Rogue athletes and recombinant DNA technology: challenges for doping control

Hassan M. E. Azzazy* and Mai M. H. Mansour

DOI: 10.1039/b707495f

The quest for athletic excellence holds no limit for some athletes, and the advances in recombinant DNA technology have handed these athletes the ultimate doping weapons: recombinant proteins and gene doping. Some detection methods are now available for several recombinant proteins that are commercially available as pharmaceuticals and being abused by dopers. However, researchers are struggling to come up with efficient detection methods in preparation for the imminent threat of gene doping, expected in the 2008 Olympics. This Forum article presents the main detection strategies for recombinant proteins and the forthcoming detection strategies for gene doping as well as the prime analytical challenges facing them.

Introduction

The race between dopers and the efforts of sports authorities to develop effective doping detection methods has been ongoing for as long as competitive sport has existed. About 1.3–2% of athletes tested for doping have had positive doping results over the past few years.[1] The exponential progress in recombinant DNA technology has made the tedious task of doping detection in athletes even more challenging, with the development of a variety of recombinant proteins with performance-enhancing effects, as well as the development of gene therapy concepts into the new prohibited doping method – gene doping.[2] It is defined as the 'non-therapeutic use of cells, genes, genetic elements, or modulation of gene expression, having the capacity to enhance athletic performance' according to the prohibited list published by the World Anti-Doping Agency (WADA).[3] The core of the detection challenge for recombinant proteins abuse is that these proteins are basically the same as their endogenous counterparts and in case of gene doping, are produced by the athlete's own cells *via* the introduced gene.

It should, however, be clear that the difference in performance between normal athletes and gene dopers is most likely to be rather significant. The report on the cross-country Finnish skier who had a mutation in his erythropoietin (EPO) receptor closely resembles the expected scenario. The mutation has caused the skier to have a truncated EPO receptor that resulted in benign erythrocytosis.[4] The skier had higher levels of haemoglobin (>200 g L^{-1} compared with the mean normal of 154 g L^{-1}) and a remarkable increase in endurance compared with his peers. This led him to win three Olympic gold medals and two world championships.[4]

Recombinant versions of proteins such as EPO and human growth hormone (hGH) are currently being abused in sports,[5] while genes for insulin-like growth factor-I (IGF-I), peroxisome proliferator-activated receptor-delta (PPAR δ), and myostatin inhibitor are also perceived as prime gene doping candidates.[6] In light of their significant performance-enhancing potential, where recombinant EPO (rEPO) is a major endurance booster and recombinant hGH (rhGH) and beta human chorionic gonadotropin (bhCG) are strength enhancers, this Forum article will focus on the challenges faced in doping detection of these recombinant proteins whether they are introduced directly into athletes or produced by their own cells *via* gene doping. As for the other gene doping candidates, IGF-I plays a role in increasing muscle mass and strength as one of the action mediators of hGH. In the case of PPAR δ, increased expression would enhance endurance as it is associated with the formation of type I (slow-twitch) skeletal muscle and can induce their conversion from type II (fast-twitch) fibers. Type I and II muscles are determinants of endurance and speed, respectively. It is also very likely to have a role in body weight control. As for myostatin gene, a negative muscle mass regulator, its inhibition would lead to limited restriction of muscle growth and thus increased strength.[1,6]

Apart from the notion that the promises of gene doping may be too great a temptation for some athletes, there are several indicators that would justify the expectations that gene doping could soon be a reality, as soon as next year's Olympics. Over 3000 patients have received some sort of gene therapy to date with minimal side effects, excluding one fatality on account of an auto-immune response.[1] There have also been several successful trials for 'gene doping' in animal models. Also, 'gene doping' trials using animal models (few of these trials are funded by WADA) were also performed as part of the efforts for the development of detection methods. Table 1 presents some examples of successful studies of gene therapy and doping in animal models. The outcome of such studies is the main cause for concern over the imminent threat of gene doping.

Although there are general proposed strategies for the detection of gene

Department of Chemistry and Yousef Jameel Science & Technology Research Center, Sci Bldg # 310, The American University in Cairo, 113 Kasr El-Aini Street, Cairo 11511, Egypt. E-mail: hazzazy@aucegypt.edu; Fax: +2 022 795 7565; Tel: +2 022 797 5918

Table 1 Animal studies of gene therapy/doping

Target gene	Study	Animal model	Results	Ref.
EPO	Gene delivery using an adenovirus	Cynomolgus macaques (n = 9)	• Successful expression of fully functional EPO protein, whose serum levels were measured by ELISA • *Side effects*: 1 Severe immune response towards endogenous and new EPO 2 High increase in blood viscosity hampering normal blood flow and heart functioning	36
EPO	Gene delivery using an adenovirus introduced by intramuscular injection	Mice (n = 7) and monkeys (n = 2, each injected with different vector concentration)	• Increase in hematocrit by 49–81% (mice) and 40–70% (monkeys) • Effects lasted for 12 weeks in monkeys and for more than a year in mice • Elevated serum EPO level determined using radio-immunoassay	37
EPO	Hydrodynamic limb vein delivery of plasmid DNA into skeletal muscle	Rat anemia model (n = 10)	• Long-term expression for more than 450 days. The expression was also proportional to the dose of injected plasmid DNA. Serum EPO levels were measured using ELISA methods	11
PPAR δ	Gene injection into mice zygotes	Mice (n = 4, for each of the control and transgenic groups)	• Running time improved by 67% while the distance improved by 92% (as determined by running of mice on a treadmill; exhaustion endpoint was when the mice could not avoid repetitive electric shocks) • Resistance to obesity, even in lack of exercise and on fat-rich diet	38
IGF-I	Gene delivery using an adeno-associated viral vector	Rats (n = 24)	• 20–30% increase in muscle strength and mass and an increase in endurance. IGF-I expression level was measured using reverse-transcription PCR. Contractile properties of flexor hallucis longus muscle were measured *in situ*	39
hGH	Gene delivery using adeno-associated virus serotype 2 *via* ductal delivery to salivary glands	Male BALB/c mice (n = 5)	• A notable increase in salivary hGH levels of 1084 $\pm$ 102 pg mL^{-1} and serum levels of 151.5 $\pm$ 17.3 pg mL^{-1}, four weeks post-gene administration. Serum and saliva hGH levels were determined using a chemiluminescence immunoassay kit	40

doping, no established methods are available yet. By contrast, established methods are available for the detection of injected recombinant proteins such as EPO, the first recombinant hematopoietic growth factor which has been available commercially as a recombinant protein drug for over 20 years,[7] and hGH. These are two of the main substances of abuse in sports that will be addressed in this paper along with recombinant human chorionic gonadotropin (rhCG).

Gene doping

Methods

Gene doping is based on the introduction and subsequent expression of a target gene or by modulating the activity of an existing gene.[1,2,5] The target gene can be introduced into the athlete's body either *in vivo* or *ex vivo*. The latter case involves gene transfer to host cells in culture and re-introduction of the genetically-modified cells into the host. In case of *in vivo* gene doping the target gene is introduced into the human body by viral vectors, direct injection using a syringe or gene gun, or liposomes.[1,6]

The use of viral vectors has proven to be the most efficient method for gene therapy and/or gene doping.[6] 'Tamed' (less immunogenic) replication-deficient vectors derived from retroviruses, adenoviruses, or lentiviruses may be genetically engineered to deliver the gene of interest into the athlete's cells.

Potential detection strategies

A conclusive test for detecting gene doping does not exist to date,[1,2,5] but extensive research is currently being undertaken to investigate some promising strategies.

Structural difference of proteins encoded by transgenes. The first approach involves the detection of minor structural differences between the recombinant proteins expressed by the transgenes and their endogenous counterparts, possibly due to different post-translational modifications in different cells.[5,6] Monkeys doped by the EPO gene at the French National Doping Laboratories produced EPO that has a slightly different glycosylation pattern from the endogenous one.[5,8] Suitable antibodies may be developed for the respective EPOs using either classical hybridomas or recombinant antibody fragments generated by phage display technology.

Assessment of immune response to delivery vectors. Viral vectors are the most popular method for gene delivery and are most likely to be used for gene doping. Therefore, a highly promising approach would be to assess the immune response to viral vectors.

DNA microarrays and expression profiling. The third approach assesses the expression profile of endogenous genes that may be altered following the expression of a foreign gene.[1,3,6,7] This can be done using DNA microarrays. DNA microarray is a comparative technique for expression profiling, which was developed to compare the expression patterns of genes in diseased conditions, *e.g.* cancer, as compared with normal or healthy conditions.

First, mRNA is extracted from blood mononuclear cells from diseased and healthy samples, and then reverse-transcribed into complementary DNA (cDNA). During this process, a radioactive label or a fluorescent tag is incorporated in the cDNA, where a different tag is chosen for each condition, *e.g.* a green tag for cDNA from the healthy sample and a red tag for the diseased one. The basis of the microarray operation is that the synthesized cDNA

binds to complementary oligonucleotides (probes) immobilized on a glass or silicon solid support,[9,10] commonly referred to as a chip. The chip has numerous spots, each containing a particular oligonucleotide sequence.[9,10] A laser is then used to scan the chip to visualize the fluorescent signal given by the cDNA bound to the complementary probes. The color of the signals obtained from the spots indicates whether a specific gene is up- or down-regulated or remains at normal expression levels in the diseased *versus* healthy condition, and the quantification of the gene expression is determined by evaluation of the fluorescence intensity. The results obtained from the DNA microarray are typically confirmed using real-time PCR.[9,10]

Based on this approach, particular gene chips could be developed for the suspected targets for gene doping, and would compare doping *versus* non-doping conditions instead of diseased and healthy states. The chip would contain probes for the target doping gene as well as the genes associated with its expression. This way the chip would assess the expression of the target gene itself as well its expression signature. For example, an EPO gene doping chip can be developed based on the knowledge that exposure of erythroid progenitor cells to EPO results in the up-regulation of 54 genes and down-regulation of 36 other genes.[7] When a reference expression signature is established, the altered expression patterns of these genes along with that of the EPO gene can be used as an indirect detection method for EPO gene doping.

DNA barcodes. Finally, DNA barcodes can be used to distinguish a particular transgene from other transgenes and its endogenous counterpart. A unique short oligonucleotide sequence could be added to each transgene and/or viral vectors that could be used to identify their respective manufacturers.[6,7] The barcode can be detected by conventional molecular detection methods such as PCR. Such barcodes are to be used by companies supplying genes and gene therapy vectors for expression in mammalian systems, both for research purposes and commercial use. However, the application of this approach is a logistical challenge more than an analytical one, as it will require the cooperation of all stakeholders for constructing a database of barcodes that would facilitate the tracking of transgenes and expression vectors, and implementing the barcodes, as well as bearing the financial cost of the process.

Challenges for detection

With regard to the various gene doping strategies, the efficiency of the detection of structural differences between the endogenous protein and the one encoded by the transgene varies with the target protein. For example, some proteins expressed by the transgene are generated intracellularly and do not show up in blood or urine. This is particularly observed with muscle-enhancing genes such as IGF-I, which is produced in muscle.[1,5]

In the case of assessing the immune response to viral vectors, there is a possibility that the tested athlete could have been infected by the virus *via* non-doping routes and therefore the detection of antibodies in his/her blood will not provide conclusive evidence of doping. Also, viral vectors used in gene therapy trials have been genetically modified to minimize the host immune response.[6] Detection of the transgenes themselves would require an invasive biopsy and is thus impossible to use in a sports setting.[1] On top of that, there are some non-viral gene delivery methods, *e.g.* hydrodynamic limb vein (HLV) technology, that are being developed for gene therapy uses[11] and which may not illicit a detectable immune response. The HLV approach involves the rapid injection of a large volume of DNA-containing fluid, in the direction of blood flow, through a peripheral vein of a limb that is temporarily isolated from the normal flow using a tourniquet. The vein use allows gene access to all muscle compartments in the limb and the HLV approach has been demonstrated as being quick, safe, and well-tolerated for repeated delivery.[11]

Experiments using DNA microarrays suffer from a lack of standardized quality controls and reference methods.[12] In an effort towards resolving this issue, and to enhance the reproducibility and reliability of data obtained using DNA microarrays, the National Institute of Standards and Technology has initiated the process of defining universal RNA standards.[9]

The development of a reference database for different genetic and expression profiles that change following doping as well as establishing corresponding normal values would be the major difficulty facing the microarray and expression profiling strategy. Nevertheless, the WADA is initiating a project known as the Athlete Passport which has one of its aims to gather individual athlete testing data, including haematological data, which, over time, would act as a baseline individual reference.[3] It may be possible to expand this concept later to include expression profiles related to suspect doping genes.

Recombinant proteins

1. Erythropoietin (EPO)

EPO is used as a drug to treat anemia associated with specific disease conditions such as renal failure and cancer.[11] It is a glycoprotein hormone with a 40% carbohydrate content which attributes to the hormone's glycosylation micro-heterogeneity. Glycosylation of EPO is species- and tissue-specific and is critical for its biological activity. Recombinant and endogenous EPO isoforms have different glycosylation patterns.[7] The normal range of EPO in urine is 0–3.7 IU L^{-1}.[13] rEPO is used as an ergogenic aid to enhance endurance with 7–8% improvement in performance with rEPO doping as compared with normal endurance training.[7,14] Several isoforms of rEPO exist, *e.g.* rEPO α, and β,[3,7] and are detectable with current methods.

Detection methods.

Indirect detection methods. It is possible to detect the abuse of rEPO by measuring five hematopoietic parameters: concentration of serum EPO, hematocrit level, percentage of reticulocytes, percentage of macrocytes, and concentration of serum soluble transferrin receptors (sTfr).[7,15] Two models were developed based on the behavior of each of the five parameters during and after controlled treatment with rEPO: the 'ON' model and the 'OFF' model.[7] For the 'ON' model: 'ON' score = haemoglobin + 9.74 ln(EPO); **or** haemoglobin + 6.62 ln(EPO) + 19.4 ln(sTfr).

For the 'OFF' model: OFF score = haemoglobin − 60(reticulocyte percentage)$^{1/2}$; **or** haemoglobin − 50(reticulocyte percentage)$^{1/2}$ − 7 ln(EPO).[16] Each of these models is most effective within a particular timeframe of rEPO abuse. The 'ON' model is applied during or shortly after rEPO treatment, whereas the 'OFF' model is used for detection several weeks after having discontinued EPO administration.[17] However, this indirect method is not accepted by WADA as a decisive criterion for EPO misuse.

The proposed concept of the athletes' Hematologic Passport would provide baseline data on hematologic parameters of individual athletes.[14] In practice this would help better interpretation of results generated by different detection techniques and could be part of the WADA's Athlete's Passport project.

Direct detection methods. In practical application, the indirect method is more of a screening method. If the five hematopoietic parameters are unusual, rEPO is directly detected in urine samples by a technique that involves isoelectric focusing (IEF), double immunoblotting and chemiluminescent detection.[3,18] This urine test, which takes up to three days to generate results, is the one that is approved by WADA to establish a positive result for EPO abuse; the indirect detection results hence do not suffice for an official result of positive EPO abuse.

The basis of the direct method for detection is the charge difference between different endogenous EPO isoforms and rEPO.[19,20] After the IEF is performed and the gel visualized, an endogenous EPO band appears in the more acidic region of the gel, while rEPO appears in the basic region.[19,20] Initially a cut-off of 80%, or sometimes 86%, of bands being in the basic region was used as a positive criterion of the IEF result, but the use of a defined value was found to be inappropriate.[19] Later, the criteria changed to using the ratio of bands in the acidic and basic areas. The current positivity criteria set by the WADA require that at least two out of three of the most intense bands, measured by densitometry, are in the basic region.[3,20]

Capillary zone electrophoresis also has the potential for detection of rEPO and is based on the different glycosylation patterns (mainly the difference in sialic acid groups) between endogenous and recombinant EPO.[21] The coupling of capillary electrophoresis with electrospray ionization mass spectrometry has shown good utility in the direct detection of rEPO with high sensitivity.[22]

Challenges for detection. In addition to the short half-life of EPO (3–4 days) in urine,[7] sample integrity is one of the main issues faced in the detection of recombinant proteins, which is particularly evident in the case of rEPO. Blood samples need to be promptly analyzed for rEPO abuse since the blood parameters analyzed, *e.g.* hematocrit and percentage of reticulocytes, decline with time and temperature.[17] Sample transportation to another testing location even at low temperatures would pose a significant risk of analyte deterioration due to mechanical stress. Additionally, there is a need to protect EPO against proteases in urine samples used in IEF.[18] The urine matrix also complicates identification of EPO bands.[17]

One of the prominent issues facing the indirect detection methods is the analytical variability among different laboratories and different methods. A number of different assays and measurement methods are available for each of the parameters used in the ON and OFF models, and each parameter brings its own variability into the equation. Therefore, inter-laboratory standardization and method validation with the used model formula are of great significance.[19]

Recently, there has been some criticism directed towards the current urine EPO test, although it is still valid and used by the WADA, and its standardization between accredited laboratories is being optimized.[3,23] The criticism included the time-consuming ultrafiltration step in sample preparation, the lack of reproducibility of carrier ampholyte gels, and the non-specific binding of secondary antibody to urinary proteins in the immunoblotting step which makes double blotting necessary.[15] An alternative method involves sample preparation by one-step acetonitrile precipitation and 2D electrophoretic analysis with the use of immobiline pH gradient (IPG) strips for establishing the pH gradient of the gel for IEF. These strips are considered more stable and reproducible than the ampholyte gels used by the WADA.[15] Other concerns included that the ionic strength conditions used are too weak to ensure dissociation of ionically-bound complexes, and that the 7 M concentration of urea in the buffer is inadequate for the denaturation of the proteins in the sample. Additionally, the monoclonal primary antibody used in the subsequent immunoblotting step is believed to be non-specific to EPO with cross-reactivity with a number of proteins which are not unusual to be present in the athlete's urine samples.[23] These include a protein from *Escherichia coli*, which is found in healthy human skin, and other proteins found in human urethial tissue extracts. These proteins have similar isoelectric points to rEPO, causing four bands to appear in the rEPO pH range as a result of the cross-reactivity of the primary antibody with these proteins. Similar cross-reactivity was also observed with *Saccharomyces cerevisiae*.[24] This lack of specificity could lead to false-positive results in legitimate athletes, especially if they experience some sort of urinary tract or genital infection or proteinuria. The manufacturer's technical document indicates the antibody to be specific for an epitope within the first 26 amino acids at the NH_2 terminus of endogenous human urinary and human rEPO, together with the applicability of the antibody in Western blot analysis.[25] It was also indicated that some issues faced with the 2D method have been unjustly extrapolated to the test approved by the WADA, without actually performing a comparative study between the two methods.[26] In spite of the criticism, the WADA has indicated that the test is reliable *per* the stringent result interpretation criteria, which take into consideration the rare incidence of overlap of recombinant and endogenous EPO zones, with independent opinion required in the case of positive results.[3,27] In defence of the WADA-approved test, it was stated that the accredited laboratory in Los Angeles, CA, USA has analyzed over 2600 urine samples for rEPO abuse. Out of the nine positive results, only two athletes have maintained a claim of innocence, but lost in the appeal. For the remaining seven cases, one had a physician indicted for rEPO distribution, three cases have publicly admitted the

abuse, and the remaining three did not challenge the subsequent penalties.[27]

As for the promising approach of capillary electrophoresis-coupled mass spectrometry, despite the method's directness and sensitivity, it is still not well-suited for practical use in sports doping detection. A highly concentrated sample is needed to achieve adequate sensitivity; therefore, sample enrichment procedures are required.[22]

One additional challenge that has been investigated on a relatively limited scale is the possibility that the doper may resort to microdosing rEPO abuse, which would lead to a very narrow detection window of about 12–18 h post-injection. Even during the microdose administration stage the isoelectric profiles showed the re-appearance of endogenous EPO bands, further masking the doping action.[28] Such a possibility is a real threat to current detection methods and should be thoroughly investigated and considered in the positivity criteria of the doping test.

2. Human growth hormone (hGH)

hGH is a peptide hormone, used medically to treat individuals with deficiency in endogenous hGH secretion and girls with Turner's syndrome.[29] hGH has a number of isoforms, the major ones having sizes of 20 and 22 kDa, and is usually present as a mixture of isoforms and fragments. Despite the lack of clinical trial evidence of the performance-enhancing effect of hGH, anecdotal evidence indicates that hGH is abused for its lipolytic and anabolic activity, and it is one of the most popular doping substances. The lack of clinical evidence may be attributed to the fact that such trials consider a mean effect over the participating population in the trial, which may not be clinically significant when considering a large number of subjects. However, in doping cases the results are considered significant for the individual and minute enhancement may be sufficient to achieve athletic excellence.[6,30]

It is worth noting that a synergistic relationship between hGH and insulin was established, where the action of hGH is significantly affected by insulin presence. As a performance enhancer, recombinant insulin facilitates the entry of glucose into cells in greater amounts. Under normal conditions, absence of insulin would significantly diminish the anabolic effect of hGH. Co-administration of insulin and hGH, however, may lead to abnormal enhancement of hGH action as insulin is essential for the anabolic action of hGH.[31]

Detection methods. Testing for rhGH was introduced by the WADA in the Athens 2004 Olympic Games.[3] There are two approaches for the detection of rhGH: one approach assesses the pharmacodynamic endpoints of hGH action, whereas the other is based on hGH isoform detection. Both approaches utilize immunoassays for the detection of different isoforms of endogenous hGH, or the markers dependent on its release. In both approaches, the WADA requires that two separate immunoassays targeting different epitopes are performed to verify the presence of the hGH isoform or marker.[30] rhGH consists only of one isoform which is 22 kDa, whereas the endogenous hGH consists of different isoforms with various sizes. Administration of rhGH (22 kDa) will repress pituitary secretion of hGH by negative feedback. Thus, if immunoassay analysis of serum shows abnormally elevated levels of the 22 kDa protein, this would indicate illegal use of rhGH.[29,30]

The assessment of pharmacodynamic endpoints of hGH action monitors changes in parameters modulated by hGH. hGH exerts most of its functions through the generation of insulin-like growth factor (IGF-I). The main advantage of this approach is the long half-life of IGF-I as compared with hGH, where IGF-I has a half-life of 89.5 h.[30] Other markers of hGH action include IGF-I binding protein and biochemical markers of bone turnover such as osteocalcin, the C-terminal propeptide of collagen type I, and the C-terminal cross-linked telopeptide of collagen type I.[30,32]

Challenges for detection. The main challenges for rhGH detection are its short half-life (*ca.* 20 min), pulsatile release pattern, and the fact that it exists in urine samples at very low concentrations. As a result, urine samples, which are easy to obtain and non-invasive, could not be used for testing. Additionally, endogenous hGH isoforms in blood are short-lived, with a maximum detection window of 36 h.[30] Repeated sampling over a 24 h period can help overcome the pulsatile release issue; however, it is inapplicable in a sports setting.

3. Human chorionic gonadotropin (hCG)

hCG is a pregnancy hormone which is normally present at low concentrations in plasma and urine in the absence of pregnancy or a secreting tumor, and is used as a drug to stimulate ovulation.[33,34] hCG is a target for dopers due to its stimulation of testicular testosterone production without affecting the urinary testosterone/epitestosterone (T/E) ratio.[33,34] It is currently prohibited in males only as it has been suggested that hCG has a minimal performance-enhancement effect in women and its testing risks invasion of privacy in the case of unrecognized pregnancy or other pathological conditions.[3,34]

Detection methods. The WADA-accredited doping laboratories may use radio-immunoassays or ELISA to screen for hCG in urine samples. The confirmation is done using an ELISA assay with chemiluminescence detection. The decision limit for hCG, the concentration above which the test for an athlete would be considered positive, is set at less than 5 mIU mL^{-1}. Testosterone abuse is usually detected by comparing its concentration with that of epitestosterone (a biological form of testosterone that does not improve performance) in urine (T/E ratio). The T/E ratio is about 1 : 1, but when it exceeds 6 : 1 it may indicate testosterone abuse. For confirmation of suspicious T/E ratios, gas chromatography coupled to isotope ratio mass spectrometry (GC–IRMS) may be used. This technique is based on the fact that natural and synthetic testosterone differ in carbon-12 to carbon-13 ratios. Although this technique is very sensitive, the instruments needed are expensive, and the method is technically demanding and requires large urine specimens.[3]

Challenges for detection. Because the alpha subunit of hCG is identical to that of luteinizing hormone,

Table 2 Summary of main analytical challenges for the detection of gene doping and recombinant protein abuse

Doping method	WADA-approved test	Main analytical challenges	Ref.
Gene doping (see Table 1 for potential targets)	None	• Need for reference databases for expression profiles • Tedious process of DNA microarray result analysis • Need for method standardization of DNA microarrays • Determination of structural differences between the endogenous protein and the one encoded by the transgene is not applicable to all doping proteins. It depends on post-translational modifications that are specific to the cells expressing the recombinant protein • For methods based on assessment of the immune response to the gene delivery vector (*e.g.* assay of anti-adenovirus antibodies) there is a chance of false-positive results if the athlete were infected with the virus *via* other means and was not undergoing gene doping. Also, the use of tamed viral vectors would abolish the use of this approach	6,8,10
rEPO abuse	Yes (IEF of urine followed by double immunoblotting and chemiluminescent detection)	• Suspected cross-reactivity of primary antibody used in the immunoblotting in the WADA-approved method with proteins commonly found in athlete urine samples, *e.g.* proteins of *E. coli*, and *S. cerevisiae* • Labor-intensive technique	3,17,24
hGH abuse	Yes (immunoassays for various hGH isoforms)	• Short half-life of hGH in urine and blood, leading to a very limited direct detection window • Possible overestimation of hGH concentration due to the presence of isoform fragments	30

thyroid-stimulating hormone and follicle-stimulating hormone, most hCG immunoassays employ antibodies that target the hCG beta subunit.[33,34] To circumvent possible cross-reactivity of the antibodies with other targets, immunoaffinity extraction followed by tandem MS may be used for hCG-doping detection.[33] According to the 2006 statistics of the WADA-accredited laboratories, more than 15 000 samples were assayed for hCG over the year, of which only 22 tested positive for hCG.[3]

Scope of the challenge

The main analytical challenges of detection methods are summarized in Table 2. The logistics involved in implementing doping detection methods in major sporting events such as the Olympic Games and World Championships complicate the challenge. Doping tests need to be minimally invasive and suitable for screening large numbers of athletes. Using the Athens 2004 Olympics as an example, more than 10 000 athletes from over 200 countries participated. This variability in ethnic origins also introduces the challenge of taking into consideration the ethnic differences, which would result in different baseline biological parameters. These 10 000 athletes competed in about 300 medal events. The anti-doping rules of the International Olympic Committee (IOC) for the selection of athletes for doping testing included the following: in individual events, the top four athletes in addition to another random athlete are selected for a doping test. In team events, testing is conducted in at least 25% of the competitions on one or two athletes and at least two athletes from each of the four top-finishing teams. This is in addition to testing any athlete establishing or breaking a world or Olympic record. On other occasions, the IOC required all medallists be tested for EPO. Not to mention the turn-around time requirements concurrent with the sample volume and rigorous determination criteria. The 2004 Olympics yielded a total of 3505 urine and blood samples analyzed in the Athens doping control laboratory. Positive doping results were required to be reported within 36 h, although 72 h was allowed for EPO.[3,35] Taking into consideration the legal and ethical consequences of a positive doping test, detection strategies for gene doping and recombinant protein abuse have to rise to more challenges than just achieving adequate sensitivity and specificity.

Conclusions

Recombinant DNA technology has put forward the ultimate detection challenge for doping in athletes: gene doping, which, along with recombinant proteins, present the current frontier of doping methods. The challenge lies in the concept that the performance-enhancing protein exerts the same effect as its endogenous counterpart, regardless of whether it was produced outside the body or within it. There are some established detection strategies for doping using administered recombinant proteins, and potential ones for gene doping. However, they are faced by a number of challenges including sample integrity issues, reagent specificity, short half-life of analytes, and the availability of reference data. Further work is needed to address these challenges and to optimize the available solutions.

References

1 H. J. Haisma and O. de Hon, 'Gene doping', *Int. J. Sports Med.*, 2006, **27**, 257–266.
2 M. Unal and D. Unal Ozer, 'Gene doping in sports', *Sports Med.*, 2004, **34**, 357–362.
3 World Anti-Doping Agency; http://www.wada-ama.org (accessed 12 July 2007).
4 A. de la Chapelle, A. L. Traskelin and E. Juvonen, 'Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis', *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 4495–4499.
5 L. DeFrancesco, 'The faking of champions', *Nat. Biotechnol.*, 2004, **22**, 1069–1071.
6 H. M. Azzazy, M. M. Mansour and R. H. Christenson, 'Doping in the recombinant

era: strategies and counterstrategies', *Clin. Biochem.*, 2005, **38**, 959–965.
7 E. Diamanti-Kandarakis, P. A. Konstantinopoulos, J. Papailiou, S. A. Kandarakis, A. Andreopoulos and G. P. Sykiotis, 'Erythropoietin abuse and erythropoietin gene doping: detection strategies in the genomic era', *Sports Med.*, 2005, **35**, 831–840.
8 F. Lasne, L. Martin, J. de Ceaurriz, T. Larcher, P. Moullier and P. Chenuaud, '"Genetic Doping" with erythropoietin cDNA in primate muscle is detectable', *Mol. Ther.*, 2004, **10**, 409–410.
9 J. D. Chaudhuri, 'Genes arrayed out for you: the amazing world of microarrays', *Med. Sci. Monit.*, 2005, **11**, RA52–RA62.
10 L. Joos, E. Eryuksel and M. H. Brutsche, 'Functional genomics and gene microarrays – the use in research and clinical medicine', *Swiss Med. Weekly*, 2003, **133**, 31–38.
11 M. G. Sebestyen, J. O. Hegge, M. A. Noble, D. L. Lewis, H. Herweijer and J. A. Wolff, 'Progress Toward A Nonviral Gene Therapy Protocol for The Treatment of Anemia', *Hum. Gene Ther.*, 2007, **18**, 269–285.
12 P. F. Macgregor and J. A. Squire, 'Application of microarrays to the analysis of gene expression in cancer', *Clin. Chem.*, 2002, **48**, 1170–1177.
13 F. Lasne and J. de Ceaurriz, 'Recombinant erythropoietin in urine', *Nature*, 2000, **405**, 635.
14 M. Ashenden, 'Contemporary issues in the fight against blood doping in sport', *Haematologica*, 2004, **89**, 901–903.
15 A. Khan, J. Grinyer, S. T. Truong, E. J. Breen and N. H. Packer, 'New urinary EPO drug testing method using two-dimensional gel electrophoresis', *Clin. Chim. Acta*, 2005, **358**, 119–130.
16 R. Parisotto, M. J. Ashenden, C. J. Gore, K. Sharpe, W. Hopkins and A. G. Hahn, 'The effect of common hematologic abnormalities on the ability of blood models to detect erythropoietin abuse by athletes', *Haematologica*, 2003, **88**, 931–940.
17 R. Kazlauskas, C. Howe and G. Trout, 'Strategies for rhEPO detection in sport', *Clin. J. Sport Med.*, 2002, **12**, 229–235.
18 F. Lasne, L. Martin, N. Crepin and J. de Ceaurriz, 'Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones', *Anal. Biochem.*, 2002, **311**, 119–126.
19 J. Segura, J. A. Pascual and R. Gutiérrez-Gallego, 'Procedures for monitoring recombinant erythropoietin and analogues in doping control', *Anal. Bioanal. Chem.*, 2007, **388**, 1521–1529.
20 J. A. Pascual, V. Belalcazar, C. de Bolos, R. Gutiérrez, E. Llop and J. Segura, 'Recombinant erythropoietin and analogues: a challenge for doping control', *Ther. Drug Monit.*, 2004, **26**, 175–179.
21 M. De Frutos, A. Cifuentes and J. C. Diez-Masa, 'Differences in capillary electrophoresis profiles of urinary and recombinant erythropoietin', *Electrophoresis*, 2003, **24**, 678–680.
22 B. Yu, H. Cong, H. Liu, Y. Li and F. Liu, 'Separation and detection of erythropoietin by CE and CE–MS', *Trends Anal. Chem.*, 2005, **24**, 350–357.
23 W. W. Franke and H. Heid, 'Pitfalls, errors and risks of false-positive results in urinary EPO drug tests', *Clin. Chim. Acta*, 2006, **373**, 189–190.
24 A. Khan and M. S. Baker, 'Non-specific binding of monoclonal human erythropoietin antibody AE7A5 to *Escherichia coli* and *Saccharomyces cerevisiae* proteins', *Clin. Chim. Acta*, 2007, **379**, 173–175.
25 R&D Systems Inc.; http://www.rndsystems.com/pdf/mab2871.pdf (accessed 12 July 2007).
26 O. P. Rabin, F. Lasne, J. A. Pascual, M. Saugy, F. J. Delbeke and P. Van Eenoo, 'New urinary EPO drug testing method using two-dimensional gel electrophoresis', *Clin. Chim. Acta*, 2006, **373**, 186–187.
27 D. Catlin, G. Green, M. Sekera, P. Scott and B. Starcevic, 'False-positive Epo test concerns unfounded', *Blood*, 2006, **108**, 1778.
28 M. Ashenden, E. Varlet-Marie, F. Lasne and M. Audran, 'The effects of microdose recombinant human erythropoietin regimens in athletes', *Haematologica*, 2006, **91**, 1143–1144.
29 M. Saugy, N. Robinson, C. Saudan, N. Baume, L. Avois and P. Mangin, 'Human growth hormone doping in sport', *Br. J. Sports Med.*, 2006, **40**, i35–i39.
30 C. M. McHugh, R. T. Park, P. H. Sonksen and R. I. Holt, 'Challenges in detecting the abuse of growth hormone in sport', *Clin. Chem.*, 2005, **51**, 1587–1593.
31 P. H. Sonksen, 'Insulin, growth hormone and sport', *J. Endocrinol.*, 2001, **170**, 13–25.
32 M. Bidlingmaier, Z. Wu and C. J. Strasburger, 'Test method: GH', *Baillieres Best Pract. Res. Clin. Endocrinol. Metab.*, 2000, **14**, 99–109.
33 L. H. Gam, S. Y. Tham and A. Latiff, 'Immunoaffinity extraction and tandem mass spectrometric analysis of human chorionic gonadotropin in doping analysis', *J. Chromatogr., B*, 2003, **792**, 187–196.
34 D. J. Handelsman, 'Clinical review: The rationale for banning human chorionic gonadotropin and estrogen blockers in sport', *J. Clin. Endocrinol. Metab.*, 2006, **91**, 1646–1653.
35 The Olympic Movement; http://www.olympic.org (accessed 12 July 2007).
36 P. Chenuaud, T. Larcher, J. E. Rabinowitz, N. Provost, Y. Cherel, N. Casadevall, R. J. Samulski and P. Moullier, 'Autoimmune anemia in macaques following erythropoietin gene therapy', *Blood*, 2004, **103**, 3303–3304.
37 E. C. Svensson, H. B. Black, D. L. Dugger, S. K. Tripathy, E. Goldwasser, Z. Hao, L. Chu and J. M. Leiden, 'Long-term erythropoietin expression in rodents and non-human primates following intramuscular injection of a replication-defective adenoviral vector', *Hum. Gene Ther.*, 1997, **8**, 1797–1806.
38 Y. X. Wang, C. L. Zhang, R. T. Yu, H. K. Cho, M. C. Nelson, C. R. Bayuga-Ocampo, J. Ham, H. Kang and R. M. Evans, 'Regulation of muscle fiber type and running endurance by PPARδ', *PLoS Biol.*, 2004, **2**, e294, DOI: 10.1371/journal.pbio.0020294.
39 S. Lee, E. R. Barton, H. L. Sweeney and R. P. Farrar, 'Viral expression of insulin-like growth factor-I enhances muscle hypertrophy in resistance-trained rats', *J. Appl. Physiol.*, 2004, **96**, 1097–1104.
40 A. Voutetakis, I. Bossis, M. R. Kok, W. Zhang, J. Wang, A. P. Cotrim, C. Zheng, J. A. Chiorini, L. K. Nieman and B. J. Baum, 'Salivary glands as a potential gene transfer target for gene therapeutics of some monogenetic endocrine disorders', *J. Endocrinol.*, 2005, **185**, 363–372.