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Bridging the Gap Between HPV Infection and Cervical Cancer:
The Future of Clinical Diagnostics

Author: Kathryn Iacono, Ph.D.

There has been a clear progression in the advancement of diagnostic testing, including cervical cancer testing. At one point cervical cancer was the leading cause of death due to cancer in women, but its frequency has drastically declined over the past forty years due to the inclusion of routine Pap testing with pelvic examinations. Identifying Human Papillomavirus (HPV) as the leading risk factor associated with cervical dysplasia in the 1990's was also a major milestone that allowed for the differentiation of normal morphologic events from those induced by HPV infection. Molecular technology has now progressed to the level where patients can be stratified even further, based upon the actual strain of HPV they harbor, allowing physicians to identify those women who need more frequent monitoring. The constant advancement of molecular techniques and their more frequent use as diagnostic tools has improved the level of medical care an individual receives.

Human papillomavirus (HPV) has been identified as the leading risk factor associated with cervical dysplasia. This virus poses a considerable health risk considering its highly associative link to cervical cancer and its status as the most common sexually transmitted disease within the United States. Currently, 20 million women in the United States are infected with HPV and every year 6.2 million more are newly infected. While most HPV infected individuals eliminate evidence of the virus without developing clinical manifestations, a few individuals will progress to cervical dysplasia and possibly on to cervical cancer. Papillomaviruses are a diverse group of viruses comprised of almost 200 different subtypes. The advent of newly developed molecular techniques has identified various subtypes in association with benign and malignant lesions. HPV subtypes designated as low-risk are associated mainly with genital warts and low-grade squamous intraepithelial lesions of the cervix (LSIL) and vulva (VIN 1), while high-risk HPV subtypes are frequently associated with invasive cervical cancer. The recent development and routine use of diagnostic assays designed to evaluate for the presence of HPV strains and identify them as either high or low risk for

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WHAT'S INSIDE ➤➤

Human Papillomavirus (HPV)

Author: Shlomo Stemmer, MD

Human papillomavirus (HPV) is the most common sexually transmitted infection in the United States. It is estimated that 20 million individuals in the US are currently infected with HPV with 6.2 million individuals becoming newly infected every year (1). Most HPV infected individuals eliminate evidence of the virus without developing clinical manifestations. However, in some instances, progression to cervical dysplasia and possibly cervical cancer does occur. Cervical cancer is a slow progressing, often symptomless disorder that is highly preventable. Although cervical cancer was the leading cause of death due to cancer in females within the United States, the advent and widespread use of screening Papanicolaou (Pap) smears has significantly reduced the number of cervical cancer cases. Despite the success such screening has produced, in 2006 the American Cancer Society estimated 9,710 new cases and 3,700 deaths were directly attributed to cervical cancer.

Human papillomaviruses are a genetically diverse group of viruses comprised of almost 200 different subtypes. Over 40 HPV subtypes infect the epithelial and mucosal lining of the anogenital tract (3). HPVs are DNA viruses whose genome is organized in three regions: The early gene (E1 to E7), the late gene (L1 and L2) regions, and the upper regulatory region (URR). HPV type is defined as a complete genome whose L1 gene sequence is at least 10% dissimilar to that of any other HPV type (4). HPV subtypes can also be classified according to their associated risk that an HPV infected individual will develop cervical cancer. Low-risk HPV subtypes (6, 11, 42, 43, 44) are found mainly in genital warts and low-grade squamous intraepithelial lesions of the cervix (LSIL) and vulva (VIN 1) (5), while high-risk HPV subtypes (i.e., 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) are frequently associated with invasive cervical cancer.

Most HPV infected individuals are asymptomatic and able to eliminate the virus. In some cases, the HPV infection persists and progresses to invasive cervical cancer. High-risk HPV has been detected in up to 99.7% of cervical-squamous cell carcinomas (2). HPV 16 alone is associated with more than 50% of all cervical cancers (6). It is estimated that worldwide HPV 16 and HPV 18 are responsible for 60% to 70% of cervical cancer cases (7). A pooled data report of 11 case-control studies from nine countries, involving 1,918 women with histologically confirmed squamous-cell cervical cancer, found that the most common HPV subtypes, in order of decreasing frequency were: HPV 16, 18, 45, 31, 33, 52, and 35 (8).

Infections with HPV in women are often transient. Younger women are better able to clear the virus while only one-third of women over thirty years of age clear their infections (9). In February 2003, a panel of experts published interim guidelines recommending the use of HPV testing in addition to cervical cytology. Women thirty years of age or older, who are negative for both high-risk HPV and cervical cytology, do not need to be rescreened for three years. However, women with normal cervical cytology that test positive for high-

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risk HPV need to be retested in 6 to 12 months. Women who persistently test positive for high-risk HPV need further evaluation with colposcopy, regardless of the cytology result.

HPV DNA testing may also be used as an adjunct to cervical cytology in the triage of women with atypical squamous cells of undetermined significance (ASCUS) (10). Women with ASCUS may be managed by repeat cytology, immediate colposcopy, or HPV testing. Adolescents with either ASCUS or LSIL cytology may be followed by a single HPV test in one year, or repeat cytology in 6 to 12 months. Women, treated with excision or ablation of the transformation zone due to CIN 2 or CIN 3, may be tested for cure with a single HPV test in 6 months, prior to returning to annual screening.

Type specific HPV testing that specifically identifies HPV 16 and 18 may be used to triage women at the greatest risk for HGSIL and more advanced cervical neoplasia (11). Conversely, women with other high-risk HPV infections could possibly be managed less aggressively. For the management of women thirty years of age or older, Khan et al proposed utilizing type specific testing (11). Women with normal cervical cytology and positive HPV 16 or 18 testing are referred for colposcopy, while women that test negative for HPV 16 or 18 but positive for other high-risk HPV subtypes, need only be followed with repeat cytology and HPV testing in 12 months.

In June of 2006, Gardasil® (manufactured by Merck and Co., Inc., Whitehouse Station, New Jersey) a quadrivalent HPV vaccine (HPV 6, 11, 16, 18), was approved for use in females aged 9 to 26 years. A bivalent HPV vaccine protecting against high-risk HPV 16 and 18 is currently in phase 3 trials. Women already infected with HPV 16 or 18 will not clear the virus any faster following vaccination and it should not be used for treatment of existing HPV 16 or 18 infections (12). The expectation is that in a few decades, widespread vaccination will almost eliminate the risk of cervical cancer. However, during this time continued prevention, screening, and treatment of HPV induced cervical neoplasia is needed.

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11. **Khan MJ, Castle PE, Lorincz AT, et al**. 2005. The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J Natl Cancer Inst*, **97**:1072-79.
12. **Hildesheim A, Herrero R, Wacholder S, et al.** 2007. Effect of human papillomavirus 16/18 L1 virus-like particle vaccine among young women with preexisting infection. *JAMA*, **298**:743-53.

Quality Assurance Q&A

Q: My patient's result reports now say HPV Type-Detect 2.0® by Bio-Plex Analysis instead of HPV Type-Detect® by PCR. What is the difference between PCR and Bio-Plex?

A: Test 152: HPV Type-Detect 2.0® by Bio-Plex Analysis actually combines PCR methodology with a liquid micro-array system called Bio-Plex Analysis. The initial PCR amplification step amplifies an area of the L1 major capsid region of HPV. This region is a suitable target, because a diverse genetic region is contained within a more conserved region for all of the 19 detected HPV subtypes. Subsequent detection and differentiation between the 19 specific subtypes occurs via Bio-Plex Analysis using specific oligonucleotide probes. Bio-Plex is a multiplex suspension array system that utilizes polystyrene 5.6 micron microspheres internally dyed with red and infrared flurophores. The microsphere beads are made with different dye intensities, and with 100 different bead types the user is able to develop up to 100 different assays simultaneously. The surface of the bead uses simple coupling chemistry to have the ability to attach an oligonucleotide probe that will hybridize to an amplified DNA template. High tech fluidics based on the principles of flow cytometry cause a stream of suspended microspheres to line up in single file before passing through a detection chamber. Detection is carried out on the instrument. As a microsphere passes through the detection chamber, a red laser excites both the internal red and infrared dyes, allowing classification of the microsphere to one of the hundred detection channels. A green laser excites any orange fluorescence associated with the binding of the DNA template. The reporter readings are made on each individual particle, and the classification can be determined by the Bio-Plex instrument.

If you have a question you would like addressed in future issues, please email your question(s) to QAQ&A@mdlab.com

Medical Diagnostic Laboratories, L.L.C.

NEW TEST ANNOUNCEMENTS

Now available on *OneSwab*®

152	HPV Type-Detect® 2.0 by Bio-Plex Analysis
1118	Methicillin resistant *Staphylococcus aureus* (MRSA) by conventional PCR
1119	Panton-Valentine Leukocidin (PVL) DNA by Real-Time PCR** **(Type IV + #1118 Req.) [Community Acquired MRSA = Type IV MRSA+ and PVL+]**

Now available on *UroSwab*®

152	HPV Type-Detect® 2.0 by Bio-Plex Analysis

Now available on *NasoSwab*™

1121	*Neisseria meningitidis* by Real-Time PCR
1118	Methicillin resistant *Staphylococcus aureus* (MRSA) by conventional PCR
1119	Panton-Valentine Leukocidin (PVL) DNA by Real-Time PCR** **(Type IV + #1118 Req.) [Community Acquired MRSA = Type IV MRSA+ and PVL+]**

As of November 3, 2008, the following test replacement took effect:

Discontinued	140	HPV Type-Detect® by PCR
Replacement	152	HPV Type-Detect® 2.0 by Bio-Plex Analysis

Recent Publications

Medical Diagnostic Laboratories, L.L.C.

1. **Fakioglu E, Wilson SS, Mesquita PM, Hazrati E, Cheshenko N, Blaho JA, Herold BC**. 2008. Herpes simplex virus down regulates secretory leukocyte protease inhibitor: a novel immune evasion mechanism. *J Virol*. **82**(19): 9337-44.

2. **Bowles RN, Yedowitz JC, Blaho JA**. 2008. Reconsideration of viral protein immunoblotting for differentiation of human herpes simplex viruses. *Diagn Microbiol Infect Dis*. **62**(2): 167-76.

3. **López MR, Schlegel EF, Wintersteller S, Blaho JA**. 2008. The major tegument structural protein VP22 targets areas of dispersed nucleolin and marginalized chromatin during productive herpes simplex virus 1 infection. *Virus Res*. **136**(1-2): 175-88.

4. **Vermitsky JP, Self MJ, Chadwick SG, Trama JP, Mordechai E, Adelson ME, Gygax SE**. 2008. A Survey of Vaginal-flora Candida species of Different Age Groups Using species-specific PCR Detection. *J Clin Microbiol*, **46**(4): 1501-3.

HUMIGEN, L.L.C.

1. **Ucisik-Akkaya E, Dorak MT**. 2008. A study of natural killer cell lectin-like receptor K1 gene (KLRK1/NKG2D) region polymorphisms in a European population sample. *Tissue Antigens*, In press.

JOURNAL WATCH

Kalantari M, Villa LL, Calleja-Macias IE, Bernard H 2008.
Human papillomavirus-16 and -18 in penile carcinomas: DNA methylation, chromosomal recombination and genomic variation. *Int J Cancer*, **123**: 1832-40.

The study suggested that HPV-dependent carcinogenesis of the penis and cervix follows similar etiological and epidemiological parameters. Penile carcinomas are frequently associated with high-risk HPV types. Investigators observed three properties in Brazilian patients: HPV-DNA methylation, junctions between HPV and cellular DNA, and genomic variation. They concluded that these mechanisms also occur during penile carcinogenesis because 95 HPV-16 molecules derived from 19 penile lesions had 58% of the CpGs in the L1 region and 22% in the 5' part of the LCR region methylated. Out of the 15 HPV-16 lesions, 11 confirmed chromosomal integration by reverse ligation inverted PCR, while 4 had samples with concatemeric integrations or episomes. They also observed that there may be a higher risk with variant AA lesions, as is suspected in cervical cancer. The authors suggest further investigation into the progression of HPV related carcinogenesis.

Saunier M, Monnier-Benoit S, Mauny F, Dalstein V, Briolat J, Riethmuller D, Kantelip B, Schwarz E, Mougin C, Pretet JL. 2008.
Analysis of Human papillomavirus Type 16 (HPV-16) DNA load and physical state for identification of HPV-16 infected women with high-grade lesions or cervical carcinoma. *J Clin Microb,* **46**(11): 3678-85.

Infection with high-risk HPV (HR-HPV) can lead to the progression of cervical intraepithelial neoplasia grade 1 (CIN 1) to more severe precancerous lesions such as CIN 2 or CIN 3. This study identifies the possibility of using the viral load of HPV-16 as an identifier for high-grade lesions by utilizing 3 sets of Real-Time PCRs targeting E2 and E6 (HPV-16) and the human albumin gene. The authors conclude that having an HPV-16 copy number normalized against the albumin cells greater than 22,000 copies/10^3 cells or an E2/E6 ratio of 0.5 was a sufficient indication of having high-grade lesions. The cutoff values found are only for the identification of HPV-16 using the above mentioned methods, rendering more cutoff values necessary for other HR-HPV types.

Dela Cruz, WP, Richardson, JY, Broestler, JM, Thornton, JA, Danaher, PJ. 2007.
Rapid Determination of Macrolide and Lincosamide Resistance in Group B Streptococcus Isolated from Vaginal-Rectal Swabs. *Inf Dis Obstet & Gynecol,* 2007: 46581. Published online in June 2007.

GBS is a serious neonatal bacterium that up to 30% of women carry and antibiotic prophylaxis is still the course of action for prevention with penicillin being the first drug considered for prevention and treatment. With penicillin allergies estimated at 3% to 10% of the general population, other treatments are sought after and have been used, with the side effects of increasing strain resistance to these drugs, particularly, drugs from the Macrolide (erythromycin) and Lincosamide (clindamycin) families. Standard disk diffusion methods to detect these resistant strains are the current gold-standard testing method, but can take 18 to 24 hours to produce a result. The authors of this paper developed a FRET PCR assay that is 93% sensitive and 90% specific for the detection of both resistances and can do so within 2 hours, but still requires prior GBS isolation.

Tiwari P, Singh D, Singh MM. 2008.
Anti-*Trichomonas* activity of *Sapindus* saponins, a candidate for development as microbicidal contraceptive. *J Antimicrob Chem,* **62(**3): 526-34.

In view of increased resistance of the parasite protozoan *Trichomonas vaginalis* to classical drugs of the metronidazole family, the need for new unrelated agents is increasing. This study demonstrates the anti-Trichomonas activity of saponins isolated from *Sapindus mukoross*, whose spermicidal activity has been well established. Saponins have long been known to have cell membrane lytic and detergent action, and they also have a spermicidal action and have been used commonly as local contraceptives.

The MIC assay in the present study show that saponins exhibit anti-Trichomonas activity at a concentration 10-fold lower (0.005%) than its minimal effective spermicidal concentration. At this concentration, saponins are neither cytotoxic to the host cells nor do they alter vaginal microflora as shown by the mitochondrial reduction potential measurement assay using florescence-activated cell sorting. Anti-Trichomonas activity of the saponins was evaluated using a cytoadherence assay, the substrate gel electrophoresis method for the estimation of the activity of CPs of Trichomonas and RT–PCR analysis. The data reported shows that saponins inhibit proteolytic activity of the parasite's cystene proteinases (CPs) that are important for adherence, nutrition acquisition and virulence of the parasites, and a 46% reduction in cytoadherence to host cells in comparison to controls. The decreased expression of adhesion proteins AP65 and TvCP2 observed in the RT-PCR studies supports the inhibition of adhesins and membrane-expressed CPs at the genetic level. Fluorescence studies on the effect of Sapindus saponins on actin cytoskeleton of *T. vaginalis* using phalloidin-FITC show that in saponin-treated parasites, fluorescence is reduced and concentrated mainly at the periphery, suggesting disruption of actin cytoskeleton underlying the cell membrane that affects membrane-mediated adherence of Trichomonas to the host cells. These data suggests the potential of saponins for development as spermicidal microbicide for human use.

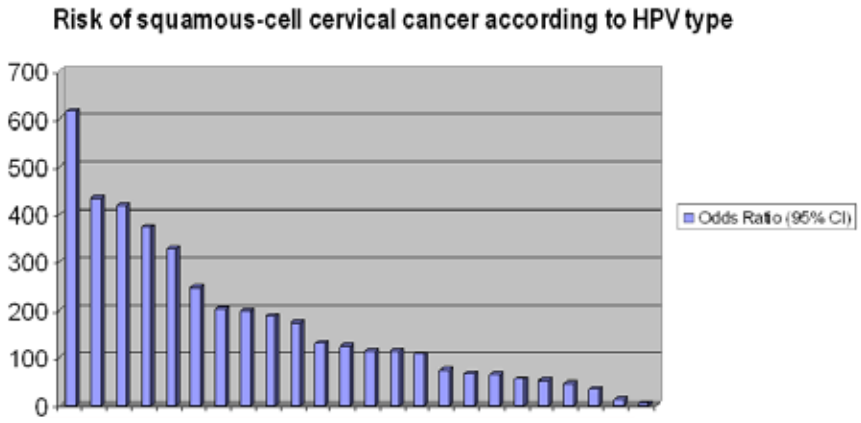
Pfaller MA, Boyken LB, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, and Diekema DJ. 2008.
Validation of 24-hour fluconazole MIC readings versus the CLSI 48-hour broth microdilution reference method: results from a global *Candida* antifungal surveillance program. 2008. *Journal Clinical Microbiology,* **46**(11): 3585-90.

The ability to determine the susceptibility of a particular species of yeast can dictate the proper course of treatment, especially with an isolate like *Candida glabrata*, where the overall treatment costs can be lowered by treating with the appropriate antifungal based on its resistance. In this study, the authors examined the gold-standard CLSI microbroth reference method for reading MIC at 24 hours versus 48 hours to determine if it would be effective. The study looked at 11, 654 isolates and their MICs at both 24 and 48 hours and the essential agreement (within 2 log2 dilutions) between the 24 hour and 48 hour results was 99.6%. However, the categorical agreement for *C. glabrata* was only 67.5%, with a majority of errors in calling an isolate susceptible at 24 hours and susceptible dose-dependent at 48 hours. This is not unusual as *C. glabrata* MICs tend to hover around the breakpoints, leading the authors to caution readings at 24 hours with this species. Early speciation and susceptibility testing is important in antimicrobial treatment, and although reading at 24 hours appears to be effective, this assay is still very time consuming.

e-Quiz

For results to the electronic Epidemiology Quiz, please visit www.mdlab.com and click on the e-Quiz link.

1. Place in order of highest to lowest odds ratio:



single infections	HR-58
LR-6	HR-59
LR-11	HR-68
HR-16	HR-73
HR-18	Multiple infections
HR-31	other 2 infections
HR-33	16 & other LR
HR-35	16 and 18
HR-45	16 and other HR
HR-51	18 and other HR
HR-52	other 3 infections
HR-56	4 or 5 infections

2. Which HPV subtype is the most common high-risk subtype and is found in almost half of all cervical cancers?

3. HPV DNA can be detected in swabs from anal canal of over _____ % of men who have sex with men (MSM).

   a. 5% b. 25% c. 45% d. 50%

4. True or false. As many as 91% of women with HPV infection will become DNA negative for HPV within 2 years.

5. Testing for HPV DNA in the mangement of women with ASCUS pap test results is recommended as an option by:
   a. American Society for Colposcopy and Cervical Pathology (ASCCP)
   b. American Cancer Society (ACS)
   c. American College of Obstetricians & Gynecologists (ACOG)
   d. All of the above

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cervical cancer provide an added level of discrimination to the diagnostic evaluation and offers a more tangible method of stratifying patients by their presumptive infectious course. MDL has been providing this information to physicians and patients through their HPV Type-Detect test, which identifies nineteen of the most common HPV subtypes in circulation, and their latest assay, the HPV Type-Detect® 2.0 by Bio-Plex Analysis, which utilizes the latest state-of-the-art technology to improve assay sensitivity and significantly reduce turn-around time.

Cervical cancer is a slow progressing, often symptomless disorder that is highly preventable. While at one point it was the leading cause of death due to cancer in females within the United States, increased vigilance through the standard employ of Pap testing and, more recently, the ability to stratify women at greater risk based upon the presence of a high-risk HPV subtype has significantly reduced the number of cancer cases. While these tests serve as a useful means to identify populations who should be monitored more closely, they are not definitive diagnostic assays as far as progression to cancer is concerned as cellular morphology within the cervix is known to fluctuate for non-cancerous reasons, including hormonal fluctuations, infections, and benign polyp growth. Therefore, the development of a sensitive and highly prognostic assay capable of discriminating between early onset dysplasia and non-cancer related morphological changes would eliminate the "watch and wait" element associated with Pap testing and allow for earlier diagnosis and initiation of treatment. MDL is working diligently to expand upon its current HPV-Type Detect assays to provide as much information as possible so that physicians and their patients can together decide upon the best course of action. Our efforts are focused upon established cellular anomalies known to occur during the transformation process, with particular emphasis on the inappropriate expression or mutation, of key cell cycle regulatory host genes that may arise as a result of HPV infection, mutation or genetic duplication, as well as looking at alterations within the virus itself. More specifically, MDL is concentrating on the transforming capabilities of the HPV E6 and E7 viral proteins and their interaction with key cell cycle regulators, including p53, pRb and the cyclin/CDK complexes from the host' point of view and evaluating the activity of viral gene promoters. In pursuing these two research branches, we hope to identify cellular or viral alterations that may serve as clues which may link the progression of HPV infection to transforming events and provide a better indication of a patient's probability of progressing to cervical cancer.

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