Introduction

• Mastitis – in most cases associated with udder infections/intra-mammary infections (IMI)
  – Any microorganism (bacteria/fungi/algae)
  – Mainly bacteria e.g.
    • Staphylococci
    • Streptococci
    • Coliforms

Example: National surveys in Sweden 2002/03 and 2008/09 – routine culturing

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Acute clinical (n=1056)</th>
<th>Subclinical (n=590)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sa</td>
<td>19 %</td>
<td>19 %</td>
</tr>
<tr>
<td>CNS</td>
<td>16 %</td>
<td>16 %</td>
</tr>
<tr>
<td>Srd</td>
<td>9 %</td>
<td>9 %</td>
</tr>
<tr>
<td>Sru</td>
<td>8 %</td>
<td>8 %</td>
</tr>
<tr>
<td>E.coli</td>
<td>3 %</td>
<td>3 %</td>
</tr>
<tr>
<td>Sra</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Ap</td>
<td>6%</td>
<td>6%</td>
</tr>
<tr>
<td>Contam</td>
<td>4%</td>
<td>4%</td>
</tr>
<tr>
<td>No growth</td>
<td>22%</td>
<td>11%</td>
</tr>
</tbody>
</table>

Why do we need microbiological diagnostics of udder infections?

• For treatment decisions
  – Eg clinical cases, dry-cow therapy
• To identify type of mastitis problem
  – Decisions regarding management activities, control programs etc
• To control health status
  – Eg buying/selling, breeding decisions

Microbiological diagnostics – How is it done? Guidelines

• Nordic Recommendations for the Laboratory Diagnostics of Mastitis, 1974
• International Dairy Federation, IDF
  – Laboratory Methods for use in Mastitis Work, 1981
  – Definition and Guidelines for Diagnosis, 1987
Microbiological diagnostics – How is it done? Guidelines

- NMC (National Mastitis Council)
  - Laboratory Handbook on Bovine Mastitis, 1999
  - Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality, 2004
- Laboratory accreditation (EN 17025)

Microbiological diagnostics – How is it done? Guidelines

- Guidelines are not complete
- Cover culture-dependent methods
- PCR-tests not included
- Update needed

Microbiological diagnostics - points to consider

- Milk sampling
- Storage and transport of milk samples
- Investigation of growth/presence of microorganisms
  - Veterinary field lab vs commercial labs
  - Culturing vs PCR
- Interpretation of findings – making a microbiological diagnosis at the lab
- Interpretation of the results – making a decision at the cow/farm

Milk sampling

- Decisions before sampling
  - Quarter/cow/herd level?
  - Type of sample? Quarter or composite sample?
    - Pros and cons, culture/PCR ...
  - Which cows/quarters to sample?
    - Based on purpose, history, SCC ...
  - When to sample?
    - In relation to milking, during the lactation cycle ...
- The objective of the investigation decides which way to go

Milk sampling: Cow level – Quarter vs composite samples

- Quarter samples = gold standard
  - Easier to sample aseptically
  - Gives the best picture of the bacterial concentration at the sampling occasion
    - Bacterial concentration varies
- Cow composite sample =
  - Larger risk for contamination
  - Dilution effect
Milk sampling: Cow composite sample - manual vs test milking sampling

- **Manual**
  - Same routine as quarter sampling – aseptic, but collect milk from all quarters in the same tube
  - Risks
    - Must open the tube several times = risk for contamination
    - Difficult to get the same amount from all quarters
    - Difficult to mix properly

- **Via test milking equipment (PCR)**
  - Easy to do
  - Risks
    - Contamination from skin and equipment
    - Risk for carry-over between cows
  - Dilution effect
    - Subclinical quarters have lower production

Aseptic quarter milk sampling

- Sample a clean and dry teat of a clean and dry cow in a clean and dry barn!
  - Clean the teats if necessary using dry/wet tissues
  - Strip out some milk
  - CMT test
  - Clean the teat end with alcohol scrubs
  - Take sample aseptically using sterile milk tubes (with/without preservative)
  - Teat dipping (if suitable/needed)

Storage/transport of milk samples

- Store milk tubes chilled
- Deliver/Send milk tubes to the lab quickly
  - Store chilled at field lab
  - Transport milk tubes chilled to lab
- Risks
  - Too high temperature if not chilled
    - Abundant growth of mixed flora => not correct diagnosis
  - Overgrowth of contaminant => interpreted as pure culture => false positive sample
  - Too low temperature – freezing
    - Some pathogens can die => false negative or false positive (should have been mixed flora)

Storage/transport of milk samples

- If PCR analysis
  - Use sterile tubes with preservative (bronopol) = kills bacteria
  - No need for chilling
  - Freezing not a problem

Investigation of growth/presence of microorganisms in milk samples

- Vet field lab vs accredited lab
- Culturing
  - Most common
  - Gold standard
- PCR (polymerase chain reaction) tests
  - Culture-independent
  - Commercial multiplex tests available
Culturing

- Milk (10µl) is spread on
  - Non-selective agar plates (blood agar)
  - Selective agar plates (eg SELMA used at field lab)
- Incubated at 37°C (aerobic) and evaluated after
  - 18-24 h
  - 48 h

Culturing

- Evaluation of colony morphology
  - Colour, size, hemolysis...
- Evaluation of numbers of colonies and type of growth (pure, contamination)
- Growth on selective agar
- Gram-staining (microscope)
  - Size, shape (coccï/rods), chains, cluster of grapes...
- Agglutination tests (S aureus, streptococci)
  - OK to use with bovine isolates?

Culturing

- Biochemical testing eg
  - Catalase
  - Potassium hydroxide
  - Coagulase
  - Carbohydrate fermentation
  - CAMP reaction
  - PI (PGUA/indol)
  - etc

Culturing – new techniques

- Species differentiation of bacteria
  - Matrix-Assisted Laser Desorption/Ionization
  - Time of Flight (MALDI-TOF) mass spectrometry
- Molecular methods identifying strains within bacterial species eg
  - PFGE
  - RAPD
  - MLST

Culturing

- Antibiotic resistance eg
  - β-lactamase production – staphylococci
    - Eq clover-leaf method
    - Microdilution method
      - Minimum inhibitory concentration (MIC)

PCR tests

- Commercial multiplex real-time PCR tests (Finnzymes Diagnostics, Finland)
- Identification of DNA
  - certain udder pathogens
  - In some tests - βlZ gene – β-lactamase production
PCR – How is it done?

• DNA extraction directly from milk (350 μl)
• Mix DNA extract with PCR solutions
• DNA amplification in 40 thermal cycles
  – real-time PCR instrument

Pros and cons PCR vs culturing

• Pros eg
  – Faster
  – Can detect smaller amounts of bacteria
  – Can detect killed and growth inhibited bacteria
  – Possible to use milk with bronopol
  – Can detect Mycoplasma bovis (difficult to culture)

• Cons eg
  – Identifies a limited number of pathogens
  – More expensive
  – Not possible to save isolates for further studies eg antimicrobial resistance, genotyping
  – Results can be difficult to interpret

Problems with interpretation of PCR results

• Staphylococci
  – If Staphylococcus spp are detected it can be due to presence of several species = contamination
  – If blaZ gene is detected and Staphylococcus spp and/or S aureus you can't tell which species that is resistant
• When several pathogens are detected in a sample
  – Difficult to decide if contamination or not
  – PCR detects more samples with presence of bacteria than culturing
    – Interpretation? Killed or alive? True infection/contamination?

• Based on today's knowledge
  – If not aseptic samples, PCR recommended mainly at herd investigations of Str agalactiae and Mycoplasma bovis

Interpretation of findings – making a diagnosis at the lab

• Culturing
  – No growth
  – Growth of pathogen
    • Amount (1-3)
    • In pure culture
    • In mixed flora
    – Contamination
• PCR
  – No presence
  – Presence of one or more pathogens and/or blaZ
    • Amount (+, ++, +++)
    • Ct values
    – Contamination?

Interpretation of the results at the cow/herd

• Combine microbiological findings with information on
  – Cow/herd history, clinical signs, SCC
  – Sampling and sample handling
  – Culturing or PCR
• Are the lab results true or false?

Interpretation of the results at the cow/herd

• No growth/presence of bacteria
  – True
    • Healthy udder
    • Non-infectious mastitis
    • Pathogen already eliminated
  – False
    • Pathogen needs special culturing conditions or is not included in panel
    • The pathogen concentration in milk is too low
    • Inhibiting substances
  – Take new sample? How good is one sample?
Interpretation of the results at the cow/herd

• Growth/presence of one pathogen
  – True
    • Infectious mastitis
    • Latent infection
  – False
    • If contaminated from start and sample handled/stored suboptimally can result in over-growth of one species

Interpretation of the results at the cow/herd

• Growth/presence of mixed flora/contamination
  – Culture: If 3 or more species = contamination
    • Exception if *S. aureus* or *St. agalactiae* are clearly identified
  – PCR: number of species?
  – Poor sampling or sample handling/transport
  – Uncertain result - may hide an infection
  – Take new sample!

Future developments and needs

• New methods and understanding of pathogenesis
• Culture-dependent methods eg
  – Quality control (field lab/commercial lab)
  – Improvements – eg MALDI-TOF
  – Cost-efficient molecular methods for isolate differentiation within species
• Culture-independent methods eg
  – Improvements of PCR
  – New techniques to detect microorganisms in milk
• Cow-side bacteriological quick tests

Thank you for your attention!