No evidence for cross-contamination of dried blood spots excised using an office hole-punch for HIV-1 drug resistance genotyping

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Sir,

In a recent report, we described a laboratory procedure to perform HIV-1 genotypic drug resistance testing from dried blood spots (DBS).1 The methodology as a whole was subjected to rigorous validation. However, it has since been pointed out to us that we had not demonstrated that the process for cleaning the hole-punch used to excise DBS from filter paper was sufficient to prevent cross-contamination between samples. Our DBS excision technique involves the use of a hand-held 6 mm diameter office hole-punch (catalogue number 272575—Staples, Doncaster, UK). This is used to punch a pair of DBS per specimen from a filter paper collection card. It is cleaned by punching five filter paper spots from a fresh, unused card, before being used again to excise from a DBS specimen. In order to address these concerns, studies were performed to investigate the possibility that this procedure could generate false positives due to contamination of the hole-punch. Such methodological concerns are important considering the use of DBS as a widespread surveillance method.

Ten DBS per specimen were prepared from three venous whole blood specimens collected with EDTA anticoagulant from HIV-infected individuals with known high viral load (>1 x 10^5 copies/mL). HIV viral load confirmation was performed using the COBAS AmpliPrep HIV Taqman Assay (Roche, Lewes, UK). A further 30 DBS were prepared from normal human whole blood collected with citrate anticoagulant. DBS were prepared, stored and tested as per our earlier report.1 Two punches from each DBS were used for nucleic acid extraction. Alternate HIV and normal whole blood DBS were excised and tested consecutively. For each DBS replicate, PCR reactions to amplify fragments of the human β-globin gene, and two HIV subgenomic regions [protease spanning nucleotide positions 2057–2979, reverse transcriptase (RT) 2813–3618 of the HIV genome, numbering as HXB2 reference strain, K03455], were carried out.

For each normal whole blood replicate DBS, only the human β-globin gene fragment was successfully amplified. HIV nucleic acids were not detected by nested PCR in any normal whole blood replicate, in either the protease or RT regions. For each HIV whole blood DBS replicate, human β-globin and HIV protease and RT genes were detected by PCR. The outcome of these studies is summarized in Table 1, along with HIV viral load data for each patient specimen.

Several studies have reported different methods to excise HIV DBS for drug resistance genotyping. The studies of Ziemniak et al.2 reported the excision of DBS using a sterile disposable razor blade. Bertagnolio et al.3 reported the use of disposable sterile scissors to excise DBS specimens. Others have reported the use of scissors and forceps sterilized between uses by spraying with a solution of 70% ethanol, before being completely dried.4,5 These methods for the excision of DBS from filter paper collection cards are cumbersome, timing-consuming, expensive and—in the case of razor blade use—potentially hazardous.

A recent study by Driver et al.6 reported the use of a manual hole-punch to excise DBS for detection of HIV DNA by PCR, for use in the diagnosis of mother-to-child transmissions. Extensive replicate testing revealed no false positives regardless of whether the hole-punch was cleaned between DBS or not. Taken together with our study, the use of the manual hole-punch is a cost-effective, labour-saving method for DBS excision for HIV drug resistance genotyping, for which specimen cross-contamination is unlikely.

**Table 1.** Consecutive excision and processing of alternate HIV whole blood and normal whole blood DBS specimens, showing amplification of human β-globin and HIV protease and RT genes for each replicate

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Human β-globin</th>
<th>HIV protease</th>
<th>HIV RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen A (113322 cp/mL)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Normal whole blood</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Specimen B (116000 cp/mL)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Normal whole blood</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Specimen C (232000 cp/mL)</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Normal whole blood</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Viral load assessments are shown in HIV RNA copies/mL (cp/mL).

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**Transparency declarations**

None to declare.

**References**

Leakage into Portuguese aquatic environments of extended-spectrum-β-lactamase-producing Enterobacteriaceae

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Sir,

Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae have been recognized as significant nosocomial pathogens during recent decades. The spread of these organisms is worrisome since they might also transfer antibiotic resistance genes among different hosts and environments. In this work, we describe the occurrence of ESBLs and integrons among Enterobacteriaceae from Portuguese aquatic environments and analyse the clonal relationship between Portuguese ESBL-producing isolates of environmental, human and animal origin.

We identified 16 ESBL-producing Enterobacteriaceae (11 Klebsiella pneumoniae, 4 Escherichia coli and 1 Enterobacter aerogenes) in eight environmental samples collected in Portugal in order to detect cephalosporin-resistant Gram-negative bacteria. The samples included: (i) raw urban wastewater directly from sewer lines downstream of four hospitals located in the Porto area (n = 5; 2001–02); (ii) river water from river Sousa in the north of Portugal (n = 1; 2003); and (iii) Porto marine water (n = 2; 2003–04). River and marine waters were collected close to clandestine discharge points of water streams contaminated by faecal coliforms. Samples were collected in sterile bottles and processed on the same day of collection. River or marine samples were submitted to a vacuum membrane filtration procedure [100 mL on 0.45 μm pore-size membranes (Millipore Corporation, Molsheim, France)] and filters were placed on MacConkey agar plates supplemented with ceftazidime (1 mg/L) or cefotaxime (1 mg/L). Wastewater samples were directly spread on these plates (0.1 mL).

ESBL characterization was accomplished by the standard double-disc synergy test, amplification of known bla genes and sequencing. Bacterial identification, antibiotic susceptibility tests, conjugation assays and plasmid analysis were performed as described previously. Clonal relatedness was established by randomly amplified polymorphic DNA analysis (RAPD)-PCR and/or PFGE. The clonal relationship between environmental strains from this study and ESBL-producing strains widely spread in both hospital (northern and central regions, 2002–04) and community Portuguese settings (northern region, 2003–05) was determined.

E. coli phylogenetic groups were identified by multiplex PCR. Class 1, 2 and 3 integrons were characterized by PCR-RFLP and sequencing.

The epidemiological characteristics of the ESBL-producing isolates studied are shown in Table 1, which includes typing results and patterns of resistance to non-β-lactam antibiotics. A diversity of ESBLs was identified from five hospital sewage samples and comprised TEM-116 from different K. pneumoniae strains collected in all five hospital wastewaters, SHV-12 or TEM-10/SHV-27 from K. pneumoniae and TEM-52 from E. coli, each collected from different hospital wastewater streams. From the River Sousa, we identified a TEM-24-producing E. aerogenes strain and a TEM-52-producing E. coli. CTX-M-14 and CTX-M-32 producers, which are commonly recovered from community and hospital settings in south Europe, were also identified in two marine coastal waters. All but TEM-116, previously detected from Ria de Aveiro (central Portugal), have not been previously recovered from environmental samples. K. pneumoniae was the species most commonly found as an ESBL producer (69%, 11/16).

Several RAPD or PFGE patterns were identified although some clones were persistently recovered, such as TEM-116-producing K. pneumoniae RAPD-type KP-2, isolated in 2001 and 2002, or were clonally related to those previously identified among clinical samples, such as the TEM-24-producing E. aerogenes, the TEM-116-producing K. pneumoniae RAPD-type KP-2 and the SHV-12-producing K. pneumoniae RAPD-type KP-5. No clonal relationships were found between ESBL-producing Enterobacteriaceae of aquatic and animal origin.

Two ESBL-producing E. coli belonged to phylogroup D (one CTX-M-14 and one TEM-52-producing isolate). The remaining two isolates belonged to phylogroup A or B1. The blaESBL genes were successfully transferred by conjugation from 31% (5/16) of the strains (Table 1).

Although the CTX-M-14-producing E. coli recovered from coastal waters was clonally unrelated to other CTX-M-14-producing strains from Portuguese hospitals or healthy