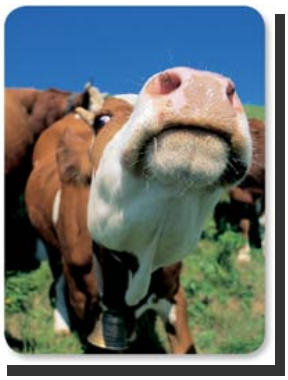
FINNZYMES DIAGNOSTICS

PathoProof™ Mastitis PCR Assay

Mikko Koskinen, Ph.D.
Director, Diagnostics, Finnzymes Oy



- Real-time PCR -based mastitis testing in milk monitoring programs
- PathoProof Mastitis PCR Assay
- Comparison of the PCR assay with conventional bacterial culture-based mastitis testing
- Brief overview of validation studies

FINNZYMES

Finnzymes Oy

- Founded in 1986
- Headquarters in Espoo, Finland
- Among the world's leading molecular biology reagent manufacturers
- Focus on PCR (Polymerase Chain Reaction) based products since 1989

FINNZYMES

Somatic Cell Count and IMI
Background

- SCC measurements are commonly used to monitor milk quality
- Elevated SCC is an indication of intramammary infection (IMI), also known as mastitis
- IMI leads to decreased milk yield and quality and is by far the economically most important infectious disease in dairy cattle
- Identification of bacteria responsible for IMI is one of the cornerstones for targeting antimicrobial therapy and for monitoring the disease prevalence and severity in the herd
- In an optimal situation, SCC score would be routinely coupled with information on bacteria responsible for IMI
- In the current mastitis testing schemes SCC measurements and IMI pathogen testing are separate practices. WHY!!??
  - milk used for SCC measurements is preserved and therefore cannot be used for bacterial culturing

FINNZYMES

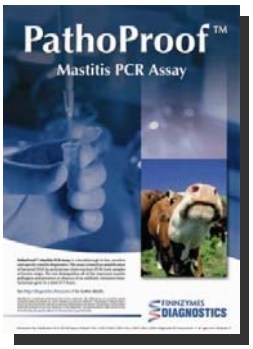
If bacteria responsible for IMI were identified in a milk monitoring program...

- In addition to SCC score, the dairy farmers would receive the following information:
  - -is the elevated SCC score due to bacteria causing IMI?
  - -what is/are the bacterial species responsible for IMI?
  - -what is the proper treatment?
  - -are there animals with still a normal SCC but signs of bacterial infection
- Immediate information on bacteria responsible for IMI would lead to (for an individual animal):
  - -improved treatment efficacy (better and faster cure rates)
  - -less unnecessary use of antimicrobials
  - -less discarded milk and faster return to normal milk
- ...and in a herd monitoring program:
  - -early detection of arising problems
  - -identification, treatment and eradication of animals causing mastitis problems in the herd
  - -confirmation that new arrivals joining the milking herd are not infected by mastitis pathogens (do not present a risk to the herd)
  - -accurate information on the response of mastitis pathogens to actions at the farm
- This practice would also eliminated the need to take separate milk samples for bacterial culturing

FINNZYMES

Real-time PCR in milk monitoring programs

- Real-time PCR –based technology to identify and quantify all major pathogens causing IMI
- Based on detection of bacterial DNA ⇒ does not rely on the ability of bacterial cells to grow in culture ⇒ can be used with preserved milk samples and therefore integrated to milk monitoring



http://diagnostics.finnzymes.fi

FINNZYMES

Bacterial species and species groups identified by the PathoProof™ Mastitis PCR Assay

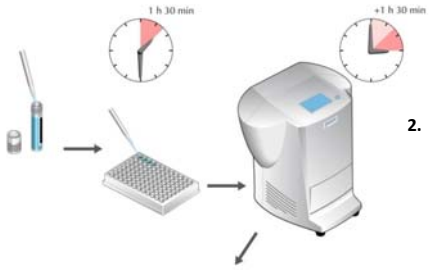
-*Staphylococcus aureus*
-Coagulase negative staphylococci (CNS)
-*Streptococcus agalactiae*
-*Streptococcus dysgalactiae*
-*Streptococcus uberis*
-*Escherichia coli*
-*Corynebacterium bovis*
-*Enterococcus faecalis, E. faecium*
-*Klebsiella pneumoniae, K. oxytoca*
-*Serratia marcescens*
-*Arcanobacterium pyogenes, Peptostreptococcus indolicus*
-Beta-lactamase penicillin resistance gene (blaZ)

⇒ Large-scale data demonstrate that the target bacteria cover >99% of all subclinical and clinical mastitis cases in Europe, as well as in North America (eg. Makovec & Ruegg 2003; Pitkälä et al. 2004; Tenhagen et al. 2006; Koivula et al. 2007)

FINNZYMES

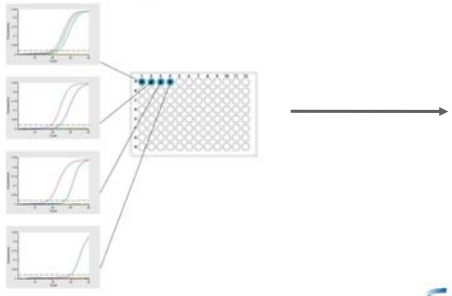
PathoProof™ Mastitis PCR Assay laboratory workflow

1. Extraction of bacterial DNA from fresh or preserved milk.



2. Real-time PCR amplification of bacterial DNA

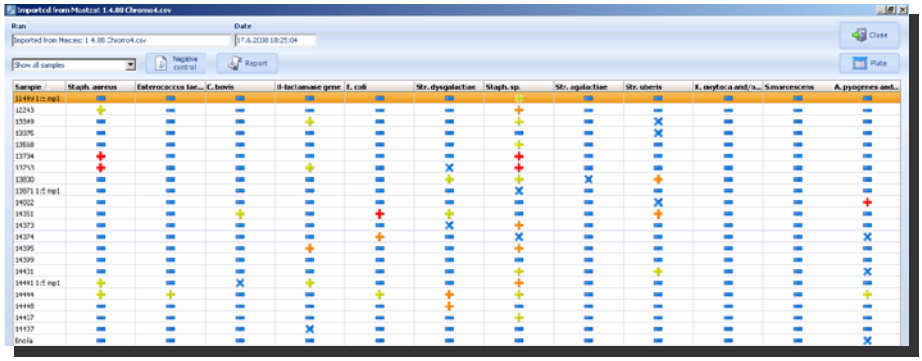
3. The PathoProof Mastitis PCR Assay identifies and quantifies simultaneously 11 most important mastitis species/groups and the most relevant antibiotic resistance gene (blaZ)



FINNZYMES

PathoProof™ Mastitis PCR Assay laboratory workflow

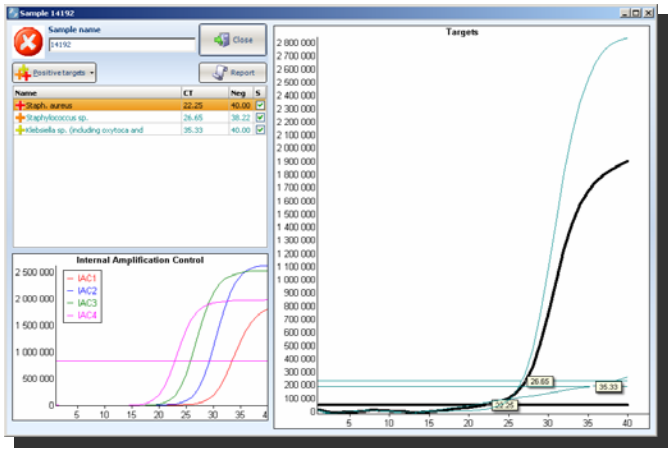
4. Automated interpretation, quality flagging and reporting of the results with the Norden Lab Mastis Studio



FINNZYMES

PathoProof™ Mastitis PCR Assay laboratory workflow

4. Automated interpretation, quality flagging and reporting of the results with the Norden Lab Mastis Studio



FINNZYMES

Comparison of the PathoProof Mastitis PCR Assay and conventional bacterial culturing		
	PathoProof Mastitis PCR Assay	**Bacterial culturing**

FINNZYMES

Comparison of the PathoProof Mastitis PCR Assay and conventional bacterial culturing

	PathoProof Mastitis PCR Assay	Bacterial culturing
Time	3-4 h for all bacterial species	24-48 (sometimes up-to 72) h depending on the situation

FINNZYMES

Comparison of the PathoProof Mastitis PCR Assay and conventional bacterial culturing

	PathoProof Mastitis PCR Assay	Bacterial culturing
Time	3-4 h for all bacterial species	24-48 (sometimes up-to 72) h depending on situation
Accuracy (analytical specificity and sensitivity)	100% specificity and 100% sensitivity for all bacterial species[1] and the beta-lactamase penicillin resistance gene.[2] Specificity is not dependent on user experience	Specificity of bacterial identification is highly dependent on user experience. Some bacteria are very challenging to identify. Nearly all conventional penicillin resistance identification methods have poor sensitivity (high frequency of false negatives)[2]

1- Koskinen et al. (2008) Analytical specificity and sensitivity of a real-time PCR assay for identification of bovine mastitis pathogens: *J. Clin. Microbiol.* In review.
2- Pitkälä et al. (2007) Comparison of tests for detection of beta-lactamase producing staphylococci. *J. Clin. Microbiol.* 45: 2031-33.

FINNZYMES

Comparison of the PathoProof Mastitis PCR Assay and conventional bacterial culturing

	PathoProof Mastitis PCR Assay	Bacterial culturing
Time	3-4 h for all bacterial species	24-48 (sometimes up-to 72) h depending on situation
Accuracy (analytical specificity and sensitivity)	100% specificity and 100% sensitivity for all bacterial species and the beta-lactamase penicillin resistance gene. Specificity is not dependent on user experience	Specificity of bacterial identification is highly dependent on user experience. Some bacteria are very challenging to identify. Nearly all conventional penicillin resistance identification methods have poor sensitivity (high frequency of false negatives)
Sensitivity with real milk samples	Viability of bacteria does not decrease sensitivity. Provides positive results from approximately 40-50% of 'no-growth' samples[1]	'No-growth' results from up-to 50% of all milk samples. Viability of bacteria to grow on culture determines sensitivity[2]

1- Pyörälä et al. (2008) Real-time PCR –based bacteriological diagnosis of milk samples with no growth in conventional culturing. 25th Congress of World Buiatrics Association, Budapest, Hungary, June 2008.
2- Makovec & Ruegg (2003) Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J. Dairy Sci.* 86:3466-72.

FINNZYMES

Comparison of the PathoProof Mastitis PCR Assay and conventional bacterial culturing

	PathoProof Mastitis PCR Assay	Bacterial culturing
Time	3-4 h for all bacterial species	24-48 (sometimes up-to 72) h depending on situation
Accuracy (analytical specificity and sensitivity)	100% specificity and 100% sensitivity for all bacterial species and the beta-lactamase penicillin resistance gene. Specificity is not dependent on user experience	Specificity of bacterial identification is highly dependent on user experience. Some bacteria are very challenging to identify. Nearly all conventional penicillin resistance identification methods have poor sensitivity (high frequency of false negatives)
Sensitivity with real milk samples	Viability of bacteria does not decrease sensitivity. Provides positive results from approximately 40-50% of 'no-growth' samples	'No-growth' results from up-to 50% of all milk samples. Viability of bacteria to grow on culture determines sensitivity
Preserved samples	Can be used (bronopol)	Cannot be used

FINNZYMES

Comparison of the PathoProof Mastitis PCR Assay and conventional bacterial culturing

	PathoProof Mastitis PCR Assay	Bacterial culturing
Time	3-4 h for all bacterial species	24-48 (sometimes up-to 72) h depending on situation
Accuracy (analytical specificity and sensitivity)	100% specificity and 100% sensitivity for all bacterial species and the beta-lactamase penicillin resistance gene. Specificity is not dependent on user experience	Specificity of bacterial identification is highly dependent on user experience. Some bacteria are very challenging to identify. Nearly all conventional penicillin resistance identification methods have poor sensitivity (high frequency of false negatives)
Sensitivity with real milk samples	Viability of bacteria does not decrease sensitivity. Provides positive results from approximately 40-50% of 'no-growth' samples	'No-growth' results from up-to 50% of all milk samples. Viability of bacteria to grow on culture determines sensitivity
Preserved samples	Can be used (bronopol)	Cannot be used
Quantitative / qualitative	Quantitative and qualitative	Mainly qualitative

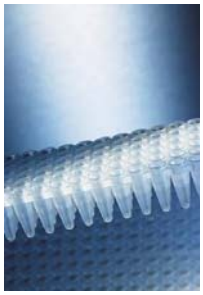
FINNZYMES

Validation studies

1. analytical specificity and sensitivity

- Primary goal: to demonstrate that the PCR assay does not provide false positive results

  ⇒ a positive result obtained using the assay is a true positive even if the sample is negative in culture



FINNZYMES

Validation studies

1. analytical specificity and sensitivity

- 650 culture isolates, representing 83 different species or species groups
- Origin: Finland, Canada, USA, Portugal, Italy, UK, Norway
- Penicillin resistance: 150 staphylococcal isolates with a known status for the beta-lactamase gene (Pitkälä et al. 2007, *J. Clin. Microbiol.* 45: 2031-3)

analytical specificity = true negatives / (true negatives + false positives)
analytical sensitivity = true positives / (true positives + false negatives)

FINNZYMES

Validation studies

1. analytical specificity and sensitivity

(results for all strains and, in brackets, for strains originating from mastitis)

Bacterial target	Analytical specificity	Analytical sensitivity
Staph. aureus	100% (100%)	100% (100%)
Staph. sp.	100% (100%)	100% (100%)
Str. agalactiae	100% (100%)	100% (100%)
Str. dysgalactiae	100% (100%)	100% (100%)
Str. uberis	99,0% (100%)	100% (100%)
E. coli	99,5% (100%)	100% (100%)
C. bovis	100% (100%)	100% (100%)
Enteroroccus sp.	100% (100%)	100% (100%)
Klebsiella sp.	100% (100%)	100% (100%)
Serratia marcescens	100% (100%)	100% (100%)
A. pyogenes	100% (100%)	100% (100%)
Penicillin resistance (blaZ)	100% (100%)	100% (100%)

FINNZYMES

Validation studies

2. field trial comparison against bacterial culturing and studies of 'no growth' samples

- Subclinical and clinical mastitis milk samples tested in parallel using bacterial culturing and the PCR assay
- 'no growth' samples tested using the PCR assay
- Results in brief:
  - -when both methods provided positive results, the results indicated the same bacterium in >99% of all cases
  - -approximately 35% of all samples yielded no growth in bacterial culture. Of these, approximately 50% were positive with the PCR assay

FINNZYMES

Validation studies

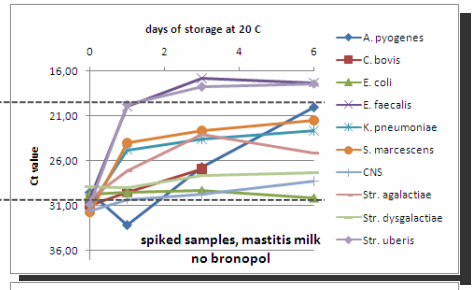
3. bronopol

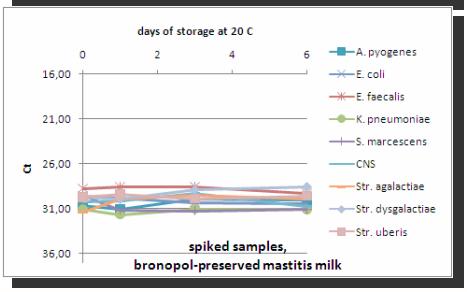
- Bronopol is used to preserve milk samples in milk testing programs
- In many countries, milk is transported to the bacterial culture laboratories without any form of preservation or refrigeration
- Questions:
  - 1) can mastitis bacteria grow during transportation and can that growth bias the culture test results?
  - 2) does bronopol have an adverse effects on the results of the PathoProof Mastitis PCR Assay?
- Methods
  - -mastitis milk samples were spiked with different bacterial species
  - -the samples were stored at room temperature with and without bronopol
  - -PathoProof Mastitis PCR Assay after: point zero, 1 day, 3 days, 6 days

FINNZYMES

Validation studies
3. bronopol

1000x increase in bacterial quantity

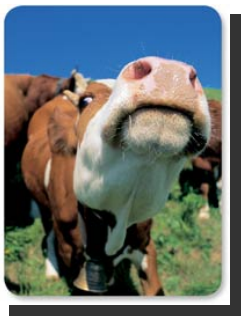




FINNZYMES

Validation studies
3. bronopol

- Conclusions:
  - -bacterial quantity can increase within 24 hours by >1000-fold in a fresh mastitis milk sample
  - -growth is not linear and can vary depending on the species, strain, sample, etc. ⇒ the effect cannot be predicted
  - -bronopol does not affect the results of the PathoProof Mastitis PCR even following 6 days of storage at RT ⇒ the assay can be used reliably with bronopol-preserved milk



FINNZYMES

Thank you for your attention!

FINNZYMES