Detection and identification of orthopoxviruses using a generic nested PCR followed by sequencing

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ABSTRACT

Some orthopoxviruses are considered to be potential biological weapons. After the smallpox eradication campaign ended, routine vaccination was stopped around the world. Consequently, a significant portion of the population is now completely unprotected from infection by variola virus and related orthopoxviruses. Some of the symptoms associated with non-variola infections can be similar to smallpox, causing alert and panic situations. These infections should be considered as real public health concerns, so suitable tools for their differential diagnosis are needed. This study aims to devise a simple and easy-to-perform method that is able to detect and identify any orthopoxvirus that might cause infection in humans. In addition, the similarity of the different genes in the genomes of several species of orthopoxviruses is investigated, and orthopoxvirus-universal primer pairs in the tumour necrosis factor receptor II homologue gene are designed, taking full account of nucleotide similarity. A strategy is devised for their sensitive, rapid and cost-effective detection and identification, based on a nested PCR followed by sequencing. The efficacy of the method is tested with samples sent by the European Network of Imported Viral Diseases as part of two external quality control assays. All human orthopoxviruses assayed were detected and identified.

KEY WORDS: Orthopoxvirus.
Polymerase chain reaction.
Smallpox.

Introduction

The family *Poxviridae* and the subfamily *Chordopoxvirinae* (poxviruses of vertebrates) contain eleven genera, the most important of which being variola virus (VARV) and the closely related cowpox virus (CXPV), vaccinia virus (VACV, the vaccine strains used for immunisation against smallpox) and monkeypox virus (MPXV), which can infect humans and are classified in the single genus *Orthopoxvirus*.

Orthopoxviruses are characterised by large brick-shaped virus particles that contain a double-stranded DNA genome of approximately 200,000 bp.[1] Among the orthopoxvirus genomes characterised, the highest sequence homology in orthopoxvirus species is found in the middle of the genome, while their terminal sections can exhibit considerable variability, probably reflecting differences in host range, tissue tropism and virulence.

In humans, orthopoxviruses cause infections ranging from the mild (VACV, CPXV and some strains of MPXV) to the fatal (VARV). These viruses have recently been discussed as biological weapons.[2,3] In particular, VARV is a major threat as an agent of bioterrorism.[4] Clinical infection is characterised by an incubation period of seven to 17 days, followed by the onset of high fever, malaise and prostration, often with severe headache, nausea and vomiting.[5]

Smallpox patients can transmit the virus, at least during the first week when lesions in the oral mucosa ulcerate and seed the saliva with VARV.[6–10] The disease causes severe mortality rates of about 30% among victims.

Initial diagnosis of VARV infection is often based on the epidemiological and medical history and clinical symptoms, but the conclusive point is the detection of the virus. Detection of orthopoxviruses has been carried out by isolation, electron microscopy and, more recently, genome amplification-based techniques. Isolation is time-consuming and requires posterior analysis for identification. Negative staining techniques used in electron microscopy may provide quick detection, but specific identification is not possible.

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In contrast, genomic amplification approaches based on the polymerase chain reaction (PCR) could provide rapid and sensitive detection and identification of orthopoxviruses. Some PCR-based methods (e.g., real-time PCR), have been described, but, as far as we know, no generic PCR followed by sequencing has been described.

Generic amplification followed by sequencing of the amplified product could result in rapid and sensitive detection and identification of a wide range of orthopoxviruses, and offer the opportunity to collect molecular data on poxvirus infections.

This study describes the development of a generic nested PCR assay, designed in the tumour necrosis factor receptor II homologue gene, followed by sequencing for sensitive and specific detection and identification of VARV and other orthopoxviruses capable of causing human illnesses. The efficacy of the method is proved on samples sent by the European Network for the Diagnostics of Imported Viral Diseases (ENIVD), as part of external quality control (EQC) assessments.[11]

Materials and methods

Viruses and their propagation

Cell monolayers (CV1; derived from the kidney of a male adult African green monkey) maintained in Dulbecco´s modified Eagle medium (DMEM), supplemented with fetal bovine serum (2%) and with a mixture of penicillin and streptomycin (1%, Gibco BRL), were infected with VACV (Western Reserve strain) and cultured for two days until cytopathic effect was evident. Infected cultures were subsequently scrapped and the infected cells harvested in DMEM. Titration was undertaken by adding a mix of 50% agar (Difco) in DMEM. Cell monolayers were fixed by adding 10% formaldehyde in phosphate-buffered saline and the plates visualised by adding crystal violet.[12]

Rabbitpox (RPXV, strain Utrecht), ectromelia (ECTV, strain Moscow) and CPXV (strain Brighton) were obtained from ATCC.

Samples from external quality assurance

A first test panel, containing freeze-dried human plasma samples spiked with some orthopoxviruses, was received by the laboratory in order for it to take part in an assurance test carried out by ENIVD in 2002.[11] Samples from camelpox virus (CMLV), MPXV (strain Lam87), VACV (Elstree) and modified virus Ankara strains, CPXV (81/02 and Brighton strains) and ECTV were prepared and sent as coded samples. Serial dilutions of titred MPXV were also included.

A second test panel similar to the previous one was sent in 2004. Samples from MPXV (in different concentrations), CPXV and other members of the family not included in the orthopoxvirus genus (tanapox virus and taterapox virus) were sent as coded samples.

Extraction of DNA

Viral DNA was isolated from the infected cell cultures from preparations of viruses, and from the samples from the first EQC panel, following a procedure described previously.[13] In brief, infected cells were disrupted and 50 µL of supernatant fluid or the preparations were incubated with 200 µL guanidinium thioisocyanate lysis buffer. Nucleic acid was precipitated with an equal volume of pure isopropanol, dried after washing with ethanol (70%) and dissolved in 10 µL ribonuclease-free water.

Viral DNA present in the second EQC was obtained using the QIAamp viral RNA mini kit (Qiagen, Spain), following the manufacturer's instructions.

Oligonucleotides

Alignments were undertaken using nucleotide sequences of DNA of different orthopoxviruses, obtained from GenBank (National Institute of Health, Bethesda, Maryland, USA), using the MACAW 2.0.5 program.[14] The abbreviations and accession numbers of the viruses that provided the sequences are indicated below.

Degenerated primers were based on conserved motifs in a region of the tumour necrosis factor receptor II homologue gene to align perfectly with known orthopoxvirus sequences. Primers selected were:
PoxS361: 5′ $_{361}$AACACKACTCACAATAGAATCTGTG $_{385}$ 3′,
PoxAS989: 5′ $_{1010}$ ATATYGCAACTASCGGGCATATGGC $_{986}$ 3′,
PoxS457: 5′ $_{457}$ TGTGGAATAGGATACGGAGTATCCG $_{481}$ 3′,
PoxAS927: 5′ $_{947}$ GTATCMKTTTCGTAGTCTTGAG $_{926}$ 3′.

PoxS361 and PoxAS989 were used in the first amplification and PoxS457 and PoxAS927 in the nested amplification. The letters 'S' and 'AS' refer to sense and antisense sequences, respectively. Indicated positions correspond with those of VACV (strain Temple of Heaven [VVU87585]).

Reaction amplification

Amplification was carried out in a PCT-200 Peltier thermal cycler (MJ Research, Watertown, MA, USA) utilising thin-walled reaction tubes (REAL, Durviz, Valencia, Spain) with no mineral oil overlay. Briefly, 5 µL nucleic acid preparation was added to 45 µL PCR mix containing 2.5 mmol/L $MgCl_2$ (Applied Biosystems, Applera Hispania, SA, Madrid, Spain), 0.2 mmol/L each dNTP (Amersham Pharmacia Biotech, Sweden), 40 pmol each primer and 2.5 units of AmpliTaq DNA polymerase (Applied Biosystems).

Samples underwent 40 PCR cycles at 94°C for 1 min (denaturation), 55°C for 1 min (annealing) and 72°C for 45 sec (elongation). A final extension step was carried out at 72°C for 5 min.

The nested PCR reaction mixture was carried out in a final volume of 50 µL and contained 2.5 mmol/L $MgCl_2$ (Applied Biosystems), 0.2 mmol/L each dNTP (Amersham Pharmacia Biotech), 40 pmol each primer, 2.5 units of AmpliTaq DNA polymerase (Applied Biosystems) and 1 µL of the product of the first amplification. The mix was subjected to the same PCR cycles as described above.

After completion of amplifications, 10 µL each reaction mixture was analysed by electrophoresis in a 2% agarose gel (MS8, Hispanlab, Spain) containing 0.5 µg/mL ethidium bromide in TBE buffer. Products were visualised under ultraviolet (UV) light. A 1 kb DNA ladder (Boehringer Manheim) was included on each gel.

Cloning

The DNA band resulting from the first generic amplification of VACV DNA was excised from the agarose gel using the QIAquick gel extraction kit (Qiagen) and ligated with the HTP TOPO TA cloning kit (Invitrogen, Barcelona, Spain) onto the pCR 4-TOPO vector, following the manufacturers' instructions. One Shot TOP10 competent cells (Invitrogen) were used to transform the resulting plasmid.

Colonies containing recombinant plasmids were identified by PCR using the generic primers so that the correct band was obtained only when the insert was present. Plasmid DNA was extracted from bacterial cells using the Wizard Plus SV Minipreps DNA purification system (Promega, Innogenetics Diagnostica y Terapeutica SA, Barcelona, Spain). Purified plasmid was quantified spectrophotometrically and digested with AflIII (New England Biolabs, Izasa, Spain) to linearise it.

Direct sequencing

Bands from the amplification were purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions on both strands were performed using PoxS457 and PoxAS927 primers with the ABI Prism BigDye Terminator Cycle Sequencing v2.0 Ready Reaction (Applied Biosystems) and analysed using an ABI 377 automated sequencer (Applied Biosystems).

Handling of sequences

The sequences obtained were compared using the BLAST

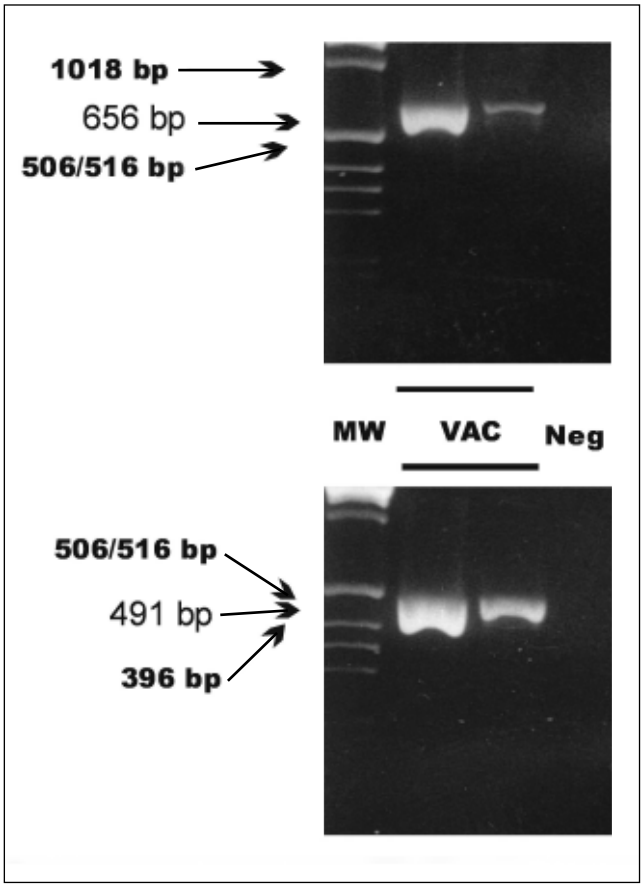
tool (National Centre for Biotechnology Information) with those recorded in databases, and this provided rapid, preliminary identification. In order to achieve more precise identification, a further analysis was carried out to study the level of homology within orthopoxvirus sequences, using the multiple sequence alignment programme ClustalW (1.8 version) that produces an optimal sequence alignment file.[15] A distance tree was built using the Jukes-Cantor parameters with the neighbour joining method in the MEGA3 program,[16] which calculated confidence bootstrapping values.

Sequences from databases

Sequences from orthopoxviruses were obtained from databases, and their accession number and strain are indicated. Those in bold letters were used in the phylogenetic analysis and the abbreviation used in it is shown in parentheses.

Buffalopox: **BVU87233**, 81, (Buff81); **U87232**, 3906, (Buff3906). *Callithrix jacchus*: AY298786. *Camelpox*: **CVU87840**, Dubai-1992 CP-5, (CamelCP5); **AF438165**, M-96 Kazakhstan, (CamelM96); **AY009089**, CMS, (Camel CMS); **U87839**, Saudi-M3, (Camel SM3); **U87837**, Somalia-1978, (CamSom78); **U87838**, Iran CP-1, (CamelCP1); AY460597, IIL/VSD/1; AY102981, CP-1231 Haut Dubai. *Cowpox*: AY102955, EP-2; **U90228**, Munich OPV 91/1cat, (Cow0901); **X94355**, GRI-90, (CowGRI90), **U90227**, Munich OPV 85 human, (CowOPV85); **U90232**, Munich OPV89/4 cat, (CowOPV8974); **U90229**, CPV58, (CowCPV85); **U90230**, Munich EP2-1975 elephant, (CowEP275); **U90226**, Munich OPV 89/2 cat, (Cow0892); **U90231**, Munich 89/1cat, (Cow891); **U90233**, Munich OPV89/5 cat, (CowO895); **AF482758**, Brighton Red, (CowBR); **U90225**, Munich OPV 90/2 human, (Cow0902); **U90234**, Munich OPV90/1 cat, (Cow0911); **U90235**, Munich OPV90/5 cat, (Cow0905). *Ectromelia*: AJ567683, Naval; AF012825, U86380, Moscow; AJ567682, MP4; AJ567681, Ishibashi I-111; AJ567680, MP5; AJ567679, Hampstead; U86381, Mill Hill. *Monkeypox*: **MVU87845**, Zaire-1977 77-0666, (MonkZ77); **MVU87841**, **AF380138**, Zaire-1996 96-16 (MonkZ9616); **MVU87847**, Zaire-1979 79-0005, (MonkZ795); **MVU88543**, Zaire-1996 96-17, (MonkZ9617); **MVU87995**, clone CV1, (MonkCV1); **MVU87994**, clone CW-N1, (MonkCWN1); **MVU87846**, Benin-1978 78-3945, (MonkB78); **MVU87844**, Nigeria-1971 71-0082, (MonkNi); **MVU87843**, Sierra Leone-1970 70-0266, (MonkSL); **MVU87842**, Liberia-1970 70-0187, (MonkLib); **MVU88144**, UTC Rotterdam-1965, (MonkUTC); **MVU88143**, WMP Washington D.C. 1961, (MonkWMP); **MVU88142**, Zaire-1970 Congo-8, (MonkZ70). *Rabbitpox*: **RVU86873**, Utrecht, (RabUtrec); **AY484669** (Rabbit). *Taterapox*: **TVU86874**, Dahomey-1968 (TateraD68). *Vaccinia*: **U86872**, Venezuela, (VacVenez); **U86871**, **Y17729**, Lister, (VacList); **M35027**, Copenhagen, (VaccCop); **U87584**, Columbia, (VacColum); AF095689, Tian Tan; **AY243312**, **Y17730**, J02422, WR, (VacWR, VaWR); **VVU87585**, Temple of Heaven, (VacTH); **U94848**, Ankara, (VacAnk); **AY603355**, Acambis 3000 Modified Virus Ankara, (VacMVA); AY102945, Patwadangar; **AJ416892**, USSR, (VacUSSR). *Variola*: **VVU88148**; **U18341**, Minor Somalia-1977, (VarSom77, VarSM77); **VVU88147**, Major, Congo-1970 (CNG-70); **VVU88152**, Whitepox Chimp 9-4, (VarChi94); **VVU88151**, 'whitepox' Chimp 9-2, (VarChi92); **VVU88149**, **Y16780**, **U18339**, **X70841**, Brazillian alastrim minor Garcia-1966, (VaGar66, VarGar66, VarG66, VaGa66); **VVU88145**, Major Harvey-1944 (VarHAR44); **L22579**, Bangladesh-1975, (VarBan75); **X67117**, **X69198**, India-1967, (Varind67); **U88150**, minor Sierra Leone-1968 (VarSLN68); **U88146**, minor Butler-1952 (VarBUT62)

Fig. 1. Gel documentation of amplified DNA from VACV. A fragment of 491 bp was obtained when nested PCR was carried out with DNA from VACV. Two serial dilutions of this DNA have been assayed. MW: DNA marker, Neg: DNA obtained from Vero cells.



Results

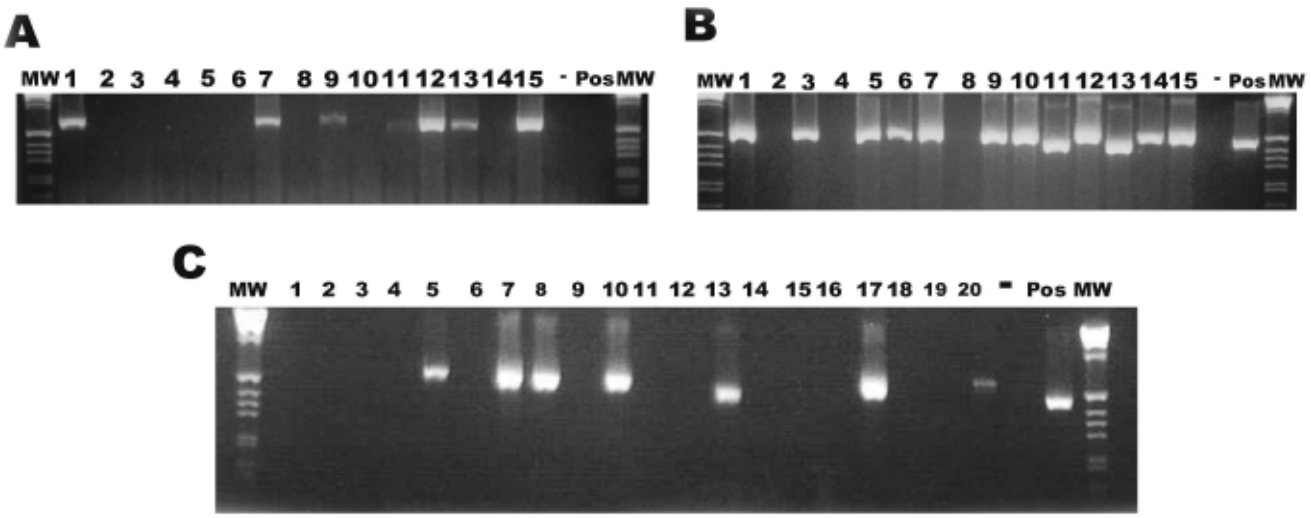
Generic amplification

Primer and Mg^{2+} concentrations and thermocycling parameters were standardised by experimentation to achieve the highest levels of sensitivity and specificity. The conditions described above were the ones selected; however, the reaction also works in a wide range of salt conditions and annealing temperatures (data not shown).

A band of the correct size (656 bp) was obtained after first amplification of VACV DNA and also a correct one after the corresponding nested reaction (491 bp) (Fig. 1).

The method also amplified purified CPXV (Brighton strain) and RPXV (strain Utrecht; not shown) and MPXV, CPXV (81/102 and Brighton strains) and CMLV spiked in human plasma samples (Fig. 2), giving rise to bands with slight differences in size according to their sequences. ECTV was not detected because of a deletion[17] in the selected region (Fig. 2). The method is specific for members of this genus, as can be seen in Figure 2, were no bands were obtained with other members of the family.

Fig. 2. Gel documentation of amplified DNAs from EQCs. Results from the first quality control are shown in the upper part. Bands obtained after first (A) and nested (B) reactions are shown. DNA from MPXV (Lam87 strain) corresponding to less than 1,6E+06, 1,7E+05, 2,8E+04, 4E+03, 4E+02, 8E+01 and less than 50 copies per tube were amplified in channels 12, 1, 10, 5, 14, 3 and 6, respectively. DNA samples from CMLV (channel 7), CPXV 81/02 and Brigthon strains (channels 9 and 15, respectively), VACV, Elstree and modified strains, (channels 13 and 11, respectively), ECTV (channel 4) and negative plasma samples (channels 2 and 8) were also assayed. MW: DNA marker, Neg: H_2O, Pos: VACV WR strain. Results from the second quality control are also shown (C). DNA from MPXV corresponding to less than 8,3E+04, 8,3E+03, 8,3E+02, 83, 83 and 8,3 genome equivalents (ge) were amplified in channels 7, 10, 8, 5, 20 and 1, respectively. CPXV (channel 17), VACV (channel 13) and other poxviruses not belonging to the orthopoxvirus genus (tanapoxvirus and parapoxvirus, channels 12 and 19, respectively), samples with inhibitors added (6, 9, 14 and 15) and negative samples (2, 3, 4, 11, 16 and 18) were also assayed. MW: DNA marker, Neg: H_2O, Pos: VACV WR strain.



Sensitivity

Sensitivity of the reaction was calculated in pfu/tube and in genome equivalents (copies)/tube. With this aim, the extracted DNA of serial dilutions of a titred VACV stock was used. It was possible to detect approximately 1 pfu/tube using the nested reaction (Fig. 3).

To establish the limit of detection in copies/tube, the product of the first amplification of VACV was cloned, as described above. Dilutions of plasmid containing this insert were assayed. The sensitivity was 5–50 copies/tube (not shown). These results are equivalent to those obtained in the first and second EQC, in which the limit of detection was below 50 copies or between eight and 83 genome equivalents, respectively (Fig. 2).

Identification and grouping of sequences

Double-stranded DNA products amplified in the nested PCR were sequenced directly. Using the BLAST tool, the sequences of 13QC2002 and the control of VACV (named 'vaccinia') showed the greatest homology with VACV strain WR; 11QC2002 and 13QC2004 showed comparable homology with VACV strain Ankara; sequences of 15QC2002, 17QC2004 and the control CPXV strain Brighton (named 'cowpox') with CPXV strain Brighton; the sequence of the control RPXV strain Utrecht used (named 'rabbitpox') showed the best score with RPXV strain Utrecht; the sequences of 1QC2002, 3QC2002, 5QC2002, 6QC2002, 10QC2002, 12QC2002, 14QC2002, 5QC2004, 7QC2004, 8QC2004, 10QC2004 and 20QC2004 showed homology with MPXV sequences; and the sequence of 7QC2002 with that of the CMLV CMS strain.

A more precise analysis was performed by selecting the sequences of the amplified products and equivalent sequences, which corresponded to the viruses indicated above, and aligning them with the Clustal programme. MEGA3 software was used to construct a tree based on their relationships. This method was able to divide orthopoxvirus sequences perfectly into different main groups and to identify them (Fig. 4). Variola virus groups showed a bootstrap value of 100, and the same result was obtained with MPXV. There is another group in which CMLV strains are grouped and another one that includes VACV and related (RPXV, CPXV and buffalopox) viruses that form a heterogeneous group.

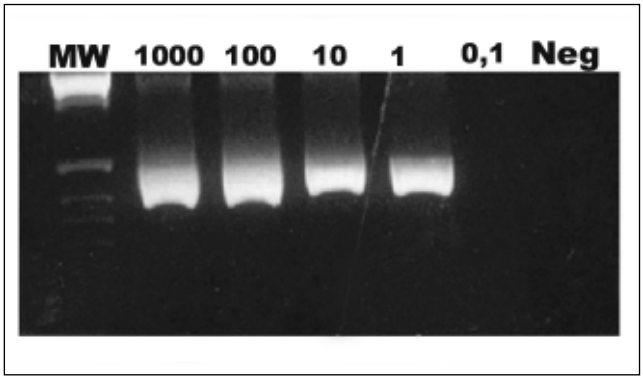


Fig. 3. Gel documentation of amplified DNA from serial dilutions of VACV. DNA obtained from serial 10-fold dilutions of a titred stock of VACV was amplified. DNA marker, Neg: H_2O

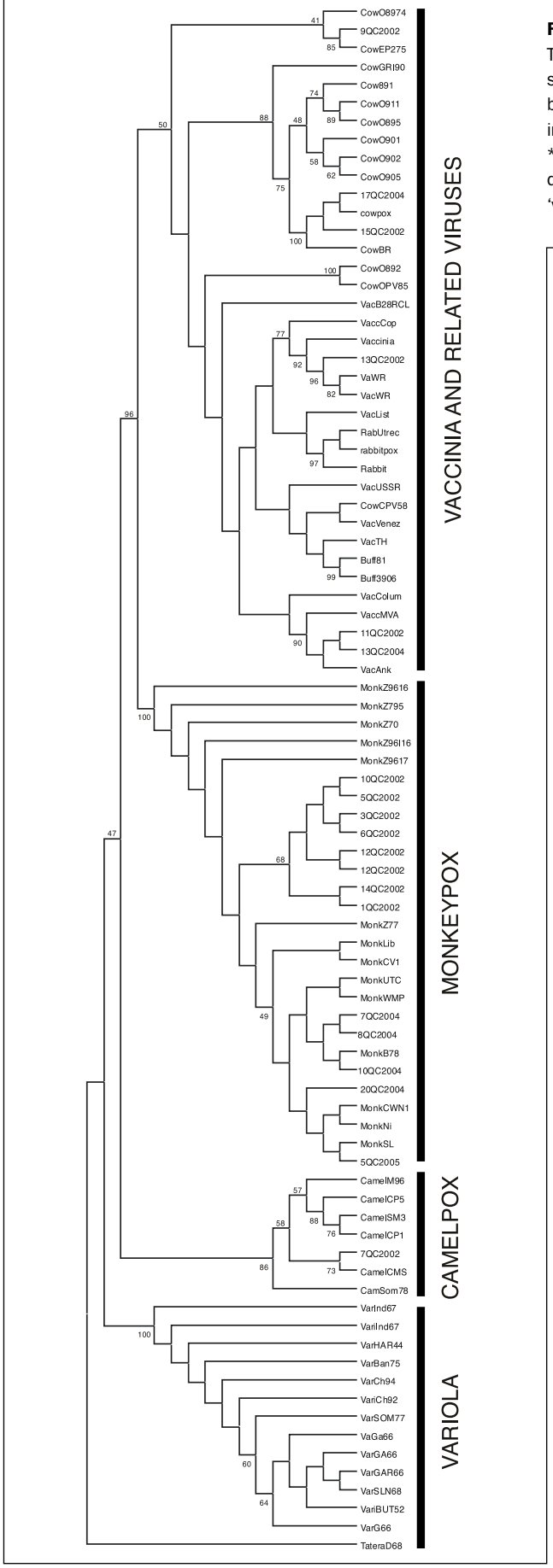


Fig. 4. Phylogenetic tree obtained with orthopoxvirus sequences. The tree shows the relationships between some orthopoxvirus strains. Each number at a node represents the value obtained by bootstrapping test. Sequences obtained from databases are identified in the text (Materials and methods). Those sequences designated *QC2002 correspond to the ones used in the first EQC, the ones designated *QC2004 correspond to the second EQC and 'rabbitpox', 'vaccinia', 'cowpox' and 'camelpox' to the ones amplified in this study.

Discussion

The most important pathogen of the Orthopoxvirus genus is the eradicated VARV virus that is now causing concern among the public health community because of its possible utilisation by bioterrorist groups. However, other agents of this genus (e.g., MPXV) have also raised an alert due to the possibility of outbreaks and spread in regions free of these agents. The outbreak in USA caused by the uncontrolled import of African pets illustrates this point.[18]

Good systems for surveillance of emerging pathogens, based on rapid and reliable techniques to achieve relatively simple, rapid and accurate detection and diagnosis, would be very useful for the identification and control of what could amount to devastating orthopoxvirus infection.

Electron microscopy provides rapid detection of orthopoxviruses. However, specific and sensitive detection and identification of members of this genus are needed, and this can be achieved through molecular approaches (e.g., methodologies based on real-time PCR).

The technique described in this paper is a rapid, sensitive and easy method that can detect a wide spectrum of orthopoxviruses. Subsequent identification by sequencing of the amplified product provides the real possibility of obtaining useful data on viral circulation for molecular epidemiology.

Although care must be taken when working with nested PCR techniques in order to avoid contamination, their higher sensitivity is very useful when clinical samples or fomites are being used. Furthermore, sequencing of the amplified product permits differentiation of strains and the ability to monitor their circulation, not only in the clinical context but also in the case of a bioterrorist attack.

Owing to the sequences in conserved regions of most orthopoxviruses in the tumour necrosis factor receptor II homologue gene, the primers used in the reaction are able to amplify a huge number of orthopoxviruses (including all those that can cause human illness) and do not amplify related poxviruses not belonging to this genus.

All orthopoxviruses tested were amplified with a good level of sensitivity, with the exception of ECTV in whose genome there is a deletion in the corresponding region.[17] Viruses representing all those orthopoxviruses able to cause human illness have been assayed and detected and identified accurately.

Many assays for orthopoxvirus detection and identification have been developed in recent years, due to increasing concern associated with these infections. Most of the assays are based on real-time PCR methods,[4,19–22]

although methods based on hybridisation[23,24] also have been described. Real-time PCR methods are usually rapid, reliable and sensitive, but higher equipment and reagent costs mean that they are not easy to implement for many laboratories. Equipment and reagents used in microarray-based technologies are also expensive and require special expertise.

Methodologies based on classic PCR technology are easier to perform, and some assays have been described for orthopoxvirus detection;[25–27] however, virus discrimination is achieved by identifying punctual differences in positions in their genome and thus the risk of virus mutation cannot be ruled out. The nested format is used in many laboratories worldwide for routine diagnosis because access to more modern technologies is not available to them. Contamination is avoided by using trained professionals and suitable equipment and controls.

The present study describes a simple technique based on a nested PCR method, followed by sequencing, which does not need special equipment or expertise. The ability to perform PCR, agarose gel electrophoresis and sequencing is all that is required.

Analysis of the data presented in this paper shows that results can be obtained in one working day. Furthermore, data from the sequences obtained are extremely valuable, as they provide the correct identification of the viruses detected.

In order to assess the level of diagnostic proficiency, an external quality assurance study was carried out through ENIVD. Results obtained using the methods described here (Fig. 2) were accurate for all samples except an ECTV (a virus that infects mice but is not considered infectious to humans),[28] which could not be amplified because of a deletion in the selected region. Samples in which inhibitors (not identified by the ENIVD) were added were not amplified, so the need for an internal control is highlighted in order to avoid false-negative results.

Comparison of results obtained with the method described here against those obtained by the other 22 participating laboratories in the EQC scheme is favourable. Participation in these external controls and the results obtained validates the technique for use in the detection and identification of orthopoxviruses. The design of the method described here makes it suitable for detecting variola virus; however, it was not available for study and thus cannot be demonstrated.

In conclusion, the nested PCR technique described can be used to detect and identify orthopoxviruses causing human infection in both the diagnostic and the surveillance setting. □

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