The evaluation of the specificity of the Acid Phosphatase test to identify semen

By

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DECLARATION

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Abstract

The burden of sexual assault cases in South Africa is exceptionally high, and analysis of biological material in these cases may provide evidence towards a criminal investigation. These analyses include the identification of biological material to: (i) presumptively identify if semen may be present in order to guide downstream DNA profiling analysis to identify the alleged assailant; and (ii) confirm if semen was indeed present to provide evidence of ejaculation. The presumptive test used to detect the possible presence of semen is the Brentamine Fast Blue (FB) test, which detects the presence of acid phosphatase (AP) by a colour change reaction. AP is an enzyme which is present in human semen in high concentrations, but is also found in animal semen at lower concentrations. The current methods of presumptive testing cannot differentiate between animal and human semen. The specificity of the Brentamine FB test was therefore explored in this study, by subjecting semen from humans (n = 16), dogs (n = 13), horses (n = 5), ostriches (n = 9), and rams (n = 13) to this test; and measuring the enzyme kinetics using a novel method: UV-Vis spectrophotometry. Reaction kinetics showed a significant difference in the AP activity between humans and each of the four different animal species in this study ($p < 0.001$). Confirmatory testing was also performed using microscopy and morphological differences were seen between human and animal spermatozoa, with significant differences between human, ram, ostrich and dog semen ($p < 0.05$) but not horse semen ($p > 0.05$). This study demonstrates that enzyme kinetics holds potential to increase the specificity of presumptive testing for human semen, which could possibly be supplemented with microscopy-based confirmatory testing. These results are of value to forensic scientists who may be faced with questions regarding semen specificity in casework. Further, the baseline of semen morphometric dimensions generated for each species' can aid in forensic investigations for comparison with evidential samples.

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Abbreviation	Definition
Abs	absorbance units
AP	acid phosphatase
CpG	5'- C-phosphate-G-3'
cm	centimeter
DNA	deoxyribonucleic acid
FSL	Forensic Science Laboratory
FB	Fast Blue
g	gram
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
ml	milliliter
mRNA	messenger RNA
s	seconds
SAPS	South African Police Service
SEVI	semen-derived enhancer of virus infection
STR	short tandem repeat
tDMR	tissue-specific DNA Methylated Regions
U	International unit to measure the activity of the enzyme
UV	ultraviolet
UV-Vis	ultraviolet visual
xg	times gravity
µm	micrometer
µl	microliter
%	percentage
µm	micrometer

Chapter 1: Introduction

1.1. Background

1.1.1. Body fluid identification

Body fluid identification involves the detection, identification and confirmation of unknown stains which are thought to be body fluids. Various body fluids can be found on crime scenes and on related evidence items. Examples of the most common biological fluids deposited on crime scenes include: blood, semen, saliva, urine, vaginal fluid and mucus (Zapata, Fernandez de la Ossa, and Garcia-Ruiz, 2015 and Virkler and Lednev, 2009). Discovery of a potential body fluid can hold significant evidential value, particularly to provide information if a physical altercation or sexual assault, had occurred at a scene (Ja Hyun An, Kyoung-Jin Shin, 2012).

Identifying semen from unknown biological evidence plays a crucial role in sexual offence cases. Determining that biological evidence from a victim is semen suggests that sexual activity took place (Johnson *et al.*, 2012). Forensic examination of the victim and the scene allows for enabling the recovery, detection, and characterisation of such evidence for analysis, which can ultimately involve DNA analysis and possible identification of the alleged perpetrator.

South Africa unfortunately reports some of the highest statistics of sexual assault in the world, with approximately 50 000 cases reported annually (South African Police Service - Department of Police, 2017). Biological evidence from these cases are sent to the Biology unit, at the Forensic Science Laboratory (FSL), South African Police Service (SAPS) for analysis. This analysis typically comprises a presumptive test to first test if semen may be present, and if this test is positive, DNA profiling follows in attempt to identify who the biological evidence belonged to.

As discussed in detail later in this chapter, presumptive tests have many limitations, including the high number of false positive reactions (Vennemann *et al.*, 2014). While confirmatory tests are available, they are not utilised in a local context, which is a limitation of this investigation workflow. Furthermore, the specificity of the presumptive test of semen was previously raised in a local court case, whereby the species of the presumptively identified semen was questioned. Since the presumptive test for semen is not species-specific, the question as to the species of the presumed semen remained unanswered by current workflows. This issue also

raised a research question regarding how the specificity of the tests to identify human semen could be improved.

This introduction will provide some background into the burden of sexual assault in South Africa, as well as discuss the principles and literature surrounding the identification of semen using different methods. This will lead up to the rationale of this research study and the chapter will end with specific aims and objectives.

1.2. Sexual offence in South Africa

In South Africa, an exceptionally high number of sexual offences are reported every year. From 1 April 2016 to 31 March 2017 a total of 49 660 cases were reported across the country (South African Police Service - Department of Police, 2017), translating to 0.097% of a population of 51.19 million reporting sexual offences. This number was slightly lower compared to the previous year's sexual offence statistics for the country, where a total of 51 895 reported cases of sexual offences were recorded. Provincially, Gauteng leads with the most reported rape cases in the 2016/2017-year with 9 566 filed cases across stations. KwaZulu Natal, Eastern Cape and the Western Cape followed closely behind with 8 484, 8 050 and 7 115 reported cases respectively (South African Police Service - Department of Police, 2017).

These numbers are higher than those reported in other Southern African countries, even taking into account the population size. For example, in Lusaka, Zambia (population size: 3 million), 1154 sexual offence cases were reported over an eight year period (2007-2014) (approximately 0.0048% per year) (Makasa and Heathfield, 2018), compared to the approximate 50 000 cases reported per year in South Africa for a population size of approximately 52 million people. In Nigeria, different university teaching hospitals have reported between 76 and 304 cases over a 5-year period which translated to an occurrence of sexual offences of less than 0.001% for those specific areas (Akinlusi *et al.*, 2014; Badejoko *et al.*, 2014). However, under-reporting may play a role in the apparent low number of cases in these countries (Kullima *et al.*, 2010; Ojo, Oliver and Louis, 2013).

In first world countries, the number of reported cases is also lower in comparison to South Africa. With a population of 8.78 million people living in London, sexual offences reported in a one year period between 31 July 2016 and 31 July 2017 presented with 19 964 cases (Data.london.gov.uk, 2018).

Numerous hypotheses exist as to why these cases remain abundant in South Africa, which has been discussed extensively by Jewkes and colleagues in various articles (Jewkes and Abrahams, 2002; Jewkes, Levin and Mbananga, 2002; Jewkes *et al.*, 2006, 2011; Seedat *et al.*, 2009). These reasons include the possibility of sexual coercion, through which the victim may be blackmailed, threatened or deceived by the perpetrator into consenting. The perpetrator may be a stranger, a known individual or have a relationship or be married to the victim (Jewkes and Abrahams, 2002). Sexual exploitation has also been seen in South Africa, where goods, money or presents are exchanged for consent. This has been seen particularly in the younger population of women who need an income or desire gifts that are beyond reach of what they are able to afford (Jewkes and Abrahams, 2002).

There is a lack of literature surrounding the progression of sexual offence cases to the courtroom and specifically, the role of forensic evidence in these cases in South Africa. Other countries have demonstrated the value of DNA evidence in the identification of alleged perpetrators, particularly of younger victims (Briody, 2002; Bermetz, Trelle and Tonia, 2013; Felson and Cundiff, 2014; McAllister *et al.*, 2016; Menaker, Campbell and Wells, 2017), while other studies have shown a lack of convictions when forensic evidence was not used in cases (although not necessarily vice versa) (Ingemann-hansen *et al.*, 2008; Makasa and Heathfield, 2018). Biological evidence has the potential to provide important information to the court: firstly, the identification of semen suggests that ejaculation has occurred, while DNA evidence may match to a suspect. Each of these aspects (semen identification and DNA profiling) provide important but different evidence towards a criminal investigation; this study will focus on the identification of semen, and not the DNA profiling aspects for human identification purposes.

1.3. Presumptive Tests

1.3.1. Principle of presumptive tests

Presumptive tests are techniques used to provide an indication as to the possible identification of a biological stain, in order to guide downstream processing (Virkler and Lednev, 2009; Ja Hyun An, Kyoung-Jin Shin, 2012). Different tests are used to detect certain components of each body fluid, by detecting enzymes or proteins which are present in these fluids (Virkler

and Lednev, 2009; Zapata, Fernandez de la Ossa and Garcia-Ruiz, 2015). Examples of enzymatic tests include amylase test for saliva and the acid phosphatase (AP) test for semen.

In general, a presumptive test is based on the addition of a particular chemical to a biological stain and observing if a colour change occurs or not. If a colour change occurred, the added solution has been converted to a different substrate by the enzyme present in the body fluid, and the substrate produced has a colour which is visible to the naked eye. The enzymes present in these body fluids are however present in other substances, and false positive reactions therefore occur, albeit usually at a slower rate. As such, time thresholds are established for presumptive tests to minimise the detection of false positives (Virkler and Lednev, 2009; Ja Hyun An, Kyoung-Jin Shin, 2012; Vennemann *et al.*, 2014).

1.3.2. Acid phosphatase test

Acid phosphatase (AP) is a water-soluble enzyme secreted by the prostate gland. It is a glycoprotein secreted and 100 kDa in size consisting of two subunits each with a molecular weight of 50 kDa (Derechin *et al.*, 1971; Luchter-Wasyl and Ostrowski, 1974; Muniyan *et al.*, 2013). The enzyme is expressed in the ACPP (Acid Phosphatase, Prostate) gene on chromosome 3q21-23 on the human genome (Winqvist *et al.*, 1989; Muniyan *et al.*, 2013). The AP enzyme is produced in the prostate glands epithelial cells and form less than 1% of protein secretions from the gland (Lilja and Abrahamsson, 1988). Medical conditions such as prostatic cancer was discovered to have increased activity in AP. Activity of the serum increased with the progression of the disease (i.e. the stages of cancer and its metastasis throughout the body) and is used as a diagnosis for the presence of the disease. The human immunodeficiency virus (HIV) infection is enhanced by the AP found in semen if the virus is transferred during sexual intercourse (Adermann *et al.*, 2007; Arnold *et al.*, 2012).The viral particles (either its genetic material or outer capsid protein) are promoted by AP for assembly into virions (a complete virus particle, that includes its genetic material as well as the capsid) using the C-proximal peptide fragments of the AP enzyme, forming amyloid fibrils. These fragments are known as a semen-derived enhancer of virus infection or SEVI. The SEVI fragments allow the assembled virions to become infectious and spread. Once spread, these virions may attach to its target cells for integration to its nucleus and replicate. However, the capturing of viral particles is only reliant on the level of AP within the individual but does not affect the level of AP in semen after infection (Adermann *et al.*, 2007; Arnold *et al.*, 2012).

The AP enzyme is found at high concentrations in seminal fluid (Davidson and Jalowiecki, 2012; Laux, 2012). The presumptive test thus aims to detect the activity of the AP enzyme within an unknown stain (Kind, 1957); thus, the test was originally termed the "AP test". A change in colour to purple indicates a presumptive positive for the presence of semen.

The colour change within the AP test works by exploiting the AP enzymes ability to catalyse the hydrolysis of organic phosphates (Virkler and Lednev, 2009; Davidson and Jalowiecki, 2012; Laux, 2012). Two common components of the AP presumptive test consists of α-naphthyl phosphate and a Brentamine Fast Blue salt. The AP enzyme hydrolyses the α-naphthyl phosphate and yield a phenol group named α-naphthol and a phosphate group. The α-naphthol then reacts with a diazotised salt, Brentamine Fast Blue and results in the development of a coloured purple azo dye. The product of the reaction forms over a period of time (Davidson and Jalowiecki, 2012; Redhead and Brown, 2013).

There are two ways in which to perform the AP test: the direct and indirect test. For the direct test, an extract of the unknown stain is tested directly with the Brentamine Fast Blue reagent (Redhead and Brown, 2013). The filter paper is then tested for the possible presence of semen. The indirect test involves placing a piece of filter paper, that has been moistened with water, over the suspected unknown stain. The paper is then pressed firmly down on the stain to be able to transfer a proportion of semen onto the filter paper. Alternatively, a swab taken from a medical examination may be made wet with some water and pressed onto a section of filter paper (Redhead and Brown, 2013; Lewis *et al.*, 2013).

According to Kind, a colour change reaction from an orange to purple colour will happen within a few seconds up to a few minutes after the addition of the reagent (Kind, 1957). Therefore, the time taken for a colour change to occur to purple to indicate a positive reaction is key to the AP test in determining if there could be a possible presence of semen. Forensic laboratories worldwide have adopted different cut off times, with the most common being two minutes (Lewis *et al.*, 2012; Redhead and Brown, 2013). Lewis *et al.* showed that semen dilutions up to 1 in 40 can be detected within two minutes if ejaculation has occurred from the perpetrator onto the victim or clothing (Lewis *et al.*, 2012). If the reaction from orange to purple occurs within two minutes, this will be indicative of a positive reaction for the possible presence of semen. Anything beyond the set cut off time of two minutes is considered as a negative result (Lewis *et al.*, 2012). In South Africa, SAPS adopt a cut off time of 65 seconds, where, the stain is considered as presumptively positive for semen if it reacts within 65 seconds.

The two minute cut off time internationally has been challenged by many researchers. Lewis *et al.* (2013) presented that utilising a ten minute cut off time can increase the detection of dilute semen samples using the direct and indirect methods (Lewis *et al.*, 2013). Redhead and Brown also showed that the two minute cut off for a positive reaction is insufficient for detecting weaker dilutions of semen. This trend was seen from the 1 in 200 dilutions up until the 1 in 1000 dilutions (Redhead and Brown, 2013). Allard *et al.* (2007) also investigated the cut off time and found that diluted and aged semen samples reacted between two to ten minutes; and thus stated that the cut off time could be increased (Allard *et al.*, 2007). These studies suggest additional research is needed to determine the optimal cut off time for a positive reaction regarding the AP test.

Further, the AP enzyme is not restricted to only human seminal fluid. Many other body fluids as well as semen from other animals contain AP at lower concentrations, which are also detectable after applying the AP test (Murdoch and White, 1967). Substances known to have been identified to result in false positives are vaginal fluid, mushrooms, tea and dog semen (Venneman *et al.*, 2014; Curry and Heathfield, 2016) Reaction of the body fluids with the AP test may provide positive results - sometimes within the time cut off threshold (Virkler and Lednev, 2009; Ja Hyun An, Kyoung-Jin Shin, 2012). These results are known as false positive results.

1.4. Enzyme Activity of Acid Phosphatase

1.4.1. Enzyme kinetics

To evaluate the kinetic activity of AP, the use of α-naphthol has been of principle importance in enzymatic assays, particularly colourmetric reactions. α-naphthyl phosphate has been tested to be specific for AP enzymes (Leslie and Raymond, 1977; Luchter-wasylewska, 1997). AP is similar to numerous other enzymes, whereby it is able to function as a catalyst. The rate of catalysis is commonly measured by the number of products, or the number of moles formed per second, that may be produced over time. The amount of enzyme and substrate present need to be equally proportional for the rate of catalysis over time to be measured accurately. The Michaelis-Menten model is used for simple enzymatic reactions (Johnson and Goody, 2012).

An ultraviolet visible (UV-Vis) spectrophotometer is an instrument that allows the detection and measuring of substances at a given wavelength of light. In biology and chemistry, this allows the measurement of a specific compound's concentration at a particular wavelength.

For the study of enzyme kinetics, the activity of the enzyme can be measured directly on its activity level and its ability to break down substrates into coloured products. The spectrophotometer has the ability to measure colour intensity within the invisible UV range. This extends the observation range for the products that are studied and allows observations of colours appearing or disappearing (Bisswanger, 2014).

Kinetic studies to evaluate the activity of the AP enzyme have been extensively performed for humans in a clinical setting. The AP enzyme has shown to be stable breaking down substrates (phosphomonoesters) at pH 5 to 7 and thus functions optimally in water and slightly acidic conditions (Luchter-wasylewska, 2001). The average specific activity for human AP was determined to include a range of 637±357 UI (international units) (Miteva *et al.*, 2010). Relatively few studies have researched enzyme activity of AP for animal semen, and these investigated this topic in relation to animal breeding. It was found that bulls, rams and pigs had AP enzyme activities of 24.7±11.8, 64.9±14.6 and 680±304 UI respectively (Roussel and Stallcup, 1966; Zakrzewska *et al.*, 2002; King and Macpherson 1966).

A study by Miteva *et al*. compared the characteristics of human and animal AP activity. The activity of each of the three species showed significant differences that may permit them to be distinguished from each other. The enzyme activity reported 1031.9±536.5 U/l for humans, which was significantly higher compared to rams (18.7±1.7 UI) and jacks (11.5±3.6). However the sample size for each set of species was small to infer statistically significant results, with samples from four humans, three jacks and three rams (Miteva *et al.*, 2010). However, this study suggests the possible use of enzyme kinetics in forensic science, to determine the species to which the semen belongs.

1.4. Confirmatory tests

1.4.1. Confirmatory tests for semen

Confirmatory tests are tests applied to verify the origin of a specific body fluid with more certainty (Virkler and Lednev, 2009; Ja Hyun An, Kyoung-Jin Shin, 2012). The most common method to confirm the presence of semen is the visual identification for spermatozoa under a microscope. Various staining procedures exists for visualisation: Papanicoloau stain, Wrights stain, Haematoxylin and Eosin (H&E) stain, Baecchi's stain, as well as the Kernechtrot-picroindigocarmine stain (or commonly referred to as the Christmas tree stain) (Virkler and

Lednev, 2009). The two most commonly used ones in forensic practice are the H&E and the Christmas tree stains. The Christmas tree stain acquired its name by the colours the stain provides various parts of the spermatozoa. The spermatozoa heads are stained red while the tails are stained green. For the H&E stain, the heads are stained purple and the tails and acrosomal caps are stained pink (Young *et al*., 2014).

1.4.2. Anatomy of the spermatozoa

A spermatozoon consists of a head, neck and an end tail piece covered by a plasma membrane. The morphology and size will differ according to species. Human spermatozoa are relatively shorter compared to other animals previously investigated (these include stallion, boar, canine, ram, bull and ostrich) (Table 1).

Table 1: Comparison of the morphological differences of spermatozoa between various animals

Species	Head width	Head length	Tail length	Staining method	Morphological observations	References
Human	2.93 - 3.04µm	4.28 - 4.98µm	Varied	Christmas tree, H&E, alkaline fuschin stain	Round-headed.	(Allery *et al.*, 2001; Soler *et al.*, 2003; Pesch and Bergmann, 2006)
Horse	2.79 - 3.26 µm	5.33 - 6.62 µm	30 µm	H&E stain	Oval elongated head that is relatively flat.	(Pesch and Bergmann, 2006; Brito, 2007)
Boar	4.5 – 4.6 µm	8.8 – 9.1 µm	40 µm	Eosin and methylene blue stain	Elongated heads.	(Hirai *et al.*, 2001; Pesch and Bergmann, 2006; Mora *et al.*, 2007)
Dog	3.77 - 4.46 µm	6.49 - 7.06 µm	Not recorded	Giemsa stain	Large sperm heads that are narrow and pyriformic.	(M.Dahlbom, M.Andersson, M.Vierula, 1997)
Bull	4.5 µm	9.15 µm	64 µm	H&E stain, toluidine blue stain	Pear- shaped heads.	(Beletti, Costa and Guardieiro, 2005; Pesch and Bergmann, 2006)
Ostrich	0.5µm	13µm	Not recorded	Fixation (formalde hyde, gluteralde hyde)	Slightly curved, cylindrical head that is tapered at the anterior section of the head.	(Soley, 1993)

Studies used various staining techniques to characterise morphology (Pesch and Bergmann, 2006). Differences in animal semen was compared for breeding purposes using the H&E stain, Giemsa stain, Hemacolor, and Papanicolau (M.Dahlbom, M.Andersson, M.Vierula, 1997; Pesch and Bergmann, 2006; Brito, 2007); but not many studies have used the Christmas tree stain. A comparative study between mammalian spermatozoa was conducted by van der Horst and Maree (2009) with a novel staining method (SpermBlue®). The stain was developed and employed to identify characteristics that allowed the differentiation between different species of mammals' semen in conjunction with analysis on computer systems (van der Horst and Maree, 2009).

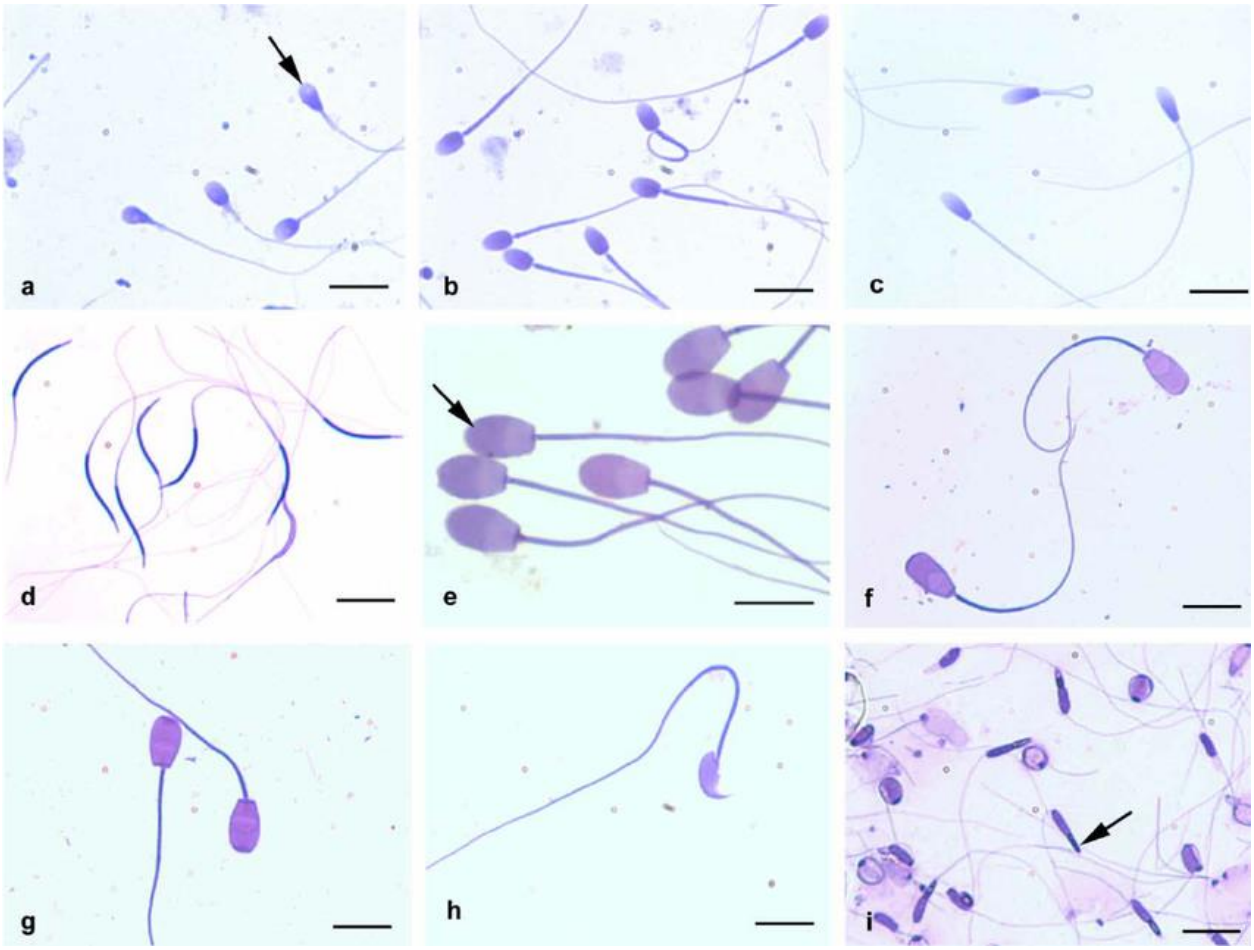

Figure 1: a-i Sperm morphology of human and eight representative species stained with SpermBlue®. a) Human b) Vervet monkey c) Horse d) Chicken e) Ram f) Boar g) Bull h) Mouse i) Abalone. Each scale bar represents 10 mm, the large arrows within a, e and i show the acrosome (van der Horst and Maree, 2009).

1.5. Rationale, aims and objectives

1.5.1. Motivation for the study

An in-house pilot study was conducted in 2015-2016 which investigated the specificity, sensitivity and reliability of the Brentamine fast blue test as well as the suitability of the cut off time that the Forensic Science Laboratory (FSL), South African Police Service (SAPS) have set for a positive semen reaction (Curry and Heathfield, 2016). The study included semen from consenting male human participants (n=15), as well as semen from dogs (n=2). In addition, various false positives were tested, this included tea, mushrooms and various plants.

Semen stains as well as false positives were seeded onto poly-cotton material in a dilution series and tested directly and indirectly using the AP test. The colour changes as well as the

reaction times were recorded. Dog semen reacted within the 65 second time cut off and no difference was found between human and dog semen when using the conventional direct and indirect methods.

The rate of the colour change reaction was then measured objectively using a UV-Vis spectrophotometer, by placing the semen dilution/false positive in a cuvette and adding the Brentamine FB reagent. The pilot study found that by using an objective means to observe colour change, a noticeable difference was found between human and dog semen, as well as the false positives tested. This study suggested that UV-Vis spectrometry could potentially serve as an alternative method for presumptive testing of semen, with increased specificity for human semen. However, due to small sample sizes, this difference could not be investigated statistically.

The results from the pilot have laid a positive foundation for this concept to be explored further. The sample size needs to be expanded and more animals need to be included to test the hypothesis that the UV-Vis spectrophotometer is a more specific method to identify human semen than the traditional direct and indirect methods of testing. The results from more objective testing methods should also be supplemented with confirmatory testing. Exploring these methods of semen identification hopes to establish a fresh approach, with increased specificity towards human semen, with the overall intention to assist with medico-legal investigations and forensic science purposes.

1.5.2. Aims

To investigate the presumptive and confirmatory tests for semen identification, regarding its kinetics, sensitivity, specificity and sperm cell morphology to differentiate between human and animal semen.

1.5.3. Objectives

- Determine the reaction time of the Brentamine FB test on human and non-human semen samples using (i) direct testing; (ii) indirect testing and (iii) enzyme kinetics.
- Investigate morphology of the semen samples of various species using H&E staining and microscopy.

- Statistically assess if there are any differences between human and non-human semen characteristics using these methods.

Chapter 2: Materials and Methods

2.1. Sample collection

2.1.1. Humans

Previously, one semen sample was acquired from each of fifteen unrelated adult male volunteers during the pilot project. Approximately 2ml of semen was collected into a 50ml centrifuge tube (Nest Biotechnology, USA) and stored at four degrees Celsius. Nine of the participants gave consent to allow the sample to be stored long term for subsequent testing. These nine samples were included in this project as well, and tested 20 months after initial collection for comparison. The data generated from these aged samples is represented in the results section as 'human (aged)' samples. In addition, one new human participant was recruited for the study, whereby the data generated from his semen sample was grouped with previously generated data (referred to as 'human (fresh)' samples in results), for comparison purposes. Ethics approval was given by the University of Cape Town, Faculty of Health Science, Human Research Ethics Committee (HREC REF: 502/2015). All participants gave informed consent to participate in the study (Appendix 1).

2.1.2. Non-humans

Semen samples were obtained from several animals; one sample per animal from each of four different animal species. The species included *Canis lupus familiaris* (dog, n=13), *Equus ferus caballus* (horse, n=5), *Ovis aries* (ram, n=13), and *Struthio camelus* (ostrich, n=9). The dog and horse samples were obtained from Klapmuts veterinary farm, while the ram and ostrich samples were obtained from Elsenburg veterinary farm. The University of Cape Town, Faculty of Health Science, Animal Research Ethics Committee confirmed that their approval was not required for this project as no animals were being killed or harmed during the process of semen sample collection.

It was challenging to control for the volume of semen obtained from each of the animals; as such, if small volumes were obtained, only some of the analyses outlined below could be performed on the sample. The final sample numbers for each analysis is included in the results section.

2.2. Sample processing overview

An overview of the methods is portrayed in Figure 2, and details pertaining to each part of the process is presented in following sub-sections.

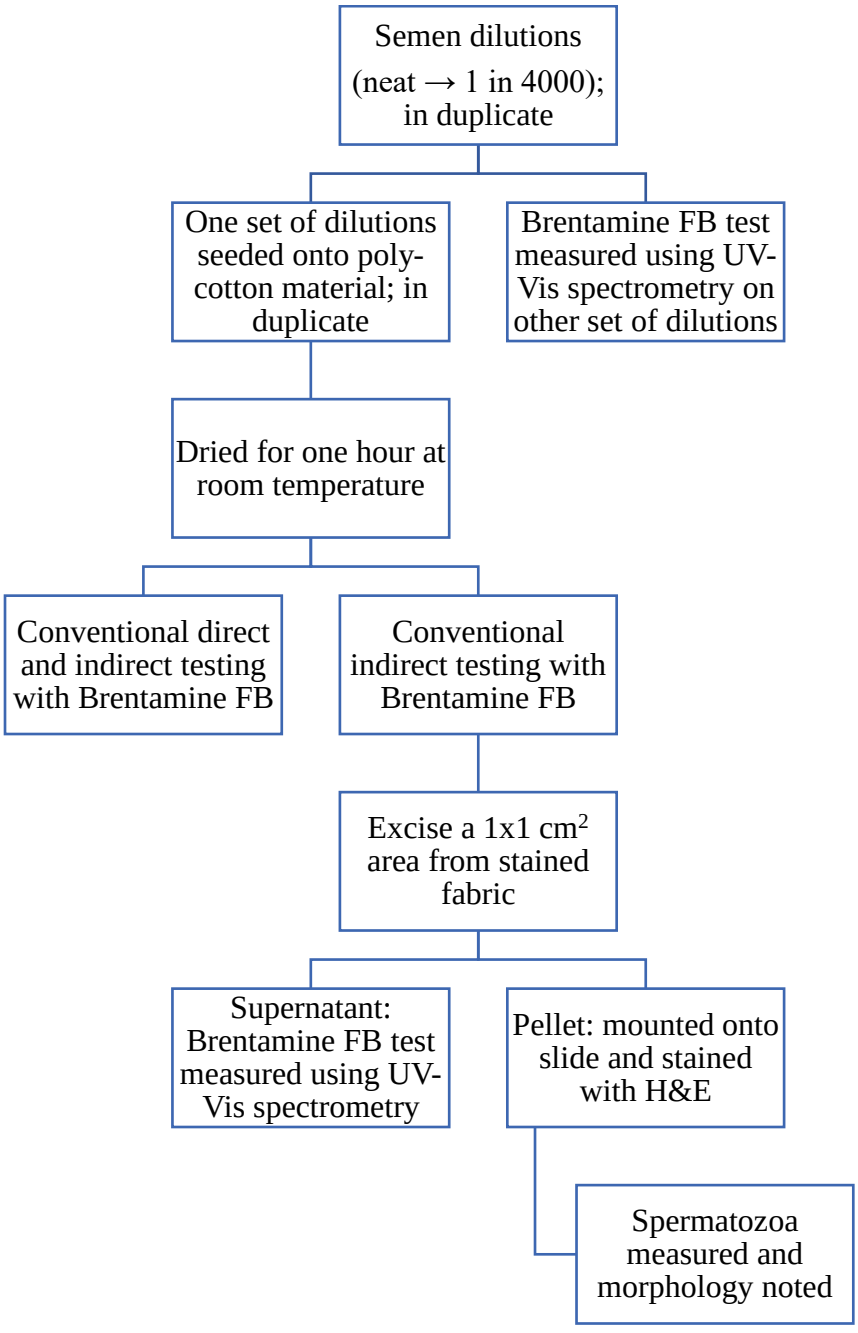

Figure 2: Sample processing overview of each sample undergoing presumptive, confirmatory and the reaction kinetics tests.

2.3. Brentamine Fast Blue reagent preparation

The Brentamine Fast Blue reagent solution was prepared by mixing two solutions, named solution A and solution B. Solution A was prepared by dissolving 0.4g of sodium acetate and 0.2g of ortho-dianisidine (Sigma-Aldrich, Germany) in 2ml of deionised water. A volume of 200µl of glacial acetic acid was added to the solution subsequently and then mixed. Solution B was made by dissolving 0.08g of sodium α-naphthyl phosphate (Sigma-Aldrich, Germany) in 1ml of deionised water and mixed. The Brentamine FB working reagent was then mixed by intermixing 1ml of solution A, 100µl of solution B and 8.9ml of deionised water. The solution was thoroughly mixed by vortexing until the contents were mixed evenly. Both solution A and the Brentamine FB reagent were freshly prepared on the day of testing each time.

2.4. Sensitivity

A dilution series was prepared with the fresh semen samples with deionised water. The dilutions stood as follows: 1 in 4, 1 in 10, 1 in 40, 1 in 100, 1 in 400, 1 in 1000, 1 in 2000 and 1 in 4000. A volume of 200µl of each dilution and a neat semen sample were spotted onto separate cutout sections of $6 \times 6 cm^2$ polycotton fabric squares for each participant, in duplicate. The spotted fabric remained left to dry at room temperature for 60 minutes.

2.4.1. Indirect test

Whatman Grade 1 filter paper (GE Healthcare, United Kingdom) was moistened with 200µl of deionised water. The filter paper was placed above the stained fabric. A firm, continuous pressure was directed onto the filter paper for a count of five seconds. The filter paper was subsequently applied with the freshly made Brentamine Fast Blue reagent by pipetting 50µl of the reagent onto the filter paper. The reaction was timed until a colour change was observed. The time taken for a colour change was recorded, if no colour change was observed after ten minutes after applying the reagent, the reaction was considered negative. The timer was stopped once the time reached ten minutes. The test was performed in duplicate, once for each set of dilutions.

2.4.2. Direct test

A volume of 50µl of Brentamine Fast Blue reagent was subsequently added by pipetting onto the seeded fabric directly. This test was performed for one set of dilutions only. The potential colour change to purple was timed until such a colour change was observed. If there was no colour change reaction observed after ten minutes after applying the reagent, the reaction was considered negative.

2.5. Spermatozoa extraction and staining

2.5.1. Extraction

Only neat seeded samples were used for the extraction and staining method. A 1 × 1 cm^2 section of the fabric was cut from the stained area of each sample using a sterile scalpel (Hi-Care Int., Cape Town). The sections were placed into a 1.5ml microcentrifuge tube and a volume of 400µl of deionised water was added. Manual agitation was applied for approximately ten seconds with forceps. The section was squeezed with the forceps to allow any excess liquid to flow back into the micro centrifuge tube. Subsequently, the cut section of material was dispensed. The tube was then centrifuged for 60 seconds at 13000xg. The supernatant was removed and placed in new microcentrifuge tubes, followed by storage at 4ºC for later use (See section 6.1.). The pellet was resuspended in 200µl deionised water. A volume of 20µl of the resuspended sample was placed onto a clean glass microscope slide (Marienfield, Germany).

2.5.2. Staining

The prepared slides were subjected to manual staining methods using the H&E staining procedure. The slides were first immersed into the haematoxylin stain for five minutes. The slides were subsequently rinsed in running tap water for one minute to remove the remaining dye. The slides were dipped once in acid alcohol and rinsed under running tap water for one minute. The slides were immersed in Scott's tap water for one minute and rinsed again under tap water for one minute. Following that, the slides were immersed in the eosin stain for five minutes. The slides were washed with water and subjected to dehydration steps of increasing alcohol concentrations of 70%, 90% and 100%. The slides were cleared of alcohol with xylol and mounted with a coverslip for preservation.

Viewing of the slides was examined under a Leica DM500 compound microscope (Leica Microsystems, Switzerland). The stained slides were viewed under 40x magnification. The microscope was mounted with a camera, with which photographs were captured and processed utilizing the Leica Application Suite LAS EZ version 3 software (Leica Microsystems, Switzerland). Spermatozoa heads, tails and the entire spermatozoa were chosen at random and measured using the software's measuring application to obtain the morphometric parameters for the human and non-human samples. Morphology of spermatozoa was observed and noted.

2.6. UV-Vis spectrophotometry

2.6.1. Semen specimens

A new series of semen dilutions was prepared using the neat semen samples using deionised water, were as follows: 1 in 100, 1 in 200, 1 in 300, 1 in 400, 1 in 500, 1 in 600, 1 in 800, 1 in 1000. These samples were included for 'direct' testing with UV-Vis spectrophotometry, to establish a baseline for this method of using Brentamine FB.

In addition, the supernatant from the semen extracted from the fabric was used for 'indirect' UV-Vis spectrophotometry testing. This method of testing was performed to simulate a more realistic 'casework' sample, as one would expect semen to be recovered from fabric in casework, rather than testing it straight from its liquid form. The following dilutions were tested in this way: 1 in 4, 1 in 10, 1 in 40, 1 in 100, 1 in 400, 1 in 1000, 1 in 2000 and 1 in 4000 were also used.

2.6.2. Measuring the reaction rate

A volume of 800µl of each semen sample dilution was pipetted in a 2ml plastic cuvette (Lasec, South Africa). The cuvette was subsequently placed in a Shimadzu UV1800 spectrophotometer with the reaction read at a temperature of 37˚C (Shimadzu, Japan). A volume of 200µl of the freshly prepared Brentamine FB reagent (section 2.3.), with a starting concentration of 0.094 mol/L (Appendix G), was added to the cuvette. The software for the instrument, the UV Probe 2.3.3 (Shimadzu, Japan), was used to obtain the absorbance readings of the reaction at one second time intervals. The absorbance acquired was performed at a wavelength of 525nm (tested in the in-house pilot study) for 120 seconds.

2.6.3. Reaction rate calculations

The raw data of the colour change absorbance readings was copied from the UV Probe software and exported as is to Microsoft Excel according to each dilution read. A line graph was plotted, with trendline and graph equation: using y=mx+c, from the start of the reaction to the saturation point. The slope of the graph was taken from the graph equation as the reaction rate (the change in absorbance value over time, Ab/s) for each sample and for each dilution. The estimated reaction rate was then added to a data sheet in Graphpad Prism and plotted into a line graph for comparison to other animals for each sample.

2.7. Statistics

Two-way ANOVA was performed on the reaction times for the direct and indirect tested semen and the enzyme kinetics to compare each species. One-way ANOVA was performed for the morphological dimensions of the spermatozoa cells to compare each species. The Bonferroni and Tukey's multiple comparison tests were performed as post-hoc tests for the two-way and one-way ANOVA tests respectively, to correct for multiple testing The data from the pilot study were included for statistical analyses, including human (n = 15 for direct and indirect AP test, n = 15 for UV-Vis spectrophotometry, n = 5 for confirmatory testing) and dog (n = 2) which were tested with the direct and indirect AP test, UV-Vis spectrophotometry and confirmatory testing during the pilot study. The analysis was performed using Graphpad Prism 5 (Graphpad, USA). A p-value of less than 0.05 was considered to be statistically significant.

Chapter 3: Results

3.1. Sensitivity and specificity

3.1.1. Direct AP testing

Semen from human (fresh: n=1 and aged: n=9), dog (n=7), horse (n=1), ostrich (n=9) and ram (n=13), were tested using the direct method of the Brentamine FB test. Each animal species showed an increase in reaction time as the dilution increased. Figure 3 illustrates the time taken for each semen dilution to react with the Brentamine FB direct test for each human and animal under study (data from the pilot study was included for statistical power and comparison purposes). Faster reaction times were seen for both fresh and aged human semen compared to dog, horse, ostrich and ram semen. however, no significance between human (fresh and aged) was seen after applying the Tukey multiple comparison post-hoc test ($p > 0.05$). Only human (aged) and ram semen were statistically different from each other with regards to time taken for a positive reaction with the Brentamine FB test to occur ($p < 0.05$), and no statistical significance in the time taken for positive reactions to the Brentamine FB test was found between human in comparison to dog, horse and ostrich semen.

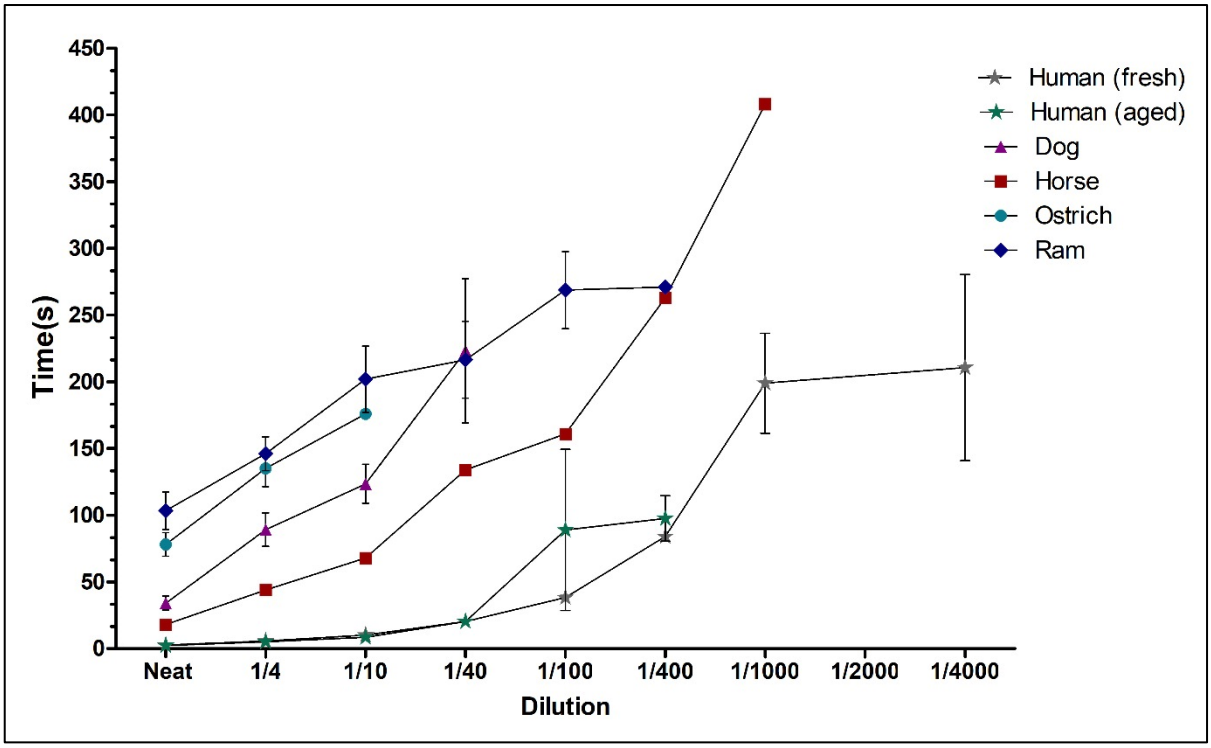

Figure 3: **The direct test of human (aged: n = 9, fresh: n = 16), dog (n = 9), horse (n=1), ram (n=13) and ostrich (n=9) semen onto cotton fabric using the Brentamine FB test**. Median reaction times (in seconds) was plotted against each dilution for each species. The more dilute the sample, the longer the time to react positively using the direct test. Mean reaction times were not significantly different between animals after corrections for multiple testing were applied.

3.1.2. Indirect AP testing

The mean time taken for positive reactions to occur using the conventional indirect method of testing is depicted in Figure 4 for each semen dilution and for each animal under study. As expected, the reaction times were considerably longer compared to the direct method of testing (Figure 3). For samples that were in the 'neat' to '1/40 dilution' range, human samples reacted significantly faster compared to the other three animal species (each $p < 0.05$), except for dog semen. No significant differences was found between fresh and aged human samples across all dilutions after applying the Tukey's post-hoc test.

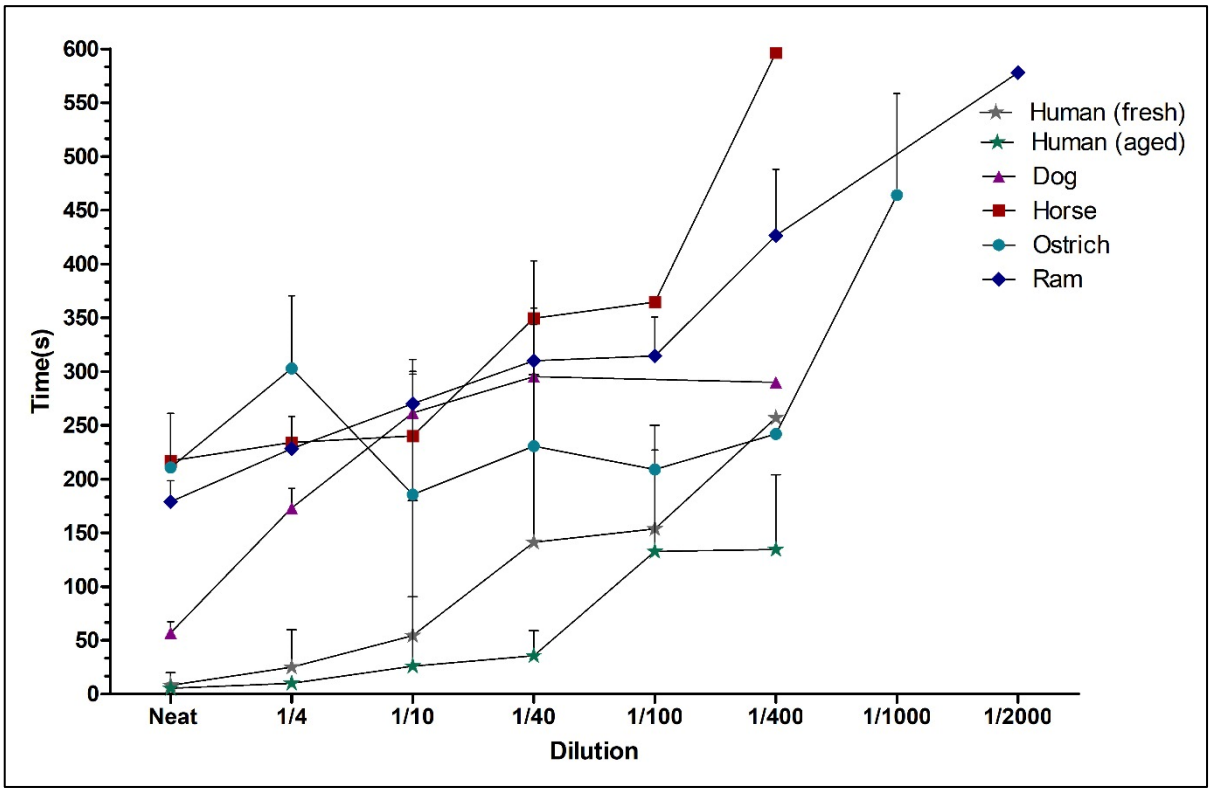

Figure 4: **The indirect test of human (aged: (n = 9), fresh: (n = 16)), dog (n = 9), horse (n = 1), ram (n = 13) and ostrich (n = 9) semen from cotton fabric using the Brentamine FB test**. Median reaction times (in seconds) was plotted against each dilution for each species. Reaction times were longer compared to direct testing.

3.2. Reaction rates using UV-Vis spectrophotometry

UV-Vis spectrophotometry was used to determine the reaction rates of the human and different animal species when the Brentamine FB test was applied to various dilutions of semen, also using a direct and indirect testing method.

3.2.1. Direct testing using UV-Vis spectrophotometry

The semen sample was subjected directly with the Brentamine FB reagent and measured using the UV-Vis spectrophotometer as described in section 2.6.2. The mean reaction rates were calculated and plotted against the dilution factors (Figure 5).

Fresh and aged human semen samples had significant differences according to their reaction rates over time at the 1 in 200, 1 to 1 in 600 dilutions (each $p < 0.05$). Further, fresh human semen reacted significantly faster compared to dog, ostrich and ram semen for all dilutions (each $p < 0.001$), and compared to horse semen for 1 in 100 to 1 in 400 (each $p < 0.01$). A similar observation was made for aged human semen samples, which had significantly different reaction rates across all dilutions compared to dog, ostrich and ram semen (each $p < 0.001$), whereas only the 1 in 100, 1 in 600 and 1 in 800 dilutions were significantly different for horses (each $p < 0.01$). Pairwise comparisons of each non human animal showed no significant difference in reaction rates between dog, horse, ram and ostrich.

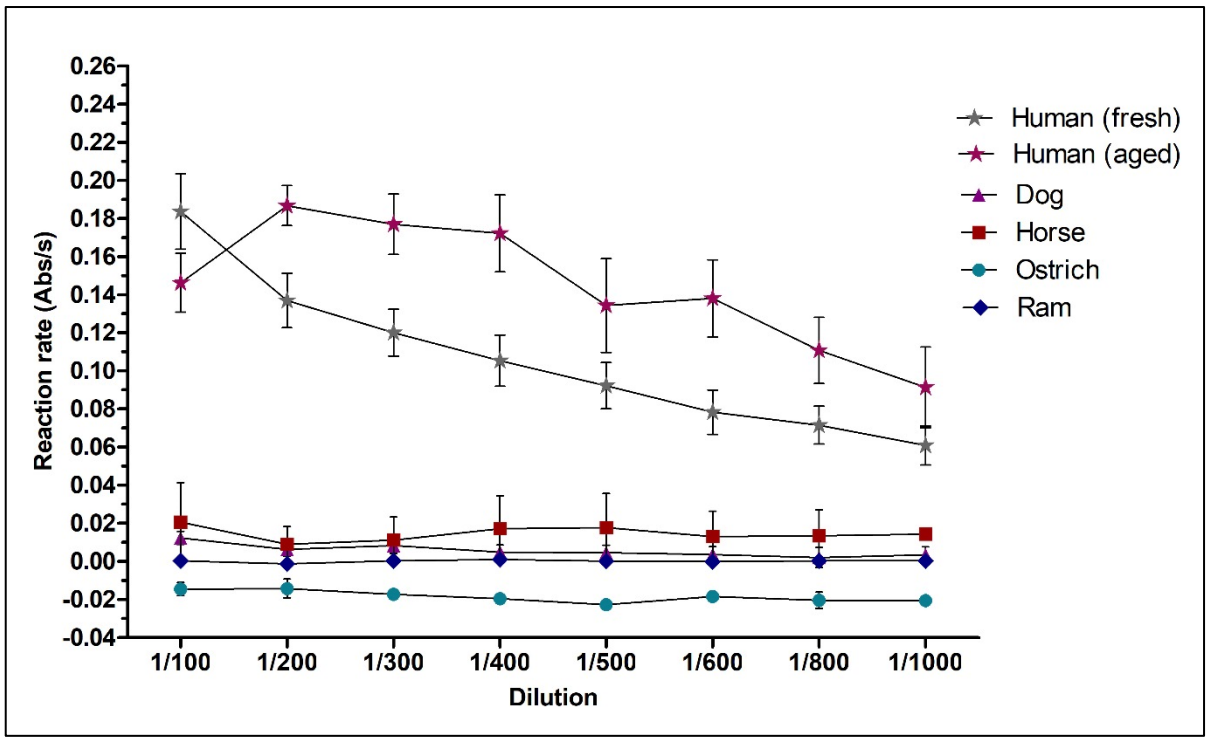

***Figure 5*: Reaction rates of different species, human (aged: (n = 9), fresh: (n = 16)), dog (n = 9), horse (n = 1), ram (n = 13) and ostrich (n = 9), tested directly using the Brentamine FB test.** Median reaction rates (in Ab/s) were plotted for each dilution of each species. Human semen had significantly higher reaction rates compared to all species.

3.2.2. Indirect testing using UV-Vis spectrophotometry

When tested indirectly, the mean reaction rates showed a similar trend as compared to the direct method of testing (Figure 6). The reaction rates of the human aged and fresh semen were significantly different at the neat dilution ($p < 0.001$) but this difference diminished in the lower dilutions (1 in 4 to 1 in 4000) where there was no significant difference. While, the neat dilution of fresh human semen had a lower reaction rate than the aged human semen samples (at 0.0639 Abs/s compared to 0.176 Abs/s), this may be explained by only one data point for the neat dilution.

The animal semen reacted slower than the human samples, particularly at dilutions lower than 1 in 40. The fresh human semen samples had significantly faster reaction rates at the 1 in 4 and 1 in 10 dilutions, when compared to dog semen (each $p < 0.01$), but were not significantly faster at the neat, 1 in 40 to 1 in 4000 dilutions. Fresh human semen had significantly fast

reaction rates at the 1 in 4 dilution when compared to horse semen. Ostrich and ram semen were compared to fresh human semen samples and showed significant differences at the 1 in 4 and 1 in 10 dilutions (each $p < 0.01$), but not at the other dilutions.

The aged human semen samples showed similar patterns of significance when compared to animal semen, however, the neat dilution had significantly faster reaction rates. Aged human semen compared to ostrich, dog and ram semen had significantly faster reaction rates at dilutions of neat to 1 in 10 (each $p < 0.01$), while when compared to dog semen had faster reaction rates at neat ($p < 0.001$), 1 in 4 (each $p < 0.01$), and 1 in 10 ($p < 0.01$) dilutions. Aged human semen had significantly faster reaction rates at the neat and 1 in 4 dilution (each $p < 0.001$). Reaction rates between the four different animal species tested (dog, horse, ostrich, and ram) were not significant.

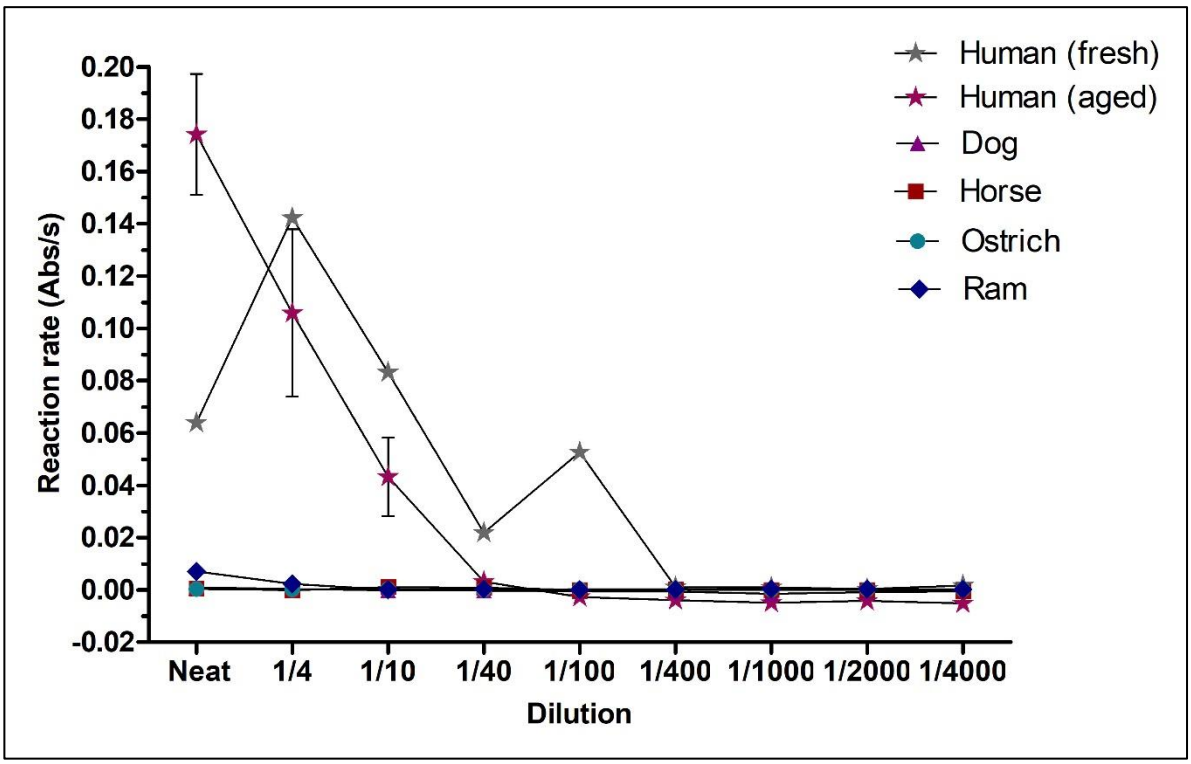

***Figure 6*: Reaction rates of different species, human (aged: (n = 9), fresh: (n = 16)), dog (n = 9), horse (n = 1), ram (n = 13) and ostrich (n = 9), tested indirectly using the Brentamine FB test.** Median reaction rates (in Ab/s) were plotted for each dilution of each species. Human semen had faster reaction rates than animal semen.

3.3. Confirmatory test – sperm cytology

Neat semen was mounted onto slides and then stained using H&E. All slides viewed exhibited spermatozoa with at least some tails intact. Figure 7 (A-E) shows representative images of semen from each of the five species under study.

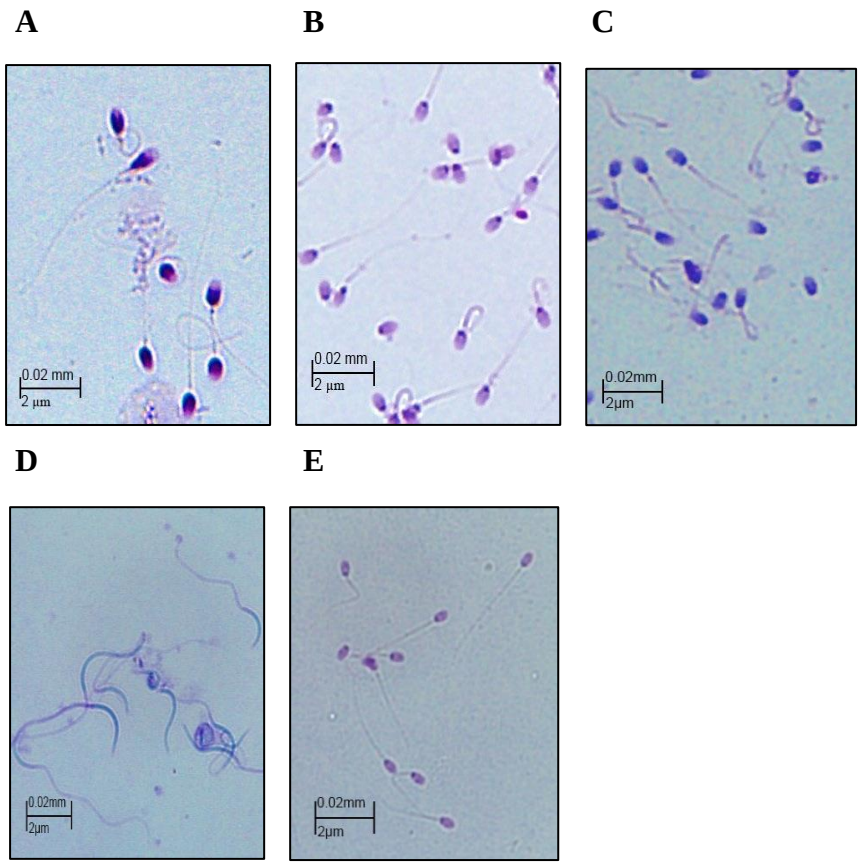

Figure 7 A-E: **Haematoxylin and Eosin stained spermatozoa extracted from seeded poly-cotton fabric:** (A) human, (B) dog, (C) horse, (D) ostrich and (E) ram semen viewed under a *Leica DM500* compound microscope at 40 X magnification.

Morphological differences were noted for each species (Figure 7). The head of the human spermatozoa (Figure 7A) was darker than that of other species, and the acrosomal cap at the anterior part of the spermatozoa head was also more diffuse and stained pink. Spermatozoa heads from the horse semen (Figure 7C) showed a similar pattern of diffusion as the human spermatozoa, however, the acrosomal caps at the anterior section of the horse spermatozoa head exhibited a darker pink colour than that of the dog semen.

The dog semen (Figure 7B) was more diffuse throughout the head apart from the neck area at the posterior section which appears a darker purple when compared to the other animal species. Ram spermatozoa (Figure 7E) heads exhibited the same type of diffusion as dog semen with lighter colour stained at the neck from the posterior part connecting to the tail. The species with the most differentiated morphological structures was the ostrich semen (Figure 7D) with no diffusion pattern found within the head, but rather a spindle shaped and curved appearance, which is characteristic to bird spermatozoa (Gee *et al.*, 2004).

The spermatozoa for each species were then measured (Table 3, Appendix D), and Figure 8 illustrates the measurements of the spermatozoa. Horse spermatozoa heads had the largest head width of the five species (Figure 8A) while ostrich spermatozoa heads length tended to be the largest (Figure 8B). Rams had the longest measured tails (Figure 8C), and overall the longest spermatozoa cells in total (Figure 8D). The length of the ram spermatozoa tail was significantly longer compared to each of the other species ($p < 0.001$, for each species).

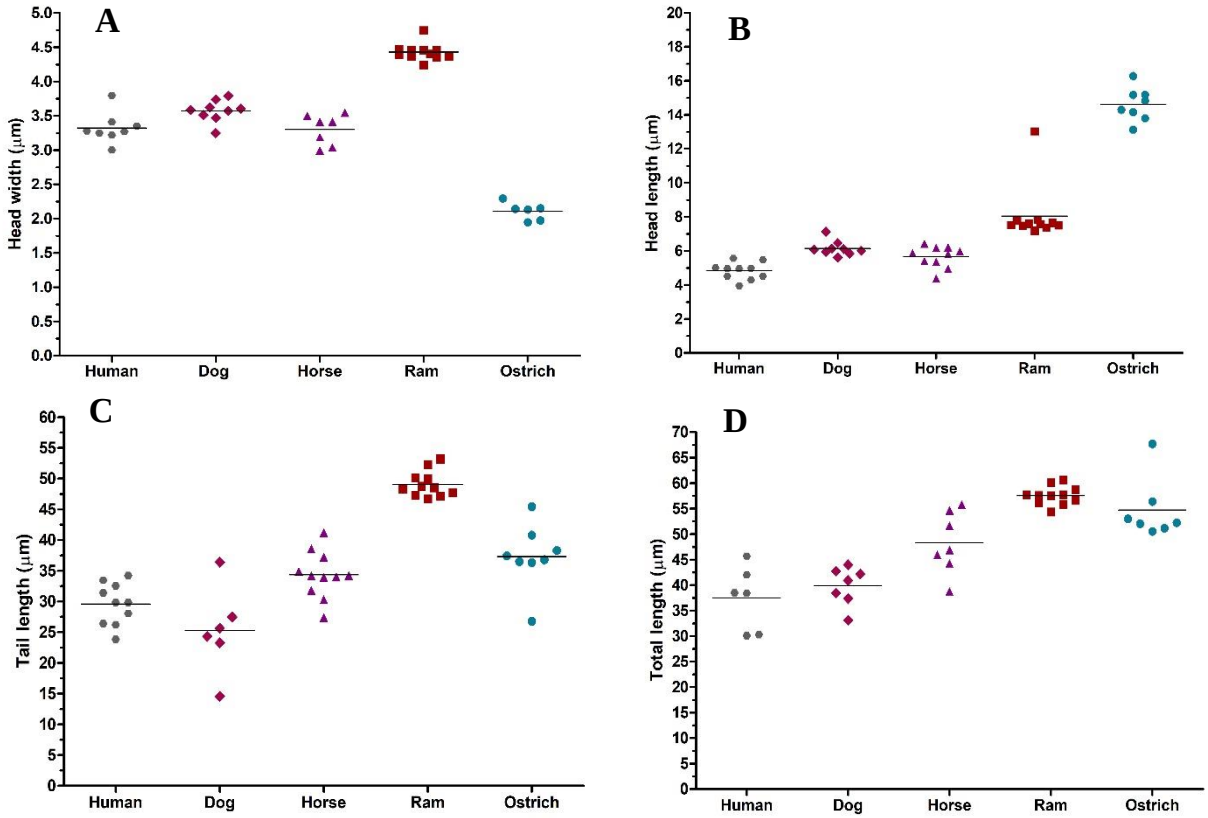

***Figure 8 A-D*: Scatter plot of the length and width measurements of spermatozoa of all species**. (A) The width of the spermatozoa heads of each species; (B) The length of the spermatozoa heads of each species; (C) The length of the spermatozoa tails of each species; (D) The entire length of the spermatozoa cells of each species.

Human spermatozoa head measurements (n=5) (Figure 8A) exhibited a great variety in size compared to the other animal species. A significant difference was found between human spermatozoa head width compared to ram, ostrich and dog species (each $p < 0.05$), but not the horse species. The length for human spermatozoa head (n=5) (Figure 8B) had significant differences compared to the ram, ostrich and dog species (each $p < 0.05$), while the horse species had no significant difference when compared to human species. Human spermatozoa tails (n=1) (Figure 8C) were different to ostrich and ram species (each $p < 0.001$), however not for horse and dog species.

An average length of 38.45 µm was recorded for human spermatozoa cells (Figure 8D), which was lower than any other animal species. No significance in length was found between dog and human spermatozoa. However, a significant difference was observed in length was found in the other three animal species (horse, ostrich, and ram (each $p < 0.01$)).

Chapter 4: Discussion and conclusions

The aim of the study was to investigate the presumptive and confirmatory tests for semen identification, regarding its kinetics, sensitivity, specificity and sperm cell morphology to differentiate between human and animal semen. Semen from human and animals was subjected to the Brentamine FB presumptive test using the conventional direct and indirect method. Reaction kinetics of the Brentamine FB test were then objectively measured using a UV-Vis spectrophotometer. Confirmatory testing was performed by mounting semen on the slides and staining with H&E and measuring the stained spermatozoa cells under a compound microscope. The data were then analysed to assess if human semen differed from animal semen using these different tests.

4.1. Conventional testing using the Brentamine FB test

The purpose of the initial part of testing was to investigate if various dilutions of animal semen reacted within the 65 second cut off time used in South African Police Service Forensic Science Laboratories. To set a baseline, various dilutions of human semen were first tested in order to establish the sensitivity of our in-house Brentamine FB test and to evaluate if the method was comparable to published literature. Once a baseline was established, the animal semen was tested with the same method and was compared to the recorded times of the human semen.

Positive reactions for the human semen were observed for dilutions up to 1 in 1000 between 3 and 4 minutes using the direct method of testing (Figure 3) and up to 1 in 1000 within 4 minutes using the indirect method of testing (Figure 4). These findings were consistent with other studies; Lewis *et al.* detected positive reactions up until a dilution of 1 in 3000 with both the direct spray and direct aerosol application of the Brentamine FB reagent (Lewis *et al.*, 2013), while Redhead and Brown detected up until a dilution of 1 in 1000 using the direct application method of the reagent (Redhead and Brown, 2013). The sensitivity of our results are comparable to the results in literature using a similar method.

Both fresh and aged human semen reacted positively with the Brentamine FB test within 65 seconds (fresh: up to 1 in 1000 for the direct test and 1 in 400 for the indirect test; aged: up to 1 in 400 for the direct test and 1 in 1000 for the indirect test) (Figures 3 and 4). Dilutions for

dog (direct: up to 1 in 1000; indirect: up to 1 in 400) and horse (direct: up to 1 in 1000; indirect: 1 in 400) also reacted positively within 65 seconds, however, none of the ostrich and ram semen dilutions reacted within this 65 second time frame (Figures 3 and 4).

A study in Brazil used the two minute cut off time to establish a positive result by testing diluted and undiluted fresh semen (Gonçalves *et al.*, 2017). Dilutions up until 1 in 200 reacted within two minutes, while aged (24 hours old) samples only reacted positively after this time frame of two minutes. The authors consequently concluded that the Brentamine FB test was of low sensitivity; however, this may be attributed to methodological differences; e.g. indirect method of testing or the use of vaginal swabs for testing.

In our study, 20 month old semen samples reacted positively within 65 seconds, which are similar to results stated in Kaye (1951). Kaye proposed that storing garments or underwear after proven positive for two years may still show positive results once tested again (Kaye, 1951). Nakanishi *et al.* has also shown that using the presumptive test for semen can provide positive results from evidence containing seminal stains aged from 2 years up to 56 years of age (Nakanishi *et al.*, 2014).

Studies using the AP tests has been commercialised into rapid detection kits for the identification of semen. The Phosphatesmo kit®, a commercialized kit, provided positive results for three classes of semen that contain various types of spermatozoa. The first class contains normal structural and functional spermatozoa, the second class contains oligospermic spermatozoa (spermatozoa that are low in concentration in the ejaculate) and azoospermic semen (semen containing no spermatozoa). Their results exhibited that positive results were found 72 hours after seeded semen samples. The kit was compared to a detection kit for prostatic specific antigen (PSA), an antigen found in the seminal fluid, and was the recommended kit for the detection of semen (Khaldi *et al.*, 2004). Some studies have tested the specificity of the test towards animal seminal fluid, and the animal seminal fluid tested negative when using the PSA test (Maher *et al.*, 2002; Miteva *et al.*, 2007). However, PSA can also be found in female urine and breastmilk, and therefore also has limitations with regards to its own false positives.

Overall, the results from the first part of this study showed that the sensitivity of the Brentamine FB test was similar to other studies. The results also support the poor specificity of the Brentamine FB test towards human semen as observed in other studies (Allard *et al.*, 2007; Lewis *et al.*, 2012, 2013; Redhead and Brown, 2013; Gonçalves *et al.*, 2017) and showed that

semen from common animals in South Africa also react positively. More specifically, semen from dogs and horses reacted within the 65 second cut off time used in South Africa, which may have implications during casework. While human semen appeared to react faster than animal semen, and was statistically significant for some dilutions for some animals.

4.2. Alternative methods for semen identification

The second part of the study was for the determination of the reaction kinetics of the AP enzyme when subjected to the Brentamine FB test. This was done with the use of the UV-Vis spectrophotometer which is able to measure colour changes objectively. Human and animal semen were subjected to the Brentamine FB test, both directly and indirectly, and the reaction rates were measured and compared (Figures 5 and 6).

Overall, both fresh and aged human semen had significantly higher reaction rates with the Brentamine FB test compared to the semen from all four different animals in this study (Figures 5 and 6). AP enzyme activity for human samples reacted between 0.05 Abs/s and 0.24 Abs/s, compared to animal enzyme activity that reacted between -0.01 Abs/s and 0.03 Abs/s. This is due to the different levels of AP activity that varies from one species to another (Mann, 1954). This illustrates that using the Brentamine FB test in conjunction with a UV-Vis spectrophotometer might increase the sensitivity of AP testing.

The kinetics of AP was briefly described by Hillman, Fabing-Byrd and Ertingshausen, for its use in clinical settings. α-naphthyl phosphate has been found to have specificity for AP and been frequently used for kinetic studies after this discovery. Fast and simple automated methods were then incorporated into various fields including forensic studies (Shaw, Brummund and Dorio, 1977). While Miteva *et al.* (2010) suggested that the significant difference in AP enzyme activity between ram and human semen may have forensic use with regards to semen specificity determination (Miteva *et al.*, 2010), this study has provided some of the first data in a forensic context to demonstrate this with other animals.

The results from UV-Vis spectrophotometry showed that human semen reacted significantly faster compared to all other animals' semen, for at least some dilutions, when using the direct method of testing. This was in contrast to the results from the traditional method of the Brentamine FB test, which showed that both ram and dog semen reacted within the 65 second cut off time, and that the time for a positive reaction was not always significantly different

between human and animal semen. The reason why human semen reacted faster compared to animal semen using the UV-Vis spectrophotometry method is most likely due to the higher levels and activity of the AP enzyme in humans (Murdoch and White, 1967; Miteva *et al.*, 2010). When using UV-Vis spectrometry as the method to detect the enzyme kinetics, objective readings of colour change were measured, as opposed to the subjective nature of the traditional tests. Subjectivity of the traditional colour change tests may introduce error into the time measurements taken for a positive reaction, which can be avoided when using UV-Vis spectrophotometry. This characteristic perhaps makes the UV-Vis spectrophotometry method a more reliable method to conduct the Brentamine FB test. While this finding needs to be verified in a larger cohort, these results add and support for this proof of concept and motivate for this method to be explored even further.

When testing human semen using the UV-Vis spectrophotometry method, the direct method of the Brentamine FB test had higher reaction rates for human semen (fresh and aged), compared to the indirect method, as expected (Figure 5 and 6). When using the direct method of testing, fresh human semen reacted significantly faster compared to aged semen, but this was not the case for the indirect method of testing. The horse semen when tested with the UV-Vis had lower reaction rates compared to human semen, in both the direct and indirect test. AP enzyme activity in horses is lower compared to humans, which may explain the slower reaction rates (Pesch, Bergmann and Bostedt, 2006; Kareskoski *et al.*, 2010). Breeding season has also reported to affect the amount of enzyme present in horse seminal fluid (Kareskoski *et al.*, 2010). Our study looked at the AP enzyme in breeding season, which may be important to the study by what activity is found within that season.

The AP enzyme activity in ostrich semen has also been studied and has shown to have AP concentration values of 12.2±5.1 U/l (Ciereszko *et al.*, 2010). The authors also found that ostriches do not produce significant amounts of AP, which explains the AP concentrations in their study, as well as the slower reaction times in this study. Yet, another factor which may influenced the low AP activity in this study is that ostrich semen was mixed with an extender at time of collection, to delay the agglutination of the semen (Ciereszko *et al.*, 2010; Smith, 2016). However, it is not clear if the extender had an effect on the AP enzyme activity, and investigating this was beyond the scope of this study.

While pigs were not included in this study, they have been reported to have similar AP activity to humans: 681±304 UI (King and Macpherson, 1966), compared to 637±357 UI for humans

(Telisman *et al*., 2000). The similarity in enzyme activity levels between humans and pigs as previously reported, might not have been easily differentiated using the UV-Vis spectrophotometry method. Thus inclusion of pigs in this study, may have affected the overall significance of these results due to their close similarity in spectrophotometric measurements to that of humans. Therefore, further work in this project must include other animals, such as pigs, for comparison purposes.

Overall, the second part of this study demonstrated that the Brentamine FB test combined with the objective measurement capability of the UV-Vis spectrophotometer allowed for significant differentiation of human and animal AP activity, which was superior to that of the traditional testing method, whereby only ostriches and rams did not react within the 65 second cut off time. These results need to be reproduced on a larger cohort, also consisting of more species, and the methods used still require extensive validation prior to use in the forensic field. However, these results have revealed that this method of objective measurement may be useful for the forensic community when faced with questions surrounding the specificity of semen.

However, new methods involving DNA typing of seminal stains are also being developed which may reduce the need to develop a UV-Vis based test such as this one. Some of these molecular methods can be used to identify if semen is present and if it is of human origin. With technology progressing, new techniques have been introduced for body fluid identification. Novel and more sensitive methods are under development that allow only small amounts of sample to be used for processing while not destroying the sample. Messenger RNA (mRNA) marker profiling is a relatively new technique that is currently been validated as a tool that can be used to identify body fluids (Haas *et al.*, 2009; Lennard *et al.*, 2012). This technique relies on the expression of mRNAs to identify body fluids and attempt to find unique expressions that can be found for each body fluid. Lennard *et al.* attempted to find markers for blood, menstrual blood, saliva, semen and vaginal secretions (Lennard *et al.*, 2012). Different sections of the genes were targeted within the mRNA to attempt and express the markers for the different body fluids. However, encounters with cross reactivity and co-expression of markers occurred between the certain fluids were problematic, such as the identification and co-expression between menstrual and semen markers.

The authors stated that this novel technology can play a critical role in the forensic science field for body fluid identification, but there was no consensus as to where and what mRNA

markers can be used for this area of forensic science. The authors concluded that much more research needs to be studied in this new area of technology (Lennard *et al.*, 2012).

DNA methylation profiling is another emerging area of study that is being applied in body fluid identification. This allows the ability to distinguish between different types of body fluids with high specificity using existing Short Tandem Repeats (STRs) procedures already in place for DNA profiling (Lee, Park and Choi, 2012; Park *et al.*, 2014). DNA methylation involves epigenetics that play a part in gene regulation and development. The principle of this technique looks at the different methylation patterns for the different tissue or cell types. These can then aid profiling to identify certain characteristics of an individual based on small amounts of DNA, such as age determination and differentiation between monozygotic twins. (Vidaki, Daniel and Court, 2013; Kayser and Vidaki, 2017).

Another study looked at in what manner aging may affect the methylation of tissue-specific DNA methylated regions (tDMRs) in forensic samples, particularly semen. The results found that a small number of semen samples made interference in its profiles that made it uncertain of what body fluid type it was (An, Choi and Shin, 2013). The authors stated that there is promise for the use of this method, however validation towards its use in body fluid identification still needs to be done (An, Choi and Shin, 2013). In later years the markers for body fluids: venous blood, saliva, vaginal fluid, menstrual blood, and semen were more defined in terms of its distinguishability in complex mixtures (Forat *et al.*, 2016; Lee *et al.*, 2016). Both studies identified the markers for each body fluid to have specific methylation patterns that can be identified to be of a single body fluids origin. CpG markers that have potential to facilitate identification of specific body fluids were tested, however not validated for use yet (Forat *et al.*, 2016; Lee *et al.*, 2016).

Automated systems are also being used to detect body fluids using thermocycling instruments with fluorescence detection capabilities. However, thorough validation and optimisation is needed for the performance parameters of new technology along with its methods (Young, Moore and Bishop, 2017).

While these assays also hold promise to increase the specificity of semen identification methods, molecular methods require specialised laboratories and personnel. The UV-Vis spectrophotometry method suggested here only requires one instrument and is easy to operate and relatively straight forward to interpret by personnel. All methods would of course require extensive validation, and could perhaps complement one another in a forensic workflow.

4.3. Sperm morphology

The purpose of the third part of the study was to create baseline measurements for the dimensions of human and animal spermatozoa and to assess if there were any differences between human and animal spermatozoa using the H&E stain (Figure 7 and 8)

The morphological dimensions under the microscope were measured according to: head (Figure 8 A and B), tail (Figure 8 C) and the whole spermatozoa cell (Figure 8D). Differences in terms of shape were found between human semen and each of the other four different animal species, as described in Chapter 3, section 3.3. Differences in measurements were observed between human and (i) dogs, (ii) ram and (iii) ostriches ($p<0.05$, for each combination) but not for horses ($p>0.05$), which was in accordance with what has previously been reported in literature (Dott, 1975; Katz *et al.*, 1986; Soley, 1993; Soley and Roberts, 1994; M.Dahlbom, M.Andersson, M.Vierula, 1997; Pesch and Bergmann, 2006; Brito, 2007; van der Horst and Maree, 2009; Soler and Esteso, 2010).

However, the studies of Katz *et al.* and van der Horst *et al,* used different staining methods of Papanicolaou and Sperm Blue® respectively. Both studies looked at human, ram, and horse (Katz *et al.*, 1986; van der Horst and Maree, 2009). Pesch *et al.* investigated dogs as well for comparison to both humans and farm animals using H&E staining (Pesch and Bergmann, 2006). The findings from this study were similar to this reported literature with regards to the head and tail dimensions measured under light microscopy.

Ostrich semen dimensions measured in a study using scanning electron microscopy and transmission electron microscopy, however the dimensions in our study were different to the reported literature, with exception of the head length (Soley, 1993; Soley and Roberts, 1994). Reasons for the difference of the measurements can be due to the instrument used to view the spermatozoa, the use of computer systems to analyse the morphometry, the difference in subspecies of ostrich bred in various farms across the country, or the quality of the ejaculate (Smith *et al.*, 2016).

Overall the study shows that human sperm morphology can be differentiated from animal sperm morphology to create a baseline of measurements for use in the forensic field (Dott, 1975; Soley, 1993; Soley and Roberts, 1994; Pesch and Bergmann, 2006; Brito, 2007; Soler and Esteso, 2010; Gadelha *et al.*, 2013). Statistical differences between the dimensions show

that measurement of spermatozoa could be incorporated into the confirmatory test for semen, for a microscopy based confirmatory test. Based on the results, human semen should have measurements within the following ranges: 2.80 – 3.59 µm for width of head, 3.95 – 5.50 µm for head length, 23.85 – 34.20 µm for tail lengths and 30.10 – 45.70 µm for the entire cell. In future work, these morphology results could possibly be used to generate a more complex statistical model, which could perhaps combine the morphology and reaction rate results to predict an integrated probability of a semen sample belonging to a human.

4.4. Limitations and future developments

The relatively small cohort limited the statistical power of the results and may have contributed to false positive and/or false negative findings in this study. To account for false positives, post-hoc tests to correct for multiple testing was performed. However, the trends found in this study must be assessed in future studies, by using more human samples, along with more species as well as more samples per species. It would be of particular interest to include animals with an AP activity closer to that of humans, such as pigs, in order to test the hypotheses which have stemmed from this project.

In any sample set of this nature, some samples will be of low volumes of ejaculate which can limit the amount of replicated testing of samples. There was also a lack of availability of horse and dog semen, particularly for the sampling of horse semen, due to breeding seasons not being within the time frame of the study. To this end the study should be conducted over a longer time period to improve chances of recruiting animals during their respective breeding season.

Future studies should build upon this study, for example to investigate the influence of the extender on the enzyme quality and quantity in ostrich semen and expansion of the baseline for presumptive testing. More complex statistical modelling should also be explored when the sample size is sufficiently powered. Validation of this method also need to be performed in order to be able to use it in the forensic field.

5. Conclusion

In this study, we investigated the specificity of the Brentamine FB test for presumptive testing of semen as well as objectively measure differences in the enzyme kinetics of human and

animal AP using a novel UV-Vis spectrophotometric approach. Confirmatory testing was performed to find differences in the morphology and create a baseline for measurements.

Differences were found between human and animal semen samples regarding presumptive and confirmatory tests. Aged semen that was stored for 20 months was also tested and compared to fresh human semen. Testing with the conventional in-house Brentamine FB test revealed that dog and horse semen reacted within the 65 second cut off time set by the SAPS Forensic Science Laboratories, again demonstrating the poor specificity of this presumptive test.

Further tests to explore the specificity of the Brentamine FB test was achieved using the UV-Vis spectrophotometry method to ascertain the enzyme activity between humans and the four different animal species. We found differences in activity of the AP enzyme that allowed us to distinguish human and animal species, as well as between fresh and aged semen that may aid in forensic casework. This study provides further support for this proof of concept that there are differences in enzyme activity between semen from human and the four different animal species, which are often found living near humans in South Africa.

Confirmatory testing also showed differences between human and the four different animal spermatozoa according to their head, tail and whole cell measurements. The measurements recorded in the study provide baseline data that could be used for comparison purposes in future forensic casework.

Further studies should build on this study with a focus on increasing sample size, inclusion of more species and building an integrated statistical model to predict the probability of semen originating from humans as opposed to other animals. Such a low-cost method would be of value to the forensic community, particularly when faced with challenges regarding specificity of semen. Overall, these results have contributed towards much needed research in South Africa regarding the identification of semen, which remains an integral part of sexual offense investigation.

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Appendix A:

Table consisting of the components of the ostrich retardant used to preserve the ostrich semen (Smith, 2016)

Table 2: Compounds contributing to the ostrich diluent adapted for macro minerals.

Component	**Empirical formula**	**Molecular Weight**	**OS1 (g/L)**
D-Glucose	$C_6H_{12}O_6$	180.16	10.00
Magnesium acetate ($4H_20$)	$(CH_3COO)_2Mg \times 4H_20$	214.46	0.18
Tripotassium citrate (H_20)	$K_3C_6H_5O_7$	324.40	4.30
Sodium glutamate (H_20)	$C_5H_8NNaO_4H_20$	187.13	16.00
Sodium Phosphate dibasic (DiSodium anhydrous)	Na_2HPO_4	142.01	0.60
TES	$C_6H_{15}NO_6S$	229.25	4.59
Calcium chloride	$CaCl_2$	110.98	0.33
Sodium Selenite	Na_2O_3Se	172.94	-
Zinc Chloride	$ZnCl_2$	136.30	-

Appendix B:

INFORMATION FORM and INFORMED CONSENT

Project Title: **Evaluation of the specificity of the acid phosphatase test to identify semen**
Researcher: Robyn Conrad
Supervisor: Laura Heathfield
Co-supervisor: Lyle Curry

We would like to invite you to participate in a research study that involves forensic presumptive testing for semen, using the acid phosphatase test. The recruitment of this study will end on the 1 September 2017.

The decision to participate is entirely your own. If you decide not to participate in the study this will not disadvantage you in any way. There is no obligation on you to participate in this study. In addition, at any point during the study you are free to withdraw without having to provide any reason for this, and with no consequences to you. There is no monetary incentive for this study.

Background:

Presumptive testing of stains at the crime scene or in the forensic lab are quick methods of possible identification of the body fluid responsible for the stain. Forensic scientists use the acid phosphatase test for the presumptive identification of semen. Acid phosphatase (AP) is an enzyme that is found in high concentrations in semen. The test for AP involves treating a stain with a mixture of reagents and if the stain is positive for AP the stain will turn purple. In the Forensic Science Laboratory in South Africa, the cut off time for a positive reaction for the AP test is 65 seconds, which in the eyes of the forensic analysts, indicates the possible presence of human semen. This cut off time however, has not be tested in the lab, nor has the AP test been compared to semen samples from animals. The aim of this study is to therefore evaluate exactly how long the AP test takes to presumptively identify semen and to investigate how specific this test is to humans.

The study will involve collecting semen from male volunteers and various animals. The donated semen samples will be tested to investigate how specific the AP test is on human semen when compared to animal semen and to determine the reaction kinetics of the AP enzyme in semen stains. This will allow us to better determine the cut off time for the AP test, to better inform forensic labs on what the cut off time should actually be. No DNA analysis will be carried out on the semen samples.

What we need from you:

To participate, you will need to donate one semen sample. You will be required to masturbate and ejaculate into the sample jar and record the time at which this was done. This can be done in the comfort of your own home. There will be no risk your health.

The donation must be done in the morning to ensure a fresh sample to be used in the study. The sealed specimen jar with ejaculate must be returned to the researcher as soon as possible after collection. If there is any time delay between sample collection and handing it to the researcher, the sample must be kept in the fridge.

You will be required to remain abstinent for 36 hours before making a donation, that is no ejaculations 36 hours before the donation. You will also be required to keep the semen from making any contact with any other bodily fluids. The samples will be anonymised and coded, and stored in a 4° C fridge within an access controlled laboratory in the Division of Forensic Medicine and Toxicology until the project is complete. If you should decide to withdraw from the study at any time, your samples will be destroyed.

All information about you will be kept strictly private and confidential. You will not be given the results of any tests done.

Contact details:

This study has gained ethics approval from the Faculty of Health Science Human Research Ethics Committee (HREC 502/2015). The UCT's Faculty of Health Sciences Human Research

Ethics Committee can be contacted on 021 406 6338 in case participants have any questions regarding their rights and welfare as research subjects on this study. You may also contact the study supervisor Ms Laura Heathfield at telephone 021 406 6569 or email: laura.heathfield@uct.ac.za.

Please read the sentences on the accompanying consent form and then tick either the yes or no box.

Thank you for your participation and your valuable time.

INFORMED CONSENT

I, _____ (full name), do willing state:

	Yes	No
I have read the above / the above has been read to me, and I understand what this study entails.		
I have had the opportunity to discuss the study and my questions have been answered to my satisfaction.		
I know what is required of me, and I understand and accept the requirements.		
I consent to participate in this study I understand that my consent is entirely voluntary.		
I understand that I may withdraw from the research study at any time without giving a reason and without any consequence to me whatsoever.		
I understand that if I withdraw from participation at any time, my samples will be destroyed.		

I understand the samples will be stored for the duration of the project in the Division of Forensic Medicine and Toxicology at the University of Cape Town. After the project, I would like (please tick one option):

- ☐ The samples to be stored for future research for any research project which is approved by the Faculty Health Science Human Research Ethics Committee.
- ☐ The samples to be stored for future research that stems directly from this research project and which is approved by the Faculty Health Science Human Research Ethics Committee.
- ☐ The samples to be destroyed.

If you sign this form, it means that you voluntarily give permission to participate in the study.

Participant:

Print Name: …….

Signature: ……..…….……

Date: |__|__|/|__|__|/|__|__|__|__|

Person obtaining consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer:

Print Name: ……....

Signature: …….……

Date: |__|__|/|__|__|/|__|__|__|__|

Witness

Print Name: ……....

Signature: …….……

Date: |__|__|/|__|__|/|__|__|__|__|

PARTICIPANT QUESTIONNAIRE*:*

Date of birth: .....

	YES	NO
To the best of your knowledge, do you have any sexually transmitted infection or diseases?		
Have you had a vasectomy?		
Are you willing to donate semen more than once?		

Date & time of first donation: |__|__|/|__|__|/|__|__|__|__| Time:

Appendix C:

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

14 February 2017

HREC REF: 502/2015

Miss Laura Heathfield
Forensic Medicine & Toxicology
Falmouth Building
Entrance 3, level 1

Dear Ms Heathfield

PROJECT TITLE: THE EVALUATION OF TESTS FOR THE IDENTIFICATION OF SEMEN

Thank you for submitting the attached fhs007 form to the Faculty of Health Sciences Human Research Ethics Committee.

The HREC acknowledge that the student, Robyn Conrad will also be involved in this study.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Form FHS007: Amendment – study staff

HREC office use only (FWA00001637; IRB00001938)			
☑ Approved			
This serves as notification that all changes to the study staff and documentation described below are approved.			
Chairperson of the HREC signature		Date	21/9/2016

Principal investigator to complete the following:

1. Protocol information

Date (when submitting this form)	2016/09/27
HREC REF Number	502/2015
Protocol title	The evaluation of tests for the identification of semen
Protocol number (if applicable)	
Principal Investigator	Laura Heathfield
Department / Office Internal Mail Address	Reception; Division of Forensic Medicine and Toxicology Falmouth building (entrance 3, level 1); Faculty of Health Sciences Anzio Road, Observatory

1.1 Does this protocol receive US Federal funding?	☐ Yes	☐✓ No

2.1 Staff changes (tick ✓)

Are new personnel being added to this research?	☐✓ Yes	☐ No
Are current personnel being removed from this research?	☐ Yes	☐✓ No
Is the principal investigator for this research being changed? If yes, please attach revised conflict of interest and PI declaration statements. (Refer: sections 7 and 8.3 in the New Protocol Application Form - FHS013)	☐ Yes	☐✓ No
Do the consent and assent forms need modification to reflect these staff changes? If yes, please attach copies of the revised forms, with all changes highlighted or tracked and listed in the documents for approval.	☐ Yes	☐✓ No

2.2 Amended study staff details

Title, first name, surname	Department/Division	E-mail	Role of new staff member
Miss Robyn Conrad	Pathology/Forensic Medicine and Toxicology	robynconrad26@gmail.com	Recruitment of participants; continuation of laboratory work; data analysis

3. List of documentation for approval

Please list below all staff documentation such as CVs, declarations, GCP certificates and revised consent forms which need approval. This information must correspond to all 'yes' answers in 2.1 above. This form will be signed and returned to the PI as notification of approval. Please add extra pages if necessary.

Miss Robyn Conrad is a second year Masters student who will be doing her minor dissertation in 2017. The purpose of this submission is to add her name to the study.

This study took longer than anticipated, due to the complex optimisation period and parallel collection of animal samples (reviewed by animal ethics committee). This project was initiated by one Masters student in 2015/2016, doing his minor dissertation. The remainder of this study will be continued by a second Masters student in 2017.

The protocol itself will not be amended; the informed consent form will stay the same, and the methods used will stay the same. Robyn Conrad will be recruiting participants and carrying our laboratory work and data analysis as described in the original proposal, in order to complete this study, which ended up being too large for one minor dissertation for one student.

4. Signature

My signature certifies that I will maintain the anonymity and/ or confidentiality of information collected in this research. If at any time I want to share or re-use the information for purposes other than those disclosed in the original approval, I will seek further approval from the HREC.

Signature of PI		Date	27/09/2016

Appendix D:

Table 3: Median measurements of the head, tail, and entire spermatozoa for human, horse, dog, ostrich and ram, with standard deviation.

	Head		Tail (µm)	Whole (µm)
	Width (µm)	**Length (µm)**		
Human (n=5)	3.21± 0.28	4.72 ± 0.51	32.41± 5.99	37.50 ± 6.25
Horse (n=5)	5.02 ± 4.44	5.39 ± 0.518	34.73 ± 3.75	37.28 ± 7.15
Dog (n=11)	4.38 ± 2.75	6.21 ± 0.43	28.59 ± 5.98	42.43 ± 3.72
Ostrich (n=9)	2.10 ± 0.13	14.60 ± 0.97	28.77 ± 12.23	45.05 ± 1.79
Ram (n=13)	4.75 ± 0.98	8.07 ± 1.75	48.67 ± 1.71	57.22 ± 1.59

Appendix E:

Table 4: Median reaction times (in seconds) of human and four different animal species subjected to the direct method of the Brentamine FB test at various dilutions.

Dilution/species	Human (fresh) (n=15)	Human (aged) (n=9)	Dog (n=9)	Horse (n=1)	Ostrich (n=9)	Ram (n=13)
Neat	3	2	39	18	87	103
1 in 4	11	5	83	44	141	146
1 in 10	20	8	121	68	176	202
1 in 40	26	20	226	134	NR	217
1 in 100	38	89	174	161	NR	269
1 in 400	84	98	NR	263	NR	271
1 in 1000	212	NR	184	408	NR	NR
1 in 2000	NR	NR	NR	NR	NR	NR
1 in 4000	NR	NR	NR	NR	195	NR

NR = No Reaction

Appendix F:

Table 5: Median reaction times (in seconds) of human and four different animal species subjected to the indirect method of the Brentamine FB test at various dilutions.

Dilution/species	Human (fresh) (n=15)	Human (aged) (n=9)	Dog (n=9)	Horse (n=1)	Ostrich (n=9)	Ram (n=13)
Neat	14.5	4	69	217	242	182
1 in 4	33.5	0	168	234	327	234
1 in 10	46	5	267	240	300	271
1 in 40	84	10	295	349.5	305	304
1 in 100	113	26	136	364.5	209	315
1 in 400	257	37	290	596.5	242	427
1 in 1000	NR	136	NR	NR	281	291
1 in 2000	NR	NR	NR	NR	242	578
1 in 4000	NR	NR	NR	NR	NR	NR

NR = No Reaction

Appendix G:

Calculations for the starting concentration of Brentamine FB reagent:

Concentration in solution A:

$$\text{Concentration} = \frac{\text{mass (grams)}}{\text{molecular weight} \times \text{volume (liters)}}$$

Mass of Brentamine FB salt (o-dianisidine): 0.4 grams

Molecular weight: 475.47 g/mol

Volume: 0.002 liters

$$\text{Concentration} = \frac{0.4}{475.47 \times 0.002}$$

Concentration (solution A) = 0.421 mol/L

Concentration in working reagent:

$C_1V_1 = C_2V_2$

C_1 = concentration in solution A

V_1 = volume of solution A

C_2 = concentration of Brentamine FB in working reagent

V_2 = volume of working reagent

$C_1V_1 = C_2V_2$

$(0.421)(0.002) = C_2 \times 0.0089$

$$C_2 = \frac{(0.421)(0.002)}{0.0089}$$

Concentration of Brentamine FB in working reagent = 0.094 mol/L