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No doubt about the validity of the urine test for detection of recombinant human erythropoietin

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To the editor:

False-positive Epo test concerns unfounded

The brief report by Beullens et al[1] is misleading regarding the urine test that the World Anti-Doping Agency (WADA) uses to detect recombinant human erythropoietin (rhEpo). The WADA-recommended test is based on immunoelectorphoresis and double blotting (IEF/DB), and was developed by Lasne and de Ceaurriz in 2000.[2]

Our WADA-accredited laboratory has performed the IEF/DB test for rhEpo on more than 6800 urine samples, including more than 2600 doping control samples from athletes. Of the latter, we have reported 9 positive cases for rhEpo: 3 of these have publicly confessed to using rhEpo, 3 have accepted penalties, the physician of a seventh has been indicted for distribution of rhEpo, and 2 maintained their innocence but lost on appeal.

We take issue with Beullens's use of the term "false positive" because, as the authors emphasize, the compound they are discussing is not rhEpo. If the compound detected can be identified as not rhEpo, then it cannot cause a false positive. This term sensationalizes an otherwise interesting case report that could in due course contribute to the body of science.

The criteria used by the WADA laboratories are well known and readily available.[3] Beullens et al do not state the criteria they used to make the "false-positive" claim. Using the WADA criteria, the "false-positive" electropherogram[1(Fig1A)] is clearly negative. Moreover, they do not include a negative or a positive urine quality-control sample. Forensic testing results are normally accompanied by a comprehensive documentation package that supports the conclusion. A report such as this that raises a profound issue (false accusations against an athlete) at a minimum requires far more documentation.

Another important but unexplained issue is the nature of the compound that appears to migrate in the same general region as rhEpo and is characterized by bands. The pH range of the ampholytes is needed in order to fully interpret the data. In the left panel of their Figure 1A, the epoietin-β lane shows 3 faint bands and possibly a very weak fourth band. The bands in the darbepoetin lane are overly dense. Knowing the pH range of the ampholytes might explain why the darbepoetin region seems closer to the rHuEPO region than we customarily observe (compare with Figure 1 here).

The right panel of their Figure 1A shows an apparent protein with bands, but it does not look like a typical rhEpo positive (Figure 1 here). Further, 1 and maybe 2 of the bands migrate more basically than the most basic epoietin-β band. Under the WADA rules, the identification criteria are not met.

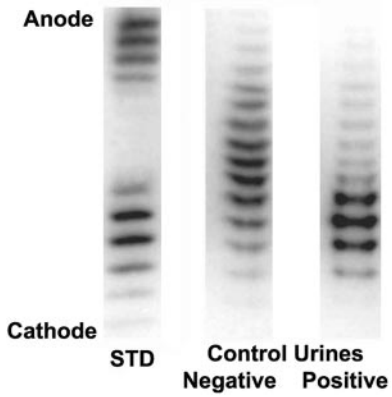

Figure 1. Electropherogram showing a darbepoetin alfa/rhEpo standard (left lane) and human quality control urines. The darbepoetin alfa/rhEpo standard is on the left, the negative human quality control urine (from a known rhEpo-free donor) is in the middle, and the positive human quality control urine (pooled from known donors on rhEpo) is on the right.

Finally, the athlete has a puzzling renal disease characterized by a concentrating defect and an excessive number of casts that apparently does not interfere with his athletic prowess. He should have a full nephrology evaluation.

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To the editor:

No doubt about the validity of the urine test for detection of recombinant human erythropoietin

Beullens et al report the "false-positive detection of recombinant human erythropoietin in urine following strenuous physical exercise."[1(p4711)] This report, based on observations conducted on urine from 1 single subject, relies in fact on serious errors of interpretation of poor-quality images. A first sample, collected just after exercise and analyzed by double-blotting following isoelectric focusing of the retentate from ultrafiltrated urine,[2,3] gives rise to a banding pattern interpreted as unrelated to Epo based on the argument that this pattern is missing in a second sample collected 1 hour later. A simple routine assay (using other antibodies than the AE7A5 used for immunoblot) of the Epo level in these 2 ultrafiltered samples before IEF would have probably shown that a high concentration of this hormone was present in the first one but not in the second one. It is quite surprising that this basic control

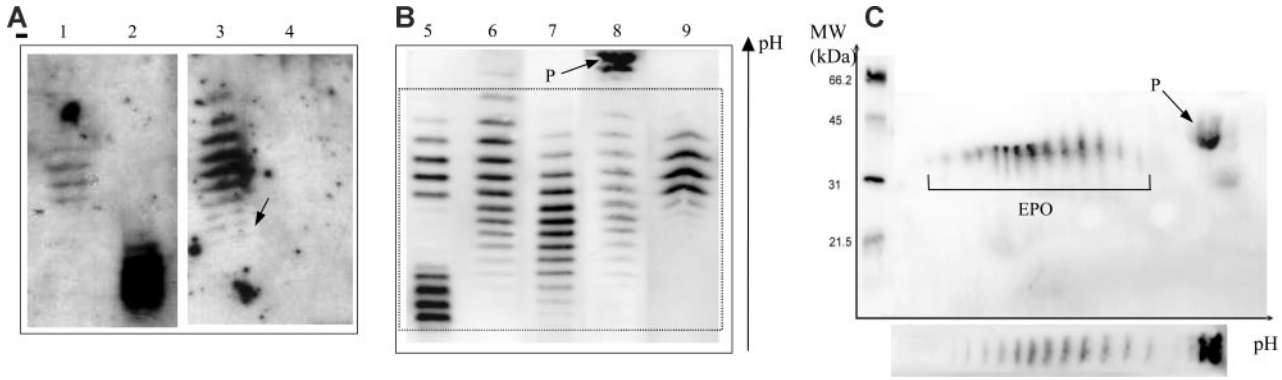

Figure 1. Epo profiles obtained by double-blotting. Panel A is reproduced from Beullens et al.[1(Fig1A)], and panels B and C are results from our laboratory. (A) Lane 1 shows epoetin β; lane 2, darbepoetin α; lane 3, urine sample considered as "false positive" (the arrow shows a white hole corresponding to ineffective transfer of proteins in this area); and lane 4, the same sample as in lane 3 1 hour later. (B) Lane 5 shows a mixture of epoetin β and darbepoetin α; lane 6, natural urinary Epo from a sample taken after strenuous exercise (note some shift toward the cathode of the banding pattern); lane 7, natural urinary Epo; lane 8, urine sample showing the protein (P) unrelated to Epo outside the window of integration (dotted box) used for interpretation of an antidoping control result; and lane 9, urine sample in case of epoetin administration (note the difference with the natural urinary Epo pattern even in the case of the post–strenuous exercise sample). (C) Two-dimensional electrophoresis of a urine sample showing both Epo and protein P.

was not performed. Did the authors fail to obtain an image of natural urinary Epo for comparison with their "interfering protein"? The SDS electrophoresis results are entirely misinterpreted due to the very different conditions chosen for preparation of the same urine sample before SDS (10-fold concentration) and IEF (200-fold concentration). Under such distorted conditions, the absence of a band corresponding to the molecular weight of Epo (39 kDa) in SDS electrophoresis cannot, in any account, be considered as a proof that there was no EPO in the sample submitted to IEF. The only band detected (42 kDa) by SDS has been incorrectly related to the bands detected by IEF. Why isn't a 2-dimensional electrophoresis shown to demonstrate such an assertion? In fact, the bands shown in the IEF figure cannot be detected in the SDS experiment due to the insufficient concentrating step. From our experience, the 42-kDa band corresponds to a protein very often present in urine in high concentrations after strenuous exercise and detected by the AE7A5 antibody used for immunoblotting. This protein is known not to interfere with the Epo pattern of an antidoping control due to a more basic isoelectric point (pI). It is unfortunate that the IEF image shown in this article has been cut just below the area corresponding to this protein. The subsequent investigations of deglycosylation were very interesting and corroborate our results about this 42-kDa protein. It is unfortunate that they were arbitrarily attributed to the bands shown by IEF.

In summary, the IEF pattern shown by Beullens et al has been interpreted as unrelated to Epo, whereas it corresponded to a bad-quality image (such a result would have been categorically rejected from any interpretation in antidoping control) of a well-known Epo pattern observed after particular conditions of strenuous exercise. The figure enables one to compare the IEF results of Beullens et al[1] with well-identified Epo patterns as observed in our laboratory from several hundreds of samples. A 2-dimensional electrophoresis has been introduced to support our statements.

It is disappointing that such poor-quality experiments and misinterpreted results led the authors to believe that the validity of the Epo antidoping test could be questioned.

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Response:

False-positive detection of rhEpo remains a real concern

Epo test results are clearly not always interpreted identically by all WADA-accredited laboratories. For example, the athlete we examined was initially found guilty of rhEpo abuse by 2 WADA-accredited laboratories. During the ensuing appeal procedure, it was disclosed by a third WADA-accredited laboratory that the original data leading to the suspension of this athlete had been interpreted wrongly by the former 2 WADA laboratories, and that the tests were actually negative for rhEPO.

According to the official WADA identification criteria for rhEPO (Figure 1A), the third lane of our IEF immunoblot[2] is positive for rhEpo, and the fourth lane is negative (Figure 1B). We disagree with the interpretation of these data according to other ad-hoc "WADA rules." Strikingly, lane 6 of Dr Lasne's figure is presented as endogenous Epo but should be interpreted according to the official WADA criteria as rhEpo (Figure 1C). As for the required controls, our IEF data show a negative urine sample as well as samples of purified rhEpo, which served as positive controls (Figure 1B).[2] We are surprised by the criticism that we did not detect endogenous Epo, since a WADA report indicates that up to 20% of the analyzed specimens do not contain endogenous Epo.[3]

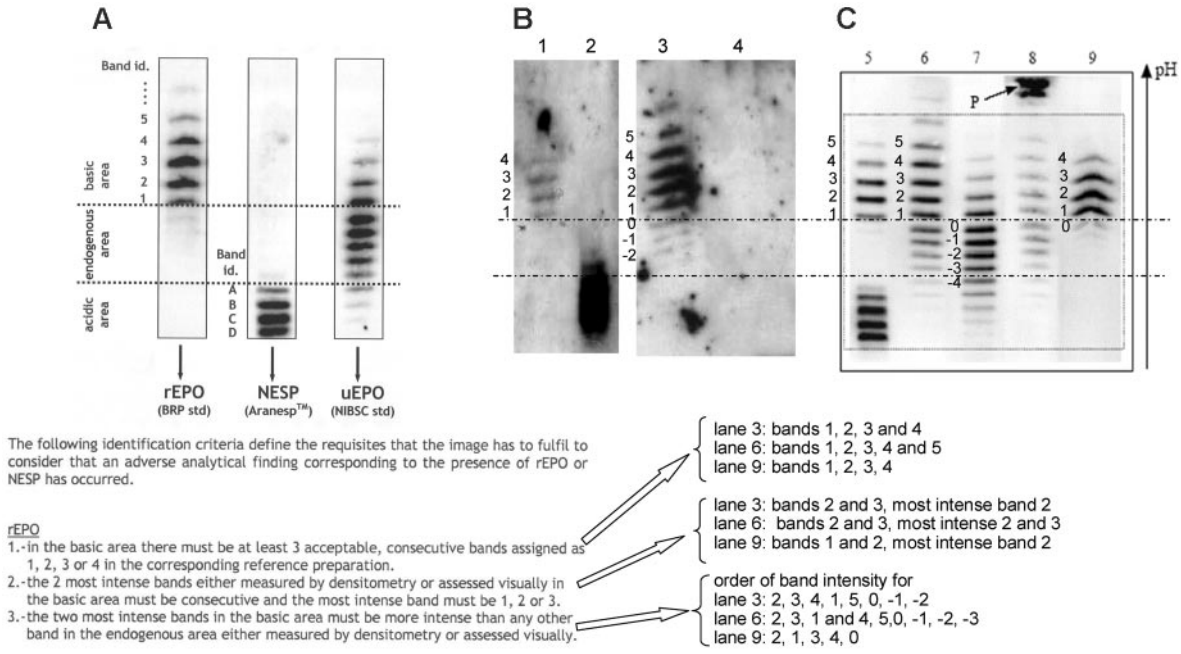

Figure 1. Epo profiles obtained by double immunoblotting. (A) Acceptance criteria for rhEpo, as defined by WADA.[1] (B) Figure 1A from Beullens et al.[2] Lane 1, epoetin β; lane 2, darbepoietin α; lane 3, "false-positive" urine sample collected immediately after exercise; lane 4, negative urine sample collected 1 hour after the sample in lane 3. (C) Figure 1 from Lasne. Lane 5, mixture of epoetin β and darbepoietin α; lane 6, urine sample after strenuous exercise; lane 7, natural urinary Epo; lane 8, urine sample showing protein P, unrelated to Epo; lane 9, urine sample in case of epoetin administration. Bands in panels B and C are numbered according to the criteria defined in panel A. Using the acceptance criteria of panel A, lanes 3 (panel B), 6, and 9 (panel C) should be considered as an adverse analytical finding corresponding to the presence of rhEpo.

The background staining in Figure 1B does not interfere with the application of the Epo identification criteria and, hence, we do not understand why our data should be rejected because they are "poor-quality images." As a recent *Nature* editorial puts it: "Slightly dirty images reflect the real world."[4(p892)] A WADA report furthermore acknowledges that protein-rich samples cause background staining.[3]

We have used the term "false positive" according to the current terminology in diagnostic medicine in the sense of a type I error (ie, when a test incorrectly reports that it has found a positive result when none really exists). The same terminology has been used in a WADA report.[3] Lasne argues that our "false-positive" result stems from endogenous Epo that under particular conditions of strenuous exercise migrates more basically. This is a speculation not supported by any evidence. In fact, a WADA-supported study concluded that strenuous exercise does not affect the Epo pattern in such a way as to require changes in the identification criteria.[5] Lasne apparently did not understand the problem generated by the detection of urinary "rhEpo" immediately after exercise but not in a sample that was obtained 1 hour later (Figure 1B). As the half-life of Epo is about 8 hours, this indicates that the detected signals at 0 hours are not derived from Epo itself.

We believe that the lack of specificity of the anti-Epo antibody (clone AE7A5) lies at the heart of the false-positive detection of rhEpo. This issue has also been raised by Khan et al,[6] and is not unexpected since this antibody is sold for research use only. We note that Lasne is the first WADA-supported investigator admitting to the nonspecificity of this antibody. It is very surprising that this cross-reactivity has not been noted in the thousands of Epo tests that have been performed so far. Lasne claims that this lack of specificity does not interfere with the Epo test because the interfering signals are from "a" protein that migrates more basically than the Epo isoforms. However, Figure 2 of our study[2] shows that the AE7A5 antibody binds to multiple polypeptides during immunoblotting, even at much lower protein concentrations than those used for the Epo test. It is incorrect that this cross-reactivity is only detected after SDS-PAGE. Lane 8 of Lasne's figure proves that this also applies to IEF. Lasne's claim that we attributed the false-positive rhEpo-signals to the 42-kDa cross-reacting band is also incorrect. Any cross-reacting protein(s) can be responsible for this.

We firmly maintain our conclusion that, in the examined athlete, strenuous exercise did result in the false-positive detection of rhEpo. The diagnostic use of an antibody that is not monospecific and the evident use of ad-hoc interpretation criteria by the WADA-accredited laboratories are worrisome and difficult to reconcile with the claim that the rhEpo test is infallible.

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