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Use of Simultaneous Multiplexed DNA Sequencing(MultiGEN) for the Detectionof C.trachomatis, N.gonorrhoea S. aglacticease G.vaginali,s Herpes Simplex 1 &2,

T.Vinayagamoorthy, D.Luevano, M. Chellani, T.Cahill, R.Hodkinson and J. Voland
MultiGEN Diagnostics Inc , San Diego, California. www.multigen-diagnostics.com
moorthy@multigen-diagnostics.com

SUMMARY

We have developed a multiplexed DNA sequencing assay, the MultiGEN Antenatal Panel to simultaneously detect in a single test one or all of the following pathogens: C. trachomatis, N. gonorrhea, G. vaginalis, HSV 1, HSV2, and Group B Streptococcus. Out of 140 clinical samples tested, each of which were positive by present methods for one of the above 6 pathogens, 126 were in concordance with the MultiGEN test. Out of the 14 that were discordant, all 14 were negative by the MultiGEN test for that pathogen. Furthermore, in some of the 14 discordant samples, the MultiGEN tests detected additional pathogens that had not been reported by the corresponding present method. MultiGEN test panel is could unify the present fragmented market that will enable the physician to have a comprehensive and verifiable (nucleotide sequence) results on a timely fashion.

INTRODUCTION

There are more than 4 million term pregnancies in the USA each year. The prevalence of traditional STDs (*C. trachomatis, N. gonorrhea, G. vaginalis, T. vaginalis and Herpes simplex 2)* in these pregnancies *is* approximately 25%, and the carriage rate for *Streptococcus agalactiae* (Group B Streptococcus GBS) is 10-40%. Clinical infections in the mother with these pathogens include intra-partum fever, arthralgias, meningitis, encephalitis, acute endocarditis, fatal septicemia, acute vulvo-vaginitis, urethritis, pelvic inflammatory disease (PID), tubo-ovarian abscess, and acute salpingitis leading to subsequent infertility. The newborn baby is also at risk for serious infections including herpes encephalitis, gonococcal conjunctivitis, pneumonitis, fatal septicemia and others.

It is therefore widely recommended that all pregnant women be tested during the first antenatal visit for *C .trachomatis, N. gonorrhea, G. vaginalis, G. vaginalis, and Herpes simplex 2.* Repeat testing for these pathogens and for Group B streptococcus during the 35-37th weeks of pregnancy is also a standard of practice. This group of organisms are from different phyla (bacterial, viral and protozoal), and presently they have to be tested for using entirely different technologies (1,2,3). Not unexpectedly therefore, due to logistics, costs and practicality they are usually not routinely tested. Unnecessary and dangerous complications therefore inevitably occur involving both mothers and their newborns.

MultiGEN is a modified Sanger sequencing platform technology that detects multiple pathogens simultaneously (4,5). We have developed a MultiGEN Antenatal Test that simultaneously detects *C. trachomatis, N. gonorrhea, Herpes simplex 2, G. vaginalis and Group B Streptococcus.* This manuscript presents a comparison of MultiGEN with present testing methods.

MATERIALS AND METHODS

Anonymous patient samples that had previously tested positive for Factor V, Factor II, mthfr –C677T and A1298C were obtained from reference laboratories. Total DNA was extracted using BugsNBeads A (NorDiag, USA). Multiple PCR of target amplicons was performed in a 50-ul volume containing 5ul of extracted DNA. The amplified multiple targets were purified using Ampure (Agencourt, USA). Purified amplicons were sequenced using MultiGEN sequencing primers by cycle sequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems, USA) on a GeneAmp 2400 thermocycler (PE Applied Biosystems, USA). Unincorporated dye terminators were removed using Clean Seq (Agencourt USA). The samples were then dried in a speed vac (DNA 120, ThermoSavant, USA) and re-suspended in 20 ul of Hi-Di formamide. Samples were analyzed by capillary electrophoresis using the ABI PRISM Genetic Analyzer 3130.

ORGANISM	PATHOGEN SPECIFIC SEQUENCE	GENBANK ACCESSION	LOCUS
C.trachomatis	CGTAAAGGGCGTGTAGGCGGA	FM872308	857256-857276
N.gonorrhea	TTGCCCGAAACTAAAGAGCCTTTCGTGA	CP001050	1797955-1797982
Group B Strep	TGTCTTCATTGGCGAAACAATCGTT	FJ752161	459-483
G.vaginalis	GTGGTGTACCACCCATAACAGTGGCCGGGTCTCCCCATTCGG	L08167	486-527
Herpes 1	ccacggcgtc gccctgaaca cggacta ttacttctcccac ctgt tggggg	FJ593289.1	66121-66170
Herpes 2	GGGGCGTTCCGCTCAACACGGACTATTACTTCTCGCACCTGCTGGGGG	AY038367	3517-3470

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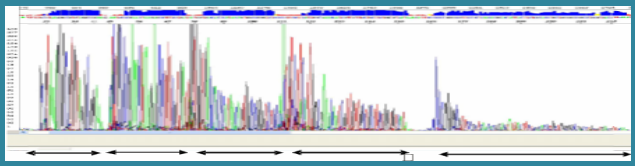
SINGLE ELECTROPHEROGRAM SHOWING ALL SIX PATHOGENS



C.trachomatis N.gonorehea G.vaginalis GroupBStrep Herpes1&2

ORGANISM	SAMPLE TYPE	COMPARATIVE METHOD	No. SAMPLE	POS BOTH	NEG BOTH	POS MULTIGEN	POS C.MTD
C.trachomatis	Swabs, thin prep	Aptima Combo2	37	21	9	0	7
	Urine	m2000	10	10	0	0	0
N.gonorrhea	Swabs, thin prep	Aptima Combo2	28	12	14	0	2
	Urine	m2000	10	9	0	0	1
Group B Strep	Swabs	Streptex Rapid kit	15	15	0	0	0
G.vaginalis	Swabs	Affirm VP	25	24	0	0	1
Herpes 2	Swabs	Elisa HSV	15	13	0	0	2

RESULTS

We tested 148 clinical samples including vaginal swabs, Thin PrepsTM and swabs collected for culture/ELISA methods. Comparison of the MultiGEN test with reference lab results are shown in Table 1. All of the discordant samples were negative by the MultiGEN test panel.

To resolve this discrepancy, DNA extracted from the discordant specimens was sent to another reference laboratory which performed re-sequencing which was then compared with MultiGEN's findings. The re-sequencing results from the second reference lab were in complete concordance with MultiGEN's results. In order to rule out any false negatives by MultiGEN, 14 samples that were negative by MultiGEN were tested for the presence of 4 cellular markers (Factor V Leiden, Prothromvbin, and mthfr C677t and A1209C). All 14 samples showed positive results for all four cellular genetic markers. Some of the negative discordant samples were spiked with the positive clinical samples for the respective pathogens, and MultiGEN then detected positive results for the respective organisms. Furthermore, four MultiGEN negatives were each repeated twice with MultiGEN testing, generating the same results. In 3 of the discordant samples that were positive by PCR probe, other/additional pathogens were detected by MultiGEN: Group B Streptococcus , G. vaginalis , and N. meningitidis . In 4 of the discordant samples that were positive by ELISA, other pathogens were also detected by MultiGEN: Group B Streptococcus , and N. meningitides.

DISCUSSION

The significance of identification of clinically relevant microbes has two components:

(a) Correlation with clinical symptoms. Although Herpes simplex 2 and Streptococcus agalactiae can be asymptomatic, their presence during pregnancy necessitates specific medical intervention and we believe that they should be routinely tested in antenatal patient screening.

(b) Accurate identification of the microbe. MultiGEN technology is a modified Sanger sequencing method, where the final step of identification is enzyme mediated, and the final step generates an electropherogram with species specific nucleotide sequences. The MultiGEN Antenatal Panel test produces a verifiable result format (Fig.1) that shows the species specific signature nucleotide sequence(s) for the pathogen(s) tested. With other current methods (PCR/probe or EL ISA) the identification step is not enzyme mediated, and does not produce a verifiable result format. Therefore the accuracy of the result generated by the MultiGEN panel should be identical to traditional DNA sequencing, which is the accepted 'Gold Standard' for identification.

All MultiGEN assays that were positive were positive by reference methods hence Mu ltiG EN did not produce false positives. In order to rule out false negatives, samples that were not positive by MultiGEN were tested (a) by spiking with positive samples showing that there was no inhibition of amplification, and (b) by detecting cellular genetic markers that were positive when tested. Furthermore, samples were shown to contain additional organisms to the ones identified by the reference methods (PCR/probe or EL ISA).

The MultiGEN Antenatal Panel (MAPTM) can cost-effectively detect a variety of pathogens from different types of clinical samples, including vaginal swabs and Thin PrepTM. For obvious medical reasons, it is vital that results for STD and GBS tests during pregnancy are accurate. False positives for GBS can result in unnecessary antibiotic treatment with risk of an adverse drug reaction in a pregnant woman. False positives for STDs in this setting could also lead to significant problems in family relationships. False negatives for any of the pathogens risk the many serious consequences listed earlier.