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# Whatever happened to mastitis pathogenesis?

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## Research Reflection

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**Abstract**

We have learned a lot about infections of the mammary gland of dairy cows from experimental investigations of the pathogenesis of the various diseases. The understanding gained has contributed to huge successes in reducing the prevalence of infection in properly managed dairy herds. Now descriptive studies using DNA technologies reject previous concepts of mammary gland sterility by default. Bacteria, at least markers of genes, of many genera are reported even from absolutely healthy mammary glands. This may be a technological artefact. No direct evidence exists because experimental studies of infection are no longer fashionable. A regeneration of the lost arts in the pathogenesis of infection is essential to separate truth from conjecture and deal with coming challenges from rapidly changing farm systems and the reduction in access to antimicrobial drugs. In this Opinion Paper I argue for a return to experimental approaches that construct hypotheses, and then test them, in intramammary disease research.

## My issue

I do not see any significant current experimental science involving infections of the mammary gland of the cow. Instead, today's thinking on mastitis is based on circumstantial findings from epidemiology, molecular technologies and modelling. Without proper understanding of the pathogenesis of intramammary disease in the dairy cow we will not be able to evolve control systems that cope with changes in farm systems and the pressure to reduce the availability of antimicrobial drugs.

As a young scientist nearly 40 years ago I was stimulated to switch to mastitis as my primary area of scientific research by the successes of many international groups investigating infection of the mammary gland of the dairy cow. We knew a great deal about the aetiology of infections with all the important pathogenic bacteria described. We needed to know more about the processes of infection to enhance the value of hygienic milking production as enshrined in the Five-Point mastitis control plan which was then, as now, the main method of udder health management. Simply, the fundamentals of control of intramammary health remain based on prevention of new infections and removal of existing infections (Dodd and Neave, 1970); the history is described by Hillerton and Booth (2018).

The science of bacteriology in isolating and identifying the bacteria that cause infections advanced through the 20th century by the development of laboratory culturing using selective media and understanding of specialised growth conditions. No advances were more significant than those of the Royal Veterinary College, London, group in the 1930s. FC Minnet, AW Stableforth and SJ Edwards (some 40 papers in the Journal of Comparative Pathology 1930 onwards) differentiated what was termed *Streptococcus mastitidis* groups I-III eventually into *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis*, but aetiology was not enough.

Key to going further includes understanding of the mechanisms of infection, the processes of infection progressing to disease and the interactions of bacterial growth with the immune defences of the host. The prospects for future mastitis control outlined by Bramley and Dodd (1984) have yet to be realised. Much of the mastitis research from 1970 to 2000 was on the pathogenesis of the various and different types of intramammary infection by classical development of hypotheses and testing by experimentation. Now there is a relative paucity of such investigations in the literature of the past two decades. Instead, mastitis research seems to focus not on experimentation but on sciences relatively remote from the dairy cow and the dairy farmer, on data, molecular technologies and modelling, requiring no muck on boots or a 4 am start in the milking parlour. My issue is that we have moved from, lost even, any significant current experimental science involving infections of the mammary gland of the cow and moved to sanitised studies lacking immediate relevance to dairy farmers, cow health and milk quality. This is a dangerous situation. We are rapidly moving into the era of severe limitations of access to antibiotics to manage udder health, further remoteness from the cow due to increased herd size and the expanding use of automation (extending from milking systems to whole farm management), and we have changes in the aetiology of mastitis consequential to changes in farm systems (staff knowledge and skills, farm scale environmental management such as housing

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systems, milking systems etc). This all means that we need to reinvent and create more understanding of the pathogenesis of the various diseases of the mammary gland in all livestock species. Different pathogens in different situations cause quite different diseases varying in attributes including clinical features, prognosis, response to therapies, and transmissibility/infection pressure.

## Some background

It has long been the tenet that the mammary gland of the dairy cow, both in lactation and in the dry period, is sterile by default and that challenges to this state are continuous, particularly by certain groups of bacteria, most notably Streptococci, Staphylococci and some Gram-negative bacteria, and that their invasion is usually met effectively by innate and induced immune mechanisms. Historically, investigations of the mammary gland focused on the problems of infection, such that abnormalities of milk and tissue were the important factors and not any sense of normality. We do not know the infection prevalence of the mammary gland in pre-industrial milk production, although we can infer that it was significant: the first town dairies of the 1800s needed to milk their cows, and distribute milk, several times daily as the keeping quality of the milk was so poor. As commercial dairy farming became more developed (including by increasing milk yields in larger herds to supply large volumes of milk to urban markets), we came to understand that intramammary infections could affect 60% or more of all lactating cows in the herd (Booth, 1997) with a consequent and significant deleterious effect on milk quality. Thus, research up to about 1970 was very much focused on reversing this situation.

Real progress has required extending knowledge on the invasion-infection-disease pathways, eg *Staphylococcus aureus* (Newbould and Neave, 1965), *Streptococcus uberis* (Thomas *et al*., 1994), *Escherichia* coli (Bramley, 1976), *Trueperella pyogenes* (Hillerton and Bramley, 1989). Along with other studies in the programmes in these laboratories, many published about the same time, came greater understanding of the processes of bacterial invasion, bacterial growth and establishment, inflammation, bacterial elimination and development of a state of chronic infection, that laid the foundation for infection models, often used later for clinical trials in the development of antimicrobials and other interventions.

One result has been that since the 1970s clinical mastitis rates and infection levels have plunged by 80% in developed dairy systems, creating animal health and milk quality levels of which all dairy farmers and veterinarians are justifiably proud. Unfortunately, development of new farming systems, including intensification, have changed the infection spectrum. Environmentally-originating pathogens now predominate, whilst overall infection prevalence is probably lower than ever before (Booth, 1997). In advanced farming systems clinical mastitis is no longer a normal and common feature, and for much of a dairy season may actually be rare. Finding a clinical case at a rate of 8 cases/100 cows/year on a 3000-cow farm is like finding a needle in a haystack. The dairy worker has to examine 114,000 foremilk samples to find one case! Thus, the task of finding each case, and extending new control measures to random events, is becoming too difficult to do manually, and may not be justifiable economically, unless an outbreak is due to an uncommon pathogen or causes significant costs or losses.

For much of the 20th century infection or sterility was determined from a combination of classical culture-based bacteriology and immune abnormalities consequent to the presence of bacteria in the mammary gland, mostly the concentration of leucocytes in milk (usually described as the somatic cell count of milk). This has extended to detection of chemical markers of inflammation. These techniques indicate viable bacteria in the mammary gland. Bacteria have to scavenge nutrients to grow and the immune system has to defend against invasion and colonisation. Various studies using pre-culture enhancement, selective media and specialised conditions have suggested that negative samples in the absence of any other information are probably negative. No detection of bacteria when milk is abnormal (often reported in up to 20% of milk samples from clinical mastitis on farm but sometimes as low as 5% in experimental studies) is usually considered as being due to limitations of the sensitivity of the routine laboratory methods, or that bacteria have been eliminated by the immune response which itself has still to decay. However, isolation of bacteria in the absence of any signs of milk or gland abnormality, even isolation of known mastitis pathogens, is usually considered as contamination or it is assumed that the bacteria did not originate from within the mammary gland. It has periodically been fashionable in bacteriology to consider that some samples may be falsely negative by culture and that a sample may contain viable but non culturable bacteria (VBNC). This was reported initially in samples from environmental sources (Xu *et al*., 1982) or induced by adverse environments (Robben *et al*., 2018) but no references have been found that VBNC exist or are induced by the immune system, or lack of nutrients etc, or that they may be a factor in non-isolation of bacteria from a mammary gland or milk.

Watts (1988) listed 137 different bacterial species or variants, many being very unfamiliar species, as reported causes of intramammary infections of the dairy cow. Even today, certain bacteria are the most recognised causes of clinical mastitis, and non-bacterial pathogens are a rarity with exceptions being viruses, such as Foot and Mouth Disease virus (Wellenberg *et al*., 2002), algae and fungi (mostly yeasts). Commensal bacteria have attracted more attention as the more frequent pathogens (especially *Staphylococcus aureus* and various Streptococci) have become better controlled and less common. These ubiquitous bacteria may, infrequently, cause local problems, eg *Corynebacterium bovis* (Bramley *et al*., 1976). Various coagulase negative staphylococci (CNS), often now described as non-aureus staphylococci (NAS), have become the subject of more study. This is despite any consistent evidence that they cause any significant health, welfare, production or economic costs (Pearson *et al*., 2013; Valckenier *et al*., 2019) and, significantly, no-one appears to have found it necessary to develop any control programmes for these bacteria.

## Epidemiology and modelling but not any proof

Epidemiology and modelling studies have contributed significantly to understanding of some aspects of mastitis, whilst never providing any proof of any hypothesis. This has not stopped a new generation of research investigators. The last decade in bacterial molecular science has included numerous descriptions of microbiomes, involving complex mixtures of nucleotide sequences in many different internal body sites of various animal species, including the bovine mammary gland (see review by Derakhshani *et al*., 2018). The 137 pathogens of Watts (1988) are being replaced with the description that many of these pathogens, and very many other organisms of various genera, may be already, and constantly, in the mammary gland prior to and in the complete absence of any classical indicators of infection.

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They cause no problems, nor induce any pathogenesis, as yet determined.

## When is a pathogen not a pathogen?

Robert Koch's postulates (Koch, 1884) were commonly used in the definition of disease and pathogenicity for many decades. His own, later, studies into cholera and tuberculosis undermined some of his own philosophy. Further progress has eroded their universality but very many experiments have confirmed postulate number 3, which states that *'the cultured microorganism should cause disease when introduced into a healthy organism'*. Fredericks and Relman (1996) have argued that this has been superseded by *'Fewer, or no, copies of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease'*. However, the microbiome concept purports that 16S RNA genes or part sequences are present in milk, but intact bacteria (VBNC?) are often not determined (Kuehn *et al*., 2013), questioning if the bacteria actually exist. This is reported as occurring even in healthy quarters, those with no evidence of infection or disease (including any response of the innate immune system). Newbould and Neave (1965) showed that as few as 9 cfu *Staphylococcus aureus* introduced into the bovine mammary gland were sufficient to cause disease; one of very many similar studies for all common pathogens, both in the lactating and involuted mammary gland.

What am I missing? When is a pathogen not a pathogen? Hanage (2014) was one of the first to question if the microbiome was simply hype. The key challenge was that the concept as presented then confused correlation with causation, with respect to health and disease. Further counter arguments have been raised to the microbiome concept. Rainard (2017) has challenged it on immunological grounds, suggesting that the presence of so many organisms is incompatible with the innate and induced immune system, with generations of experimental infections and with the efficacy of validated mastitis control programmes. Now Taponen *et al*. (2019) have concerns about contamination of samples, and reagents and kits, having found the genera in the microbiome of the bovine mammary gland mostly represent bacteria associated with water and soil.

The bovine milk microbiome is a more complex issue than expected (Taponen *et al*., 2019). It may eventually be recognised as most likely a technological artefact rather than due to viable non-culturable bacteria that have absolutely no effect on the gland, the milk composition and the immune system. To resolve the dilemma will require controlled studies, encompassing the variables of environment and production systems to demonstrate how the microbiome influences the dynamics of the health status of the mammary gland. In short, experiments in pathogenesis.

## My needs

We live in a time when it is unfashionable to carry out experimental infections, as necessary to understand pathogenesis. This is very often due to ethical restrictions or lack of funding, but I worry that it may sometimes be due to lack of the desire to truly understand. Mastitis research in the last 10 years has become based on circumstantial evidence, models and statistics, but never facts related to pathogenesis. At some stage the hypotheses and consequences of bacteria, or their remnants, being present constantly in the mammary gland (validity of the microbiome of the mammary gland) have to be tested experimentally or their supposed presence will remain simply conjecture with no clinical evidence of support.

Further implications of a developing generation of scientists without the ability to understand pathogenesis and create infection/disease models will be felt in several essential areas, from efficacy testing of new therapeutic agents to essential field trial evaluations, and even to understanding the implications of epidemiological studies. My issue is, in short, that we no longer appear to construct hypotheses, and then test them, in intramammary disease research. Thus, we never have absolute proof as might be delivered by applying Koch Postulate number 3. The new molecular technologies and epidemiology can only ever provide circumstantial evidence, if it is evidence. We need some old-fashioned pathogenesis to really know when a pathogen is a pathogen.

Maybe all that we really have to deal with in the mastitis world are the same 'classical' significant pathogens which have been our priority of the past 100 years. Thankfully, we know most of what to do about them, although we will now have to do it using fewer antimicrobials as we combat the supposedly unnecessary use in amount and type, meaning restricted access to antimicrobials for animal health (OIE, 2019). That requires another Reflection!

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