Malaria Diagnosis

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Why The Concern?

• Most prevalent disease in the world
  – 2.1 billion live in MALARIOUS areas
  – 200-300 million new cases annually
  – 1 million deaths annually

• Sever malaria occurs in children and pregnant women

• In area with lower exposure, malaria affects all age groups
Distribution of Malaria
Epidemiology in Iran

• About 11460 and 6122 cases have been reported in 2008 and 2009 respectively in Iran.

• 90% malaria patients are suffered from Plasmodium vivax, 52% cases belonged to Sistan and Baluchistan province.
• Iran is situated in the Eastern Mediterranean Region
• Southeast of Iran is located in Oriental zone
Etiologic agents of human malaria

- *Plasmodium falciparum*
- *Plasmodium vivax*
- *Plasmodium ovale*
- *Plasmodium malariae*
- *Plasmodium knowlesi*
Life cycle of the malarial parasite
Plasmodium Life Cycle
Relapsing malaria

- *P. vivax* and *P. ovale* hypnozoites remain dormant for months
- They develop and undergo pre-erythrocytic Schizogony
- The schizonts rupture, releasing merozoites and produce clinical relapse
Malarial Paroxysm

• Can get prodrome 2-3 days before
  – Malaise, fever, fatigue, muscle pains, nausea, anorexia
  – Can mistake for influenza or gastrointestinal infection
  – Slight fever may worsen just prior to paroxysm

• Paroxysm
  – Cold stage - rigors
  – Hot stage – Max temp can reach 40-41\degree C, splenomegaly easily palpable
  – Sweating stage
  – P. falciparum and P. vivax tertian
  – P. Malariae quartan
Clinical presentation

• Varies in severity and course
• Parasite factors
  – Species and strain of parasite
  – Size of inoculums of parasite
• Host factors
  – Age
  – Immune status
  – General health condition and nutritional status
Cerebral malaria - pathophysiology

It is believed to result from sequestration of parasitised red cells in the small blood vessels in the brain.

- release of cytokines • which in turn induce the release of nitrous oxide, a known depressor of consciousness
Parasitemia reapperance

- 1-recrudescence
- 2-reinfection
- 3-relapse
Immunity

• Innate
  • Hemoglobin S  sickle cell trait or disease
  • Hemoglobin C and hemoglobin E
  • Thalessemia – α and β
  • Glucose – 6 – phosphate dehydrogenase deficiency (G6PD)
  • Absence of certain Duffy coat antigens improves resistance to *P.v.*
• Acquired immunity
Immunity against P. falciparum
Malaria Diagnosis

- Clinical Diagnosis
- Malaria Blood Smear
- Fluorescent microscopy
- Antigen Detection
- Serology
- Polymerase Chain Reaction
WHO recommendation on malaria diagnosis

• Parasitological confirmation (microscopy or RDT) before treatment

• Exceptions:
  • children under 5 years of age in areas of high transmission - treatment should be based on clinical diagnosis
  • suspected severe malaria, if parasitological confirmation is not immediately possible
Plasmodium falciparum (trophozoite stage in thin smear)

Plasmodium falciparum (trophozoite stage in thick smear)
The Malaria Parasite

Three developmental stages seen in blood films:

1. Trophozoite
2. Schizont
3. Gametocyte
• *Plasmodium vivax*:
  Gametocytes
  Fig. 28 and 29: Nearly mature and mature macrogametocyte (female); Fig. 30:
  Microgametocyte (male)

• *Plasmodium falciparum*:
  Gametocytes
  Figs. 27, 28: Mature macrogametocytes (female); Fig. 29, 30:
  Mature microgametocytes (male)
Quantified Buffy Coat

- Acridine orange
- UV Microscope
- Hematocrit tube
- Centrifuge 5 min 12000 RPM
<table>
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<tr>
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<th>HRP2</th>
<th>pLDH</th>
<th>Aldolase</th>
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<tr>
<td><strong>P. falciparum</strong> -specific antigen</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pan-specific antigen</td>
<td></td>
<td>+</td>
<td>+</td>
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<tr>
<td><strong>P. vivax</strong> -specific antigen</td>
<td></td>
<td>+</td>
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RDTs: test formats

The products come in a number of formats:
- Plastic cassette
- Card
- Dipstick
- Hybrid cassette-dipsticks
RDT procedure

1. Collect blood
Current RDT formats:

- Card/cassette/dipstick
- HRP2
- HRP2 & aldolase
- pLDH
- Pf & pan
- pLDH Pf & Pv
- "COMBO" tests
- pHRP2, pLDH pan & pLDH Pv
- aldolase
- Price range: Up from 0.50 $ for dipstick, 0.60 $ for cassette.

2

Mix blood with lysis buffer and detection antibodies on strip (or in well or test tube)
RDT procedure

Lysed blood and reagents migrate up strip
Antigen Detection

Antigen-antibody complex + Immobilized monoclonal antibody = Antibody-antigen-antibody complex
COMPONENTS OF ANTIGEN DETECTION TEST BEFORE USE

- **P. falciparum**
  - Control

- **P. vivax**
- **P. ovale**
- **P. malariae**
- **P. falciparum**
  - Control

- Negative
Antigen Detection
Malaria Immunochromatographic Dipstick

OptiMAL Assay

*P. falciparum* specific monoclonal antibody

*Plasmodium* pan specific monoclonal antibody

Control
RDT

1. PLDH/HRP2 Combo Card Test

Company: Premier Medical Corporation Ltd. Made In INDIA. Website: www.premiermedcrop.com European Representative: Transnational Technologies Inc. United Kingdom.

2. Care Start Malaria RAPYD TEST

Company: DiaSys. Made In England
Problems with RDTs

• The cost of the RDT also varies from test to test and from country to country

• *Cross-reactions with auto antibodies*: Studies have reported cross reactivity of the various RDTs with auto antibodies such as rheumatoid factor (3-6%)

• pLDH and HRP2 may remain positive 5-6 days and 2-3 weeks after treatment respectively.
Sensitivity and Specificity

- Overall for HRP-2, the meta-analytical average sensitivity and specificity were 95.0% (93.5% to 96.2%) and 95.2% (93.4% to 99.4%), respectively.
- Overall for pLDH, the meta-analytical average sensitivity and specificity were 93.2% (88.0% to 96.2%) and 98.5% (96.7% to 99.4%), respectively.
Detection Of Antimalarial Antibodies

• Malarial antibodies can be detected by immunofluorescence or enzyme immunoassay.
• It is useful in epidemiological surveys, for screening potential blood donors and occasionally for providing evidence of recent infection in non-immunes. Malaria vaccines.
• Assessment of eradication
Indirect Fluorescent Antibody (IFA)
Indirect immuno-cytochemistry: ELISA

ELISA = Enzyme-linked Immunosorbent Assay
پروب‌های DNA

• توالی نوکلئوتیدی ژن‌های مختلف یک گونه با هم یکسان نیست. در آین روش سکانس شناخته شده اسید نوکلئیک (نیکوپنیک) ژن‌های متفاوت با مواد رادیواکتیو اکتیو (P32) يا معرف رنگی غیرradiowaکتیو نشان دهنده تأثیر این پروب برای مشخص کردن اسیدنوکلئیک انگل به کار می‌رود و این مزیت را دارد که سکانس‌های مکمل ساخته خواهند شد.
Polymerase chain reaction (PCR)

- The PCR test is reportedly 10-fold more sensitive than microscopy, with one study reporting a sensitivity to detect 1.35 to 0.38 parasites/µL for *P. falciparum* and 0.12 parasites/µL for *P. vivax*. 
30 - 40 cycles of 3 steps:

**Step 1: denaturation**
1 minute 94 °C

**Step 2: annealing**
45 seconds 54 °C
forward and reverse primers !!!

**Step 3: extension**
2 minutes 72 °C
only dNTP's
PCR تکنیک‌های با اساس

• Nested PCR
• SCCP (single strand conformational PCR)
Principle of the nested –PCR method

1. From the sequences of strain-specific segment of DNA determine a specific segment.
2. Design the sequences of external and internal primer pairs.
Nested PCR

- Plasmodium 18 S ribosomal ribonucleic acid (rRNA) gene
- Primers
  - rPLU5: 5CCT GTT GTT GCC TTA AAC TTC 3
  - rPLU6: 5TTA AAA TTG TTG CAG TTA AAA CG 3

  Initial denaturation 95°C 5 min
  Denaturation 94°C 30 s ↘
  Annealing 58°C 120 s → 25 cycle
  Extension 72°C 120 s ↗
  Post extension 77°C 5 min
Outer PCR
Plasmodium vivax
(Multiplex-PCR) PCR مولتی پلکس

در این نوع PCR، به طور همزمان از چند جفت پرایمر استفاده می‌شود و چندین قطعه زنی تکثیر می‌یابد. در این تکنیک سرعت کار افزایش می‌یابد اما راهاندازی این روش مستلزم وقت بیشتری است تا شرایط کار به صورت مطلوب مشخص شود. امروزه این تکنیک در تشخیص بیماری‌های زنتیک، عوامل عفونی، میکروپی و... جایگاه مهمی را در مراکز تحقیقاتی و آزمایشگاه‌های طبی بی خود اختصاص داده است.
بررسی محصول PCR از طریق آنزیم‌های محدود‌الاثر بر روی بسیار مطلوب و نسبتاً سریع و ساده است. برای این کار با آنزیم‌های محدود اثر، محصول PCR (DNA) به را به قطعاتی تقسیم کرده و قطعات مورد نظر را از طریق الکتروفورز در ژل مشاهده می‌کنند. با در نظر گرفتن محل اثر آنزیم‌های بر شده دهنده (محدود اثر) وجود یا عدم وجود قطعه مورد نظر مشخص می‌گردد.

(Restriction Fragment Length Polymorphism) RFLP
1) In intact probes, reporter fluorescence is quenched. (2) Probes and the complementary DNA strand are hybridized and reporter fluorescence is still quenched. (3) During PCR, the probe is degraded by the Taq polymerase and the fluorescent reporter released.
Plasmodium falciparum antigens
Some tested vaccines

• The SPf66 vaccine against the blood stages showed a small effect in some studies, but not in Africa. Spf66 (Patarroyo 1987)
  Trial 1: In Colombia it gave an efficacy of 33% over all ages and 77.2% over ages < 5 years
  Trial 2: In Gambia it gave an efficacy of 8% in 6-11 month babies and 9% over ages 2-15 years
• Another vaccine (MSP/RESA) did not prevent malaria attacks, but meant the density of parasites in the blood was lower.
• NYVAC-p7
RTS,S Trial

- A recombinant protein of the 19 NANP CSP repeats (R), along with T-cell epitopes (T) fused to the hepatitis B surface antigens, with infused S antigen (RTS,S) with adjuvant.

- New adjuvants; QS21 (Saponin extract of Quillaja saponaria) + MPL (Monophosphoryl Lipid A) with Oil-in-water emulsion (= AS02) or Liposome suspension (= AS01).
RTS,S/ASO2

• In 2003 in a trial in Mozambique 2022 children from one to 4 years received three dose of either RTS,S ASO-2 or control vaccine. The vaccines efficacy was 35% and demonstrating 49% relative efficacy rate against severe malaria.

• In recent trial in 214 Mozambican infants (3 to 5 months) vaccine efficacy against new P.falciparum infection was about 65% after 6 moths follow-up period and 33% over 14 months of follow-up
RTS,S/ASO1

• In 2008 a recent trial in infants (5 to 17M) in Kenya and Tanzania (3 doses immunization with RTS,S ASO1ε) efficacy was 60% against all episodes of P. falciparum malaria

• After 6 months follow-up the incidence in those vaccinated was 8% (32 out 402) whereas the incidence in control group was 16% (66 out 407)
Fig-1 Gel photograph showing PCR amplified products of EBA-175 from different *Plasmodium falciparum* infected isolates in the southeast of Iran. The DNA size marker is a 100 bp ladder shown on the left and right side. Lane2 and 10 are multiple infection and negative control respectively.
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<tr>
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<th>Chloroquine</th>
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<tr>
<td>Anti-ES</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
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<tr>
<td>Anti-EE</td>
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<td>Anti-Gametocyte</td>
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Prevention

• Chemoprophylaxis
  - Chloroquine / pyrimethamine
  - used for
  - prophylaxis of malaria

Chemotherapy: 1 week before entry into the endemic area; for 4 weeks after returning from the endemic area.