Influenza Virological Diagnosis and Surveillance

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Scope

• Objectives of virological influenza surveillance,
• How it is organised in order to achieve
  – a representative sampling of circulating viruses,
  – as well as ability to rapidly
    • detect,
    • understand,
    • respond to emerging changes.
• The relevant
  – virus characteristics
  – laboratory methods used
• Current and future opportunities and challenges for the virological surveillance of influenza
Rationale for virological influenza surveillance

• To tell true flu apart from other infections
  – The symptoms of influenza virus infection overlap with other infections.
    • Many other infections can result in influenza-like illness (ILI)
    • Influenza virus infection frequently does not give influenza-like illness
  – Laboratory verification is therefore an essential complement to any epidemiological influenza surveillance scheme.

• To tell different influenza viruses apart since they may have:
  – different antigenic properties,
  – different age profiles/risk groups
  – different pathogenicity,
  – different susceptibility to antiviral drugs.

• The viruses also constantly and rapidly evolve.
Objectives of virological influenza surveillance

- ILI and verification of real flu
- Occurrence of types and subtypes
- “True epidemiology”
- Monitoring evolution, drift and shift
- Providing viruses, vaccine antigen, reagents
- Maintaining effective diagnostics
- Early warning
Key topics

1. Importance of virological surveillance to confirm influenza disease
2. Importance of virological surveillance for the WHO network and biannual vaccine strain selection process
3. Laboratory methods for diagnosis and subtyping of influenza viruses
4. Need for virus isolation and current problems for isolation and growth of influenza viruses (especially H3N2)
5. RT-PCR method and current uses in NICs
6. Laboratory detection of variant viruses
7. Characterization of influenza viruses at the national level (NIC)
8. Principles of antigenic and genetic characterization of influenza viruses for vaccine strain selection (HI antigenicity; molecular evolution of HA and NA; human serology)
9. Influenza A and B virus molecular evolution
Key topic 1

• Importance of virological surveillance to confirm influenza disease
Objective: ILI and verification of “true flu”

- Usually, but not always good correlation
ILI and verification of “true flu”: ILI summer wave in Norway, 2009 pandemic

- ILI peak in week 35-36 was not supported by virological surveillance
- ’Hypersensitivity’ due to public and professional concern?
- A rhinovirus epidemic took place in August-September
Key topic 2:
Importance of virological surveillance for the WHO network and biannual vaccine strain selection process

• **Timely** information about virus trends and evolution
• Provide viruses for vaccine
• Reagents
• Maintenance of diagnostic capability
The WHO-coordinated Global Influenza Surveillance Network

- has been in operation since the late 1940s, when the need to monitor the rapid changes in the virus in order to provide up-to-date, efficacious influenza vaccine was discovered.
- National Influenza Centres collect virus-containing specimens, identify influenza viruses, report their findings, and forward representative virus specimens or isolates to WHO Collaborating Centres.
- CCs perform detailed characterisation, particularly on the antigenic properties of the viruses.
- One important outcome of this process is the twice-yearly WHO recommendations for the composition of influenza vaccines, together with the virus strains needed for vaccine production.
Objective: Occurrence of types and subtypes

- Monitor circulation of all relevant types/subtypes (and lineages)
  - Type A, B
  - Type A: subtypes H1 (pre-2009 seasonal & 2009 pdm), H3, ...
  - Type B (Vic/2/87 & Yam/16/88 lineages)
  - Unbiased surveillance algorithm

  - Timely for usefulness
Objective: Providing viruses, vaccine antigen, reagents

- Northern hemisphere flu season often comes underway only in January/February
- Things must be in place in February to have vaccine for the next season
Process of influenza vaccine virus selection and development

Seasonal

1. Collection of specimens and disease/epidemiological data - all year round

2. Diagnosis, virus isolation in MDCK, preliminary analysis - hours - 3 weeks

3. Ferret antiserum production - 3-5 weeks

4. Thorough antigenic and genetic analysis - 1-3 weeks

5. Review and selection of candidate viruses for vaccine use - 1-3 weeks

6a. Classical reassortment of high-growth viruses for H1N1 & H3N2 - 3-4 weeks

6b. Reassortment of high-growth viruses using reverse genetics (and full safety testing) - 6 weeks

7a. Antigenic and genetic characterization of reassortants - 4 weeks

7b. Development of standardized reagents for inactivated vaccines - 6 weeks

8. Evaluation of growth property - 3 weeks

9a. Development of standardized reagents for inactivated vaccines - 6 weeks

9b. Development of standardized reagents for inactivated vaccines - 6 weeks

Availability of vaccine viruses and standardized reagents

W Zhang, WHO
The 2002/2003 Season – an example of role for genetic analysis at national level

- Incidence of ILI remains low, but a rising trend is apparent in week 7
- Sporadic circulation of A(H3N2) since mid-January; early detections mostly in hospitalised infants
- Sporadic influenza B since Late December
Viruses from the 2002/2003 season are in bold; reference/vaccine strains are underlined.

Aligned partial HA gene sequences, neighbour-joining of Kimura-corrected genetic distances. The genetic distance between two strains are represented as the sum of the length of horizontal branches connecting them.
The reporting of novel patterns

Differences in HA1 antigenic sites
- emergence of Fujian/411/02 drift variant

First Fujian/411/02-like identification in Europe, January 2003:

Differences in HA1 antigenic sites
Geographic differences

- Epidemics differ!
- Virol. surveillance must have good geographical coverage
- Different epidemics leave different population immunity
- thus the susceptibility will differ in the next season
  - Likely that geographical differences will continue
Monitoring B lineages

- Distinct and diverging lineages since the 1970s(?)
- Recognised in late 1980s
- B/Victoria/2/87 and B/Yamagata/16/88
- Vic-lineage absent from global scene cca 1991-2001 (but persisted in east Asia)
  - Re-emerged 2002 and has co-circulated since
- Defined by HA genetic lineage
  - Genetic reassortment has occurred repeatedly, viruses are lineage ‘mosaics’
- Trivalent vaccines has only one B virus
- Evolution toward subtypes?
B virus characterised by variant, 2005/06, by patient age

- The most numerous virus may not be the one affecting risk groups the most
Monitoring flu B lineages

• Best possible information needed for risk assessment and vaccine composition recommendations
• Different age distribution
• WHO FluNet database captures B virus lineage data,
  – But limited data entered
• Surveillance should provide adequate and timely information
  – Traditionally discriminated by antigenic characterisation of cultured virus (takes time)
  – Molecular rapid tests have been missing – but are coming now!
    • Real-time duplex RT-PCR, conventional PCR, others..
• Weekly up-to-date information should be feasible
Influenza virological surveillance at national level (Norway)

Norwegian laboratories (ca 20)

Sentinel physicians (ca 70)

Samples with virus

Samples from patients with influenza symptoms

Weekly reports

NIPH

Viruses from Norway

Weekly reports

WHO ref.lab

Vaccine virus etc.

www.fhi.no/influenza

WHO

ECDC
Key topic 3: Laboratory methods used for diagnosis and subtyping of influenza viruses

- Viral RNA detection and identification by RT-PCR (prim.lab; NIC; CC)
- Virus isolation in mammalian cells (MDCK) or embryonated eggs (NIC; CC)
- Identification / Antigenic characterisation through haemagglutination-inhibition, virus neutralisation, IF.. (NIC; CC)
- [Genetic characterisation by sequence analysis (NIC; CC)
  - Conventional (Sanger) sequencing; focused genotyping (pyrosequencing/SNP PCR etc); “Next-Generation Sequencing”]
Objective: Maintaining effective diagnostics

- Influenza viruses (can) evolve rapidly
- Antigenic, functional, and genetic changes can affect the functionality of tests and protocols
  - Antisera/mAbs can fail
  - PCR primers and probes can fail
    - Review sequence match continuously
    - Good to have ‘fall-back’ alternative protocols
  - Substrates in functional tests can fail
    - RBCs in HA / HI testing (particularly recent H3N2 viruses)
More on lab methods

Key topic 4: Need for virus isolation and current problems for isolation and growth of influenza viruses (especially H3N2)

- Virus isolates are essential for phenotypic analysis and vaccine
- Virus isolation is becoming ‘unfashionable’ in diagnostic labs
  - Need for virus isolation remains, NICs encouraged to provide isolates
  - Primary isolates in eggs still needed for vaccine – even fewer labs do this routinely
  - The maintenance of competence is becoming a challenge

- Current problems for isolation and growth of influenza viruses (especially H3N2)
  - **Virus changes**: A(H3N2) receptor binding characteristics have changed over the last decade
    - More difficult to grow – bind less well to cellular receptors
    - More difficult to verify growth – bind less well to indicator cells (chicken/turkey/even guinea pig or human red blood cells)
  - **Sampling changes**: less fresh samples & less suitable samples (e.g. in lysis buffer for PCR/antigen test)
Key topic 5: RT-PCR method and current uses in NICs

RT-PCR = Reverse Transcription – Polymerase Chain Reaction
- Extremely powerful molecular biology tool
- Method of choice for influenza virus detection and identification
- Trend: RT-PCR becoming the front line diagnostic; virus isolation a follow-up method for PCR positives
RT-PCR use in NICs

• Flu A/B Detection methods
  – Should be robust and broadly reactive (e.g. cover all animal and human influenza A viruses)
  – Conserved internal genes are suitable targets, e.g. Flu A matrix protein gene

• Subtype (and flu B lineage) identification
  – HA (and NA) target is highly heterogeneous and rapidly evolving – but can’t be substituted for subtyping
    – H1pdm09; H3; (H1pre-09); H5; (flu B VIC/YAM lineage)

• Realtime RT-PCR protocols most popular

• Amplification of any gene for sequencing etc.
Influenza diagnostic RT-PCRs: WHO guidance

WHO information for molecular diagnosis of influenza virus in humans - update

It is strongly recommended that all un-subtypable influenza A specimens should be immediately sent for diagnosis and further characterization to one of the six WHO Collaborating Centres for Reference & Research on Influenza.

August 2011

This document provides information on molecular diagnostic protocols available as of the above date for influenza viruses in humans.

This is a revision of the document published on WHO’s website on 23 November 2009 with updated molecular protocols.

Specimens

The most appropriate specimens for the diagnosis of influenza are upper respiratory tract specimens. Samples should be taken from the deep nostrils (nasal swab), nasopharynx (nasopharyngeal swab), and nasopharynx. Appropriate precautions should be taken to avoid exposing the collector to respiratory secretions.
Key topic 6: Laboratory detection of variant viruses

- **Detection of variant viruses**
  - Drift variants among seasonal influenza viruses
  - Non-seasonal variants that we look for: A(H5N1)HPAI; A(H9); A(H3N2)v etc
  - Variants we do not see coming (e.g. emergence of A(H1N1)pdm09)

- **Expect the unexpected!**
  - Set up the testing so that unusual viruses are likely to be noticed
A NIC director’s nightmare

really strange H3 virus

Really robust fluA typing PCR

+ Really broad & robust subtyping PCR

Really nice A & H3 positive

All is well, no follow up indicated

All is NOT well!

really strange H3 virus epidemic

really strange H3 virus

Really robust fluA typing PCR

+ “Narrow” & less robust subtyping PCR

A positive & subtyping negative

Follow up indicated, including shipment to WHO CC

Early warning & response achieved

really strange H3 virus epidemic
H3 RT-PCR (Current version)

- Human viruses since ca 2008 have 1 probe mismatch
- Additional mismatch affects performance
  - Including several 2011/12 viruses (5-10% in Norway)
  - More anomalous than H3v
  - 1 more difference might lead to failure(?)
Modified probes

- Current H3 PCR can be adapted to discern better between current majority seasonal H3 and variant viruses e.g. H3N2v(2011)
  - Exploit conserved T983C since 2008

**Current H3 PCR**

- H3N2 pre-2008 pos ctrl
- H3N2 “Stockholm” grp3a
- H3N2 “Iowa” grp6
- H3N2v Indiana/8/11

**Updated human H3 probe**

- H3N2 pre-2008 pos ctrl
- H3N2 “Stockholm” grp3a
- H3N2 “Iowa” grp6
- H3N2v Indiana/8/11

**H3v matched probe**

- H3N2v Indiana/8/11
- H3N2 pre-2008 pos ctrl
- H3N2 “Stockholm” grp3a
- H3N2 “Iowa” grp6
How robust subtyping PCRs?

• Aim for full within-subtype reactivity?
• Or «canary in a coal mine» type of test?
  – (First to fall over when the atmosphere is less than perfect)
• We probably want both
• Can they be combined (e.g. twin probes?)
Tools & strategies for discovering unusual ("variant") influenza viruses

Conclusions:
• Testing strategy must include a robust fluA test
• Not bad if subtyping signals a problem with unusual virus
• No shame to refer problem to WHO CC
• (But there will be «false alarms»)
Key topic 7: Characterization of influenza viruses at the national level (NIC)

- Subtype and lineage identification – subtyping is NIC ‘duty’
- Antigenic characterisation – recommended in Europe
- Genetic characterisation – optional but increasingly popular
- (Antiviral resistance) – optional but increasingly popular

- Important that characterisations work has a clear objective and that data are being used – locally and globally
Genetic characterizations in different periods of the season 2010/11

Norwegian Institute of Public Health
Capturing European Characterisation data – ECDC/CNRL
Key topic 8: Principles of antigenic and genetic characterization of influenza viruses for vaccine strain selection

- HI antigenicity;
- molecular evolution of HA and NA;
- Human serology
Vaccine strain selection

• Are there new antigenic variants?
  – Are they ‘real’?
• Are they emerging and likely to predominate within their subtype/lineage?
• Is there a suitable candidate vaccine virus?
Is it a new antigenic variant?

– Is the reduced reactivity to reference ferret antisera consistent?
– Does the difference in ferret antiserum reactivity in HI go both ways?
– Does the antigenic properties have a plausible genetic correlate?
– Is the human immune response to the current vaccine significantly less reactive to the new variant?
  • Human post vaccination serology data is routinely used in the vaccine strain selection process
Are they emerging and likely to predominate within their subtype/lineage?

- Is there evidence of emergence of a distinct group of viruses that is likely to be better covered by a vaccine strain from this group?

- Is there a suitable candidate vaccine virus?
  - Must be an egg isolate with the right antigenic characteristics
  - A high growth version must be provided (wild type or reassortant)
Key topic 9: Influenza molecular evolution

• Influenza A and B virus molecular evolution

• Generation of genetic variation:
  – Error prone RNA polymerase and no proofreading: high mutation rate
    • Insertions/deletions are less common
  – Segmented genome: Facilitates gene segment reassortment
  – Homologous recombination: not playing a (significant) role

• Natural selection:
  – Fitness in general (ability to replicate and spread within and between host individuals)
  – Immune selection (population based) – a major force in human influenza
  – Host species adaptation
  – Resistance to natural and man-made antiviral compounds and mechanisms
  – Response to changes in ecological conditions – e.g. population density and pathogenicity/transmissibility in poultry – emergence of high-path avian influenza
  – Selection for compatibility of viral gene constellation; e.g. balance between receptor binding (HA) and receptor destruction (NA)
Influenza virus evolution in humans: “Trunk” phylogenies – extinction of side branches

- "Trunk" pattern strongest with H3, less pronounced with H1 and B
- The evolutionary basis not entirely understood
- Interference among related viruses a likely factor

Ferguson et al. (2003) Nature 422, 428-33
Human Immunodeficiency virus 1 (HIV-1)

- ‘star’ phylogenies
  - Increasing genetic diversity over time
- (Influenza A(H5N1) shows similar pattern)

Tee K K et al. J. Virol. 2008;82:9206-9215
Virus evolution at RNA, protein, & antigen levels

Evolution at the nucleotide level is gradual; Resulting antigenic drift occurs in steps

Objective: “True epidemiology”

- Understanding the “true epidemiology” is necessary for optimal response and prevention.
- Genetic analysis of the very heterogeneous influenza viruses allows dissecting the relationships between viruses (and thus infections) in great detail.
- Demonstrates that a flu outbreak can consist of many sub-epidemics.
- Some apparent patterns (e.g. spread of epidemic between neighbouring countries) can be refuted by virus genotyping evidence.
Genetic analysis complements and adds to phenotypic analyses, but cannot replace them

- Genetic analysis does not measure directly the phenotypic trait
Genetic analysis can be more timely and ‘reliable’

• **Antigenic analysis depends on reagents**
  – Takes time to produce/provide; in limited amounts
  – Output of analysis is particular to virus-reagent interaction
  – Can be hard to compare/interpret

• **Genetic analysis relies on “universal” method and principle**
  – Rapid and robust
  – Output (the sequences) is ‘absolute’ information
  – But genotype/phenotype relationships incompletely understood
Picking up changes in pathogenicity

• Determinants for pathogenicity to be covered later in the course (prof. Klenk)
• An example from Norway on picking up a candidate pathogenicity marker
Picking up changes in pathogenicity
Is the HA1 Asp222Gly mutation changing receptor specificity?
(pos 225 in H3)

- Documented for the 1918-virus
- Only one mutation in the HA was sufficient to revert the HA receptor preference to that of classical avian strain.
- Same effect in the 2009-virus?

Stevens J et al
Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities.
J Mol Biol. 2006 Feb 3;355
Experts Say Swine Flu Mutations Do Not Warrant New Alarm
Apparent pattern / Hypothesis

- The mutation is not in circulation
- The mutation occur sporadically
- Altered binding specificity and cellular tropism make the virus prone to infect a wider range of cells in the lower respiratory tract
- Correlation between occurrence of the D222G mutation and severe disease
Infection with the recently emerged pandemic influenza A(H1N1) virus causes mild disease in the vast majority of cases, but sporadically also very severe disease. A specific mutation in the viral haemagglutinin (D222G) was found with considerable frequency in fatal and severe cases in Norway, but was virtually absent among clinically mild cases. This difference was statistically significant and our data are consistent with a possible causal relationship between this mutation and the clinical outcome.

in any of 205 mild cases investigated (Table), thus the frequency of this mutation was significantly higher in severe (including fatal) cases (p<0.001, Fisher’s exact test, two-sided) than in mild cases. D222G mutants were detected throughout the sampling period, from the first recorded severe cases in July until early December. The frequency of another substitution in the same position, D222E, did not differ significantly between mild and severe cases (p=0.772). Yet another substitution, D222N, was observed in a very few cases (n=4), and at a higher rate than expected among severe
The difference in frequency of the mutation between the severe/fatal and mild cases is statistically highly significant ($p < 0.001$, Fisher’s Exact Test, two-sided) and indicates a strong correlation.

Consistent with hypothesis that this mutation may contribute to severe illness

But not necessary for severe illness (and maybe not sufficient for severe illness?)

Apparently not well transmitted
Summary D222G

• The mutation is still only found in patients with severe illness
• The same mutation was not found in any of the 205 analysed cases with mild disease
• This mutation has been found sporadically throughout the period of pandemic virus circulation, since the first severe and fatal cases in the summer. There is no indication that the mutated virus has increased in frequency during this period.
• The mutation seem to occur sporadically in single cases, with no or little onward transmission (mixtures, switch in serial samples)
• Since the mutation was not found in the majority of severe and fatal cases it is clear that non-mutated viruses also are capable of causing severe disease
• Further investigations needed to clarify if this mutation alter the virulence and transmissibility of A(H1N1)pdm09
  – Increasing body of laboratory based evidence on D222G as a determinant of pathogenicity and receptor specificity
• Approx 100 publications on this topic have ensued
The relevant virus characteristics that vary

- Antigenic characteristics
- Genetic makeup / relatedness to other virus strains
- Fitness (ability to replicate and spread)
- Pathogenicity
- Susceptibility to antiviral drugs
- Suitability to be captured / analysed by diagnostic tests and characterisation protocols in use
Picking up novel developments

Success factors:

- Have an eye for new developments
- Do analyses timely
- Keep in touch with network and reference laboratory
- Follow up research
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Thanks, and greetings from the National Influenza Centre, NIPH, Oslo, Norway

http://www.fhi.no/influensa

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