MULTIPLEX PCR TESTING FOR INFECTIOUS DIARRHEA

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Outline

• What is acute infectious diarrhea and what causes it?
• How has acute infectious diarrhea been diagnosed using laboratory tests?
• What is the culture-independent multiplex PCR test for acute infectious diarrhea, and what are its advantages and disadvantages?
• Published studies and Bronson experience
Clinical relevance

• Diarrheal disease is a **common** condition
• The differential diagnosis of diarrheal illness is broad and includes non-infectious conditions
• The differential is easily narrowed by taking a good patient **history** and a few laboratory tests
• Many diarrheal diseases are **self-limited** and do not require treatment
• A few infections require treatment, so rapid diagnosis is helpful
Definitions

- **Diarrhea**: increase in water content, volume or frequency of stools
  - More than 200 grams or 200 ml in 24 hours
  - For epidemiologic surveillance; consistency of less than soft and frequency of ≥3 per day
- **Infectious diarrhea**: caused by a microorganism
- **Acute diarrhea**: ≤14 days duration
- **Persistent diarrhea**: >14 days duration
- **Chronic diarrhea**: ≥30 days duration
- **Secretory diarrhea**: the intestine secretes rather than absorbs fluid (>1 liter/day)
- **Exudative diarrhea (dysentery)**: fresh blood, protein or pus in stool
What you need to know to diagnose infectious diarrhea

• Severity and time course (history)
• Characteristics
  – Febrile (physical examination)
  – Hemorrhagic (laboratory or history)
  – Nosocomial (history)
  – Persistent (history)
  – Inflammatory (laboratory)
• Exposures (history)
• Immunocompromised (history)
History of current illness

• Most of the information you need to make the diagnosis is in the patient history
  – When and how did your symptoms begin?
  – What is your stool like (grade it using the Bristol scale)?
  – How many bowel movements do you have per day normally and after your condition started?
  – Do you have fever, do you notice blood in your stool (dysentery) or do you always feel like you have to go (tenesmus)?
  – Are you dehydrated?
  – Do you have any other symptoms?
  – Are you on any medications?
### Bristol Stool Chart

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Separate hard lumps, like nuts (hard to pass)</td>
</tr>
<tr>
<td>Type 2</td>
<td>Sausage-shaped but lumpy</td>
</tr>
<tr>
<td>Type 3</td>
<td>Like a sausage but with cracks on its surface</td>
</tr>
<tr>
<td>Type 4</td>
<td>Like a sausage or snake, smooth and soft</td>
</tr>
<tr>
<td>Type 5</td>
<td>Soft blobs with clear-cut edges (passed easily)</td>
</tr>
<tr>
<td>Type 6</td>
<td>Fluffy pieces with ragged edges, a mushy stool</td>
</tr>
<tr>
<td>Type 7</td>
<td>Watery, no solid pieces. <strong>Entirely Liquid</strong></td>
</tr>
</tbody>
</table>
Other risk factors in the history

- Travel to a seacoast or developing country
- Day-care center
- Consumption of unsafe foods, dairy, drinking or swimming in surface water
- Animal exposure (farm, petting zoo, pets)
- Group exposure (dormitory, workplace)
- Medications (antibiotics, proton pump inhibitors)
- Underlying medical conditions
- Receptive anal intercourse
- Occupation as a food-handler
- HIV-positive
Antibiotic treatment of infectious diarrhea

It is important to determine the cause of infectious diarrhea because some agents should be treated, some are self-limited but may be treated under certain conditions, and some should not be treated with antibiotics.

1. Tend to treat: *Shigella, Campylobacter, Vibrio, parasites*
2. Sometimes: *Salmonella, C. difficile, Aeromonas/Plesiomononas*
3. Do not treat: *Enterohemorrhagic E. coli, Yersinia, viruses*

Generally you withhold antibiotic treatment until you identify the cause.
Evaluate severity and duration
Obtain history and physical examination**†‡§∥
Treat dehydration
Report suspected outbreaks¶
Check all that apply**: 

** A. Community-acquired or traveler’s diarrhea 
(especially if accompanied by significant fever or blood in stool) 

- Culture or test for:
  - Salmonella
  - Shigella
  - Campylobacter
  - *Escherichia coli* O157:H7 (if blood in stool, also test for Shiga toxin and refer isolates if toxin-positive)
  - *Clostridium difficile* toxins A ± B (in suspected nosocomial outbreaks; in patients with bloody stool and in infants, also add tests in panel A)

- Consider quinolone for suspected shigellosis in adults (fever, inflammation); macrolide for suspected resistant Campylobacter; avoid antimotility or certain antimicrobial drugs if suspected STEC (afebrile, bloody diarrhea).††

** B. Nosocomial diarrhea 
(onset after >3 days in hospital) 

- Test for *C. difficile* toxins A ± B (in suspected nosocomial outbreaks; in patients with bloody stool and in infants, also add tests in panel A)

- Discontinue antimicrobials if possible; consider metronidazole if illness worsens or persists.

** C. Persistent diarrhea >7 days 
(e especially if immunocompromised)

- Consider parasites††
  - Giardia
  - Cryptosporidium
  - Cyclospora
  - *Isospora belli*
    - plus
  - Inflammatory screen**

** If HIV-positive, add:**
  - Microsporidia (Gram-chromotrope)
  - *M. avium*-intracellulare complex
    - plus
  - Panel A

- Treat per results of tests.
The “3-day rule”

• “Fecal specimens from patients with diarrhea that develops after 3 days of hospitalization ...should not be submitted for routine stool culture...or ova and parasites”

• The only cause of infectious diarrhea that starts after 3 days of hospitalization is Clostridium difficile colitis

• Laboratories reject stool cultures after the patient has been hospitalized for more than 3 days (sometimes 5 days)
Stool specimen collection

- **Cary Blair medium** for culture and multiplex PCR transport; important for *Shigella*
- **PROTO-FIX™** for ova and parasite transport;
  - Formalin fixative
- **Fresh stool** for *C. difficile* PCR, lactoferrin, rotavirus antigen
  - Good for 2 hours
Testing for inflammatory diarrhea

- The “big four” bacterial pathogens (Salmonella, Shigella, Campylobacter and C. difficile, plus Yersinia) all produce **inflammatory diarrhea**; neutrophils in the stool
  - **Viral and parasitic agents are generally not inflammatory** except for Entamoeba histolytica
- Testing for inflammatory diarrhea can help to narrow the differential diagnosis (exclude viruses)
- Two tests for inflammatory diarrhea in the stool are lactoferrin and calprotectin tests
  - Antigen tests for proteins found in neutrophils
  - They are equivalent, lactoferrin seems to be more popular in the US, calprotectin in Europe
- Non-infectious inflammatory diarrhea diseases will also be positive, including ulcerative colitis and Crohn’s Disease
Stool lactoferrin test

• Microscopic examination of stool for leukocytes (inflammatory diarrhea) has problems
  – WBCs die and disappear within minutes
  – WBCs look like other things

• Preferred test; qualitative enzyme immunoassay test for stool lactoferrin or calprotectin antigen
  – Takes 10 minutes
  – Antigen is stable
  – Doesn’t work in leukopenic patients
Testing for bacterial pathogens; the standard stool culture

• Includes Salmonella, Shigella, Campylobacter and shigatoxin (enterohemorrhagic E. coli)

• Media:
  – MacConkey agar
  – MacConkey broth
  – Xylose lysine deoxycholate agar (XLD)
  – Campy Blood agar (special atmosphere)
Campylobacter culture

- Campylobacter are microaerophilic, usually in low numbers, and grow slowly
- You need a selective medium, special atmosphere, higher temperature (42° C) and longer incubation (48 hours) to grow them
- Campylobacter agar is blood agar with trimethoprim, vancomycin, and polymyxin B to inhibit normal enteric bacteria and is made and incubated anaerobically
Testing for enterohemorrhagic *E. coli*

- The detection of EHEC has evolved
- The first EHEC strains we discovered failed to ferment sorbitol, so early tests used **Sorbitol MacConkey agar** and looked for non-fermenting colonies; *wrong*
- Later, we thought that all EHEC were serotype **O157:H7**, so we looked for those; *wrong*
- Now we look for the shiga toxin directly
• May be potential pathogens
  – Heat-labile (LT) E. coli toxin
  – Heat-stable (ST) E. coli toxin
  – Enteroinvasive E. coli (EIEC)
  – Enteroaggregative E. coli (EAEC)
  – Enteropathogenic E. coli (EPEC)

• We don’t know if there are carrier states for these *E. coli* strains

• We are seeing these as co-infections with other pathogens
Rapid testing using antigen tests

- **Shiga toxin for enterohemorrhagic *E. coli* (EHEC)**
  - Test the MAC broth for toxin antigen with an EIA
  - Takes 20 minutes
  - Differentiates toxin 1 and 2

- **Salmonella and Shigella**
  - Use a latex agglutination test on non-fermenting MAC or XLD colonies to identify
    - *Shigella*; identifies to species (*sonnei, dysenteriae, flexneri and boydii*)
    - *Salmonella*; identifies to serogroup (A, B, C, D, E, G and Vi)
Testing for *Clostridium difficile*

- The #1 cause of hospital-acquired and antibiotic-associated diarrhea
- Tested if the patient has been hospitalized or has taken an antibiotic within 3 months
- The current standard for *C. difficile* is a nucleic acid amplification test
Optional testing for unusual bacterial stool pathogens

- Requires additional request:
  - *Vibrio cholerae* and *parahaemolyticus*
    - Use thiosulfate-citrate-bile salts-sucrose (TCBS) agar
  - *Yersinia enterocolitica*
    - Use cefsulodin-irgasan-novobiocin (CIN) agar
  - Aeromonas and Plesiomonas
    - Add a blood agar plate, screen with oxidase
  - Unusual stuff if found in pure culture:
    - Yeast
    - *Staphylococcus aureus*
    - *Pseudomonas aeruginosa*
Susceptibility testing and treatment

- Antibiotics for enteric infections are limited
  - CLSI says report only **ampicillin, a fluoroquinolone and trimethoprim/sulfamethoxazole**
  - **Cephalosporins do not work** for gastroenteritis
    (may report 3rd generations for Salmonella bacteremia)
  - Comment; “Antimicrobial therapy of *Salmonella* noninvasive gastroenteritis is not indicated unless the patient is younger than 3 months, immunocompromised, has severe colitis, is septic, or has chronic GI disease.”
- **Shigella**: test and report antibiotics
- **Campylobacter**: no susceptibility testing, treat with a macrolide empirically
Testing for viral pathogens

• **Rotavirus**
  – Antigen or PCR test
  – Common cause of seasonal (late winter) diarrhea in children; see the epidemic curve

• **Norovirus**
  – Most common cause of diarrhea in adults
  – Antigen or PCR test

• **Sapovirus** and **Astrovirus**
  – Cause epidemics in adults
  – New for the US, available only with PCR
Multiplex testing for stool pathogens

- Multiplex PCR is ideal for detecting stool pathogens
  - You are really looking for only a dozen things
  - Detects low numbers of specific pathogens
- Several versions are available
  - Combines bacterial, viral and parasite testing
- Detects co-infections
- Does not replace culture for epidemiology typing
- Detects things we have not detected before
Multiplex testing for stool pathogens

- Take 200 ul sample directly from the Cary-Blair transport vial
- Mix with buffer, inject into the test container ("pouch")
- Insert the pouch into the machine
- 2 minute hands-on time, 1 hour test time
- Machine uploads the result into the computer
Targets in multiplex PCR test

- **Bacteria**
  - *Campylobacter* genus
  - *Clostridium difficile* toxin
  - *Plesiomonas shigelloides*
  - *Salmonella* genus
  - *Yersinia enterocolitica*
  - *Vibrio* genus
  - *Vibrio cholerae*
  - EAEC
  - EPEC
  - ETEC
  - STEC
  - EIEC/Shigella

- **Viruses/parasites**
  - Adenovirus 40/41
  - Astrovirus
  - Norovirus
  - Rotavirus
  - Sapovirus
  - *Cryptosporidium*
  - *Cyclospora*
  - *Entamoeba histolytica*
  - *Giardia lamblia*
Comparison of workflow

- **Conventional culture**
  - Time to negative result; 44 hours
  - Time to positive result; 97-145 hours
  - Number of process steps/decisions; 141
  - Technologist labor; 15 minutes for negative, 40 minutes more for positive

- **PCR**
  - Time to all results; about 2 hours
  - Number of process steps/decisions; 25
  - Technologist time for all steps; 2 minutes
Example of data

<table>
<thead>
<tr>
<th>WEEK</th>
<th>Positive Specimens</th>
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<tbody>
<tr>
<td>1</td>
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<td>15</td>
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</tbody>
</table>

Legend:
- Campylobacter
- E. coli
- Salmonella
- Shigella/Enteroinvasive E. coli
- Yersinia enterocolitica
- Norovirus
- Rotavirus
- Sapovirus
• PCR cannot distinguish between *Shigella* and Enteroinvasive *E. coli*

• **Positivity rate** is much higher than conventional testing
  – >50% positive compared with <10% positive for culture
  – The most common positive targets were things physicians may not expect or know what to do with; EPEC, EAEC
  – Viral agents are common; as common as bacterial, and include Astrovirus and Sapovirus, which are new

• **Coinfection** is common; >30% of positives contained >1 target, in all combinations

• *C. difficile* carriage is common (13%); what does it mean?
Conventional enteric pathogen testing is à la carte for bacterial, viral, *C. difficile* and parasite testing; PCR is all-inclusive; the syndrome approach to testing

- The panel includes targets you may not need, but have to take them
- Total cost will increase, although cost per reportable result is lower for PCR

PCR is much more sensitive in detecting most pathogens; detects colonization as well as infection

Managing appropriate utilization and provider interpretation
Thank you!
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