Laboratory preparedness for detection and monitoring of Shiga toxin 2-producing Escherichia coli O104:H4 in Europe and response to the 2011 outbreak

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E. coli (Escherichia coli, E.coli) - a rod-shaped bacterium, most strains are harmless and are part of the intestinal flora of animals and humans. However, E.coli can penetrate into the urethra, and then into the bladder, after attachment to the epithelium it begins to actively multiply, causing an infection.
Laboratory diagnosis of diseases caused by Escherichia coli, Shiga toxin-producing (STEC-culture), and the detection of pathogens STEC-infections in food.

Methodological guidelines define the allocation scheme of clinical material and food products of the three groups of Escherichia producing Shiga toxins, and their identification:

a) E. coli O104: H4 epidemic strain that caused the outbreak of STEC-infection in Germany and other countries in the spring of 2011


A key factor affecting the three groups of Escherichia are Shiga toxins - Stx1 and Stx2.

Acute intestinal infections with hemorrhagic manifestations of colitis (HC) and hemolytic uremic syndrome (HUS), caused by Escherichia that produce Shiga-like toxins (STEC), are common in many countries around the world and recorded in the form of sporadic cases or outbreaks of covering large numbers of people, from tens to thousands.

Among strains of E. coli are particularly important pathogenic strains capable of synthesizing verotoxins (shigapodobnye toxins). There is a group enterohemorrhagic strains of E. coli (EHEC) with highly pathogenic serovars O157: H7, O26, O103, O111, O145 and other verotoxin production is the most common criterion for the determination of this group of bacteria.

Escherichia coli O104:H4 is an enteroaggregative strain of the bacterium Escherichia coli, and the cause of the 2011 Escherichia coli O104:H4 outbreak. The “O” in the serological classification identifies the cell wall lipopolysaccharide antigen, and the “H” identifies the flagella antigen.

Analysis of genomic sequences obtained by BGI Shenzhen show that the O104:H4 outbreak strain is an enteroaggregative E. coli (EAEC or EAggEC) type that has acquired Shiga toxin genes, presumably by horizontal gene transfer. Genome assembly and copy number analysis both confirmed that two copies of the Shiga toxin stx2 prophage gene cluster are a distinctive characteristic of the genome of the O104:H4 outbreak strain. The O104:H4 strain is characterized by the following genetic markers:

- Shiga toxin stx2 positive,
- terE positive (tellurite resistance gene cluster),
- eae negative (intimin adherence gene),
- β-lactamases ampC, ampD, ampE, ampG, ampH are present.

Micrograph of two E. coli
What Happened in Europe in 2011

Between May and August 2011, an outbreak of Shiga toxin 2-producing Escherichia coli (STEC) affected over 4,000 individuals in Europe. It was associated with the highest number of cases of hemolytic uremic syndrome (HUS) reported to date (782 confirmed and 119 suspected cases) in the European Union (EU)/European Economic Area (EEA). The first cases were reported from Germany, where the laboratory characterization of the causative bacterial strain was conducted. The outbreak strain was identified as STEC with unusual characteristics. These included the rare serotype O104:H4, lack of attaching/effacing pathogenicity island of virulent STEC strains, as indicated by the lack of the eae gene, but harboring virulence markers of enteroaggregative E. coli, e.g. presence of aggR gene, and exhibiting a multidrug resistance phenotype, including production of CTX-M-15 extended spectrum beta-lactamase (ESBL). At the beginning of June 2011, the European Centre for Disease Prevention and Control (ECDC) published an EU epidemic case definition for this outbreak strain to allow standardized reporting by the EU/EEA countries and comparison of data at EU level for outbreak monitoring.
Of note, six of nine countries with clinical microbiology Stx/stx detection capacity had reported STEC O104:H4 epidemic cases as compared with two of 12 countries with no capacity.

Table

<table>
<thead>
<tr>
<th>Shiga toxin diagnostic testing at clinical laboratories</th>
<th>Number of countries (n=32)</th>
<th>Reporting</th>
<th>Not reporting</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available in more than 15%</td>
<td>6</td>
<td>3</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Available only in some, in case of outbreaks</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>2</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

1 For the United Kingdom, data were received for England and Wales, and for Scotland. No data were received for Northern Ireland. Therefore, England and Wales, and Scotland were considered as separate countries, hence the survey comprised 32 instead of 30 European Union and European Economic Area countries.
Next figure explains:
Capabilities of national reference laboratories in the European Union and European Economic Area for case detection and identification of Shiga toxin 2-producing Escherichia coli O104:H4 before and after the 2011 outbreak, March 2012

Data obtained revealed that prior to the STEC O104:H4 outbreak in April 2011, 11 of 32 countries had NRL capacity to confirm STEC O104:H4 cases. In December 2011, five months after the outbreak, 22 countries reported such NRL capacity.
Causes of Contamination

People become ill with STEC-infections after consuming low-quality meat products, unpasteurized milk, yoghurt, cheese, vegetables, spinach, various salads, sprouted grains beans, juice and other food and water, STEC-colonization of bacteria. Infection with the people in contact with farm and domestic animals, as well as by direct contact with sick STEC-infection.
EHEC strains represent a serious threat to life especially for the elderly and children under 5 years of age. The most common route of infection - the fecal-oral. The incubation period for STEC-infection - an average of three to eight days. The main clinical symptoms in STEC- infections are acute abdominal pain, diarrhea, often with blood (hemorrhagic colitis), nausea, vomiting, temperature can be significantly improved. Most cases recover within 5-10 days without any complications. Some patients (30%) one week after the onset of diarrhea may develop hemolytic uremic syndrome (HUS). The main symptoms of HUS - reduction in the frequency of urination, feeling very tired, anemia, skin and mucous membranes. In patients with HUS, acute renal failure, hemolytic anemia and thrombocytopenia.

It is proved that a deadly E.coli infection created in the lab

The genetic code reveals the secrets
When German scientists from the Robert Koch Institute, have deciphered the genetic makeup of the strain O104, they found that it is resistant to all of the following combinations of antibiotics:

* penicillin
* tetracycline
* Nalidixic acid
* Trimethoprim - sulfamethoxazole
* cephalosporin
* Amoxicillin / clavulanic acid
* Piperacillin - sulbactam
* Piperacillin - tazobactam

In addition, this strain has the ability to produce O104 special enzymes which give it "bacterial super strength" technically known as ESBL: Beta-lactamase spread spectrum (ESBL) are enzymes which can be released by bacteria making them resistant to cephalosporins, such as cefuroxime, cefotaxime and ceftazidime - which are the most widely used antibiotics in many hospitals, "explains the Health Protection Agency in the UK.
When considering the genetic evidence that is now before us, it is hard to imagine how this could happen in the wild. While resistance to a type of antibiotics—often a common phenomenon—the emergence of a strain of E. coli, immune to eight different classes—in combination—simply contrary to the law of genetic permutations and combinations in vivo. Simply put, this is a super strain of E. coli that could not have been born in the nature. And so we are left with only one explanation as to where he really came from: the lab!
Is a condition that is caused by a missing part (also referred to as a deletion) of the short arm (p) of chromosome 4. This missing material can result from minor to severe delays in development, alteration in facial appearance, and can also include other birth defects.

Affects at least 1 per 50,000 newborns.

More common in females resulting in a male 1:2 ratio.
Causes

Unbalanced translocations, either de novo or inherited from a balanced rearrangement

“Pure” de novo terminal or interstitial deletions in 4p16

Chromosome 4 ring, del (4p) mosaicism, or a duplication/deletion rearrangement derived from a chromosome 4 inversion

Normal chromosome

A B C D E F G H

Terminal deletion

A B C D E F G H

Interstitial deletion

C D E F G H

A B E F G H
The sperm and egg come together at conception.

The fertilized egg has two chromosomes 21.

The fertilized egg begins dividing, making more cells (mutant).

The cell with 45 chromosomes does not make enough copies (cannot survive).

During mitotic division, there is non-disjunction of the chromosome 21, causing one cell to have 47 chromosomes (three 21s), while the other has only 45 (two 21s).

Both cell types continue making more cells.

This results in monosomy (two types of cells) for trisomy 21 in the developing baby.

Paracentric Inversion

Centromere

Breaks in Chromosome

Reinserted Piece of DNA

Pericentric Inversion

Centromere

Breaks in Chromosome

Reinserted Piece of DNA with Centromere
**Size Matter?**

<table>
<thead>
<tr>
<th>Small Deletion (≤ 3.5 Mb)</th>
<th>Large Deletion (5-18 Mb)</th>
<th>Very Large Deletion (&gt; 22 Mb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild phenotype lacking major malformations. Most of the time people who fall into this category are not diagnosed</td>
<td>Most common Widely recognizable syndrome phenotype</td>
<td>Severe case causing severe phenotype that can barely be recognized as Wolf-Hirschhorn syndrome</td>
</tr>
</tbody>
</table>

**PHENOTYPE TRAITS**

- 5-18 Mb deletion
  - Hypotonia
  - Seizures
  - Mental Retardation
  - Pre- and Post-Natal Growth Delay
  - Microcephaly
Typical Facial Dysmorphisms
- High forehead
- Round broad face
- Cranial symmetry
- Ptosis
- Exophthalmos

Ocular Coloboma
- Strabismus
- Hypertelorism
- Short philtrum
- Prominent philtrum columns

Cleft lip
- Micrognathia
- Prominent ears
- Low deformed ears
- Hearing loss

TECHNIQUES
- Conventional Cytogenetic
- FISH
- Microarray
- Genomic
CONVENTIONAL CYTOGENETIC TECHNIQUE

Detect numerical and structural abnormalities in metaphase cells.

Unable to detect loss, gain or genetic imbalances of genetic material with a megabase smaller than 5 million base pairs (4-6 Mb).

Time consuming
Labor expensive
Require cultured cells

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

Genetic Mapping technique using fluorescent tags for analysis of chromosomal aberration and genetic abnormalities.

Detecting DNA sequence can be compared to looking for a needle in a haystack.
- Needle = Targeted DNA sequence
- Haystack = Set of chromosomes

The search for the needle will be easier if the person looking for the needle had a powerful magnet.
- Powerful magnet = Fluorescent copy of targeted DNA sequence
- Hybridization = When magnet meets the needle
Indirect Denatured Mix - hybridization Extra Step Signal becomes visible after application of a fluorescent antibody against the hapten
WHAT IS MICROARRAY ANALYSIS?

Can perform an experiment on thousands of genes at the same time.

Each individual spot on a microarray is composed of multiple strands of DNA.

The DNA sequence in each spot is unique.

Thousands of spots are arranged in rows and columns on a solid surface (usually glass).

The precise location and sequence for each spot is recorded in a computer database.
DNA sequence variations that occur when a single nucleotide (A, G, T, C) in the genome sequence is altered

- Act as chromosomal tags to specific regions of DNA. These regions can be scanned for variations that involve disease and disorders.

Scanning a genome in search of where SNPs occur can help scientists make chromosome maps so we can identify genes contributing to specific traits.

- 10,000,000 SNP sites have been identified in the human genome.
It was generally thought that genes were almost always present in two copies in a genome. Recent studies show that large segments of DNA can vary in copy number. Their sizes can be anywhere from thousands to millions of base pairs. Copy number variations can lead to a dose imbalance. There are cases where a gene can have one or more than three copies instead of two. In rare cases, there are genes missing altogether.
COMPARATIVE GENOMIC HYBRIDIZATION MICROARRAY ANALYSIS (ACGH)

CGH contributes in a genome wide screening for CNVs.

Uses two genomes, a test and a control, which are labeled and hybridized to metaphase chromosome.

Fluorescent signal of test will then be linearly plotted across each chromosome. This is where we can identify copy number changes.

Can be used to quickly scan the genome for imbalances.

Instead of using metaphase chromosomes, this new method known as array CGH, uses slides arrayed with small segments of DNA as the targets for analysis.

Array CGH: The Complete Process

Step 1-3 Patient and control DNA are labeled with fluorescent dyes and applied to the microarray.

Step 4 Patient and control DNA compete to attach, or hybridize, to the microarray.

Step 5 The microarray scanner measures the fluorescent signals.

Step 6 Computer software analyzes the data and generates a plot.
GTG banding revealed 4p16 deletion in both sibs
9 years and 9 months of age

Displays facial features of WHS

Born by cesarean due to fetal distress (37.5 weeks)
4lbs 10.96 oz
1 foot 5 inch

Hypotonia was noted at birth. 7 months later tonic-clonic seizures developed

Psychomotor retardation
Global IQ of 25

7 years 5 months

Showed clinical findings similar to her brother

Kidney malrotation

Global IQ 30

Born by cesarean section at 37.2 weeks
4lbs 10.8 oz
1 foot 4.5 inches
Chromosome analysis on lymphocytes by GTG (400-700 bands) and NOR banding. FISH was performed using LSI WHSCR1 Spectrum Orange and CEP 4 Spectrum Green probes.

Table 1

<table>
<thead>
<tr>
<th>Chromosomal Abnormality</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translocations</td>
<td>2</td>
</tr>
<tr>
<td>Inversions</td>
<td>5</td>
</tr>
<tr>
<td>Aneuploidies</td>
<td>10</td>
</tr>
<tr>
<td>Isochromosomes</td>
<td>3</td>
</tr>
<tr>
<td>Endocentric divisions</td>
<td>4</td>
</tr>
</tbody>
</table>

Cytogenetic and FISH Analysis

ToTelVysion Mixtures
- number 4 (4p Spectrum Green, 4q Spectrum Orange, 21q Spectrum Green/Orange, and LSI AML1 Spectrum Aqua) and number 10 (10p Spectrum Green, 10q Spectrum Red, 15q Spectrum Green/Orange and LSI PML Spectrum Aqua)
SNP array

- Genomic mapping was performed on the affected children and parents using Genome-wide human array 5.0 set (Affymetrix Inc.)
- Genotyping Console 4.1 was used for quality assessment and genotyping of the data.

CNV analysis

- Performed using SNP and Variation Suite 7.5.6 software (Golden Helix Inc.)
- Reference set was generated in the lab consisting of 71 healthy subjects including parents.
- The copy number analysis method was used to identify the CNV segments.

Microarray Analysis

CYTOGENETIC AND FISH RESULTS

- High resolution GTG banding revealed a 4p16.1 deletion.
- FISH using WHSCR1 and subtelomeric probes confirmed the loss of both sequences.
- Mother’s karyotype was normal.
- Father carries a derivative chromosome 4 and an apparent heteromorphism in both chromosomes 15.
- ToteTelVysion Mixtures showed that 4p subtelomeric signal was located on the short arm of chromosome 15.
- Ag-NOR banding was negative on 4p and nothing on der(4) with acrocentric chromosomes.
- Ag-NOR positive on der(15) and acrocentric association confirming and insertion from 4p to 15p.
- Other chromosome 15 showed an increased stalk on short arms.
- Both children inherited the chromosome 15 stalk (15pstk+)
• CNV analysis confirmed a similar 4p deletion in both siblings, 6.48 Mb in boy and 6.50 Mb in girl.
• Telomeric break points affected two genes in both affected children (ZNF718 and ZNF595)
• The difference between the telomeric breaks were 11.1 Kb amongst the two
• Both children inherited a different chromosome 4 from their mother which was documented by SNP genotyping analysis (data not shown)
• Centromeric break points differ by 14 Kb between the two.
DISCUSSION

- Children revealed a similar 4p deletion. From 4p16.1 to 14p16.3
- Deletion affects at least 70 genes, including the 200 kb critical region associated with WHS phenotype.
- Genes- LETM1, FGFR11 and WHSC1 have been associated with seizures, facial findings, growth delay and distinctive facial features.
- Researchers identified an isolated 4p deletion due to a paternal balanced insertion.
- FISH showed that these genomic imbalances resulted from the segregation of a parentally balanced insertion.
- The NOR of chromosome 15 are often cryptic when present in an unbalanced form.
- The affected children are the product of an adjacent imbalance from the paternal insertion. Non-affected girl received both chromosomes by alternate segregation.
CONCLUSION

• Results reinforce the importance of thorough clinical analysis, as well as conventional and molecular karyotyping of patients and their parents for proper genetic diagnosis.

• SNP arrays for CNV analysis can determine deletion size with higher precision and can detect cryptic partial trisomies. (extra chromosome)

• Identified novel type of chromosome rearrangements in siblings and its mechanism is more frequent than anticipated.

• Importance of the combined application of classical and molecular techniques to clarify chromosomal structural rearrangements.
OPTIMIZING FROZEN SAMPLE PREPARATION FOR LASER MICRODISSECTION ASSESSMENT OF CRYOJANE TAPE TRANSFER SYSTEM

DR: SHEN
NAME: GOWSIKA JEEVANANTHAN
BIO: 325
NOVE 6TH 2013

WHAT IS OPTIMIZING FROZEN SAMPLE

- Cryosection which require a high degree technique skill to acquire intact sections
- This test immediately done in a surgery room while the patient is on anesthetic condition
- They take the sample tissue and do frozen sample test to diagnosis.
PREPARATION PROTOCOLS USED FOR LASER CAPTURE MICRODISSECTION (LCM) REGION OF NORMAL BREAST EPITHELIUM

- Dissection on the left, and after dissection/collection of cells on the right
- Affected both quality and quantity of RNA yield
- Total RNA yield ranged from 28.3% to 97.1% of expected (0.10-0.34 µg)

HOW TO DO OPTIMIZING FROZEN SAMPLE PREPARATION

- Firstly, PET-CJ slide should be placed on a MMI SupportSlide
- Secondly, PET-CJ slides should be kept at RT before sectioning and cooled down on the UV Flash Unit before 2 min cooling
- Thirdly, the position of the tape on PET-CJ slide during transfer is crucial for membrane integrity.
- Special care should be taken during roller application to the PET-CJ slide positioned on top of the plastic MMI Support Slide

* http://www.youtube.com/watch?v=WfGo2lwI5Q8
CRYOJANE TECHNIQUE CONSUMABLES FOR METAL FRAME PET MEMBRANE SLIDES.

- (A) MMI SupportSlide
- (B, C) Cryo.Jane Solution A and Solution B, respectively
- (D) metal frame PET membrane slide
- (E, F) Simulation of Transfer Adhesive Tape application
- (E) “PEEL THIS OFF” window rolled on the OCT block,
- (F) Tape with the attached cryosection after cryotomy
- (G) PET slide-SupportSlide assembly prior to transfer of CryoJane section to the membrane
- (H) Cryo.Jane Hand Roller for application of Cryo.Jane section to the membrane. Scale bars correspond to 1000 µm.

WHAT IS LASER MICRODISSECTION (LM)

- Laser microdissection (LM) is an invaluable tool in to facilitate collecting specific cell populations for molecular analysis
- If the patient have any tumor and do the full surgery he will do a frozen sample of the tissue and find out the diagnosis
WHY THE SURGEON DO LASER MICRODISSECTION

- If there is any cancer or any other disease they will take out the full tissue for the patient, if not they will leave it alone.
- Benefit the patient from spreading all over the body.
- If the tumor is normal they don’t need a full surgery.

WHAT IS CRYOJANE TAPE TRANSFER SYSTEM

- CryoJaneTape-Transfer System for Cryosectioning makes it easy to produce high-quality frozen sections of difficult tissues.
- The Cryosectioning Tape Transfer System it’s an easy way to make high quality frozen section of difficult tissue.
- They have two different type of section, one is Glass – CJ and the other method is metal frame PET slide. Mostly they used the PET method.
WHAT CAN BE PREVENT

- By the Laser it’s easy to find the high quality frozen section of difficult tissue.

- It will prevent the patient from spreading if there any type of cancer.

- Therefore cryojane technique standard method of laser microdissection slide preparation from the frozen tissue is the test for the different type of laser to evaluate the different type of RNA stability.

Baseline RNA quality control of samples obtained from different tissue preparation protocols using laser capture microdissection (LCM).

- A core biopsy sample was taken from the fresh frozen tissue block.
UV-LM ON PET-CJ SLIDES (METHYL GREEN STAIN), AND QUALITY OF RNA RETRIEVED FROM LM TARGETS.

- [A, B] dissecting screen at 10x and 40x magnification the circle area of dissected target for RNA retrieval.
- [C, D] Representative Agilent electropherogram of LM RNA extracted from mouse liver on slides without (contaminant is present in RNA sample) and with pretreatment solution (high quality RNA).
- [E, F] Mouse skin morphological details on the MMI CellCut Plus dissecting screen at 10x and 40x magnification, respectively; inset shows an area of papilloma dissected for RNA retrieval.
- [G, H] Representative Agilent electropherogram of LM RNA extracted from mouse skin papilloma on slides without (contaminant is present in RNA sample) and with pretreatment solution (high quality RNA), respectively.
- A, E, F: Scale bars correspond to 100 µm; B: Scale bar corresponds to 50 µm.

SUMMARY

- Laser microdissection because it’s easy to find the high quality frozen section of difficult tissue.
- Prevent the patient from spreading if there any type of cancer.
- We tested the CryoJane for use with different types of lasers to evaluate its applicability to complex LM projects involving tissues with different RNA stability.

http://www.youtube.com/watch?v=DmcCrq-qZas&list=TLjtRqI5NKs8LCg0YeXP1aorLCmzfmS
REFEREANCE:

HTTP://WWW.NCBI.NLM.NIH.GOV/PMC/ARTICLES/PMC3689705/

NONINVASIVE PRENATAL KARYOTYPING FROM MATERNAL PLASMA
• Noninvasive prenatal screening is used for determining the genetic risks of a fetus. Such as trisomies 13, 18, 21 and sex chromosome abnormalities.
• The presence of fetal DNA in the plasma of the mother is used for fetal DNA karyotyping.
  • Karyotyping: Procedure by which chromosomes are examined, either by chromosome counting or looking for structural deformities. Useful in looking for genetic abnormalitie.
INVASIVE PROCEDURES:

- Amniocentesis: The use of amniotic fluid from the fetal sac.
  - Poses small risk to mother and baby.
  - Only offered to women at high risk for genetic defects.
- Cordocentesis: Blood is removed from the umbilical cord of the baby.
  - Good for blood conditions and infections.
  - Used to deliver blood transfusions and medication.
  - Poses a high risk for miscarriage
- Chorionic Villus Sampling: Samples chorionic villi from the placenta.
  - Good for detecting genetic conditions.

NONINVASIVE PROCEDURES:

- Ultrasound: Use of high frequency sound waves to create an image of organs and child in the case of pregnancy.
- Maternal Blood Sampling for Fetal Blood Cells: Using the fetal cells known to be in the maternal plasma to determine genetic compositions.
MASSIVELY PARALLEL SEQUENCING (MPS)

- MPS has been used to detect chromosomal abnormalities such as trisomies 13, 18, and 21, as well as sex chromosome aneuploidies.
- Though popular in clinical settings, MPS has not been proven as a strong enough tool to perform a genomewide analysis for noninvasive fetal karyotyping, microdeletions or microduplications. The assessment of the affinity of MPS to subchromosomal deletions and duplications didn’t prove to be adequate.

Fluorescent in Situ Hybridization (FISH): A technique used to localize the presence or absence of a specific DNA sequence on a chromosome. This involves the use of a fluorescent probe on the desired location.
- It works on the basis of nucleic acid base pairs.
- Only the sequence that compliments the probe will be recognized and bound by the fluorescent probes.
- Different probes can be found for different locations on the chromosome.

Quantitative Fluorescence polymerase chain reaction (QF-PCR): Similar to PCR it is for the amplification, and analysis of chromosomal DNA by the usage of fluorescent markers. Unlike PCR it quantifies the number of chromosomes present per screening.
- The total number of markers correspond to the total number of chromosomes present.
• Array Comparative Genome Hybridization (aCGH): Involves co-hybridization of a sample DNA and a control DNA. The sample is fluorescently labelled.
  • They are spotted on a glass slide.
  • Hybridized clones contain DNA present in human DNA and spaced out evenly.
  • It functions on the basis of gain, loss. More signal emanating from the sample means there’s a gain in that genomic region whereas higher signal from the control signifies a loss of that region.
  • Gains and loses are then mapped and depending on the ration of gain, loss or normal aberant regions can be accurately identified.

FETAL SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)

- Mutations or alleles that are inherited by the fetus from the mother that may cause genetic defect.
- Can be genotyped by using maternal plasma DNA sequencing.
- Can be detected by relative haplotype dosage analysis.
- Relative haplotype dosage analysis confirmed to be strong enough for genomewide genotyping.
- Method used to detect ~2.5Mb deletion inherited from the mother.
CONCERNS OF RELATIVE HAPLOTYPE ANALYSIS:

• Requires extra analytical assessment from the mother, either by studying the medical family history or submitting the mother to a haplotype screening.
• It is still unclear if the method could be used for detecting in novo subchromosomal deletion or duplications.

METHODS AND MATERIALS:

• SAMPLE:
  • Pregnant women were recruited and given informed written consent forms.
  • Six cases were screened for and an additional eight to be used as control groups.
    • Cases 01, 02, and 03: Maternal peripheral blood obtained after an invasive procedure was performed.
    • Case 04, 05, and 06: Maternal peripheral blood obtained before any invasive procedure was performed.
      • Blood samples drawn at 12 3/7 to 28 4/7 weeks
DNA was quantified by real-time PCR from the extracted maternal plasma. The target for the PCR was the leptin gene.

A DNA library was prepared by using 13-20 ng of the DNA extracted from the maternal plasma. For test subjects and control groups.

The DNA was then enriched by 12-cycle PCR.

**CALCULATIONS:**

For subchromosomal aberration detection:
- 1Mb bins were merged with 100kb bins and the genomic representation (GRx-y) was calculated.
- RCx-y is the read count

\[
GR_{x-y} = \frac{RC_{x-y}}{RC_{total}}
\]
• Mean and standard deviation of the control group was determined. The z-score for each 1Mb of the test sample was calculated by:

\[
\text{z-score}_{GRx-y} = \frac{GR_{x-y}\text{test} - \text{mean}GR_{x-y}\text{reference}}{SD_{x-y}\text{reference}}
\]

• GRx-y is the genomic representation of the 1Mb
• GRx-y reference and SDx-y reference are the standard deviation and mean of the reference samples.

• The fetal % was determined by:

\[
\text{Fetal\%} = \frac{(GR_{x-y}\text{test} - \text{mean}GR_{x-y}\text{reference}) \times 2}{\text{mean}GR_{x-y}\text{reference}} \times 100\%
\]

• Expected proportion of total molecules (E) was

\[
E = \left(1 + \frac{f}{2} \times d\right) \times \frac{1}{T}
\]

• F= fetal DNA %
• D= change in chromosome number in aberration (d=-1 for microdeletions and +1 for microduplications)
• T= total number of bins for the entire genome
RESULTS:

- Maternal plasma of the eight control samples as well as the six test samples were analyzed.
  - Average of 211 million DNA fragments from each sample was screened.
  - The genome was divided into 2,687 1Mb bins.
  - The genomic representation of each was compared to the genomic representations obtained from the control groups.
  - The z-score was calculated and about 0.3% of the bins were found to fall outside the set interval.
    - From a total 2,687 1Mb bins sequenced, about 8 bins would fall outside the range.
    - To prevent false-positives the only ones considered duplications would require to show three consecutive bins with the same z-score outside the reference interval and with the same direction.

- The z-scores were plotted using the Circos plot.
Circos plot showed that 94.9%-98.7% of the 1Mb bins were normal. Duplicates would only be considered if three consecutive bins showed the same aberration, and the circos plot identified three aberrations marked by red arrows on the Circos plot.

**CASES:**

- **Cases 01, 02, 03:** Underrepresentation was found on the long arm chromosome 22.
- **Cases of 04 and 05:** Overrepresentation also on the long arm of chromosome 22.
  - **Case 04:** Had microduplications in the same region
  - **Case 05:** The mother had the microduplication which was detected in the maternal plasma. Confirmed by CGH.
- **Case 06:** Five consecutive underrepresentations on the long arm of chromosome 3 and underepresentations on the long arm of chromosome 4. Confirmed by full karyotyping.
- The aberrations detected were similar to those seen by CGH, FISH and QF-PCR.
FETAL DNA PERCENTAGE:

- Fetal % was estimated from DNA sequenced at the regions with underrepresentation and overrepresentation.
- Chromosome Y based method was used to compare fetal % on the three cases with male fetuses.
- Fetal de novo fetal %: 9.2% - 17.8%
- Case 05: microduplication fetal % at 96.7%. Suggests that the majority of the circulating DNA would have the change.

SIMULATION ANALYSIS FOR DIAGNOSTIC SENSITIVITY:

- Diagnostic sensitivity of shotgun MPS was found to be:
  - About 96% detection of a 3Mb chromosomal aberration when the fetal % was at 5%.
  - Increased to 99% when fetal % was 6%.
  - Chromosomal aberrations of smaller size would require a larger number of DNA molecules for analysis.
DISCUSSION:

- The possibility of using shotgun MPS for noninvasive prenatal molecular karyotyping at 3Mb resolution has been shown.
  - In five cases subchromosomal deletions or duplications on chromosomes 3q, 4q and 22q have been identified.
  - The sixth case involved maternally inherited microduplication of chromosome 22q

- In three cases maternal plasma was obtained after a noninvasive procedure.
  - Fetal DNA were similar to the fetal DNA percentage of plasma obtained before any invasive procedure.
  - For the diagnostic to be considered valid, an aberration needed to have a z-score of about 0.3%.
  - The aberration needed to be present at least 3 times continuously within a 3Mb bin.
• Current protocol advantage:
  • About 20% of the known pathogenic copy number variants are detected.
  • At 2Mb and 1Mb resolution, sensitivity could reach 99%
    • At this resolution shotgun MPS may be expected to cover about 50%-80% of the known pathogenic copy number variant in the maternal plasma.
  • The cost of screening would drop significantly to a range acceptable by healthcare providers.
    • Sequencing has been reduced
    • May improve diagnostic accuracy in time

OVERALL:

• Shotgun MPS is a possible procedure for detection of prenatal chromosome aneuploidies, subchromosomal changes, and fetal mutations for single gene disorders.
• This method can detect in novo duplications or deletions, unbalanced translocations and maternal copy number changes.
• Any abnormalities revealed by the noninvasive method can be further studied by known invasive methods.
• It may provide a safer form of prenatal screening.
• FISH, Array CGH and QF-PCR are useful methods for DNA detecting chromosomal abnormalities.
• Each case matched the percent of aberrations with their z-scores.
  • The case with the maternally-inherited abnormalities were expected to have a larger percent of abnormal circulating DNA, which was confirmed to be 96.7%
  • In comparison the de novo cases ranged between 9.2% to 17.4%

REFERENCES:
GOAL: TO DEVELOP AND VALIDATE A PATHOGEN DNA ISOLATION METHOD FROM LARGE VOLUMES OF BLOOD THAT ALLOWS OPTIMAL PCR DETECTION

BY: PROFESSOR CLAUDE V. SYLVAIN

BLOODSTREAM INFECTION?

- The presence of pathogenic organisms (i.e., bacteria, mycobacteria, fungi, and viruses) in the bloodstream. It’s mainly found in people who have been recently hospitalized, those with weak immune system, and elderly people with common health issues, and it is the primary origin of septic shock due to inflammatory reactions (Rapid breathing, Rapid heartbeat, fever, inadequate blood flow)
- BSI is one of the leading cause of death in the U.S
- The mortality rate ranges from 10% to 40% and healthcare cost total in the range of billions of dollars per year
- Fast and adequate antibiotic therapy reduces mortality rates
A critical part of the treatment for BSI patients is rapid/immediate identification of the pathogenic organism in order to administer appropriate treatments to eradicate the underlying cause of infection.

Various diagnostic technologies exist for the detection of pathogens in BSI patients.

We will focus on a non-enzymatic and more rapid DNA enrichment method for blood samples that was recently developed, called Polaris vs. two previous methods. One that is enzymatic, involved the removal of human DNA from blood samples called MolYsis, and another that does not entail removal of human DNA (Triton-Tris-EDTA - EasyMAG).

**SUMMARY OF DIFFERENCES BTWN THE 3 METHODS**

- **Polaris**: Blood cell lysis + non-enzymatic DNA degradation + chemical bacterial lysis
- **MolYsis**: Blood cells lysis + enzymatic DNA degradation + enzymatic bacterial lysis
- **TTE**: only red blood cell lysis, no DNA degradation + chemical bacterial lysis
QUANTITY OF PATHOGENS & REMOVAL OF HUMAN DNA

• Because pathogens levels in blood cultures of BSI patients can be extremely low, therefore low quantities of pathogens in the blood result in lower rate of detection for clinically relevant sensitivity
• Also contributing to the reduction sensitivity of the bacteria is human DNA, it gives rise to false-positive signals in PCR assays and inhibit the pathogen-specific PCR.
• Therefore removal of human DNA and amplification of the pathogen DNA by pathogen DNA enrichment method must be performed before the identification PCR, it allows the easy processing of larger volumes of blood and increases the sensitivity of detection of the bacteria.

MATERIALS AND METHODS - PREPARATIONS

• EDTA blood of healthy human volunteers was collected.
• Staph. Aureus (gram positive), Pseudomonas aeruginosa (gram negative), and Candida albicans were cultured overnight on blood agar plates (TSA plates w/ 5% sheep blood)
• After this, a ten-fold serial dilution was made in PBS,
• and the blood was spiked with the pathogenic microorganisms with the different dilutions, yielding 0-1000 CFU/ml blood
Flowchart of Experimental Set-up

DNA ISOLATION METHODS

- For the process of DNA purification, 3 different methods were used:
  I. Triton-Tris-EDTA (TTE) + EasyMAG method (200 µl)
  II. Polaris enrichment + QIAamp (1 ml and 5 ml protocol)
  III. Molysis complete 5 (1 ml and 5 ml protocol)
POLARIS PT. 1 - DEGRADATION OF HUMAN CELLS & DNA

- 1 or 5 ml blood was mixed with an equal volume of selective lysis buffer for 3 minutes
- to lyse blood cells and fragment the released human DNA and then 1 or 5 ml neutralization buffer was added.
- The selective lysis is based on a mild detergent to degrade the human cell membranes but not the bacterial and fungal cell walls.
- An elevated pH will ensure degradation of the released nucleic acids. Therefore, this method focuses on the enrichment of the intact bacteria and fungi from blood and not potential free pathogen DNA.
- The selective lysis reaction needs to be controlled in time as Gram-negative bacteria might be lysed upon prolonged exposure. Therefore an equal volume of neutralization buffer is added after 3 min. This buffer will ensure a complete arrest of the selective lysis treatment by lowering of the pH and dilution of the detergent to an ineffective concentration. At this moment in time, the pathogens will remain intact.

POLARIS PT. 2 - CENTRIFUGATION

- suspensions were centrifuged for 15 minutes (5 ml protocol) or 10 minutes (1 ml protocol)
- Pellets were resuspended in 1 ml washing buffer and centrifuged for 10 minutes at maximum speed in a Eppendorf centrifuge.
- Resulting pellets were thoroughly resuspended in 200 µl bacterial lysis buffer (BLB) and incubated for 10 minutes at 95°C on a thermomixer set at 1000 rpm.
- After addition of 20 µl neutralization buffer 2, lysates were further processed for DNA purification using QIAamp blood mini kit columns (Qiagen, Venlo, The Netherlands) or the generic program of the EasyMAG device.
SUMMARY OF THE POLARIS METHOD

- Selective lysis of blood cells, degradation of DNA (1-5ml)
- Capture Pathogens
- Lyse Pathogens
- DNA Purification
- PCR

REAL-TIME PCR: DETECTION OF PATHOGENIC ORGANISMS

Real-time PCR allows the scientist to actually view the increase in the amount of DNA as it is amplified.
TABLE 2. DETECTION RATES (PERCENTAGE OF POSITIVE PCRS) OF 3 DIFFERENT DNA ISOLATION METHODS IN DILUTIONS SERIES OF 1–1000 CFU/ML.

<table>
<thead>
<tr>
<th>CFU/ml</th>
<th>1000</th>
<th>100</th>
<th>10</th>
<th>1</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>Polaris 5 ml</td>
<td>100% (12/12)</td>
<td>100% (12/12)</td>
<td>100% (12/12)</td>
<td>75% (9/12)</td>
</tr>
<tr>
<td></td>
<td>MoYis 5 ml</td>
<td>83% (10/12)</td>
<td>92% (11/12)</td>
<td>58% (7/12)</td>
<td>17% (2/12)</td>
</tr>
<tr>
<td></td>
<td>TTE-EasyMAG</td>
<td>100% (6/6)</td>
<td>92% (11/12)</td>
<td>70% (11/15)</td>
<td>36% (5/14)</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Polaris 5 ml</td>
<td>100% (12/12)</td>
<td>83% (10/12)</td>
<td>100% (12/12)</td>
<td>75% (9/12)</td>
</tr>
<tr>
<td></td>
<td>MoYis 5 ml</td>
<td>92% (11/12)</td>
<td>83% (10/12)</td>
<td>67% (8/12)</td>
<td>25% (3/12)</td>
</tr>
<tr>
<td></td>
<td>TTE-EasyMAG</td>
<td>100% (10/10)</td>
<td>100% (10/10)</td>
<td>60% (6/10)</td>
<td>30% (3/10)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Polaris 5 ml</td>
<td>100% (6/6)</td>
<td>100% (10/10)</td>
<td>100% (12/12)</td>
<td>70% (7/10)</td>
</tr>
<tr>
<td></td>
<td>MoYis 5 ml</td>
<td>100% (6/6)</td>
<td>75% (6/8)</td>
<td>50% (4/8)</td>
<td>50% (4/8)</td>
</tr>
<tr>
<td></td>
<td>TTE-EasyMAG</td>
<td>100% (6/6)</td>
<td>90% (9/10)</td>
<td>50% (7/12)</td>
<td>30% (2/10)</td>
</tr>
</tbody>
</table>

Fisher's exact test performed on 1 CFU/ml samples, statistically significant when p<0.05. a: Polaris versus MoYis; b: Polaris versus TTE-EasyMAG; c: MoYis versus TTE-EasyMAG.

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PERFORMANCE OF POLARIS: EFFECT OF SAMPLE VOLUME

<table>
<thead>
<tr>
<th></th>
<th>1ml</th>
<th>5ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staph. Aureus</td>
<td>12.5%</td>
<td>70%</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>44%</td>
<td>75%</td>
</tr>
<tr>
<td>C. albicans</td>
<td>75%</td>
<td>75%</td>
</tr>
</tbody>
</table>

- a higher detection rate was observed for the 5 ml samples compared to those derived from 1 ml, indicating that a 5 ml sample provides a higher sensitivity than a 1 ml sample.
COMPARISON OF POLARIS, TTE-EASYMAG AND MOLYSIS

- Calculations of detection rates, i.e., percentages of positive PCRs, demonstrated a detection rate of 100% for all pathogens at a concentration of 10 CFU/ml for the Polaris procedure.
- The TTE-EasyMAG procedure performed much worse in this respect with a detection rate of only 58%, 60%, and 79% for 10 CFU/ml S. aureus, C. albicans, and P. aeruginosa.
- MolYsis resulted in a detection rate of 50%, 67%, and 58% for 10 CFU/ml S. aureus, C. albicans, and P. aeruginosa.
- Processing samples containing 1 CFU/ml never resulted in a 100% detection rate for the tested methods.
- The best results were obtained with Polaris as a 70% detection rate was obtained for S. aureus, and 75% for both C. albicans and P. aeruginosa. MolYsis detection rates at this pathogen concentration varied between 17 and 50%, and TTE-EasyMAG between 20–36%.

CONCLUSION

- In conclusion, Polaris and MolYsis enrichment followed by DNA isolation and real-time PCR enables reliable and sensitive detection of bacteria and fungi from 5 ml blood. However, Polaris is slightly more sensitive and faster providing pathogen identification within 3 hours.
- The Polaris procedure does not use chaotropic agents nor enzymes, but only chemicals that should remain stable over time. Furthermore, it was demonstrated that Polaris pathogen enrichment can be combined with both QIAamp and EasyMAG (generic) DNA purification.
- Polaris and MolYsis have shown to be valuable in spiked blood samples since they can handle large blood volumes. Clinical evaluation of Polaris is presently ongoing in comparison to MolYsis, which is clinically validated. Preliminary results of this ongoing study (Emergency Care Unit, Jeroen Bosch Hospital) show that both pathogen enrichment procedures work for clinical samples.