Biomarkers to predict severity of bovine *E.coli* mastitis in the periparturient period: bridging the gap between genotype and phenotype

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The causes of periparturient (PP) *E.coli* mastitis in lactating cows are complex and multifactorial (Burvenich et al., 2007). The disease is accompanied by a large variation in clinical symptoms varying from mild/moderate, to severe life-threatening sepsis. There is a small subpopulation of severely affected cows that suffer from unbalanced inflammation. It is now accepted that severity of PP *E.coli* mastitis is mainly determined by cow factors (Burvenich et al., 2003). Studies on isolated blood neutrophils (PMN) of healthy cows before intramammary infection with alive *E.coli* bacteria, showed that chemotaxis (Lohuis et al., 1990; Kremer et al., 1993) and the capacity to produce reactive oxygen species (Heyneman et al., 1990) before challenge is negatively correlated with severity and positively with pathogen elimination. At least three major issues can be discerned from these studies: (I) the role of the alteration of pre-infection PMN function in the outcome of PP *E.coli* mastitis. Since the nineties many studies have contributed to the understanding of the alteration of PP PMN function and viability. In vitro effects of non-esterified fatty acids, beta hydroxybutyrate, estradiol, progesterone, glucocorticoids and IGF were studied (Lamote et al., 2004; Scalia et al., 2006; Sander et al., 2011). (II) The study of potential links with other diseases during the same period. The PP period is a critical period for animal welfare and dairy economics. (III) Identification of one or more biomarkers (BM) to characterize *E.coli* mastitis specifically and their potential role in the management and health care of cows in general. In contrast to the afore-mentioned issues only a few studies are dealing with predictability of severity of PP diseases. This review will analyze some historical studies for potential BM discovery based on PMN function and relating gene expression to its phenotypic outcome (e.g.: CD11/CD18, alkalin phosphatase by van Werven et al., 1997, CD25-expression by Zoldan et al., 2014, and serum proteomics by Cairoli et al., 2006). It will also focus on genome- and epigenome- based tools and discusses advantages, limitations and future prospects. The potential utility of BM in experimental research and/or field studies will also be highlighted (see Figure 1).

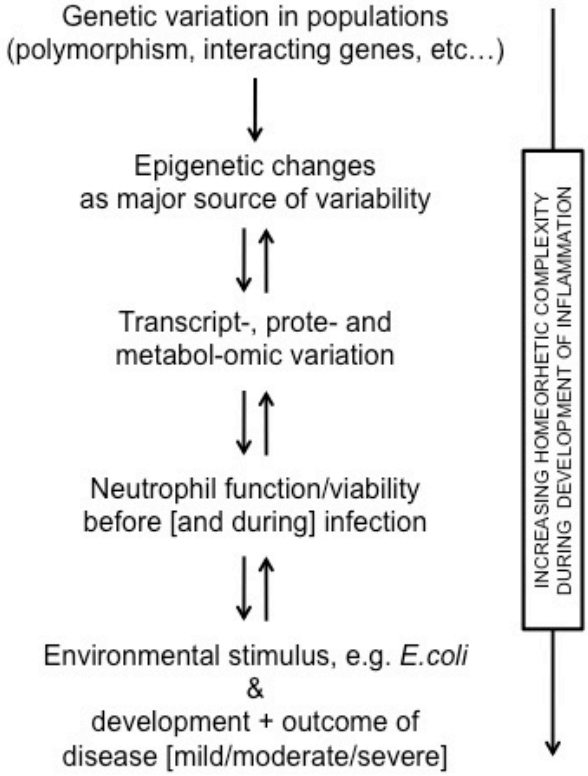

Figure 1
Hypothetical and simplified scheme illustrating the concept of severity of periparturient *E.coli* mastitis in lactating cows. Unidirectional and reciprocal (double arrows) interactions are shown (adapted from C. Burvenich et. al, 2003 Veterinary Research 34, 521-564; C. Burvenich et. al, 2004 Koninklijke Academie voor Geneeskunde van België, 66/2, 97–150; M. Rambeaud, 2006 PhD dissertation, University of Tennessee). Variation in genome, epigenic regulation and milieu intérieur (Claude Bernard, 1857) increases complexity. The inflammatory process is controlled by homeostatic (W. Cannon, 1926) and homeorhetic mechanisms (C. Waddington, 1957). This scheme is a compilation of considerable amount of work executed by scientists worldwide. It can be used as a working hypothesis to detect, develop and validate potential biomarkers to predict the outcome of periparturient *E.coli* mastitis and other related infectious diseases.

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INTRODUCTION

Environmental *E.coli* bacteria are one of the most common etiologic agents isolated from clinical periparturient (PP) mastitis in well-managed farms. More than 50% of all clinical mastitis cases occur during the first eight weeks after calving. Although *E.coli* strains may influence the severity of infection, the primary determinant of severity is the physiological state of the cow. Severe PP *E.coli* mastitis is associated with a pre-infection history of neutrophil (PMN, Table 1 & 2) and mononuclear cell (MN) compromisation. Experimental challenge of the PP mammary gland with opportunistic *E.coli* bacteria has shown to be a good model to estimate suppressed immune competence during the first weeks of lactation (1). Next to this, reduced liver function has also been detected (3) as well as changes in serum proteomics (6). PP environmental mastitis is also linked with other infection diseases during the same period (e.g. metritis). The PP period is therefore a critical period for animal welfare and dairy economics.

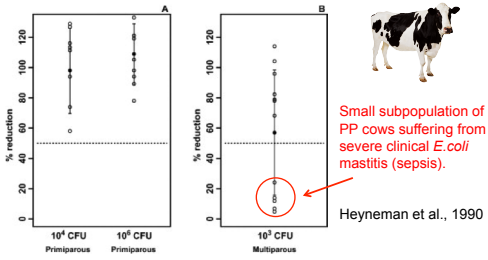
OBJECTIVE

An overwhelming amount of evidence of PP immune dysfunction has been generated by many researchers worldwide. A large amount of data have been collected since 1990. Nevertheless, only a small subpopulation of PP cows with *E.coli* mastitis suffer from unbalanced inflammation (sepsis, see Fig. 1). The data can be used to discover and to evaluate potential PP biomarkers. The goal would be to provide relevant information about: 1) PP immune dysfunction (risk biomarker) and/or 2) outcome of PP infection diseases (prognostic biomarker). The *in vivo* mammary *E.coli* challenge and *in vitro* stimulation of isolated neutrophils are assays that provide accurate data within a large phenotypic variation. In this "simplified preliminary report" we show how the afore-mentioned data of PMN function, gene expression and phenotype can be integrated into a valuable model to study BM (Fig. 2).

RESULTS

Phenotypic severity variation (Fig. 1)

PP *E.coli* mastitis (2 cfu loads; A heifer, B multiparous)



Small subpopulation of PP cows suffering from severe clinical *E.coli* mastitis (sepsis).

Heyneman et al., 1990

Correlation severity phenotype & pre-infection function

PMN reactive oxygen species, ROS (Table 1)

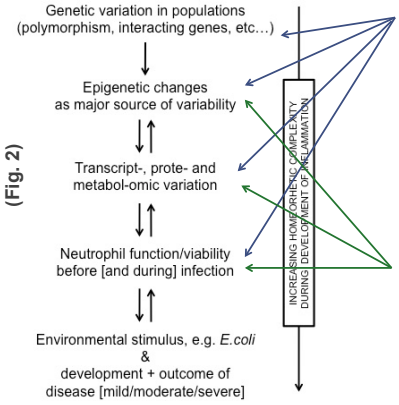
Day +1 (O_2^- nmoles x PMN)
Zymosan induced ROS synthesis R = - 0.90
PMA induced O_2^- ROS synthesis R = - 0. 77

Heyneman et al., 1990

PMN chemotaxis (Table 2)

Day 0	R = - 0.60
Day +1	R = - 0.72
Day +2	R = - 0.74
Day +5	R = - 0.92
Day +6	R = NS

Kremer et al., 1993



(Fig. 2)

Genes related to production of ROS during phagocytosis, and chemotaxis

Perturbations in PMN functions during PP are accompanied by modulation of the expression of TLR4 pathway genes (TRAF6, ATF3, RELA, IL8, and C5aR are lower during PP). C5a and TLR4 signaling in PMN may provide positive feedback promoting severe mastitis. C5a seems to be a critical early mediator in the development of severe *E. coli* mastitis (5). Boulougouris et al. (2015) Poster presentation "Innate Immune Memory Conference, March 18-20, 2015, Welcome trust Cambridge, UK" is studying the expression of eight genes involved in ROS-production: CAT, SOD2, CYBB, CYBA, NCF1, RAC1, RAC2 and NCF4 and their epigenetic regulation. Multiple genes or their isoforms can potentially alter gene function and thus be used as BM (4). However, no isoforms were detected in the afore-mentioned eight genes.

Hormones and metabolites affect several PMN functions and epigenesis

Neutrophils from PP cows have altered gene expression profiles which are linked to inappropriate responses upon an intramammary *E.coli* infection. Epigenetic mechanisms such as DNA methylation, histone modification and microRNAs have key functions in the regulation of gene expression (2). Growth hormone, estradiol, progesteron, glucocorticosteroids, insuline like growth factor - IGF, cathecholamines, nonesterified fatty acids and beta-hydroxybutyrate affect PMN function and viability (1).

CONCLUSION

This simplified model shows potential adoption of gene, epigene and/or phenotype data as BM. More data have to be included to obtain a complete picture. To what extent identification of cows at risk for a short period would enable a more targeted intervention have to be discussed. The model can be adapted to non-neutrophil cells (e.g. mononuclear cells) and BM in blood or milk as far as they participate in the pathogenesis of *E.coli* mastitis.

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(6) Cairoli F et al., 2006 Serum protein pattern during cow pregnancy: acute-phase proteins increase in the peripartum period. Electrophoresis. 27:1617–1625.