CEPHEID
ON-DEMAND REPORT

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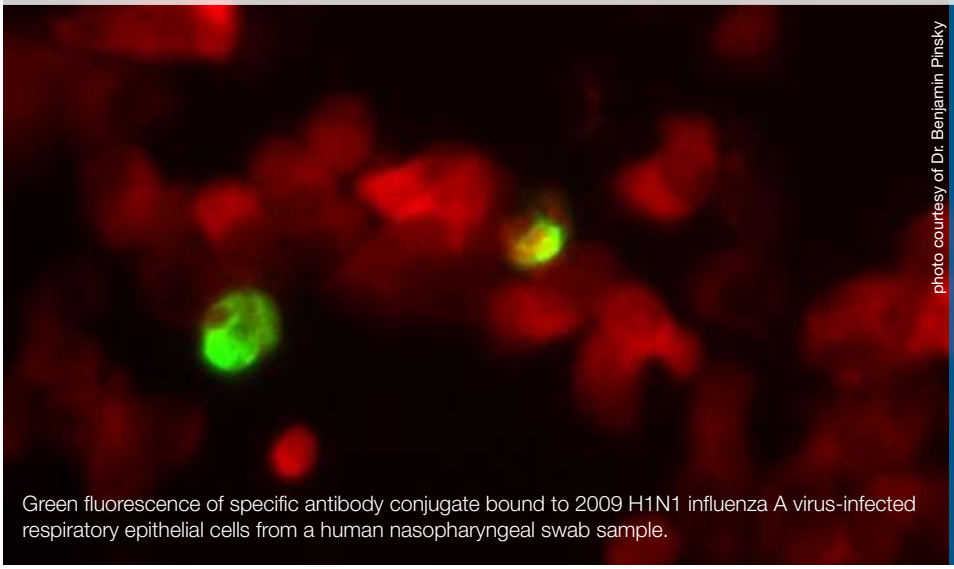

photo courtesy of Dr. Benjamin Pinsky

Green fluorescence of specific antibody conjugate bound to 2009 H1N1 influenza A virus-infected respiratory epithelial cells from a human nasopharyngeal swab sample.

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Influenza Hijacks Laboratory Efforts Worldwide

The next great wave of influenza developed in our own backyard

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Since late April, 2008, laboratories worldwide have experienced unprecedented volumes of respiratory samples. While most western scientists were waiting for the impending new pandemic of influenza to spread to the world from Asia and considering Avian influenza and related strains to be the top contenders, it appears that the next great wave of worldwide influenza developed in our own backyard. A rapid increase in influenza cases reported near Mexico City beginning in March, 2008, was quickly followed by unusual influenza activity in the U.S. California noted cases starting in late March and confirmed in April to be the same H1N1 strain as that seen in the Mexican outbreak[1]. As can be seen from the graph (See Figure 1) based on data reported by the U.S. World Health Organization (WHO) and National Respiratory and Enteric Virus Surveillance System (NREVSS), almost all of the following influenza isolates identified were a previously unknown strain, now designated 2009 influenza A (H1N1), also known as "novel H1N1" because it differed in several genetic ways from the already circulating influenza A (H1N1).

The virus seems to have been generated by a unique combination of six genes from influenza A strains from both human and avian origins that had managed to meet and recombine their RNA genetic elements in swine (hence the early designation of "swine flu," an unfortunate choice of names that suggested that pigs were a potential source of the infection and sent pork demand tumbling unnecessarily), and from there move into humans. In fact,

*See **LABORATORIES** on page 2*

CEPHEID
ON-DEMAND
REPORT

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Laboratories Worldwide Have Experienced Unprecedented Volumes

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some reports have suggested that the basis for this apparent "genetic promiscuity" may have originated in U.S.-based industrial pig farms in the late 1990's[1]. The genes included existing H1N1 matrix, neuraminidase, and hemagglutinin gene segments from Eurasian swine, neuraminidase and hemagglutinin genes from H3N1 and H3N2 segments circulating in birds, nucleoprotein and viral matrix genes from classic swine influenza H3N2, polymerase protein genes from avian and swine strains H1N1 and H3N3, and nonstructural protein genes from H1N1 (See Figure 2)[2].

This virus is unusual in a number of aspects. Its appearance in laboratories has continued rather steadily since this spring, whereas in previous years the "flu season" usually ended around May. In fact, in the second week of September of this year, 99% of all viruses typed by the reporting laboratories were 2009 influenza A (H1N1) (http://www.cdc.gov/flu/weekly/). Surprisingly, the disease in most patients seems to be less severe than disease caused by the previously circulating influenza strains [Influenza A (H1N1) and (H3N2)], although certainly there are sporadic reports of severe cases and even deaths. However, the virus appears to be unusually contagious, spreading rapidly among family members or close contacts. Even during the summer months, when most influenza virus activity in the temperate U.S. almost disappears, this virus has continued to proliferate and cause disease (See Figure 1). Because of the rapidly changing influenza pandemic situation, many sources for this information were only accessible on-line, and not yet available in formal publications.

Since the beginning of the epidemic in March, the World Health Organization has reported that >60,000 specimens have tested positive for influenza, as reported on FluNet, the international web-based database (http://gamapserver.who.int/GlobalAtlas/home.asp). Of these, almost 60% were determined to be the novel strain, although that number is an underestimate as not all strains were typed

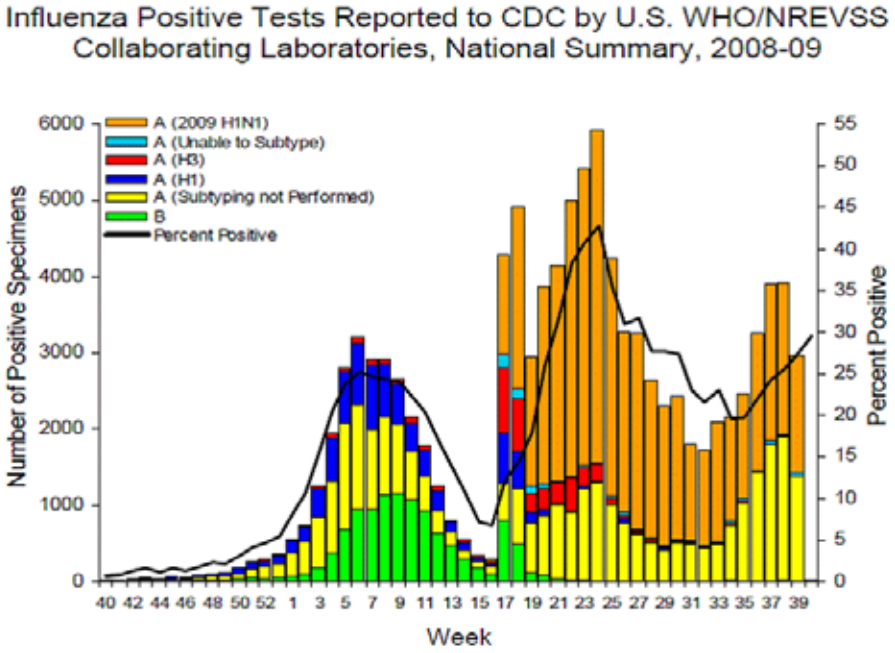

Figure 1: Number of specimens positive for various influenza strains from October, 2008 until mid-August, 2009."
http://www.cdc.gov/flu/weekly/pdf/External_F0939.pdf

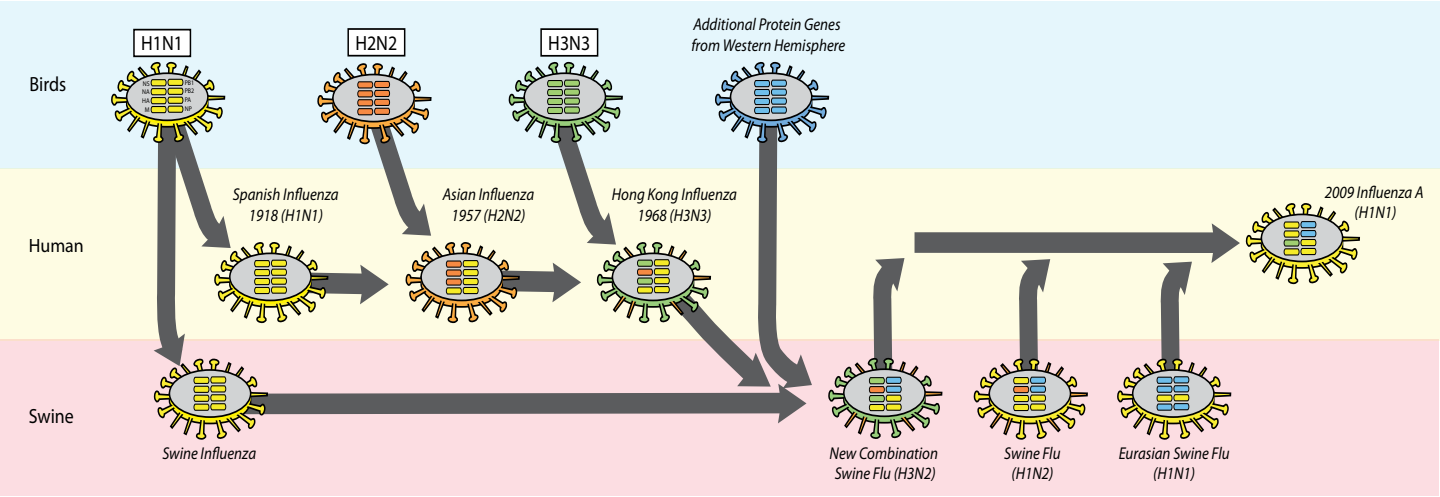

Figure 2: H1N1's Genetic Family Tree — The genes included existing H1N1 matrix, neuraminidase, and hemagglutinin gene segments from Eurasian swine, neuraminidase and hemagglutinin genes from H3N1 and H3N2 segments circulating in birds, nucleoprotein and viral matrix genes from classic swine influenza H3N2, polymerase protein genes from avian and swine strains H1N1 and H3N3, and nonstructural protein genes from H1N1.

(WHO Weekly Epidemiological Record, September 4, 2009; http://www.who.int/wer/). Of the >8,000 samples received for influenza testing by participating laboratories, 76% were positive and more than half of those harbored the new 2009 influenza A strain. In the world at large, parts of Asia and Africa still showed a large percentage of cases were subtypes other than 2009 influenza A (H1N1) but the Western Hemisphere and Europe had much less diversity (http://www.cdc.gov/h1n1flu/updates/international/map.htm).

Patients who are experiencing influenza-like-illness (ILI), and often those with simple upper respiratory symptoms not likely to represent influenza, have presented themselves for testing in doctors' offices, emergency departments, and outpatient clinics in record numbers. The constellation of symptoms associated with influenza disease includes fever, headache, fatigue, cough, sore throat, runny or stuffy nose, body aches, and gastrointestinal symptoms such as diarrhea (more common in children). These symptoms are not different from seasonal influenza, but the patient groups at highest risk for novel H1N1 are slightly different this year. Older adults (>65) are not at higher risk as they are for seasonal influenza, but when they do become ill, their disease can be serious. The groups at higher risk this flu season include young children < 5 years, pregnant women, particularly those carrying multiple fetuses, diabetics, obese people, and those with asthma or other respiratory problems[5]. Laboratories that usually stopped testing around May have been forced to continue to test for influenza all summer, causing havoc with summer vacation plans and laboratory workload.

Why is it important to know if a patient's influenza-like-illness is caused by the previously circulating influenza A strains or the 2009 novel influenza A H1N1 strain? There are two good reasons: (1) antiviral treatment recommendations are based on the infection strain and (2) patients in the higher risk groups infected with novel 2009 H1N1 should be managed more aggressively. The novel 2009 H1N1, now the dominant strain in the U.S., is resistant to amantidine and rimantidine, but still primarily susceptible to oseltamivir, although there are now several reports documenting the isolation of resistant strains (http://www.who.int/csr/disease/swineflu/notes/h1n1_antiviral_use_20090925/en/index.html). It has been suggested that widespread use of oseltamivir prophylaxis in the absence of disease is more likely to result in development of antiviral resistance (http://www.cdc.gov/h1n1flu/recommendations.htm). In contrast, the previously circulating seasonal influenza A (H1N1) strains were resistant to oseltamivir but remained susceptible to an older oral drug class, the adamantanes. Both versions of influenza A are susceptible to zanamivir (Relenza), but because zanamivir must be administered as an aerosol, it may not be appropriate for some severely ill patients. For current treatment and prophylaxis guidelines, which are changing rapidly, consult the CDC website at http://www.cdc.gov/h1n1flu/recommendations.htm.

Despite the need for rapid information based on diagnostic test results, there are currently no diagnostic tests that are both reliable and rapid. Types of assays available include rapid antigen tests appropriate for point-of-care testing, both membrane enzyme immunoassays (MEIAs) and immunochromatographic tests (ICTs), the most commonly used tests worldwide. Although performance, especially sensitivity, in the past was not ideal, the rapid availability of positive results was thought to be helpful for clinical decisions. A recent paper evaluating 20 rapid antigen tests with culture-obtained viruses (unfortunately the novel influenza A H1N1 strain was not tested) showed widely variable sensitivity among the tests, with the most sensitive test requiring

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Neonatal Early Onset Group B Streptococci Disease May Have Met Its Match

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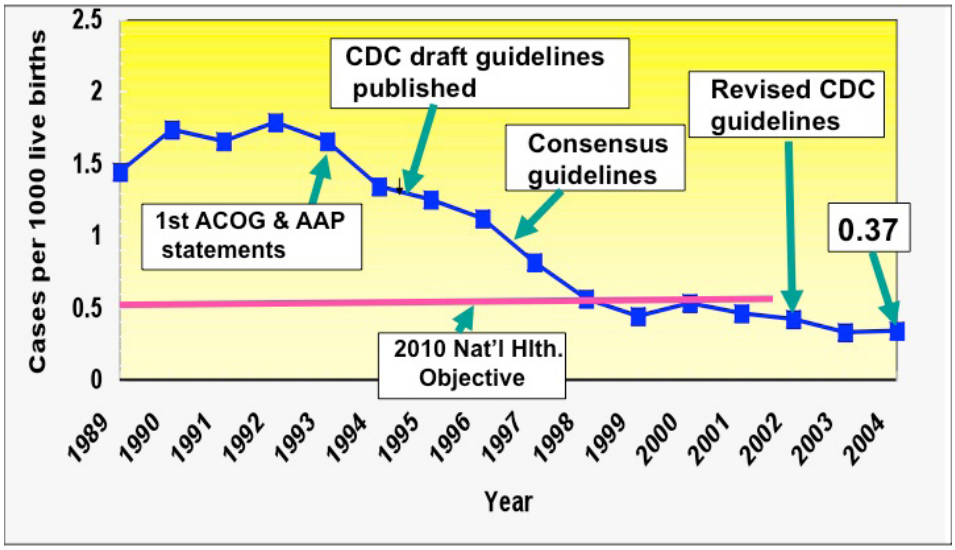

Figure 3: Rates of early-onset GBS disease in the U.S.

Something surprising has happened with the epidemiology of early-onset group B streptococcal (GBS) disease in the last few years. While initial efforts to reduce the incidence of GBS disease in infants were very successful, disease rates have subsequently leveled off. What happened? Since the revised Centers for Disease Control and Prevention (CDC) recommendations for antenatal culture-based surveillance to detect colonization of pregnant women were issued in 2002[23], the overall rates of early onset disease (EOD), defined as GBS occurring in babies < 7 days old, dropped steadily until around 2003, when the rates leveled off (See Figure 3)[3]. True enough, the national rates had already fallen below the 2010 National Health Objective, but even a few cases of early-onset GBS disease is too many for a preventable disease[1]. A disturbing aspect of the lack of additional progress since 2003 may relate to a subset of American women who seem to account for a disproportionately large proportion of EOD (See Figure 4)[2]. A close look at the statistics for EOD indicates that African-American babies appear to be experiencing a rising incidence of EOD, increasing 70% from 2003 to 2005[2].

Overall, 10-30% of pregnant women are colonized with group B streptococci[11]. The organism resides in the gastrointestinal tract, reaching the vagina by traversing the moist skin of the perineum, where it can be detected by culture of vaginal or vaginal/rectal swabs. Maternal colonization with GBS carries with it a risk of stillbirth, miscarriage, and prematurity[24]. When the newborn passes through the birth canal during vaginal delivery, the baby can become colonized with GBS on its mucous membranes[16,22]. Invasive strains of the organism can easily breach the weak protective barrier of the newborn to reach the bloodstream, traveling from there to the cerebrospinal fluid, causing septicemia and meningitis. Maternal colonization can also be detected by the presence of GBS in the urine, again presumably from the gastrointestinal source[27]. The recommendations that appeared in the Morbidity and Mortality Weekly Report (MMWR) state that all women with GBS bacteriuria at any time during pregnancy are also in a high risk group for transmitting GBS disease to their infant[23]. This recommendation regarding a urine screening component to detect GBS colonization poses a problem for laboratories, as they struggle to determine how many suspicious colonies in a urine culture full of multiple morphologies of gram-positive cocci and other skin flora must be further worked up so as not to miss any GBS. The problem is compounded by the fact that laboratories do not know which of the many urine samples that arrive in the laboratory for analysis are from pregnant women. Thus, a vast amount of unnecessary work may be performed. On the other hand, if a laboratory reports out "mixed gram-positive organisms" on a urine culture from a woman who later gives birth to a baby that develops EOD, that laboratory may be named in a lawsuit.

Approximately half of the babies born to colonized women will become colonized on their skin or mucous membranes. Fortunately, only 2% of those babies develop overt disease, but of those that do, 4-23% die from sepsis, pneumonia, or meningitis, and an additional 13-29% suffer from lifelong sequelae, including bone and joint involvement, developmental delay, blindness, deafness, and neurological impairment[1]. The disease is prevented in infants by administering an active antimicrobial agent to the mother during the intrapartum period[9]. Mothers in labor are treated with intravenous

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ampicillin, penicillin, or, in the case of penicillin allergy, clindamycin or erythromycin[23]. An astonishing >30% of women in the United States receive intrapartum prophylactic antibiotics during labor and delivery[26]. The antimicrobial agents are not without their own risks, such as anaphylaxis in unsuspected allergic woman, increasing antimicrobial resistance in the organisms causing infections in the babies and mothers, and the general risks of receiving intravenous antibiotics during labor[19], including the risk of acquiring *Clostridium difficile* infection.

Current recommendations from the American College of Obstetrics and Gynecology and the American Academy of Pediatrics and the 2002 MMWR include performing a culture to detect GBS at 35–37 weeks of gestation. Both vaginal (not cervical) swab and anal swab samples must be collected to maximize the sensitivity of the test. In fact, during the third trimester, as many as 18% of women carry GBS only in the gastrointestinal tract and their status will be reported falsely as negative if only a vaginal swab sample is tested[5,11]. The culture procedure is described clearly in the MMWR article, and it has been a common source of malpractice litigation over the years when a baby is born to a woman whose cultures were mishandled by a physician or a laboratory that did not follow the guidelines. Cultures must include overnight broth enrichment in Lim broth or other specified selective broth, usually containing antimicrobial agents such as nalidixic acid to inhibit growth of enteric organisms. The broth is subcultured onto blood agar and the plates are incubated overnight before the colonies are identified. Overall, the approach works well, but it has shortcomings. First, non-beta hemolytic group B streptococci may not be detected. Second, results may not be available to the patient's caregivers at the time of delivery, since some women deliver their babies before 35–37 weeks and have not been screened. Third, culture may fail to detect women with low numbers of GBS. Finally, the colonization status of women can change from the time of the screening culture to the time of delivery (See Figure 4). The "holy grail" of prevention of GBS that would prevent both GBS disease and the overuse of unnecessary antimicrobial agents would be a sensitive and specific rapid test that yielded accurate and dependable results for clinical decision-making at the time the woman presents in labor[26]. Direct antigen detection on vaginal swabs was used widely for a period of time, but when the results of such tests were examined critically in comparison with enriched culture methods, the sensitivity fell far short of that necessary for proper clinical decision making[4]. In fact, the 2002 MMWR document stated that rapid tests at time of delivery were not reliable and should not be used[23].

Longitudinal Study of Group B Streptococcus Carriage in Pregnancy (n=735; low prev ~13%)
Goodman JR et al. Infect Dis Obstet Gynecol 5:237-243 (1997)

1st trimester / 26-28 wks / 37 wks / delivery				#	%
-	-	-	-	564	77
+	+	+	+	28	4
+/-	+/-	+/-	+	50	7
+/-	+/-	+/-	-	82	11
+/-	+/-	-	+	32	5

Should have been treated at delivery but were NOT

Figure 4. Culture results obtained at four different times during pregnancy.

Attempts to enhance the sensitivity and timeliness of the culture system include the use of Granada broth and Granada-like media, which develop an orange pigment in the presence of hemolytic GBS[20,21]. Unfortunately, non-hemolytic strains are also non-pigmented and the broth requires several hours of growth to produce results[17,21]. Some laboratories have implemented molecular methods to speed up and enhance detection of GBS directly from the broth[8]. More recently, chromogenic agar media have been developed for selective growth and morphological differentiation of GBS from other bacterial flora[21]. These methods are still culture-based, and not appropriate for intrapartum testing.

Dr. Michel Bergeron introduced a major advance in GBS detection in pregnant women with the development of the commercially available IDI GBS real-time PCR kit. Developed in Canada, Bergeron's test performed very well during a study of women in labor[7]. The test is now marketed as the BD GeneOhm Strep B test, and its sensitivity and specificity have been reported as 95.3% and 99.1%[25]. After a number of sample preparation steps, the PCR amplification is performed in a SmartCycler (Cepheid). Since each of the samples undergoes a series of processing steps, it is more cost effective to run the test in batch mode on a set schedule. This may increase the turn around time of the test results, although it is still faster than culture-based testing. Studies have shown that

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Gold-Standard For GBS Testing Has Moved To Molecular Diagnostic Detection

25% of women deliver their infant in less than 3.5 hours from the time that they arrive at the hospital. It was widely held that this may not allow sufficient time for bactericidal levels of an antimicrobial agent to be achieved in the mother's blood stream before the baby is born. However, a 2007 study that evaluated GBS colony counts in vaginal swabs from pregnant women in labor, reported that colony counts dropped by more than 80% within the first 2 hours after intravenous penicillin was given[18]. In addition, Barber and colleagues measured the levels of penicillin in umbilical cord blood of infants born to mothers given the standard dose of intravenous penicillin prophylaxis during labor[6]. They found that babies born within 4 hours of penicillin exposure had higher serum levels than babies born after 4 hours. In fact, levels of penicillin in the babies' bloodstream was highest at just one hour after the penicillin was given to the mother, and all levels measured were above the minimal inhibitory concentration (MIC) of GBS, suggesting that even in women who deliver very quickly, prophylactic antimicrobial agents would still be effective[6].

Current estimates based on extracting data from >7500 patient charts from a 10-state sample are that 85% of all women in the United States now receive antepartum screening cultures for GBS[26]. So why are EOD rates not dropping further? One explanation for the leveling off of the curve of the numbers of babies born with EOD is the 13% of women whose GBS status is unknown at the time of delivery. This group includes 7-11% of women who deliver preterm, i.e., before the GBS screening cultures were collected. Sadly, the risk of EOD with GBS is even higher in preterm babies than in babies delivered at term. In one large study, 36% of all babies who develop EOD were born before 40 weeks of gestation, although only 11% of all babies were born preterm[26]. Another reason is that even when cultures were performed, the results may not be known to the attending physician when the woman presents for delivery. A more relevant observation was made in a 1997 study performed using culture methods which documented the intermittent nature of GBS carriage in pregnant women (See Figure 4); among women with a GBS positive culture at any point during their pregnancy, intermittent positivity was more common than consistent culture positivity[15]. But the most important statistic with regard to prevention is the number of women who change colonization status from negative (on their 35-37 week screening test) to positive at time of delivery[10,15]. Around 5-9% of the women who were culture-negative at antenatal testing become culture positive at the time of labor; since overall, most women test negative by culture at this time point, the number of "new positives" is fairly large compared to the total number of positives. Indeed, a frightening 61.4% of 254 women whose babies developed early onset GBS disease had tested negative for GBS carriage in their antenatal screening culture[15,26]. Thus, a rapid and reliable PCR test that can be used when women arrive in labor at the hospital would be beneficial, particularly in this population. Fortunately, such a test now exists and a series of recent publications has shown its efficacy.

The Xpert GBS assay was cleared by FDA in 2006. This system was recently improved to allow the instrument to report a positive result based on achieving a threshold level of fluorescent signal, indicating presence of GBS genetic elements, rather than waiting for all cycles of polymerase chain reaction to finish. This newest version of the software can report a positive result in as little as 35 minutes. The recent resurgence of interest in intrapartum testing was probably heralded by the ground breaking work of Gavino and Wang in Chicago[14]. They evaluated whether the results of PCR assays performed by obstetric personnel during delivery were equivalent to the results of a culture of the same sample type done by trained laboratory personnel. They also compared the intrapartum test results with results of cultures performed antenatally. Their first conclusion was that intrapartum PCR tests were >10% more sensitive for presence of GBS at delivery than the results of the antenatal screening cultures. In addition, they discovered that the overall results generated by the nursing staff who were performing the Xpert GBS assay on the wards were comparable to the results generated in the microbiology laboratory (only one

> Studies have shown that 25% of women deliver their infant in less than 3.5 hours from the time that they arrive in the hospital.

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false negative PCR result from the nurses' testing group among 55 subjects)[14].

A multisite study comparing the two FDA-cleared molecular tests for GBS was reported last year by Jordan and colleagues, representing sites in Phoenix, Arizona; North Hollywood, California; Winston-Salem, NC; Indianapolis, IN; Houston, TX; and Pittsburgh, PA[12]. Vaginal/rectal swabs were collected from 418 eligible women and tested by two PCR assays and standard enriched culture at two times: between 35-37 weeks of gestation and at the time of delivery. The intrapartum Xpert GBS assays were performed by nurses in the Labor and Delivery suites, while the BD assays were performed in the microbiology laboratory. Compared with culture, the BD GeneOhm Strep B PCR test showed sensitivity of 79% and the Xpert GBS assay showed a sensitivity of 91%[12]. In addition to better performance, the ability to test each sample individually as it arrived and the rapid time to positivity were cited by the authors as benefits of the Xpert GBS assay. All samples were analyzed together for this study, but results from intrapartum testing were reported immediately to clinicians, who made their own decisions on treatment.

An even more recent study from France compared results of the Xpert GBS assay performed during labor and the standard antenatal culture[13]. Results showed a relatively poor positive predictive value for antenatal cultures (58.7%). In their study of 968 births, 75% of women delivered >3.5 hours after the tests were performed, allowing sufficient time for active prophylaxis. Importantly, the authors concluded that if the results of the Xpert GBS test had been delivered (they were not released since testing was performed under a study protocol), four cases of neonatal infection and nine episodes of GBS colonization could have been prevented. The Xpert GBS test performed on intrapartum women was 98.5% sensitive and 99.6% specific compared with a reference standard culture[13].

The authors of the article on the outcomes of the MMWR 2002 guidelines espousing universal antenatal screening for GBS stated that "Rapid PCR based testing at admission for delivery may improve the accuracy of screening by identifying colonization status at the time of labor and delivery."[26] This forward-looking statement should now be slightly modified. Today, it seems that one could instead say "Rapid PCR based testing at admission for delivery WILL improve the accuracy of screening by identifying colonization status at the time of labor and delivery."

Rapid And Accurate H1N1 Test Still Elusive

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3 times the initial volume as some other tests[10]. With regard to this year's circulating strain of H1N1 novel 2009, the problem appears to have intensified; the rapid tests' sensitivities range from 10-70%3, a sensitivity so low that many clinicians and microbiologists are choosing not to offer the test. Although positive results may still be helpful (even though false positives are relatively common), especially in low-prevalence areas, negative result cannot be used to rule out infection. With the advent of 2009 influenza A H1N1, recent studies have shown that individuals who test negative with these assays can still be infectious and should not be encouraged to reenter public settings such as schools, churches or hospitals without confirmation from more sensitive tests. The CDC's recent interim guideline on rapid tests (http://www.cdc.gov/h1n1flu/guidance/rapid_testing.htm) suggests that laboratories add a statement about the limitations of the tests and not make infection control decisions on the basis of these tests (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5837a1.htm?s_cid=mm5837a1_e)

Aside from the rapid antigen tests, all other available assay types are high complexity. Culture has always been quite sensitive, but its turnaround time is too slow for clinical use[9]. Direct fluorescent antibody testing of respiratory secretions is specific, but its sensitivity is dependent on the expertise of the laboratory performing the test. A recent study showed poor sensitivity in one laboratory but good sensitivity has been seen in other settings[4, 9]. The current gold standard for testing is use of a molecular diagnostic method for detection of viral nucleic acids[4, 6]. Of the commercial molecular assays currently available, only the Focus assay is FDA-cleared for specific identification of novel 2009 H1N1, although other platforms (Prodesse ProFlu+ and Luminex xTag) do detect the novel strains along with other influenza A strains, so it would not be missed. A number of laboratories have developed their own in-house molecular tests on varying platforms, based on sequences or procedures published by CDC or others, available on the WHO website (http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf)[7, 8]. Faster new assays are on the horizon.

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