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The Crux

BIMONTHLY FORUM FOR THE LABORATARIANS

CONTENTS

Editorial

In keeping with our tradition of not forgetting problems of current relevance, we take up a much-dreaded intercontinental disease - a disease that is spread by those who do not require passports when they cross international boundaries. In fact they come to India from places as far as Siberia. It is a zoonotic disease. Yes, you have guessed it right, we are talking about Avian Bird Flu. It has gained immense importance so much so that WHO runs a complete department dedicated to it completely with multiple international stations located almost in every continent. The risk from bird flu is generally low to most people because the viruses occur mainly among birds and do not usually infect humans. However, during an outbreak of bird flu among poultry (domesticated chicken, ducks, turkeys), there is a possible risk to people who have contact with infected birds or surfaces that have been contaminated with excretions from infected birds. The outbreaks of avian influenza A (H5N1) among poultry in Asia are an example of bird flu outbreaks that have caused human infections and deaths. In such situations, people should avoid contact with infected birds or contaminated surfaces, and should be careful when handling and cooking poultry. DISEASE DIAGNOSIS segment of this issue delves deep into the clinico-diagnostic aspects of this profession related hazard.

At the request of our readers we are commencing a series on urinary crystals. Various kinds of crystals and the reasons for their appearance (even the rarer ones) are discussed at length. A rather common laboratory investigation occupies the space dedicated to INTERPRETATION portion. All related normal, abnormal, iatrogenic, metabolic aspects are laid out for your benefit. It will be covered over multiple issues.

We received a letter from overseas about Laboratory Acquired Brucellosis and the diagnostic approach utilized. The letter is reprinted as it sheds sufficient light on all bacteriologic diagnostic modalities related to Brucellosis. The TROUBLESHOOTING part of this issue is dedicated to this letter alone.

BOUQUET has sweet and sour (you decide the taste) jokes, a few international proverbs and in Brain Teasers you shall tell not what is right but what is wrong. It is equally important (in life and otherwise) to know what is right and also to know what is wrong!

DISEASE DIAGNOSIS

AVIAN (BIRD) FLU

Avian influenza in birds: Avian influenza is an infection caused by avian (bird) influenza (flu) viruses. These influenza viruses occur naturally among birds. Wild birds worldwide carry the viruses in their intestines, but usually do not get sick from them. However, avian influenza is very contagious among birds and can make some domesticated birds, including chickens, ducks, and turkeys, very sick and kill them. Infected birds shed influenza virus in their saliva, nasal secretions, and feces. Susceptible birds become infected when they have contact with contaminated secretions or excretions or with surfaces that are contaminated with secretions or excretions from infected birds. Domesticated birds may become infected with avian influenza virus through direct contact with infected waterfowl or other infected poultry, or through contact with surfaces (such as dirt or cages) or materials (such as water or feed) that have been contaminated with the virus. Infection with avian influenza viruses in domestic poultry causes two main forms of disease that are distinguished by low and high extremes of virulence. The "low pathogenic" form may go undetected and usually causes only mild symptoms (such as ruffled feathers and a drop in egg production). However, the highly pathogenic form spreads more rapidly through flocks of poultry. This form may cause disease that affects multiple internal organs and has a mortality rate that can reach 90-100% often within 48 hours.

Human infection with avian influenza viruses: There are many different subtypes of type A influenza viruses. These subtypes differ because of changes in certain proteins on the surface of the influenza A virus (hemagglutinin [HA] and neuraminidase [NA] proteins). There are 16 known HA subtypes and 9 known NA subtypes of influenza A viruses. Many different combinations of HA and NA proteins are possible. Each combination represents a different subtype. All known subtypes of influenza A viruses can be found in birds. Usually, "avian influenza virus" refers to influenza A viruses found chiefly in birds, but infections with these viruses can occur in humans. The risk from avian influenza is generally low to most people, because the viruses do not usually infect humans. However, confirmed cases of human infection from several subtypes of avian influenza infection have been reported since 1997. Most cases of avian influenza infection in humans have resulted from contact with infected poultry (e.g., domesticated chicken, ducks, and turkeys) or surfaces contaminated with secretion/excretions from infected birds. The spread of avian influenza viruses from one ill person to another has been reported very rarely, and transmission has not been observed to continue beyond one person. "Human influenza virus" usually refers to those subtypes that spread widely among humans. There are only three known A subtypes of influenza viruses (H1N1, H1N2, and H3N2) currently circulating among humans. It is likely that some genetic parts of current human influenza A viruses came from birds originally. Influenza A viruses are constantly changing, and they might adapt over time to infect and spread among humans. During an outbreak of avian influenza among poultry, there is a possible risk to people who have contact with infected birds or surfaces that have been contaminated with secretions or excretions from infected birds. Symptoms of avian influenza in humans have ranged from typical human influenza-like symptoms (e.g., fever, cough, sore throat, and muscle aches) to eye infections, pneumonia, severe respiratory diseases (such as acute respiratory distress), and other severe and life-threatening complications. The symptoms of avian influenza may depend on which virus caused the infection. Studies done in laboratories suggest that some of the prescription medicines used worldwide for human influenza viruses should work in treating avian influenza infection in humans. However, influenza viruses can become resistant to these drugs, so these medications may not always work. Additional studies are needed to demonstrate the effectiveness of these medicines.

Avian influenza A (H5N1) in Asia and Europe: Influenza A (H5N1) virus also called "H5N1 virus" is an influenza A virus subtype that occurs mainly in birds, is highly contagious among birds, and can be deadly to them. Outbreaks of avian influenza H5N1 occurred among poultry in eight countries in Asia (Cambodia, China, Indonesia, Japan, Laos, South Korea, Thailand, and Vietnam) during late 2003 and early 2004. At that time, more than 100 million birds in the affected countries either died from the disease or were killed in order to try to control the outbreaks. By March 2004, the outbreak was reported to be under control. Since late June 2004, however, new outbreaks of influenza H5N1 among poultry were reported by several countries in Asia (Cambodia, China [Tibet], Indonesia, Kazakhstan, Malaysia, Mongolia, Russia [Siberia], Thailand, and Vietnam). It is believed that these outbreaks are ongoing. Influenza H5N1 infection also has been reported among poultry in Turkey Romania, and Ukraine. Outbreaks of influenza H5N1 have been reported among wild migratory birds in China, Croatia, Mongolia, and Romania. As of January 7, 2006, human cases of influenza A (H5N1) infection have been reported in Cambodia, China, Indonesia, Thailand, Vietnam, and most recently, several cases in Turkey. For the most current information about avian influenza and cumulative case numbers, see the World Health Organization (WHO) website.

Human health risks during the H5N1 outbreak: H5N1 virus does not usually infect people, but more than 140 human cases have been reported by the World Health Organization since January 2004. Most of these cases have occurred as a result of people having direct or close contact with infected poultry or contaminated surfaces. So far, the spread of H5N1 virus from person-to-person has been rare and has not continued beyond one person. Of the few avian influenza viruses that have crossed the species barrier to infect humans, H5N1 has caused the largest number of detected cases of severe disease and death in humans. In the current outbreaks in Asia and Europe, more than half of those infected with the virus have died. Most cases have occurred in previously healthy children and young adults. However, it is possible that the only cases currently being reported are those in the most severely ill people, and that the full range of illness caused by the H5N1 virus has not yet been defined. So far, the spread of H5N1 virus from person to person has been limited and has not continued beyond one person. Nonetheless, because all influenza viruses have the ability to change, scientists are concerned that H5N1 virus one day could be able to infect humans and spread easily from one person to another. Because these viruses do not commonly infect humans, there is little or no immune protection against them in the human population. If H5N1 virus were to gain the capacity to spread easily from person to person, an influenza pandemic (worldwide outbreak of disease) could begin. No one can predict when a pandemic might occur. However, experts from around the world are watching the H5N1 situation in Asia and Europe very closely and are preparing for the possibility that the virus may begin to spread more easily and widely from person to person.

Treatment and vaccination for H5N1 virus in humans: The H5N1 virus that has caused human illness and death in Asia is resistant to amantadine and rimantadine, two antiviral medications commonly used for influenza. Two other antiviral medications, oseltamavir and zanamavir, would probably work to treat influenza caused by H5N1 virus, but additional studies still need to be done to demonstrate their effectiveness. There currently is no commercially available vaccine to protect humans against H5N1 virus that is being seen in Asia and Europe. However, vaccine development efforts are taking place. Research studies to test a vaccine to protect humans against H5N1 virus began in April 2005, and a series of clinical trials is under way.

Notable Epidemiologic Findings:

H7N7, United Kingdom, 1996: One adult developed conjunctivitis after a piece of straw contacted her eye while cleaning a duck house. Low pathogenic avian influenza A (H7N7) virus was isolated from a conjunctiva specimen. The person was not hospitalized and recovered. **H5N1, Hong Kong, Special Administrative Region, 1997**: Highly pathogenic avian influenza A (H5N1) infections occurred in both poultry and humans. This was the first time an avian influenza A virus transmission directly from birds to humans had been found to cause respiratory illness. During this outbreak, 18 people were hospitalized and six of them died. To control the outbreak, authorities killed about 1.5 million chickens to remove the source of the virus. The most significant risk factor for human H5N1 illness was visiting a live poultry market in the week before illness onset. **H9N2, China and Hong Kong, Special Administrative Region, 1999**: Low pathogenic avian influenza A (H9N2) virus infection was confirmed in two hospitalized children and resulted in uncomplicated influenza-like illness. Both patients recovered, and no additional cases were confirmed. The source is unknown. Several additional human H9N2 virus infections were reported from China in 1998-99. **H7N2, Virginia, 2002**: Following an outbreak of low pathogenic avian influenza A (H7N2) among poultry in the Shenandoah Valley poultry production area, one person developed uncomplicated influenza-like illness and had serologic evidence of infection with H7N2 virus. **H5N1, China and Hong Kong, Special Administrative Region, 2003**: Two cases of highly pathogenic avian influenza A (H5N1) virus infection occurred among members of a Hong Kong family that had traveled to China. One person recovered, the other died. How or where these two family members were infected was not determined. Another family member died of a

respiratory illness in China, but no testing was done. **H7N7, Netherlands, 2003**: The Netherlands reported outbreaks of highly pathogenic avian influenza A (H7N7) virus among poultry on multiple farms. Overall, 89 people were confirmed to have H7N7 virus infections associated with poultry outbreaks. Most human cases occurred among poultry workers. H7N7-associated illness was generally mild and included 78 cases of conjunctivitis (eye infections); five cases of conjunctivitis and influenza-like illness with fever, cough, and muscle aches; two cases of influenza-like illness; and four cases that were classified as "other." One death occurred in a veterinarian who visited one of the affected farms and developed complications from H7N7 infection, including acute respiratory distress syndrome. The majority of H7N7 cases occurred through direct contact with infected poultry. However, Dutch authorities reported three possible instances of human-to-human H7N7 virus transmission from poultry workers to family members. **H9N2, Hong Kong, Special Administrative Region, 2003**: Low pathogenic avian influenza A (H9N2) virus infection was confirmed in a child in Hong Kong. The child was hospitalized with influenza-like illness and recovered. **H7N2, New York, 2003**: In November 2003, a patient with serious underlying medical conditions was admitted to a hospital in New York with respiratory symptoms. The patient recovered and went home after a few weeks. Testing revealed that the patient had been infected with a low pathogenic avian influenza A (H7N2) virus. **H7N3, Canada, 2004**: In March 2004, two poultry workers who were assisting in culling operations during a large influenza A (H7N3) poultry outbreak had culture-confirmed conjunctivitis, one of whom also had coryza. Both poultry workers recovered. One worker was infected with low pathogenic H7N3 and the other with high pathogenic H7N3. **H5N1, Thailand and Vietnam, 2004**: In late 2003 and early 2004, severe and fatal human infections with highly pathogenic avian influenza A (H5N1) viruses were associated with widespread poultry outbreaks. Most cases had pneumonia and many had respiratory failure. Additional human H5N1 cases were reported during mid-2004, and late 2004. Most cases appeared to be associated with direct contact with sick or dead poultry. One instance of probable, limited, human-to-human spread of H5N1 virus is believed to have occurred in Thailand. Overall, 50 human H5N1 cases with 36 deaths were reported from three countries. **H5N1, Cambodia, China, Indonesia, Thailand and Vietnam, 2005**: Severe and fatal human infections with highly pathogenic avian influenza A (H5N1) viruses were associated with the ongoing H5N1 epizootic among poultry in the region. Overall, 98 human H5N1 cases with 43 deaths were reported from five countries. **H5N1, Azerbaijan, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Thailand, Turkey, 2006**: Severe and fatal human infections with highly pathogenic avian influenza A (H5N1) viruses occurred in association with the ongoing and expanding epizootic. While most of these cases occurred as a result of contact with infected poultry, in Azerbaijan, the most plausible cause of exposure to H5N1 in several instances of human infection is thought to be contact with infected dead wild birds (swans). The largest family cluster of H5N1 cases to date occurred in North Sumatra, Indonesia during May 2006, with seven confirmed H5N1 cases and one probable H5N1 case, including seven deaths. Overall, 115 human H5N1 cases with 79 deaths were reported in nine countries. **H5N1, Cambodia, China, Egypt, Indonesia, Laos, Nigeria, Vietnam, 2007**: Severe and fatal human infections with highly pathogenic avian influenza A (H5N1) viruses occurred in association with poultry outbreaks. In addition, during 2007, Nigeria (January) and Laos (February) confirmed their first human infections with H5N1. **H7N2, United Kingdom, 2007**: Human infection with low pathogenic avian influenza A (H7N2) virus resulting in influenza-like illness and conjunctivitis were identified in four hospitalized cases. The cases were associated with an H7N2 poultry outbreak in Wales. **H9N2, China, 2007**: In March 2007,Hong Kong, Special Administrative Region, confirmed low pathogenic avian influenza A (H9N2) virus infection in a 9-month-old girl with mild signs of disease.

Symptoms of Avian Influenza in Humans

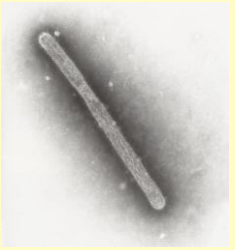
Symptoms: Although the exact incubation period for bird flu in humans isn't clear, illness seems to develop within one to five days of exposure to the virus.

Common signs and symptoms: Most often, signs and symptoms of bird flu resemble those of conventional influenza, including: Cough, Fever, Sore throat, Muscle aches. A relatively mild eye infection (conjunctivitis) is sometimes the only indication of the disease.

Severe signs and symptoms: People with bird flu also may develop life-threatening complications, particularly: Viral pneumonia. Acute respiratory distress the most common cause of bird flu-related death.

LABORATORY DIAGNOSIS

Specimen collection: Standard and appropriate barrier precautions should always be taken when collecting specimens from patients. Specimens should be obtained as soon as possible after the onset of symptoms. In general, a nasopharyngeal swab or aspirate is considered the preferred specimen for seasonal influenza testing; however, recent data suggest that oropharyngeal and lower respiratory tract specimens (i.e., sputa and bronchoalveolar lavage fluid) are superior to nasopharyngeal specimens for the detection of avian influenza A (H5N1) infection in humans. Specimens from multiple sites may yield the best results. In cases of atypical presentations, such as gastroenteritis and encephalopathy, stool and cerebrospinal fluid specimens, respectively, are advised.



H5N1 virus, electron microscopy - photomicrograph.

Specimen storage and transportation: Specimens should be collected in the appropriate viral transport medium and shipped immediately to the testing laboratory (on ice, if possible) in accordance with regulations of the Transportation of Dangerous Goods Act. Specimens may be refrigerated at 4°C (± 2°C) for up to 48-72 hours; after that, specimens should be frozen at -70°C. Swabs and transport media intended for bacteriologic testing are not suitable for influenza testing. In addition, swabs with calcium alginate or cotton tips and wooden shafts are not recommended. Swabs for influenza testing should have a dacron tip and an aluminum or plastic shaft.

STANDARD DIAGNOSTIC RECOMMENDATIONS

During epidemics, a presumptive diagnosis can be made on the basis of the clinical symptoms. However, influenza A and B can co-circulate, and mixed infections of influenza and other viruses have been reported. Isolated cases of suspected influenza should be investigated for these may represent the first cases of an impending epidemic. **Virus Isolation:** Throat swabs, NPA and nasal washings may be used for virus isolation. It is reported that nasal washings are the best specimens for virus isolation. The specimen may be inoculated in embryonated eggs or tissue culture. 10-12 day embryonated eggs are used for virus isolation. The specimen is inoculated into the amniotic cavity. The virus replicates in the cells of the amniotic membrane and large quantities are released back into the amniotic fluid. After 2-3 days incubation, virus in the amniotic fluid can be detected by adding aliquots of harvested amniotic fluid to chick, guinea pig, or human erythrocytes. Pathological specimens can be inoculated on to tissue cultures of kidney, chicks or a variety of other species. Rhesus monkey cells are the most sensitive. Although no CPE (Cytopathic effect) is produced, newly produced virus can be recognized by haemadsorption using the cells in the tissue culture, and haemagglutination using the culture medium which contains free virus particles. Influenza B virus and occasionally influenza A will produce a CPE in MDCK cells. Influenza viruses isolated from embryonated eggs or tissue culture can be identified by serological or molecular methods. Influenza viruses can be recognized as A, B, or C types by the use of complement fixation tests against the soluble antigen. (A soluble antigen is found for all influenza A, B or C type virus but antibody against one type does not cross react with the soluble antigen of the other. The further classification of influenza isolates into subtypes and strains is a highly specialized responsibility of the WHO reference laboratories. The HA type is identified by HAI tests, the NA type is also identified. **Rapid Diagnosis by Immunofluorescence and other immunologic techniques:** cells from pathological specimens may be examined for the presence of influenza A and B antigens by indirect immunofluorescence. Although many workers are convinced of the value of this technique, others have been disappointed with the specificity of the antisera and the level of background fluorescence that makes the test difficult to interpret. EIA tests for the detection of influenza A viral antigens are available that are easier to interpret than immunofluorescence. PCR assays for the detection of influenza RNA have also been developed but there usefulness in a clinical setting is highly questionable. The test uses technology known as Reverse Transcription--Polymerase Chain Reaction (RT-PCR) to amplify and detect genetic material from the influenza A/H5 from the Asian lineage, the same virus that has been associated with bird flu outbreaks in animals and humans in east Asia, Turkey, and Iraq. **Serology:** Virus cannot be isolated from all cases of suspected infection. More commonly, the diagnosis is made retrospectively by the demonstration of a rise in serum antibody to the infecting virus. CFT is the most common method used using the type specific soluble antigen. However, the CF

test is thought to have a low specificity. A more specific test is the HAI test. Infection by influenza viruses results in a rise in serum antibody titre, but the requirement for a 4-fold or greater rise in titre of HI of CF antibody reflects the inaccuracy of these tests for detecting smaller increases in antibody. A more precise method for measuring antibody is by SRH. SRH is more sensitive than CF or HAI tests and has a greater degree of precision. A 50% increase in zone area represents a rise in antibody and is evidence of recent infection. Sera do not have to be pretreated to remove non-specific inhibitors which plaque the HAI test. SRH may well replace CF and HAI tests in diagnostic laboratory in future.

WHO GUIDELINES

Specimen testing: Rapid antigen testing is not currently recommended for the detection of avian influenza A (H5N1): a negative result does not exclude avian influenza, and a positive result of an antigen test (including immunofluorescence methods) does not differentiate between seasonal and avian influenza A viruses. Confirmatory testing and subtyping must be performed by molecular methods (e.g., reverse transcriptase polymerase chain reaction), virus culture or both. Culture of this high-risk pathogen is restricted to certified containment level 3 facilities. All specimens that test positive for influenza A (H5N1) must be confirmed by the National Microbiology Laboratory or its designate. Given the increased likelihood of seasonal influenza infections, these guidelines for H5N1 testing in humans should be applied only to patients who have a history of travel, or contact with a traveller, to areas affected by outbreaks of avian influenza *and* a significant clinical and exposure history. The need exists for increased vigilance for the surveillance, recognition, reporting and prompt investigation of patients with severe influenza-like illness or severe respiratory illness.

Recommended laboratory tests to identify avian influenza A virus in specimens from humans (All kits/ devices are available from WHO offices/ outlets)

General information: Highly pathogenic avian influenza (HPAI) caused by certain subtypes of influenza A virus in animal populations, particularly chickens, poses a continuing global human public health risk. Direct human infection by an avian influenza A(H5N1) virus was first recognized during the 1997 outbreak in Hong Kong Special Administrative Region of China. Subsequently, human infections with avian strains of the H9 and H7 subtypes have been further documented. The current outbreak in humans of avian A (H5N1) and the apparent endemicity of this subtype in the poultry in southeast Asia require increased attention to the need for rapid diagnostic capacity for non-typical influenza infections. Laboratory identification of human influenza A virus infections is commonly carried out by direct antigen detection, isolation in cell culture, or detection of influenza-specific RNA by reverse transcriptasepolymerase chain reaction. These recommendations are intended for laboratories receiving requests to test specimens from patients with an influenza-like illness, in cases where there is clinical or epidemiological evidence of influenza A infection. The optimal specimen for influenza A virus detection is a nasopharyngeal aspirate obtained within 3 days of the onset of symptoms, although nasopharyngeal swabs and other specimens can also be used. All manipulation of specimens and diagnostic testing should be carried out following standard biosafety guidelines. The strategy for initial laboratory testing of each specimen should be to diagnose influenza A virus infection rapidly and exclude other common viral respiratory infections. Results should ideally be available within 24 hours.

Procedures for influenza diagnosis

Assays available for the diagnosis of influenza A virus infections include:

Rapid antigen detection: Results can be obtained in 15-30 minutes. *Near-patient tests for influenza*. These tests are commercially available **Immunofluorescence assay**. A widely used, sensitive method for diagnosis of influenza A and B virus infections and five other clinically important respiratory viruses *Enzyme immunoassay*. For influenza A nucleoprotein (NP). **Virus culture:** Provides results in 2-10 days. Both shell-vial and standard cell-culture methods may be used to detect clinically important respiratory viruses. Positive influenza cultures may or may not exhibit cytopathic effects but virus identification by immunofluorescence of cell cultures or haemagglutination-inhibition (HI) assay of cell culture medium (supernatant) is required. **Polymerase chain reaction and Real-time PCR assays:** Primer sets specific for the haemagglutinin (HA) gene of currently circulating influenza A/H1, A/H3 and B viruses are becoming more widely used. Results can be available within a few hours from either clinical swabs or infected cell cultures. Additionally several WHO Collaborating Centres are developing PCR and RT-PCR reagents for non-typical avian/human influenza strains. Any specimen with a positive result using the above approaches for influenza A virus and suspected of avian influenza infection should be further tested and verified by a designated WHO H5 Reference Laboratory ii. Laboratories that lack the capacity to perform specific influenza A subtype identification procedures are requested to: (1) Forward specimens or virus isolates to National Institute of Virology (NIV), National Institute of Immunology (NII) or to a WHO H5 Reference Laboratory for further identification or characterization. (2) Inform the WHO Office in the country or WHO Regional Office or WHO HQ Global Influenza Programme that specimens or virus isolates are being forwarded to other laboratories for further identification or further characterization.

Identification of avian influenza A subtypes

Immunofluorescence assay: Immunofluorescence assay (IFA) can be used for the detection of virus in either clinical specimens or cell cultures. Clinical specimens, obtained as soon as possible after the onset of symptoms, are preferable as the number of infected cells present decreases during the course of infection. Performing IFA on Inoculated cell cultures is preferable as it allows for the amplification of any virus present. **Virus culture:** Virus isolation is a sensitive technique with the advantage that virus is available both for identification and for further antigenic and genetic characterization, drug susceptibility testing, and vaccine preparation. MDCK cells are the preferred cell line for culturing influenza viruses. Identification of an unknown influenza virus can be carried out by IFA using specific monoclonal antibodies (see above) or, alternatively, by haemagglutination (HA) and antigenic analysis (subtyping) by haemagglutinationinhibition (HAI) using selected reference antisera. Unlike other influenza A strains, influenza A/H5 will also grow in other common cell lines such as Hep-2 and RD cells. Standard biosafety precautions should be taken when handling specimens and cell cultures suspected of containing highly pathogenic avian influenza A.

GOLDEN RULE. Clinical specimens from humans and from swine or birds should never be processed in the same laboratory.

Polymerase chain reaction: Polymerase chain reaction (PCR) is a powerful technique for the identification of influenza virus genomes. The influenza virus genome is single-stranded RNA, and a DNA copy (cDNA) must be synthesised first using a reverse transcriptase (RT) polymerase. The procedure for amplifying the RNA genome (RTPCR) requires a pair of oligonucleotide primers. These primer pairs are designed on the basis of the known HA sequence of influenza A subtypes and of N1 and will specifically amplify RNA of only one subtype. DNAs generated by using subtype-specific primers can be further analysed by molecular genetic techniques such as sequencing. **Laboratory confirmation:** All laboratory results for influenza A/H5, H7 or H9 during Interpandemic and Pandemic Alert periods of the WHO Global Influenza Preparedness Plan should be confirmed by a WHO H5 Reference Laboratory ii or by a WHO recommended laboratory. Influenza A/H5, H7 or H9 -positive materials, including human specimens, RNA extracts from human specimens, and influenza A/H5, H7 or H9 virus in cell-culture fluid or egg allantoic fluid, should be forwarded to a WHO H5 Reference Laboratory ii or a WHO recommended laboratory. Communication and publication of analysis results should be according to the WHO Guidance for the timely sharing of influenza viruses/specimens with potential to cause human influenza pandemics. **Serological identification of influenza A/H5 infection:** Serological tests available for the measurement of influenza A-specific antibody include the haemagglutination inhibition test, the enzyme immunoassay, and the virus neutralization tests. The microneutralization assay is the recommended test for the measurement of highly pathogenic avian influenza A specific antibody. Because this test requires the use of live virus, its use for the detection of highly pathogenic avian influenza A specific antibody is restricted to those laboratories with Biosafety Level 3 containment facilities. **Antiviral Agents for Avian Influenza A Virus Infections of Humans:** CDC and WHO recommend oseltamivir, a prescription antiviral medication, for treatment and chemoprophylaxis of human infection with avian influenza A viruses. Analyses of available H5N1 viruses circulating worldwide suggest that most viruses are susceptible to oseltamivir. However, some evidence of resistance to oseltamivir has been reported in H5N1 viruses isolated from some human H5N1 cases. Ongoing monitoring for antiviral resistance among avian influenza A viruses is critical. **Prevention of Avian Influenza A Virus Infections of Humans:** Persons exposed to avian influenza A-infected or potentially infected poultry are recommended to follow good infection control practices including careful attention to hand hygiene, and to use personal protective equipment. In addition, they should be vaccinated against seasonal influenza and should take influenza antiviral agents for prophylaxis. Exposed persons should be carefully monitored for symptoms that develop during and in the week after exposure to infected poultry or to potentially avian influenza-contaminated environments.

INTERPRETATION

URINARY CRYSTALS

Crystals precipitate in urine due to high concentrations of solutes. There are four factors that enter into crystal formation: [**1**] Solute concentration: factors include dehydration, dietary excesses, and medications. [**2**] pH: Solubility is pH dependent. Crystals that precipitate in neutral or alkaline urine are less soluble than the crystals that precipitate in acidic urine. As a rule, inorganic salts (calcium, phosphate, ammonium, and magnesium) precipitate in alkaline urine. Organic solutes (uric acid, cystine, bilirubin, and x-ray dye) tend to precipitate out in acidic urine. [**3**] The rate of flow through the tubules affect crystal formation. A slow rate of flow produces a concentrated urine and promotes crystal formation. A rapid flow rate produced a more dilute urine and decreased crystal formation. [**4**] Temperature: If warm, the solutes remain in solution better and crystallization is retarded or does not occur. If cold, then solutes become less soluble and crystallization occurs readily. Normal, healthy urine seldom contains crystals. The presence of crystals in urine is called *crystalluria.* If crystals are present in the urine at the time of voiding, it is possible that this may be clinically significant. Most crystals observed in urine precipitate out after sitting (especially if the urine is refrigerated before testing), because the solute concentration is high, a super saturated solution, and the solubility threshold is exceeded as the urine cools.

AMORPHOUS SEDIMENT IN URINE AND ITS CLINICAL SIGNIFICANCE.

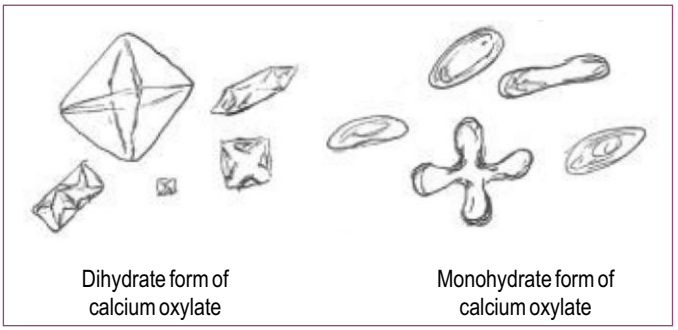
There are three types of amorphous sediments. Each has the following details: [1] precipitated salts, [2] not clinically significant, [3] are coarse granular in appearance. If the amount of amorphous sediment is abundant, it can make the microscopic evaluation difficult, hence they become a nuisance and hindrance. The term "amorphous" literally means "*without any form*". These crystals are shapeless and formless, resembling sawdust or sand. Amorphous sediment is the most commonly encountered types of crystals in urine. If urine is tested within the first hour after collecting (without refrigeration), amorphous formation is minimized. In neutral to alkaline urine, there are two types of amorphous crystals. The most common type is "amorphous phosphates". As you observe urine specimens in lab, a urine with a moderate amount (or larger) of amorphous phosphates present, the macroscopic, cloudy appearance will be white. The are soluble in dilute acids and will not dissolve when heated to 60 $^{\circ}$C. These are made up of magnesium and calcium phosphates. The other type of formless crystal is amorphous carbonate. This type is found infrequently in urine and tends to be composed of rod-shaped calcium carbonate. Because of their small shape, they have been confused with bacteria. If large calcium carbonate crystals are present, they will take on a dumbbell-like shape. If a dilute acid is added, effervescence will be observed as CO_2 is given off. Both of these crystals appear colourless when viewed with the microscope. In neutral to acid urine, amorphous urates are observed. Macroscopically, these crystals will appear as a pink, brick-like dust. Because of their chemical nature, they readily absorb the urinary pigments. It is uroerythrin that imparts the reddish colour. In the microscope, they will appear colourless or sometimes a brownish colouration. These crystals are uric acid salts of sodium, potassium, magnesium, or calcium. If these crystals are warmed to body temperature or to 60 $^{\circ}$C, they will dissolve. They will also dissolve in dilute alkali. If you add a strong mineral acid like HCl or glacial acetic acid to the sediment, allow the mixture to "sit" for a period of time. uric acid will crystallize out.

NORMAL URINARY CRYSTALS AND THE pH AT WHICH THEY ARE FOUND.

Amorphous urates (pH: acid to neutral), Calcium oxalate (pH: acid to neutral, sometimes can be observed in a slightly alkaline pH), Uric acid (pH: acid to neutral, sometimes can be observed in a slightly alkaline pH, Monosodium urate (pH: acid to neutral), Calcium oxalate, both di- and monohydrate forms, (pH: acid to neutral, sometimes can be observed in slightly alkaline pH), Amorphous phosphates (pH; neutral to alkaline), Triple phosphate (pH: neutral to alkaline), Dicalcium phosphate (pH: neutral to alkaline), Calcium phosphate (pH; neutral to alkaline), Calcium carbonate (pH: neutral to alkaline), Ammonium biurate (neutral to alkaline), Calcium sulfate (pH; acidic).

CALCIUM OXALATE CRYSTALS AND THEIR SIGNIFICANCE IN URINE.

Also called envelope crystals. They are colourless and do not absorb pigments from the urine. The most common shape is the octahedral form which appears as two pyramids joined at their bases. When focusing on this crystal, there is the appearance of a refractile cross or star in the center of a cube. If there is an increased number of these crystals, then it is called "oxaluria". The persistent finding of numerous calcium oxalate crystals could be an indicator of small bowel disease, urinary calculi, renal failure disease, diabetes mellitus, high milk intake, bone fractures, CNS injuries, ethylene glycol poisoning, or acetazolamide therapy. These crystals are formed from the calcium salts of oxalic acid and other oxalates. Foods high in oxalic acid and oxalates are: oranges, cabbage, rhubarb, asparagus, brussel sprouts, tomatoes, spinach, broccoli, and berries. There are two basic types of calcium oxalate crystals; dihydrate and monohydrate forms. The dihydrate form tends to form squares and rectangles, whereas the monohydrate form tends to form oval and dumbbell shapes. Biconcave disk forms have been reported. Caution: Monohydrate may form long ovals and closely resemble acetaminophen crystals. Because there is a variety of forms, these crystals may be described as being pleomorphic. Regardless of the type, either form is generally considered to be clinically insignificant. Calcium oxalate crystals are soluble in dilute hydrochloric acid but not dilute acetic acid.

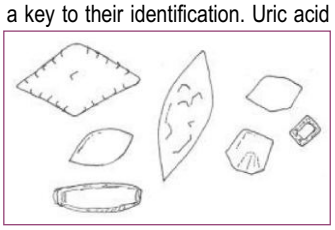


Dihydrate form of calcium oxylate

Monohydrate form of calcium oxylate

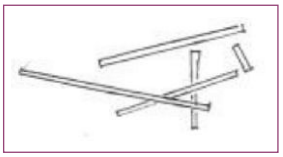
URIC ACID CRYSTALS AND THEIR SIGNIFICANCE.

This is the most pleomorphic of the crystals found in urine. Forms/patterns include rhombic (diamond), cubes, rosettes (when multiple crystals cluster and fuse), needles, wedge, dumbbells, hexagons, and irregular plates/shapes. Uric acid crystals, when first formed are colourless, but because of their chemical properties, will adsorb pigments from the urine. Uric acid crystals will appear in varying shades of yellow or yellow-brown, dependent upon the amount of pigment in the urine. This colouration is a key to their identification. Uric acid crystals are quite variable in size. These crystals are soluble in alkali but are insoluble in acids or alcohol. Generally, the presence of uric acid crystals are considered to be clinically insignificant. In any condition (examples: gout, leukemia, lymphoma) in which there is an increase in the cellular turnover rate, uric acid crystals will be increased. If a patient is on cytotoxin therapy, there will be an increase in cell destruction. This means that purine metabolism will be increased and uric acid crystal formation will occur.



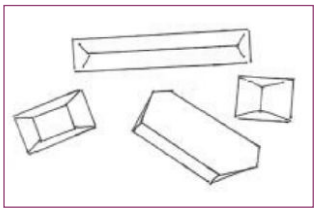
SODIUM URATE CRYSTALS AND THEIR CLINICAL SIGNIFICANCE.

Sodium urates are a variant of uric acid crystals. These are colourless (most often observed) to slightly yellow rods or slender prisms. Some laboratorians call these uric acid spears. They are found singly or in clusters. They will dissolved at 60 $^{\circ}$C. They are clinically insignificant and may be reported as "urate crystals". In the presence of hydrochloric acid, they will change to the uric acid form.



TRIPLE PHOSPHATE CRYSTALS AND THEIR CLINICAL SIGNIFICANCE.

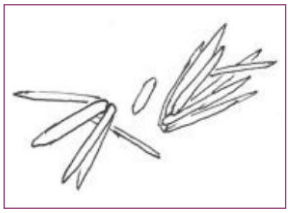
Also called ammonium, magnesium phosphate crystals, "coffin lids", and "hip-roof" crystals, they are colourless and exhibit much variation in size. They often



appear as three- or six-sided prisms, colourless, and very refractile. Other pattern variations are "feathery star-shapes" and fern-like forms, but these forms are uncommon. There presence is generally non-significant, however they are observed with chronic UTI, obstructive uropathy, and urinary calculi. These crystals are characterized by imperfections (not perfectly formed). There are very variable in size and are soluble in 10% acetic acid.

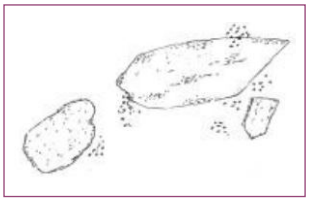
DICALCIUM PHOSPHATE CRYSTALS AND THEIR SIGNIFICANCE.

This is a uncommon variation of calcium phosphate and may be found in slightly acidic urine. The correct designation is dicalcium hydrogen phosphate. They tend to be long slender prisms, with one end pointed. They are often found in clusters and for this reason may be called "stellar phosphates". They are colourless, soluble in dilute acetic acid, and are clinically insignificant.

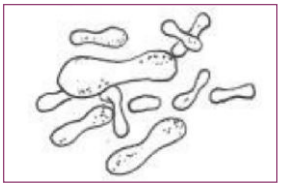


CALCIUM PHOSPHATE CRYSTALS AND THEIR SIGNIFICANCE.

These crystals are usually observed as large, colourless, irregular, thin plates that are granular in appearance. They float on top of the urine and resemble a type of "scum". They have been reported as wedge shaped prisms. These are soluble in 10% acetic acid. CAUTION: Small plates may resemble a degenerate squamous epithelial cell.



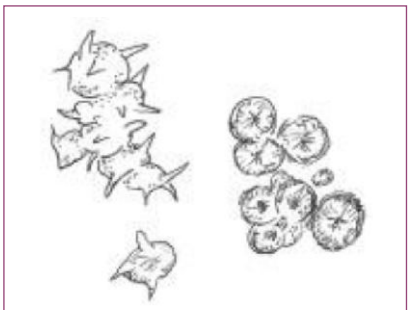
CALCIUM CARBONATE CRYSTALS AND THEIR SIGNIFICANCE.



These crystals appear most often in an amorphous form. On occasions, they will appear in crystalline form and then they are dumbbell in shape. It has been suggested that this shape is due to clumping and fusing of the amorphous crystals. They are soluble in dilute acetic acid and will effervesce.

AMMONIUM BIURATE CRYSTALS AND THEIR CLINICAL SIGNIFICANCE.

Synonyms are: thorn apple crystal and starfish crystal. Their shape is peculiar for it fused spheres, tortuous shape, and the presence or absence of spiny projections. They are yellow brown in colour, rarely occurring in fresh urine. If alkaline urine is allowed to stand, then these crystals may precipitate out. They are not clinically significant and will dissolve at 60 ^{0}C, in acetic acid, or sodium hydroxide. They have been confused with yeast cells and leucine crystals. If concentrated HCl is added, these crystals can reform to uric acid crystals.



[with and without spicules (thorns)]

CALCIUM SULPHATE CRYSTALS AND THEIR CLINICAL SIGNIFICANCE.

This is a very rare crystal that occurs as long, thin needles and prisms. It is found only in acidic urine.

To be continued...

BOUQUET

In Lighter Vein

A woman wanted a pet so she went to the local pet shop. She looked at the dogs and the cats but finally settled on a parrot that was perched in the back of the store for $50.00.

She asked the shopkeeper why the parrot was so cheap, to which he replied, "Well, I have to tell you, the birds last owner was a madam at a whorehouse and he occasionally makes off colour remarks that may offend some people." Thinking that the price was right and she could handle anything he might say, she took him. When she got home she set the bird down on the table. He looked around and said, "New house, new madam".

"That's not so bad," she thought.

A little while later, her daughters got home from school, and the parrot spoke again, "New house, new madam, new whores."

Even though she felt a little insulted, she thought that wasn't so bad either.

Later that evening, her husband Ray came home.

The parrot again spoke out...

This time it said, "Hi Ray!"

The woman met with a divorce attorney the next day.

Two women that are dog owners are arguing about which dog is smarter....
First Woman : "My dog is so smart, every morning he waits for the paper boy to come around and then he takes the newspaper and brings it to me.

Second Woman : "I know..."

First Woman : "How?"

Second Woman : "My dog told me."

Wisdom Whispers

- "The road to happiness lies in two simple principles: find what interests you and that you can do well, and put your whole soul into it - ever bit of energy and ambition and natural ability you have."
- "To me, fair friend, you never can be old For as you were when first your eye I eyed, Such seems your beauty still."
- To put off repentance is dangerous."
- "The more the universe seems comprehensible, the more it also seems pointless."
- "To beg of the miser is to dig a trench in the sea."
- "Nothing falls into the mouth of a sleeping fox."
- Cut your coat to suit your cloth."
- "The dead open the eyes of the living."
- He keeps watch over a good castle who has guarded his own constitution."

Brain Teasers

WITH EACH HEADING (KEYWORD) ARE GIVEN FOUR SUPPLEMENTARY STATEMENTS, IDENTIFY THE INCRRECT ONE/ STATEMENT THAT IS LEAST OR NOT RELATED TO THE HEADING.

1. Local signs of acute inflammation
   A. Pallor B. Dolor C. Tumor D. Calor
2. Lymphocytosis
   A. Visceral larva migrans B. Whoopping cough
   C. German measles D. Infectious monocytosis
3. Indications for doingg a liver biopsy
   A. Granulomatous hepatitis B. Hydatid cyst of liver
   C. Post-necrotic cirrhosis D. Hepatocellular carcinoma
4. Low ESR
   A. Sickle cell anaemia B. Multiple myeloma
   C. Severe dehydration D. Polycythaemia vera
5. Mixed tumours
   A. Teratoma B. Haemangioendothelioma
   C. Fibroadenoma D. Pleomorphic adenoma
6. Special forms of macrophages
   A. Siderophges B. Lipophages C. Epithelioid cell D. Neutrophil

Answers. 1. A., 2. A., 3. B., 4. C., 5. B., 6. D.

TROUBLESHOOTING

(Letter to the Editor from Turkey)

Laboratory-acquired Brucellosis

Dear Editor,

Brucellosis is a serious disease seen worldwide and has been historically known as undulant fever, Bang's disease, Gibraltar fever, Mediterranean fever, and Malta fever. Brucellosis has a limited geographic distribution but remains a major problem in Mediterranean and Middle Eastern countries.

In 2001, a total of 15,510 brucellosis cases were reported in Turkey.

In our centre there are approximately 20 to 25 new cases each year. Brucella is most commonly transmitted by the consumption of contaminated raw or unpasteurised milk and cheese. Laboratory-acquired brucellosis has also been documented and is considered the most important laboratory-acquired bacterial infection. All *Brucella* spp. have been implicated in laboratory-associated infections, and they may account for up to 2% of all laboratory-associated infections.

In this paper, we report 3 laboratory-acquired brucellosis.

Case 1 was a 26-year-old female laboratory worker, who works as microbiology technologist. She presented with joint pain and fever of 1 week's duration. There was no history of trauma. The patient had given birth 2 weeks prior. Physical examination and haematological parameters were normal. However, the levels of aminotranferases were elevated [aspartate transferase (AST), 45 U/L; alanine transferase (ALT), 55 U/L].

The past history of the patient did not reveal any raw cheese consumption. She had been working on the determination of subgroups of *Brucella* positive cultures. She was tested for brucella using the Rose Bengal microagglutination test (Tulip Diagnostics Ltd. Goa, India) as well as serological titre of anti-*Brucella abortus* antibodies were evaluated by using a standard tube agglutination test (Seromed Laboratory Products, Turkey).

The Rose Bengal microagglutination test was positive. The *Brucella* serum agglutination test was reactive (1/640). Two sets of blood samples were obtained for culture. The blood cultures showed bacterial growth (Bactec 9050, Becton Dickinson, USA) following 72 hours of incubation. Bacteria were isolated in 5% sheep blood agar. Grams' stain revealed small gram-negative coccobacilli. The organism was confirmed to be *B. melitensis* by standard biochemical reactions (production of urease, catalase - positive, oxidase-positive, H_2S and indole negative, the dyes basic fuchsin, thionine, thionine blue are positive).

In addition, biochemical identification using an API 20 NE (BioMerieux, France) was done. Treatment was initiated with a combination of 2 g of ceftriaxone plus 600 mg of rifampin every day for 6 weeks. The patient had a full recovery without any coexisting problem.

Case 2 was a 28-year-old female laboratory worker who presented with non-specific symptoms of malaise, vomiting and fever. She had been working in the same microbiology laboratory as Case 1 and Case 3. She had worked with the same *Brucella* samples as the patient in Case 1 two weeks prior. She did not have any risk factors for brucellosis exposure such as ingestion of unpasteurised milk products.

The results of her physical examination and haematological and biochemical studies were normal. Brucella serology was positive at 1/160. The blood culture revealed *B.melitensis* growth after 3 days. She received doxycycline at a dosage of 100 mg po twice daily and rifampin at a dosage of 600 mg po qd for 6 weeks. She recovered completely.

Case 3 was a 24-year-old female microbiology technologist who was suffering from fever and pain of the lower extremities. Her physical examination and routine laboratory tests were normal. Brucella serology was positive at 1/640. *B.melitensis* was isolated from the blood culture. She received the same treatment as the second case and recovered fully. Like the preceeding 2 patients, she denied other exposure to brucellosis.

Brucellosis has been considered the most important laboratory-acquired bacterial infection. Aerosol transmission generated accidentally or during microbiologic techniques from contaminated materials are the proposed routes of transmission. Our present report includes 3 patients with an exposure history of working *Brucella* bacteria in a microbiology laboratory. No accident occurred in the laboratory during the time they were exposed. All 3 cases were found to be working on the specimen of the index case patient. The index case was a 45-year-old man who had a *Brucella* serology of 1/640 and blood culture that grew *B.melitensis*. Only 3 of the microbiology staff work on the specimen.

The other personnel in the laboratory who did not work with the specimen were also examined with Rose- Bengal test, brucella antibody and brucella-related symptoms. The results were negative for brucellosis. Brucellosis is an endemic disease in our country.

The 3 patients presented did not have any suspicious history of unpasteurised milk consumption or animal contact. The absence of this kind of contamination led us to believe that the transmission to our patients was through the laboratory route. The patients were thought to have been infected during subculturing for collecting bacteria.

These laboratory workers were unaware of the hazards of aerosol transmission of *Brucella* spp. They handled the biosafety cabinet, and used gloves and masks. Sniffing culture plates is another risk factor, and is a common practice in bacteriology laboratories in Turkey, as in other countries. *Brucella* spp. are highly infectious because the infectious dose by an aerosol is only 10 to 100 organisms. Laboratory-acquired brucella infections are very important in developing countries and in countries with endemic disease, such as Turkey.

In our country, biosafety cabinets do not exist in most hospital laboratories. Therefore, using gloves, masks and goggles and also continuous education on biosafety where brucellosis is endemic is very important. The transmission in our cases was probably due to aerosol contamination because of the current practice of sniffing culture plates. Additionally, catalase test used for bacteria identification might have produced aerosol.

In 1986, the World Health Organization recommended the use of doxycycline in combination with rifampin for 6 weeks as the preferred treatment for adult acute brucellosis.

The second and third cases received doxycycline and rifampin for 6 weeks. However, the first case received rifampin + ceftriaxone for 6 weeks. It is known that tetracyclines accumulate in fetal bones and teeth, and furthermore, pass into breast milk.1 For this reason, the combination of rifampin + ceftriaxone was preferred instead of the combination of rifampin + doxycycline for Case 1, who had given birth 2 weeks prior and was breastfeeding. Laboratories should be aware of laboratory-associated hazards and take adequate safety precautions even after the use of biosafety cabinet, gloves and masks.

In addition, all laboratory workers should be educated periodically for occupational risks in the laboratory. In our hospital, all microbiology laboratory staff were educated concerning laboratory-acquired bacterial infections. The laboratory staff are discouraged from smelling the culture plaques. In order to prevent future infections, close collaboration between clinicians and laboratory staff was initiated.

Biosafety level 3 has to be advocated and used whenworking with micro-organisms such as *Brucella* spp. Clinicians should alert laboratory personnel if they suspect Brucellosis in patients so they can take extra precautions.

TULIP NEWS

Tulip sets its foot into ELISA automation

Tulip's Instrumentation Division was introduced in the year 2000 Since then, a range of instruments (both semiautomatic and fully automated) for various IVD testing have been successfully placed in the Indian market. Each of these instruments were introduced selectively into the market based on market requirements and their suitability to Indian work conditions. Apart from these, instruments launched by Tulip Group are backed by an excellent after sales service support system.

Now Tulip partners with Dynex Technologies Inc., in introducing the most appropriate instrument for Indian IVD market

DYNEX DS2, Fully Automated 2-Plate ELISA Processor

Step Up to Automation… With DS2 modules.

DYNEX Technologies, Inc. has a long and distinguished track record of bringing break through products to market and the company has been a leading innovator specifically in microplate analysis. DYNEX introduced the first manual microplate ELISA reader and molded immunoassay microplates. In 1990s, DYNEX entered the market with the first contiguous ELISA processing system consisting of reader, incubator, multi-reagent dispenser, and washer.

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