Gram Stain Bench Guide

A reference guide to Gram stain preparation, interpretation and troubleshooting

APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES

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Intended Use

The Gram Stain Bench Resource Guide is designed to assist laboratory scientists in the accurate preparation and analysis of Gram-stained slides including the Gram staining procedure, typical staining, troubleshooting chart and atypical staining examples. This document is intended for laboratorians of all skill levels and is meant to supplement formal training. It is not intended to replace institutional protocols.

Chart Key

ANO$_2$ .....Anaerobic environment

BAP .....Blood Agar Plate

CHOC .....Chocolate Agar Plate

CO$_2$ .....5-10% Carbon Dioxide

CVA .....*Campylobacter* Blood Agar

MAC .....MacConkey Agar Plate

N$_2$ .....85% Nitrogen environment

O$_2$ .....Oxygen/ambient air

SDA .....Sabouraud Dextrose Agar

TSB .....Tryptic Soy Broth

Gram Stain Reagents

Step	Reagent	Action	Bacterial Cell Color After Step
Primary Stain	Crystal violet	Crystal violet penetrates the peptidoglycan in the bacterial cell wall.	Purple
Mordant	Gram's Iodine	Iodine binds to the crystal violet, forming a complex that facilitates binding to cell wall.	Purple
Decolorizer	Either: • 95% Ethanol • 95% Ethanol/ Acetone Mix*	• Bacteria with a thick peptidoglycan layer will resist decolorizing and retain the crystal violet-iodine complex. • Bacteria with a thin peptidoglycan layer will lose the crystal violet-iodine complex.	• Purple (Gram-positive) • No color (Gram-negative)
Counterstain/ Secondary Stain	Safranin	Safranin will enter the cell walls which have lost the crystal violet-iodine complex.	• Purple (Gram-positive) • Pink (Gram-negative)

**Follow your SOP for the specific formula used in your laboratory.*

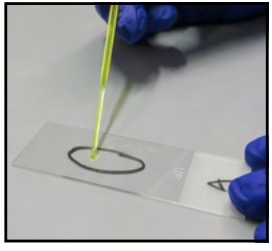
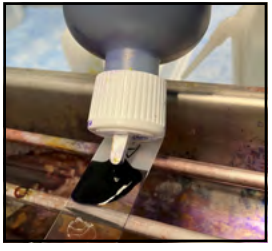
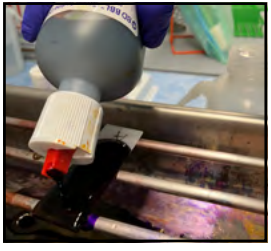
Written Step-by-step Guide

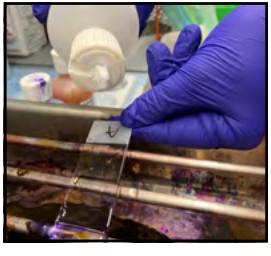
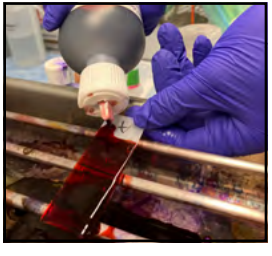
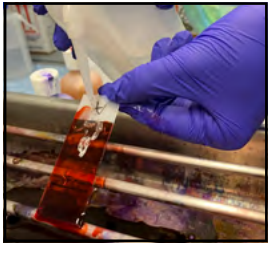
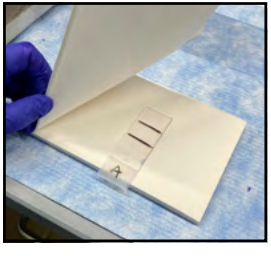
See "Visual Step-by-step Guide" on page 8 for corresponding images to these instructions.

Step	Action
1. **Slide preparation**	• If not using slides with imprinted circles, outline an area of the slide with a wax pencil. • **From an agar plate:** Add a drop of sterile saline or water inside the designated area on the slide. Using one end of a sterile wooden applicator stick or disposable needle touch one single, isolated colony and emulsify it into the water drop on the slide. • **From a broth:** Use a small disposable loop or needle to transfer a small amount of the liquid to the designated area on the slide. • Set the slide aside to dry fully.
2. **Heat or Methanol Fix**	• **Heat Fix:** Once the slide is fully dry, place the slide onto a slide warmer set to 37°C. The heat will fix the cells to the slide so they don't wash off during staining. If the slide has not fully dried prior to heat fixing there is a risk of disrupting the cell walls or aerosolizing the cells, which is a potential biosafety risk. • **Methanol Fix:** Once slide is fully dry, flood slide with methanol for 60 seconds. Rinse methanol from slide using a wash bottle or running water while holding the slide at an angle.
3. **Primary stain**	Flood the designated area of the slide with crystal violet and leave on for 60 seconds.
4. **Rinse**	Using a wash bottle or running water and while holding the slide at an angle, rinse the slide to remove the crystal violet stain.

Step	Action
5. **Fix with mordant**	Flood the slide with iodine and leave on for 60 seconds.
6. **Rinse**	Using a wash bottle or running water and while holding the slide at an angle, rinse the slide to remove the iodine.
7. **Decolorize**	• Decolorize the slide by applying decolorizer and almost immediately rinsing with water. • Repeat this process until the purple color stops coming off while holding the slide at an angle. Note: *If this is repeated beyond the time necessary to remove the crystal violet stain there is a risk of over-decolorizing which will result in Gram-positive cells appearing Gram-negative or Gram-variable. Proper timing will depend on decolorizer formulation.*
8. **Counterstain**	Flood the designated area of the slide with safranin and leave on for 30-60 seconds.
9. **Rinse**	Using a wash bottle or running water and while holding the slide at an angle, rinse the slide to remove the counterstain.
10. **Blot dry**	Gently blot excess liquid from slide and allow to air dry.

Visual Step-by-step Guide

1. Slide preparation	2. Fix	3. Primary stain	4. Rinse	5. Fix with mordant
				

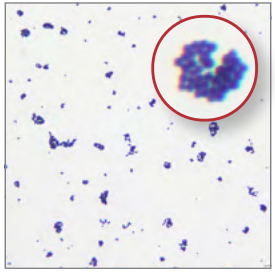
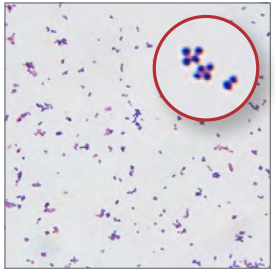
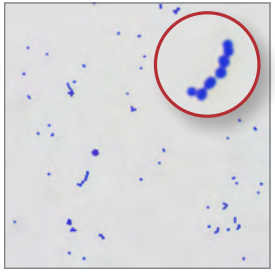
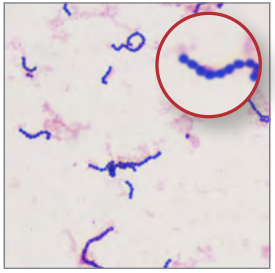
6. Rinse	7. Decolorize	8. Counterstain	9. Rinse	10. Blot dry
				

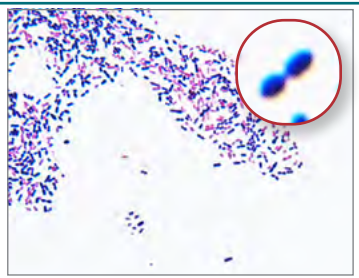
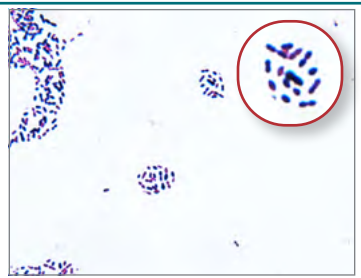
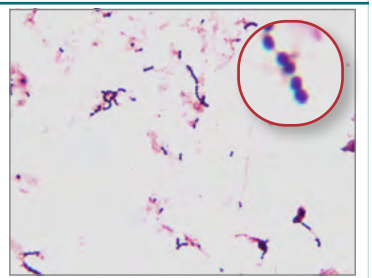
Step	Action
1. **Scan on low power**	Scan the slide at 10x to identify a well-stained area.
2. **Scan on high power**	Scan the slide at 100x to be sure the proper area is still in focus and visible.
3. **Add oil**	• Turn the nosepiece so no objective lens is touching the slide. • Without moving the slide or the stage, add a drop of immersion oil to the slide.
4. **Read on high power oil immersion**	Observe the previously identified area of the stained slide with a 100X high power objective lens.

If the image goes out of focus or the well stained area is lost: Wipe the oil from the lens and drop back to 10X to find the area of interest. The imprinted circle on the slide or the wax pencil mark can serve as a guide.

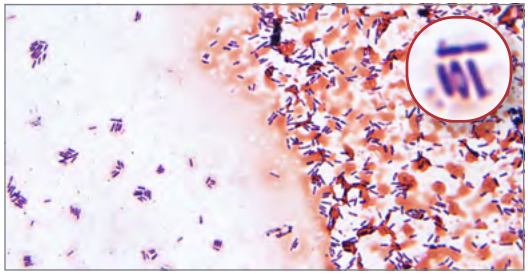
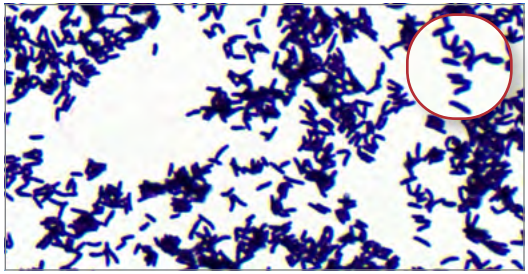
Reading Stained Slides

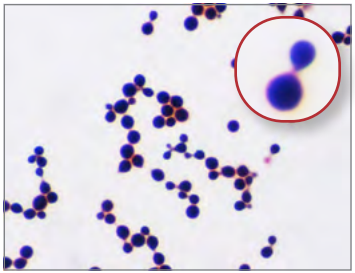
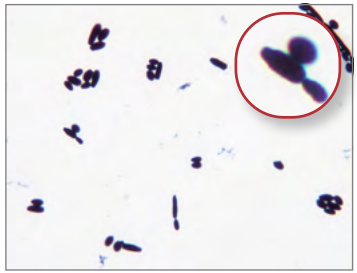
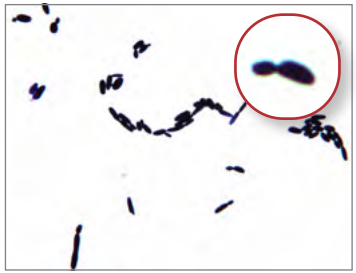
Gram-positive Organisms

Characteristics		Cocci in Clusters		Cocci in Pairs and Chains	
Organism		*Staphylococcus aureus*		*Streptococcus pyogenes* (Gp A)	
Growth Conditions	Media State	Solid Media	Liquid Media	Solid Media	Liquid Media
	Media	BAP	TSB	BAP	Blood Culture Bottle
	Environment	O_2	O_2	O_2	O_2
	Temperature	37°C	37°C	37°C	37°C
	Time to Growth	24 Hours	18 Hours	24 Hours	24 Hours
Staining					
Notes		Typically appear in clusters, similar to a cluster of grapes; detail in inset. Stains purple.		Typically appear as chains, similar to a string of beads or pearls; better chaining morphology in broth. Detail in inset. Stains purple.	

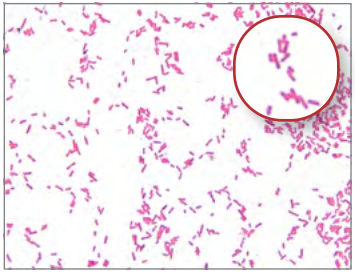
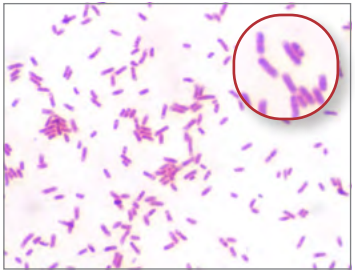
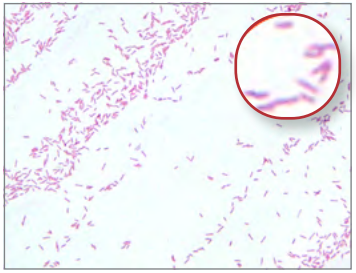
Characteristics		Cocci in Pairs and Chains		
Organism		*Streptococcus pneumoniae*		
Growth Conditions	Media State	Solid Media	Solid Media	Liquid Media
	Media	BAP	BAP	TSB
	Environment	CO_2	CO_2	CO_2
	Temperature	36°C	36°C	36°C
	Time to Growth	18 Hours	24 Hours	24 Hours
Staining				
Notes		Typically appear in pairs, almost oval with the pointier ends together (lancet-shaped); detail in inset. Stains purple.	**Capsule**—appears as a clearing around cells; detail in inset. Stains purple.	More defined morphology from broth; detail in inset. Stains purple.

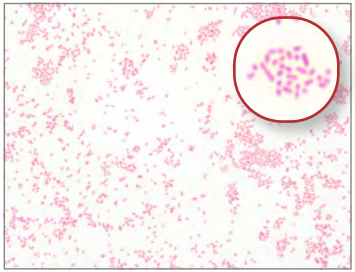
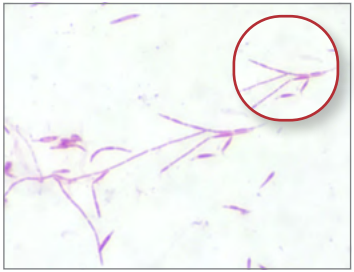
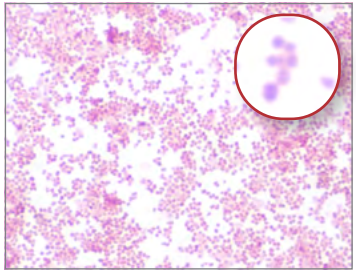
Gram-positive Organisms

Characteristics		Rods with Spores	
Organism		*Bacillus subtilis*	*Corynebacterium diphtheria*
Growth Conditions	Media State	Solid Media	Solid Media
	Media	BAP	BAP
	Environment	O_2	O_2
	Temperature	36°C	37°C
	Time to Growth	18 Hours	24 Hours
Staining			
Notes		Look for spores and their placement within the cell (terminal, subterminal or central); detail in inset. Stains purple.	Look for palisading/picket fence arrangement; detail in inset. Stains purple.

Characteristics		Yeast		
Organism		*Cryptococcus* spp.	*Candida tropicalis*	
Growth Conditions	Media State	Solid Media	Solid Media	Liquid Broth
	Media	SDA	SDA	TSB
	Environment	CO_2	O_2	O_2
	Temperature	37°C	37°C	7°C
	Time to Growth	48 Hours	72 Hours	24 Hours
Staining				
Notes		See budding detail in insets. Stains purple.		

Gram-negative Organisms

Characteristics		Rods		
Organism		*Escherichia coli*		*Campylobacter jejuni*
Growth Conditions	Media State	Solid Media	Liquid Media	Solid Media
	Media	BAP	TSB	CVA
	Environment	O_2	O_2	N_2
	Temperature	37°C	37°C	42°C
	Time to Growth	24 Hours	24 Hours	48 Hours
Staining				
Notes		Stains pink.		Displays gull-wing and “s” shapes. Stains pink.

Characteristics		Rods		Diplococci
Organism		*Haemophilus influenzae*	*Fusobacterium* spp.	*Neisseria gonorrhoeae*
Growth Conditions	Media State	Solid Media	Solid Media	Solid Media
	Media	CHOC	BAP	CHOC
	Environment	CO_2	ANO_2	O_2
	Temperature	37°C	37°C	37°C
	Time to Growth	24 Hours	48 Hours	24 Hours
Staining				
Notes		Displays pleomorphic morphology (coccobacilli). Stains pink.	Cells have pointed ends. Stains pink.	Displays a "coffee or kidney bean" arrangement with the long, curved edges together in each cell of the pair. Stains pink.

References for Atypical Staining (1)

This section outlines common issues that arise during Gram staining, along with possible causes and remediation techniques. The issue numbers are referenced in the Atypical Staining section.

1. Gram-positive appears pink/morphology inconsistent with Gram stain reaction

e.g., Gram-negative cocci in chains

Problem	Possible Causes	Remediation Options
Technique	Over decolorized	• Verify decolorizing time • Use a timer • Consult SOP to confirm proper technique • Ensure acetone/alcohol ratio of decolorizer is appropriate for procedure times (the higher percentage of acetone, the shorter the decolorization time) • Ensure proper timing of exposure to iodine • Concurrently stain a QC slide
Growth issues/ other	Culture is old	• Check date of subculture (encourage dating of media when inoculated) • Use fresh bacterial growth

2. Gram-negative appears purple/morphology inconsistent with Gram stain reaction

Problem	Possible Causes	Remediation Options
Technique	Smear is too thick	Prepare smear using a small drop of water and a loopful of organism
	Under decolorized	Verify decolorizing time
Growth issues/ other	Culture is old	• Check date of subculture (encourage dating of media when inoculated) • Use fresh bacterial growth

3. Inorganic material on slide/unable to see organisms or see pieces of organisms

Problem	Possible Causes	Remediation Options
External factors (environment/ reagents)	Stain precipitate	Filter stains, particularly crystal violet
	Slide is contaminated	• Keep box of slides covered when not in use • Avoid potential splashing when making slides • Ensure rinsing does not run over on other slides

References for Atypical Staining (2-3)

References for Atypical Staining (3)

Problem	Possible Causes	Remediation Options
External factors, cont'd	Old staining reagents	Use in-date reagents
	Improperly prepared/stored reagents	• Confirm proper reagent preparation, if made in-house • Confirm reagents are stored properly
	Immersion oil is contaminated	• Confirm sterility of immersion oil/open new reagent • Clean objective • Review proper application of oil—do not touch slide
Technique	Staining slide before it is dry	Ensure slide is fully air-dried prior to fixation
	Excessive heat fixation	Ensure slide is not held in heat too long (disrupts cell walls)
Growth issues/ other	The patient may be undergoing antimicrobial therapy	If possible, confirm sample was collected prior to antibiotic treatment
	Organism not cultured in ideal growth environment/media	Confirm organism is growing in appropriate growth environment
	Organism too old/cell wall compromised	Subculture organism to confirm viability/stain fresh growth

4. Unusual morphology/unable to determine morphology

Problem	Possible Causes	Remediation Options
Technique	Staining slide before it is dry	Ensure slide is fully air-dried prior to fixation
	Excessive heat fixation	Ensure slide is not held in heat too long (disrupts cell walls)
Growth issues/ other	The patient may be undergoing antimicrobial therapy	If possible, confirm sample was collected prior to antibiotic treatment
	Organism not cultured in ideal growth environment/media	Confirm organism is growing in appropriate growth environment
	Organism too old/cell wall compromised	Subculture organism to confirm viability/stain fresh growth

5. Unable to see individual cells

Problem	Possible Causes	Remediation Options
Technique	• Smear too thick • Smear too thin, prepared with too much water/not enough organism	Prepare smear using a small drop of water and a loopful of organism
	Focused on heavy part of smear	Move microscope stage to thinner section of smear

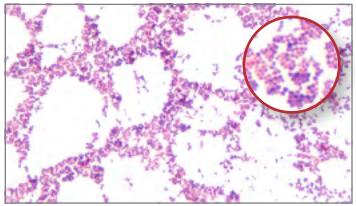
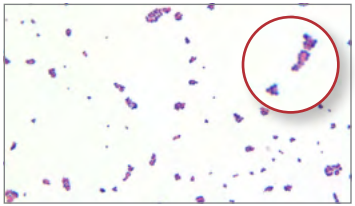
References for Atypical Staining (4-5)

References for Atypical Staining (5-6)

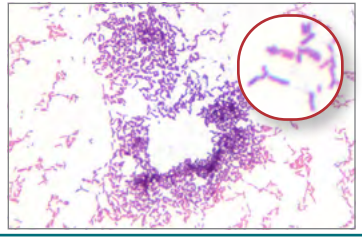
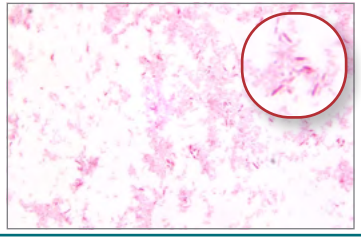
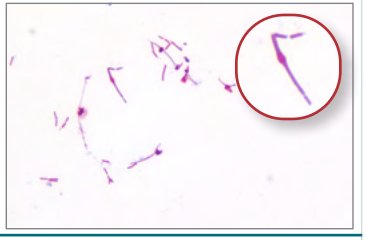
Problem	Possible Causes	Remediation Options
Technique, cont'd.	Smear not fixed	Fix organism to slide before staining
	Organism washed away	Ensure proper rinsing/washing technique
	Smear area not clearly delineated	Delineate reading area with a wax pencil to help find correct plane
	Slide is upside down (organism facing down) on microscope stage	Flip slide on microscope stage
Growth issues/ other	Organism too old/cell wall compromised	Subculture organism to confirm viability/stain fresh growth

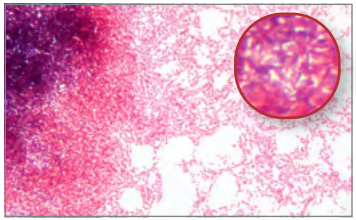
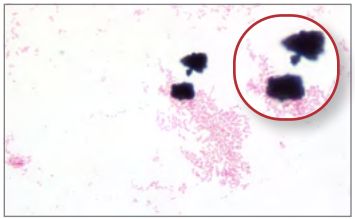
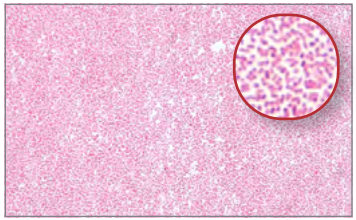
6. Organism appears out of focus/unable to “zoom” in

Problem	Possible Causes	Remediation Options
Technique	Slide is upside down (organism facing down) on microscope stage	Flip slide on microscope stage
	No immersion oil used	Add immersion oil
Growth issues/ other	Organism too old/cell wall compromised	Subculture organism to confirm viability/stain fresh growth

Characteristics		Gram-positive Cocci		
Issue	Problem	Over-decolorized	Too Old	Antibiotic-treated
	Troubleshooting Reference #	1	1, 2, 3, 5	3, 4
Organism		*Staphylococcus aureus*		
Growth Conditions	Media	BAP	BAP	BAP
	Environment	O_2	O_2	O_2
	Temperature	37°C	37°C	37°C
	Time to Growth	24 Hours	3 Days	24 Hours
Staining				
Notes		Cells can look Gram negative	Cells can look inconsistently decolorized; they can appear stretched out or scant cells may be seen due to cell death	Displays bulbous/irregular shapes and scant number of cells

Gram-negative Organisms

Characteristics		Gram-negative Rods		
Issue	Problem	Over-decolorized	Too Old	Antibiotic-treated
	Troubleshooting Reference #	2	1, 2, 3, 5	3, 4
Organism		*E. coli*		
Growth Conditions	Media	BAP	BAP	BAP
	Environment	O_2	O_2	O_2
	Temperature	37°C	37°C	37°C
	Time to Growth	24 Hours	7 Days	24 Hours
Staining				
Notes		Cells can look Gram negative	Cells can look inconsistently decolorized; they can appear stretched out or scant cells may be seen due to cell death	Displays bulbous/irregular shapes and scant number of cells

Characteristics		Gram-negative Rods		
Issue	Problem	Smear Too Thick	Stain Precipitate	Smear Too Thick
	Troubleshooting Reference #	2, 5	3	2, 5
Organism		*E. coli*		*Haemophilus influenzae*
Growth Conditions	Media	BAP	BAP	CHOC
	Environment	O_2	O_2	CO_2
	Temperature	37°C	37°C	36°C
	Time to Growth	24 Hours	24 Hours	18 Hours
Staining				
Notes		Displays heavy section that looks Gram positive.	Displays excess “blobs” of Crystal Violet	Displays darker staining than usual, making it difficult to see morphology.

24 Additional Resources

The following are additional helpful resources when determining best practices for performing a Gram stain:

Gram Stain Protocols

American Society for Microbiology (ASM)

How to Perform a Gram Stain

US Centers for Disease Control and Prevention (CDC)

Gram Stain Workshop for the Laboratory Generalist

Nebraska Public Health Laboratory

The Gram Stain

Virtual Interactive Bacteriology Laboratory, Michigan State University

Association of Public Health Laboratories

The Association of Public Health Laboratories (APHL) works to strengthen laboratory systems serving the public's health in the US and globally. APHL's member laboratories protect the public's health by monitoring and detecting infectious and foodborne diseases, environmental contaminants, terrorist agents, genetic disorders in newborns and other health threats.

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