Mare reproductive loss syndrome: septic setae or toxin?

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Abstract

Abortion is characteristic of mare reproductive loss syndrome and equine amnionitis and fetal loss syndrome. Exposure to Lepidopteran caterpillars has been associated with these syndromes and 2 hypotheses were proposed. Penetrating septic setae hypothesis asserted that caterpillar setae served as a mechanical means of introducing pathogenic bacteria into fetoplacental unit causing abortion. A second hypothesis suggested that an unidentified toxin was responsible for inciting inflammatory tissue changes, resulting in abortion. Paper will summarize historical perspectives, to evaluate whether oral bacteria can induce an epithelial or endothelial response, to discuss inflammatory cascade process (implicated in abortion) and provide information for lack of evidence for setae or bacteria as causative agents (not able to induce inflammation) for these syndromes.

Keywords: Caterpillars, abortion, septic setae, toxin, bacteria

Introduction

Mare Reproductive Loss Syndrome (MRLS) was described during 2001 and 2002 breeding seasons in Ohio River Valley involving Ohio, Kentucky, Tennessee and West Virginia. In decreasing order of frequency, MRLS had 5 clinical presentations: early pregnancy loss, late pregnancy loss, fibrinous pericarditis, panophthalmitis and Actinobacillus meningoencephalitis.[1] Resulting progeny loss altered a 50 year economic model of Thoroughbred marketing when largest sales company in Lexington, KY terminated its summer sale of most elite yearlings due to a high rate of fetal loss in mares with early conception dates (February and March of 2001) that would have provided more mature yearlings for July sales. Further expenses incurred in search for MRLS etiology totaled, from all sources, $1,903,553 ($694,615 from the Kentucky Thoroughbred Association/Kentucky Thoroughbred Owners and Breeders and Agriculture Development Board; $295,938 from Grayson-Jockey Club Research Foundation; $80,000 from University of Kentucky; and $423,000 in 2002 plus $410,000 in 2003 from the USDA Agriculture Research Service).[2]

Affected tissues usually had some degree of inflammation. No lesions were identified as pathognomonic. Fifteen to 20% of MRLS late pregnancy losses had no identifiable lesions (Harrison 2005 review of MRLS case records of University of Kentucky Veterinary Diagnostic Laboratory ([UKVDL] in 2001 and 2002, unpublished data). Septic setae and toxin hypotheses remain unproven[1] and it has not been determined if causative factor is mechanical transport of bacteria into gravid uterus, noxious effect of a toxin, or combination of both.[3]

Septic setae hypothesis asserts that horses consume live Eastern Tent Caterpillars (ETC; *Malacosoma americanum)* that are covered by fine hairs (setae). Setae separate from caterpillars in mare's digestive tract and penetrate intestinal mucosa, mechanically carrying with them pathogenic bacteria. Setae have small barbs that interact with motile tissue, enabling them to "ratchet" their way through epithelium and carry pathogenic bacteria into blood stream. Septic material is then spread throughout body. Small setae fragments also enter circulation and these contaminated fragments penetrate capillary endothelium to introduce bacteria into the placenta, pericardium or ocular globe. Bacteria proliferate in invaded sites and cause early and late pregnancy losses, pericarditis and panophthalmitis, collectively called MRLS.[4]

As oral commensal bacteria (alpha Streptococcus and Actinobacillus) were isolated in MRLS late abortions, perhaps these bacteria were transported hemotogeneously from mare's oral cavity to fetoplacental unit. Hence, an experiment was designed to determine if intravenous administration of oral bacterium can cross protective capillary endothelial barrier in pregnant mares.

Bacteria

Isolation of bacteria from late pregnancy losses was a most consistent finding; it occurred in 90% of MRLS cases examined.[5] Alpha Streptococci were isolated from 223 cases (51%), Actinobacillus from 74 (17%) and a combination of these 2 organisms from 8 (1.8%) cases. In total, these organisms were associated with 70.4% of all MRLS abortions in 2001.[6] Records on 3527 abortions submitted to UKVDL revealed that 628 abortions were caused by bacterial infections. Presence of alpha Streptococci in 26, and Actinobacillus in 14 cases,[7] a frequency of only 1.6%. Thus, presence of alpha Streptococci and Actinobacillus represents a unique component of MRLS rarely observed in other cases of bacteria-induced equine abortion.

Alpha Streptococci and Actinobacillus spp. are common oral commensal microflora that act as biological barriers to infection by competing with pathogens for food and space on digestive tract mucosa.[8] These organisms are adapted to live on tissue surfaces, including oral mucosa, where they easily outcompete pathogens.[9] Cell walls of these 2 species of bacteria do not contain pathogen associated molecular patterns (PAMPS) that are associated with pathogenic microbes. Absence of these patterns allows these bacteria to live on oral mucosa without stimulating an inflammatory response from oral mucosal epithelium.

Materials and methods

An experiment in compliance with USDA Terrestrial Animal Health Standards Commission and Chapters 7 and 8 under The Use of Animals in Research and Education (p. 1 - 12), was designed to evaluate effects of an alpha streptococcal septicemia on vascular endothelium and fetal survival.

A dental wedge was used to expose oral cavity of a pregnant mare to obtain swab samples. Samples were collected with a covered swab (Kalayjian Industries Inc., Signal Hill, CA) passed over her soft palate mucosa and streaked onto blood agar (Becton, Dickinson and Company, Sparks, MD). This mare had a previous history of MRLS and possibly harbored a unique strain of alpha Streptococcus not present in normal population. Alpha Streptococcus colonies were selected from resulting mixed growth based on morphology, chemical analysis and incomplete hemolysis of red blood cells on culture plates. These colonies were recultured in pure form on blood agar. Individual colonies of alpha Streptococccus were then removed from the surfaces of 4 culture plates using a sterile swab and mixed in 250 ml of sterile saline. Three pregnant mares were given 10, 20, and 40 ml of this stock bacterial suspension intravenously, daily for 3 consecutive days, whereas 1 control mare received 40 ml sterile saline intravenously. Mares at 40, 47, 55, and 60 days of pregnancy were selected for this experiment, as ultrasonically observable fetal fluid echogenicity from days 30 - 85 is a reliable indicator for agent's presence causing MRLS.[1] Immediately after first injection, stock suspension was streaked onto blood agar using a 1 µl calibrated loop. Concentration of colony forming units in suspension was calculated to be $4 - 6 \times 10^9$/ml. An intradermal injection of 0.2 ml of this bacterial suspension was followed by a biopsy 96 hours after injection. Each mare's rectal temperature was recorded at 12 hour intervals and complete blood counts and fibrinogen concentrations were done every 24 hours for first 5 days. Transrectal ultrasonography (5 MHz transducer) was performed every 48 hours over 20 days to evaluate fetal heartbeat and fetal fluid echogenicity.

Results

Biopsy revealed that dermis contained multifocal perivascular cuffs of low numbers of neutrophils and minimal to low numbers of macrophages, lymphocytes, and eosinophils. Inflammatory cells infrequently moved into adjacent dermis. Endothelial cells were occasionally hypertrophied and vascular lumen contained marginated neutrophils. Infectious agents were not overtly evident. Fetal fluids remained anechoic in both untreated control mares and all 3 mares injected with alpha Streptococcus. Mares had no febrile responses nor hematological changes. Fibrinogen, a marker for inflammation, did not increase in any mare and none had clinical signs of septicemia nor response to intravenous infusion of bacterial suspension. All remained pregnant 30 days after last administration of alpha Streptococcus suspension and delivered normal foals.

Discussion

Unaffected endothelial barrier prevented transfer of bacteria to fetoplacental unit. Alpha Streptococcus from oral cavity must somehow breach oral mucosal epithelium to gain entry into bloodstream for transport to distant sites in the body where MRLS is expressed. Neutrophils in oral cavity possess IgA Fe receptors at higher levels than neutrophils in blood. Azurophilic granules contain myeloperoxidase, defensins, neutral serine proteases, lysozyme and bactericidal/permeability increasing protein. These azurophilic granules are primarily designed for killing microorganisms, although some specific granules also have ability to solubilize collagen.[10] Increased oral mucosal permeability is caused by this neutrophilic activity, but alpha Streptococccus isolated from MRLS abortions did not appear to have ability to initiate such a neutrophilic response. An increased mucosal permeability or physical penetration is necessary for alpha Streptococcus on mucosal surface to cross through mucosal epithelium. This altered permeability was not induced. Pure cultures of alpha Streptococcus at this dosage and duration were unable to affect vascular endothelium or increase capillary permeability. Unaffected endothelial barrier prevented transfer of bacteria to fetoplacental unit.

Conclusion

Alpha Streptococcus was unable to penetrate vascular endothelium and enter the fetoplacental unit to cause MRLS unless transported by some other means.

Setae

Urticating agents associated with Lepidopteran setae may reduce ability of gastrointestinal mucosa and epithelial barriers of placenta to prevent bacteraemia.[11] Septic setae hypothesis suggests that ETC setae serve as mechanism that breaches oral mucosal epithelium and vascular endothelium to allow bacteria to move from mare's oral cavity to fetus. Setae of ETC are a visual and tactile cue that caterpillar is both toxic and unpalatable.[12] A second function of these setae is to disrupt airflow over cuticle, thereby reducing heat loss by convection (Fitzgerald 2004 personal communication). ETC setae are soft and are not shed. They are covered by microscopic projections oriented toward tip of setae. Thus, setae would have to migrate through tissue blunt end first for these projections to act as "barbs", unlike Processionary caterpillar (PC; *Ochrogaster lunifer*) where projections face base of setae, enabling tip to penetrate moving tissue while barbs prevent retrograde movement.[13] These setae are hollow, filled with an unidentified toxin and easily shed. Setae of ETCs are composed of inert substances that, by themselves, induce minimal inflammatory tissue changes. "Urticating hairs" on caterpillars are so because of presence of some irritant causing molecules produced by the caterpillar, not by setae alone.[14] Setae alone are unable to stimulate an acute inflammatory response (Diaz 2006, personal communication).

ETC setae were ground to a powder to allow passage through a 25 gauge needle and resuspended in sterile saline. This mixture was then sterilized by 5kGy ionizing radiation. A series of intradermal injections of this material containing 2 µg ETC setae (0.2 µg setae protein) and 20 µg ETC setae (2 µg seatae protein) were administered. Subsequent welt size was evaluated to demonstrate an inflammatory response at 0, 6, 24, 30, 48, and 54 hours post injection and biopsies were obtained 24 hours post injection. None of injections containing setae alone induced skin welts, thereby indicating that no acute inflammatory response was produced by exposed tissue cells. Setae fragments caused some necrosis and elicited a foreign body response.[15]

Tissue migration of ETC setae in experimental situations appeared to be limited to no deeper than mucosa or submucosa of intestinal tract, as observed in horse, pigs,[16] goats, and rats.[1] Presence of setae in mucosa and submucosa induced formation of microgranulomas which are defined as "an inflammatory lesion characterized by chronic inflammation with mononuclear cell infiltration and extensive fibrosis".[17] There was no evidence of deeper migration of ETC setae into the tissue. Presence of setae-induced microgranulomas in intestinal mucosa and submucosa has only been demonstrated in experimental settings when ETCs were fed or gavaged. No microgranulomas containing setae were reported in any adult equine gastrointestinal tracts examined at UKVDL in 2001 and 2002. Caterpillar setae are inert and cannot induce inflammation beyond a foreign body response. Circulating setae fragments would have to

escape from the microgranulomas and enter circulation to then breach the vascular endothelium and allow entry of bacteria into placenta, pericardial fluid and anterior and posterior chambers of the globe. Setae alone have no ability to induce active inflammation.

Setae must gain entry into the oral cavity before they can serve as mechanical means to breach oral epithelium and vascular endothelial barriers. It is suggested that horses ingest caterpillars unknowingly. However, equids are highly selective feeders, able to sort out individual components of mixed feeds that an individual may not prefer. This is accomplished by using highly sensitive olfactory receptors and a vomeronasal organ (in nasal passages) that is very responsive to pheromones and other airborne molecules. Horses have sensitive vibrissae on their muzzles that enable them to identify small plants for ingestion and avoid consumption of foreign materials. Their sense of taste has not been as fully investigated, but palatability preferences are known. Sense of taste, texture and smell would suggest ability to sense and avoid consuming foreign substances (Gustafson 2016, personal communication) such as 1 g caterpillars, unless they were a preferred item. There are no reports of horses in their natural environment willingly consuming ETCs, in spite of widespread exposure to them in 2001 and 2002. Furthermore, horses chose to avoid consuming water contaminated by ETCs if a source of fresh water was also available. Horses will consume macerated or whole ETCs that are mixed with sweet feed in an experimental setting,[16] but this does not represent natural exposure or confirm ingestion in their normal environment. It was unlikely horses would readily consume ETCs in a field setting.[18]

Conclusion

Setae of ETC are soft, inert and not shed. They appear unable to migrate through tissue any deeper than the submucosa. There is no evidence to support consumption, unless mixed with feed. Therefore, we inferred that setae do not contribute to the etiology of MRLS. Some other explanation must be sought to identify method of passage of bacteria through epithelial/endothelial barrier, possibly inflammation.

Inflammation

Because some degree of inflammation in affected tissues was most frequently observed, it was widely held that etiological agent(s) must be capable of inducing an inflammatory response. Pathogenesis of this syndrome may be found among inflammatory cascade of molecular and cellular responses that lead to inflammation. Evaluation through this matrix may clarify relative role of setae, bacteria or toxin(s). A brief review of events that occur during an inflammatory cascade will aid in evaluation of both hypotheses.

Increased capillary permeability can be caused by inflammation. Any inflammatory process must start with an insult to epithelial or endothelial cells. This insult may be traumatic, hypothermic, hyperthermic, and toxic or infectious agents.[8] Damaged cells produce multiple molecular signals that initiate inflammatory response. These include free heparin sulphate, normally restricted to intact cell membrane and mast cell granules. This plus fibrinogen and cellular proteins are released which activate macrophages to produce proinflammatory cytokines. These substances are recognized by toll-like receptors (TLRs) located on cell membranes of multiple cell types such as leucocytes, epithelial cells and endothelial cells. Thrombin is released by damaged cells, initiating procoagulation activity and acting on fibrinogen to produce insoluble strands of fibrin that are deposited within capillaries. Histamine is released by mast cell granules that stimulate production of glycoprotein P-selectin receptors from endothelial cells. This protein binds with L-selectin on neutrophils' surface, causing them to slow down within capillaries and eventually stop and become bound to vascular endothelium by platelet activating factor (PAF). Dead or damaged cells release adenosine that activates mast cells to degranulate and release heparin and proinflammatory cytokines. Damaged cell walls release phospholipids that react with phospholipase to produce arachidonic acid. This interacts with 5-lipooxygenase to create 4 leukotrienes, all potent proinflammatory molecules. Three of these are vasoactive and increase vascular permeability. Arachidonic acid also reacts with cyclooxygenase to produce 4 proinflammatory prostaglandins. Molecules released by damaged tissue serve as signals to immune system's "sentinel" cells (macrophages,

mast cells and dendritic cells), vast majority of which are in connective tissue and/or just under any epithelial or endothelial barrier. These sentinel cells are the primary source of inflammatory cytokines and chemokines.[9]

Tumor necrosis factor alpha (TNF_α) is key mediator of acute inflammation. This family of inflammatory cytokines contains at least 18 related proteins that regulate cellular activation. TNF_α family has a broad systemic effect, as TNF_α receptors are on almost all nucleated cells. This family of cytokines is main stimulant to macrophages and mast cells to produce interleukin 1 (IL1) and interleukin 6 (IL6), both of which are potent inflammatory cytokines. TNFα in combination with IL1 triggers critical changes in endothelium of small blood vessels to induce microvascular thrombosis and capillary leakage. Chemokines are proinflammatory cytokines, with 50 chemokines identified. Their primary roles are as chemotactic factors and leukocyte activators.

Production of inflammatory cytokines, leukotrienes, and prostaglandins serve as overlapping and redundant signals to immune system that tissue insult and damage has occurred. This inflammatory cascade is similar in response to any tissue damage, either infectious or noninfectious. All tissue damage must be managed by innate branch of immune system. Introduction of infectious agents extends inflammatory response to involve acquired immunity branch of immune system. Main difference between these 2 branches of immunity lies in their use of receptors to recognize invaders. Innate system uses pre-existing receptors on macrophages, neutrophils, dendrite cells and natural killer cells that are able to bind to molecular patterns common to many microbes. Cell walls of gram positive organisms are characterized by complex peptidoglycans, whereas cell walls of gram-negative organisms contain peptidoglycans covered by a layer of lipopolysaccharides. Innate system receptors bind to these molecular patterns that are not present in mammalian tissues. These arrangements are called PAMPS, recognized by receptors as foreign and "dangerous". Important receptors are toll-like receptors (TLRs) on macrophages, mast cells, dendritic cells, eosinophils and epithelial cells in respiratory and intestinal mucosa. There are at least 10 pre-existing TLR types that have specific binding sites for specific PAMPS. Known TLRs, alone or in combination, can recognize almost all known pathogens. Binding of TLRs and PAMPS causes cell genes to form and release cytokines in various quantities, qualities and combinations in response to specific antigens.[17] It is this genetic control that makes an organism pathogenic in 1 species, but not in another. This sequence of events is summarized in the flow sheet that describes the inflammatory cascade (Figure). Notice how many paths lead to increased capillary permeability and leakage; this paradigm can explain all unusual components observed in MRLS.

Conclusion

Some toxic irritant(s) that could induce the signs of inflammation, resulting in increased capillary permeability, would allow bacteria to enter the fetoplacental unit. Unidentified irritants associated with lepidopteran caterpillars may provide this toxin.

Other observations

Twenty eight mares aborted in Australia in 2004 following exposure to PC. These abortions were named equine amnionitis and fetal loss syndrome (EAFL). Mares involved were mid - late pregnancy and clinical presentation and necropsy findings were identical to MRLS cases observed 2 years previously in US.[19] Most mares aborted with no signs of impending abortion, but some had signs of mild colic prior to onset of delivery. Inflammatory changes in amnion and amniotic portions of umbilical cord, and inner surface of allantoic lining of allantochorion were observed. There were variable pathological changes in fetus and frequent recovery of multiple species of environmental bacteria, usually nonpathogenic, from fetus and/or placenta.[19] Nonpathogenic isolates were also characteristic of MRLS.

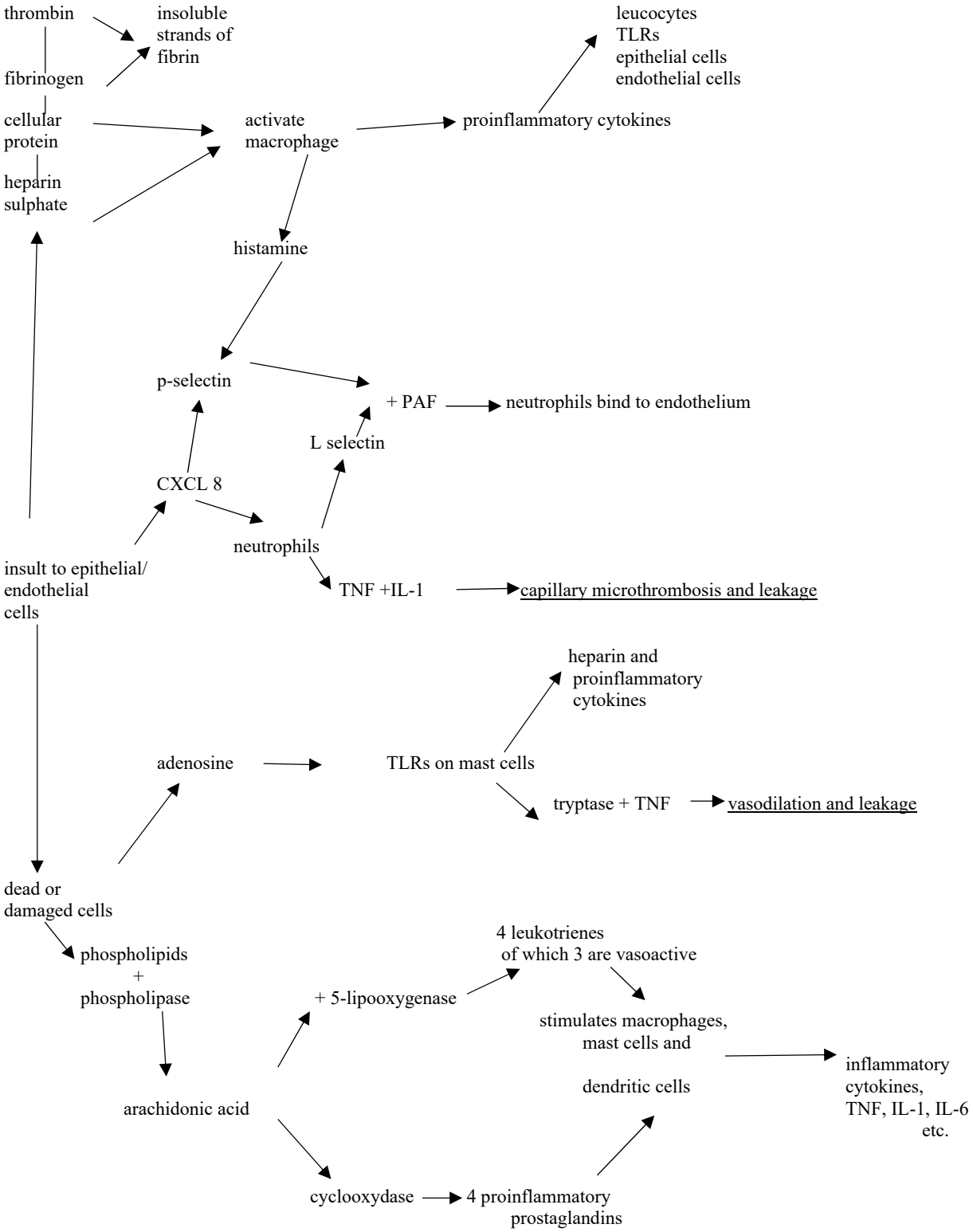

Figure. Inflammatory cascade (adapted[9] with permission)

Processionary caterpillar was confirmed as causative factor of EAFL experimentally by gavaging 100 g of whole PCs into 4 mares between 194 and 205 days of pregnancy daily for 5 days;[3] 3 mares aborted within 11 days after first administration. Subsequently, 4 groups (3 mares per group) were gavaged with 0, 1, 2, or 5 g of shed PC exoskeleton daily for 5 days. One mare of each group given 1 or 2 g of PC exoskeleton aborted and 2 of 3 mares given 5 g exoskeleton aborted.[3] Furthermore, early pregnancy loss, a feature of MRLS but not of field cases of EAFL, was produced in 2 of 6 mares between 45 and 55 days of pregnancy, on days 6 and 7 following gavages of 5 g PC exoskeleton daily for 5 days.[3] Tissues were examined from 4 mares, 1 untreated control and 3 gavaged with 50 g of whole PCs daily for 5 days.[11] Setae and/or fragments were present in all regions of gastrointestinal tract of treated mares, but none in control. Endometritis was present in treated mares and setae fragments were observed in 1 mare's uterus. Tissue response to presence of setae ranged from none to microabcesses.

Horses are not only animals sensitive to etiological agent(s) of MRLS. Pregnant pigs could also be affected by exposure to ETCs.[16] Five gilts in mid-pregnancy were each fed 40 g/day of macerated ETCs for 10 days; 2 of 5 treated gilts aborted their entire litters, whereas none of 5 untreated control gilts aborted. Treated gilts were necropsied 1 - 3 days after abortion. Streptococci were isolated from fetuses of 2 litters that aborted and also from those in 3 litters that did not abort. Caterpillar setae were observed in alimentary tract of all 3 gilts fed ETCs.

A syndrome similar to MRLS and EAFL was reported in dromedary camels in Western Sahara.[20] Rainfall is scarce in this desert region but when it occurs, it is accompanied by rapid growth of Acacia trees. Even more infrequently, this plant growth supports an outbreak of caterpillars of Lasiocampipae family (ETCs and PCs belong to this family) responsible for MRLS and EAFL. Camels ingest these caterpillars while browsing on Acacia leaves, following which, pregnant camels have acute abortions or deliver weak calves. Some of these newborn calves exhibit bilateral hyphemia, also observed in some foals affected by MRLS. Many of these compromised newborn calves suffer a failure of passive antibody transfer, followed by sepsis and death. This syndrome, called *Duda* by native pastoralists, has been recognized for many years, though apparently no formal investigations have been undertaken.

Mice were evaluated as a potential animal model suitable for MRLS research.[21] Mice do not abort their conceptuses but resorb them in response to a stressor. Mice were 5 days pregnant and each test group was treated for 14 days. Group 1 (n = 4) received 0.5 g saline by gavage and Group 2 (n = 4) received 0.4 saline by intraperitoneal injection. Group 3 (n = 7) received 19 mg fresh caterpillar frass by gavage. Group 4 (n = 8) received 200 mg frozen ETCs by gavage and Group 5 (n = 7) received 20 setae plucked from frozen ETCs and injected intraperitoneally. Gilmer type gland located at base of each setae may serve as a storage site for a chemical toxin. Mice were euthanized on day 19 of pregnancy and necropsied. Mice in Groups 1, 2, 3, and 4 had no abnormalities of uterus or fetuses, whereas 3 of Group 5 mice that received intraperitoneal injections of setae had resorbed all their fetuses and had acute suppurative inflammation at implantation sites; bacterial isolates from fetuses were *Seratia marcescens* and *Lantoea agglumerans*. This experimental design could not determine if resorptions were in response to setae alone or to a toxin associated with setae.

Conclusion

"Septic setae hypothesis" specifies that bacteria that become septic are pathogens. Furthermore, 70% of isolates associated with MRLS were nonpathogenic oral commensals that do not stimulate an inflammatory response from a normal immune system. If MRLS is an infectious disease, it should comply with Koch's postulates. More than 20 microorganisms were isolated from various tissues in MRLS cases of 2001and 2002. No bacteria could be isolated from 16% of MRLS cases.[6] Proposed infectious etiology failed to comply with Koch's postulates in that syndrome can apparently occur with no bacteria present. It is further unlikely that 20 bacteria could all induce an identical clinical and tissue response to qualify as multiple etiological agents. Evidence therefore suggests that bacteria are not etiological agents of MRLS as they, alone, appear unable to cross an intact epithelial or endothelial barrier. Hence, they would require

mechanical assistance from penetrated setae. Septic setae hypothesis was questionable, as it appeared that setae, bacteria, or a combination thereof induced inflammation associated with MRLS. Increased capillary permeability caused by inflammation would allow septic bacteria to cross both epithelial and endothelial barriers.

Setae, bacteria, and/or unidentified toxin associated with ETC appear to induce inflammation when consumed by horses, pigs, and camels and when injected intraperitoneally in mice**.** Absence of an identified toxin, observations that camels will ingest whole caterpillars, that both MRLS and EAFL can be reproduced by ingestion or gavage of Lepidopteran caterpillars, that clinical and tissue responses to this exposure are identical and that setae were present in 1 mare's uterus suffering EAFL, all lend support to septic setae hypothesis currently favored for MRLS/EAFL. However, evidence suggests that neither bacteria or setae, or a combination thereof, is the etiological agent(s) of these syndromes. Tissues affected by MRLS have an extensive capillary supply adjacent to anatomical structures that provide an enclosed compartment containing fluid i.e. placenta with allantoic and amniotic fluids, pericardium with pericardial fluid and globe containing vitreous. This, coupled with increased capillary permeability and microthrombosis caused by inflammatory cascade, are the foundation of MRLS.

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Conflict of interest

Authors have no conflicts of interest.

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