Tracing movement of African buffalo in southern Africa

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Summary
Genetic characterisation of two pathogens, namely foot and mouth disease (FMD) virus and Mycobacterium bovis, isolated from African buffalo (Syncerus caffer) in southern Africa was used to determine the origin of buffalo in situations where the source of infection was obscure. By determining the phylogenetic relatedness of various FMD virus isolates using partial sequencing of the main antigenic determinant, VP1, the origin of buffalo moved illegally to the non-endemic region of South Africa was traced to the Kruger National Park (KNP) where FMD is endemic in the buffalo population. Comparative analysis of the ‘genetic fingerprints’ of bovine tuberculosis isolates from buffalo and cattle has aided in tracing the original source of infection of buffalo populations in the KNP. Furthermore, these analyses have assisted in tracing the origin of infected animals that have been moved to other parts of South Africa.

Keywords

Introduction
The use of techniques that enable analysis of the genomes of infectious agents combined with statistical methods for deriving relationships between them is being used increasingly to advance the understanding of the epidemiologies of a number of important livestock diseases (13, 22, 27, 29, 34, 48, 61, 66). The previously painstaking and often unreliable process of determining the origin of infections such as foot and mouth disease (FMD) and classical swine fever (CSF) that suddenly appeared in new locations can now be a rapid and reliable process, if adequate background data are available (28).

Infectious agents, and ribonucleic acid (RNA)-containing viruses in particular, are able to mutate rapidly. This can result in geographically separated populations evolving independently, allowing representatives of such separated microbial populations to be identified (so-called toptypes) (7, 12, 25, 42, 53, 65). Subsequent spread of these toptypes to new locations can therefore be traced by genome analysis. Thus, for example, the recent intra- and intercontinental spread of the pan-Asian O toptype of FMD virus, which has caused serious epidemics in locations as far apart as the Indian subcontinent, South-East Asia, South Africa and the United Kingdom, is well known (40, 54).

This approach is being used increasingly for other important infections of animals, some of which are discussed here. However, these techniques provide another possibility that was not originally anticipated, namely the use of genome analysis of infectious agents to identify the origin of animals that harbour them. The rate at which the genomes of infectious agents mutate is several orders of magnitude faster than the rate of mutation of mammalian genomes (33). This means that differentiable mutations occur much faster than in the mammalian hosts. Therefore, the identification of the probable origin of animals may be possible by studying the infectious agents harboured by animals where more usual methods for animal identification have failed. This method is particularly useful where particular populations of animals have been isolated for relatively long periods of time, e.g. within wildlife reserves or on closed-herd farms. So far, the infectious agents that have been used are principally those that are potentially pathogenic, because information on these agents is usually more detailed than that on non-pathogenic agents. However,
This paper will focus on the use of FMD viruses and Mycobacterium bovis to determine the probable origin of African buffalo (Syncerus caffer) using specific examples. These animals are a valuable resource in southern Africa where their status as one of the ‘big-five’ game species makes them highly sought after for both hunting and eco-tourism. Unfortunately, these buffalo can maintain the three South African Territories (SAT) types of FMD viruses, Theileria parva (the causative agent in southern Africa of Corridor disease (CD)) and M. bovis (which causes bovine tuberculosis), making the animals potentially dangerous for domestic cattle with which they may come into contact. For this reason, the prices paid for buffalo differ, depending on whether or not the animals are infected with these agents (which they may harbour without apparent ill effect). In 1998, breeding animals that were infection-free were valued above US$16,500 each, whilst FMD-infected buffalo were valued at between US$500 and US$1,000. Those infected with T. parva were worth between US$4,300 and US$7,200 (64). At that time, the population of buffalo in South Africa was estimated at 31,500, of which 67.1% were in sub-populations infected with FMD viruses and T. parva (Kruger National Park (KNP)), 25.2% were infected with T. parva only (Hluhluwe-Umfolozi Game Reserve (HUP)) and 7.7% were free from infection with these agents (referred to locally as ‘disease-free’) (64).

The buffalo populations in HUP and KNP are also the only populations in South Africa known to be infected with M. bovis. Bovine tuberculosis (BTB) is becoming a significant problem in wildlife world-wide (11, 14, 15, 19, 47, 55) and has severely affected nature conservation and ecotourism in South Africa (35, 36, 37). Since the mid-1980s, BTB has become established as an important disease of wildlife in the major game reserves of South Africa (18, 26). African buffalo serve as effective maintenance hosts because of their relatively high susceptibility to M. bovis, herd structure and characteristic behavioural patterns (49). Once a buffalo herd becomes infected with M. bovis, the prevalence can exceed 90% (49). Under these conditions, spill-over of the infection to other species occurs and has been demonstrated in a number of species, such as lion, kudu, leopard, cheetah, baboon (9, 35), hyena, genet and bushpig (D.F Keet and D. Cooper, personal communication). Furthermore, infected buffalo pose a threat to cattle, and therefore also their owners, when the fences separating livestock and wildlife areas are breached.

In South Africa, buffalo from FMD- and CD-infected herds may be moved only within veterinary control areas that are registered with the Directorate of Veterinary Services, Department of Agriculture and that comply with the fencing regulations for premises holding buffalo (Animal Disease Act 1984, Act No. 35). Most African buffalo in the KNP become infected with the three SAT-type FMD viruses at an early age. Following initial sub-clinical infection, a high proportion become carriers of the disease (58). Single buffalo can maintain the virus for five years or longer in the oesophago-pharyngeal region, while an isolated herd of buffalo was shown to maintain infection for up to twenty-four years (17). These carriers can, albeit rarely, infect susceptible livestock, notably cattle, after direct and prolonged contact between the two species (16, 24, 32, 63).

In persistently infected African buffalo, SAT-type FMD viruses have a mutation rate of between $1.54 \times 10^{-2}$ and $1.64 \times 10^{-2}$ substitutions per nucleotide per year in the region coding viral protein 1 (VP1) (63). Furthermore, SAT-type viruses evolve independently in different regions (7, 39, 62), in accordance with the topotype concept (53). This localised, yet highly variable evolution of FMD viruses is useful for tracing the origin of epizootics and has previously been applied to demonstrate interspecies transmission under both experimental (24, 63) and natural conditions (6, 23).

In South Africa, genome typing of M. bovis isolates by restriction fragment length polymorphism (RFLP) has been useful for tracing the origin of individual infected animals as well as in investigating the transmission of BTB within and between animal species (44). Generally, RFLP analysis is an accepted method for characterising strains of Mycobacterium tuberculosis and the closely related M. bovis (20, 46, 56, 60). The most commonly used marker is the insertion sequence IS6110. Gel patterns are produced with fragments generated by treatment of bacterial deoxyribonucleic acid (DNA) with restriction endonucleases. This provides information on both the number of insertions and the relative sites of insertion within the genome. In studies on human tuberculosis, epidemiologically unrelated isolates have a high degree of polymorphism of the IS6110 RFLP gel pattern, while epidemiologically related isolates have identical or similar (one band variation) patterns (60). Furthermore, the IS6110 gel patterns of M. tuberculosis have sufficiently high degrees of stability to allow epidemiologically valid studies to trace outbreaks of M. tuberculosis strains with high copy numbers of IS6110 (45, 57). In strains with only a few copies of this insertion sequence, including M. bovis, the accuracy and discriminatory power of strain classification must be increased by including an additional genetic marker, such as the highly repetitive polymorphic guanine- and cytosine-rich repeat sequence (PGRS) (2, 21, 51) in the analysis.

This paper expands on this theme by detailing a case that demonstrated the usefulness of sequencing data of the main antigenic determinant of FMD virus, to determine the geographical origin of FMD-infected buffalo moved illegally. This case also illustrates the accuracy with which illegally moved buffalo can be traced. Furthermore, the authors describe the potential use of RFLP analysis for M. bovis isolates from infected buffalo, to trace the origin of these animals.
Materials and methods

Study area and background case details: foot and mouth disease

In August 1998, seven juvenile African buffalo were transported to a farm in the Potgietersrus district in the Northern Province of South Africa (Fig. 1). The buffalo, which were sold as ‘disease-free’ animals, were allegedly purchased from a farm in the Free State Province. Both farms are outside the FMD control area. Suspicion was raised when only photocopies of movement permits were presented. The buffalo were immediately quarantined and serum and probang samples collected for serological screening and virus isolation. As soon as infection with FMD virus was confirmed, the buffalo were destroyed and intensive serological surveys conducted in the area surrounding the purported source farm to ensure that the disease had not spread from the buffalo to other susceptible animals in the vicinity. For comparative purposes, SAT-3 viruses recovered from FMD-infected buffalo populations in South Africa, Zimbabwe and Zambia were also selected. These included viruses from the KNP in South Africa, Chikwarakwara and Gona-re-zhou in southern Zimbabwe, Hwange National Park in western Zimbabwe, Matusadona National Park, the Urungwe Safari Area in northern Zimbabwe and Kafue National Park in Zambia.

Virus isolation

Primary porcine kidney (PK) cells were inoculated with 10% suspensions of ground tissue and secretions recovered from probang samples or lesions and studied for the development of cytopathic effect (CPE). The viruses recovered were typed by enzyme-linked immunosorbent assay (ELISA) (50).

Serology

Antibodies to FMD virus SAT 1, 2 and 3 were measured using a liquid phase blocking ELISA (30).

Molecular characterisation methods

The C-terminal half of the 1D (VP1) region of the FMD virus genome was amplified and sequenced using primers developed for molecular epidemiological studies of SAT-type viruses in southern Africa, as described previously (3). Nucleotide sequences were generated by direct sequencing of the purified polymerase chain reaction (PCR) product, after which the data were aligned (31) and used to infer a phylogeny (41). The phylogenetic analysis methods employed followed those advocated for genetic resolution of FMD viral relationships based on partial VP1 sequence data (43).

Study area and background case details: bovine tuberculosis

The following discussion focuses on two separate outbreaks of BTB in which molecular typing was useful in identifying the origin of disease.

First case

In 1990, the first case of BTB in African buffalo was diagnosed in the southern part of the KNP, from which location the disease spread in a northerly direction. Infected cattle may have been the origin of the epidemic; according to official reports of the Directorate of Veterinary Services, tuberculous cattle had been identified on various farms south of the KNP in the period between 1955 and 1987 (38). Circumstantial evidence pointed to the involvement of a specific farm (Farm A) in the Barberton district of Mpumalanga Province where cattle had reportedly mingled and shared grazing with buffalo during the 1950s and 1960s (Fig. 1). The farm was subsequently depopulated in 1992. Isolates of M. bovis from several cattle from Farm A and buffalo from the KNP were analysed by RFLP in 1997.

Second case

Before tuberculosis infection was diagnosed in the HUP (the second largest game reserve in South Africa), buffalo from this conservation area were used to stock other game parks and private farms in various parts of the country. In two of these operations, buffalo were diagnosed with tuberculosis six months after translocation from the HUP (P. Morkel and N.P.J. Kriek, personal communication). In both cases, the...
buffalo were moved to properties in the Northern Cape Province (Fig. 1). Traceback of the origin of this infection was considered crucial for two principal reasons. Firstly, if the buffalo had carried the infection at the time of leaving the HUP, this implied that the single (negative) intradermal tuberculin test performed before translocation was insufficient to ensure effective movement control. Alternatively, the buffalo could have contracted the infection after reaching their various destinations, in which case the unidentified source of infection could still pose a risk to the remaining wildlife populations in the new conservancies.

**Bacterial culture and restriction fragment length polymorphism analysis**

*Mycobacterium bovis* was cultured from tissue samples submitted for routine diagnosis and processed as described previously (8). Subcultures of the primary isolates in either 10 ml of 7H9 Middlebrook broth or on Löwenstein-Jensen medium with pyruvate were treated with a final concentration of 0.2M glycine on the penultimate day of incubation. Isolation of DNA was performed as described by van Soolingen *et al.* (59). For hybridisation with the IS6110 and PGRS probes, 1.5 µg of DNA was digested with PvuII and AluI respectively. Electrophoresis and DNA transfer to a nylon membrane was performed as described by Skuce *et al.* (56). The entire region encoding IS6110 was amplified by PCR using a digoxigenin (DIG)-labelled primer (56). Prehybridisation, hybridisation and detection were performed according to the instructions supplied by the manufacturer of the DIG-labelled probes. DIG-labelled DNA molecular weight marker VII was used on one central and both outer lanes as an external marker. The DNA patterns generated with the IS6110 probe were analysed using commercial software (similarity coefficient of Dice, unweighted pair group method with arithmetic averages [UPGMA], maximum tolerance 1.2%), while the PGRS fingerprints were analysed manually.

**Results**

**Foot and mouth disease**

Serology and isolation of foot and mouth disease virus

Sera obtained from 1,295 cattle, 784 sheep and one buffalo in the vicinity of the source farm in the Free State Province were negative for the three SAT-types (results not shown). Serum samples recovered from the seven juvenile buffalo indicated the presence of antibodies to the three SAT-types with high titres against SAT-3 (results not shown). SAT-3 FMD viruses were isolated from three of the seven buffalo allegedly moved from the Free State to the Potgietersrus area.

Genetic characterisation of foot and mouth disease viruses

Nucleotide sequence determination and phylogenetic analysis based on partial VP1 sequences revealed that SAT-3 viruses cluster within four distinct genetic lineages (I-IV) (Fig. 2), that correspond with geographically discrete sampling localities (4). These genetic lineages correspond to the following geographical regions:

- lineage I: southern Zimbabwe and South Africa
- lineage II: Botswana and western Zimbabwe
- lineage III: Zambia
- lineage IV: northern Zimbabwe.

Within lineage I, five discrete genotypes are discernable, labelled A-E (Fig. 2). Genotype A comprises viruses exclusively from Chikwarekwarara (100% bootstrap support), genotype B contains viruses from two different buffalo herds occurring within the Gona-re-zhou National Park. Included within the latter genotype is a virus obtained from a buffalo from Hwange

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**Fig. 2**

Neighbour-joining tree (Jukes and Cantor method) based on partial VP1 gene nucleotide sequence data, depicting genetic relationships of SAT-3 foot and mouth disease viruses from southern Africa

Bootstrap values based on 1,000 replications and ≥ 95% are indicated in bold. Viruses recovered from illegally-moved buffalo are indicated in bold and italic. The major phylogeographical lineages are indicated as I-IV, in accordance with previously identified SAT-3 buffalo topotypes (4), whilst lineage I genotypes are denoted A-E. Genotype A contains viruses from Chikwarekwarara; genotype B contains viruses from two different herds in the Gona-re-zhou National Park; genotype C contains viruses from the buffalo found at Potgietersrus; genotype D contains viruses from the northern Kruger National Park (KNP) and genotype E contains viruses from the southern parts of the KNP. Lineage II contains viruses from Botswana and western Zimbabwe; lineage III viruses from Zambia, while lineage IV contains viruses from northern Zimbabwe.
Genetic characterisation of bovine tuberculosis

First case

Molecular typing of *M. bovis* isolates from domestic cattle from Farm A (TB871) and wild animal species: buffalo (TB1795, TB435, TB428, TB442, TB643, TB664), lion (TB571), baboon (TB600) and kudu (TB1773) from the KNP demonstrated that all isolates shared an identical gel pattern, indicating that the BTB infection in wildlife in the KNP had probably originated from that farm (Fig. 3). *Mycobacterium bovis* isolates from cattle on Farm A and wildlife in the KNP had identical fingerprint patterns using both the IS6110 (Fig. 3) and PGRS (results not shown). The single kudu isolate from a locality close to Farm A (TB636) was unrelated to any of the other isolates.

Second case

Buffalo that were translocated from the HUP to the Northern Cape and showed clinical signs of BTB after movement, had identical RFLP patterns to other isolates from the HUP (TB1006, TB1042, TB1044) (Fig. 3). This was a clear indication that the buffalo were sub-clinically infected at the time of movement and that the skin test had failed to detect that infection.

Fig. 3

Dendogram based on computer-assisted comparison of IS6110 restriction length polymorphism patterns from *Mycobacterium bovis* isolates obtained from different domestic and wildlife species

The animals sampled were: 1) free-ranging wild animals in the KNP: TB435, TB428, TB442, TB1773, TB1795, TB571, TB643, TB664 and a domestic cow (TB871) from Farm A; 2) greater kudu (TB636) from a farm in close proximity to Farm A; 3) buffalo from the HUP, including those translocated to the Northern Cape Province: TB1006, TB1042, TB1044; 4) cattle from different geographical regions within South Africa: TB1226, TB1302, TB681, TB631, TB1729, TB1048, TB709, TB1130, TB999, TB810, TB1057, TB1311, TB781
Discussion

A major driving force in the illegal trade in African buffalo is the difference in price between ‘clean’ and ‘diseased’ buffalo, which results from the limited availability of infection-free buffalo and the increasing demand for animals of this status. Given the role of this wildlife species in transmitting diseases to livestock, the trade in African buffalo must be closely monitored to enable irregularities to be identified rapidly and parties found to be transgressing laws ensuring disease control brought to justice. Furthermore, the traceback of possible sources of outbreaks in domestic cattle is important for more efficient disease control.

Most buffalo in southern Africa occur within wildlife reserves and have been separated from other sub-populations for many years. The same applies to the pathogens found in these animals which have been evolving at a much faster rate than their hosts, as already explained. The genetic diversity (or lack thereof) of pathogens isolated in particular situations can therefore be used to trace the origin of the animals, as long as an extensive database on these pathogens is available.

Phylogenetic analysis of the SAT-type FMD viruses derived from African buffalo in southern Africa indicates that viruses evolve independently in discrete geographic localities and that in some cases the virus populations are specific to a game park (4, 6, 62). This provides a powerful tool for tracing the origin of FMD-infected buffalo, as demonstrated by the fact that a virus isolated from the Gona-re-zhou National Park in southern Zimbabwe, was grouped with viruses from the Hwange National Park in north-eastern Zimbabwe (100% bootstrap support) (Fig. 2). This grouping was unexpected as the Hwange National Park viruses had previously been demonstrated to differ significantly from those isolated in the south of the country. However, further investigation showed that buffalo from which the virus was recovered had previously been moved from the Hwange National Park to Gona-re-zhou.

The genetic characterisation of viruses obtained from buffalo suspected as having been moved illegally in South Africa in 1998 clearly indicated that these buffalo were likely to have originated from the northern half of the KNP. Seropositivity of these buffalo to all three FMD viruses and T. b. orixnalis originated from the northern half of the KNP. Seropositivity of 1998 clearly indicated that these buffalo were likely to have suspected as having been moved illegally in South Africa in

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The gel patterns of M. bovis isolates from buffalo in the KNP and HUP differ significantly (Fig. 3). Therefore, by establishing the RFLP pattern of BTB isolates obtained from buffalo whose origin is suspect, it would be possible to establish whether the animals originated from the KNP or HUP. Due to the restricted occurrence of FMD in South Africa, FMD-free buffalo would not be expected to originate from the KNP, but may have been obtained from HUP or various infection-free regions in South Africa. Should FMD virus be detected in buffalo in South Africa, the region in the KNP (or other location outside the country) from which the buffalo originate could be determined by comparing the FMD virus isolated with known representatives of topotypes identified in southern Africa. If infected with M. bovis, it is possible to determine whether the buffalo originated from the HUP or KNP.

Conclusions

The wider use of genetic characterisation of animal pathogens and the information available in genome databases has led to the identification of other applications for this information. The use of genetic information to trace the possible origin of African buffalo in southern Africa is a positive spin-off from the databases that have greatly assisted the understanding of the epidemiology of FMD and BTB in southern Africa. However, this novel application cannot be used in isolation, and further epidemiological information should be considered before reaching conclusions on the possible origins of animals. Nevertheless, the technique offers the exciting possibility of tracing the origin of buffalo, thereby playing a role in the prevention of the spread of animal pathogens in South Africa.

Acknowledgement

The authors would like to thank Ms E. Kirkbride for the graphics in Figure 1.
Le traçage des déplacements de buffles en Afrique australe

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Résumé
Le typage génétique de deux agents pathogènes, à savoir le virus de la fièvre aphteuse et Mycobacterium bovis, isolés chez le buffle d’Afrique (Syncerus caffer) en Afrique australe, a été utilisé pour déterminer l’origine de buffles dans des situations où il était difficile de déterminer la source de l’infection. En établissant la relation phylogénétique de divers isolats du virus de la fièvre aphteuse à l’aide du séquençage du principal déterminant antigénique, VP1, on a pu tracer l’origine de buffles introduits illégalement dans la région non enzootique de l’Afrique du Sud, et remonter ainsi jusqu’au Parc national Kruger où la fièvre aphteuse sévit à l’état enzootique parmi la population de buffles. L’analyse comparée des “empreintes génétiques” d’isolats de l’agent de la tuberculose bovine provenant de buffles et de bovins a permis de tracer la source de l’infection dont sont atteints les buffles du Parc national Kruger. De plus, ces analyses ont contribué à tracer l’origine d’animaux infectés qui ont été déplacés vers d’autres régions d’Afrique du Sud.

Mots-clés

Rastreo del movimiento de búfalos en África austral

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Resumen
La caracterización genética de dos patógenos (el virus de la fiebre aftosa y Mycobacterium bovis) aislados en búfalos africanos (Syncerus caffer) en África austral sirvió para dilucidar el origen de búfalos en situaciones en que la fuente de infección resultaba difícil de determinar. Gracias a la secuenciación parcial del principal determinante antigénico (VP1) de varias cepas del virus de la fiebre aftosa, pudo determinarse el grado de parentesco filogenético entre esas cepas, lo que a su vez sirvió para rastrear el origen de búfalos transportados ilegalmente a la zona no endémica de Sudáfrica. Esos animales resultaron provenir del Parque Nacional Kruger, donde la fiebre aftosa es endémica entre la población de búfalos. Por otro lado, el análisis comparativo de la “huella genética” de diversas muestras del bacilo de la tuberculosis bovina obtenidas en búfalos y ganado vacuno ayudó a localizar la fuente original de infección de las poblaciones de búfalos del Parque Kruger. Esos análisis resultaron de ayuda también para determinar el origen de animales infectados que han sido transportados a otras partes de Sudáfrica.

Palabras clave
References


