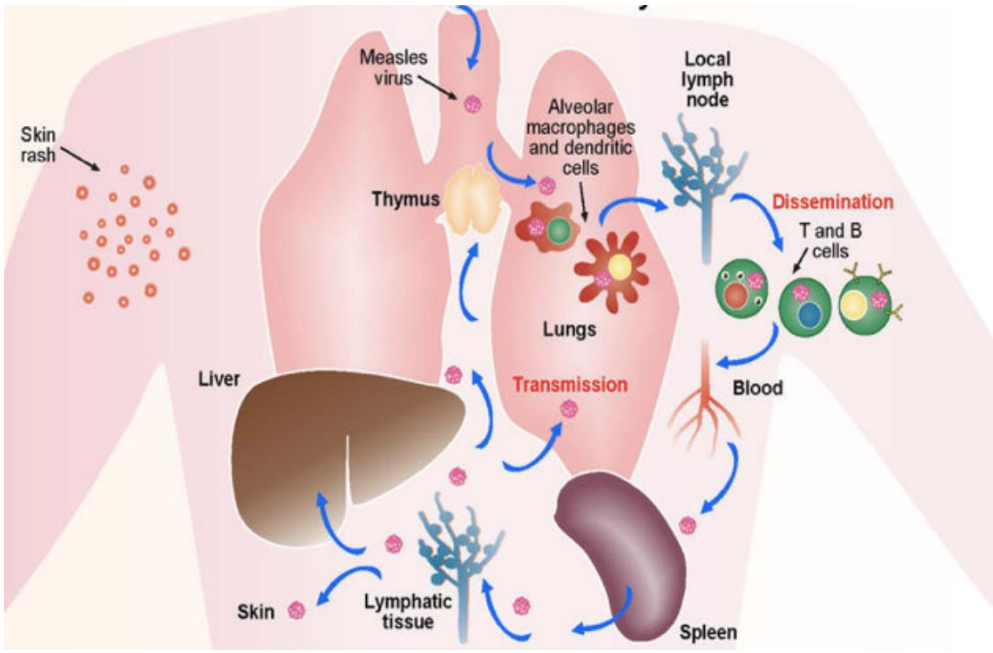


other VIRAL pneumonia

MEASLES VIRUS INFECTION

Measles virus replicates in the lower respiratory tract and can cause:

- Damage leading to **secondary bacterial pneumonia**
- **'Giant cell' pneumonia** in frail hosts



DIAGNOSIS

- Clinical diagnosis

Confirmatory laboratory diagnosis:

- Detection of specific IgM responses
- Viral RNA detection

CMV INFECTION

INTERSTITIAL PNEUMONIA

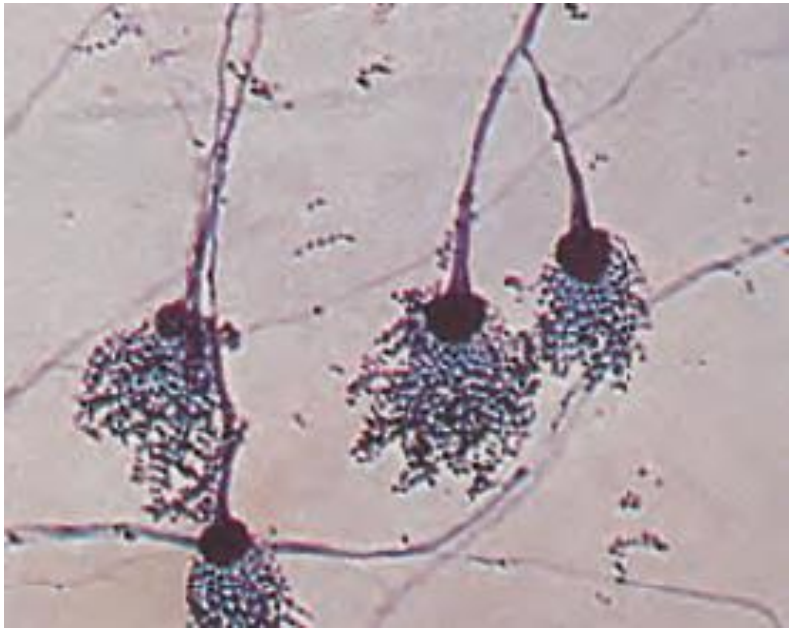
in immunocompromised patients, and in particular allogeneic bone marrow transplant recipients

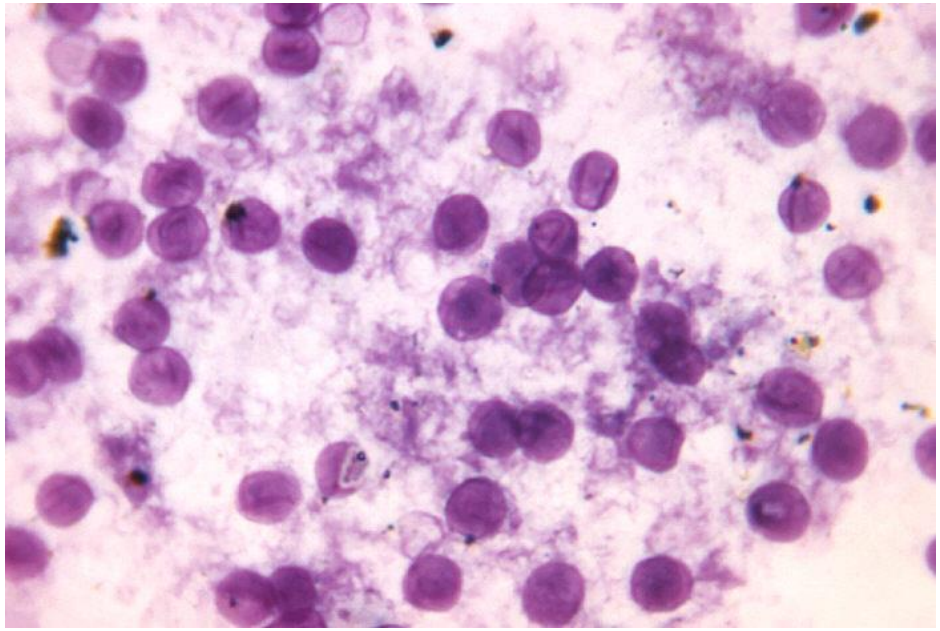
FUNGAL INFECTIONS

Most commonly seen in patients with defective immunity

Two species are of particular importance:

Aspergillus fumigatus and ***Pneumocystis jirovecii***





PROTOZOAL INFECTIONS

Various species of parasites pass through or localize in the lungs at some stage in their life cycle. Damage is limited unless the parasite load is high

Nematodes (*Ascaris, Strongyloides,* hookworms)

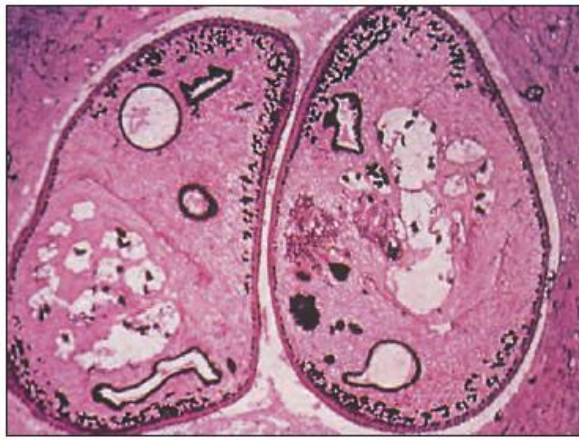
Schistosome larvae

Microfilariae of filarial nematodes (*Wuchereria, Brugia*)

Echinococcus granulosus

Entamoeba histolytica

Paragonimus westermani



Two adult *Paragonimus* contained within a fibrous cyst in the lung

Pneumonia - DIAGNOSIS

Arguments for determine the etiology of CAP

- *1*) antimicrobic resistant pathogen may be identified; *2*) therapy may be narrowed; *3*) identification of atypical pathogens, eg *Legionella*, that may have public health implications; *4*) therapy may be adjusted when patients fail initial therapy; *5*) the constantly changing epidemiology of CAP requires ongoing evaluation.

- **Sputum Gram stain and culture recommended** in hospitalized patients with severe CAP, and when strong risk factors for MRSA and *P. aeruginosa* are identified

Pneumonia - DIAGNOSIS

SAMPLING

Expectorated sputum → Collection is not invasive
Contamination with oral microbial flora can occur

Transtracheal aspiration
Bronchoscopy
Bronchoalveolar lavage
Open lung biopsy

} Invasive collection
Yield more useful results

Samples should be transported and processed as soon as possible

After 2-3 hours from sampling, a delay in the processing could:

- Allow the growth of Gram- bacilli that could mask the presence of pathogens
- Increase the mortality of *Haemophilus* and *S. pneumoniae*

Samples can be refrigerated and processed within 48 h from the sampling

General Guidelines Respiratory Specimens

Specimen good quality is very important!!!

A. Lower respiratory tract:

Bronchoalveolar lavage, tracheal aspirate, Sputum (expectorate deep cough)

Collect 2-3 mL into a sterile screw-cap sputum collection cup or sterile dry container.

B. Upper respiratory tract

Nasopharyngeal swab AND/OR oropharyngeal swab

Use synthetic fiber swabs with plastic shafts. Do not use calcium alginate swabs or wooden shafts, as they may contain **substances that inactivate some viruses and inhibit PCR testing**. Viral transport media (broth and bovine albumin fraction sterile distilled water 400 ml, gentamicin sulfate solution and 3.2 ml amphotericin, Sterilized)

Nasopharyngeal wash (Sterile saline, 0.85% NaCl)**/aspirate or nasal aspirate (infants)**

Collect 2-3 mL into a sterile, sterile dry container.

BACTERIAL DIAGNOSIS

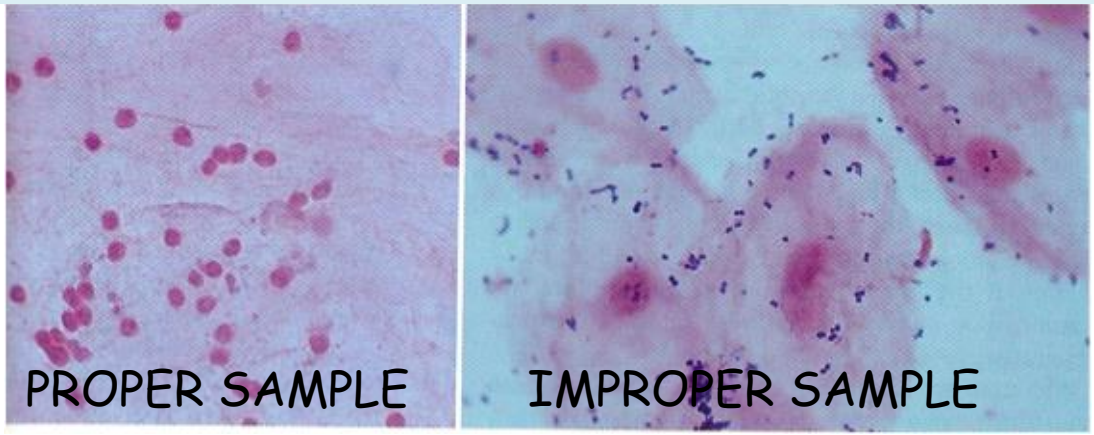
MICROSCOPIC EXAMINATION after GRAM-STAIN

1. SAMPLE QUALITY EVALUATION

Necessary for sputum which could be contaminated by microbial flora

Cell count: n. PMN/n. epithelial cells for field
(Bartlett or Murray Evaluation System)

A **positive score** is assigned to **neutrophils** (sign of infection)
A **negative score** to **epithelial cells** (sign of oropharyngeal contamination)
A final value ≤ 0 suggests the lack of active infection or saliva contamination → a new sample is required

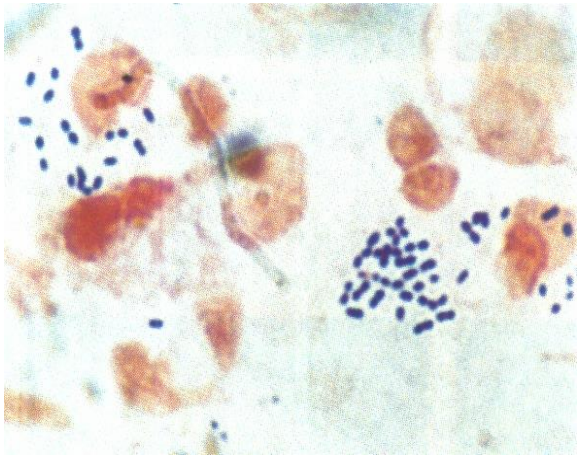


BACTERIAL pneumonia - DIAGNOSIS

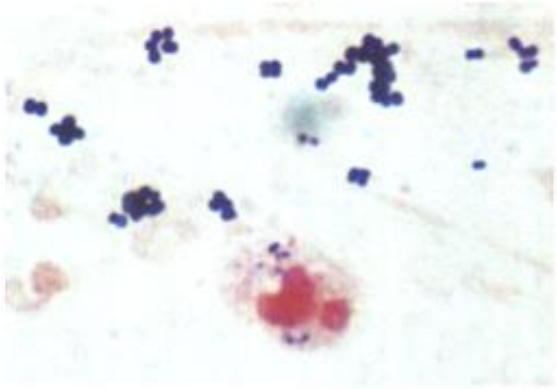
MICROSCOPIC EXAMINATION after GRAM-STAIN

2. PRESUMPTIVE DIAGNOSIS

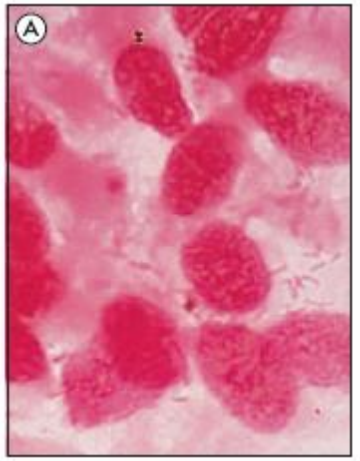
Observation of abundant polymorphs and the putative pathogen
e.g. Gram-positive diplococci characteristic of *Strep. pneumoniae*



Presence of Gram positive diplococci (*S. pneumoniae*)



Presence of clusters of Gram positive cocci (*Staphilococci*)



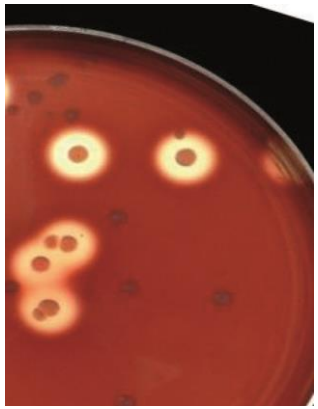
Presence of Gram negative bacilli (*L. pneumophila*)

The causative agents of atypical pneumonia will not be seen in Gram-stained smears

BACTERIAL DIAGNOSIS

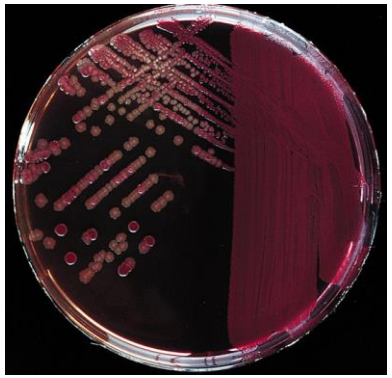
CULTURE

Standard culture techniques for:
Streptococcus pyogenes
Strep. pneumoniae
Staph. aureus
H. influenzae
Klebsiella pneumoniae
Other non-fastidious Gram-negative rods



Streptococcus pyogenes

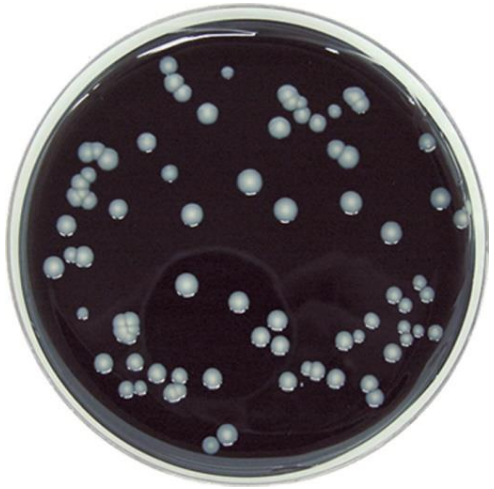
Strept pneumoniae
(alfa-emolisis).



Gram- bacteria

Special media or conditions are required for the causative agents of **atypical pneumonia**
e.g. Buffered charcoal yeast extract medium for *Legionella* growth

White colonies of *L. pneumophila* on BCYE



BACTERIAL DIAGNOSIS

- **Biochemical identification**:

eg card VITEK® Carrier Station™

MALDI-TOF Mass Spectrometer:
identification and structural characterization of bacteria

BACTERIAL DIAGNOSIS

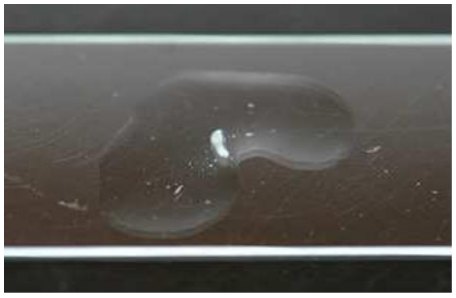
RAPID NON-CULTURAL TECHNIQUES

- **Rapid Latex agglutination assay**

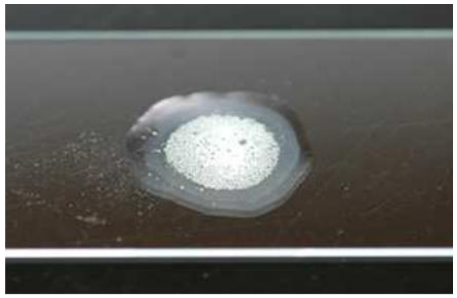
Detection of **antigen** by agglutination of antibody-coated latex particles

Pathogen	Test
Mycoplasma pneumoniae	Complement fixation test (CFT), IgM by latex agglutination or ELISA
Legionella pneumophila	Urinary antigen test or rapid microagglutination test
Chlamydophila pneumonia *Chlamydophila psittaci*	Microimmunofluorescence or ELISA using species-specific antigens
Coxiella burnetii	CFT (phase I and phase II antigens)

ATYPICAL PNEUMONIA



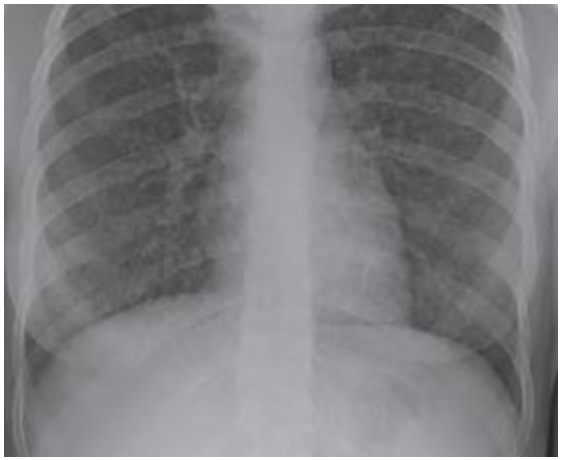
***Strept. pyogenes*: catalase neg**

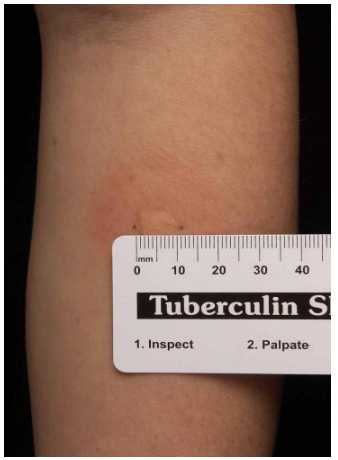


Staph. aureus: catalase pos

Tuberculosis

DIAGNOSIS



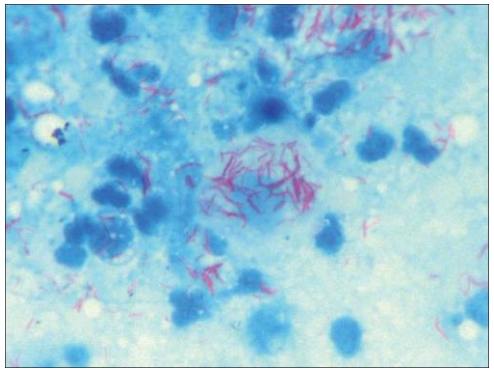


Diagnosis of TB is suggested by the clinical signs and symptoms, supported by:

- characteristic changes on **chest radiography**
- positive **tuberculin (Mantoux) test**

LABORATORY CONFIRMATORY DIAGNOSIS

Sputum sample → MICROSCOPIC EXAMINATION



Ziehl–Neelsen's staining
Microscopic demonstration of **ACID-FAST RODS**

Auramine–rhodamine stain
Microscopic demonstration of **FLUORESCENT RODS**

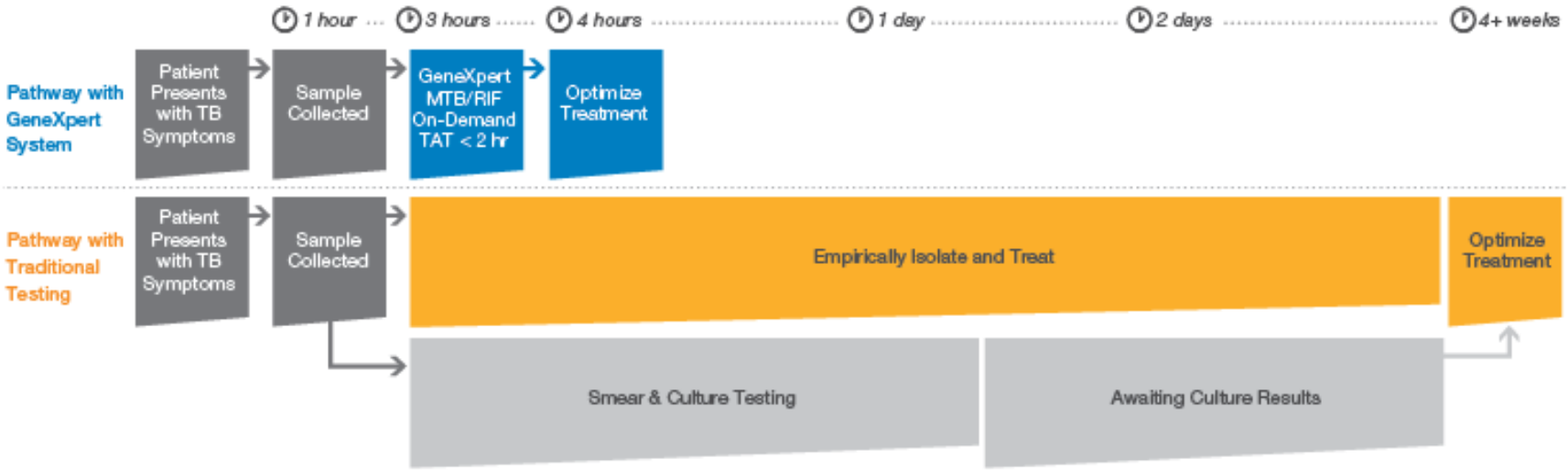
Complex media and
long time required for culture results

Tuberculosis

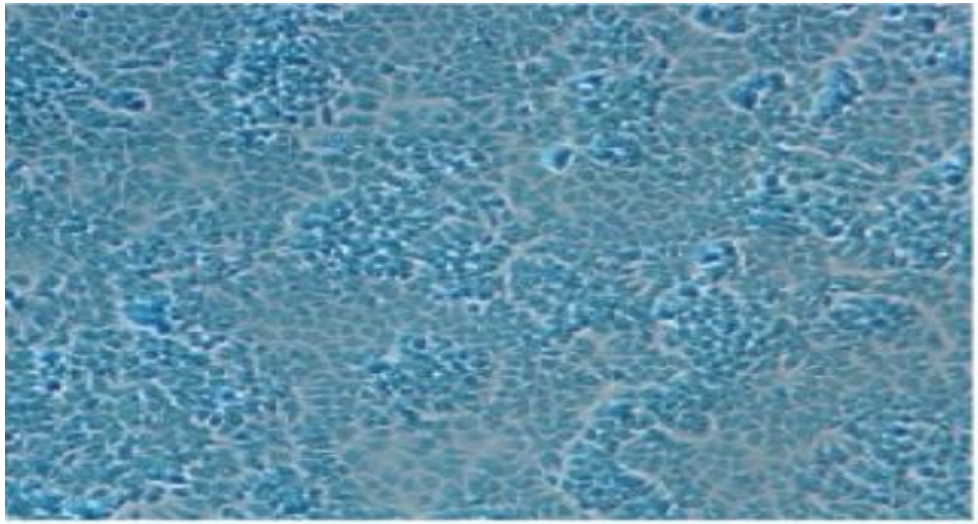
DIAGNOSIS

RAPID MOLECULAR METHODS

- ✓ PCR
- ✓ Xpert MTB-RIF molecular test:
  detects TB and rifampicin resistance



Cell culture growth for respiratory viruses



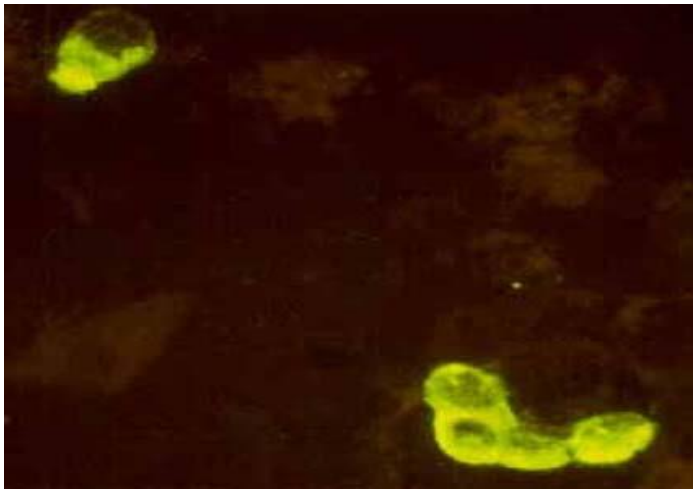
Commercial MixedCellsTM R-MixIn shell vials ready for inoculation; incubated for 48 hours and then screened using a respiratory virus monoclonal “cocktail”. If positive using the “cocktail” multiple smears are prepared and stained using monoclonal antibodies to specific respiratory viruses

Immunofluorescence

- **SimilFluor respiratory screen Chemicon**:

Respiratory Panel I: RSV (**yellow-gold**) vs Adeno, Flu A /B, Para 1-3 (apple green). Panel II per differenziare Adeno, Flu A/B, Para1-3

- **D3 Respiratory Virus Screening ID Microgen**
- **Seven Respiratory Virus Biotrin**

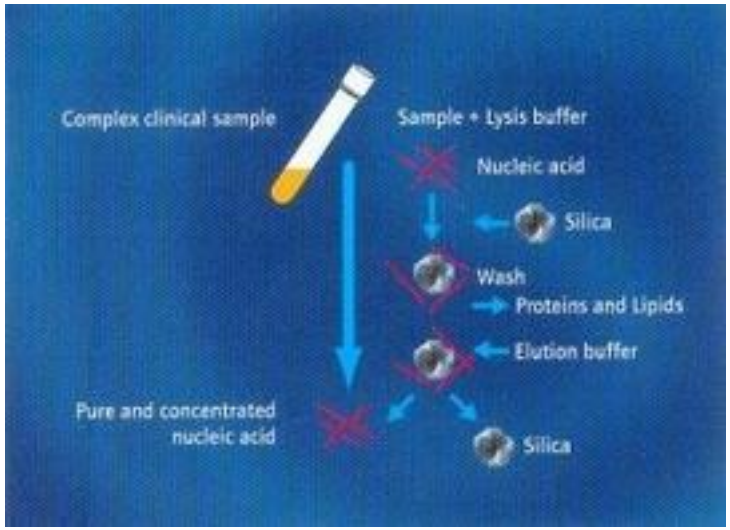


THE FIRST RAPID TESTS FOR INFLUENZA AND RSV

- **Rapid immunochromatographic tests** identify **antigens** in respiratory specimens
- Specificities are high (90-95%)
- Sensitivities are low to moderate (50-70%) and much lower in case of pandemic Influenza virus (Morbidity and Mortality Weekly Report 6/8/2009)

MOLECULAR METHODS: **Nucleic Acid** extractions

Automated **DNA** and **RNA** extraction from many samples



The first commercial Real-time for respiratory viruses: limited targets, not quantitative

- Prodesse ProFlu (the first real-time to receive FDA clearance in 2008)
- Real-time multiplex RT-PCR Artus Infl A/B RG RT-PCR Kit IVD test
- InfA and B Rotor-Gene Q MDx Qiagen
- ARGENE Influenza A/B r-gene®
- Roche RealTime ready Influenza A

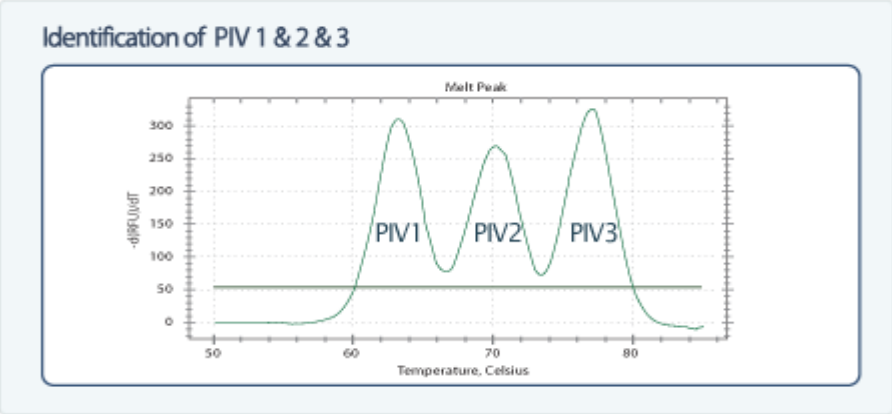
- Hexaplex® :
  Influenza A Virus
  Influenza B Virus
  Parainfluenza 1 Virus
  Parainfluenza 2 Virus
  Parainfluenza 3 Virus
  RSV
- Q-Hexaplex® Plus:

added with Metapneumovirus

A molecular diagnosis in real-time with limited targets for viruses and bacteria leave most LRTI cases without an etiological agent detected

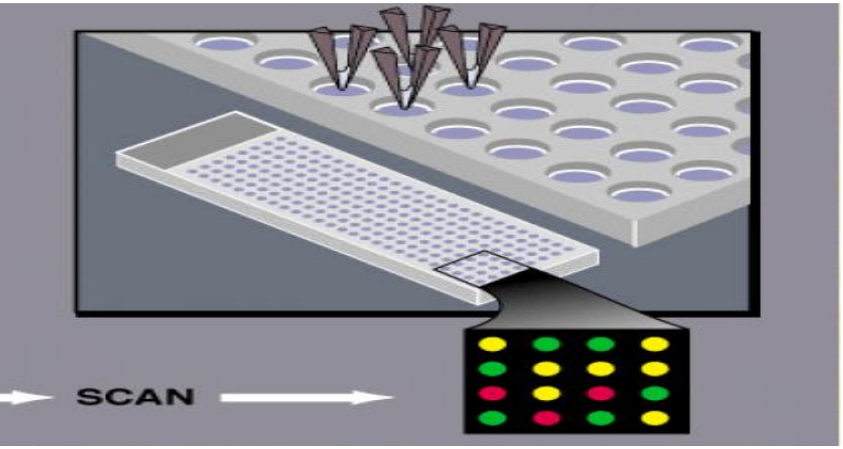
NOVEL MOLECULAR METHODS CE-IVD
Multiplex real-time PCR

New technologies with enhanced multiplexing in real-time PCR
e.g. The Anyplex™ II **16 respiratory viruses** influenza A and B virus, RSVA and B, adenovirus, metapneumovirus, coronavirus 229E, NL63, OC43, Parainfluenza 1-4, Rhinovirus A/B/C, Enterov and Bocav.
Capillary electrophoresis as the detection platform: semi-quantitative analysis performed on the melting peak due to amount of infecting pathogens

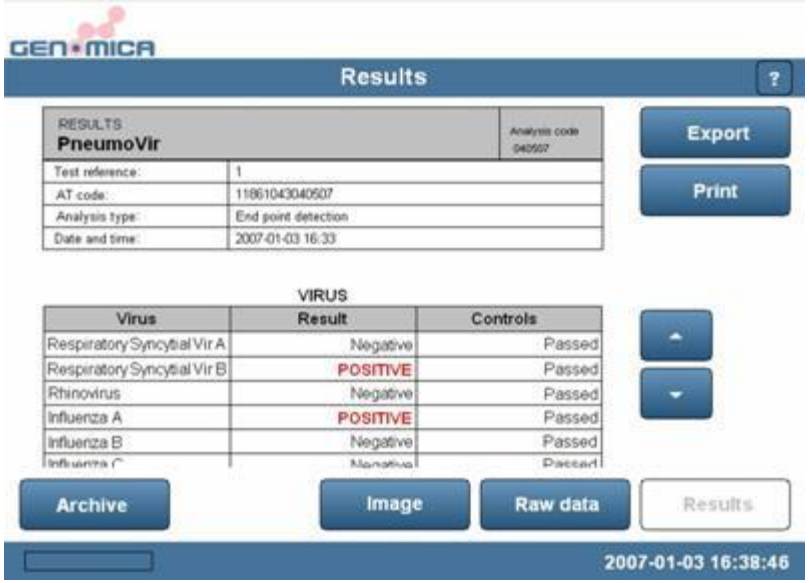


FilmArray™ Respiratory Panel (BioFire Diagnostics, Salt Lake City, UT) FDA cleared
Biomerieux

Micro-arrays for multi pathogen detection



- **CLART® PneumoVir** a low density array 120 spot specific identification of multiple probes



Syndromic diagnosis:

nearly all respiratory viruses and bacteria, including several recently detected viruses and atypical bacteria up to 35 microbes

TABLE 1] Characteristics of Commonly Used Multiplex Viral Testing Platforms

Product	Manufacturer	Technology	Fully Automated	Throughput	Turnaround Time (h)	Viruses Detected
CLART PneumoVir	Genomica	Multiplex RT-PCR, low-density microarray	No	Moderate-high	> 6	AdV, bocavirus, CoV (229E), Ev, hMPV A/B, Flu-A, Flu-A H1, H1 2009, Flu-A H3, Flu-B, Flu-C, PIV 1-4, RhV, RSV-A, RSV-B
eSensor Respiratory Viral Panel[a]	GenMark Diagnostics	Multiplex RT-PCR, hybridization, electrochemical detection	Yes	Low	1.5	AdV-B/E, AdV-C, Flu-A, Flu-A H1N1, Flu-A H1 2009, Flu-A H3, Flu-B, hMPV, PIV 1-3, RhV, RSV-A, RSV-B,
FTD Respiratory Pathogens 33	Fast Track Diagnostics	Multiplex qPCR	No	Moderate-high	> 6	AdV, Bocavirus, CoV (4), Ev, Flu-A, Flu-A H1, Flu-B, hMPV A/B, parechovirus, PIV 1-4, RhV, RSV-A, RSV-B
FilmArray respiratory pathogen panel[a]	BioFire Diagnostics	Nested multiplex RT-PCR, melting temperature analysis	Yes	Low	1	AdV, bocavirus, CoV (4), Flu-A, Flu-A H1, Flu-A H1-2009, Flu-A H3, Flu-B, Flu-C, hMPV, PIV 1-4, RhV/Ev, RSV
Infiniti respiratory pathogen panel	AutoGenomics	Multiplex PCR and RT-PCR, solid array analyzer	No	Moderate-high	> 6	AdV, CoV, Ev, Flu-A, Flu-B, PIV 1-4, RhV-A, RhV-B, RSV-A, RSV-B
RespiFinder 22	PathoFinder	Multiplex qPCR, melting temperature analysis	No	Moderate-high	> 6	AdV, bocavirus, CoV (4), Flu-A, Flu-A H1 2009, Flu-B, hMPV, PIV 1-4, RhV/Ev, RSV-A, RSV-B
ResPlex II	Qiagen	Target-enriched multiplex PCR with Luminex suspension array	No	Moderate-high	5-6	AdV (B/E), bocavirus, CoV (4), CV/echovirus, Flu-A, Flu-B, hMPV-A, hMPV-B, RSV-A, PIV 1-4, RSV-B
xTAG Respiratory Viral Panel[a]	Luminex Molecular Diagnostics	Multiplex PCR and RT-PCR with Luminex suspension array	No	Moderate	8	AdV, Flu-A, Flu-A H1, Flu-A H3, Flu-B, hMPV, PIV1-3, RhV/Ev, RSV-A, RSV-B
Verigene Respiratory Virus Plus Nucleic Acid Test[a]	Nanosphere	Multiplex RT-PCR, hybridization to gold nanoparticles	Yes	Low	2	AdV, Flu-A, Flu-A H1, Flu-A H3, Flu-B, PIV 1-4, RhV, RSV-A, RSV-B

AdV = adenovirus; CoV = coronavirus; CV = coxsackievirus; Ev = enterovirus; Flu = influenza; hMPV = human metapneumovirus; PCR = polymerase chain reaction; PIV = parainfluenza virus; qPCR = quantitative real-time polymerase chain reaction; RhV = rhinovirus; RSV = respiratory syncytial virus; RT-PCR = reverse-transcriptase polymerase chain reaction.
[a]Approved by the US Food and Drug Administration.

FROM: JM. Walter and RG. Wunderink, CHEST 2019

Pros and cons syndromic diagnosis

- High analytical sensitivity and specificity
- Minimal hands-on time
- Full automation from extraction to data analysis and reports
- Scalability

However:

- These platforms are often medium-high throughput
- High costs for few samples in a single run
- Relatively long turnaround time 3-6 h
- Uncertain pathogenic role of common agents (e.g HRV, BocaV, *S. aureus*)

SARS-CoV-2 molecular diagnosis

- **First emergency use: real-time RT-PCR (**reverse transcriptase polymerase chain reaction), amplifying N gene (protocol CDC USA), RdRp and/or E gene (protocol Charitè Berlin), validated by OMS
- **Can detect low numbers of viral genomic RNA**

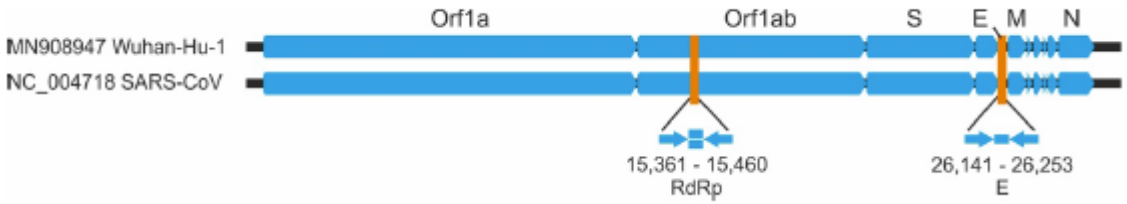


Table 1A Summary table of in-house protocols published by public health and research labs at the time of discovery of COVID-19 (https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance/laboratory-guidance)

Country	Institute	Gene targets	Reference
China	China CDC	ORF1ab and N	http://ivdc.chinacdc.cn/kyjz/202001/t20200121_211337.html
Germany	Charité	RdRP, E, N	https://www.who.int/docs/default-source/coronaviruse/protocol-v2-1.pdf?sfvrsn=a9ef618c_2
Hong Kong SAR	HKU	ORF1b-nsp14, N	https://www.who.int/docs/default-source/coronaviruse/peiris-protocol-16-1-20.pdf?sfvrsn=af1aac73_4
Japan	National Institute of Infectious Diseases, Department of Virology III	Pancorona and multiple targets, spike protein	https://www.who.int/docs/default-source/coronaviruse/method-niid-20200123-2.pdf?sfvrsn=fbf75320_7
Thailand	National Institutes of Health	N	https://www.who.int/docs/default-source/coronaviruse/conventional-rt-pcr-followed-by-sequencing-for-detection-of-ncov-rirl-nat-inst-health-t.pdf?sfvrsn=42271c6d_4
USA*	US CDC	Three targets in N gene	https://www.fda.gov/media/134922/download
France	Institut Pasteur, Paris	Two targets in RdRP	https://www.who.int/docs/default-source/coronaviruse/real-time-rt-pcr-assays-for-the-detection-of-sars-cov-2-institut-pasteur-paris.pdf?sfvrsn=3662fcb6_2

*CDC update effective from 15 March 2020.
CDC, Centers for Disease Control and Prevention; ORF, open reading frame.

Diagnostic molecular Tests for SARS-CoV-2

Rapid development of numerous molecular Tests based on real-time RT-PCR or on no-PCR based amplifications approved CE-IVD EUA/FDA

Table 2 Commercial molecular diagnostic tests that received EUA from the Food and Drug Administration of the USA as listed on their website at the time of this review. The website should be checked regularly for updates. (https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#covid19ivd)

Date EUA was issued	Manufacturer	Diagnostic (letter of authorisation)	Fact sheet for healthcare providers	Fact sheet for patients	Manufacturer instructions/package Insert	Other documents
2 April 2020	Becton, Dickinson & Company	BioGX SARS-CoV-2 Reagents for BD MAX System	Healthcare providers	Patients	IFU	None
1 April 2020	Ipsum Diagnostics, LLC	COV-19 IDx Assay	Healthcare providers	Patients	EUA summary	None
1 April 2020	Cellex*	qSARS-CoV-2 IgG/IgM Rapid Test	Healthcare providers	Patients	IFU	None
30 March 2020	QIAGEN GmbH	QIAstat-Dx Respiratory SARS-CoV-2 Panel	Healthcare providers	Patients	IFU	None
30 March 2020	NeuMoDx Molecular	NeuMoDx SARS-CoV-2 Assay	Healthcare providers	Patients	IFU	None
27 March 2020	Luminex Molecular Diagnostics	NxTAG CoV Extended Panel Assay	Healthcare providers	Patients	IFU	None
27 March 2020	Abbott Diagnostics Scarborough	ID NOW COVID-19	Healthcare providers	Patients	IFU	None
26 March 2020	BGI Genomics Co	Real-Time Fluorescent RT-PCR Kit for Detecting SARS-2019-nCoV	Healthcare providers	Patients	IFU	None
25 March 2020	Avellino Lab USA	AvellinoCoV2 test	Healthcare providers	Patients	EUA summary	None
24 March 2020	PerkinElmer	PerkinElmer New Coronavirus Nucleic Acid Detection Kit	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (1 April 2020)
23 March 2020	Mesa Biotech	Accula SARS-Cov-2 Test	Healthcare providers	Patients	IFU	None
23 March 2020	BioFire Defense, LLC	BioFire COVID-19 Test	Healthcare providers	Patients	IFU	None
20 March 2020	Cepheid	Xpert Xpress SARS-CoV-2 Test	Healthcare providers	Patients	IFU for labs IFU for point of care	None
20 March 2020	Primerdesign	Primerdesign Ltd COVID-19 genesig Real-Time PCR Assay	Healthcare providers	Patients	IFU	None
19 March 2020	GenMark Diagnostics	ePlex SARS-CoV-2 Test	Healthcare providers	Patients	IFU	None
19 March 2020	DiaSorin Molecular LLC	Simplexa COVID-19 Direct Assay	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (26 March 2020)
18 March 2020	Abbott Molecular	Abbott RealTime SARS-CoV-2 Assay	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (1 April 2020)
17 March 2020	Quest Diagnostics Infectious Disease	Quest SARS-CoV-2 rRT-PCR	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (26 March 2020)
17 March 2020	Quidel Corporation	Lyra SARS-CoV-2 Assay	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (23 March 2020)
16 March /2020	Laboratory Corporation of America	COVID-19 RT-PCR Test	Healthcare providers	Patients	EUA summary	None
16 March 2020	Hologic	Panther Fusion SARS-CoV-2	Healthcare providers	Patients	IFU	None
13 March 2020	Thermo Fisher Scientific	TaqPath COVID-19 Combo Kit	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (24 March 2020)
12 March 2020	Roche Molecular Systems	cobas SARS-CoV-2	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (31 March 2020)
29 February 2020	Wadsworth Centre, New York State Department of Public Health's (CDC)	New York SARS-CoV-2 Real-Time Reverse Transcriptase (RT)-PCR Diagnostic Panel	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (15 March 2020)
4 February 2020	CDC	CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (CDC)	Healthcare providers	Patients	IFU	Letter granting EUA amendment(s) (30 March 2020)

Authorization Documents include the Healthcare Provider (HCP) and Patient Fact Sheets and either th
*Antibody rapid test.

Venter M, Richter K. *J Clin Pathol* 2020;**0**:1–8. doi:10.1136/jclinpath-2020-206685

Important limitations of molecular tests

- a) **Real Time PCR workflow is of high complexity**, need trained personnel, is endangered by availability and price of instruments and reagents;
- b) time to diagnosis is of hours from sampling to tests results (turnaround time of 12−24 hours);
- c) positive results may be due to the presence of acting replicating virus or residual viral nucleic acid (i.e., non-infectious virus);
- d) to avoid false negative results in low-copy number samples, 2/3 viral genes are to be targeted; this can generate discrepant reports among genes, due to their differential transcriptional efficiency, thus complicating the reports;
- e) though using high-quality dedicated reagents, some assays yield false negative results due to inhibitors of the amplification steps;
- f) specimen collection, transport and processing are slowed due to safety requirements (saliva or other self-collected samples instead of physician-collected respiratory secretions are being evaluated for diagnostics).

- FROM: Antonelli G et al. The need for innovative solutions in SARS-CoV-2 diagnostics 2020

Usefulness of a **RAPID** CAP diagnosis

- Infected cases may need separate management e.g. in case of pandemic viruses!
- Early and rapid diagnosis leads to effective treatment of critical illness
- Testing offers a potential way forward combating antibiotic overuse

Rapid molecular assays

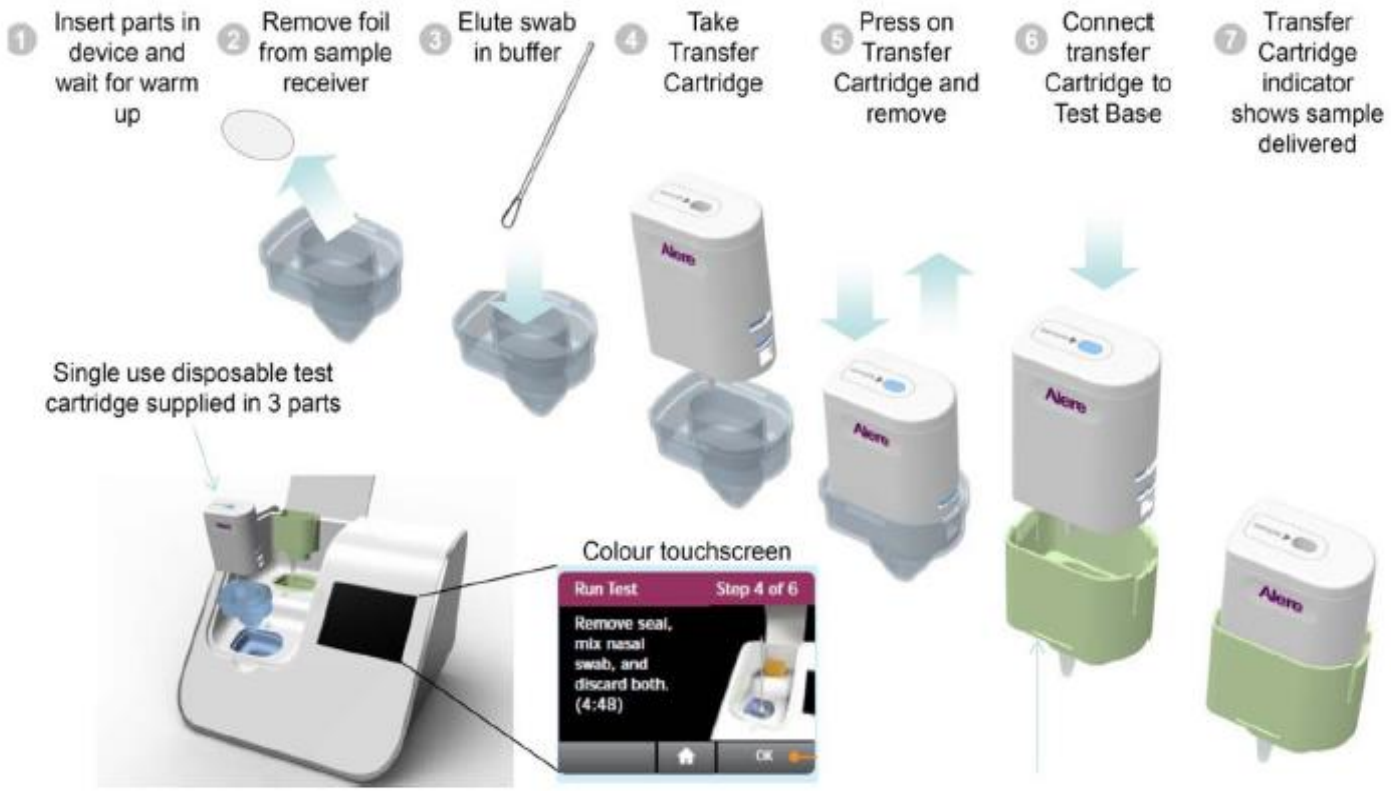
- Rapid molecular assays extract RNA from upper respiratory tract specimens and test for influenza viruses in approximately 15-30 minutes
- Based on RT-PCR or isothermal amplification.
- Sensitivities 70-100% respect to standard PCR-based kit
- Redundancy in gene target (e.g. Xpert Flu) may detect mutated Influenza
- Rapid molecular multi-tests (e.g Xpert® Xpress Flu/RSV)
- Rapid molecular assays reduced hospitalizations and other diagnostic tests but the impact on antibiotic prescribing is less marked up to now

(Braybrook et al J. of Hospital Infect , 2018; Walter et al, Chest 2018; Vos et al Clinical Infectious Disease , 2019)

RAPID MOLECULAR TESTS

Rapid detection of the current pandemic coronavirus SARS-CoV-2 in as soon as 30 minutes for positive results with less than a minute of hands on time to prepare the sample.

It can detect SARS-CoV-2, Flu A and B, RSV in the same cartridge



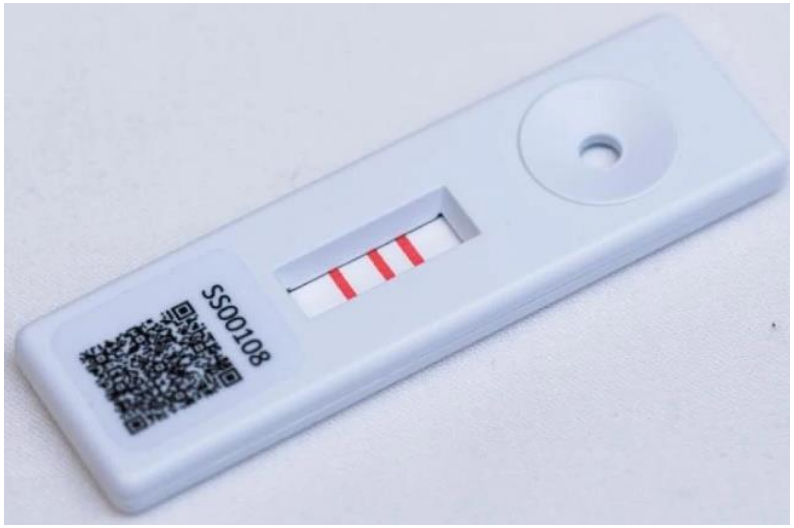
Rapid molecular diagnostic tests

- Simple to operate; no need for trained professionals; rapid turn-around-time; low throughput; Ct values can be obtained; cost-effective.

However, PCR inhibitors found in crude samples can cause failed reactions; target detection at low copies varies widely

ANTIGENIC TESTS SARS-CoV-2

- **LATERAL FLOW TEST**



- LFT use immunoassay technology using nitrocellulose membrane, coloured nanoparticles (or labels), and antibodies toward antigens contained in the infected sample.
- When a sample is added, the sample will flow along the test device passing through the conjugate pad into the nitrocellulose membrane and then onto the absorbent pad.
- As the sample moves along the device the binding reagents situated on the nitrocellulose membrane will bind to the target at the test line. A coloured line will form and the density of the line will vary depending on the quantity of the target present. Some targets may require quantification to determine target concentration.
- Chromatographic rapid tests have relatively lower **sensitivity and specificity**