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Amplicon Based Next Generation Sequencing for Parasitology

54th Annual Region I ASM Meeting
November 7, 2019

How Parasitologists Work(ed)



"Whatever it is, it's very, very little."

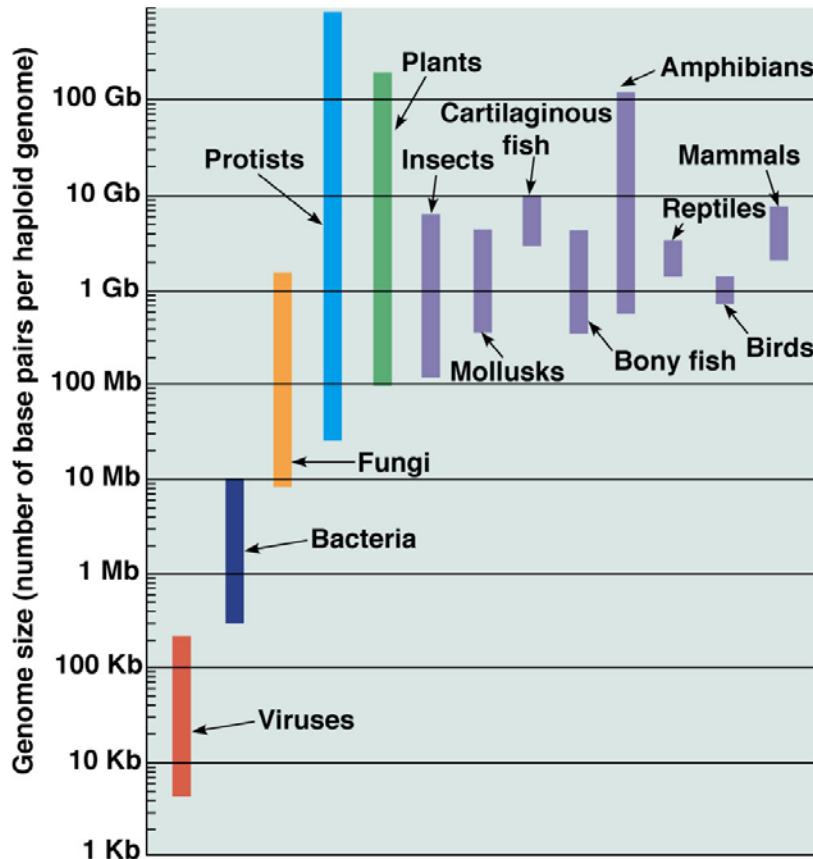
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Can Parasitology Use Next Generation Sequencing ?



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Genomes are larger

Most parasites cannot be cultured
primary specimens

Some genomes are very AT rich
P. falciparum 80%

Eucaryotes



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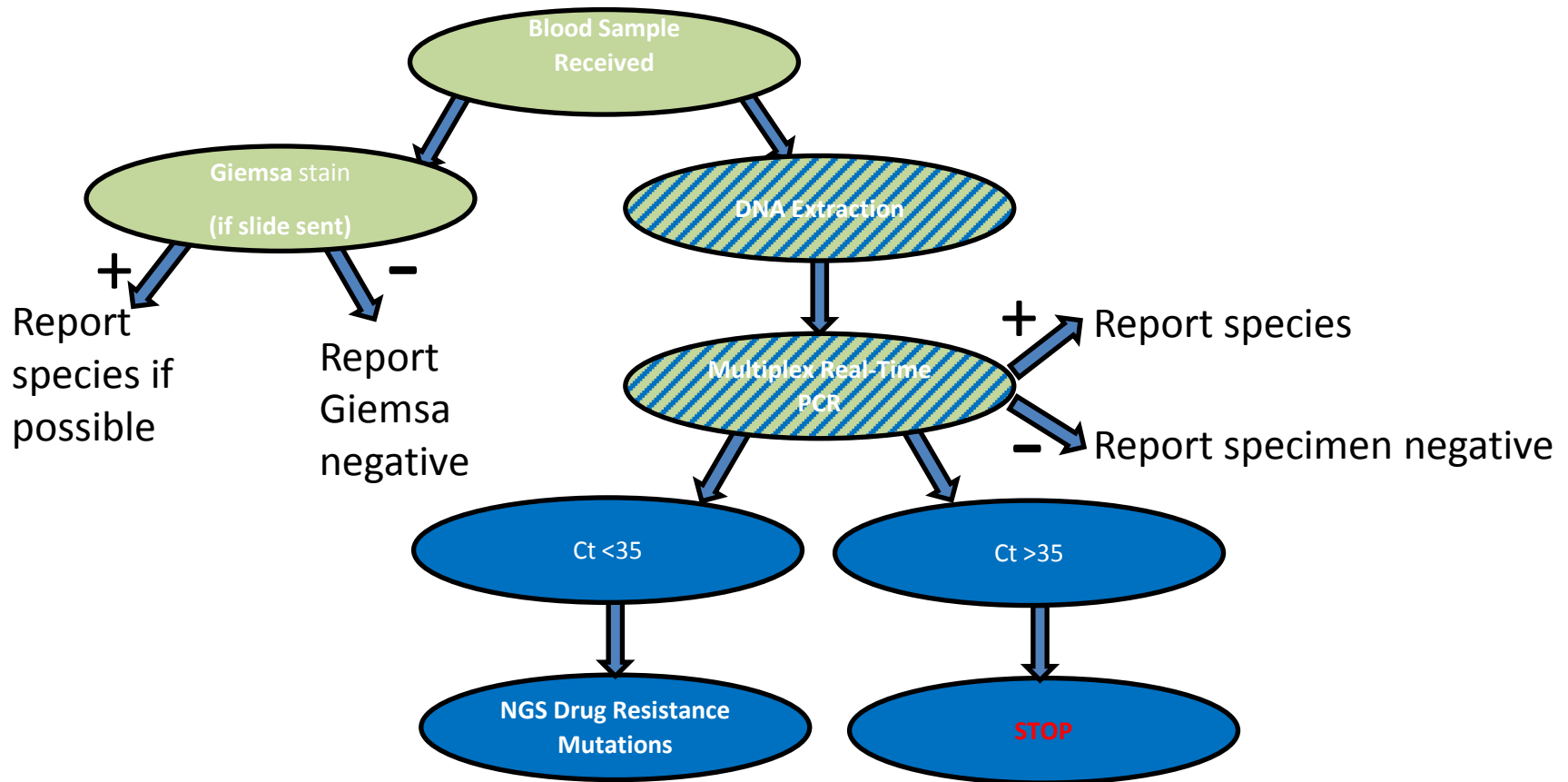
P. falciparum
**Targeted Next
Generation
Sequencing for
Drug Resistance**



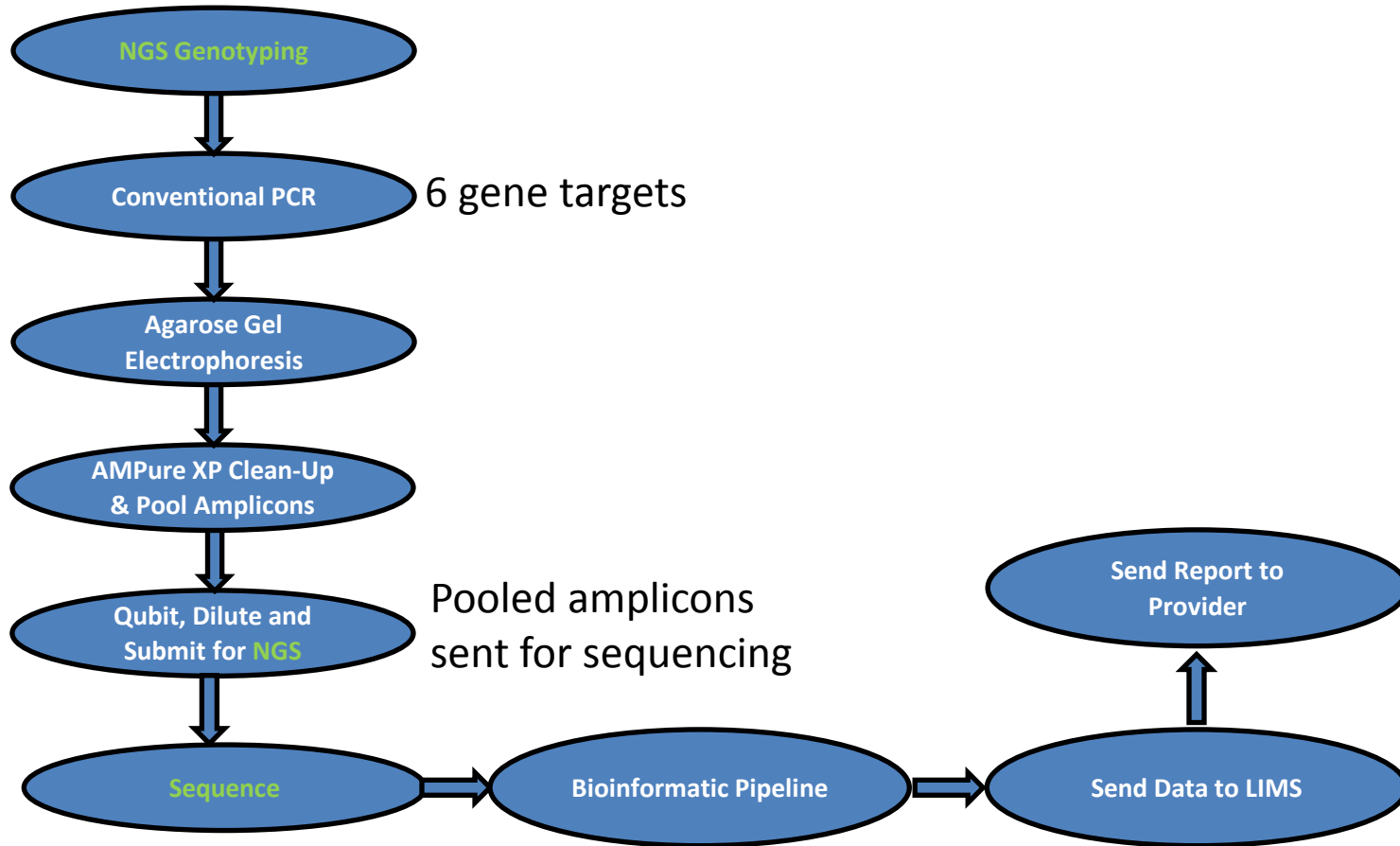
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What is NYS Doing Currently to Test for Malaria Infections?



P. falciparum NGS Workflow



Gene	Amplicon Length (bp)	Resistance Mutations	Drug(s)
<i>Pfcrt</i>	3109	M74I, N75E, K76T , A220S, Q271E, N326S, I356T, R371I	Chloroquine
<i>Pfdhfr</i>	2067	A16V, N51I, C59R, S108N/T , I164L	Proguanil, Pyrimethamine
<i>Pfdhps</i>	2817	S436A/F, A437G , K/G540E , A581G, A613S/T	Sulfadoxine
<i>Pfmdr1</i>	4155	N86Y, Y184F, S1034C, N1042D, D1246Y	Amodiaquine, Lumefantrine, Quinine
<i>Pfcytb</i>	937	I258M , Y268S/C/N	Atovaquone
<i>Pfk13</i>	2120	F446I, N458Y , M476I, Y493H , R539T , I543T , P553L, R561H , C580Y	Artemisinin



Example Report

Plasmodium falciparum Molecular Drug Resistance Prediction- Targeted Next Generation Sequencing

Predicted Resistance Profile*

Chloroquine:	RESISTANT (predicted)
Atovaquone:	Susceptible (suggested)
Artemisinin:	Susceptible (suggested)
Pyrimethamine/Proguanil:	Susceptible (suggested)
Sulfadoxine:	Susceptible (suggested)
Multi-drug:	Susceptible (suggested)

High-Confidence Mutations Detected*

crt (chloroquine):	High-confidence mutation
cytb (atovaquone):	No high-confidence mutation
k13 (artemisinin):	No high-confidence mutation
dhfr (pyrimethamine/proguanil):	No high-confidence mutation
dhps (sulfadoxine):	No high-confidence mutation
mdr1 (multi-drug):	No high-confidence mutation

In the literature, mdr1 (multidrug resistance 1), is a gene associated with resistance for several antimalarials.

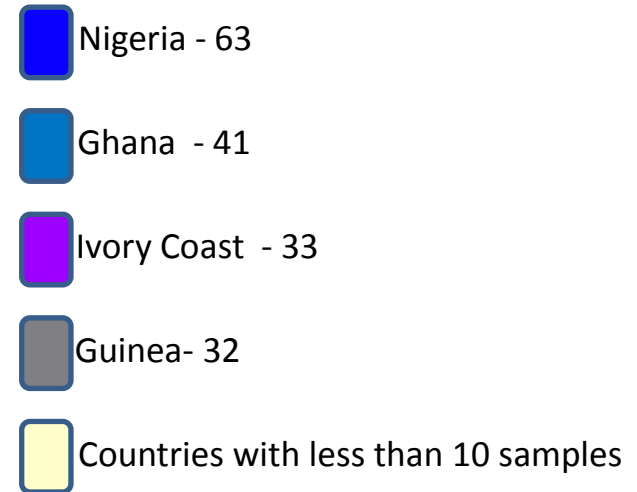
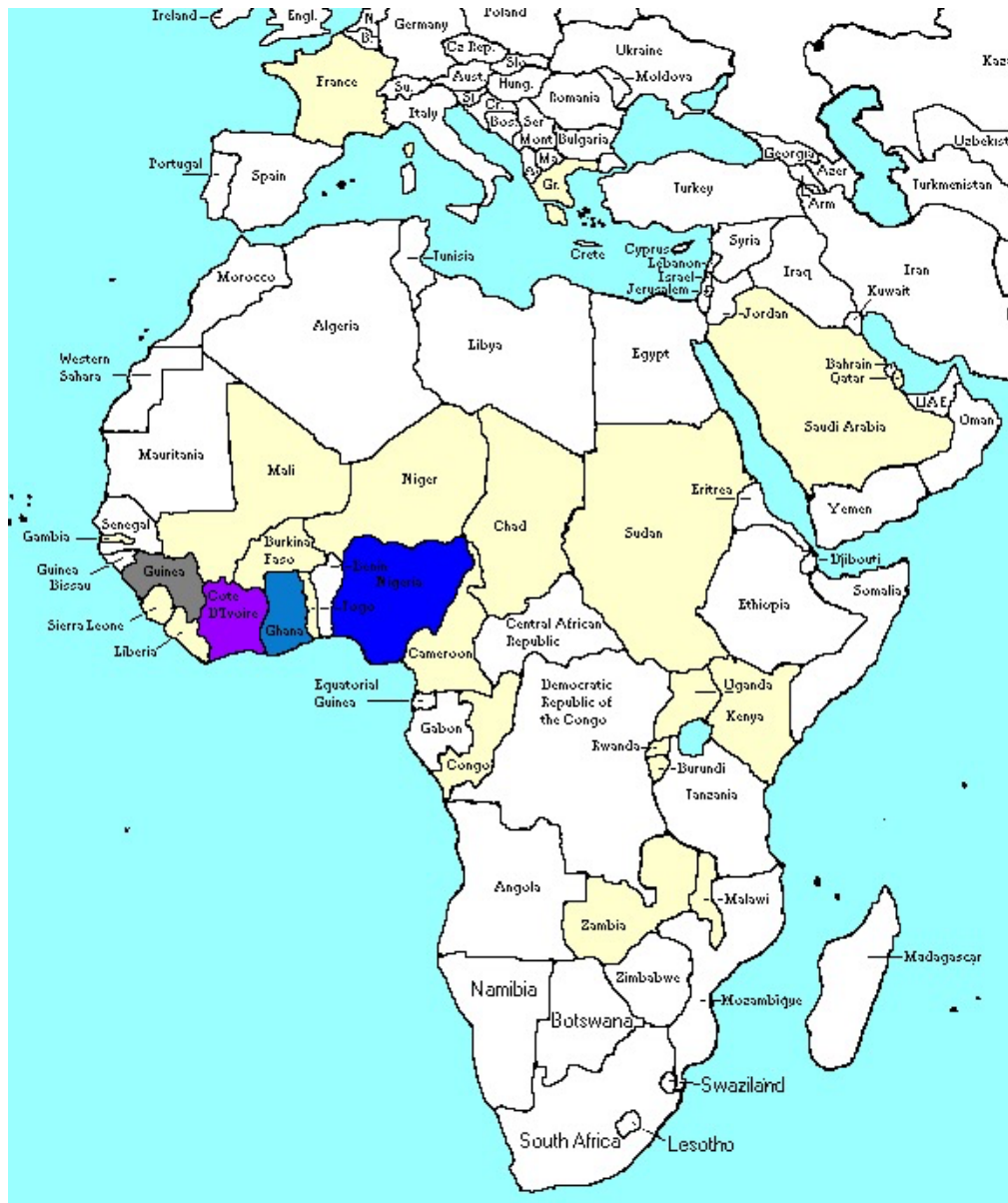
A result of "No high-confidence mutation" does not rule out resistance due to unknown contributory mutations present elsewhere in the genome.



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Majority of Malaria is Coming From West Africa



Combine the drug resistance sequences with epidemiologic data

- travel
- prophylaxis
- treatment



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Percentage of Drug Resistance Mutations

Gene	% Mutant	Drug(s)
PfCrt	30	Chloroquine
PfDhfr	95	Proguanil, Pyrimethamine
PfDhps	98	Sulfadoxine
PfMdr1	63	Amodiaquine, Lumefantrine, Quinine
PfCytb	0	Atovaquone
PfK13	0	Artemisinin

In 2018 and the first half of 2019 254 *P. falciparum* specimens were collected from patients who travelled to 30 different countries.

WHO recommendation

- artemether + lumefantrine
- artesunate + amodiaquine
- artesunate + mefloquine
- dihydroartemisinin + piperaquine
- artesunate + sulfadoxine-pyrimethamine (SP)



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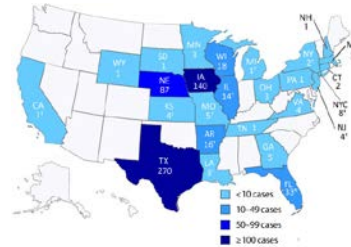
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Cyclospora NGS Genotyping

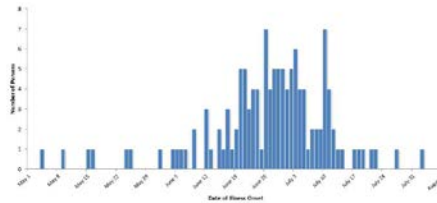


Foodborne Outbreaks of Cyclosporiasis

2013 – Salad mix and cilantro – 631 cases



2014 – Cilantro in Texas – 304 cases



2015 – Unknown vehicle & cilantro – 546 cases



2016 – Unknown vehicle – 384 cases

2017 – Unknown vehicle – 1,065 cases



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Foodborne Outbreaks of Cyclosporiasis

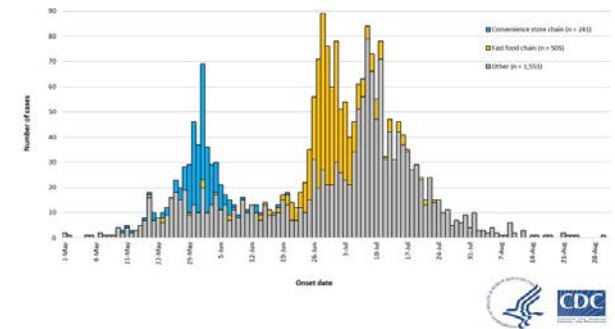
2018 – Multiple Outbreaks– 2,299 cases

Prepackaged vegetable trays (broccoli, cauliflower, and carrots)

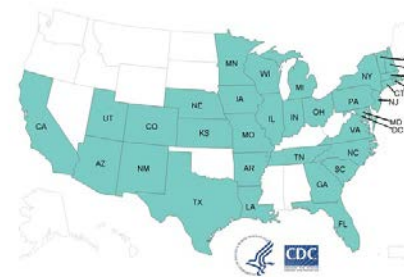
Salads at a fast food chain salads (carrots, romaine, spinach, kale, and varietal red leaf lettuce)

2 clusters associated with basil

Multiple clusters associated with cilantro



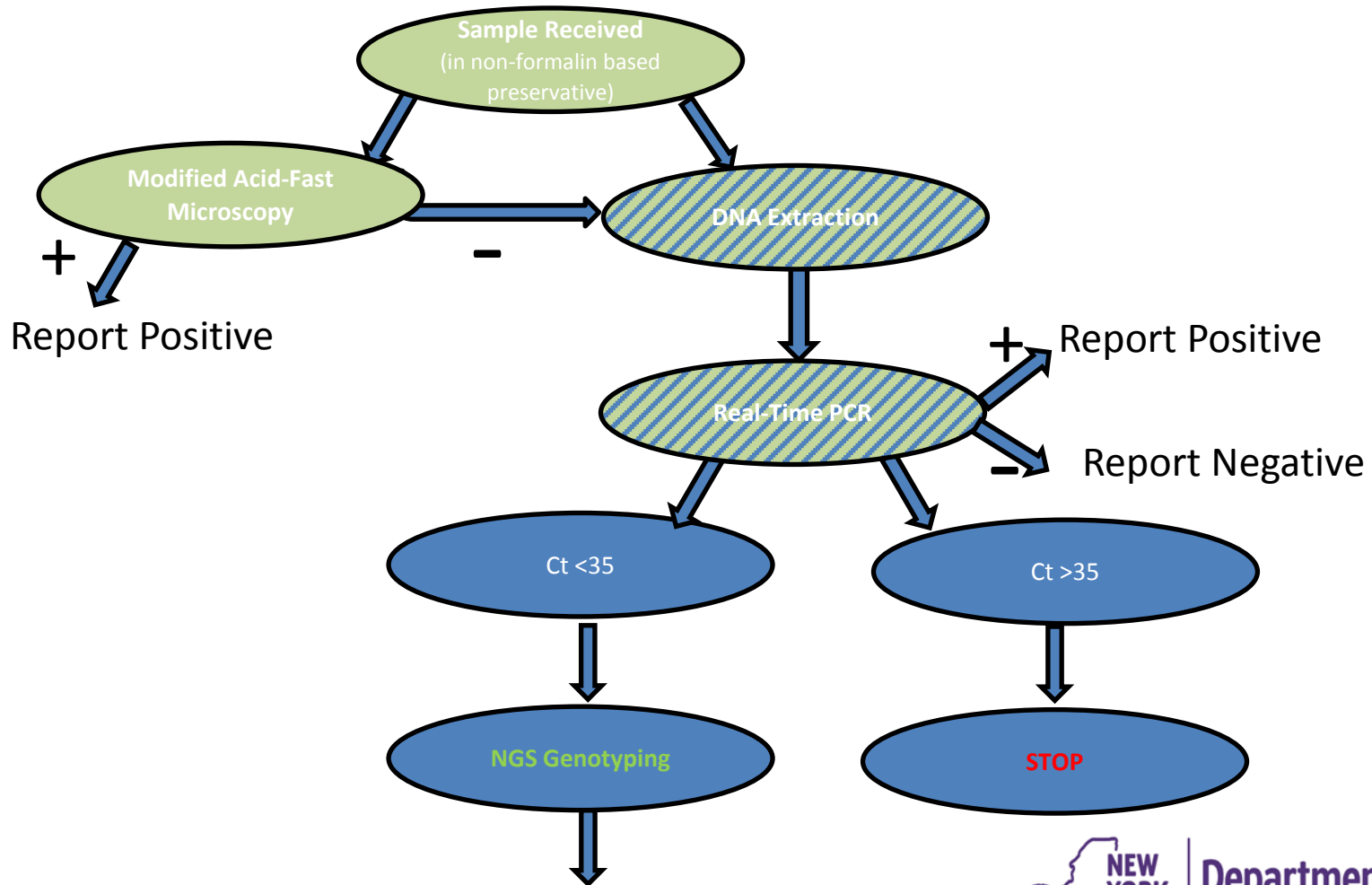
2019 – Multistate basil outbreak - 1,696 cases



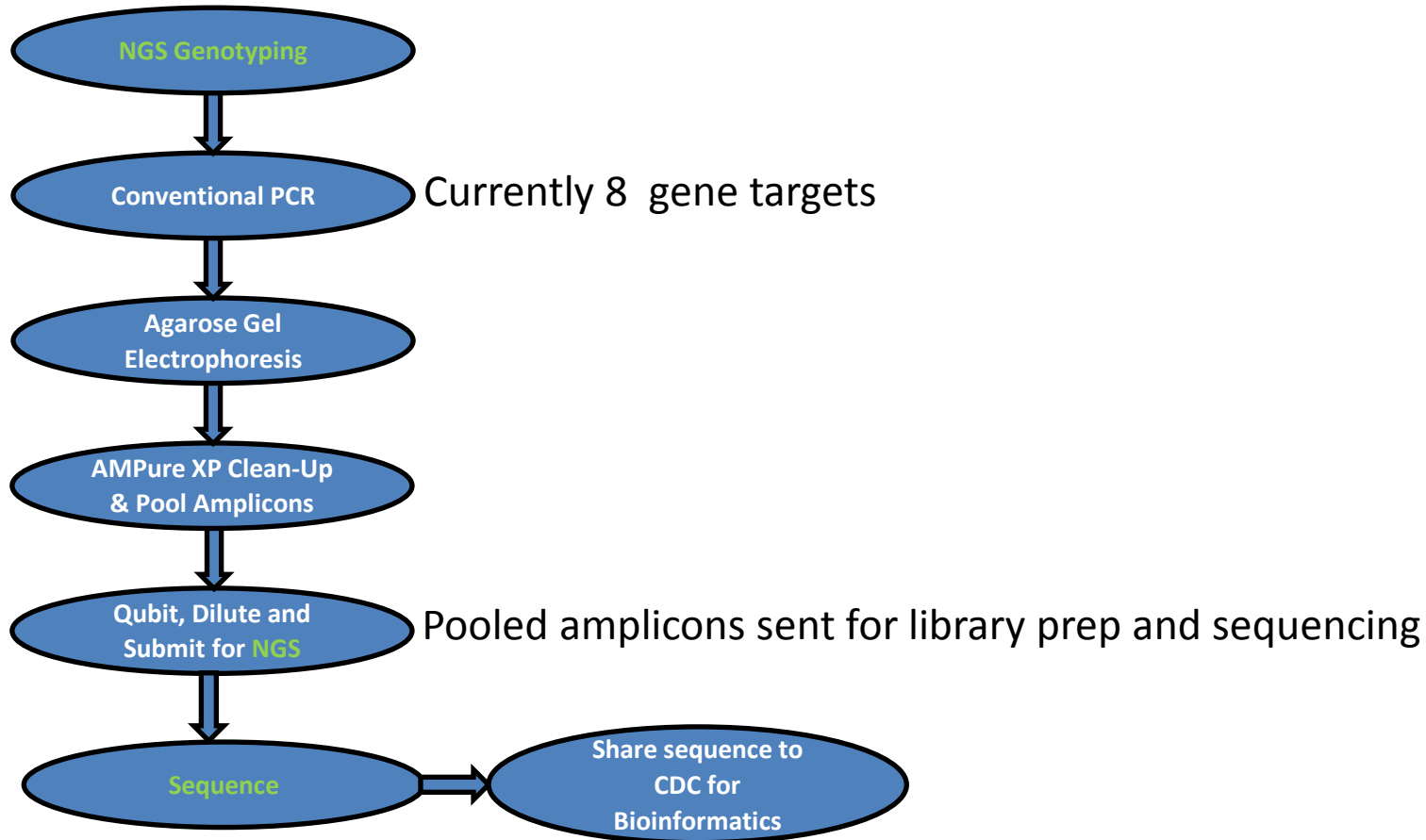
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What is NYS doing currently for *Cyclospora*?



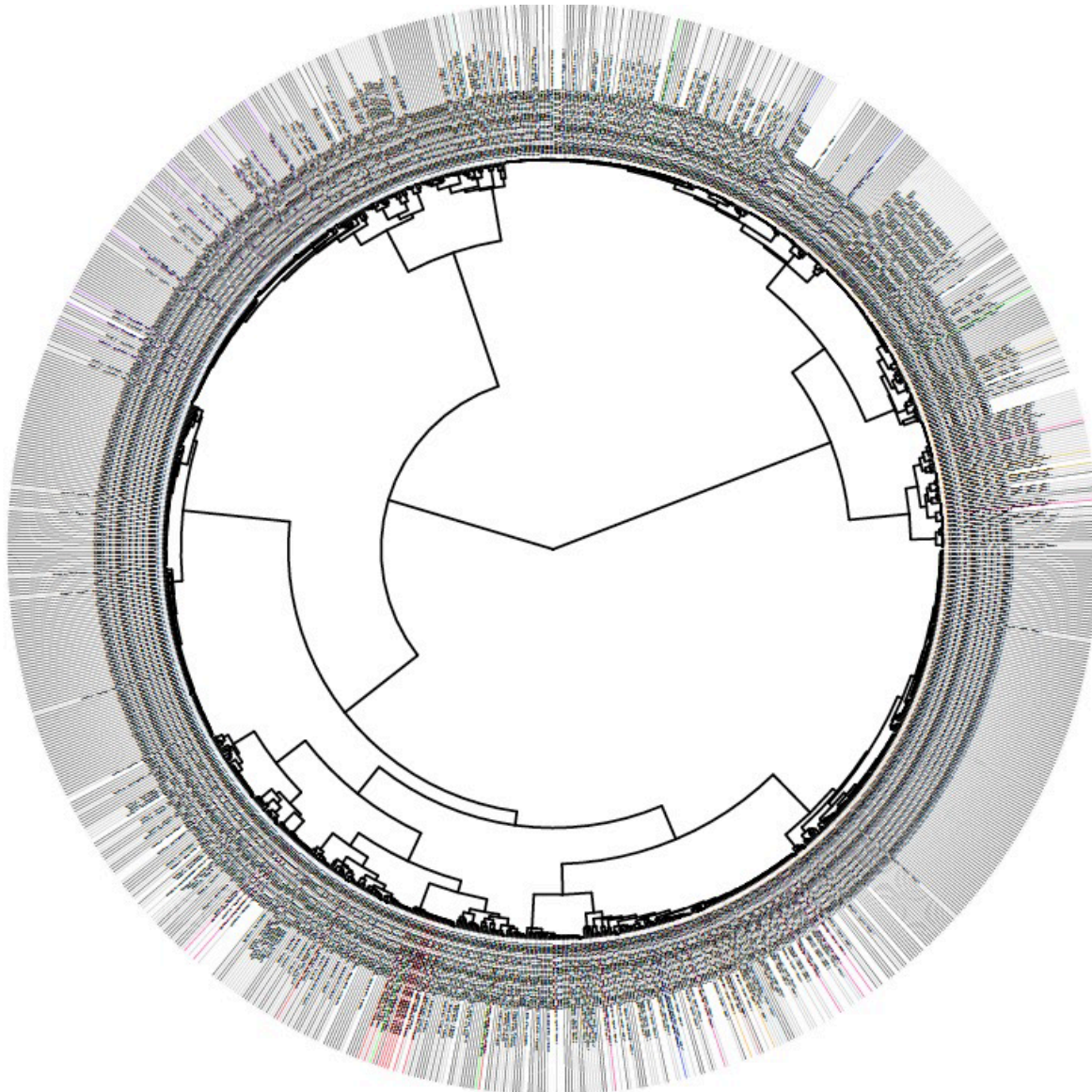
Cyclospora NGS Workflow



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Dendrogram of *Cyclospora* Samples



Dark grey – 2018

Light Grey/White – 2019

Colors - Outbreaks

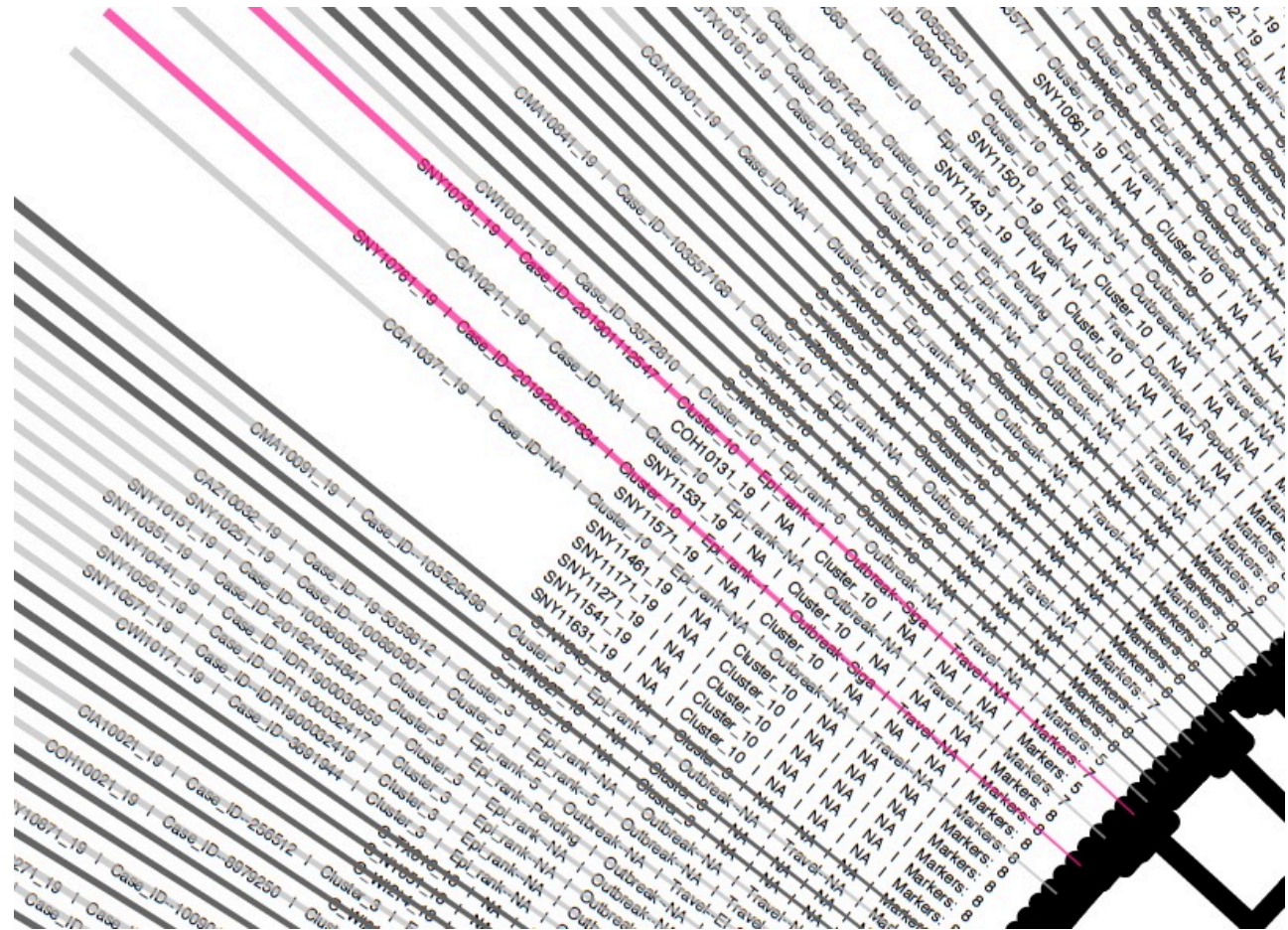


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Genotyping Links Cases Without Epi Data

Cases linked to basil consumption in upstate NY

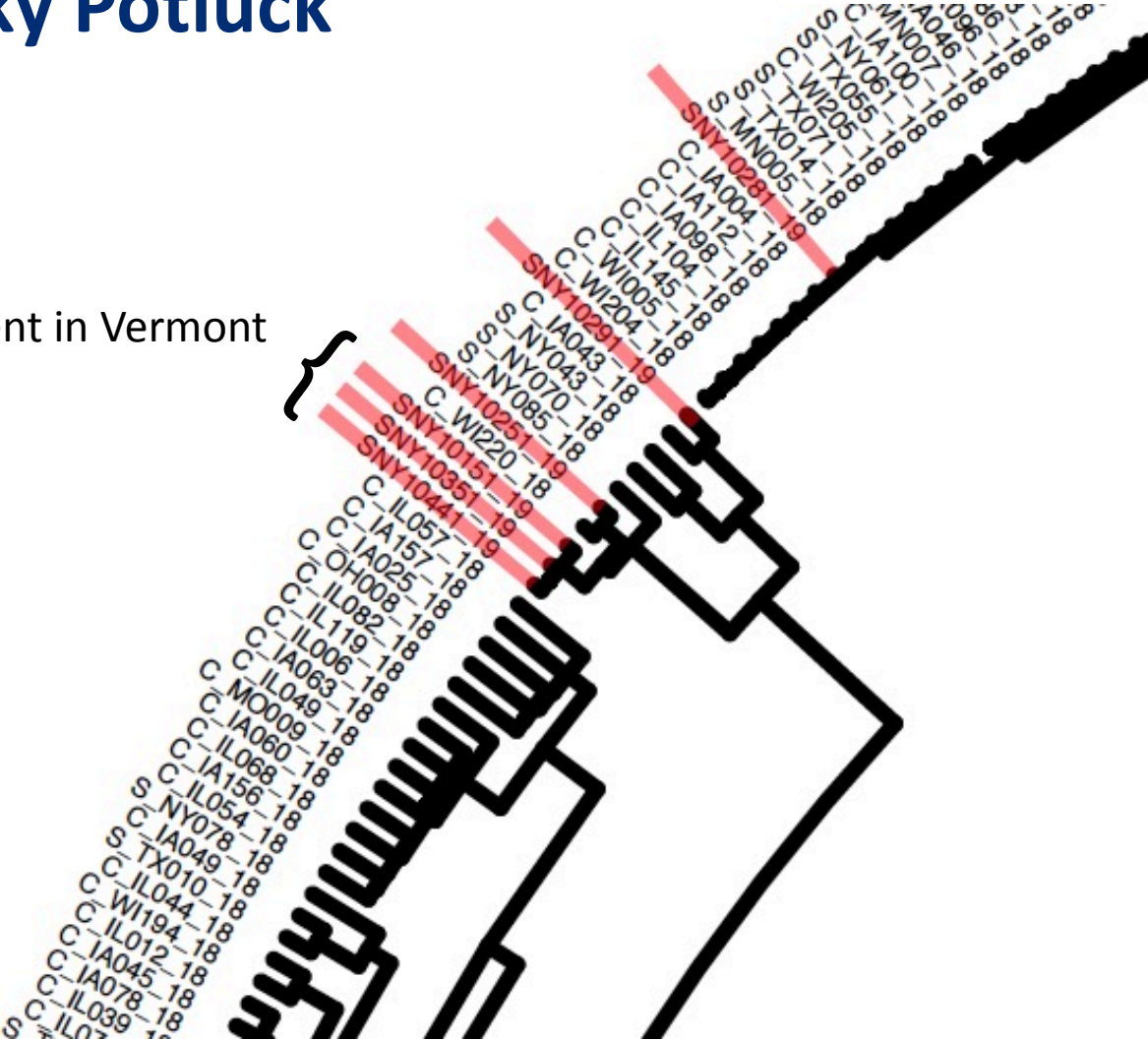


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The Unlucky Potluck

Potluck event in Vermont



Benefit of *Cyclospora* Genotyping

- Distinguish travel related cases from foodborne illness
- Link cases to help focus identification of the food vehicle
- Reduce the number of cases by identifying the source faster
- Link the contaminated food, or contaminated water where the food was grown, to cases



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NGS Universal Parasite Diagnosis (UPDx)

Metagenomic Sequencing of
Primary Samples

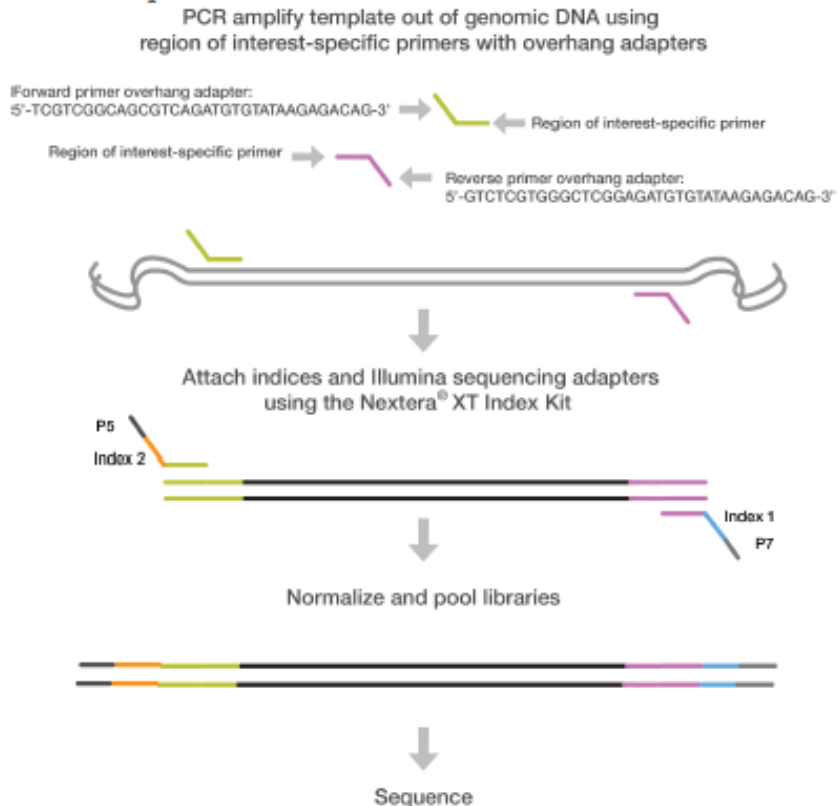


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~~16S~~ Metagenomic Sequencing Library 18S Preparation for MiSeq

~~16S~~ 18S V3 and V4 Amplicon Workflow



Target the gene of interest

- ✓ Common primer sequence for all parasites
- ✓ Primers don't recognize human, plant, bacteria, yeast,
- ✓ Different between primers to distinguish parasites

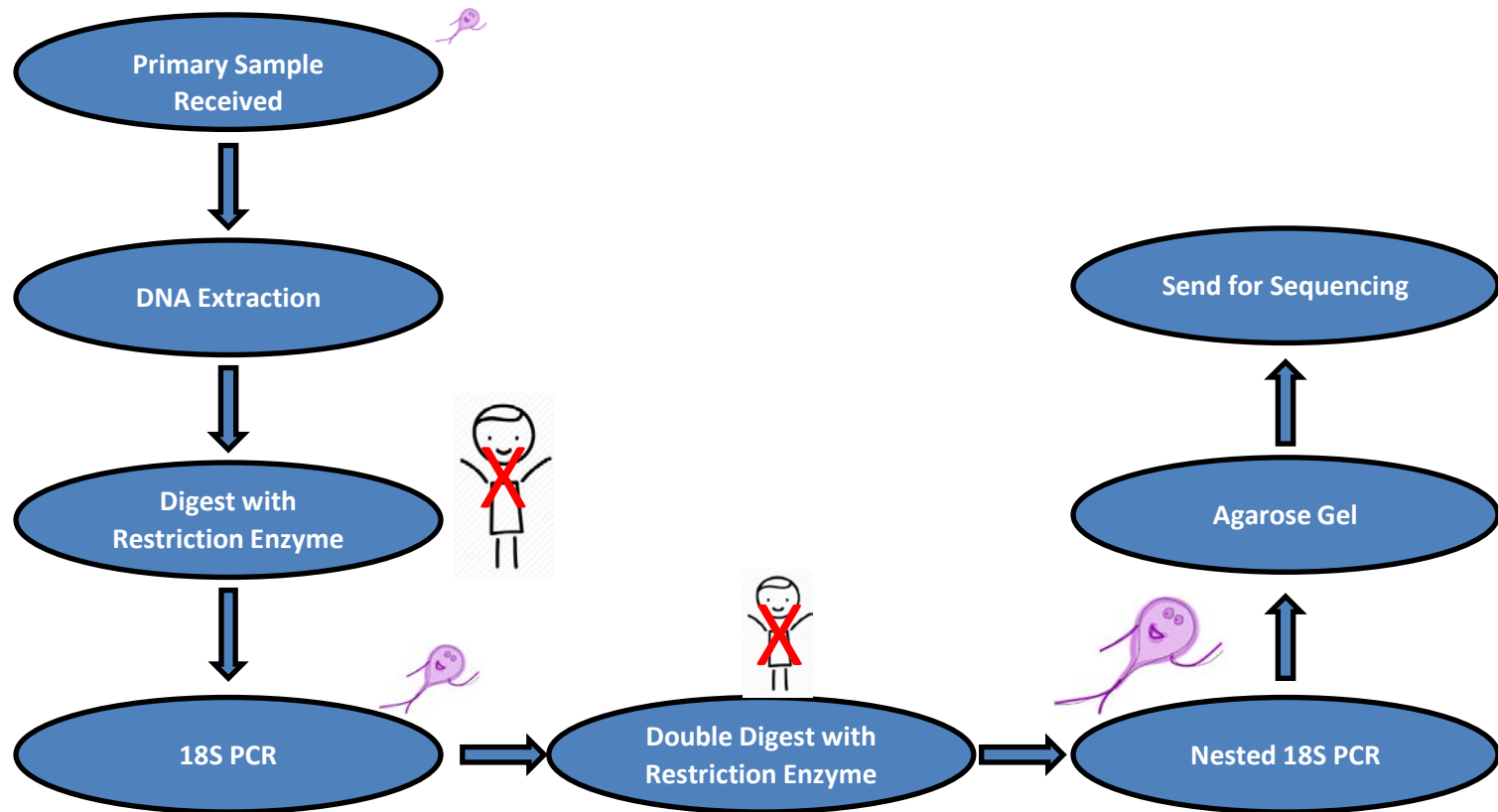
User-defined forward and reverse primers that are complementary upstream and downstream of the region of interest are designed with overhang adapters, and used to amplify templates from genomic DNA. A subsequent limited-cycle amplification step is performed to add multiplexing indices and Illumina sequencing adapters. Libraries are normalized and pooled, and sequenced on the MiSeq system using v3 reagents.



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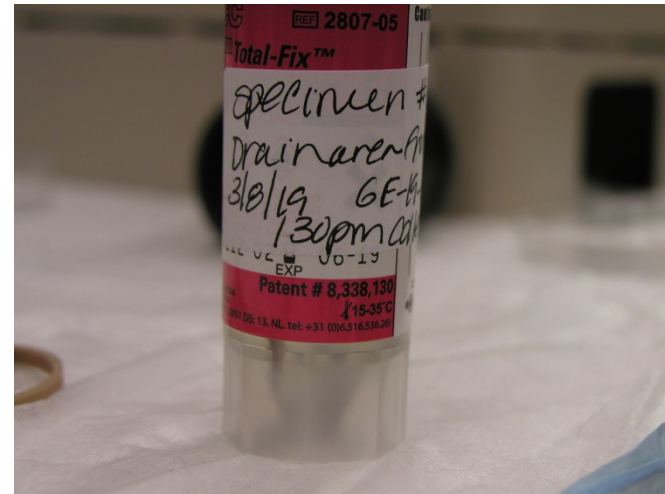
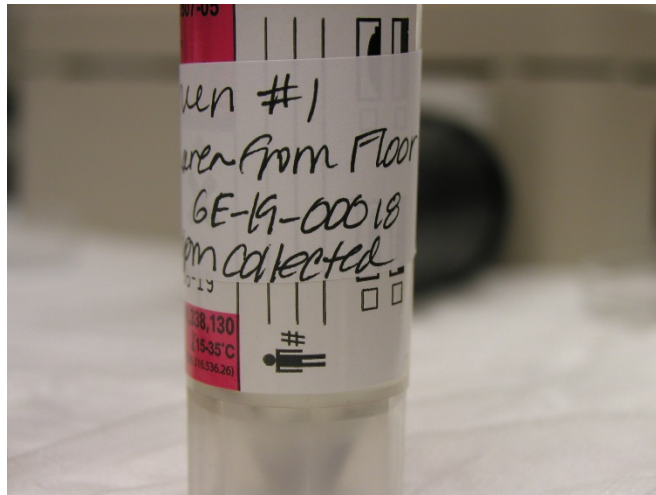
Universal Parasite Diagnostics (UPDx)



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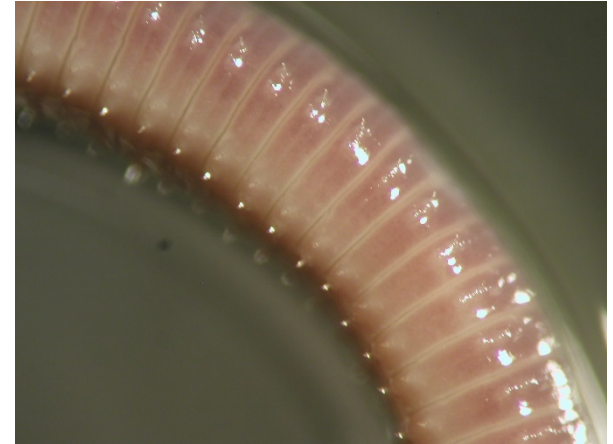
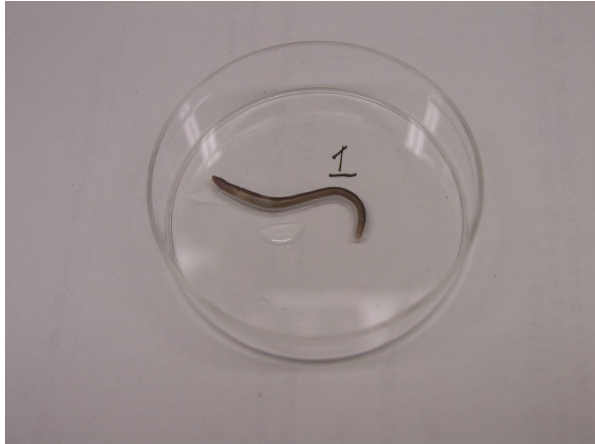
UPDx Utilized on Parasitology Mystery Samples!



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Worm reportedly sucked up in a straw at a fast food restaurant and similar ones found in the ice and a floor drain



- UPDx 18S Primers

Eisenia sp. - “redworm”, a species of earthworm adapted to decaying organic material

Description	Max score	Total score	Query cover	E value	Perc. ident	Accession
<i>Eisenia fetida</i> 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1762	1762	100%	0.0	100.00%	EF534709.1
<i>Eisenia andrei</i> isolate SR9H 18S ribosomal RNA gene, partial sequence	1757	1757	100%	0.0	99.90%	KF205933.1
<i>Eisenia fetida</i> gene for 18S ribosomal RNA, partial sequence	1757	1757	100%	0.0	99.90%	AB558505.1
<i>Eisenia fetida</i> gene for 18S rRNA, partial sequence	1757	1757	100%	0.0	99.90%	AB076887.1
<i>Eisenia fetida</i> 18S rRNA gene	1753	1753	100%	0.0	99.79%	X79872.1
Aporrectodea trapezoides isolate SR9P 18S ribosomal RNA gene, partial sequence	1751	1751	100%	0.0	99.79%	KF205934.1
<i>Eiseniona gerardoi</i> voucher UCMLT 60001 18S ribosomal RNA gene, partial sequence; mitochondrial	1751	1751	100%	0.0	99.79%	KF737140.1
Aporrectodea trapezoides isolate AT 18S ribosomal RNA gene, partial sequence	1748	1748	100%	0.0	99.69%	HQ621897.1
<i>Eisenoides carolinensis</i> 18S small subunit ribosomal RNA gene, partial sequence	1746	1746	100%	0.0	99.69%	HQ728903.1
<i>Lumbricus terrestris</i> 18S ribosomal RNA gene, partial sequence	1746	1746	100%	0.0	99.69%	HQ691211.1
<i>Dendrodrilus rubidus</i> isolate CE522 18S ribosomal RNA gene, partial sequence	1746	1746	100%	0.0	99.69%	GU901868.1



Larvae found in Breast Milk



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And the larvae are from..... Yeast?

Organism	Blast Name	Score	Number of Hits	Description
Eukaryota	eukaryotes		104	
• Fungi	fungi		98	
• Saccharomycetales	budding yeasts		94	
• Candida/Lodderomyces clade	budding yeasts		91	
• Candida	budding yeasts		90	
• Candida metapsilosis	budding yeasts	1557	1	Candida metapsilosis hits
• Candida parapsilosis	budding yeasts	1557	26	Candida parapsilosis hits
• Candida orthopsilosis	budding yeasts	1552	7	Candida orthopsilosis hits
• Candida orthopsilosis Co 90-125	budding yeasts	1552	1	Candida orthopsilosis Co 90-125 hits
• Candida tropicalis	budding yeasts	1546	33	Candida tropicalis hits
• Candida viswanathii	budding yeasts	1546	3	Candida viswanathii hits
• Candida neerlandica	budding yeasts	1546	3	Candida neerlandica hits
• Candida sojae	budding yeasts	1546	2	Candida sojae hits
• Candida tetragidarum	budding yeasts	1546	2	Candida tetragidarum hits
• Candida labiduridarum	budding yeasts	1546	3	Candida labiduridarum hits
• Candida frijolesensis	budding yeasts	1546	3	Candida frijolesensis hits
• Candida albicans	budding yeasts	1546	3	Candida albicans hits
• Candida sp. NYNU 14772	budding yeasts	1546	1	Candida sp. NYNU 14772 hits
• Candida cf. neerlandica NRRL Y-27939	budding yeasts	1546	1	Candida cf. neerlandica NRRL Y-27939 hits
• Candida buenavistaensis	budding yeasts	1546	1	Candida buenavistaensis hits
• Lodderomyces sp. BG090819.10.1.1.3.32	budding yeasts	1546	1	Lodderomyces sp. BG090819.10.1.1.3.32 hits
• Candida sp. Y574	budding yeasts	1546	1	Candida sp. Y574 hits
• Candida aquae-textoris	budding yeasts	1546	1	Candida aquae-textoris hits
• Candida sp. S42-12	budding yeasts	1546	1	Candida sp. S42-12 hits



Deep Sequencing Reveals *Megaselia scalaris*

Descriptions | Graphic Summary | Alignments | Taxonomy

Sequences producing significant alignments Download Manage Columns Show 100

select all 100 sequences selected GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
<input checked="" type="checkbox"/> Megaselia scalaris 18S ribosomal RNA gene, partial sequence	464	464	100%	8e-127	100.00%	KC177299.1
<input checked="" type="checkbox"/> Phoridae sp. Phor_H4 18S ribosomal RNA gene, partial sequence	460	460	100%	1e-125	99.60%	KM975518.1
<input checked="" type="checkbox"/> Phoridae sp. Phor_H2 18S ribosomal RNA gene, partial sequence	459	459	100%	4e-125	99.60%	KM975517.1
<input checked="" type="checkbox"/> Uncultured eukaryote 18S ribosomal RNA, partial sequence	414	414	100%	8e-112	96.43%	KU657712.1
<input checked="" type="checkbox"/> Uncultured eukaryote partial 18S ribosomal RNA, clone O1RBH070700041						FN393195.1
<input checked="" type="checkbox"/> Uncultured eukaryote clone nco40h10c1 18S ribosomal RNA gene, partial sequence						KC670883.1
<input checked="" type="checkbox"/> Uncultured eukaryote clone nco40d04c1 18S ribosomal RNA gene, partial sequence						KC670830.1
<input checked="" type="checkbox"/> Episyrphus balteatus 18S ribosomal RNA gene, partial sequence						KC177297.1
<input checked="" type="checkbox"/> Oreogeton scopifer 18S ribosomal RNA gene, partial sequence						KC177291.1
<input checked="" type="checkbox"/> Hermetia illucens 18S ribosomal RNA gene, partial sequence						GQ465779.1
<input checked="" type="checkbox"/> Euscelidia pulchra voucher Asil-39 18S ribosomal RNA gene, partial sequence						EF650108.1
<input checked="" type="checkbox"/> Syrphidae sp. Syrph_H1 18S ribosomal RNA gene, partial sequence						KM975521.1
<input checked="" type="checkbox"/> Spheginobaccha macropoda partial 18S rRNA gene, specimen voucher MZH:S155						HF559384.1
<input checked="" type="checkbox"/> Lasiocnemus lugens voucher Asil-40 18S ribosomal RNA gene, partial sequence	390	390	100%	1e-104	94.55%	EF650109.1
<input checked="" type="checkbox"/> Leptogaster cylindrica voucher Asil-1 18S ribosomal RNA gene, partial sequence	390	390	100%	1e-104	94.55%	EF650101.1

16, 184 reads



coffin fly, scuttle fly

“thrive predominately in moist unsanitary vicinities such as dumpsters, trash containers, rotting meat, vegetable remains, public washrooms, homes, and sewer pipes”



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UPDx Metagenomic Sequencing for Complex Samples

Can we do deep sequencing on blood specimens to detect *Babesia*, *Plasmodium sp.*, *Trypanosoma cruzi*, *Leishmania sp.*, filarids....?

What about something even more complex like a stool sample?

- inherently variable
- plant, yeast (etc.) DNA present in addition to human DNA
- PCR inhibitors



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Thank you

Parasitology Lab



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CDC Malaria Group

Eldin Talundzic

CDC *Cyclospora* Group

Yvonne Qvarnstrom
Joel Barratt

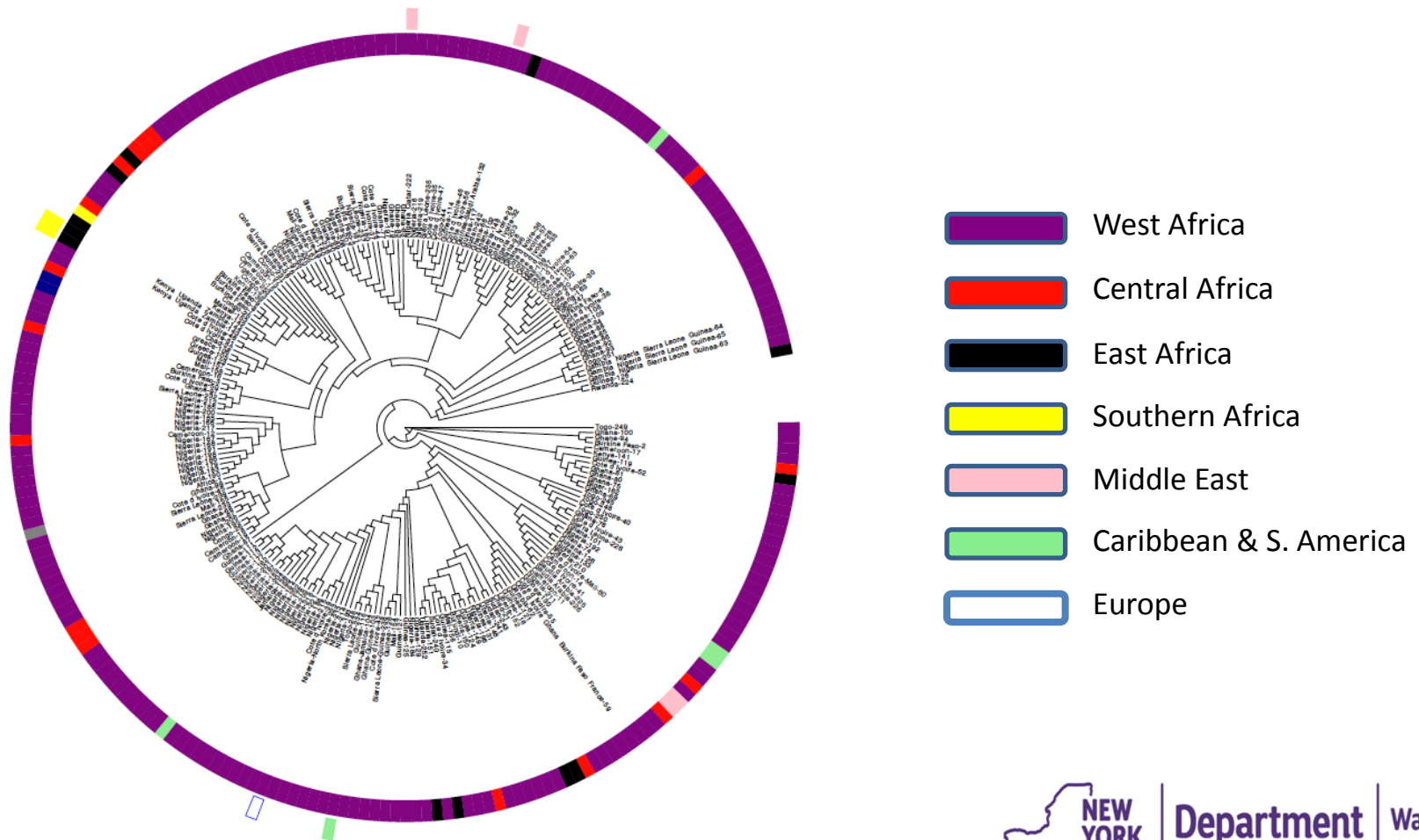
Funding from the CDC - Epidemiology and Laboratory Capacity
Advanced Molecular Detection and Parasitology Sections



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Cladogram using Approximate Likelihood Algorithm for *P. falciparum* Samples



NGS performed by Advanced Genomics Technology Center

- NexteraXT library kit - half volume reactions, with 2.5uL amplicon pool @ 0.2ng/ul
- Sequenced on a Miseq using a 300 cycle v2 sequencing kit (150bp paired ends) - nano, micro or standard flowcell
- Loaded onto Miseq at 7-8pM, avg lib size ~420
- Samples included in runs with other organisms - bacterial whole genomes, viral samples, 1% PhiX spike-in



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