



Validation of three multiplex real-time PCR for Diagnosis of six Major Respiratory Pathogens in Cattle



Three Multiplex PCR for simultaneous Diagnosis of Six Major Respiratory Pathogens in Cattle



Birthplace of Charolais breed



Bovine Respiratory Disease (BRD)

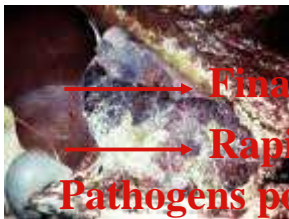
Most important health problem in Burgundy

Young cattle < 12 months, meat

Stress, contacts with adult

Viruses and/or *Mycoplasma*

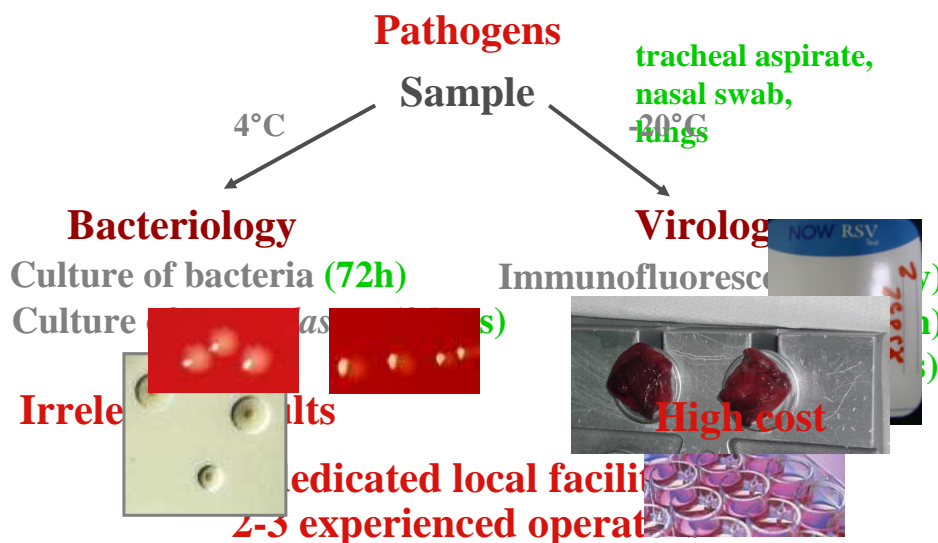
Primary bacterial surinfection



→ Financial losses

→ Rapid and accurate diagnosis of ALL Pathogens potentially involved

Classical Methods of Diagnosis of Respiratory



Six respiratory pathogens = « one » molecular tool

Sample **tracheal aspirate,
nasal swab, lungs**
 -20°C ↓
 one NA extraction **viral RNA and
bacterial DNA**

↓
 three multiplex PCR running simultaneously

RT-PCR **bRSV** *prot.F-6-FAM* - **PI-3** *prot.M-VIC* - IPC *GAPDH-NED*

PCR *P.multocida* *16SRNA* - *M.haemolytica* *plpE* - IPC

PCR *H.somni* *rpoB* - *M.bovis* *16S-ITS-23S* - IPC

1 operator, 1 laboratory facility, 1 day

Six pathogens = « one » molecular tool

one NA extraction ?

samples found positives for the six pathogens
 and from the three different types of samples

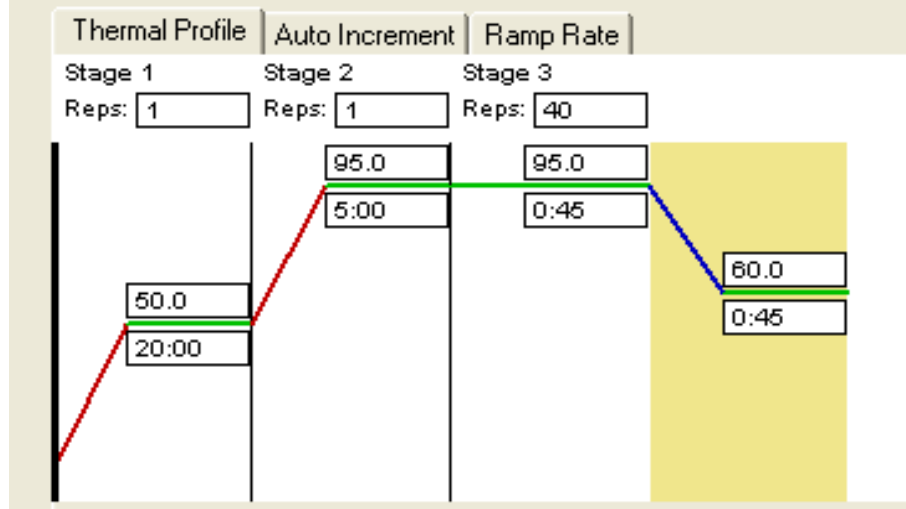
extraction-type « RNA » RNeasy kit (Qiagen)

Six pathogens = « one » molecular tool

one NA extraction ?

Sample	Ct <i>P.multocida</i>	Ct <i>M.haemolytica</i>
Lung	33.76 (31.55)	36.59 (38.01)
Tracheal aspirate	17.69 (17.11)	34.28 (31.38)

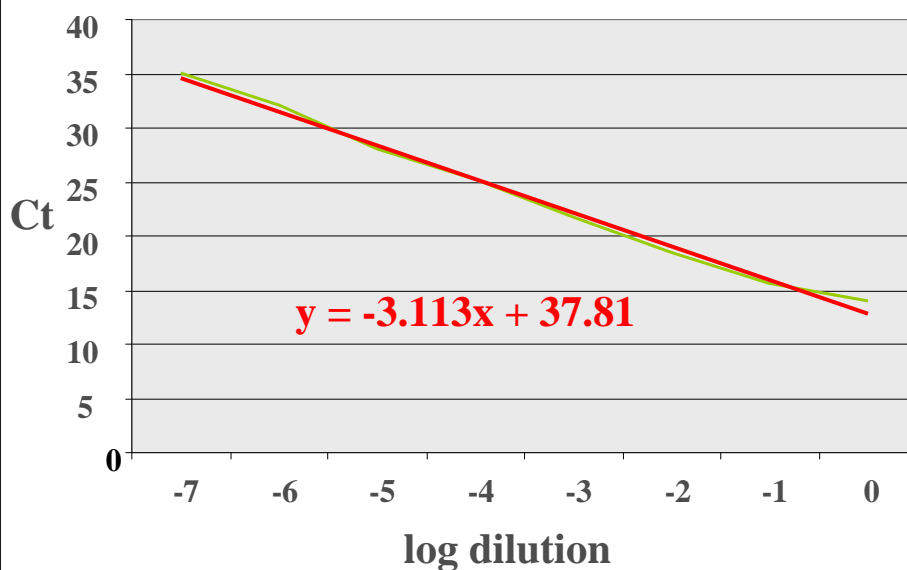
Thermal Cycler Protocol



Six pathogens = « one » molecular tool

One run?

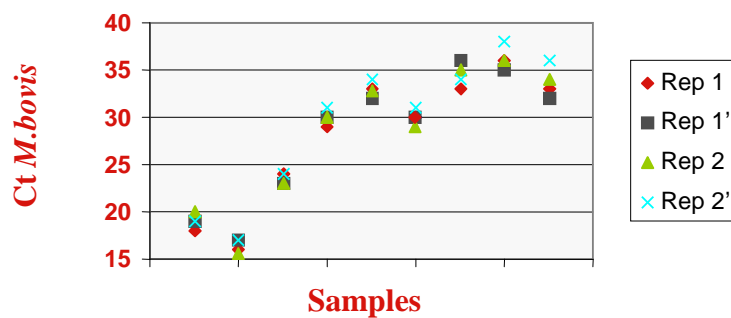
NA extract	Ct <i>P.multocida</i>	Ct <i>M.haemolytica</i>
1	26.11 (23.03)	37.34 (33.59)
2	30.04 (32.92)	35.78 (37.53)
3	34.79 (37.01)	–
4	–	27.54 (28.39)



Six pathogens = « one » molecular tool

Repeatability of PCR assay?

Replicates of positive samples are treated as independent sample by two operators on two thermocyclers



Analytical specificity assessed for each PCR

Inclusivity

3 strains of *P.multocida*
 3 strains of *M.haemolytica*
 4 strains of *H.somni*
 23 strains of *M.bovis*
 5 strains of RSV

24 strains of PI-3
 9 from Burgundy
 15 from Brittany

Exclusivity

No cross-reaction with the others targets

No cross-reaction with bacteria, viruses or fungi found in respiratory samples: *E.coli*, *Salmonella*, *A.pyogenes*, *Enterococcus*, *S.aureus*, *Moraxella*, *Pseudomonas*, *Pantoea*, *Proteus*, *Bacillus*; *M.bovirhinis*; BVDV, BHV-1, Adenovirus; *Aspergillus*, *Mucor*;

Comparison with traditionnal techniques

Determination of Diagnostic Sensitivity (**DSe**) and Diagnostic Specificity (**DSp**) on samples found positive or negative with traditionnal techniques

	« Positives » samples	« Negatives » samples
Positives PCR results	True POS.	False POS.
Negatives PCR results	False Neg.	True Neg.
	DSe = TP/(TP+FN)	DSp = TN/(TN+FP)

Comparison with traditionnal techniques

RT-PCR RSV-PI-3- IPC

14 samples

DSe = 100% DSp = 90%

PCR *H.somni-M.bovis*-IPC

29 samples PCR *H.somni*

DSe = 100% DSp = 84%

32 samples PCR *M.bovis*

DSe = 100% DSp = 73%

Comparison with traditional techniques

PCR *P.multocida*-*M.haemolytica*-IPC

49 samples PCR *P.multocida*

DSe = 94% **DSp = 66%**

49 samples PCR *M.haemolytica*

DSe = 55% **DSp = 73%**

Discrepancies on 46% of samples

18% of samples = **inaccuracy of bacterial identification**

14% of samples = **invasive-competitor germs in culture**

14% of samples = **delayed signal in PCR**

Conclusions

Advantages of molecular tool after 8 months of utilisation in LDA71

For the lab: improvement of feasibility, reduction of Cost analysis

For the veterinarians: improvement of bacterial diagnosis

Positives samples	PCR	Culture
<i>P.multocida</i>	63.8%	20.9%
<i>M.haemolytica</i>	19.4%	7.3%
<i>H.somni</i>	17.4%	3%
<i>M.bovis</i>	14%	4.5%

Conclusions

Limits of molecular tool

No bacterial sensitivity profiles to antibiotics
Relevance of weakly positive results?

Perspectives in LDA71

Other targets?

DNA microarrays?

Perspectives for LSI

Validation of Commercial kits from
PCR *P.multocida*-*M.haemolytica* and *H.somni*

Validation of NA extraction on magnetic beads

**Multiplex diagnostic kits for all bovine
respiratory pathogens at the end of this year**

TaqVet bRSV-PI3+IPC

TaqVet *Mycoplasma bovis*+IPC

TaqVet *Histophilus somni*+IPC

TaqVet *P.multocida*-*M.haemolytica*
+IPC





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Approch of analytical sensitivity

Detectability of each PCR

Serial dilutions of bacterial or viral suspensions
(positive sample for RSV)

NA extraction in duplicate and PCR

Detection limits = last dilution with positive results
for both replicates

Virus = 10^{-5}

Pasteurellacea = 10^{-6} - 10^{-7}

M.bovis = 10^{-6}