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(54) Title: ASSAYS

(57) Abstract: A DNA profiling method which comprises (i) contacting a sample believed to comprise spermatozoa and/or epithelial cells with antibodies specific for antigens presented on the spermatozoa and/or epithelial cells which antigens are not readily degraded, so as to form antibody-spermatozoa and/or antibody-epithelial cell complexes, (ii) separating the complex(es) from other material in the sample, (iii) isolating DNA from the complex(es) and (iv) determining the specific characteristics of the isolated DNA.

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ASSAYS

The present invention relates to improvements in forensic science and in particular to analytical methods for determining the identity of DNA from mixed samples.

5 The forensic biologist is mainly concerned with identifying trace evidence such as blood, semen, saliva and hairs found at the scene of crimes such as murder, rape and assault. DNA profiling has provided a means of identifying the source of such material with a very high degree of certainty. The possibility of a chance match between samples is often presented in court as "less than 1 in a 1000 million", although in reality it can be even rarer. More
10 importantly it is possible to conclusively exclude a suspect as the possible source of a body fluid thus eliminating them from a police inquiry.

Most of the DNA profiling methods in use today use the high degree of variability in tandem repeat regions of the human genome. In these regions, a core sequence is repeated many times, laid end to end. The exact number of repeats varies greatly between people and
15 consequently the length of the minisatellite region containing the repeats are different in different people. By analysing a series of different tandem repeat loci it possible to generate a DNA profile which is highly specific.

Many forensic samples contain a mixture of body fluids from different individuals. This is commonly found in sexual assault cases where vaginal swabs taken from the victim
20 will have a mixture of semen and vaginal epithelia present. To simplify the analysis it is very useful to separate the different cell types and obtain a separate DNA profile for the semen.

Currently the only practical method available for isolation of DNA from mixtures of spermatozoa and vaginal epithelial cells is based on differential (preferential) lysis. This method relies on the greater resistance of sperm heads to digestion over other cellular
25 material. The cellular material is extracted from the sample eg. swab into an extraction buffer. The preparation then undergoes relatively mild digestion to lyse cellular material other than sperm. Centrifugation isolates the intact sperm from the other digested cellular components. The sperm then undergo a more aggressive digestion with a long incubation with Proteinase K and dithiothreitol (DTT) to release the DNA.

30 The above method can be inefficient and often does not produce complete separations. Subsequent typing of genetic markers will reveal three or four alleles rather than the expected one or two that would result from complete separation of the cells within the mixture.

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The need therefore exists for further improved methods.

We have now devised novel analytical methods which use immuno-capture of male and/or female genetic material from a mixed sample followed by isolation and analysis of the DNA comprised in the male and/or female genetic material.

5 Therefore in a first aspect of the present invention we provide a DNA profiling method which comprises (i) contacting a sample believed to comprise spermatozoa and/or epithelial cells with antibodies specific for antigens presented on the spermatozoa and/or epithelial cells which antigens are not readily degraded, so as to form antibody-spermatozoa and/or antibody-epithelial cell complexes, (ii) separating the complex(es) from other material in the sample,
10 (iii) isolating DNA from the complex(es) and (iv) determining the specific characteristics of the isolated DNA.

The development of the above method allows efficient separation of spermatozoa from epithelial cells prior to DNA characterisation, more easily interpretable typing patterns and the successful individualisation of the spermatozoa and epithelial DNA.

15 By "not readily degraded" we mean that the antigens can be specifically recognised by the antibodies used in the analytical methods of the invention. That is to say the (target) antigens have not been adversely affected at any time prior to or during their use in the methods of the invention.

20 By "other material in the sample" we include uncomplexed cellular material and other DNA as may be present in the sample.

By "specific characteristics of the isolated DNA" we include all characteristics of the DNA and nucleotide sequence(s) therein, such as STRs or VNTRs as may be used to determine the identity of the isolated DNA.

25 The epithelial cells are generally female epithelial cells and usually vaginal epithelial cells.

Any convenient DNA profiling method may be used. These include methods such as short tandem repeat (STR) profiling or profiling using multilocus or single locus probes such as disclosed in European patents nos. 186271 and 238329 respectively. Alternatively the minisatellite variant repeat (MVR) profiling method disclosed in European patent no. 530009
30 may be used. Convenient and preferred methods include the use of commercially available STR kits such as Profiler Plus™ and PowerPlex16™ designed to fulfil the requirements of, for example, the US and European courts.

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Any convenient antibodies may be used provided they are specific for their target antigens. A particular antigen is the FA-2 antigen, this is a sperm specific protein to which monoclonal antibodies (Vic-1) have been raised. (R.K. Naz et al. (1993) *Biol. Reprod.*, 49, 1236-1244). The Vic-1 monoclonal antibody completely blocks human SPA (Sperm Penetration Assay of zona free hamster oocytes), and reduces acrosome reaction and release of acrosin activity. Binding of the antibody does not affect motility of the sperm. Alternatively, the antibody S19 (target epitope SAGA-1) (A.B. Diekman *et al.* (1999) *FASEB J.* 13, 1303-1313) may be used to bind to all surfaces of the human sperm.

Separation of the antibody-spermatozoa and/or antibody-endothelial cell complexes from other DNA in the sample may be achieved by any convenient technique. These include agglutination and solid phase capture methods. By way of non-limiting example a particular complex may be targeted and captured by antibodies bound directly or indirectly to a solid phase. The solid phase may for example be part of a reaction vessel such as a PCR reaction vessel or it may be a bead or particle such as a magnetic bead.

Alternatively sperm heads may be induced to agglutinate through use of, for example an IgG antibody targeted against the antigen and an IgM antibody raised against the invariant portion of the IgG antibody. The complex formed may be harvested by centrifugation or filtration.

The use of magnetic beads to separate complexed material is a preferred aspect of the present invention. The use of such beads allows for example complexed material to be concentrated or drawn closer to an immobilised secondary antibody.

In a particular aspect of the invention we provide a DNA profiling method which comprises (i) contacting a sample believed to comprise both spermatozoa and epithelial cells with antibodies specific for antigens presented on the spermatozoa and epithelial cells respectively which antigens are not readily degraded, so as to form antibody-spermatozoa and antibody-epithelial cell complexes respectively, (ii) separating the complexes from other material in the sample, (iii) isolating DNA from one or both complexes and (iv) determining the specific characteristics of the isolated DNA.

The invention will now be illustrated but not limited by reference to the following specific description, Example and Figures wherein:

Figure 1 shows an intact sperm complete with tail;

Figure 2 shows a discrete head alone;

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Figure 3 shows the situation where the acrosome at the tip of the sperm head has burst, releasing lytic activity;

Figure 4 shows a primary antibody interacting with the antigen, which in turn provides a binding site (biotin) for the Streptavidin coated magnetic particles;

5 Figure 5 shows a primary antibody interacting with the antigen, which in turn provides a binding site for the secondary antibody;

Figure 6 shows the primary antibody linked directly to a magnetic bead, simplifying the linking of the antigen and the magnetic bead;

10 Figure 7 shows the gel electrophoresis results of the DNA profiling method of the invention illustrated for convenience at a single genetic locus. Lane 1 is a control profiling reaction using blood taken from the male suspect, lane 2 is from spermatozoa from the male suspect, lane 3 is from epithelial material from the female victim and lane 4 is a control profiling reaction using blood from the female victim. It can be seen there is positive identification of the male and female DNA.

15

Specific description:

We describe here a novel technique for the separation of spermatozoa from a mixture of seminal and epithelial material. The method takes advantage of surface-presented antigens on the sperm head which are absent from other cell types.

20 Using (preferably) a high affinity monoclonal or (more likely to cause cross reactivity) a polyclonal serum, a primary antibody is applied to the cell mixture. The antibodies used as the primary will be either (a) biotinylated, or (b) of a species and sub-class, for which there is a commercially available antibody conjugated directly to a magnetic bead, or (c) linked directly to a solid support such as the wall of a tube, or a magnetic bead.

25 The primary antibody binds to the sperm antigen to form a stable conjugate. The conjugate is then retrieved from solution by means of (a) Streptavidin linked magnetic beads, or (b) magnetic beads coated with antibodies with affinity for the invariant portion of the primary antibody, or (c) directly harvested by removal of the supernatant of the tube, or harvest of the magnetic bead through an applied magnetic field.

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The Primary Antibody

Research based around the search for a male contraceptive pill has identified interference of sperm/oocyte fusion as being a promising mechanism. This is being achieved

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through identification of surface presented proteins which are absent from other cell types. Several such proteins, which have been implicated in sperm/egg fusion, have been identified and reported in the literature (Naz and Vanek. *Frontiers in Bioscience* 3, e39-48, April 30, 1998).

5 In order for a candidate antigen to be of maximal use, it must be present on the surface of the sperm head regardless of the state of preservation of the sperm head. If the swab is taken quickly after the offence and the sample stored under appropriate conditions, the sperm will be intact, complete with tails (Figure 1). If however the sampling is significantly delayed, the tails may separate, leaving discrete head alone (Figure 2). In extreme cases, it may be that
10 the acrosome at the tip of the sperm head will have burst, releasing lytic activity (Figure 3).

If a immunogenic species unique to the sperm, and is present on all the surfaces above, then it may be used as the target for the primary antibody. Alternatively, an antigen presented on the main body of the sperm head may be adequate.

The antigen is bound by the primary antibody which, in turn, provides a binding site
15 for the Streptavidin coated magnetic particles (Figure 4) or a secondary antibody bound to magnetic particles (Figure 5).

If the primary antibody is linked directly to a solid support such as a magnetic bead, the target antigen and magnetic bead are effectively linked directly (Figure 6).

20 Antigens

Published research lists a number of proteins which may be of use in immuno-contraception, and which may therefore be useful in the immuno-capture of sperm from a seminal/epithelial mixture. The most important step in the fertilisation process is binding of the sperm with the zona pellucida. Although important for a contraceptive vaccine, an unique
25 surface presented antigen of significant immunogenicity will be a candidate for development as an anchor site for immuno-selection of the sperm head.

Other antigens exist which may not have a direct role in binding of the sperm head to the zona pellucida, but which may be of potential use as sperm specific targets. One such antigen is the carbohydrate moiety of a glycoprotein, called SAGA-1 (Sperm Agglutination
30 Antigen-1).

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Magnetic Beads

Dynal UK produces microscopic, uniform, superparamagnetic, monodispersed polymer beads (Dynabeads™). The beads are available with a streptavidin coating for conjugation to biotinylated molecules. These beads may be linked to biotinylated antibodies raised against human semen. Alternatively, the beads may be purchased in an activated form which allows the direct coupling of the antibody to the bead. The antibody - bead complex will link to the sperm cells and a magnet is then used to isolate them from the other cell types present in a swab extract.

This system has the advantage over current methods of being more specific in isolating spermatozoa prior to DNA extraction. It also produces far cleaner DNA which is less likely to contain PCR inhibitors. Additionally the method requires fewer tube to tube transfers minimising sample loss, contamination and the possibility of operator error. These are all important considerations with forensic samples.

Procedure

We have purchased antibodies against pooled human semen which have been prepared in sheep. The manufacturers (Biogenesis) have purified this product and biotinylated it. This material is incubated with the streptavidin coated Dynal beads to produce the antibody - bead complex. An aliquot of the complex is added to mixed cell suspension which has been extracted from the vaginal swab. This is incubated for a short while to allow the bead - antibody complex to link on to spermatozoa in the suspension. The new bead-antibody-sperm complex is then pulled to the side of the tube using a magnetic concentrator (Dynal). The pellet is re-suspended in fresh buffer and washed several times to remove all traces of epithelial cells. The purified sperm component then undergoes digestion with proteinase K and DTT to liberate the DNA.

Example:**Characterisation of male and female DNA from a mixed forensic sample**

A post coital swab is taken from an alleged victim and treated as follows. Cellular material is extracted by vortexing the swab in phosphate buffered saline and the cells harvested by centrifugation. These are re-suspended in a small volume (300µl) of phosphate buffered saline and incubated with an excess of an anti-sperm antibody. The antibody-sperm

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complex formed is then targeted by an excess of the secondary antibody/ magnetic bead complex. (This may also be achieved by having a biotinylated primary antibody being bound by an excess of streptavidin coated magnetic beads). The complex is harvested magnetically and undergoes a series of washes to remove the unbound cellular material.

5 The complex is heated to 80°C to denature the antibodies and release the sperm heads. The DNA can then be extracted by a range of established methods. (Phenol/chloroform, Chelex 100 etc.).

STR profiling is then effected using a PE Profiler Plus™ (Profiler Plus Handbook) kit according to manufacturers instructions. The primers, buffer and thermostable DNA
10 polymerase (such as AmpliTaq Gold™) are added to the PCR tubes (as above). Conveniently 28 cycles of PCR amplification are effected using a 9700 thermal cycler (Perkin Elmer).

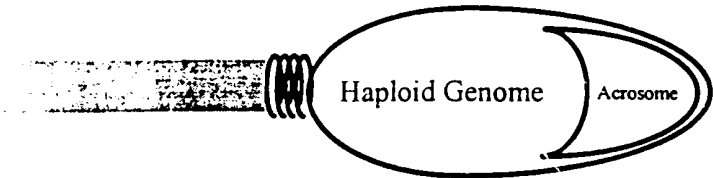
The resulting material is separated using gel electrophoresis and the results are shown in Figure 7 (for convenience these illustrate the results at one locus only). Lane 1 is a control profiling reaction using blood taken from the male suspect, lane 2 is from spermatazoa from
15 the male suspect, lane 3 is from epithelial material from the female victim and lane 4 is a control profiling reaction using blood from the female victim. It can be seen there is positive identification of the male and female DNA.

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Claims:

1. A DNA profiling method which comprises (i) contacting a sample believed to
comprise spermatozoa and/or epithelial cells with antibodies specific for antigens presented
5 on the spermatozoa and/or epithelial cells which antigens are not readily degraded, so as to
form antibody-spermatozoa and/or antibody-epithelial cell complexes, (ii) separating the
complex(es) from other material in the sample, (iii) isolating DNA from the complex(es) and
(iv) determining the specific characteristics of the isolated DNA.
- 10 2. A method as claimed in claim 1 wherein the antibodies are biotinylated for separation
in step (ii).
3. A method as claimed in claim 1 wherein the antibodies are conjugated to magnetic
beads for separation in step (ii).
- 15 4. A method as claimed in claim 1 wherein secondary antibodies are used for separation
in step (ii).
5. A DNA profiling method which comprises (i) contacting a sample believed to
20 comprise both spermatozoa and epithelial cells with antibodies specific for antigens presented
on the spermatozoa and epithelial cells respectively which antigens are not readily degraded,
so as to form antibody-spermatozoa and antibody-epithelial cell complexes respectively, (ii)
separating the complexes from other material in the sample, (iii) isolating DNA from one or
both complexes and (iv) determining the specific characteristics of the isolated DNA.
- 25 6. A method as claimed in any one of the previous claims wherein the antibodies for at
least one antigen are monoclonal.
7. A method as claimed in any one of the previous claims wherein the antibodies have
30 been raised to the FA2 antigen.
8. A method as claimed in any one of claims 1-6 wherein the antibodies have been raised
to the Sperm Agglutination Antigen-1 (SAGA-1).

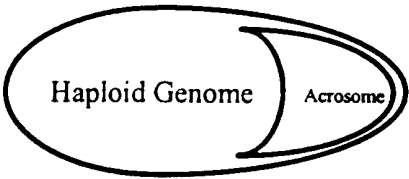
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FIGURE 1

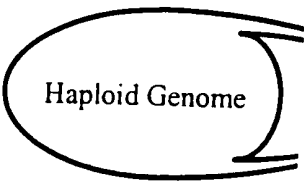
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FIGURE 2

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FIGURE 3

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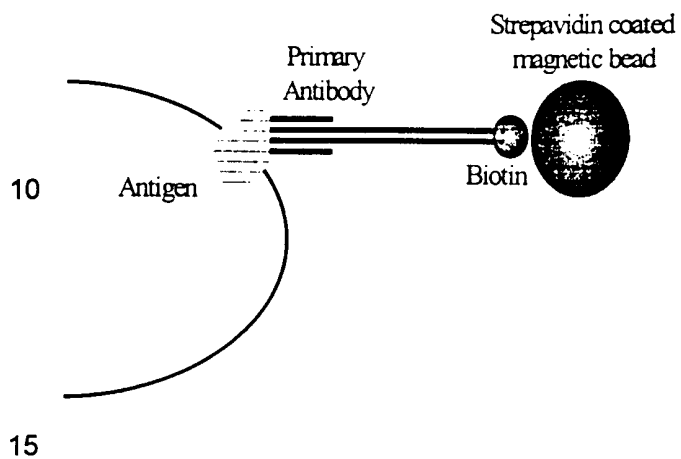


FIGURE 4

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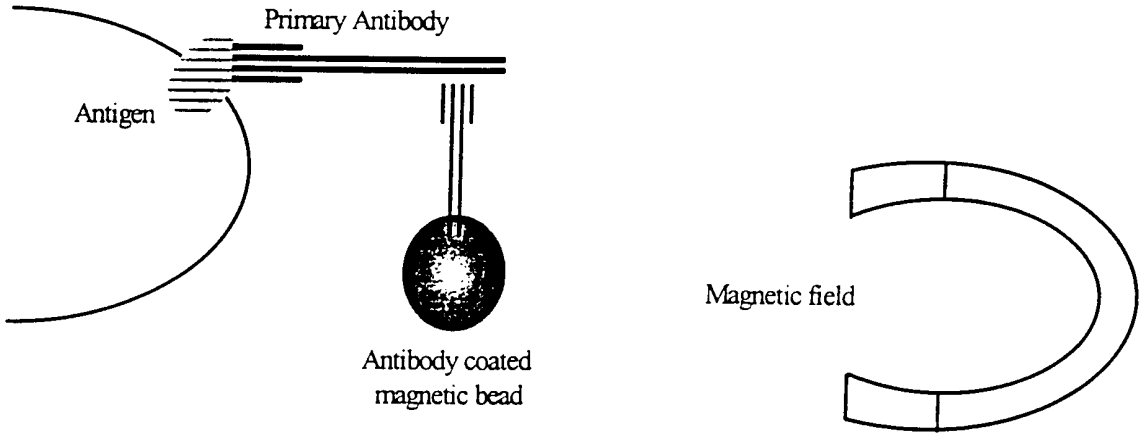


FIGURE 5

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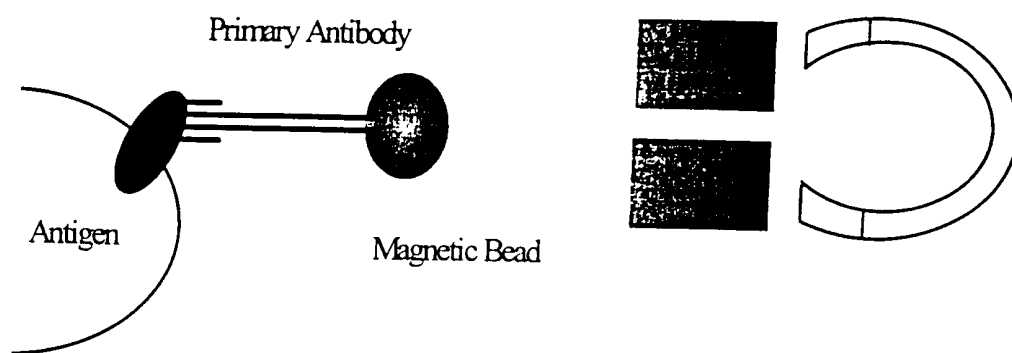


FIGURE 6

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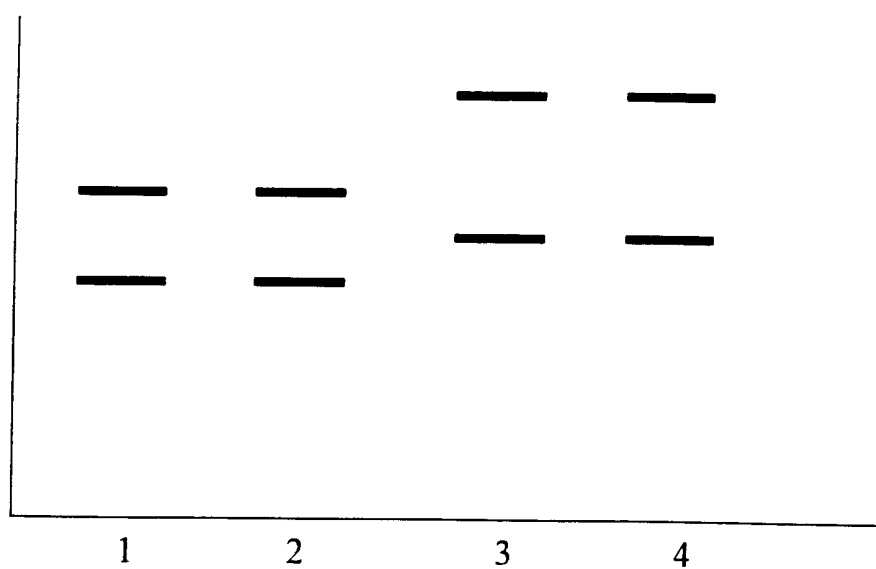


FIGURE 7