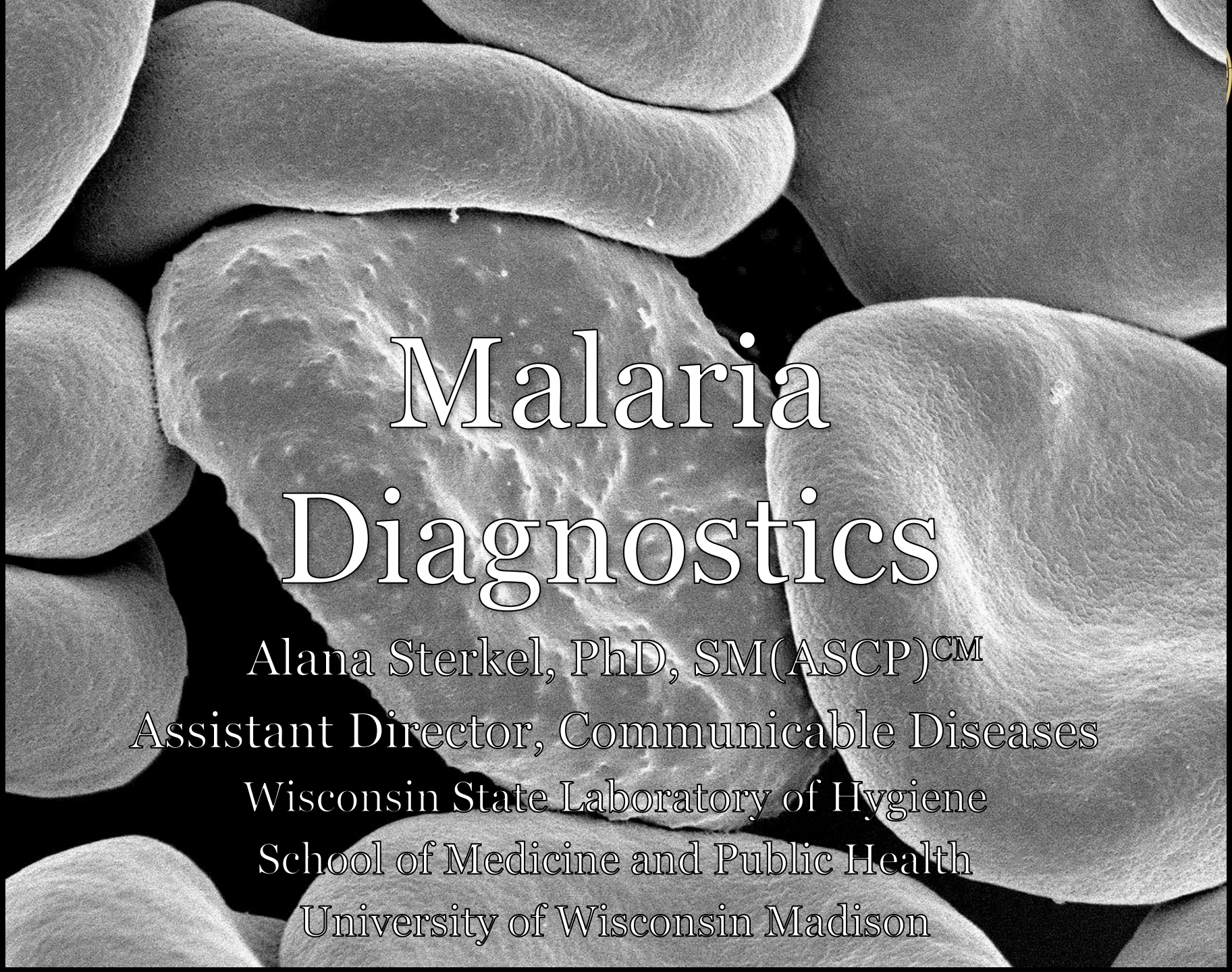
Wisconsin State Laboratory of Hygiene

UNIVERSITY OF WISCONSIN–MADISON



Malaria Diagnostics

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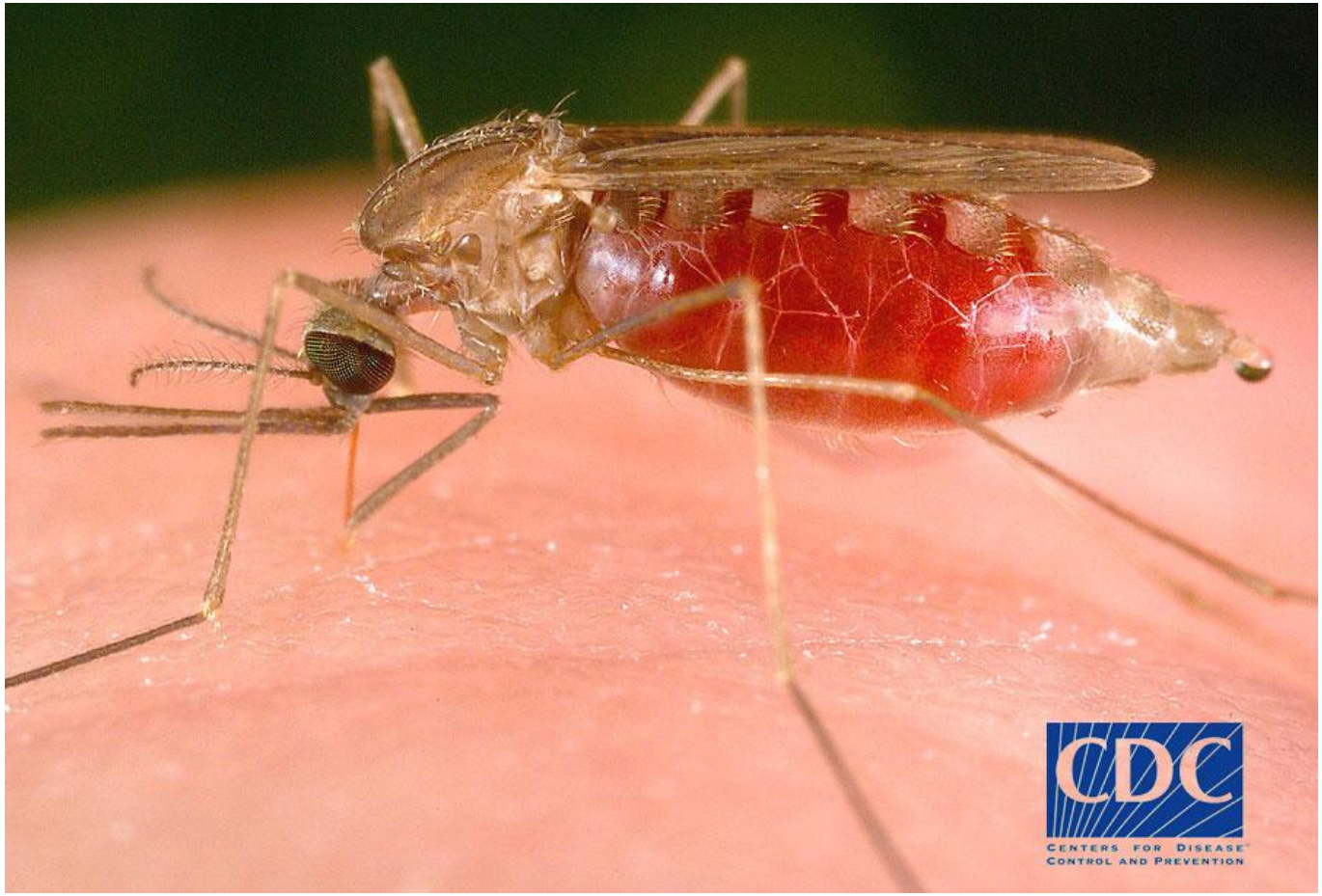
No relevant conflicts of interest to disclose



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  - Staining
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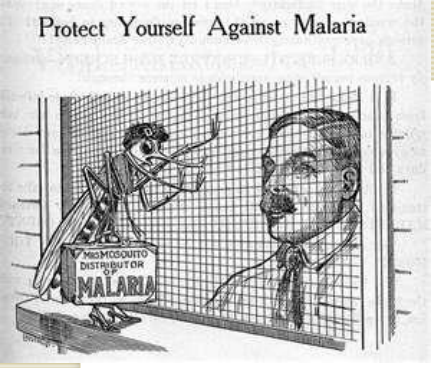
- New and emerging causes of Malaria

Disease Background



Incidence

- The World Health Organization estimates that in 2015 malaria caused 212 million clinical episodes, and 429,000 deaths.
  - 90% of deaths were in sub-Saharan Africa
  - 77% of deaths were in children under 5 years of age
- Approximately 1,700 cases of malaria are reported every year in the United States

Malaria in the USA

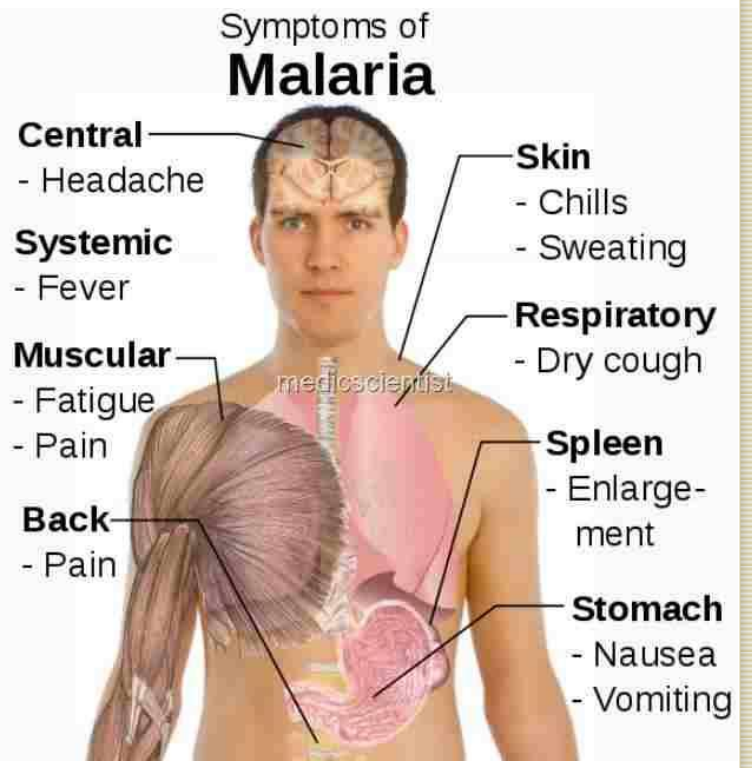
- Malaria was declared eliminated from the United States in the early 1950's.
- Between 1957 and 2015, in the United States, 63 outbreaks of locally transmitted mosquito-borne malaria have occurred
- During 1963-2015, 97 cases of transfusion-transmitted malaria were reported in the United States

Plasmodium

- Members of the *Plasmodium* genus infect a wide range of birds, mammals, reptiles, and amphibians worldwide
- Transmitted by the Anopheles mosquito
  - Can also be transmitted through transfusion or congenitally, although this is very rare.
- Over 200 species, but only 5 routinely cause human infections
  - *falciparum, vivax, ovale, malariae,* and *knowlesi*

Disease

- Infection can range from asymptomatic to life threatening.
- Immune compromise, young or elderly age, pregnancy, and fetuses are at greatest risk for severe disease
- Paroxysms of Fever, rigors, chills, and sweating
- Headache, malaise, and myalgia
- Convulsions, respiratory distress, hypoglycemia, circulatory collapse, renal failure, and coma



Plasmodium Disease Features

	falciparum	*vivax*	*ovale*	*malariae*	*knowlesi*
Incubation period	8-11	10-17	10-17	18-40	9-12
Fever cycles	36-48 hours "Tertian"	44-48 hours "Tertian"	48 hours "Tertian"	72 hours "Quartan"	24 hours "Quotidian"
Infection duration	6-17 months	5-8 years	12-20 months	20-50 years	Information lacking
Degree of parasitemia	High	Low, <2%	Low, <2%	Low, <2%	High
Average severity of disease	Moderate to Severe	Moderate to Severe	Mild	Mild to moderate	Moderate to Severe
Relapse	No	Yes	Yes	No	Not likely
Area of endemicity	Large range; Tropics and subtropics	Large range; Tropics and subtropics except west Africa	Tropics; sub-Saharan Africa and Southeast Asia	Narrow range; tropics	Narrow Range; Southeast Asia

Diagnostics

Diagnostics

- Malaria can be immediately life threatening. Testing should be ordered upon admission
- Malaria testing should be available on a 24-hour STAT basis
- Requests for testing should be accompanied by information related to clinical signs and symptoms, travel history, and receipt of malaria chemoprophylaxis or therapeutic antimalarial agents.
- Testing methods
  - Microscopic examination of blood films
  - Molecular analysis
  - Antigen detection

Need for Trained Specialists

- Accurate interpretation relies on the availability of trained and experienced microscopists, high quality reagents, and well-maintained light microscopes.
- The average laboratorian does not perform this test regularly, and may not be maintaining optimal proficiency.
- WSLH offers STAT testing by experienced staff
  - M-F 7:45am-4:30pm
  - STAT testing also available

608-263-3280 WSLH
608-258-0099 WDPH

Blood films

- The optimal time to test is between chills
  - Increases the likelihood of identifying advanced morphologies that will aid in species identification
  - Do not delay an initial exam upon admission
- The Clinical and Laboratory Standards Institute (CLSI) recommends preparation of two thin and two thick blood films for each test
- One negative set of blood films does not eliminate *Plasmodium* infection
  - This should be clearly relayed back to the physician
- If malaria remains a possible diagnosis, after the first set of negative smears, samples should be taken for at least 3 successive days.
  - As often as intervals of 6 to 8 h
- Positive patients should have additional testing at 24, 48, and 72 hours after initiating therapy to evaluate for drug resistance.
  - Parasitemia usually drops within 24 hours, often by 50% or more

Blood Collection- Finger prick

- Blood is ideally collected via finger prick with immediate (bedside) preparation
- Prick a patient's finger with a sterile, nonreusable lancet
- Let the blood flow freely
  - blood that has to be “milked” from the finger is diluted with tissue fluids, which decrease the number of parasites per field.
- Touch a slide to the drop of blood

Blood Collection- Finger prick

FIGURE A-1. Blood collection for thin or thick blood films

1
Wear gloves.

2
Clean slides with 70%–90% alcohol, dry them, and label them. Do not touch the surface of the slide where the blood film will be made.

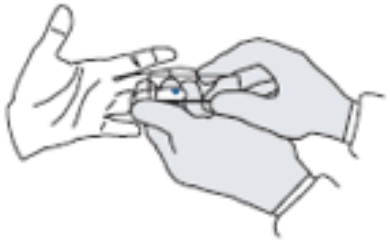
3
Select the finger to puncture, usually the middle or ring finger. In infants, use the heel.

4
Clean the area to be punctured with 70% alcohol; let dry.

5
Puncture the ball of the finger or in infants, the heel.

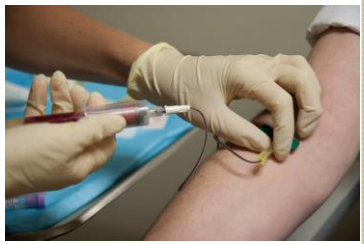
6
Wipe away the first drop of blood with gauze.

7
Touch the next drop of blood with a clean slide. Repeat with multiple slides if multiple films are needed. If blood does not well up, gently squeeze the finger. Be careful not to touch the blood films when handling the slides!



Blood Collection- Venipuncture

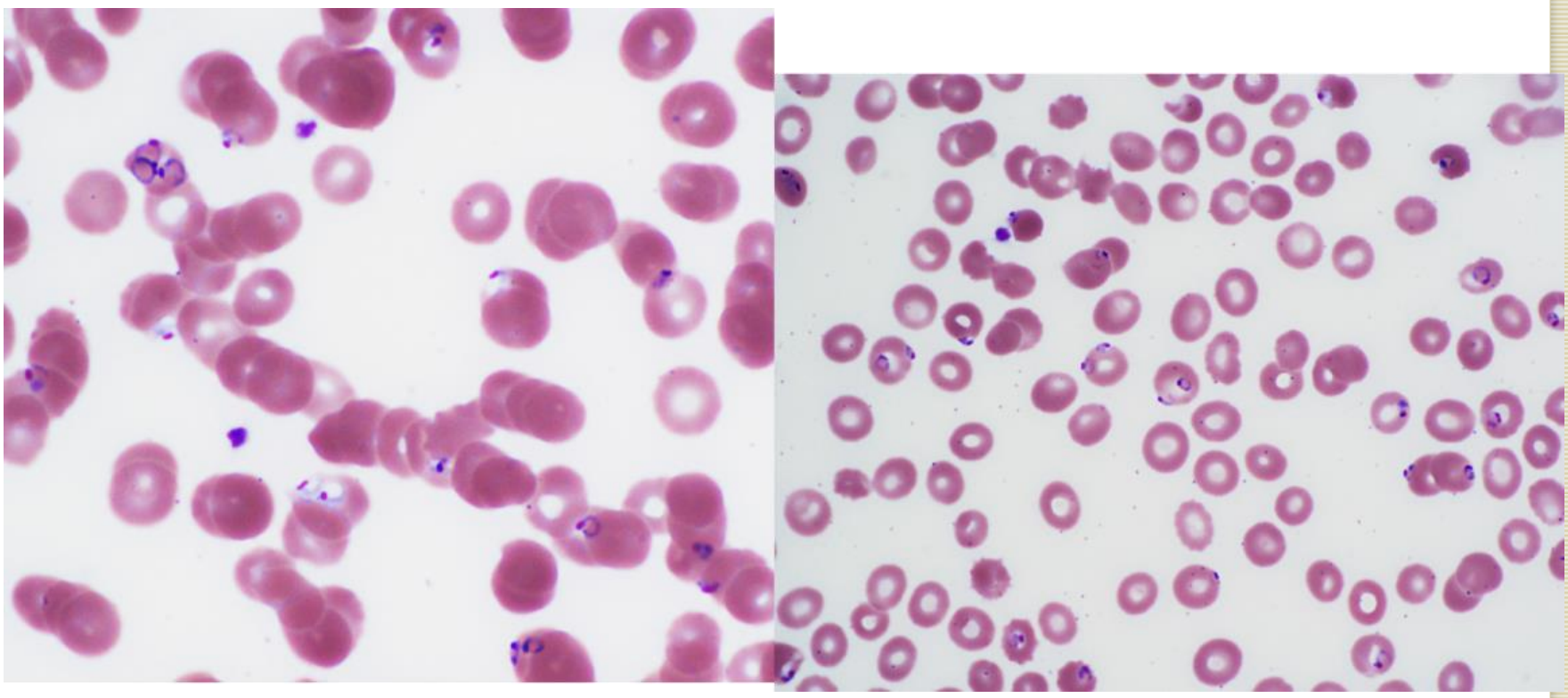
- Not ideal, but a possible alternative.
- More common in nonendemic settings for rapid transport to the laboratory
- If possible, use the blood remaining in the needle prior to mixing with anticoagulants
- EDTA is the preferred anticoagulant
  - the use of other anticoagulants such as heparin may cause significant parasite distortion
- EDTA blood (vs finger prick)
  - May take longer to dry
  - More likely to flake or peel off the slide (low filled tube)
  - RBCs have reduced stippling
  - RBCs may become crenated making it difficult to determine size and fimbrination



Case

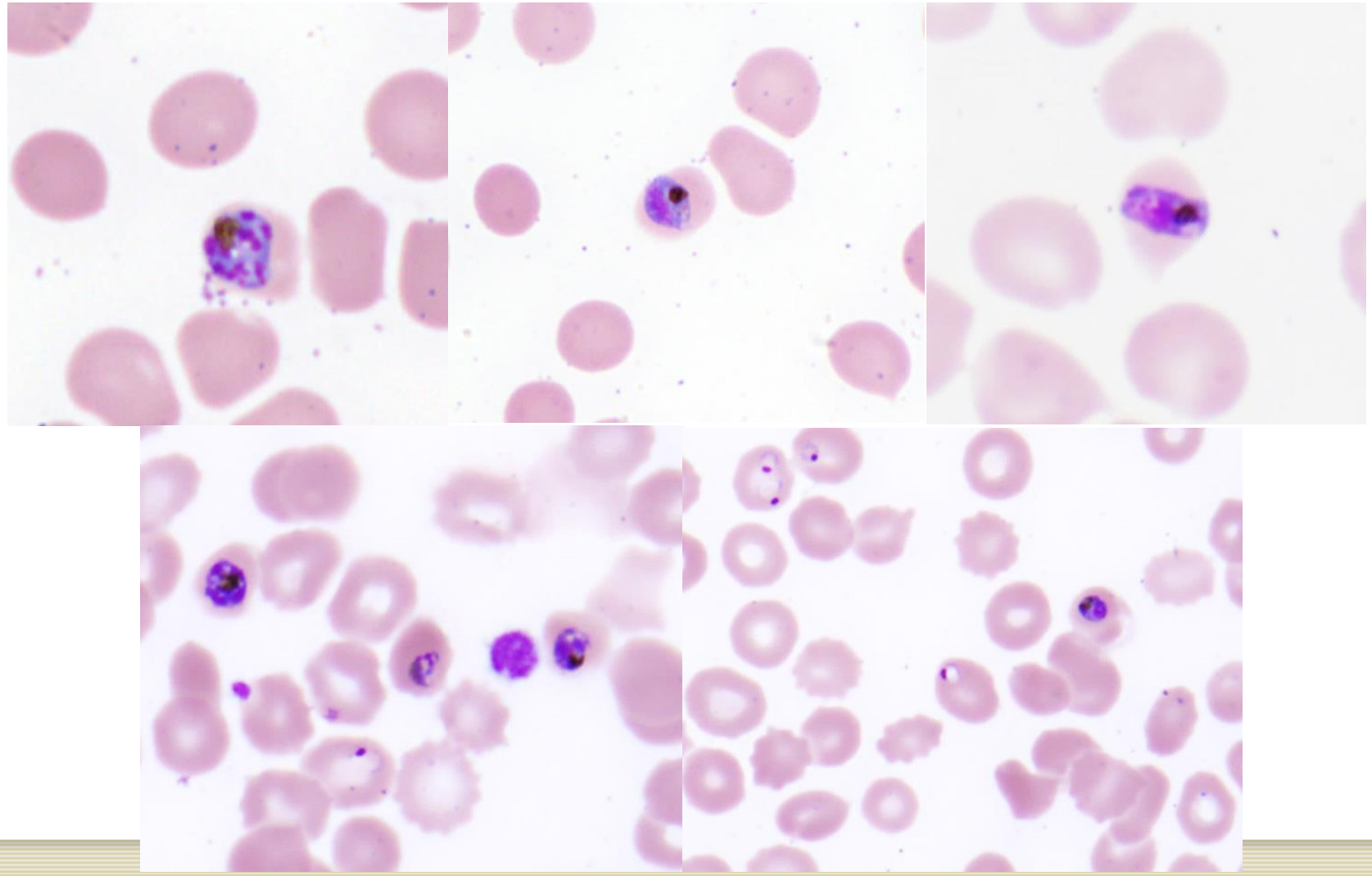
- 28 year old male present to the ER with fever and recent travel to Africa.
- He is admitted to the hospital and blood is drawn.
- Infectious Disease is consulted and Malaria testing is ordered

Thin Blood Smears



8% parasitemia

Advanced Morphologies



Case Continued

- Patient parasitemia rises to 12%
- State lab testing is requested
- CDC is consulted

- All results come back as *P. falciparum*
- An investigation reveals the slides were made 4 hours after venipuncture.
- Nurses didn't want to subject their patient to another draw so soon.

Blood Collection- Venipuncture

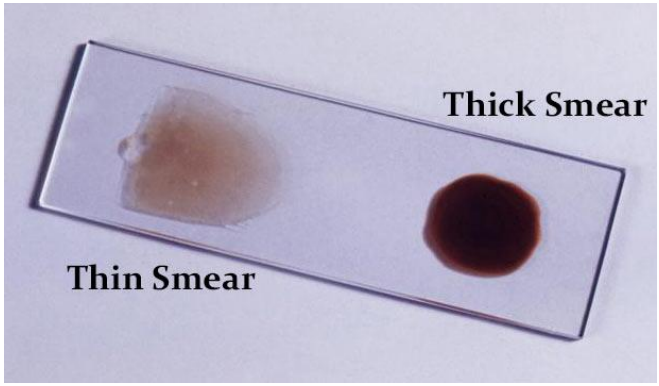
- Ideally, the smears should be prepared within 30-60 min after the specimen is drawn.
- A delay in slide preparation may complicate species identification
  - reduce stippling
  - morphologic changes
  - maturation into sexual life cycle stages

Slide types

- Thin Films: A drop of blood is spread over a large surface area
  - Species identification is better because parasite and RBC morphologies are more clear
  - Parasitemia measurements are more accurate
  - Sensitivity lower than thick films
- Thick films: A larger amount of blood is examined in a smaller area
  - 10 to 20 times more sensitive
  - Can be examined faster
  - Species identification is more difficult

Slide Preparation

- Thin film
  - One drop of blood (~50uL) near the end
  - Spread the drop using a second slide held at 45 degrees
- Thick film
  - 1-2 drops of blood should be spread to the size of a dime or nickel (1.5-2.0 cm)
  - Should be just barely able to read newsprint through the wet slide
    - Most common error is to use too much blood
  - Stir the slide for 30 second to remove fibrin
    - Not necessary if using EDTA blood

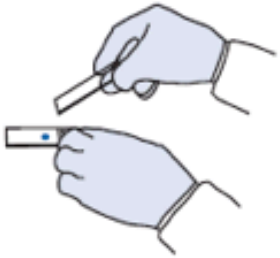


Slide Preparation

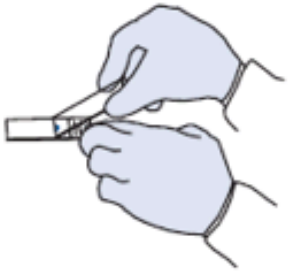
FIGURE A-2. Preparation of thin and thick blood films

1
Whenever possible, use separate slides for thick and thin films.

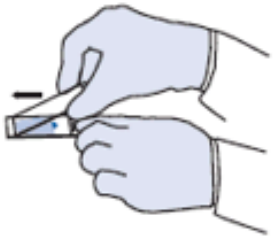
2
Thin film (a): Bring a clean spreader slide, held at a 45-degree angle, toward the drop of blood on the specimen slide.



3
Thin film (b): Wait until the blood spreads along the entire width of the spreader slide.

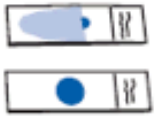


4
Thin film (c): While holding the spreader slide at the same angle, push it forward rapidly and smoothly.



5
Thick film: Using the corner of a clean spreader slide, spread the drop of blood in a circle the size of a dime (diameter 1–2 cm). Do not make the smear too thick or it will fall off the slide (you should be able to read newsprint through it).

6
Wait until the thin and thick films are completely dry. Fix the thin film with 100% (absolute) methanol. Do not fix the thick film.



7
If both the thin and thick films must be made on the same slide, fix only the thin film with 100% (absolute) methanol. Do not fix the thick film.

8
When the thin and thick films are completely dry, stain them. Thick smears might take ≥1–2 hours to dry. Protect unstained blood smears from excessive heat, moisture, and insects by storing in a covered box.

CDC DPDx

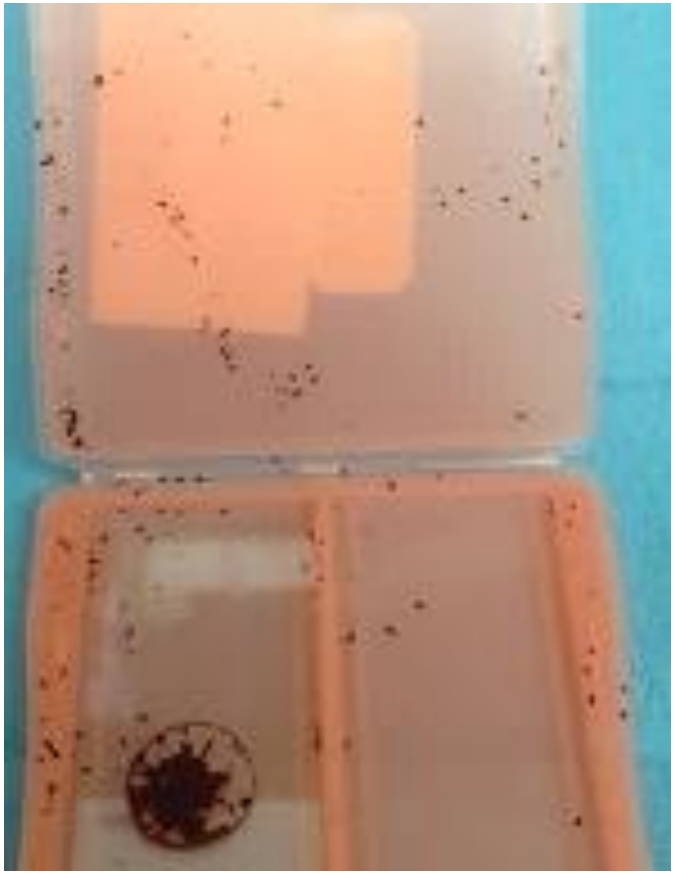
Slide Preparation

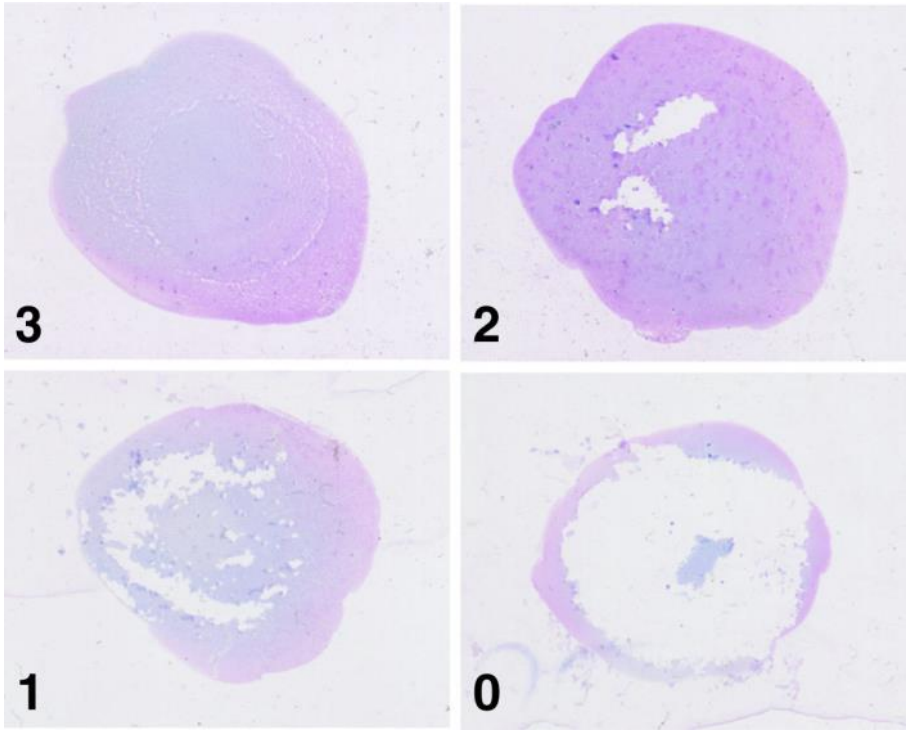
- Allow the film to air dry (room temperature) in a dust-free area.
  - Thin- at an angle
  - Thick- horizontal (8-12 hours)
  - Slides may be placed under a light fan to hasten drying (1-4 hours)
- Never apply heat to a film
  - Heat fixes the blood, causing the erythrocytes to remain intact during staining
  - This leads to stain retention and an inability to identify the parasites
- If thick films are to be stained at a later time, they should be lysed (laked) before storage
  - 10 min in Giemsa buffer
  - **Or**, quickly dipped in H_2O (up to 10 sec)

Troubleshooting

- Avoid fingerprints by only holding the sides
- Store in a dust proof container
- Use absolutely clean, grease-free slides
  - Best results are obtained when slides are cleaned
  - Immerse in 70% Ethanol, drain on a paper towel, polish with a lint free cloth

Troubleshooting- Flaking

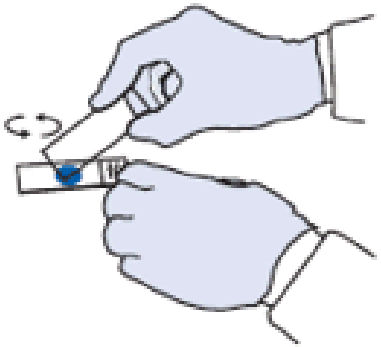




Troubleshooting- Flaking

- If too much blood is used or any grease remains on the slide, the blood may flake off during staining.
- At the correct thickness you should be just able to read text through the blood

- Lyse the smear immediately after drying to reduce flaking
- Scraping the slide surface with a second slide during preparation improves adherence
- Fresh blood is less likely to flake



Norgan, et. al. A method for reducing the sloughing of thick blood films for malaria diagnosis. Malaria Journal 2013 **12**:231

Other Points to Remember

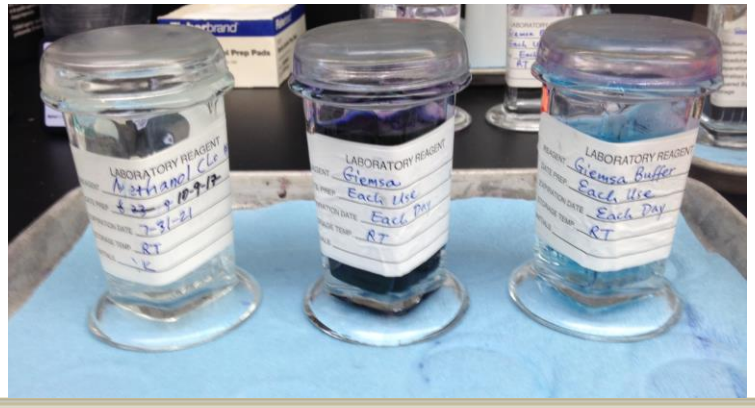
- Blood films should be stained on the same day or within a few days of collection
  - Prolonged storage may result in stain retention
- It is not recommended to combine thick and thin on the same slide
  - It can delay reporting while waiting for drying
- Detection of blood parasites by automated instruments is not recommended
  - Failure to detect parasitemias have been reported

Staining

- Giemsa is the stain of choice
  - Color descriptions in texts and at DPDx are based on Giemsa and may not be the same with other stains
- Wright or Wright-Giemsa can be used but species determination may be more difficult
  - Schuffner's dots and Maurer's clefts may not visible or as clear
- It is better to do one stain well than several stains poorly.

Giemsa Staining Protocol

1. Prepare fresh Giemsa
2. Pour 40 ml of Giemsa buffer into a second jar. Add 20 µl (2 drops) of Triton X-100.
3. Thin smears only: Fix slides in absolute methanol, 1-5 seconds and air dry
4. Place slides into the Giemsa stain for 45-60 minutes.
5. Remove thin smear slides and rinse by dipping 3-4 times in the Giemsa buffer. Thick smears should be left in buffer for 5 minutes.
6. Air dry slides upright in a rack. Fan may be used to shorten dry time.



Alternate Giemsa Protocols

Reagent	15-minute stain (7.5%)	45-minute stain (2.5%)	60-minute stain (2.0%)
Buffered water	37 mL	39 mL	49 mL
5% Triton X-100	2 drops	2 drops	2 drops
Stock Giemsa (filtered)	3 mL	1 mL	1 mL

Examination of Slides

- Examination of blood films for parasites should always be considered a STAT procedure.
- All results should be relayed by telephone to the physician as soon as possible.
- All positive results should be reported to public health agencies.

Examination of Slides

- Blood smears should first be examined at low power (100x) to evaluate for microfilariae.
- Thin films:
  - Smears should be examined in an area where there is a single layer of distinctive red blood cells.
  - Depending on the experience of the microscopist, examination usually takes 15-20 min.
  - Due to variations in speed a minimum of 300 oil immersion fields at 1000X should be examined.
  - More fields should be examined if something suspicious is found.
- Thick films:
  - Examination of a thick film usually requires 5-10 min (~100 oil immersion fields)

Determination of Parasitemia

- **The number of parasite per 100 RBCs or per uL of blood**

- **Thin smears**: Preferred
  - The percent of infected RBCs is determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs. A minimum of 500 RBCs or 10 fields total should be counted.

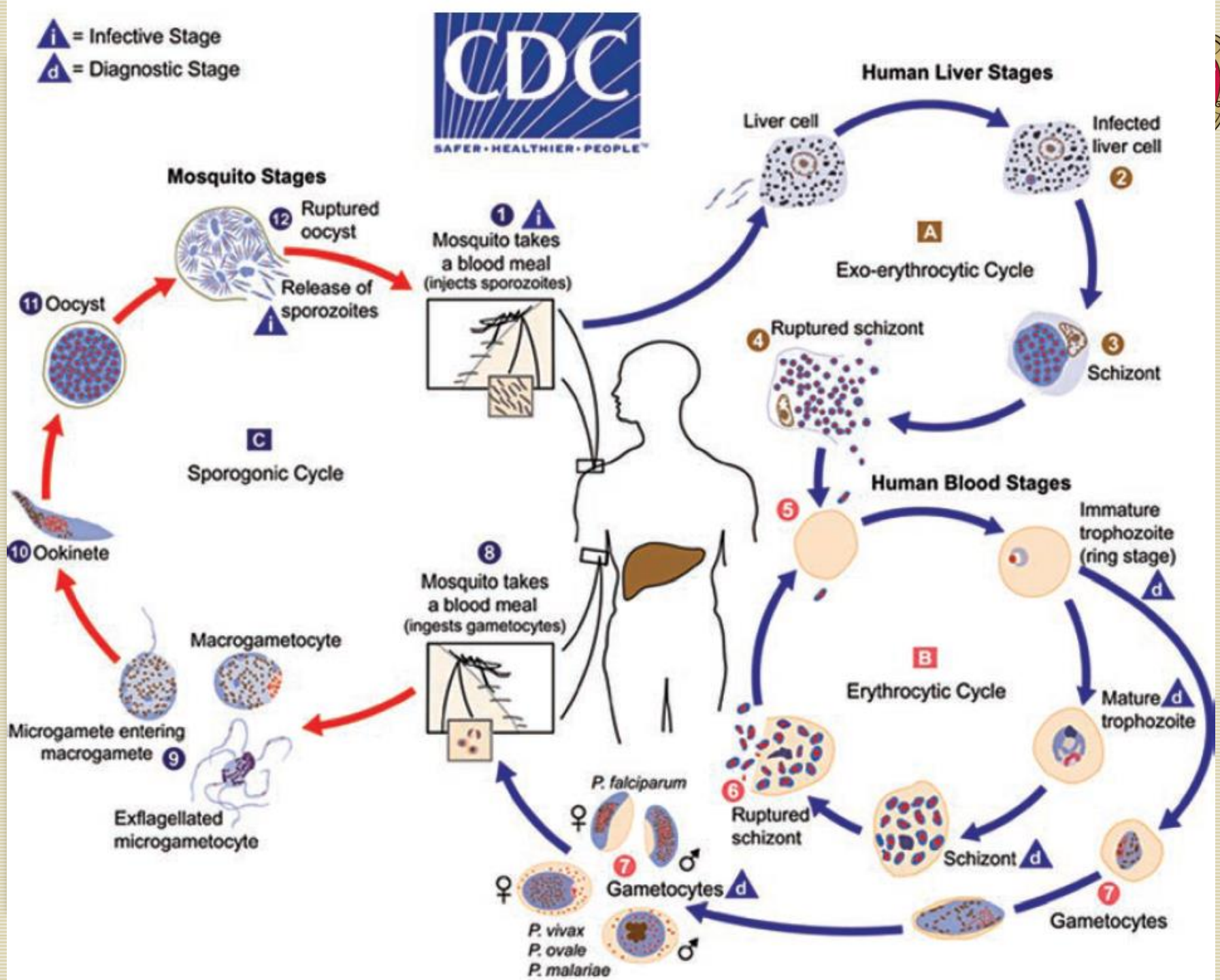
    (# infected RBCs ÷ Total # RBCs counted) × 100

    = Percent Infected RBCs
  - Notes:
    - Multiply-infected RBCs are counted as one.
    - Gametocytes are not figured in calculations.

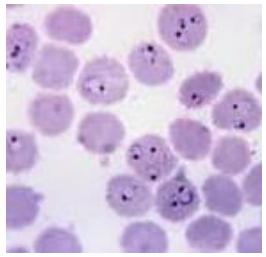
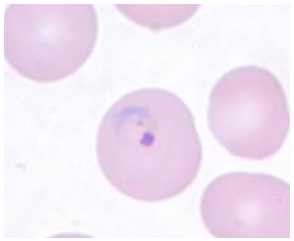
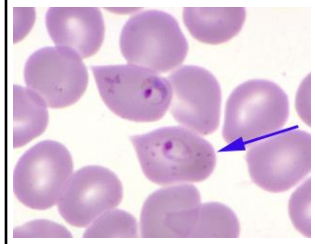
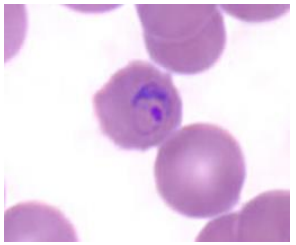
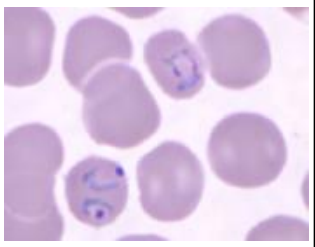
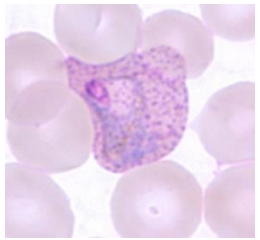
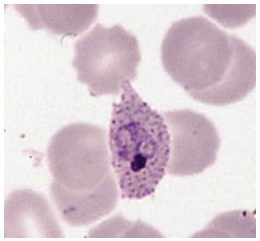
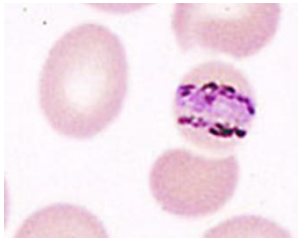
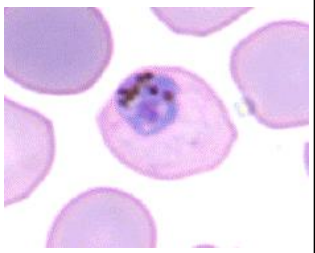
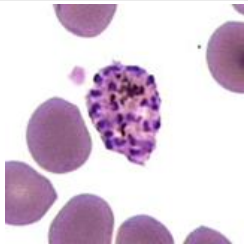
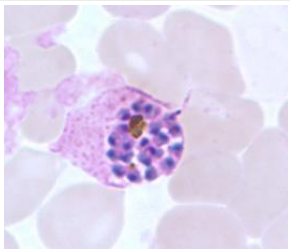
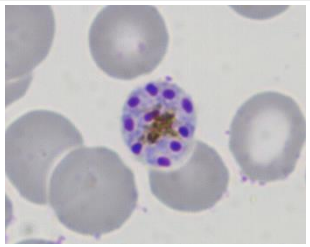
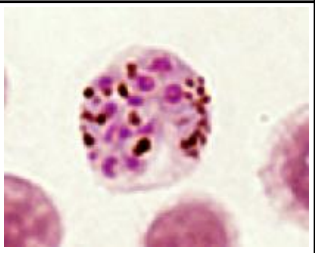
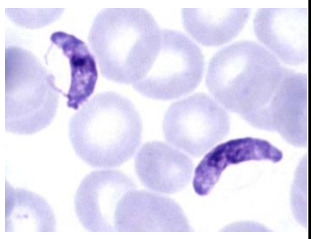
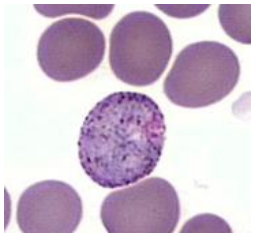
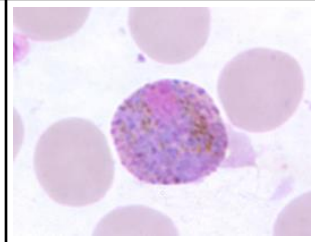
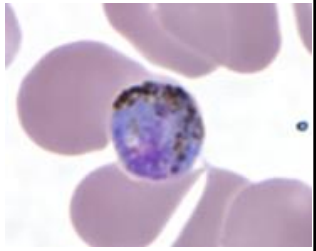
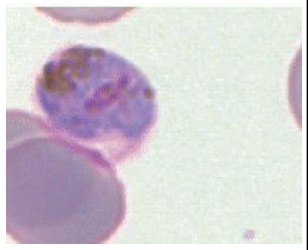
- **Thick smears**: Not ideal
  - The number of parasites/µl of blood is determined by enumerating the number of parasites in relation to the standard number of WBCs/µl (8000).

    # Parasites × (8000 ÷ # WBCs counted)

    = # parasites per µL of blood


i = Infective Stage
d = Diagnostic Stage
CDC
SAFER·HEALTHIER·PEOPLE™
Human Liver Stages
Liver cell
Infected liver cell
2
A
Exo-erythrocytic Cycle
3
Schizont
4
Ruptured schizont
Human Blood Stages
5
Immature trophozoite (ring stage)
d
Mature trophozoite
d
B
Erythrocytic Cycle
6
Ruptured schizont
Schizont
d
7
Gametocytes
d
Gametocytes
P. falciparum
P. vivax
P. ovale
P. malariae
8
Mosquito takes a blood meal (ingests gametocytes)
Macrogametocyte
Microgamete entering macrogamete
9
Exflagellated microgametocyte
10
Ookinete
11
Oocyst
12
Ruptured oocyst
Release of sporozoites
i
Mosquito Stages
1
Mosquito takes a blood meal (injects sporozoites)
C
Sporogonic Cycle

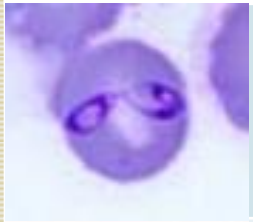
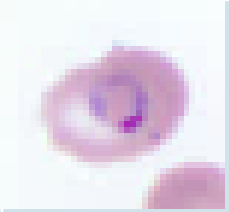
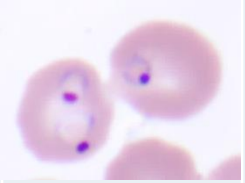
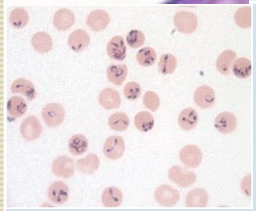
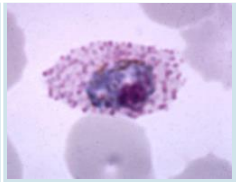
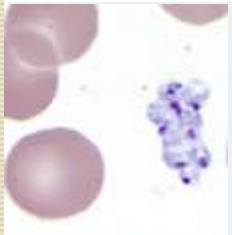
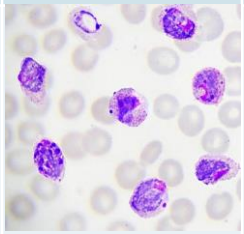
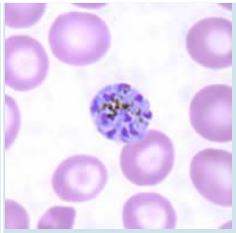
Plasmodium morphological features in peripheral blood

	falciparum	*vivax*	*ovale*	*malariae*	*knowlesi*
Immature Trophozoite (ring form)					
Mature Trophozoite (amoeboid form)	Very rarely seen				
Schizont	Very rarely seen				
Gametocyte					

Plasmodium morphological features in peripheral blood

	falciparum	*vivax*	*ovale*	*malariae*	*knowlesi*
Immature Trophozoite (ring form)	Small, delicate, 1-2 chromatin dots; occasional appliqué forms; normal RBC; occasional Maurer's clefts	Large cytoplasm, large chromatin dot; occasional Schuffner's dots; normal to large RBC	Average ring; normal to large RBC; occasional Schuffner's dots	Average ring; normal RBC; rarely, Ziemann's stippling	Delicate cytoplasm; 1 to 2 chromatin dots; occasional appliqué forms; normal RBC; rarely, Sinton and Mulligan's stippling
Mature Trophozoite (amoeboid form)	Very rarely seen; compact cytoplasm; dark pigment; normal RBC	Large amoeboid; yellowish-brown pigment; Schuffner's dots; fine, large RBC	Compact; dark-brown pigment; Schuffner's dots; normal to large RBC	Occasional band forms; dark-brown pigment; normal RBC; rarely, Ziemann's stippling	Occasional band forms; coarse, dark-brown pigment; rarely, normal RBC; Sinton and Mulligan's stippling
Schizont	Very rarely seen; 8 to 24 small merozoites; dark pigment, clumped in one mass; normal RBC	12 to 24 merozoites; yellowish-brown pigment; Schuffner's dots; large RBC	4 to 14 merozoites with large nuclei, clustered around dark-brown pigment; Schuffner's dots; normal to large RBC	6 to 12 merozoites with large nuclei, clustered around coarse, dark-brown pigment; rarely, normal RBC; Ziemann's stippling	Up to 16 merozoites with large nuclei, clustered around coarse, dark-brown pigment; normal RBC; rarely, Sinton and Mulligan's stippling
Gametocyte	Rare; banana shaped; dark pigment	Large and round; brown pigment; Schuffner's dots; large RBC	Round to oval; brown pigment; Schuffner's dots; normal to large RBC	Round to oval; rarely, Ziemann's stippling	Round to oval; rarely, Sinton and Mulligan's stippling

Babesia vs Malaria

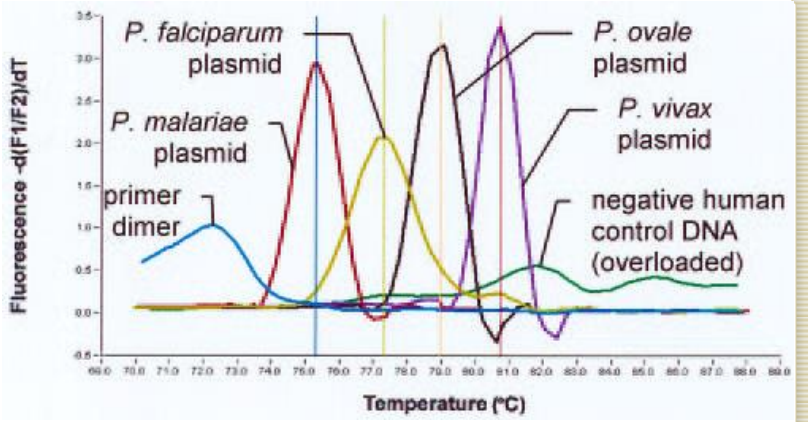
Babesia		Malaria	
	Tear drop shaped ring forms		Round signet ring forms
	Size variability White central vacuole		Fewer parasites per red cell
	Higher parasitemias		Schuffner's dots
	Extracellular forms		Advanced forms
	Maltese cross		Hemazoin pigment

When You Find a Positive

- All positive Malaria patients should be reported to WSLH and WDHS.
- Please send a thick and 2 thin smears along with EDTA blood to WSLH
- Confirmatory testing is fee exempt and provides percent parasitemia, species identification, and for new patients, molecular confirmation of ID.
- WSLH is also able to send blood on to the CDC if drug susceptibility testing is needed.

Molecular Detection of Malaria

- WSLH Protocol
  - EDTA blood
  - Extract genomic DNA
  - Real-Time PCR
  - Targets the 18S rRNA gene
  - Melt-curve analysis
  - Able to detect mixed infections

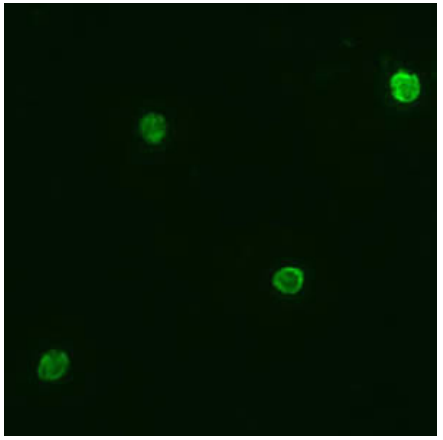


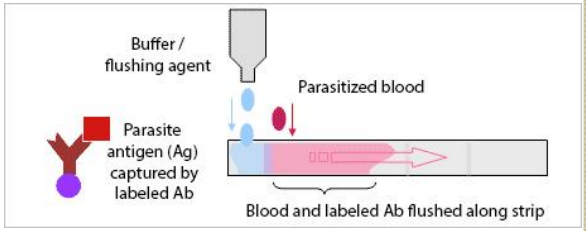
- New probe based PCR currently in development- greater specificity
- Any sample that is smear positive but PCR indeterminate will be sent to the CDC

Mangold *et al* . Real-Time PCR for Detection and Identification of *Plasmodium* spp. Journal of clinical Microbiology, Vol. 43, No. 5, May 2005, p. 2435-2440,

Antigen Detection of Malaria

- Malaria antigens used for these rapid diagnostic tests are histidine-rich protein 2 (HRP- 2), parasite lactate dehydrogenase (pLDH), and *Plasmodium* aldolase
- These tests may have reduced sensitivity to microscopy (0.002% parasitemia)
- Most only detect *P. falciparum* or *Plasmodium* species. Further testing may be needed to identify the species





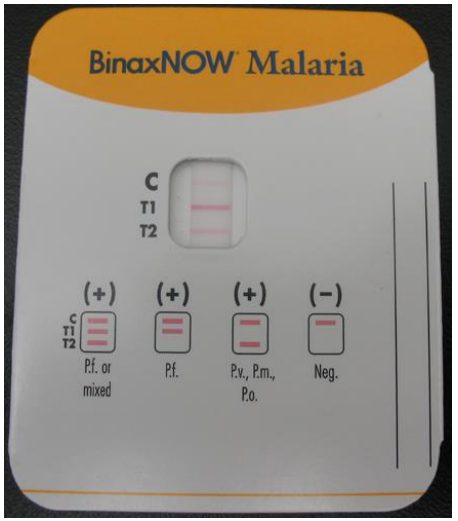
Sulzer AJ, and Wilson M. The fluorescent antibody test for malaria. Crit Rev Clin Lab Sci 1971;2:601-609.

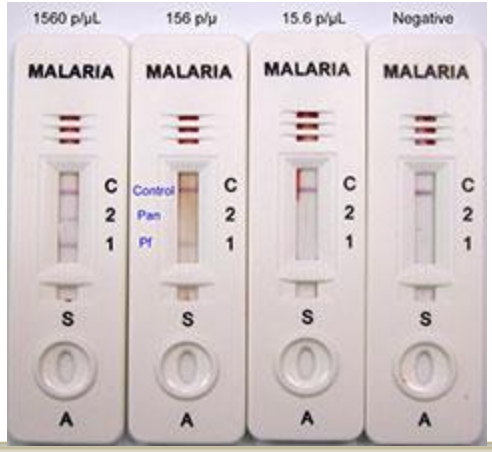
Antigen Detection of Malaria

- Negatives should be confirmed by microscopy
- Cross reactions often occur between *Plasmodium* species and *Babesia*
- Rare false positives for patients with rheumatologic disorders
- The general recommendation is to use these tests only in addition to the microscopic examination of thick and thin blood smears.

Antigen Detection of Malaria

- May be useful for:
  - Field testing when microscopy is not available
  - screening blood donors
  - testing a patient who has been recently treated for malaria but in whom the diagnosis is questioned
  - labs with low incidence and difficulty in maintaining proficiency on the microscope.
  - 3rd shift to identify disease faster when trained personnel are not available for microscopy

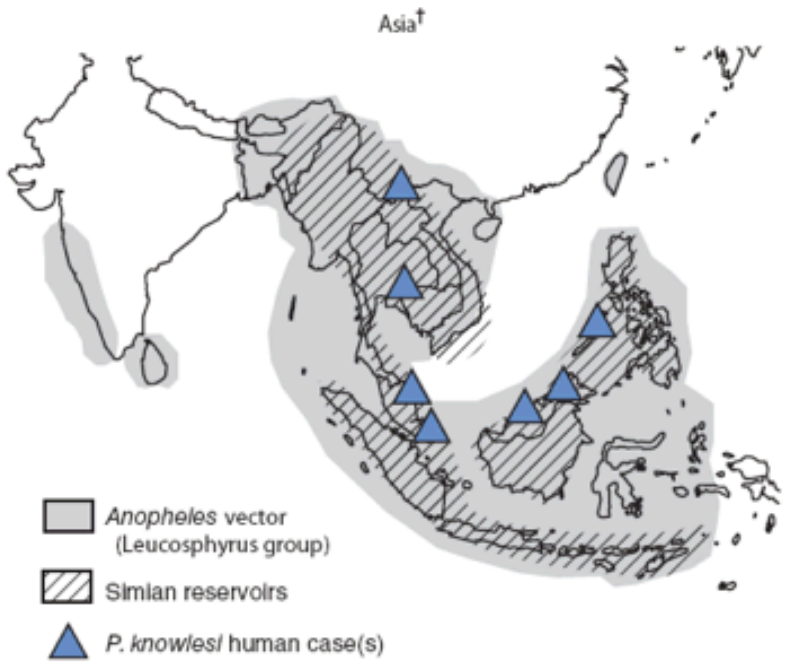




New and emerging causes of Malaria

P. knowlesi

- Geographically confined to Southeast Asia
- Predominantly infects macaque monkeys
- First naturally acquired human case 1965
- Reporting has greatly increased since 2004
- Cause of up to 70% of malaria cases in some areas



Other Simian *Plasmodium* species and their look alikes

TABLE. Simian malaria species in Asia and South America with their associated geographic distribution and morphologic similarity to one of four human *Plasmodium* species*

Simian *Plasmodium* species	Geography	Human species they resemble
Asia		
P. coatneyi	Malaysia, Philippines	*P. falciparum*
P. cynomolgi	India, Indonesia, Malaysia, Sri Lanka, Taiwan	*P. vivax*
P. eylesi	Malaysia	*P. vivax*
P. fieldi	Malaysia	*P. ovale*
P. fragile	India, Sri Lanka	*P. falciparum*
P. hylobati	Indonesia	*P. vivax*
P. inui	India, Indonesia, Malaysia, Phillipines, Sri Lanka, Taiwan	*P. malariae*
P. jeffreyi	Indonesia, Malaysia	*P. vivax*
P. knowlesi	China, Indonesia, Malaysia, Philippines, Singapore, Thailand, Taiwan	*P. malariae*, *P. falciparum*
P. pithreci	Malaysia	*P. vivax*
P. simiovale	Sri Lanka	*P. ovale*
P. silvaticum	Malaysia	*P. vivax*
P. youngi	Malaysia	*P. vivax*
South America		
P. brasilianum	Brazil, Colombia, Mexico, Panama, Peru, Venezuela	*P. malariae*
P. simium	Brazil	*P. vivax*

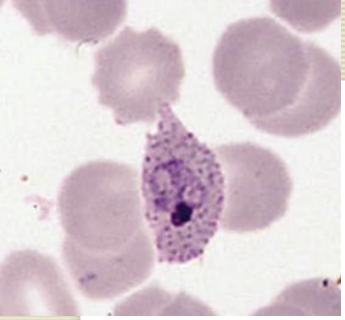
*Four species of intraerythrocytic protozoa of the genus *Plasmodium* (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) are known to cause malaria in humans.

MMWR. March 13, 2009 / 58(09);229-232

P. simium

- Recent outbreak of *P. simium* in Brazil
- In 2015-2016 over 49 cases of *P. vivax* reported in in Rio de Janeiro
- All related to jungle exposure
- *P. simium* is only distinguishable from *P. vivax* through two single-nucleotide polymorphisms in the mitochondria

Brasil et al. bioRxiv. 2017
Brasil et al. The Lancet. 2017
Grigg and Snounou. The Lancet. 2017



P. ovale

- In 2010 multilocus sequence analysis revealed that *P. ovale* is actually 2 related species
- They geographically overlap
- They do not interbreed
- Can only be distinguished through sequencing
- To avoid confusion caused by a name change the names *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* have been proposed
- *P. o. wallikeri* may have
  - Reduced or absent Schuffner's stippling
  - A shorter latency period
  - Worse disease

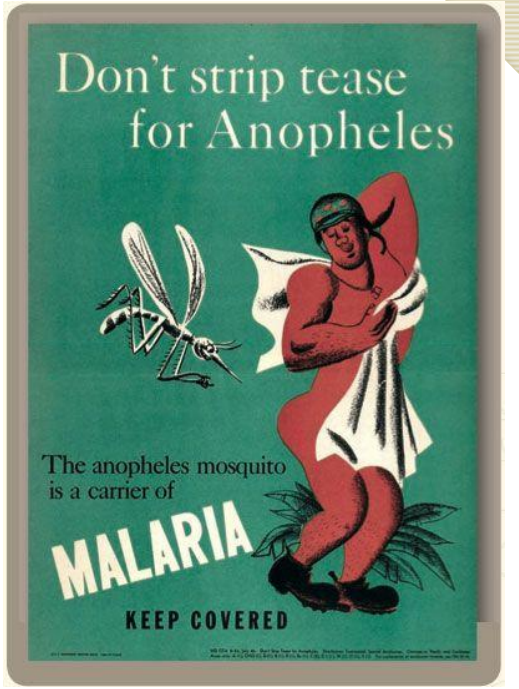
MCM

Phoung et al (2016) Malaria Journal 15:550

Conclusions

- Malaria is still a prevalent and dangerous pathogen.
- Lab diagnosis can be challenging and requires well trained staff
- New technologies are developing to reduce subjectivity and enhance sensitivity.
- Simian *Plasmodium* species are able to cause disease in humans and may emerge as major causes of Malaria

References



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