# Canine-specific tail-in, head-out sperm agglutination

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## ABSTRACT

An interesting pattern of tail-in, head-out sperm agglutination was identified in a *Brucella canis* seronegative subfertile dog. Centrifuged seminal plasma from this dog could induce a similar pattern of agglutination in six other dogs, but not in ejaculates from a single stallion and two rams. The agglutination pattern was short-lived and appeared to depend on motility of spermatozoa, although intensity of agglutination may have been affected by concentration of agglutinating factor.

**KEYORDS**: agglutination, canine, spermatozoa

## 1 INTRODUCTION

Spermatozoa are antigenically foreign to somatic cells of the male animal in which they are produced and may induce an immune response in tissues within and outside of the reproductive tract of that animal, a finding first noted by Landsteiner (1899) and Metchnikoff (1899). Agglutination of spermatozoa in mammalian ejaculates may occur in response to bacterial or viral antigens or anti-sperm antibodies in the semen, resulting in the sperm-to-sperm adherence of sperm tails, sperm heads, or a combination of tails and heads. Such agglutinated sperm are functionally immobilized, cannot penetrate cervical mucus and therefore render the male subfertile or infertile (Rümke, 1968). In addition to functional immobilization, agglutination may induce complement-mediated sperm cell membrane damage or occlusion of zona pellucida binding sites on the sperm head (Wallach et al., 1984).

Bacterial and viral infections of the reproductive tract may induce antibody responses to spermatozoa that can result in decreased fertility. Human papillomavirus has been associated with anti-sperm antibodies in men (Garolla et al., 2013), and sheep infected chronically with *Brucella ovis* may develop an anti-sperm immune response (Paolicchi et al., 2000). George and Carmichael (1984) found that seminal plasma from dogs with chronic *Brucella canis* infection caused head–head agglutination of non-agglutinated sperm from non-infected dogs, but did not determine the mechanism behind this observation. In an asthenozoospermic

mongrel dog, Kawakami et al., (2003) found head–head agglutination of sperm in association with an epididymal sperm granuloma, and however, they found no clinical evidence of scrotal inflammation or injury. Testicular biopsy and epididymal aspiration in dogs were found to cause a transient increase in anti-sperm IgG expression on the surface of spermatozoa, but this increase did not affect long-term fertility (Attia et al., 2000).

A six-year-old American pit bull terrier (‘Archie’) was presented by its owner for veterinary examination, with a complaint of no conceptions in multiple bitches despite multiple observed matings. On microscopic semen evaluation performed within five minutes of semen collection, sperm were seen to spontaneously agglutinate by adherence at the distal flagellae, forming spherical polyspermic complexes with the appearance of dandelion seed clusters (Figure 1). A single reference to such a pattern of agglutination exists in an early research paper (Henle et al., 1938), where it was described in bull spermatozoa.

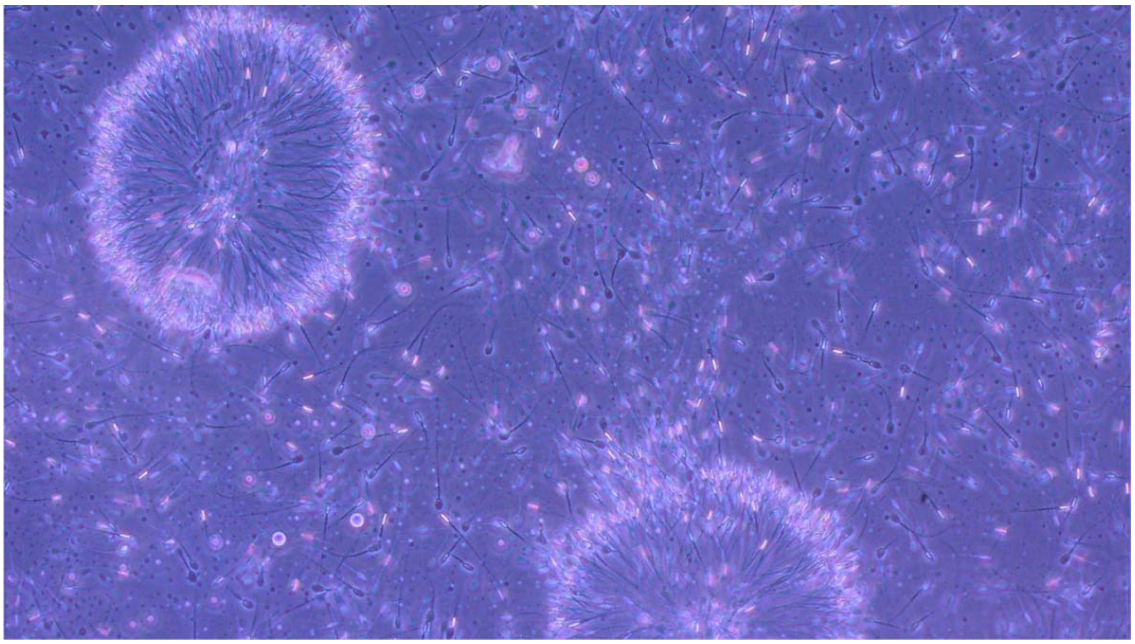

**FIGURE 1**. Characteristic tail-in, head-out sperm agglutination in an ejaculate from ‘Archie’ (100× magnification, image capture of motile unstained spermatozoa)

Since a prior description of such an agglutination pattern in dog semen could not be found, a small investigation was devised using samples obtained from the case animal and from healthy animals which produced semen showing normal motility and no agglutination. The investigation's first aim was to determine whether centrifuged sperm-free seminal plasma obtained from the affected dog could induce a similar pattern of sperm agglutination in the otherwise normal ejaculates of other dogs, thereby indicating that the agglutination-inducing agent was present in the seminal plasma and not on, or in, the spermatozoa themselves. The second aim was to determine whether seminal plasma from the affected dog could induce a similar pattern of agglutination in the normal ejaculates of other species (sheep and horses), which may indicate species specificity of the agglutination-inducing agent.

## 2 MATERIALS AND METHODS

This study was approved by the University of Pretoria Research Ethics Committee (approval number REC044-20).

The affected dog was subjected to a clinical examination including palpation of external and internal genitalia. Cephalic venepuncture was performed to draw blood for *B. canis* serology (slide agglutination test, performed at a laboratory accredited for export certification), and an ejaculate was collected, comprising the entire pre-sperm and sperm-rich fractions and approximately 5 ml of the post-sperm fraction, all of which were collected together into a single warmed pyrogen-free plastic vial. Ultrasonography was performed on its testes, epididymides and prostate. Sperm morphology was assessed by eosin–nigrosin stained smears at 1,000× magnification. The ejaculate was centrifuged at 300 *g* for 10 min, and multiple 0.2 ml aliquots of largely sperm-free supernatant were transferred to 0.5 ml polypropylene screw-top containers to be frozen at –18°C pending further analysis. Diff-Quik stained sediment smears were made to assess the foreign cell population of the ejaculate. A sample of ejaculate was submitted for general *Mycoplasma* species and specific *Mycoplasma haemocanis* antigen detection by polymerase chain reaction (PCR).

Ejaculates were collected from healthy dogs ($n = 6$), rams ($n = 2$) and a stallion ($n = 1$) using manual stimulation, electrostimulation and an artificial vagina, respectively. 0.1 ml of fresh sperm-rich fraction (dogs) or whole ejaculate (rams and stallion) was added to 0.2 ml of warmed frozen-thawed seminal plasma derived from the case dog and examined using phase contrast light microscopy (Carl Zeiss Axio Lab A1, Carl Zeiss Vision SA, Randburg, South Africa) at 100× and 200× magnification under a coverslip. Digital still photographs and videos were captured with a Carl Zeiss Axiocam ERC 5s camera. Eosin–nigrosin stained air-fixed smears were made in an attempt to obtain a fixed image of the agglutination pattern.

## 3 RESULTS

The affected dog ‘Archie’ was clinically healthy. Testes, epididymides, spermatic cords and prostate were normal on palpation, and no ultrasonographically visible lesions were identified. The dog was serologically negative for *B. canis,* and no *Mycoplasma*-associated DNA was detectable using PCR. No inflammatory cells or bacteria were identified on Diff-Quik stained semen sediment smear. Marked clumping of spermatozoa was seen on eosin–nigrosin stained smears. Due to superimposition, it was not possible to assess the morphology of clumped spermatozoa, even in a diluted sample, but the clumps appeared to contain a mixture of living and dead sperm. Free spermatozoa found between large clumps of agglutinated sperm had no obvious head or flagellar defects that were visible by phase contrast light microscopy. The addition of seminal plasma from the affected dog to separate aliquots of semen of six healthy dogs induced the same characteristic agglutination pattern. Neither the rams nor the stallion exhibited such an agglutination pattern.

The agglutination pattern did not form immediately after addition of Archie's seminal plasma to the aliquot of semen obtained from experimental dogs: in fact, a variable period of between two and ten minutes was required for maximum agglutination to occur. The agglutination process was dynamic, with new clusters continually forming and dispersing. The agglutination appeared to affect only actively motile sperm (with time, as sperm under the coverslip became less motile, the dandelion-like agglutination formations collapsed and sperm drifted apart). In contrast to what was seen in whole semen from the affected dog, the

mix of healthy experimental dog semen and seminal plasma from the affected dog failed to form permanent clumps visible on eosin–nigrosin stained smears.

A video of the agglutination can be found at the following link:

https://www.youtube.com/watch?v=wYpaNZmD_rc

This video shows the sperm-rich ejaculate from a healthy dog mixed in a 1:2 ratio with frozen-thawed seminal plasma from the case dog. A microscope artefact can be seen in the left lower half of the screen at 100× magnification (it appears to be an eosin–nigrosin stained bovine spermatozoon). Each agglutination cluster appears to be formed around debris composed of non-sperm cellular or acellular material, or immotile spermatozoa, or the interlocking distal flagellae of the motile agglutinated spermatozoa.

The formation, fusion and dispersion of clusters can be best appreciated when played back at high speed (refer to one minute five second mark in the video).

## 4 DISCUSSION

Addition of seminal plasma from the affected dog repeatably induced the agglutination pattern in ejaculates of otherwise unaffected dogs. From limited trials in other species, the condition appears dog-specific, suggesting involvement of a canine-specific factor. A failure to detect *B. canis* antibodies in serum samples or isolate *Mycoplasma* spp. DNA in seminal plasma from the affected dog suggests that these pathogens are unlikely causes of sperm agglutination in this case. No attempt was made to search for other novel infectious causes of sperm agglutination. The characteristic tail-in, head-out agglutination pattern suggests that the agglutination-inducing factor in the seminal plasma interacts with a binding factor on the portion of the sperm cell plasma membrane surrounding the flagellum. Since the ejaculate from the case dog was centrifuged but not filtered, there is a possibility that small quantities of cellular debris remaining in the supernatant were the source of the agglutinating factor. The binding of flagellae was strong enough to form distinct dandelion-like sperm clusters, but the intensity of binding diminished markedly as motility decreased, until eventually all clusters dispersed. The ephemeral nature of the agglutination was highlighted by the observation that air-fixed eosin–nigrosin stained morphology smears of mixed semen from the affected and unaffected dogs failed to demonstrate the characteristic agglutination clusters, despite clearly demonstrating agglutination on motility preparations. The marked clumping of sperm on morphology smear of the original ejaculate, versus the absence of such clumping seen in mixtures of semen from healthy dogs and the affected dog, may be a function of dilution of agglutinating factor in serial semen collections, both within the affected male's reproductive tract, and from admixture with semen from the six unaffected dogs. In the present case, the biochemical nature of the agglutinating factor and the pathophysiology underpinning its appearance is unknown. Techniques such as gel electrophoresis could be used to compare seminal plasma from the affected dog with that of unaffected dogs in an attempt to identify potential agglutination-inducing proteins.

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## CONFLICT OF INTEREST

No conflict of interest exists.

## AUTHOR CONTRIBUTIONS

G.J. Brown performed data collection and wrote the manuscript. J.O. Nothling provided editorial assistance and provided scientific insight during manuscript preparation. K.G.M. De Cramer performed data collection and provided scientific insight during manuscript preparation.

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