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The following bodies provided support for the conference:

Company	Contact details
Afrivet Business Management (Pty) Ltd	Dr Charles Gilfillan Tel: +27 (0)12 991 6416 Cell: +27 (0)82 573 0288 E-Mail: charles.gilfillan@afrivet.co.za Web address: www.afrivet.co.za
Botswana Vaccine Institute	Ernest Bagopi Tel: +267 3912711 Cell: +267 71373747
GIMS (Pty) Ltd	Senzo Nkonyane Tel: +27 (0)11 238 6300 Cell: +27 (0)82 889 0271 E-mail: snkonyane@gims.com Web address: www.gims.com
Intervet	Tel +27 (0)86 183 8838 Fax +27 (0)11 974 9320 Web address www.intervet.co.za
Kingfischer Conferences & Events cc	Ms Adrie Fischer Tel: +27 (0)12 991-8788 Cell: +27 (0)83 633-4500 E-mail: kingfischer@lantic.net
OBP	Mr. Willie Botha Tel: +27(0)12 522 1562 Cell: +27(0)82 8575885 E-mail: willie@obpvaccines.co.za Web address: www.obpvaccines.co.za
Pfizer Laboratories (Pty Ltd	Dr Chris van Dijk Tel: +27 (0)11 320 6000 Cell: +27 (0)82 789 4499 Web address: www.Pfizer.com
Quantec Research (Pty) Ltd	Me. Mariette van der Merwe Tel +27 (0)12 361 5154 Cell: +27 (0)82 415 4426 E-mail: mariette@quantec.co.za Web address: www.quantec.co.za

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Gauteng Chief Director of Veterinary Services and Sustainable Resource Management

PAPERS

STRENGTHENING VETERINARY SERVICES AS A MEANS TO TACKLE (RE)EMERGING ANIMAL DISEASES IN SOUTHERN AFRICA.

Mtei, B. J[1]., Bastiaensen, P.[1] & Funes G.[2]

[1] OIE Sub Regional Representation for Southern Africa, P. O. Box 25662, Gaborone, Botswana .Tel. + 267 391 44 24, Fax. + 267 391 44 17, E-mail b.mtei@oie.int
[2] OIE Regional Activities Department, 12 rue de Prony, 75017 Paris, France Tel. + 33 1 44 15 18 88, Fax. + 33 1 42 67 09 87, E-mail: g.funes@oie.int

1. INTRODUCTION

In recent years, emerging and re-emerging diseases, and especially zoonoses such as *Highly Pathogenic Avian Influenza* (HPAI) and *Rift Valley Fever* (RVF) have led to considerable efforts by the international community to prevent and control these diseases, in particular when these outbreaks occurred in less-developed African countries. Failing to do that it could be reasonable to assume that neither the veterinary nor public health services would be in a position to adequately prevent and or control such diseases. While the avian influenza crisis has led to a mobilisation of resources of an unprecedented scale, and has attracted public opinion's attention on animal diseases in general, it is unfortunate that this mobilisation phases-out when the public opinion looses interest. This phenomenon is currently happening as regards HPAI.

The *World Organisation for Animal Health* (OIE), as an inter-governmental organisation endowed with the mandate to develop international standards for health, welfare, safety and trade of animals and animal products worldwide, has increasingly become involved in the active support to less-developed countries worldwide, and especially in Africa, to meet international standards and to strengthen their capability to prevent and control animal diseases at source.

Currently, the OIE is working in close coordination and collaboration with other international organisations and donors in a new broader concept of "One World – One Health" which is beyond the Avian Influenza Crisis, focusing on other emerging and re-emerging diseases, including zoonoses.

In the OIE's view, diseases such as RVF will continue to emerge and re-emerge as international trade further develops and climate change leads to environmental distortions. Beyond emergency interventions, as a result of outbreaks, the OIE strongly advocates that Governments should first and foremost strengthen (in some cases, rebuild) their Veterinary Services, both for the public and the private sector veterinary delivery systems. No one can predict what animal disease will appear next to threaten the

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Throughout this paper, reference to the terms :Animals", Animal product(s), and animal diseases should be taken to mean both terrestrial and aquatic animals unless otherwise stated.

livestock sector and/or public health, but what is certain is that the intervention of efficient Veterinary Services will be required to prevent its introduction or spread and successfully control or eradicate the disease before it establishes itself in the animal population.

Deep awareness of all sectors directly or indirectly involved or affected by the activities of Veterinary Services is of a paramount importance in order to support their work

Without efficient Veterinary Services there can be no reliable information-gathering to justify international veterinary certification, which is a prerequisite for export of animals and/or their products.

At national level, in particular with specific reference to less developed countries, Veterinary Services play a crucial role in increased food production and food security, decreased qualitative and quantitative malnutrition and income generation.

Since 1924, when the OIE was established, tremendous advances have been made in terms of setting international standards and guidelines on animal health and welfare. These are contained in the Terrestrial and Aquatic Animal Health Codes and Manuals (1).

In this paper, we would like to present highlights of some of the provisions in the OIE Code with regards to quality and good governance of Veterinary Services, with special reference to Southern Africa.

2 DEFINITIONS AND CONCEPTS

OIE standards can be categorised as either horizontal or vertical. Horizontal standards are those dealing with generic aspects, such as ethics of international trade and quality of national Veterinary Services as conditions for importing countries to trust and recognise health certificates accompanying consignments of animals or their products in cross-border trade.

Vertical standards are those dealing with recommendations on OIE listed diseases. These include methods to be applied by national Veterinary Services to conduct surveillance to detect, respond, control and contain animal diseases and eventually gain recognition of disease freedom. In the specific disease chapters, guidelines are stipulated aimed at avoiding transboundary spread of diseases during exports of live animals or animal products.

Under the general provisions, section one of the *Terrestrial Animal Health Code (TAHC)* deals with definitions of which the most important relating to Veterinary Services are *Veterinary Authority*, *Official Veterinarian*, *Veterinary Para- professional*, and *Veterinary Statutory Body* (VSB).

Veterinary Authority is the Governmental Authority of a Member Country, comprising veterinarians, other professionals and para-professionals, having the responsibility and competence for ensuring or supervising the implementation of animal health and welfare measures, international veterinary certification and other standards and guidelines in the Terrestrial Animal Health Code in the whole country.

Official veterinarian is a veterinarian authorised by the Veterinary Authority of the country to perform certain designated official tasks associated with animal health and/or public health and inspections of commodities and, when appropriate, to certify in conformity with the provisions of Section 1.2. of the Terrestrial Animal Health Code.

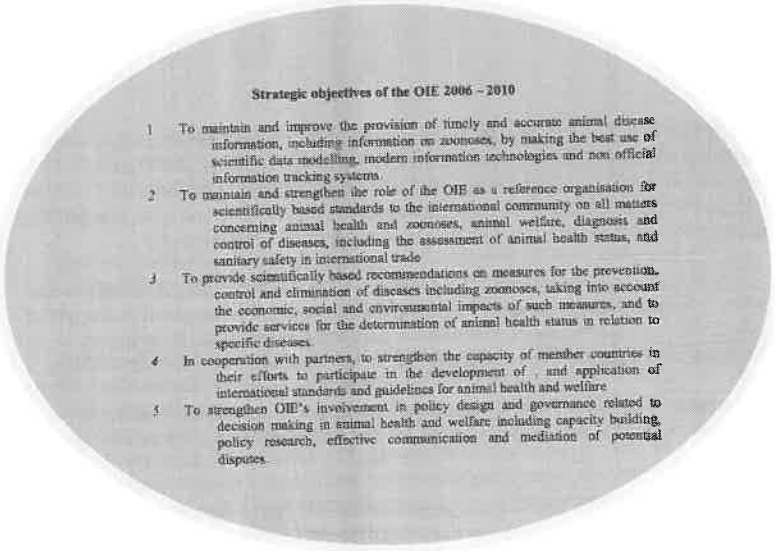
Veterinary Statutory Body is an autonomous authority established with legal mandate to licence and regulate veterinarians and veterinary para-professionals in a give country.

Veterinary para-professional is a person authorised by the Veterinary Statutory Body to carry out designated tasks in a country delegated to him or her under the supervision an official veterinarian. Tasks authorised for each category of *Veterinary para-professionals* are defined by the Veterinary Statutory Body depending on qualifications, training and according to need.

The national Veterinary Services consists all of the above, including government and non governmental organisations as well as all persons registered and licensed by the VSB. It is the sovereign right of a country to adopt the most appropriate structure depending on the Veterinary Services' legal status, development of the private sector and organisation of the Ministry responsible for livestock and or other competent authorities. The previous differentiation made between *Veterinary Administration* and *Veterinary Authority*, which sometimes led to confusion in matters of certification, was abolished by the OIE *International Committee* in May 2007.

3. OIE POLICY REGARDING VETERINARY SERVICES

OIE objectives are enshrined in its vision and mission to improve animal health throughout the world as stipulated in the current strategic plan 2006 – 2010 (2). In relation to veterinary services, the OIE's concentration is on their good governance through improved legal framework and availability of resources for national animal health services as well as provision of expertise and promoting international solidarity in the prevention and control of animal diseases and zoonoses. This requires transparency in global animal disease notification and reporting, as a result of efficient surveillance systems and dissemination of scientific veterinary information.



Standards and guidelines published by the OIE are aimed at avoiding the introduction of pathogens through international trade of animals and animal products, while at the same time preventing countries from setting up unjustified sanitary barriers as stipulated in the *World Trade Organisation's* (WTO) *Sanitary and Phyto-Sanitary* (SPS) agreement (3).

In its mission, the OIE strives to influence global governance of animal health by campaigning, advocating, lobbying and engaging with national and multinational organisations to make informed investment decisions in animal health and welfare.

Priority investment areas are surveillance and diagnostic services, which should lead to early detection, identification and rapid response to protect countries and given animal and human populations from diseases. In so doing, considering that a proper partnership between official and private sector is crucial for the Good Governance of Veterinary Services, the private sector has an important role to play and should be delegated several functions such as animal health services delivery to including treatments, vaccinations and support to the public services during emergencies.

Like any other international technical agency, the OIE, being the leading organisation entirely devoted to animal health issues and in charge of animal health and welfare standard setting, has had to adjust with time in terms of its mandate and responsibilities Currently new concepts and opportunities are being addressed including animal welfare, food safety, and bioterrorism.

4 QUALITY OF VETERINARY SERVICES ACCORDING TO THE OIE *CODE*

Article 1.3.3.1 in the OIE *Terrestrial Animal Health Code* stipulates that Veterinary Services should conform to fundamental principles of 1) ethical, 2) organisational and 3) technical nature regardless of the political, economic and social situation of a country. These principles apply equally to other competent authorities other than the Veterinary Services such as those responsible for aquatic animal health or structures acting on behalf of the Veterinary Services.

In terms of ethics, professional judgement, independence, impartiality, integrity and objectivity have to be respected. Veterinary Services must be staffed by qualified personnel with skills and scientific knowledge, which is continuously updated through on the job training. Such personnel should be able to work without pressure or prejudice of political, commercial, financial or undue hierarchical nature.

Veterinary Services should also be delivered under non-discriminatory conditions and elements of fraud, corruption and or falsification must be identified and corrected without delays. Last but not least Veterinary Services must at all time act in an objective and transparent manner.

Well organised Veterinary Services should verifiably show that their management is based on quality assurance policies and a strategic plan indicating activities with timeframes of expected results. Other important components of a good organisational structure are effective communication, proper documentation as well as monitoring and evaluation (M&E) mechanisms.

The importance of a national chain of command within the VS hierarchy can't be overemphasized. Priority in terms of procedures and standards should be directed towards International Veterinary Certification (IVC) activities, epidemiological investigations, zoning and compartmentalisation where applicable. Zoo-sanitary border controls and import regulations must also be given due consideration with possible privatisation of veterinary laboratory diagnostic services and delivery of animal health care.

Responsible authorities should ensure that adequate human and financial resources are available and effectively used to implement Veterinary Services activities.

5 EVALUATION OF VETERINARY SERVICES

Evaluation of VS is a recent development within the OIE tasks but it is already imbedded into the *Terrestrial Animal Health Code*, Chapter 1.3.3.5. There are two major reasons why Veterinary Services could be evaluated: 1) every importing country has the right to evaluate the Veterinary Services of a potential trading partner; 2) Veterinary Services of a country may want to be evaluated to assess its strengths

and weaknesses with the aim of improving internal efficiency and/or to justify increased allocation of resources for investments.

The scope of Veterinary Services' evaluations according to the *OIE Tool for the evaluation of Performance of Veterinary Services* - the OIE PVS Tool (**4**) encompasses the following core components:-

- Human, physical and financial resources,
- Technical authority and capability,
- Interaction with stakeholders, and
- Access to markets.

Each of these components encompasses several critical competencies which can be rated depending on the level of advancement, which refers to the compliance of VSs with the relevant OIE standards regarding such specific topics. The PVS document is available on the OIE website http://www.oie.int/eng/oie/organisation/en_vet_eval_tool.htm?e1d2

The outcome of a PVS evaluation should demonstrate the Veterinary Services' capacity and capability for effective prevention and control of diseases affecting animals and animal products for the benefit of safe trade and that of consumers. In most cases quantitative data may be available but the ultimate outcome of the evaluation is qualitative because the tool focuses on evaluation of quality of outputs and performance of the Veterinary Services.

The benefits and outcomes of using the OIE *PVS Tool* include:

- An indication of overall performance for each of the four components

- A relative performance rating within each of the critical competencies

- A basis for exploring areas of cooperation between veterinary services in the region or globally,

- Identifying differences in the responses of stakeholders in order to arrive at shared points of view, and to strengthen the private public partnerships (PPP)

- Fostering a common understanding in order to achieve greater levels of advancement

- Helping to determine the benefits and costs of investing in VS and, when necessary, obtaining assistance from government and financial and technical cooperation agencies

- Providing a basis for establishing a routine monitoring and follow up mechanism on the overall level of performance of the Veterinary Services over time

- Helping to identify and present justifications and specific needs when applying for national and/or international financial support (loans and/or grants)

- Providing the basis for carrying out a process of verifying compliance with the OIE standards and assessments of Veterinary Services by external or independent bodies under the guidelines and auspices of the OIE.

Essentially there are three levels of evaluation depending on who conducts the evaluation. Self-evaluation is commonly referred to as "first party" while "second party" evaluation is normally conducted by a trading partner. A "third party" evaluation is done on request by a team of accredited experts under the auspices of the OIE. The latter is meant to assist developing and transitional countries to identify gaps, which can later justify priority investments by public and private sectors, either using national or external (donor) resources. Several donors have recognised the OIE-PVS as the official mechanism for evaluating VSs when countries apply for fund requests.

Next step, after the diagnosis from the OIE-PVS Evaluation, is a gap analysis process (based on findings and outcomes of such an evaluation) followed by preparation of priority investment projects.

6. CONCLUSION

Over the years, all SADC Member States have made efforts to comply with the OIE standards, rules and guidelines. Major advances have been made in terms of animal disease notification obligations through the online *World Animal Health Information System.* This transparency and improvement in data sharing benefits the international community at large in its task of animal disease surveillance and response. SADC Member States are represented by their respective OIE Delegates, who participate in the standard setting and the decision making processes of the Organisation. In addition, SADC Member States actively engage in the activities of the OIE Regional Commission for Africa, the OIE Regional Representation for Africa based in Bamako, Mali and the OIE Sub Regional Representation for Southern Africa based in Gaborone, Botswana.

The latter, was established to assist SADC Countries, and is currently carrying out capacity building activities aimed at improving national Veterinary Services and animal disease prevention and control policies in the region. Seminars and or workshops are designed involving stakeholders from public and private sectors, not only on terrestrial animal diseases, but also on aquatic animal diseases such as Epizootic Ulcerative Syndrome, which currently threatens commercial and subsistence fish farming in large parts of the Zambezi Chobe river basin.

Even in less-developed countries in Africa and elsewhere, OIE objectives are largely achievable if only Veterinary Services and in particular Veterinary Authorities, would endeavour to comply with the agreed international standards on animal health and

welfare. OIE considers Veterinary Services to be both an international and a national public good and hence improving their quality and good governance should be given due consideration. Thanks to the OIE, many financial institutions and donors have endorsed this position and it is now largely accepted as a condition for financing regional and national interventions in animal health.

It is recommended that the *Chief Veterinary Officer* (CVO) who is normally the national delegate of the OIE, should be as close as possible to the political decision making processes in order to ensure efficiency in the design and implementation of animal health and welfare policies. For the purpose of continuity, sustainability and protection of national interests, OIE member countries should avoid changing their delegates too frequently.

The importance of a single national chain of command under the Veterinary Authority, from the central level down to the lowest administrative level, remains a challenge in a lot of countries. The Veterinary Services' mission to manage animal diseases, zoonoses and food safety issues related to products of animal origin, should ideally be managed under one umbrella within the same authority to be able to implement the gold standard "from farm to fork".

7. REFERENCES

1. OIE 2007 - Terrestrial Animal Health Code
2. OIE 2005 - 4th Strategic Plan 2006 – 2010
3. WTO - Agreement on the Application of Sanitary and Phytosanitary measures.
4. OIE 2007 - Tool for the evaluation of Performance of Veterinary Services

LESSONS FROM BCG VACCINATION TRIALS AGAINST *MYCOBACTERIUM BOVIS* IN AFRICAN BUFFALO (*SYNCERUS CAFFER*)

L. de Klerk [1], A.L. Michel[2], R.G. Bengis[3], N.P.J. Kriek[4], J. Godfroid[4]

[1] South African National Parks, Private Bag X402, Skukuza, 1350 South Africa.
[2] ARC-OVI, Private Bag X05, Onderstepoort, 0110 south Africa.
[3] Office of the State Veterinarian, Kruger National Park, P.O. Box 12, Skukuza, 1350, South Africa.
[4] Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 South Africa. Tel: +27 12 529 9111.

ABSTRACT

Vaccination has been discussed as a practical option to control bovine tuberculosis in countries where a wildlife reservoir of the disease is present. African buffalo (*Syncerus caffer*) are the main wildlife reservoir of *Mycobacterium bovis* in South African game parks and vaccination is not only the most promising but the only ethically acceptable control measure currently available. Two independent studies on the use of bacillus Calmette Guerin (BCG) vaccine in African buffalo followed by experimental infection with *M. bovis* are described. The studies were conducted in captive and semi-free ranging buffalo, respectively. In both trials the BCG vaccine was administered twice intramuscularly, six weeks apart. All vaccinates and control buffaloes were euthanazed and necropsies performed nine months after the challenge. Standard sets of lymph nodes from the head, the thoracic cavity and abdomen were cultured and examined histopathologically. No significant reduction in number of lesions or severity of disease was noted in any of the two studies, concluding that the BCG vaccine did not induce sufficient protection able to limit the shedding of organisms. The age of the buffaloes, route of infection and prior exposure to environmental mycobacteria have been considered possible reasons for vaccine failure.

1. INTRODUCTION

The African buffaloes are the main reservoir and maintenance hosts of bovine tuberculosis in the Kruger National Park (KNP) ecosystem [1,2]. The disease has also spilled over into several other sympatric mammalian species since its discovery in 1990 [3-6]. Bovine tuberculosis (BTB) is a multi-host disease in a multi-species system and because of the large number of species in which BTB has already been detected, it is reasoned that vaccination currently appears to be the most practical, promising and ethically acceptable option.

When attempting to control this disease by vaccination, the immediate aim is to reduce the transmission of infection among the reservoir hosts and as well as spread to other species. To achieve this, the vaccine does not necessarily have to prevent primary infection, but more importantly, it should rather reduce the severity of the disease and subsequent mycobacterial shedding and contamination of the environment [9]. It has been shown that under cool, moist

and shady environmental conditions, *M. bovis* can survive outside its host for long enough to significantly increase the likelihood that other animals may become infected and develop disease [10,11]. Reduced contamination of the environment may therefore also limit the subsequent spread of disease.

BCG (Bacille Calmette-Guérin) is the only vaccine currently available for the control of tuberculosis in humans and animals that has proven safety and efficacy [12]. In the past, this vaccine has been tested in a number of domesticated and wildlife species, with a marked variability in efficacy [7,8,13-15], which may limit its suitability for use as a standard vaccine in domestic livestock and wildlife. Factors that have been shown to affect the efficacy of BCG vaccine in experimental protocols include the age of the animals when vaccinated, the method of vaccine formulation, delivery system and dosage, and whether single or multiple booster doses were used [16-18].

The efficacy of a vaccine for wildlife should preferably be evaluated in the target species itself [7]. When evaluating vaccine efficacy the experimental challenge should mimic natural transmission, in both route and dose under controlled conditions. Likewise, lesions produced should resemble those found in natural infections. This approach has the additional advantage of excluding complications associated with prior *M. bovis* infection, an intangible factor that may confound field studies of vaccine efficacy [19]. The intratonsilar method has been shown to be effective in creating BTB lesions that mimic natural infection in cattle [20], red deer [21] and African buffaloes [22]. With a suitable challenge model in place for buffalo, it was possible to aim for a study to evaluate the efficacy of the BCG (Pasteur 1173P2) vaccine in this species. A first vaccination trial in captive buffalo was considered unsuccessful. It could not be ruled out that external factors played a decisive role [23].

2. MATERIALS AND METHODS

The design of the vaccination study aimed at documenting natural transmission patterns of *M. bovis* from adult infected buffalo cows to the treatment groups of buffalo yearlings under semi-free ranging conditions. Given that no indication of transmission of *M .bovis* to the yearlings could be demonstrated after one year of contact, it was decided to conduct an experimental infection. This report describes the design and findings of the second vaccination trial, carried out under semi-free ranging conditions and providing opportunities for natural as well as experimental *M. bovis* challenge.

2.1 Animals, husbandry and monitoring

Thirty buffalo yearlings, varying in age from 10 – 20 months were captured during one week in October 2002 from four different BTB-negative buffalo herds in the northern districts of the Kruger National Park. Blood in heparin was collected from each buffalo for the interferon-gamma (IFNg) assay as well as bovine and avian tuberculin PPD injected intradermally for the comparative skin test as described previously [22]. The yearlings were transferred to an enclosure near the Shingwedzi camp (northern region of KNP) where they were kept for seven days until test results for the IFNgγ assay and skin test were available. All animals tested negative and were transported to the State Veterinary Unit enclosure in Skukuza, where they were randomly divided into two groups of 15 animals each. One group received a defined dose of BCG vaccine while animals in the control group were sham vaccinated (PBS). After a period of three months in these enclosures the yearlings were released into a larger camp (100 hectares) under semi-free ranging conditions, where they joined 27 adult buffalo cows that originated from known BTB infected buffalo herds in the south of the KNP [1,24] in the Crocodile Bridge region of the Park. All cows were skin tested while inside a smaller enclosure within the semi-free range camp. The test positive buffaloes were intended to serve as natural source of infection to the yearling buffalo.
Altogether 16 calves were born during the two-year study, of which ten survived.

2.2 Anaesthesia

A combination of etorphine hydrochloride (M99; Novartis SA Animal Health) and xylazine (Chanazine 2%; Centaur, Bayer Animal Health) at standard dosages used for routine buffalo immobilization was used to anaesthetize the yearlings for intratonsilar infection. Animals were revived by administering the antidote diprenorphine hydrochloride (M50/50; Novartis SA Animal Health) at twice the dosage of the M99.

2.3 Blood specimens and weight gain

During 2003 (the first year of the semi-free range study), blood samples were collected at capture (two months prior to primary vaccination) (-2 mths), at primary vaccination (0 mths), at booster vaccination (1.5 mths) and six months after primary vaccination (6 mnths). These samples were collected from all experimental buffalo yearlings, the calves as well as from cows that tested negative in the interferon-gamma assay.

After the experimental groups were challenged in 2004 (14 months after primary vaccination), blood samples were collected every eight weeks (16 mths, 18 mths, 20 mths, 22 mths) until one month prior to slaughter. Body conditions were scored throughout the study and all the carcasses were weighed at the abattoir after euthanasia at conclusion of the study.

2.4 BCG vaccine

The BCG seed stock (Pasteur 1173P2) was grown in 7H9 Middlebrook broth (Difco Laboratories, Detroit, USA) supplemented with 10% OADC (3.83g NaCl, 25g BSA, 15ml sodium oleate and 20ml of 50% glucose in 465ml of distilled water) and 0.05% Tween 80 (VWR International, Merck House, UK) at 37°C, without shaking, to the mid-logarithmic growth phase. Bacteria were harvested by centrifugation, washed three times and the re-suspended bacteria were enumerated using a phase contrast microscope. The actual number of colony-forming units was determined retrospectively by plating out serial dilutions of the inoculum dose. Plate counts for the semi-free range study reflected a dosage of 3.2×10^7cfu for the primary and of 4.4×10^7cfu for the booster vaccination.

2.5 Experimental infection procedure

After one year of the study no evidence of natural *M. bovis* transmission was evident by IFNg assay and it was hence decided to challenge the buffaloes via the intratonsilar route. All the buffalo yearlings were experimentally infected on two consecutive days with the first group

receiving 1 x 10^3 cfu and the second group 6 x 10^2 cfu of *M. bovis*, respectively, using the method as previously described [22].

2.6 *Mycobacterium bovis* strain

The challenge strain (TB 1088), a field isolate from KNP, possessed a unique IS*6110* RFLP pattern, different from the genotypes commonly found prevalent in the buffalo herds of the southern region of KNP (Michel, in prep.).

2.7 Laboratory tests

Interferon-gamma (IFNg) assay

A modified protocol for the IFNg assay applicable in buffaloes was used [25,26]. The responses to mycobacterial antigens were used as markers of response to BCG vaccination [27,28].

2.8 Comparative intra-dermal tuberculin test

The method for the intra-dermal tuberculin test and its interpretation was applied as described previously [22] on all cows and both treatment groups of buffalo yearlings on the day of capture.

2.9 Euthanasia

At the end of the study, all experimental and in-contact buffaloes were euthanased using 2 - 3ml of a saturated solution (850mg/ml) of succinyl dicholine chloride (Scoline) intramuscularly by darting. The jugular veins were cut for exsanguination.

2.10 Necropsy procedure

The buffalo carcases were taken to the abattoir and immediately subjected to a detailed meat inspection and post mortem examination. Specimens of all lesions detected in any of the other lymph nodes or organs, were collected and processed for mycobacterial culture and histopathology. Specimens from head lymph nodes, thoracic lymph nodes, abdominal lymph nodes and carcass lymph nodes were pooled, respectively.

2.11 Bacterial isolation

Pooled specimens were collected as indicated and stored in sterile containers at -20°C. At the end of the study the frozen specimens were transferred to the Tuberculosis Laboratory of the ARC-Onderstepoort Veterinary Institute. The specimens were processed and mycobacterial culture and identification of isolates were performed as described by Bengis et al. [29]. Cultures were considered negative for *M. bovis* if no growth was detected after 10 weeks incubation.

2.12 Statistical methods

Analysis of variance (ANOVA) was the method of choice for statistical analyses performed on the IFNg data [30].

3. RESULTS

3.1 Laboratory tests

Interferon-gamma assay

All the experimental yearlings tested negative for BTB on the IFNg assays at capture. Two months later, at the time of administering the primary vaccination with BCG, the buffaloes were tested again and all were found negative after stimulation with bovine PPD. Differences between the optical density values (bovine PPD stimulated plasmas only) of the two treatment groups (vaccine and control), are indicated in Fig. 1.

Five months after the booster vaccination a significant difference could be detected between the IFNg responses to bovine PPD ($P < 0.01$) between the vaccinated and control animals and was still detectable one-year post vaccination when the animals were challenged (Fig. 1). Following challenge with live *M. bovis*, an increase in IFNg responses was observed in vaccinated and control animals. The responses in control animals were higher than in vaccinated ones. Simultaneous IFNg reactivity to avian PPD was observed sporadically in some animals but at a significant level, 12 months post vaccination in six vaccinated and two control buffaloes (results not shown).

3.3 Comparative intra-dermal tuberculin test

All buffalo yearlings were found to be negative in the comparative tuberculin skin test at capture. Fifteen of the 24 in-contact cows had positive results on the intra-dermal tuberculin test (results not shown).

3.4 Pathology and culture

Control animals

Eight of the thirteen animals had macroscopic lesions while nine were culture positive for *M. bovis*. For one buffalo (LM1~) with macroscopic lesions, confirmed by histopathology to be typical for bovine tuberculosis, the culture result was negative.

Vaccinated animals

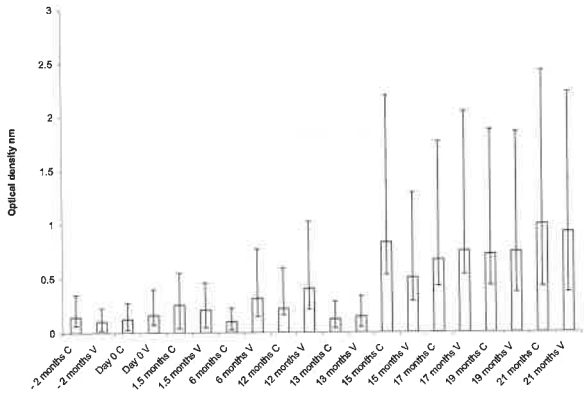
Seven of the fourteen animals had macroscopic lesions while eight were positive for *M. bovis* on bacterial culture. One male buffalo (LM12~) which showed no macroscopic lesions and was culture negative for *M. bovis*, histopathological examination revealed a typical granulomatous mycobacterial reaction. The strain used for experimental infection, which showed a unique IS*6110* RFLP profile, was isolated from both control and vaccinated animals.

In-contact cows

Fourteen of the 24 cows were found to be positive for *M. bovis* on culture . *Mycobacterium bovis* was cultured from eleven of the thirteen cows with macroscopic lesions consistent with

bovine tuberculosis. In addition, *M. bovis* was isolated form three cows with non-visible lesions. The strains were typed and showed the same IS*6110* RFLP profile typical for strains isolated from buffaloes in the southern region of KNP (strain C8, Michel in prep.). This profile was indeed different from the profile of the strain (TB 1088) used for experimental infection. Histopathological examination of two cows with macroscopic lesions revealed granulomatous lesions consistent with those of bovine tuberculosis.

Figure 1: Comparison between the optical densities of bovine PPD stimulated plasmas in control (c) and vaccinated (v) animals from capture until slaughter



-2 months: Day of capture
Day 0: Primary vaccination
13 months: Day of experimental challenge with live *M. bovis*

4. DISCUSSION

Horizontal disease transmission between the in-contact cows and the experimental buffaloes failed, despite the fact that the majority of in-contact cows could be considered as potent source of infection. Eleven of the 17 culture positive cows presented with macroscopic lung lesions, of which nine represented cases of disseminated tuberculous pneumonia. One of these cows even had to be euthanased due to severe emaciation because of generalised tuberculosis (LM 47) half-way through the study. The distribution of lesions confirms that aerosol is the most important mode of natural disease transmission between buffaloes and this is strongly supported by the presence of macroscopic lesions and isolation of *M. bovis* from pulmonary tissues of very young calves born to the in-contact cows during the study. But it also demonstrated that the opportunity for close contact with infected animals was, on its own, not sufficient to effect disease transmission. It should be noted here that the yearlings did not interact closely with the infected in-contact cows, because they were introduced from different social groupings and this was aggravated by the fact that the majority of cows had their own offspring they were associated with. Essentially the same observation was reported for natural transmission of *M. bovis* between captive brushtail possums [31]. We therefore conclude that regular and close physical contact i.e. by licking, grooming, suckling between animals over a longer period of time is necessary for successful disease transmission to occur. Our results suggest that after one year of contact, no transmission from infected cows to in-contact calves occurred, whereas transmission from infected cow to offspring took place. Typing of the strains isolated after experimental and natural infection in the different groups of animals strongly supports this.

If vaccination of animals can lead to a lesser degree of disease resulting in a reduced risk of animals becoming shedders of *M. bovis,* there may be merit in pursuing vaccination as the most or only feasible option for controlling BTB in free-ranging buffalo populations. The establishment of an infection model for BTB in African buffaloes [22] enabled us to conduct a vaccination trial to assess the efficacy of BCG in this reservoir species. The outcome of this study indicates that the BCG vaccine does not protect buffaloes under semi-free-ranging conditions from BTB infection and disease.

In trying to identify the potential causes and contributing factors for the failure, we reviewed those with a reported impact on vaccine efficacy including route of administration, booster applications, infection dose, immunogenicity, exposure to non-tuberculous mycobacteria, age and type of target species [32]. The intramuscular vaccine delivery has been preferred over the more commonly used subcutaneous route for reasons of practicality in vaccinating large buffalo herds from the air. Boostering was previously found necessary to protect against establishment of infection in deer [19] and since no data were available for buffalo the same concept was adopted. The infectious dose of *M. bovis* that was used, induced typical lesions of tuberculosis in our previous study, establishing the *M. bovis* experimental infectious model in buffalo. The use of an *M. bovis* strain showing a different RFLP profile than the anticipated natural infection strain was critical in assessing transmission patterns.

Recently, it has been shown that different BCG strains (lyophilised or in solution formulations) induced the same level of protection although the immune responses measured in the IFNg test was different. This particular study suggested that there is no correlation between the amount of IFNg release after in vitro stimulation and the degree of protection conferred [27].

We compared the increase in IFNg response between the vaccinated and control animals at pre- and post-vaccination intervals. There was a significant difference between IFNg response in the bovine PPD stimulated plasmas of the vaccinated and control groups at six months post vaccination, demonstrating a biological activity of BCG in the vaccinated buffalo. This was still persistent one year after vaccination. It must, however, be mentioned that IFNg reactivity in the vaccinated animals was not only detected to bovine PPD but also to avian PPD, Twelve months after BCG vaccination responses to both bovine and avian PPD occurred in six animals in the vaccination group as compared to two animals in the control group (results not shown), Buddle et al. [33] reported similar observations for the skin test in BCG vaccinated cattle.

A strong IFNg response does not necessarily mean protection to subsequent challenge [27], but may be the result of non-specific stimulation following infection with environmental mycobacteria [34]. We have shown in a parallel investigation to the vaccination study that the water in the buffalo camp contained various environmental non-tuberculous mycobacteria (NTM) [24], which could have negatively influenced the immune response of the buffaloes to *M. bovis*. Buddle et al [35] in fact reported that sensitisation to *Mycobacterium avium* adversely affected the protective efficacy of the BCG vaccine. Similar findings have been published on the effects of other environmental mycobacteria [36,37]. All together these findings suggest that sensitisation to NTM took place and may have adversely affected the protective efficacy of the BCG vaccine.

Literature reports provide indications that the age of the vaccinates has a direct influence on the vaccine efficacy. Neonates and very young animals are usually better vaccine responders [12, 38], which may be due to younger animals having less exposure to environmental mycobacteria and because maternal immunity may have protected them from infection. The ages of the experimental animals were generally underestimated during selection of the groups for capture and the majority were older than 12 months at the start of the study, which could have compromised vaccine efficacy.

The virulence of the KNP *M. bovis* strain used for challenge might have had an influence on vaccine efficacy but unfortunately very little is known about the virulence of different *M. bovis* strains throughout the world [39].

Because no vaccine is currently available which meets these requirements, vaccination alone cannot be expected to successfully limit the spread of the disease and should ultimately be used in combination with other control measures. A combined strategy of vaccinating the young cohorts and culling the more severely affected older individuals could be considered as a possible future solution to limit the spread of BTB and to reduce the prevalence of the infection in the KNP buffalo populations.

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Notes

SUMMARY OF FOOT AND MOUTH DISEASE VIRUS ACTIVITY (FMD OUTBREAKS) IN MPUMALANGA PROVINCE, LIMPOPO PROVINCE AND KRUGER NATIONAL PARK SINCE 1970 TO 2007.

Dyason, E.

140 Wessel Street, Polokwane, 0699, South Africa. Cell phone: 082 889 380, Fax: +27 15 294 4537, Email: dyasone@agric.limpopo.gov.za

INTRODUCTION

A large number of Foot and Mouth (FMD) outbreaks occurred since 1970 up to 2007 within the provinces of Limpopo, Mpumalanga as well as within the Kruger National Park (KNP), South Africa. These outbreaks were reported in various single, weekly, monthly and annual reports by various offices and government institutions within these described areas. The aim of this paper is to summarise all available data into one report. This data could be used to evaluate trends, patterns etc. for specifically epidemiology research purposes.

Foot and Mouth disease is an important economic disease in that it is a trade sensitive disease and the lack of efficient control could lead to trade embargos on susceptible products. In the affected area three strains of FMD occur namely SAT1, SAT2 and SAT3. The African buffalo (Syncerus caffer) which occurs within the KNP and many of the adjacent private nature reserves on the western borders of the KNP are carriers of all three strains of FMD. These carrier buffalo are the possible source of FMD outbreaks in impala within the KNP and adjacent nature reserves and in cattle within farming areas bordering the KNP complex.

The need for reliable recording of FMD outbreaks and proper safe keeping of data is of utmost importance.

MATERIALS AND METHODS

Annual Reports of the Assistant Director Eastern and North Eastern Transvaal, Director Northern Province (Limpopo province) and Limpopo FMD outbreak reports (1-21, 23, 24, 36, 37,39,40,41, 43, 44, 45) were studied and all relevant information regarding FMD outbreaks since 1970 up to 2007 were collected and summarised into a table Information collected include the date of confirmation of the outbreak (which is confirmed by the typing and isolation of the specific SAT strain), the SAT strain, the farms or areas affected in each outbreak in sequence with dates affected where information was available. The property affected was also summarised according to its current local municipality. The species affected and the time it took to control the disease was collated. The control usually continued two to three months after the last clinical case was seen to ensure the disease was under control and to comply with international disease control standards. The outbreak was classified as a focal or local infection when less than 5 areas/farms where affected or as spreading or extensive when more than 5 areas/farms were affected.

The information collected was verified and rectified/adjusted with information from reports from various files with the Director Veterinary Services Limpopo Province (42, 46, 47, 48, and 82). Annual Reports Director Veterinary Services available on the National Department of Agricultures website (25-35, 38) and Annual Reports from the State Veterinarian Skukuza (49-80). It must be noted that self governing states were in place during the reporting period and outbreaks within these self governing

states like Venda and Gazankulu were reported as a new outbreak even if it was a continuation of an existing outbreak from a bordering area. This is seen in their reports and in the Director Veterinary Services reports. In these cases the information regarding the outbreak from the different reports were collated into one outbreak.

The information was compared with known publications (22, 81 and 83) and again adjusted where discrepancies or differences occurred.

In three incidences the SAT strain could not be confirmed namely in January 1975, in November 1980 and April 1983 all within the KNP and in impala. In the January 1975 FMD outbreaks in the centre of the KNP it is assumed for the purpose of this summary that both SAT1 and SAT2 were present within the outbreak as both strains were confirmed during December 1974 in the southern parts of the KNP and it could even be argued that the outbreak could be a continuation of the FMD outbreak from the southern parts. The November 1980 outbreak is assumed for the purpose of this summary to be SAT1 as three FMD outbreaks occurred in the following two years within the KNP and all were confirmed to be SAT1 and there were no SAT2 outbreaks prior, during or after this outbreak. The April 1983 outbreak is assumed to be a SAT2 virus as there was both prior and subsequent outbreaks within the KNP that were confirmed to be SAT2 while no activity of any other strains was detected.

Data collected were compared with each other to determine any patterns. The strains were tabled against the year of occurrence and against the months of a calendar year. In both comparisons a distinction was made between an outbreak within the KNP and an outbreak outside the KNP. This was to determine any spread between the two distinct areas.

RESULTS OBSERVATIONS

The data collected is summarised in Table 1 according to the month and year of the FMD outbreak. Fifty two outbreaks occurred during this time period in the target area. Seventeen were SAT 1 of which ten occurred in the KNP in impala and seven outside the KNP in mainly cattle. Thirty two outbreaks of SAT 2 occurred of which eighteen were within the KNP in impala and fourteen outside the KNP in mainly domestic stock. Only three outbreaks of SAT 3 occurred and all three were outside the KNP and two were in cattle and one in a buffalo breeding project.

TABLE 1 SUMMARY OF FMD OUTBREAKS SINCE 1970 TO 2007 IN LIMPOPO, MPUMALANGA PROVINCES AND THE KNP.

DATE CONFIRMED	SAT STRAIN	NUMBER OF AREAS / FARMS AFFECTED	LOCAL MUNICIPALITIES AFFECTED	ANIMALS AFFECTED	DURATION OF CONTROL
24 04 70	2	ENTIRE KNP	KNP	IMPALA	11 m
11 02 71	1	ENTIRE KNP	KNP AND NKOMASI	IMPALA	17 m
10 73	1	ENTIRE NKP	KNP	IMPALA	8 m
07 11 73	2	2	BUSHBUCKRIDGE	CATTLE	?
29 04 74	1	5	MARULENG	IMPALA CATTLE	?
14 06 74	2	7	BUSHBUCKRIDGE KNP AND MARULENG	CATTLE OVINE CAPRINE IMPALA	?
09 74	2	3	KNP	IMPALA	Shortly
10 74	1	SEVERAL	KNP	IMPALA	15 m
12 74	1; 2	SEVERAL	KNP	IMPALA	13 m
01 75	1,2	SEVERAL	KNP	IMPALA	11 m
29 02 75	1	1	NKOMASI	CATTLE	?
14 06 77	2	5	NKOMASI	CATTLE	5 m
14 06 1977	2	13	MBOM BELA	CATTLE	12 m
25 06 77	2	28	PHALABORWA AND GIYANI	CATTLE	7 m
10 77	2	SEVERAL + 1	KNP AND BUSHBUCKRIDGE	IMPALA	11 m
21 07 78	2	2	KNP	IMPALA	6 m
10 04 79	2	5	GIYANI AND THULAMELA	CATTLE	4 m
14 06 79	2	1	BUSHBUCKRIDGE	CATTLE	5 m
19 06 79	2	23	GIYANI, MAKHADO AND THULAMELA	CATTLE	5 m
12 07 79	2	5	MARULENG	CATTLE	3 m
22 11 79	1	12	MUTALE AND THULAMELA	CATTLE	?
13 12 79	3	32	GIYANI AND THULAMELA	CATTLE	9 m
21 07 80	1	2	THULAMELA	CATTLE	4 m
11 80	UNKNOWN	2	KNP	IMPALA	5 m
02 04 81	2	2	MUSINA	CATTLE	3 m
06 81	1	2	KNP	IMPALA	6 m
10 81	1	1	KNP	VARIOUS	8 m
08 82	1	3	KNP MARULENG AND BUSHBUCKRIDGE	IMPALA	5 m
02 83	2	SEVERAL	KNP	IMPALA	5 m
04 83	UNKNOWN	1	KNP	IMPALA	4 m
06 07 83	2	1	PHALABORWA	CATTLE	4 m
07 83	2	2	KNP AND BUSHBUCKRIDGE	IMPALA	4 m
21 11 85	2	1	KNP	IMPALA	5 m
26 09 88	2	4	KNP AND BUSHBUCKRIDGE	IMPALA	11 m
07 92	2	SEVERAL	KNP	IMPALA	6 m
09 93	2	1	KNP	IMPALA	4 m
12 93	2	1	KNP	IMPALA	2 m
09 95	2	SEVERAL	KNP AND NKOMASI	IMPALA	17 m
05 98	1	3	MARULENG, KNP AND BUSHBUCKRIDGE	IMPALA	9 m
12 99	2	1	KNP	IMPALA	7 m

TABLE 1 Continuation

DATE CONFIRMED	SAT STRAIN	NUMBER OF AREAS / FARMS AFFECTED	LOCAL MUNICIPALITIES AFFECTED	ANIMALS AFFECTED	DURATION OF CONTROL
01 09 00	1	1	PHALABORWA	BUFFALO CATTLE	1 m
24 11 00	1	1	MIDDELBURG	CATTLE	5 m
15 12 00	1	61	NKOMAZI	CATTLE	4 m
01 02 01	2	30	BUSHBUCKRIDGE	CATTLE	7 m
09 01 02	3	1	PHALABORWA	BUFFALO	2 m
03 02	2	2	MARULENG AND KNP	IMPALA	9 m
01 03	2	2	MARULENG AND KNP	IMPALA	7 m
01 02 (07 02)	1	1	NKOMAZI	BUFFALO	2 m
08 08 03	2	3	MUTALE	CATTLE	4 m
10 03	1	1	NKOMAZI	BUFFALO	2 m
26 06 04	2	38	PHALABORWA AND GIYANI	CATTLE	8 m
08 04	2	1	MARULENG	BUFFALO	12 m
01 08 06	3	2	THULAMELA	CATTLE	4 m

TABLE 2 Comparison of the different SAT strains to the month that the disease was confirmed.

MONTH	SAT 1		SAT 2		SAT3	
	KNP	ADJ	KNP	ADJ	KNP	ADJ
JAN	1	1	2	0	0	1
FEB	1	1	1	1	0	0
MRT	0	0	1	0	0	0
APR	0	1	2	2	0	0
MAY	1	0	0	0	0	0
JUN	1	0	0	7	0	0
JUL	0	1	3	2	0	0
AUG	1	0	1	1	0	1
SEP	0	0	4	0	0	0
OCT	3	1	1	0	0	0
NOV	1	2	1	1	0	0
DEC	1	0	2	0	0	1
	10	**7**	**18**	**14**	**0**	**3**

KNP = Kruger National Park
ADJ = Areas adjacent to the KNP which could be private nature reserves and/or farming areas.

DISCUSSION

The main aim of this document is to summarise recent FMD outbreaks into one document. In many recorded outbreaks within the KNP throughout the study period it was not determined for certain that a specific outbreak was a new outbreak or that was it a continuation of a previous outbreak that was just not seen for some time or that it had spread to a different area without being detected. In the study of Thomson (81) certain outbreaks were clustered together as one outbreak and others seen as separate outbreaks. This study did the same but ended up with different clustering and separation of outbreaks. The reason for this is the complexity of a FMD outbreak in a large nature reserve like the KNP where it is difficult to monitor disease outbreaks efficiently especially when the disease changes into a clinical form that is not easily detected. Virus identification will probably answer some of these discrepancies. This however does not change the fact that a FMD outbreak did occur and that veterinary control was needed to monitor and control the outbreak.

Most of the outbreaks are clearly a separate outbreak but some could easily be a continuation of an existing outbreak. Evaluating outbreaks that occurred close to each other and could have been one outbreak are the detailed below.

The June 1974 and September 1974 could be an SAT2 outbreak that started in the communal cattle of Bushbuckridge and then spread to the KNP through the Sabi Sabie complex. The September 1974 SAT2 outbreak could be the same outbreak that was confirmed in December 1974 in the south of the KNP and could also be the same that was confirmed in January 1975 in the centre of the KNP.

The October 1974 SAT1 outbreak in the KNP and the December 1974 outbreak could be one outbreak and could also be the source of the February 1975 outbreak in the Nkomazi area.

The two SAT2 outbreaks in June 1977 which occurred in the Nkomazi and Mbombela areas could easily be one outbreak because of their relative proximity and population linkages.

The June 1977 SAT2 outbreak in Phalaborwa, the August 1977 outbreak in Giyani and the September 1977 outbreak in Letaba was described as three outbreaks being in three different governments jurisdiction but these are one outbreak and is reflected as one outbreak.

The two SAT2 outbreaks during April and June 1979 in the Giyani and Thulamela areas could easily be the same outbreak.

The two outbreaks within the KNP during February 1983 and April 1983 could be one outbreak.

No pattern could be seen between the different strains both inside and outside the KNP over the study period of thirty seven years. It seems that a certain strain does predominate for a time and than another strain takes over.

Regarding spreading of FMD from within the KNP to areas outside the KNP and vice versa the following could be highlighted:

A FMD outbreak started in the KNP during February 1971 and both cattle and impala outside the KNP where infected with the same strain during August 1971. The nine cattle where together with impala in a game camp and both species became infected. It is not known which were infected first.

The SAT2 outbreak during June 1974 was first diagnosed in cattle and later in impala within the Sabie Sabie and Manyeleti Nature Reserves. This could also be the same virus that spread into the KNP that was confirmed during September 1974.

The two SAT1 outbreaks during September 1974 and December 1974 within the KNP could be one outbreak but one of these caused an outbreak in nineteen cattle that was confirmed during February 1975 outside the KNP.

The SAT2 outbreak that was confirmed during October 1977 within the KNP spread and infected cattle bordering the KNP during February 1978.

Since the early 1980 the fences between the KNP and adjacent private nature reserves were removed, resulting that FMD outbreaks that started in the one area did spread the other but no domestic livestock were infected. These outbreaks were seen in August 1982, July 1983, September 1988, November 1995, May 1998, March 2002 and January 2003.

It can be stated that there was no serious FMD outbreak during the last 37 years that commenced within the KNP and spread to domestic livestock outside the KNP. There was no FMD outbreak within the KNP that spread to domestic livestock since 1977. The more serious FMD outbreaks in domestic livestock during these 37 years occurred unrelated to any outbreaks within the KNP and the causes of these outbreaks are an investigation on their own.

Comparing the different strains to the month in which they was confirmed is seems that SAT1 occurs more in summer and SAT2 in winter while SAT3 had no pattern.

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ANTHRAX OUTBREAK IN THE NORTHERN CAPE PROVINCE

Kegakilwe, P.S., De Vos, V., Reardon, T., Dekker, A. & Oosterhuizen, J.

1. INTRODUCTION

Anthrax is primarily a disease of herbivorous animals, although all mammals, including humans can contract it. Mortality can be very high, especially in herbivores. The aetiological agent is the spore-forming, Gram-positive rod-shaped *Bacillus anthracis,* the only obligate pathogen within the genus *Bacillus.* Most of the other species of *Bacillus* are common ubiquitous environmental saprophytes, although a number, notably *B. cereus, B. licheniformis* and *B. subtilis*, are occasionally associated with food poisoning in humans and with other clinical manifestations in both humans and animals.

The initiation of an outbreak depends on interrelated factors, which include specific properties of the bacterium, environmental factors, factors affecting dissemination of the organism, and certain human activities. Sporulation and subsequent dissemination of the spores are dependent on the carcass being opened, as the bacteria are rapidly killed by putrefaction.

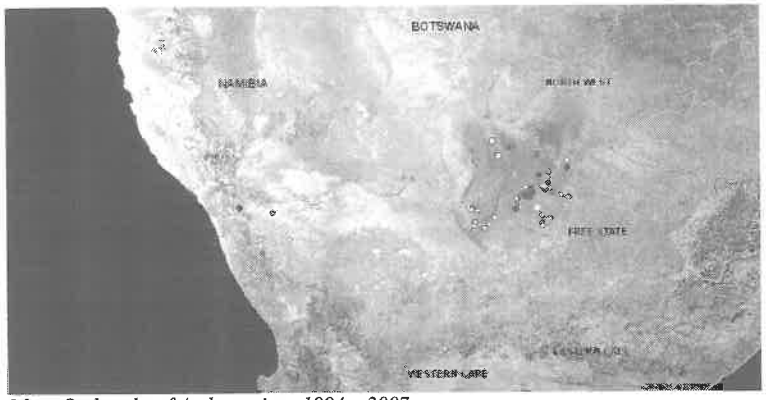
2. HISTORY IN THE N. CAPE

The first report of anthrax in animals in South Africa was in 1838 at Henkries in the Northern Cape. The first description in humans was by Robert Moffat in 1842 and the Batswana people even had a name for the disease which they called Kwatsi.

All reports reviewed have indicated that Anthrax has been a feature of the Northern Cape and in recent times, there were epidemics in 1995/96, 2000/01 and now in 2007/08 in the same areas. This suggests a 5-10 year cycle which is suspected to be linked to an increase in the Kudu (*Tragelaphus strepsiceros*) population (Trek bokke). There are still free-roaming Kudu in certain parts of the province and anecdotal evidence and recent observations by farmers and officials is that Kudu population has increased.

In the 1995/96 outbreak of Anthrax the province invited Dr MM Henton from Onderstepoort Veterinary Institute to especially investigate whether burning carcasses can aid in the spread of the disease. The conclusion was:

> "Burning creates up-draughts by which performed spores could be swept upward. The fire would destroy some of the spores, but others will remain suspended in the air or dispersed by the wind. The spores could have originated from the carcass itself or the soil underneath the carcass. The number of viable spores cultured was small."

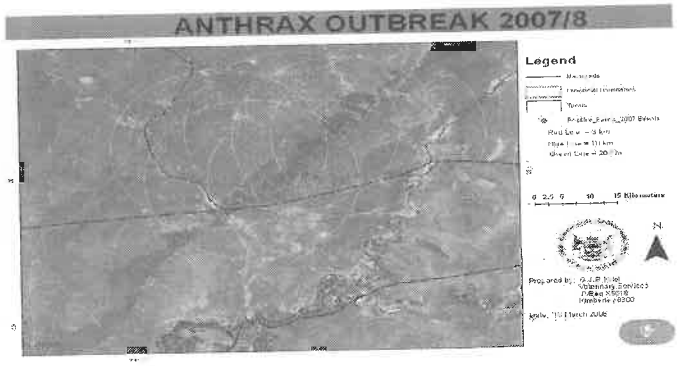


Map: Outbreaks of Anthrax since 1994 – 2007

3. CURRENT OUTBREAK (2007/08)

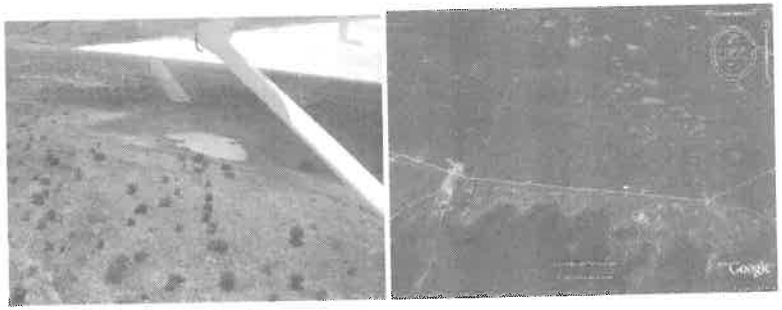
On the ***18th of October 2007*** a farmer reported a dead Eland in the Kimberley area. An ear sample was collected and the results came back positive for Anthrax. No other animals had died on this farm since then and the farm was put under quarantine. On the ***29th of October 2007***, a farm in the Herbert area, about 50 km from the first case, reported several game species dead among which was 2 Zebra, 8 Kudu, 2 Eland and 1 Waterbuck. This farm has had Anthrax in previous years and was therefore highly suspect. All samples except samples from the Waterbuck were positive for Anthrax. The farm was placed under quarantine.

…..On the ***1st of November 2007***, the same farm above reported a dead horse which was suspected to have died from Anthrax (African Horse Sickness was not necessarily ruled out). No further mortalities were reported from the 1st of November 2007 until the 4th of January 2008 when more farmers reported deaths on their farms with more deaths reported in February 2008. In total, 13 farms were eventually quarantined and deaths don't seem to have spread to other areas. The following was more surprising; on the ***29th of January 2008***, a farmer that had been warned around the 11th of January 2008 and vaccinated on the 18th of January 2008 reported 69 goats and 1 kudu dead. An ear sample of one goat found was positive for Anthrax. In total, the following animals were vaccinated on the 18th of January, 223 sheep, 257 goats and 202 cattle. Was it the vaccine that caused the above deaths? In total, 1308 wild game died of an estimated wild game population of 21063.



4. DISCUSSION

The area consists of the Ghaap plateau which has hard rocky areas with thick "swart haak" bush with poor drainage and known to be phosphate deficient. The plateau has these obvious pools or basins where water can collect and concentrate.



There were significant rains in December 2007 and a relatively short dry spell in January 2008 followed by more rains from February onwards. On certain farms, it was clear that the grazing / browsing were under pressure due to overstocking.

It is thought that spores were concentrated in these pools / basins during short dry spells followed by heavy rains. The spores are then ingested by Zebra, which are known to consume copious amounts of water. The infection then spread in two ways: mechanical transmission by the horse fly (Hippobosca) to other zebra or kudu and also by the blowfly – tree – Kudu cycle.




5. CONCLUSION

The area of the Northern Cape, currently affected, has a history of cyclic anthrax epidemics. The affected area has largely converted from cattle farming to wildlife ranching. A significant numbers of farms have part time farmers as owners. The cyclic epidemics appear to be driven by absolute or relative high population densities of wildlife, especially kudus. Heavy rain in early and mid summer also appears to be a risk factor.

The soil in the outbreak area has significant limestone formation, with high Ca++ levels and an alkaline pH. These areas are also notoriously Phosphate deficient, and pica may play a role in triggering outbreaks from dormant spores in old skeletons. Kudus, zebras and other equids were over-represented in the carcass counts. In this outbreak, blowflies appear to play an important role in contaminating leaves and transmitting infection to browsers.

In addition, large numbers of louse flies (Hippobosca) were also observed biting the hairless areas of kudu and zebras, and may be an important mechanical vector of infection in equids, kudu and other species. Almost all carcasses observed were intact except for damage around the eyes, inflicted by crows. The faeces of these birds were found to be positive for Antrax on culture. Their role as well as the role of Hippobosca in the spread of Anthrax spores and vegetative bacteria will have to be investigated thoroughly.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form a significant part of the outbreak, especially in the latter half. The reason for this lies in the very significant role of biting flies in the transmission of anthrax in the region.

Very few farms have kudu proof fencing with the result that kudus virtually roam at will. Food is therefore less of a limiting factor than with other resident animals. Add the absence of large predators, then the kudu population of the Ghaap region has the ability to attain a high density in a relatively short time.

The authorative book with the title "Game ranch management" (Bothma, 1996) state that "professional wildlife management is lacking on most wildlife ranches in South Africa, and what little wildlife management is being done, often occurs in a haphazard way". The same impression was gained for some of the farms/game ranches visited in the Ghaap region. To compound the problem further, some of the farms are owned by non-residents for personal pleasure, resulting in low key management and little or no supervision at times.

Farmers were also complaining that the Department of Nature Conservation did not recognise the fact that kudus were overabundant and was reluctant to issue permits to take off kudu and actually shortened the hunting season from five to two months.

The end-result was that the game populations, with special reference to kudus, were allowed to exceed a critical abundance threshold which is necessary for an isolated point outbreak to progress to a full scale major epidemic.

When an anthrax carcass is opened by scavengers, predators, carrion-eating birds or by man, spores form and are liberated to the environment; many may be deposited in the soil at the primary site, while wider contamination can be brought about by water runoff, scavengers (mammals, birds and insects) and man.

The dormant phase in the anthrax cycle is started by resistant spores being formed. These spores are then washed down drainage channels, ending up in flowing river systems where spores are diluted and ultimately eliminated from the ecosystem; or it may end up in low-lying poorly drained or stagnant areas, such as pans or flood plains, which act as filter and catchments for spores; the so-called "concentrator areas". These concentrator areas are usually high in calcium with a high pH. The Ghaap plateau and lower lying flats are bisected by a few shallow drainage channels, but are mostly flat and poorly drained, with numerous large and small shallow pans. The soils of the Ghaap area are also predominantly calcareous, which is ideal for anthrax spore preservation. All requirements for concentrator areas are therefore met by the Ghaap region.

The soils of the Ghaap region are also known for its phosphorus deficiency, leading to osteophagia by herbivores, which make them very vulnerable to anthrax. It has been the experience in the present outbreak that a relatively low percentage of carcasses were located in the field. Poor supervision in some areas and dense brush vegetation are contributing factors. In the KNP it has been the experience that with good supervision, full-time vehicles and teams in the field and a full-time helicopter, only 50% of carcasses were located! It is therefore virtually impossible to clean up the area where anthrax occurred. Infective bones lie around in the field for many years and can at any time strike out to form new outbreaks.

In the KNP scavengers and predators play an important role in the dissemination of anthrax organisms by not only opening up carcasses, which is necessary for good spore development; but also by dismembering, dragging around and scattering portions of contaminated carcases. In the Ghaap region large predators, scavengers and vultures were mostly eliminated. It was however witnessed that pied crows entered part of this vacated niche by locating and taking out upper eyes of fresh carcasses. All carcasses that were found had their upper eyes removed, but for the rest were mostly intact.

Studies in the KNP revealed that blowflies feed on body fluids of opened carcasses and then deposit discard faecal and vomit droplets on nearby vegetation, making browsers especially vulnerable to anthrax. It was also found that the height of these deposits coincides with the preferred browsing level of kudus, making the kudu extremely vulnerable to anthrax. The same blowfly-kudu-anthrax cycle was observed in the Ghaap region.

In contrast to the KNP, observations in the Ghaap region indicate that biting flies (*Hippobosca rufipes*) played a major role in the transmission and propagation of anthrax during the 2007/2008 epidemic. *B anthracis* was isolated from a large percentage of free-flying flies. Combine this with relatively large inoculums (large flies) and the cumulative effects of extraordinary large numbers of flies, then there can be no doubt that *H. rufipes* must be considered a potential formidable transmitter of anthrax.

The flies are known to be most frequent in summer and to bite more particularly in sunny weather. This is probably one of the main reasons why anthrax epidemics within the Ghaap region occur only in summer, coinciding with the time the flies appear in swarms. Rainfall may have an effect on their abundance.

It was observed that they would sit, and presumably feed, on a fresh anthrax carcass for a few hours and then fly off, presumably in pursuit of a new host. This explains why free-flying flies were found to be much more abundant in "anthrax areas" than in "clean" areas. It also explains their potential to infect new hosts.

The natural hosts of these flies seem to be the larger game such as kudus, zebra and wildebeest, accounting for their relatively high anthrax mortality in the Ghaap region. All equids, including horses, are also high on their preference list. Horses in the area are therefore highly vulnerable to anthrax. They will however attack any other animal or even any moving object, such as a vehicle, when disturbed from their host.

State veterinary control measures were drawn up before the dramatic increase of game ranches in South Africa and are mostly aimed at domestic livestock. Vaccination and quarantine measures, which are the main tools available for veterinary regulatory services, are very difficult and unpractical to enforce in free-ranging game, resulting in an epidemic running its own, virtually unrestricted course. Land use problems, as described, further complicate prophylactic and control measures.

Conclusions.

- Anthrax is indigenous and endemic to the Ghaap region with sporadic point outbreaks going over into cyclic epidemics when the necessary driving forces are present. It is expected that this situation will remain for the foreseeable future.
- Anthrax was an integral part and in dynamic balance with the other elements of the ecosystem before man entered the scene.
- Man's power to change the environment also had an effect on the anthrax cycle, changing it to the decimating disease which is currently the case.
- Biting flies were for the first time identified as one of the main driving forces of an anthrax epidemic in South Africa.
- Current prophylactic and control measures are ineffective, considering the fact that the area is fast changing from livestock farming to game ranching.

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INVESTIGATION OF THE PATHOGENESIS OF WEST NILE VIRUS AND OTHER ZOONOTIC FLAVI AND ALPHA VIRUSES IN HUMANS AND HORSES IN SOUTH AFRICA

Venter, M.[1]; Human, S.[1]; Gerdes, T.[2], Williams, J.[3], Leman, P.[4]; Kemp, A.[4]; Paweska, J.[4]; Setzkorn, H.[5]; Rous, G.[6], Stroebel, J. C.[7], Steyl, J.[3] Murray S.[8], Parker R.[9] & Donnellan, C.[10]

[1]Department of Medical Virology,Faculty of Health Sciences,Pathology Building, Room 3.35-9, University of Pretoria/NHLS Tswhane Academic division, 5 Bophela road, Cr Dr Savage, PO BOX 2034, Pretoria, 0001, South Africa. Tel: +27 12 3192660, Fax: +27 12 325 5550, E-mail: marietjie.venter@up.ac.za

[2]Onderstepoort Veterinary Research Institute, Pretoria, South Africa; [3]Section of Pathology, Faculty of Veterinary Science, Onderstepoort, South Africa; [4]Special Pathogens Unit, National Institute for Communicable Diseases; [5]Chartwell equine clinic, Midrand; [6]Karoo Veterinary clinic, Colesburg; [7]Western Cape Provincial Veterinary Laboratory; [8]For Witbos Clinic, Blue hills Midrand; [9]For Glen Austin Equine Clinic, Midrand South Africa; [11]Section of Equine Medicine, Faculty of Veterinary Science, Onderstepoort, South Africa.

ABSTRACT

West Nile virus (WNV) is a mosquito-born flavivirus that is widely distributed throughout Africa, the Middle East, Asia, parts of Europe, Australia, North and South America and the Caribbean. Strains of WNV have been shown to cluster into 2 main viral lineages with lineage 1 occurring predominantly in the Northern hemisphere and Australia, and lineage 2 in Southern Africa. Members of the flavi and alpha virus families have been associated with emerging and re-emerging disease outbreaks in humans and animals in recent years. Humans and horses are incidental hosts of WNV and may experience severe disease ranging from a febrile Dengue-like illness with a proportion of cases progressing to meningoencephalitis, encephalitis, hepatitis, and death. Since the introduction of WNV in the USA in 1999, > 25000 human cases have occurred across the country of which approximately 40% resulted in neuroinvasive disease. 15 000 cases were reported in horses in 2002 alone with neuroinvasive disease frequently identified. WNV is endemic in Southern Africa and has caused one of the largest outbreaks ever recorded in humans in the Karoo in 1974 affecting tens of thousands of people, although no deaths were recorded. The low number of WNV cases reported in South Africa has led to the assumption that WNV strains which belong to lineage 2 are not associated with severe disease. We have however shown by mouse neuroinvasive experiments and gene expression analysis that lineage 2 strains isolated from human cases of meningoencephalitis and a fatal case of hepatitis are as neuroinvasive as lineage 1 WNV strains associated with disease in humans and horses in the USA. In order to determine if cases of WNV or other flaviviruses are being missed in horses in South Africa, cases that resembles flavivirus symptoms that tested negative for other causes of disease have been screened by virological and molecular techniques. The molecular characterisation of pathogenic WNV strains and investigation of suspected cases in horses will be described. Detection of WNV outbreaks in horses in South Africa may serve as an early warning system for human outbreaks. Knowledge of the pathogenesis and incidence of neurological cases in horses in South Africa will help to determine if vaccination should be implemented or recommended for imported and local horses.

INTRODUCTION

West Nile virus (WNV) is a mosquito-born flavivirus that is widely distributed throughout Africa, the Middle East, Asia, parts of Europe, Australia, North and South America and the Caribbean. Members of the *flaviviridae* have been associated with emerging and re-emerging disease outbreaks in humans and animals in recent years. The transmission cycle consists mainly of birds as the vertebrate hosts and ornithophilic mosquitoes as the maintenance vectors. Humans and horses are incidental hosts and although most infections are benign, approximately 20% of human cases present as a febrile illness that is characterized by fever, a rash and arthralgia and myalgia while approximately 1% of these may develop severe disease including meningoencephalitis, encephalitis, polio-like flaccid paralysis, hepatitis, and death (Hayes *et al.* , 2005). WNV received renewed attention in the last decade following its introduction into New York in 1999 from where it rapidly spread to the rest of the USA, parts of Canada and Mexico. More than 25000 human cases have been reported of which approximately 40% presented as neuroinvasive disease (http://www.cdc.gov/ncidod/dvbid/westnile/). In 2002 alone 15 000 cases were reported in horses and neuroinvasive disease was frequently identified. The number of horse cases was reduced significantly following the introduction of an inactivated animal vaccine, but cases are continuing in humans (Dauphin and Zientara, 2007, Beasley, 2005, Schuler *et al.* , 2004). WNV is endemic in Southern Africa and has caused one of the largest outbreaks ever recorded in humans in the Karoo in 1974 affecting tens of thousands of people. During this outbreak thousands of people visited their local clinicians, although no cases of neurological disease were reported. An epizootic involving WNV and Sindbis virus also occurred in the 1980's in the Witwatersrand that affected hundreds of people. The number of confirmed human cases in recent years has been approximately 5-15 per annum; although only a proportion of cases are subjected to laboratory investigation. Severe disease has been recognised in South Africa including a fatal hepatitis and non-fatal encephalitis in humans as well as deaths in ostrich chicks, a foal and a dog (Burt *et al.* , 2002). Following the increase in cases of severe disease associated with WNV in the Northern hemisphere the question arose as to whether WNV strains with increased virulence has appeared in the northern hemisphere or whether we have previously underestimated the clinical significance of WNV in humans and animals in South Africa.

Isolates of WNV fall into two major genetic lineages, lineage 1 which is found in North America, North Africa, Europe and Australia while lineage 2 is found exclusively in Southern Africa (Burt *et al.*, 2002). To determine if differences in pathogenic potential exist between North American and South African strains, we and others have been conducting pathogenesis studies of South African and North American strains of WNV and have shown by sequencing, neuroinvasive experiments in mice and gene expression studies that differences in neuroinvasiveness exist in both lineages that are genotype- and not lineage-associated and that South African strains exist that compare in pathogenic potential to those causing outbreaks in the Northern hemisphere (Beasley *et al.* , 2004, Venter *et al.* , 2005).

A recent serological survey in thoroughbred horses has indicated that the WNV is still widely distributed in South Africa with 11% of yearlings seroconverting over a period of 1 year and up to 75% of their dams showing some exposure. In this study it was concluded that WNV is not a cause of neuroinvasive disease in horses in South Africa based on the fact that none of these horses had shown any signs of neuroinvasive disease and since 3 seronegative horses that were inoculated with a WNV strain that had recently been isolated from a human with benign illness did not result in clinical disease (Guthrie *et al.* , 2003). The strain used in these experiments has however subsequently been shown to be of low neuroinvasiveness in mice in comparison to certain other South African strains (Venter *et al.*, 2005). Furthermore, of twelve horses infected experimentally in the USA with the highly neuroinvasive NY99 strain only one developed neuroinvasive disease and although the rest seroconverted none developed clinical disease and no virus could be isolated from any of their organs suggesting that the pathogenesis of WNV is not clear-cut (Bunning *et al.* , 2002).

In South Africa the most common flaviviruses are WNV and Wesselsbronvirus which are transmitted by *Culex* and *Aedes* species of mosquitoes respectively. In order to determine if cases of WNV or other flaviviruses are being missed in South Africa, equine cases of neurological disease or fever symptoms that tested negative for other causes of disease have been screened over a period of 1 year by virological, serological and molecular techniques. The molecular characterisation of pathogenic WNV strains and investigation of suspected cases in animals and humans are discussed.

MATERIALS AND METHODS

Clinical cases

Serum and/or post mortem brain specimens from horses with neurological disease or acute fever were collected over a period of one year (March 2007-May 2008) in South Africa. Specimens from horses with unexplained disease were sent to the department of Medical Virology, University of Pretoria by the Onderstepoort Veterinary Institute, as well as by private veterinarians from the Gauteng province and the Western and Northern Cape. In total 78 specimens were received from horses that presented with fever and/or neurological symptoms. Thirty one of these had shown neurological signs while the rest were cases of acute fever or unexplained deaths.

RT-PCR screening

A Flavivirus specific nested RT-PCR specific for the NS5 region was used for screening specimens for both wesselsbron and WNV. First round RTPCR products were also subjected to a nested realtime PCR using WNV specific probes for confirmation of WNV positive specimens (Zaayman and Venter unpublished data). All positive specimens were confirmed by sequencing of the NS5 region and analysed on an ABI 3130 sequencer (Applied Biosystems, Foster City, Ca, USA).

Phylogenetic analysis

Sequences were aligned with ClustalX (v1.83) using the multiple sequence alignment option. Maximum Likelihood trees were generated with FastDNAML (Olsen *et al.* , 1994). Bootstrap statistics for 1000 replicates were calculated using Minimum evolution phylogenetic analysis with a CNI (level=2) search option and initial tree sampling by Neighbor-joining analysis with the Kimura 2-parameter model and a gamma parameter of 2.0.

Serology

Sera from horses were screened for flavivirus specific antibodies by hemagglutination inhibition assay (HAI). Positive specimens were subsequently tested for WNV IgM by IgM capture ELISA (Special Pathogens Unit, National Institute for Communicable Diseases). African horse sickness (AHSV) and equine encephalosis virus (EEV) infections were identified by viral culture followed by antigen detection assays and/or compliment fixation of serum. Immunoperoxidase stains of brain, spleen, liver and lung sections were used to identify EEV and EHV.

Virus culture:

All specimens were inoculated onto African green monkey kidney cells (Vero) grown on T25 flasks and in Eagle Minimum Essential Medium (Gibco BRL) containing 2% fetal bovine, serum and10 mg/mL of penicillin, and streptomycin (Gibco), and 1mg/ml L-Glutamin. Cultures were passaged once after 3 days and observed for 7-10 days for cytotoxic effect.

RESULTS

Screening of Specimens

A total of 78 specimens from horses of which 31 had neurological symptoms were screened by RT-PCR for cases of flavivirus infection. In total 5 specimens were positive by Flavivirus RT-PCR. WNV specific realtime PCR assays and sequence confirmation identified WNV in 3 of these cases and Wesselsbron virus in 2 (Table 1). A flavivirus specific HAI test was used to screen all PCR negative specimens. This process identified an additional 9 symptomatic horses that were seropositive for Flaviviruses. Of the Flavivirus HAI positive cases, WNV IgM ELISA confirmed 2 of these as WNV infections. In total 5 WNV cases and 2 Wesselsbronvirus cases could thus be confirmed as acute infections. All of these were associated with neurological disease (Table 1). This suggests that in total 7/31 undetermined neurological cases were due to flaviviruses in horses. A Wesselsbronvirus IgM ELISA is not yet available for further confirmation of the other flavivirus seropositive cases, but where possible convalescent serum will be followed up to detect an increase in IgG.

Neurological cases were also tested for AHSV, EEV and Equine herpes virus. For both fatal WNV and Wesselsbron virus cases where WNV was isolated or detected by RT-PCR in the brain, this was the only virus detected. In one fatal case, AHSV was also isolated from the spleen. All were negative by tissue antigen stains for EHV. The brain of the fatal Wesselsbron virus case tested negative for EEV, AHSV and EHV. Affected horses' ages ranged from 6months (fatal case) to 8years (fatal case) and included Thoroughbred, Arabian and Lipizzaner breeds. Cases were identified in Gauteng, the Northern- and Western Cape. Confirmed cases were detected from March through to May of 2008 and in May 2007. Seropositive cases were identified from January to May of this same year (2008).

Clinical description of WNV and Wesselsbron virus cases

All confirmed WNV and Wesselsbron virus cases resulted in neurological symptoms in horses. Four of the five WNV cases died or had to be euthanized after becoming paralysed. Symptoms included ataxia in all cases, weak hind and or forelimbs, and seizures and chewing in one fatal case that was confused with rabies but tested negative for this virus. Jaundice and/or hepatitis were identified in two cases, myosis of the pupils were described by one veterinarian; two cases became completely paralysed before death. Fever was not reported in all cases. Upon post mortem investigation of case HS101/08, marked generalised subcutaneous oedema was visible involving the trunk, proximal forelimbs, periaortic and coronary groves, neck and intermuscularly. Histologically, lesions were visible on central nervous sections, especially in the lumbar spinal cord with similar but less prominent lesions in the rest of the spinal cord, mid brain and white matter. A mild leptomeningitis and occasional mild spinal neuritis was also visible. EEV and EHV immunoperoxidase stains were negative. The one WNV case that survived had experience symptoms for approximately 21 days but made a full recovery.

Microscopic brain lesions were also visible on the fatal Wesselsbron virus case that was clinically consistent with encephalitis. The Wesselsbronvirus case that survived was severely ataxic and had both front and hind limb incoordination. Symptoms persisted for 20 days, but the patient made a full recovery.

Sequence confirmation

Three of the WNV RTPCR positive strains were subjected to sequence analysis and confirmed to be WNV by blast searches. Two of these strains were included in the phylogenetic analysis (HS101/08 and HS125/08). A 280 bp region of the NS5 gene was aligned with representative sequences of all 5 current recognised WNV lineages. Both clustered with lineage 2 strains (Figure 1). Both of the other two isolates (SAE122_08, and SAE118_08) that amplified with the Flavivirus specific primers but not with the WNV specific realtime PCR were confirmed to be Wesselsbron virus by blastsearch analysis and phylogenetic comparison to other Flaviviruses (results not shown). Further phylogenetic analsysis and whole genome sequencing is in process.

DISCUSSION

WNV has been associated with significant morbidity and mortality in humans and horses in the northern hemisphere over the past 10 years. WNV and certain other flaviviruses such as Wesselsbron virus are known to be endemic to South Africa. Few cases of WNV have been reported in recent years. Although many Wesselsbron virus cases were identified in sheep in the western Cape in the late 2008 summer season. Wesselsbron virus is frequently associated with abortion and disease, including hepatitis and death, in sheep and a few cases of nonfatal encephalitis in humans have been described (Jupp and Kemp, 1998, Heymann *et al.* , 1958, Bres, 1965). The role of these viruses as horse pathogens in South Africa is not yet known. To address this, cases of both unexplained fever and neurological clinical signs were investigated in horses over a period of one year (March 2007 to May 2008 summer). Of 31 neurological cases tested WNV was identified in 5 and Wesselsbron virus in 2, suggesting that as many as 22% of unexplained neurological infections in horses may be due to flavivirus infections. These infections were associated with a high mortality rate (80% for WNV and 50% for Wesselsbron virus). Furthermore the WNV cases that could be amplified by RT-PCR were all shown to belong to lineage 2, suggesting that lineage 2 WNV may cause fatal encephalitis in horses in South Africa. Wesselsbron virus has never before been described as a cause of neurological or other disease, or fatal encephalitis in horses.

All cases were identified in late summer to autumn, which is the same time that AHSV outbreaks occur in South Africa and this may contribute to the reason why flavivirus cases are being missed. Some of the WNV cases reported here were submitted as query AHSV and EEV cases. The 2008 season has had an unusually high rainfall and AHSV has been particularly severe suggesting that an increase in *Culicoides* and other arbovirus vectors such as *Culex* and *Aedes* mosquitoes may have occurred. 2008 also saw the first Rift Valley fever virus outbreak in 30 years. in South Africa, another arbovirus transmitted by *Aedes* mosquitoes.

Our results suggest that Flaviviruses may be significantly undersuspected, underdiagnosed and therefore underreported as causes of disease in horses in South Africa. Detection of cases in horses may also serve as an early warning system for human WNV outbreaks. Flavivirus diagnosis should include an RT-PCR and/or virus isolation from serum or CSF in the first 5 days of disease while the viremia is high, or from brain in fatal cases. Serological tests that detect IgM or paired acute and convalescent serum may be used to confirm cases from days 8-20 (Dauphin and Zientara, 2007, Kleiboeker *et al.* , 2004). Further investigations are needed to determine if the lineage 1 horse vaccine will protect against

lineage 2 infections. If so, this may be recommended especially when importing horses to South Africa, as well as in local horses during seasons of high rainfall.

Table 1 Confirmed flavivirus cases identified in horses in South Africa in 2007-2008.

Specimen	Date of collection	Origin	Symptoms	Outcome	Specimen type	Flavivirus RTPCR	Flavivirus Serology	Confirmatory test results
SAE122_08	08/03/01	Western Cape, Somerset-wes	Neurological symptoms	Died	Formalin fixed brain	Positive	Not done	Wesselsbron, sequencing
SAE118_08	08/05/06	Midrand	Neurological symptoms	Survived	plasma	Positive	Not done	Wesselsbron, sequencing
SAE12_07	07/04/23	Johannesburg, Sandton	Neurological symptoms	Survived	plasma	Negative	HAI positive	WNV IgM+
SAE89_08	08/03/03	Western Cape, Colesburg	Neurological symptoms	Died	plasma	Negative	HAI positive	WNV IgM+
SAHS101_08	08/04/15	Tiegerpoort, Pretoria	Neurological symptoms	Died	brain	Positive	Not done	WNV PCR+, Sequence, L2,Virus isolation: WNV PCR positive
HS123_08	08/05/08	Pretoria	Neurological symptoms	Died	brain	Positive	Not done	WNV PCR +, Sequencing, WNV L2
HS125_08	08/05/08	Pretoria, Hammanskraal	Neurological symptoms	Died	brain	Positive	Not done	WNV PCR, Sequencing, WNV L2

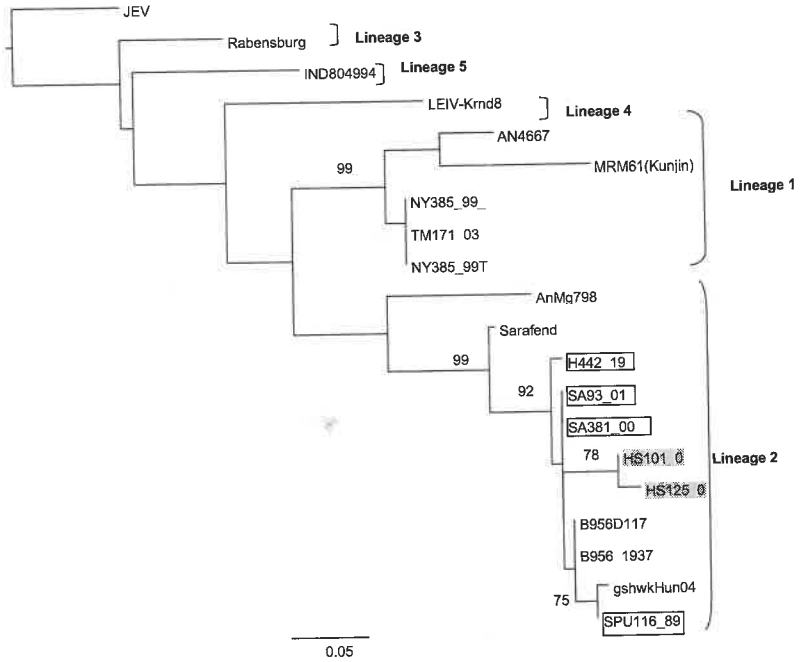

Figure 1: Maximum Likelihood tree comparing WNV strains identified in horses in South Africa with isolates belonging to lineage 1, 2, 3, 4 and 5 as indicated by brackets to the right. Bootstrap values greater than 70 are shown on the branch nodes. The scale depicts 0.05 nucleotide changes. The horse sequences identified in this study are highlighted in grey. Other South African isolates are outlined.

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DETECTION AND GENOTYPING OF WEST NILE VIRUS BY REAL-TIME PCR

Zaayman, D.[1,2] & Venter, M.[1,2,*]

[1]Department of Medical Virology, Faculty of Health Sciences, University of Pretoria
[2]National Health Laboratory Services, Tshwane Academic Division
*Principle Investigator, Corresponding author. Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, PO BOX 2034, Pretoria 0001 South Africa
Tel: +27 12 319 2660, Fax: +27 12 325 5550, Email: marietjie.venter@up.ac.za

ABSTRACT

West Nile virus (WNV) is an arthropod-borne Flavivirus, primarily transmitted by the *Culex* mosquito species. Humans and equines are considered to be incidental dead-end hosts, likely because they do not sustain viremic levels high enough to spread infection. The virus is classified into 5 genetic lineages, with lineage 1a occurring in the Northern hemisphere, lineage 1b, Kunjin virus, occurs in Australia. Lineage 2 strains are found exclusively in Southern Africa and Madagascar. Lineage 3, named Rabensburg virus was isolated from Culex mosquitoes at the border between Austria and the Czech Republic. Lineage 4 represents a unique virus isolated in the Caucasus, whereas lineage 5 strains occur in India. Most major recent outbreaks of WNV have been associated with the introduction of lineage 1 strains in the Western hemisphere, leading to the exposure of immunological naïve populations, resulting in an increase incidence of severe disease. This introduction of WNV into the Western hemisphere in 1999 and the recent emergence of lineage 2 WNV in Central Europe have highlighted the potential for spread of pathogenic WNV strains beyond its geographical boundaries. For this reason, genotyping of WNV strains may have important implications for surveillance and epidemiological studies. We report here the development of a real-time PCR method for the simultaneous detection and genotyping of WNV on the basis of dissociation-curve analysis. Hybridization probes directed at the NS5 gene were designed to be sequence specific to lineage 1a strains of WNV. Minor sequence mismatches between the probe and strains represented in the other 4 lineages allows for clear differentiation between lineages, due to differences in probe melting temperatures. Eight WNV strains, collectively representing three lineages were tested and correctly genotyped, at a detection limit of 10^{-1} $TCID_{50}$/ml. Lineage 1a strains display melting peaks at ~62°C, lineage 1b strains show peaks at ~42°C and lineage 2 strains display peaks at ~48°C. The specificity of this assay was confirmed by a negative PCR result on closely related flaviviruses, including Yellow fever and all four Dengue serotypes. To our knowledge, this is the first assay designed for the rapid, sensitive detection and genotyping of WNV and may be used as a diagnostic and epidemiological tool.

INTRODUCTION

West Nile virus (WNV) is an arthropod-borne *Flavivirus* that is spread by mosquitoes, primarily *Culex* species, with wild birds serving as natural hosts (1). Humans and horses are considered incidental hosts and because of low viremic levels, they do not play a role in the transmission cycle of the virus (2). WNV received worldwide attention when it was first recorded in the Western hemisphere following an outbreak in New York City, USA, in August of 1999 leading to several human deaths (3). The current impact of WNV in South Africa is largely unknown, partly because of a lack of clinical awareness among clinicians and veterinarians as to the pathogenic potential of WNV. Although people of all age groups and of both sexes are considered to be susceptible to WNV infection, the incidence of severe disease and death increases with age (4). An incubation period of approximately 5-15 days

normally precedes disease, but only a fifth of cases present with clinically apparent illness (5). In cases where disease does indeed occur, symptoms are usually non-specific and mild which will include fever, rash, headache and myalgia that resolves in a week (6). Less than 1% of cases progress to severe disease such as meningitis, encephalitis, poliomyelitis-like syndrome and death (7, 8). Equines are also very susceptible to WNV infection, frequently displaying signs of neurological infection including ataxia, muscle fasciculations, abnormal gait and depression (9). Early reports of outbreaks of WNV in equines could serve as a possible warning sign that corresponding infections in humans may occur (10).

Strains of the virus cluster into two main phylogenetic lineages, with lineage 1 strains largely being restricted to the northern hemisphere, South America and Australia. Lineage 2 strains are in turn thought to exclusively exist in Southern Africa and Madagascar (11). More recent phylogenetic studies suggest that lineage 1 strains sub-divide into at least 3 sub-clades. Clade A represents strains from Africa, Asia, the Middle East and America, whereas clade B is represented by the Australian isolate of the virus, named Kunjin (12). Clade C is postulated to include isolates from India (12), although recent publications support the notion of this clade representing a separate fifth lineage (13). Apart from the sub-Saharan strains that belong to lineage 2, researchers have proposed the addition of 2 new genetic lineages of WNV. This includes Rabensburg virus (lineage 3), isolated from a *Culex* mosquito near the Austrian border and a proposed lineage 4 strain identified in the Caucasus (12). It is also plausible that these viruses represent individual flaviviruses that belong to the Japanese Encephalitis serocomplex (14), although phylogenetic clustering groups them with WNV (11). In Hungary in 2004, a goshawk fledgling with neurological symptoms succumbed to what was later identified as West Nile virus. Complete genome sequencing and subsequent phylogenetic analysis revealed that this strain of WNV most closely resembled strains that belong to lineage 2, which is the first documented case of lineage 2 WNV in Europe (12).

The rapid spread of lineage 1 WNV throughout the continental United States and the report of lineage 2 WNV occurring in Central Europe, illustrates the ability of different WNV strains to spread beyond its expected geographical boundaries. This in turn, may have important consequences for both epidemiological studies and surveillance programs. After the initial identification of WNV circulating within a given population, the virus genotype may be determined by nucleic acid sequencing and phylogenetic analysis. As this is a time-consuming process requiring a degree of expertise, the development of a method capable of detecting and genotyping a given strain of WNV will positively contribute towards these programs. This report describes the development of a sensitive Real-Time PCR assay incorporating Fluorescent Resonance Energy Transfer (FRET) probes, designed for the rapid detection and genotyping of WNV that may be implemented as a diagnostic and epidemiological research tool.

MATERIALS AND METHODS

Primer and probe design

A WNV-specific reverse primer (WN9317R) was designed with the use of Primer 3 (15), for use in Real-Time PCR together with a previously published flavivirus genus-specific primer (MAMD) (16). Following multiple sequence alignments of WNV strains representing each genetic lineage in Clustal X (version 1.83) (18), a hybridization (FRET) probe set (flanked by the appropriate primers) targeting the NS5 gene of WNV was designed with the aid of the Lightcycler Probe design software package (Roche, Mannheim, Germany). The probe set was designed to be highly conserved with lineage 1a

strains of WNV, but displays minor sequence variations as compared to other WNV strains. These primer and probe sequences are available upon request.

Virus specimens and RNA extraction

Eight WNV strains were tested, representing three phylogenetic lineages (10). These include NY385/00, Eg101 and AN4766 representing lineage 1a; MSM16 (Kunjin) representing lineage 1b; obtained from Drs RE Shope and DWC Beasley of the University of Texas Medical Branch, Galveston. Lineage 2 strains, represented by SA381/00, SPU 116/89, SPU 93/01 and H442, were obtained from the Special Pathogens Unit, National Institute of Communicable Diseases, South Africa. Viruses were previously propagated in vero cells and diluted to the same titre of 10^4 $TCID_{50}$/ml. RNA from titrated WNV strains was extracted with the QIAamp viral RNA mini kit according to supplier's instructions (Qiagen, Valencia, CA).

Real-Time PCR and Nested Real-Time PCR

One-step reverse transcription Real-Time PCR was carried out with the Quantitect Probe RT-PCR system (Qiagen, Valencia, CA). Each PCR reaction contained 7µl RNA template, 0.2µM of each probe (WN 9177S and WN 9201A), 0.5µM of each primer (MAMD and WN9317R), 10µl probe mastermix and 0.2µl Reverse Transcriptase enzyme in a final reaction volume of 20µl. Real-Time PCR was carried out in a Lightcycler 1.5 instrument (Roche, Mannheim, Germany). Cycling commenced at 50°C for 30mins, followed by a single step of 95°C for 15 min. This was followed by 45 cycles of 95°C for 0s, 48°C for 30s and 72°C for 30s, yielding a product of approximately 270bp and was ended off with melting curve analysis, carried out at a temperature ramp-rate of 0.1°C/s.

The Real-Time PCR was also modified to a nested format, by performing a conventional first round PCR with primers MAMD and WN9317R, with the Titan One-Tube RT-PCR system according to supplier's instructions (Roche, Mannheim, Germany). Cycling was carried out at 50°C for 30 min, followed by one step of 94°C for 2 mins. PCR proceeded with 35 cycles of 94°C for 10s, 50°C for 30s and 68°C for 1min and one step of 68°C for 7min. Nested Real-Time PCR was carried out in a Lightcycler 1.5 instrument and by using the Lightcycler FastStart DNA Master Plus Hybprobe kit (Roche, Mannheim, Germany). Each reaction contained 2µl of first round RT-PCR product, PCR mastermix, each probe (WN 9177S and WN 9201A) and nested flavivirus genus-specific primers (FS778 and CFD2) in proportions as indicated by the manufacturer's instructions. Cycling started at 95°C for 10 min, followed by 45 cycles of 95°C for 10s, 53°C for 8s and 72°C for 8s, yielding a product of approximately 214bp and was ended off with melting curve analysis between 30°C and 80°C, carried out at a temperature ramp-rate of 0.1°C/s.

RESULTS

Specificity and sensitivity

Eight WNV strains, representing three phylogenetic lineages were amplified and correctly genotyped (figure 1). These include NY385/00, Eg101 and AN4766 representing lineage 1a; MSM16 (Kunjin) representing lineage 1b; SPU 381/00, SPU 116/89, SPU 93/01 and H442 representing lineage 2. Strains from each of the three represented lineages yielded three distinct melting curve peaks. All lineage 2 strains tested exhibited a melting peak at approximately 6°C higher than that seen for lineage 1b (Kunjin) during Real-Time PCR. Lineage 1a strains exhibited melting peaks at ~14°C higher than expected for lineage 2 strains.

In order to assess the specificity of this assay the PCR was carried out on RNA templates extracted from other important flaviviruses represented in Africa, which included Yellow fever virus (YFV), Wesselsbron and Dengue virus (serotypes 1-4). The WNV specific primer (WN9317R) failed to amplify YFV and Dengue serotypes 1-4, but was able to amplify Wesselsbron. These pathogens were however not detected by the hybridization probes during melt-curve analysis.

The sensitivity of the one-step and nested PCR assays were determined by performing PCR on a dilution series of titrated WNV strains that were selected to be representative of each of the three lineages tested. The detection limit for one-step Real-Time PCR was determined to be at a titration of 10^{-1} $TCID_{50}$/ml for MSM16 (Kunjin), Eg101 and SPU 381/00, when employing these methods. When compared to a commonly used Taqman based assay (18), the sensitivity was found to be 1 log higher (data not shown). After expanding this assay into a nested Real-Time PCR approach, titrations of 10^{-15} $TCID_{50}$/ml could be detected.

Testing of clinical specimens

The real-Time PCR in nested format was shown to be able to detect and accurately genotype WNV RNA in clinical specimens from a human patient with meningoencephalomyelitis, as well as from three fatal cases in horses that could not be detected with the commonly used Taqman assay (18) or with single round Real-Time PCR (results not shown).

DISCUSSION

The PCR assays described here is to our knowledge the first designed to identify and genotype WNV strains based on phylogenetic lineage, which could have important potential applications in diagnostics as well as in epidemiology and surveillance programs, following the report that WNV strains previously restricted to certain geographic regions have been identified elsewhere (12). Applying a one step Real-Time PCR approach has the advantage of enhancing the sensitivity of the assay, combined with a quicker turn-around time by negating the need for pre-PCR cDNA synthesis. By further limiting sample handling, the risk of sample contamination is also reduced. These advantages make such a one-step approach valuable in the diagnostic setting. By expanding this assay into a nested Real-Time PCR greatly enhances the sensitivity, which will be useful in surveillance programs such as the screening of mosquito pools, investigating bird deaths and screening of blood donations. The risk of encountering contamination is however increased when applying the nested-PCR approach and is therefore less suited to diagnostic applications. This approach was however successfully applied to amplify WNV from clinical specimens that could not be detected with single round Real-Time PCR or Taqman PCR, but was also positive in conventional nested-PCR. One human case (known to be positive for lineage 2 WNV) and three cases of WNV identified in brain tissue from horses that succumbed to neurological infection was identified and accurately genotyped.

The use of hybridization probes (FRET probes) furthermore combines the advantages of Real-Time PCR with the ability to concurrently genotype samples, eliminating time-consuming sequencing and phylogenetic analysis. Hybridization probes are also highly sensitive and specific, but are capable of tolerating minor sequence variations allowing for genotyping to be carried out based on melt-curve analysis. Figures 1 illustrates that very distinct melting-peaks can be generated with hybridization probes based on sequence divergence between different genotypes or lineages.

The relative difference between peak melting temperatures generated by the probes always remains constant across the different genotypes, regardless of PCR system used or the relative composition of PCR reagents. Therefore, lineage 2 strains will be identified by melting peaks 6°C higher than lineage 1b strains (Lineage 2 = Lineage1b + 6°C), whereas lineage 1a strains will exhibit melting peaks 14°C higher than Lineage 2 strains (Lineage 1a = Lineage 2 + 14°C). It is however necessary to run appropriate control specimens with each assay in order to determine exact melting temperatures. Sequence variations at the probe binding site for these lineages are suitable to assign genotype to lineage 3, 4 and 5 strains. The sequence mismatches at these sites also differ between these lineages, which is expected to yield distinguishable melting peaks, but needs to be experimentally determined. Even though PCR amplicons were generated for Wesselsbron, it was not detected during real-Time PCR, thereby illustrating the discriminatory potential of the probe to exclusively identify WNV. The application of Real-Time PCR as a sensitive diagnostic tool and as an effective supplement to epidemiological and surveillance projects, could further aid in the identification of WNV strains outside of its expected geographical boundaries.

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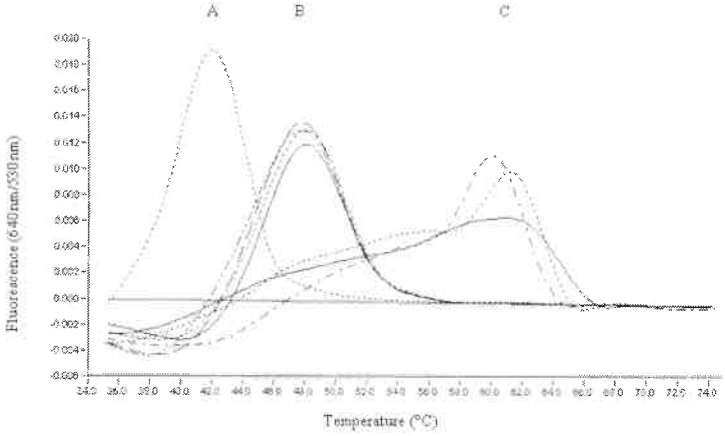

Figure 1: Melting peak data illustrating the identification and correct genotyping of the 8 strains tested, at a titration of 10^2 $TCID_{50}$/ml following one-step Real-Time PCR.
A : MSM 16 (Kunjin virus) with a melting peak at ~42°C (representing lineage 1b).
B : WN SA381/00, SPU381/00, SPU93/01 and H442 with a melting peak at ~48°C (Kunjin + 6°C) (representing Lineage 2).
C: Eg101, NY385/00 and AN4766 displaying a melting peak at 62°C (Lineage 2 + 14°C) (representing Lineage 1a).

MOLECULAR CHARACTERIZATION OF WESSELSBRON VIRUS FROM ANMALS AND HUMANS IN SOUTH AFRICA

Human S.[1*], Leman P[3], Kemp A. [3], Paweska J. [3], Venter M.[1,2#]

[1]Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, South Africa
[2]National Health Laboratory Services, Tswhane Academic division
[3]Special Pathogens Unit, National Institute for Communicable Diseases.
 # Contact person: Dr M.Venter. Senior Lecturer, Department of Medical Virology, Faculty of Health Sciences, University of Pretoria/NHLS Tswhane Academic division, PO BOX 2034, Pretoria, 0001, South Africa. Tel: +27 12 3192660, Fax: +27 12 325 5550,
E-mail: marietjie.venter@up.ac.za; stacyhuman@gmail.com

ABSTRACT

Wesselsbron virus is a zoonotic virus that affects livestock, in particular lambs, goats and cattle and is widespread and common in the warmer regions of South Africa as well as other parts of Africa. In sheep it causes a febrile illness associated with hepatitis, haemorrhage, abortion and death in new-borne lambs. It is able to spread from livestock to man, and may cause encephalitis although few cases have been described. Wesselsbron virus has also been implicated as a potential pathogen of ostriches. The importance of Wesselsbron virus as a cause of human and animal disease in South Africa is not yet well defined partly because of a lack of clinical awareness of the disease potential of arboviruses and the unavailability of simple diagnostic tests to determine their presence in infections.

Characterization of these viruses will aid in diagnosis of human and animal cases of disease. As yet there is still no known treatment for WNV and Wesselsbron virus flaviviruses. Although WNV has been characterised extensively, presently there is limited sequence information and no PCR diagnostic test available for Wesselsbron virus. Confirmation and diagnosis of this disease relies on serological techniques such as haemagluttination inhibition (HI) or immunohistochemical staining techniques, which are laborious and can not distinguish between different flaviviruses. Due to the threat of interspecies transmission of Wesselsbron virus from livestock to man and to other susceptible animals, as well as the fact that many Wesselsbron virus infections could be missed, a better understanding of the host range and molecular biology of the virus is needed. To facilitate this we will determine the sequence of a human isolate of Wesselsbron virus and compare this sequence to that of known flaviviruses, and conduct a phylogenetic comparison of the E and NS5 genes between several other strains isolated during an outbreak of Wesselsbron virus in animals. This information will facilitate a better understanding of the molecular pathogenesis of Wesselsbron relative to West Nile virus and facilitate the design of specific diagnostic techniques that can accurately distinguish Wesselsbron and WNV cases in humans and animals in South Africa.

INTRODUCTION

Wesselsbron virus was first isolated from a lamb in 1955 in the town of Wesselsbron in the Free State province, South Africa (SA) (10, 11). Invertebrate hosts of the virus, as well as viral isolations, have been found throughout Sub-Saharan Africa, particularly in South Africa where it causes febrile illness in sheep and is associated with hepatitis, haemorrhage, abortion and mortality in new-borne lambs. Isolations have also been made from ostriches and a foal in SA (14) however the importance of Wesselsbron as an ostrich or horse pathogen is not yet known.

The primary invertebrate vector of Wesselsbron virus is the *Aedes* mosquito species, with Wesselsbron virus having been isolated from pools of *Aedes* mosquitoes following outbreaks in animals in the 1980's in the Free State province in SA. The major vector in sheep is the *A. caballus-juppi* species of mosquito (11). Few human cases have been reported and have mainly been associated with a mosquito bite or handling carcasses or tissues of animals that have died of the disease and to date no human-to-human transmission has been reported. Seroprevalence in domestic and wild ruminants in the warmer regions suggest that these animals may frequently be exposed (3). Birds are also suspected to be the maintenance host although viremia in cattle is high enough to infect mosquitoes suggesting that they may be potential viral reservoirs (17).

Human Infection

Human infection by this virus is rare with only one case of encephalitis being recorded in SA where an entomologist contracted the virus during vector collection (9). The disease is usually benign with fever and mylagia (11, 16, 17). Patients have also experienced severe headaches, chills, insomnia and retro-orbital pain associated with photophobia and hyperaesthesia of the skin with an evanescent skin rash frequently present. Muscle and joint pain is also common (10). Laboratory infection or infection of laboratory field workers has accounted for 11 of the 23 cases reported (11). In severe cases, encephalitis may develop and includes symptoms of blurred vision and mental impairment. Patients usually recover within a few days to a week and up till now no permanent sequelae have been reported (10). As only a few human cases of Wesselsbron virus have been reported, routine diagnostic testing does not include this virus.

Animal Infection

Incubation in new borne lambs is approximately three days, with non-specific signs of illness including fever, anorexia, listlessness, weakness, and increased respiration becoming evident during this time (17). Wesselsbron disease and Rift Valley fever share many clinical and pathologic features, however, Wesselsbron disease is usually milder, producing much lower mortality and abortion and less destructive liver disease. The virus appears to be more neurotropic than that of Rift Valley fever because severe foetal teratology of the CNS is seen after experimental infection (16).

Viral Diagnosis

Diagnosis of Wesselsbron virus disease is confirmed by isolating virus from the blood as well as serological tests. Baba *et al* (2) reported that preliminary tests on the use of solid-phase immunosorbent techniques (SPIT) incorporated with the HI test were able to detect early Wesselsbron virus infections in Flavivirus endemic regions. However because of cross-reaction between Flaviviruses, the haemagglutination inhibition (HI) tests cannot differentiate between individual genuses (1, 4) making these tests unreliable for specific diagnosis. Neutralization tests are currently used for differentiation; however these tests are labour intensive and few specialist laboratories have access to antiserum and live virus for use in these techniques. Immunohistochemical staining has also been used to diagnose a lamb in the Free State province in SA (12).The polymerase chain reaction (PCR) is a sensitive and specific method for virus identification, however specific Wesselsbron virus RTPCR is not yet available due to a lack of sequence information of this virus. In addition limited information is available about the molecular pathogenesis of Wesselsbronvirus that determines the differences to WNV and other Flaviviruses.

In order to fully understand this virus and the disease that it causes as well as the severity of the disease, the Wesselsbron virus genome sequence will be compared to other known flavivirus sequences. Flavivirus genomes are believed to have conserved regions at the beginning and ends of the genome; which is useful when designing primers to amplify an unknown sequence. This will aid in identifying conserved regions within the genome, which will facilitate the development of diagnostic tests for both medical and veterinary purposes, help in outbreak investigations and pathogenesis studies (15) and to clarify differences in vector competence, organ tropisms and disease association relative to other Flaviviruses. To date no vaccine or preventative treatment is available for Wesselsbron disease (7). Characterization of this virus will aid in development of specific diagnostic tests, treatments and prevention of infections in humans and animals.

MATERIALS AND METHODS

Wesselsbron virus strains

Six strains of Wesselsbron virus were obtained as vero isolates from the Special Pathogens Unit (SPU) at the National Institute for Communicable diseases (NICD), Johannesburg, South Africa. These strains were isolated from arthropods (AR778, AR2209, AR11189) and animals (AN16210, TAR100) from various regions in South Africa as well as a strain isolated from a human patient (H177) that contracted Wesselsbron disease.

Preparation of virus strains

Wesselsbron strains were amplified in Vero cells until cytopathic affect was visible. Virus stock was prepared by repeat freeze-thawing of cultures and clarification of cell debris by centrifugation. Virus culture stocks were stored at $-70^{\circ}C$ until RNA extraction.

Viral RNA extraction and amplification

Viral RNA was extracted from 140µl virus stock using the QIAmp mini viral RNA extraction kit (Qiagen, Valencia, California) according to the manufacturer's recommendations. The viral RNA was amplified by RTPCR using the Titan One Step RTPCR (Roche, Mannheim, Germany) kit with flavivirus specific primers. PCR products were visualised following electrophoresis on a 1.5% gels by UV transillumination using the BioRad (BioRad, Hercules, California, USA) gel documentation system.

PCR amplicon purification and sequencing

PCR products were gel purified using the Wizard gel and PCR SV clean up system (Promega,Madison, Wisconsin, USA) following manufacturers instructions. The amplicons were sequenced in both directions using the BigDye terminator kit (Roche, Mannheim Germany). according to manufacturers instructions before running on a ABI 3130 sequencer (Applied biosystems, Foster city, California, USA).

Sequence analysis and phylogenetic analysis

Sequences were compiled using Sequencher (version 4.6) and aligned with ClustalX (v1.83) using the multiple alignment option. Maximum Likelihood trees were generated using FastDNAML and bootstrap analysis by using a consensus tree generated for 1000 bootstrap replicates following Minimum Evolution phylogenetic analysis with a CNI (level=2) search option and initial tree sampling by Neighborjoining analysis with the Kimura 2-parameter model with a gamma parameter of 2.0. Nucleotide P-distance analysis (i.e. the number of differences over the total number of sites investigated) was carried out with Mega Version 4.

RESULTS

RTPCR and nucleotide sequencing

Using the degenerate flavivirus primer MAMD and West Nile specific primer WNV9317R, an amplicon of approximately 300 base pairs was amplified for each of the Wesselsbron strains. (Fig. 1). A 288 base pair region of the NS5 gene of the Wesselsbron virus strains were sequenced and aligned with known arthropod borne flaviviruses. Maximum Likelihood phylogenetic analysis suggested that Wesselsbron virus strains were closest related to members of the unassigned and Yellow fever virus group and clustered with Sepik virus with a bootstrap statistic of 79%. It formed a separate branch to the Yellow fever virus and related viruses such as Banzi. The yellow fever virus group was distinct to the Japanese encephalitis and Dengue virus group. South African West Nile virus strains all grouped in the Japanese encephalitis group.

The average number of nucleotide differences within and between each group is shown in Table 1. Wesselsbron strains had an average of 2.9% differences between them and differed with 26%, 29%, 27% and 35% with the prototype Yellow fever viruses, Dengue viruses, the Japanese encephalitis group and Tickborne encephalitis groups respectively. The average number of nucleotide differences per site between the groups is shown below the diagonal in Table 1. Wesselsbron virus strains differed with 21%-22% to Sepik virus (results not shown).

DISCUSSION

Wesselsbron virus is an uncharacterised flavivirus in the family *Flaviviridae*. Little to no sequence information, and data of the molecular relation to other flaviviruses, is available. In order to determine the genetic variation between Wesselsbron strains, as well as the relationship to other flaviviruses the highly conserved NS5 protein of the flavivirus genome was targeted for sequencing. The NS5 protein encodes the two important enzymes, namely the RNA-dependent-RNA polymerase and methyltransferase (11), which are crucial for the replication of the virus and has been shown to be associated with differences in strain pathogenesis for West Nile virus (5, 6). Changes or genetic diversity within this region should therefore theoretically be minimal to maintain the function of these enzymes. On the basis of this hypothesis, published flavivirus primers as well as an "in-house" West Nile virus specific primer pair was used to amplify a short region of 288bp of the NS5 protein of the six Wesselsbron strains as seen in Fig.1. Amplification of this region using a West Nile virus specific primer indicated that the NS5 primer region within the flavivirus group is conserved.

Phylogenetic analyses of the NS5 region for each of the different strains are shown in Fig. 2. A Maximum Likelihood tree was generated for flavivirus strains from the Yellow fever virus group, Japanese encephalitis virus group (which include members of the West Nile lineage 1 and 2 strains) (13), the Dengue virus group, Tick borne encephalitis group and Flaviviruses that are currently unassigned. The Wesselsbron strains fell into their own branch and was closest related to Sepik virus which was isolated in New Guinea (11). The unassigned viruses together with Wesselsbron strains appear to be closest related to the Yellow fever virus group as seen in Fig.2. Sequence analysis of additional genome areas is needed to clarify this grouping. Within the Wesselsbron virus group, two monophyletic groups is visible indicating that these two groups are slightly divergent from one another. Wesselsbron strains H177 and AR2209 were closer related to one another, whilst AN61210, AR778, AR11189 and TAR100 show a higher degree of homology. This suggests that insect, human and animal isolates are similar.

The average pairwise nucleotide differences within and between the flavivirus groups indicated little differences at nucleotide level within the Wesselsbron virus group (2.9%) while it differed with 26-30% with viruses in the other groups and with 21% with Sepik virus. This confirms that Wesselsbron is a unique cluster of Flaviviruses. The differences with Sepik virus may be due to geographical separation although further sequence analysis will determine if these viruses belong to the same subgroup.

CONCLUSION

Wesselsbron virus are closest related to Sepik virus and the Yellow fever virus serological group based on sequence information of the NS5 protein. Analysis of additional genome regions especially the neutralising antigens will determine if areas exist that can be used to develop specific diagnostic test and that can provide cross protection to all strains if used in a vaccine or as therapeutic targets.

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Figure 1: Amplicons of different strains of Wesselsbron virus. Each strain was isolated from various regions in South Africa, either from arthropod vectors (AR778, AR2209, AR11189, TAR100), animals (AN16210) or humans (H177). A short section of the NS5 region of the genome was targeted for amplification. A 288 base pair amplicon was amplified using the primers MAMD and WNV9317R as indicated in the figure above.

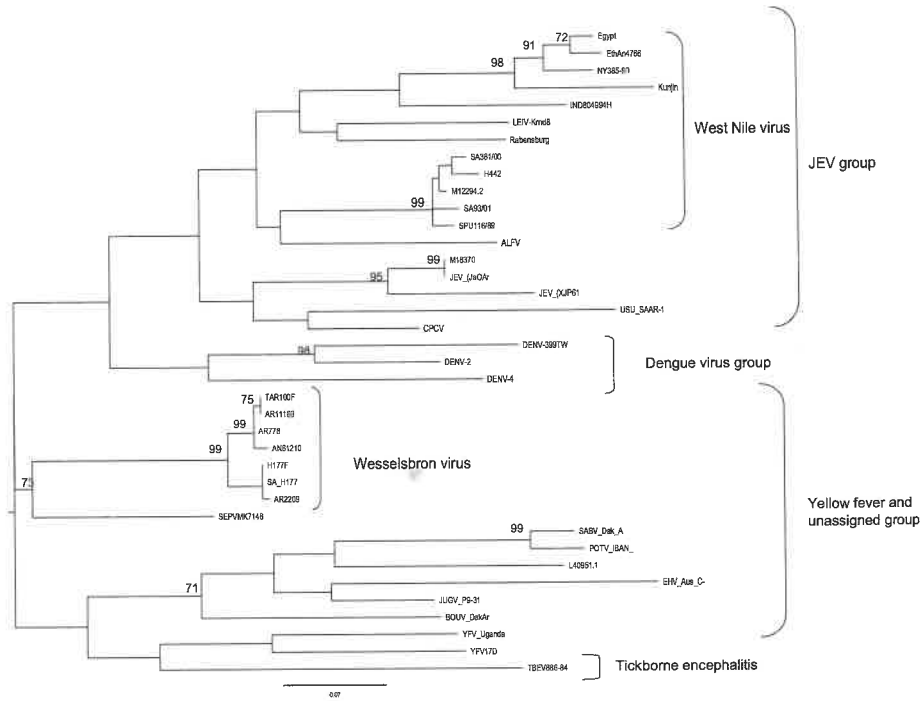

Figure 2: Maximum Likelihood tree illustrating the genetic relationship between flaviviruses based on a 288 base pair section of the NS5 region. The scale bar on the left represents the number of differences per length of branch. Bootstrap support values are shown next to each branch as calculated for 1000 replicates in a Minimum evolution based analysis. The Flavivirus serogroup assignment is shown on the far right with the Wesselsbron and West Nile virus genus shown inside the main serogroup.

Table 1: The average Pairwise evolutionary divergence between and within the Flavivirus groups shown in Figure 1. Distances between groups are shown below the diagonal and within groups in bold on the diagonal.

	1	2	3	4	5	6
[1] Wesselsbron	**0.029**					
[2] Unassigned	0.29	**0.25**				
[3] Yellow_fever_virus	0.26	0.31	**0.22**			
[4] JEV group	0.27	0.30	0.3.	**0.21**		
[5] Dengue_virus_group	0.29	0.31	0.31	0.29	**0.24**	
[6] TBE	0.35	0.36	0.29	0.35	0.34	**NC**

Notes

EPIDEMIOLOGICAL FACTORS THAT INFLUENCE CONTROL OF AFRICAN SWINE FEVER

Penrith, M.-L.

Department of Veterinary Tropical Diseases, University of Pretoria
TADScientific,40 Duncan Street, Colbyn, 0083 Pretoria, South Africa.
Tel: +27 12 342 1514, Fax +27 12 430 2192, Mobile +27 83 457 5973
E-mail: marylouise@sentechsa.com

INTRODUCTION

African swine fever (ASF) is a highly contagious and fatal haemorrhagic fever of domestic pigs caused by a unique DNA virus (*asfivirus*, Asfarviridae). It is arguably the most important constraint for pig production in sub-Saharan Africa, as well as in other regions into which it has been introduced (Penrith et al. 2004a). Since there is no vaccine, prevention of ASF depends entirely upon creating effective barriers between domestic pigs and potential sources of infection. Control of outbreaks has traditionally been achieved by 'stamping out', i.e. compulsory slaughter of all infected and in-contact pigs, with destruction of the carcasses by deep burial and/or burning. Such draconian approaches are increasingly rejected by the public for ethical, environmental and socio-economic reasons (Willis, 2007). They have always been unpopular, and mostly ineffective, in poorer countries, where they are usually applied without compensation to the owners under circumstances in which evasion of the measures is comparatively easy (Penrith & Thomson, 2004). The emphasis is therefore shifting from control to prevention, in order to avoid the challenges of outbreak control in the absence of acceptable and achievable control methods.

This paper examines the epidemiological factors that provide the guidelines for effective prevention.

HISTORY AND DISTRIBTUTION OF AFRICAN SWINE FEVER

ASF was first described by Montgomery (1921) as an entity separate from classical swine fever that caused outbreaks among settlers' pigs in Kenya, and in the next decades was identified in several more southern and eastern African countries. Its arrival in Portugal in 1957 and again in 1960, probably from Angola, demonstrated its potential for rapid spread over long distances. After its second incursion it became firmly established in both Portugal and Spain, with subsequent spread over the next 30 years to various western European countries as well as to the Caribbean islands of Cuba and Dominican Republic/Haiti and to Brazil (Figure 1). Mostly it was rapidly eradicated, although at great expense, but it remained endemic in the Iberian Peninsula until it was finally eradicated in 1993-1994 apart from a single outbreak in Portugal in 1999, and it remains endemic in the island of Sardinia (Italy). It has also spread to, and in several cases become endemic, in a number of West African countries, several of which were apparently infected for the first time during the pandemic that has unfolded in sub-Saharan Africa since the mid 1990s. It reached Madagascar in 1997, where it has become well established. In 2007, ASF was reported for the first time in the Republic of Georgia, from where it spread to neighbouring countries, most importantly Armenia. In October 2007 it was diagnosed in Mauritius. In all cases, delayed diagnosis and rapid spread of the disease hampered control measures and prevented quick eradication. All

of these countries are concerned as to how to control ASF, whether ASF is likely to evade control and become endemic, and if so, how it will be possible to maintain pig production.

MAINTENANCE AND TRANSMISSION OF ASF VIRUS

Asfivirus is the only known DNA arbovirus. In southern and eastern Africa it is maintained in an ancient sylvatic cycle between warthogs (*Phacochoerus aethiopicus*) and argasid ticks of the *Ornithodoros moubata* complex. Other wild African suids (bush pigs, *Potamochoerus* spp., and giant forest hog, *Hylochoerus meinertzhageni*) are susceptible to infection and like the warthog show no ill effects, but their role if any in the epidemiology of ASF has not been demonstrated. ASF has also become established in cycles that involve either domestic pigs and tampans, or only domestic pigs. In the absence of the sylvatic cycle, the most important factor for maintenance of the virus appears to be a supply of sufficient naïve pigs to infect to prevent the outbreak from dying out naturally, sometimes assisted by the presence of ticks of the genus *Ornithodoros* in close association with pigs.

Maintenance by Arthropod Vectors (*Ornithodoros*)

When Spain and Portugal were infected with ASF, it was eventually found that the persistence of the disease was sometimes exacerbated by the fact that pig shelters were inhabited by a species of argasid tick, *Ornithodoros erraticus*, which was able to maintain the virus for long periods and to transmit it to pigs when feeding on their blood (Sanchez Botija 1963). After this discovery, the sylvatic cycle in eastern and southern Africa was clarified by identifying the role of *O.moubata* complex ticks in maintaining the virus and transmitting it from warthogs to domestic pigs (Plowright et al. 1969, Thomson 1985), and demonstrating that these ticks are able to transmit the virus horizontally, vertically, trans-stadially, and sexually. Studies in Malawi indicated that ASF virus is maintained in a domestic cycle between domestic pigs and tampans of the *O. moubata* complex that inhabit pig shelters as well as human dwellings in the area (Haresnape et al. 1988). Tampans are long-lived arthropods that can remain dormant for several years until a blood meal becomes available (Basto et al. 2006). Portugal was considered to be free of ASF by 1993, yet a small outbreak occurred in November 1999 that was attributed to *O. erraticus* in rural pig shelters (Basto et al. 2006). Experimental infection of various species of *Ornithodoros* suggested that several species of the genus may be capable of maintaining and transmitting ASF virus (Endris et al. 1991; Groocock et al. 1980; Hess et al. 1987; Mellor & Wilkinson 1985). It can therefore be expected that if a species of *Ornithodoros* occurs in pig sties in a country that becomes infected with ASF, it may be able to maintain the virus and transmit the infection to pigs.

Maintenance of ASF Virus in The Absence Of Natural Hosts

The balance of evidence indicates that a long-term carrier state in domestic pigs does not exist (Penrith et al. 2004b), although it is often offered as a convenient explanation for persistence of ASF in pig populations. Few pigs survive infection with highly virulent viruses, which appear to be far more common than viruses of lower virulence, perhaps because their effects are dramatic. The ability of a small percentage of pigs to survive appears to be due to an inherent resistance to the virus similar to that observed in wild African suids, as they develop few or no clinical signs, and the fact that infection occurred is reflected only by the presence of antibodies. Viraemia is of short duration, probably not more than 30 days, and they are unable to infect naive in-contact pigs even when stressed (Penrith *et al.* 2004b, Valadão 1962, Wilkinson *et al.* 1983), although infective virus may be found in tissues for up

to 60 days, and viral DNA may be detectable for much longer. Subacute and chronic forms of ASF, with lifelong intermittent shedding of virus, have been described only when viruses of lower virulence were involved (Penrith et al. 2004a). Healthy survivors of outbreaks caused by highly virulent viruses such as the virus that infected the Caucasus and Mauritius are therefore not likely to play a role in ASF becoming endemic.

In endemic areas sub-Saharan Africa where the sylvatic cycle has not been demonstrated, ASF is maintained by circulation in domestic pigs. Investigations into the possible involvement of alternative natural hosts are ongoing (Vial et al. 2007, Luther et al. 2007). However, ASF has become endemic in the island of Sardinia where, unlike Spain and Portugal, *Ornithodoros* does not occur (Wilkinson 1984). The alternative to carrier pigs for maintaining ASF in domestic pig populations is the existence of sufficient pigs to permit continuous circulation of the virus (Penrith et al. 2004b, 2007). In Sardinia the susceptible pig population is extended by the existence of large numbers of European wild boar. Many domestic pigs in Sardinia are kept under free range conditions (Wilkinson 1984), so that contact with wild boars is likely. European wild boar populations have long been known as a reservoir of classical swine fever (CSF) (Kern et al. 1999; Laddomada 2000). Wild boars share the susceptibility of domestic pigs to the pathogenic effects of ASF (Laddomada et al. 1994), and are not long-term carriers of either CSF or ASF. However, it has been demonstrated that outbreaks of ASF in Sardinia occur in areas where wild boars and domestic pigs are in contact (Laddomada et al. 1994). This is likely due to the fact that the wild boar population is able to sustain the infection by continuous circulation, and the free-ranging domestic pigs have access to the carcasses of wild boars that die of ASF, or are fed the offal of sick wild boars shot by hunters. Because the ASF virus is able for survive for long periods in frozen or fresh chilled pork, as well as in uncooked or under-cooked processed pork products (Penrith et al. 2004a), persistence may be enhanced by the storage or processing of meat from pigs that died of ASF or were slaughtered during the incubation or immediate recovery period. This meat may subsequently be fed to pigs in the form of swill, or be fed upon as household waste by scavenging pigs.

Transmission of ASF Virus to Domestic Pigs

ASF virus is directly transmitted through close contact with infected pigs, bites of infected tampans, and consumption of infected meat, and indirectly through contact with fomites. Because the infective dose is high (Penrith et al 2004a), water-borne transmission is highly improbable; aerosol transmission has been shown to be possible only over short distances (2 metres or less) in a closed house (Wilkinson et al. 1977), and the movements of small animals such as rodents, birds, cats and dogs are not likely to transmit the virus effectively, as has been demonstrated for CSF (Dewulf et al. 2001). Of a large number of blood-sucking arthropods investigated, only stable flies of the genus *Stomoxys* have proven able to maintain the virus for up to 48 hours and transmit it mechanically to pigs (Mellor et al. 1987).

USING EPIDEMIOLOGY TO CONTROL ASF

Because there is no vaccine, eradication of ASF depends on eliminating the source of infection. This is obviously impossible in countries where the sylvatic cycle occurs. It is far from easy even where this cycle is absent. In 1993 Sardinia embarked upon an eradication programme that involves intensive surveillance in both domestic and wild boar populations (Dr Domenico Rutili, personal communication, 2008), but an actual increase in outbreaks was recorded in 2007. Even where domestic pigs apparently provide the only source of

infection, their eradication may be impossible for either financial or socio-economic reasons. The only hope of eradicating ASF in these cases lies in mitigating the risk posed by epidemiological factors that are present in the target area.

The principle of prevention rather than control has been established for a long time. The only way in which pigs can be farmed safely in areas where the sylvatic cycle occurs is by excluding any possibility of contact of the natural hosts with domestic pigs. The use of double fencing to ensure separation has been implemented successfully in South Africa since 1935, and it permitted Kenya to remain free of ASF for more than 30 years (Penrith et al 2004a). This approach, considerably refined by the application of biosecurity measures to exclude multiple sources of infection, is embodied in the concept of compartmentalisation that is recognised by the World Organisation for Animal Health (OIE) as a legitimate method for an epidemiological unit to maintain freedom from one or more diseases. Since implementation depends on the owner of the pigs, although monitoring by the veterinary services is required for official recognition, it is likely to prove more sustainable in the long term than government-based control.

Because of its direct transmission and limited host range, the measures to prevent ASF are relatively simple: pigs should be permanently confined, they should not be fed or have access to swill that could contain uncooked or under-cooked pork or pig remnants, access to the pig-keeping area should be limited and decontamination and disinfection practised to exclude fomites. For commercial or larger scale pig farmers these measures are so simple and logical that there should be no problem in implementing them. However, acceptance is likely to be more difficult for pig owners whose forebears have for generations farmed pigs in traditional free-ranging systems and for whom the necessity to provide food for their pigs would cancel any profit to be gained from selling them. An innovative approach is required that combines farmer education, identification of affordable and locally available housing material and feed ingredients, and improving marketing incentives to make the exercise worthwhile.

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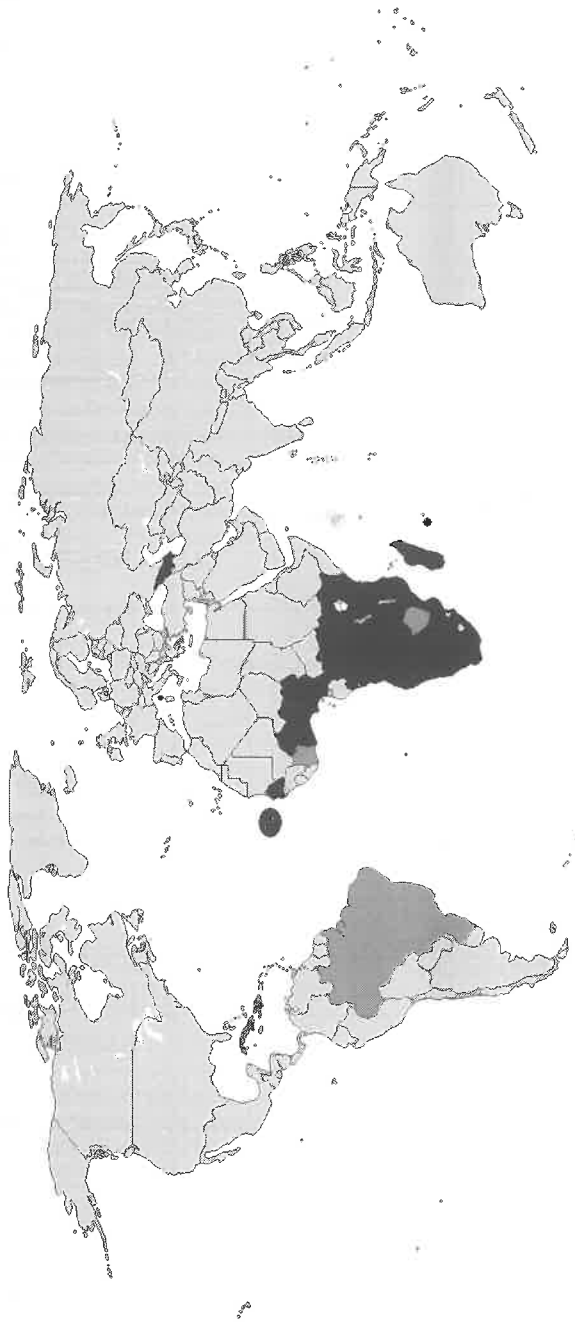

Figure 1. World distribution of African swine fever. Black = endemic or frequent sporadic infection, Dark grey = Historic infection (infection not reported for >10 years), Light grey = infection not reported officially.

Notes

RABIES CHALLENGES IN LIMPOPO PROVINCE, 2005 – 2007

Geertsma, P.J.

Department of Agriculture, Limpopo Province, Private Bag x4028, Tzaneen 0850, South Africa. E-mail: drpjg@mweb.co.za

ABSTRACT

An extensive outbreak of Rabies in the north eastern region of Limpopo Province, Republic of South Africa between July 2005 and May 2007 is studied. A total of 179 cases were seen in the outbreak which has been phylogenically typed as being most closely related to Zimbabwe dog type. 76 % of cases were in dogs. The human rabies picture is of note due to poor communication between veterinary and health authorities which caused a delay in diagnosis on the human side. There have been 26 human cases to date. The relationship on a local level between veterinary and health is seen as a necessity between these authorities in an area. The poor vaccination coverage is highlighted as a problem in the Limpopo Province with an estimated 16.9% coverage of the estimated 750,000 dogs in the province. To address this various strategies are discussed. It is noted that house-to-house vaccination is the method of choice with a 71% increase in vaccinations from 2001 to 2007 seen in a ward after implementing this method.

INTRODUCTION

Rabies, a fatal disease of humans and all other mammals, is caused by a virus which has been associated with animal bites for more than 3,000 years and is the oldest disease known to medical science.[1] There is no recorded evidence to show that canid (canine) rabies was of any importance before the late 1940s. Limpopo Province, however, experienced an outbreak of canid rabies in the 1950. This outbreak extended northwards into Zimbabwe within that year. Since that time the western portion of the Limpopo Province has experienced sporadic out breaks associated with the Black-backed Jackal (*Canis mesomelas*). This cycle in the Black-backed Jackal often involves spread to bovine and less often to dogs in this area. [2] Rabies was declared endemic to South Africa in 1999 and it is now compulsory to vaccinate dogs at least every three years.

This is borne out by the Annual Reports of Veterinary Services, Limpopo, with 123 laboratory-confirmed cases from 2001 to 2004. The majority of these cases being in bovines (64 out of 123, or 52%). [3,4,5,6,7] The annual average incidence being 30 rabies cases.

THE OUTBREAK

The north-eastern portion of the Limpopo Province is a densely populated area with 988 villages in the area. In this area there are 2,117,893 people with an average village size of 2,143 people (Stats SA, 2001.)[8] The area borders on Zimbabwe in the north. It is includes the Vhembe and Mopani District Municipalities.

In July of 2005 there were 5 cases of rabies in dogs reported in the Thulamela Municipality of Vhembe District Municipality, a maximum of 41 kilometers apart. This was the first recorded cases of rabies in that area since 1993 – 1994 where 2 cases in dogs were reported in the same area. Over the next 22 months the disease spread rapidly in the area. Up

until May 2007 there had been a total of 179 cases in the area(See figure 1). The majority of the cases were recorded in dogs (137 canine cases out of 179 cases, or 76%)(See Figure 2). The area of the outbreak was now 184 kilometers east to west and 166 kilometers from north to south.

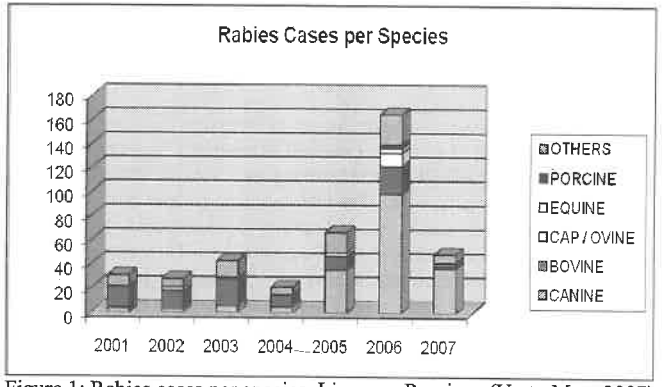

Figure 1: Rabies cases per species, Limpopo Province (Up to May, 2007)

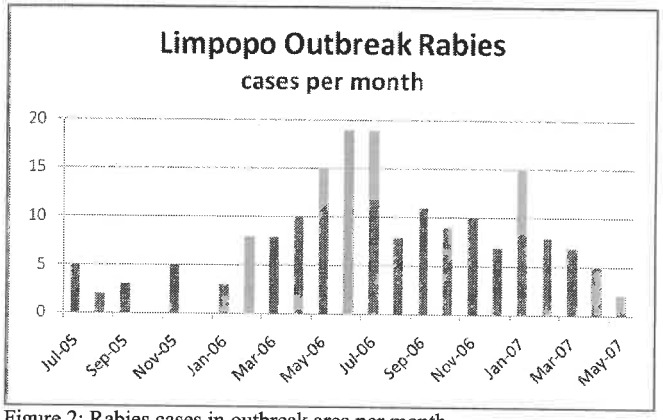

Figure 2: Rabies cases in outbreak area per month.

ORIGIN

.As can be seen from the molecular epidemiology of the virus as typed from the outbreak in Figure 3 below, the virus is more closely related to the Zimbabwe Dog strains than to the Limpopo and Zimbabwe jackal strains.[9] Many people have been crossing over from Zimbabwe into Limpopo, many illegally without passing through any veterinary checkpoint. This could easily have been the source of the infection with this outbreak. This is further seen graphically by showing all the Black-backed jackal and dog cases from 2002 to 2005. There is no contact between the western jackal cycle and the north-eastern canine outbreak. (See figure 4, below)

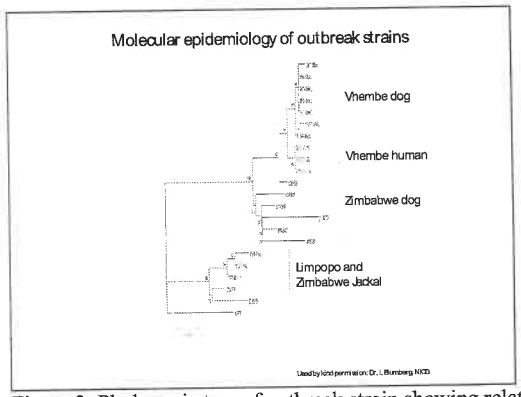

Figure 3: Phylogenic tree of outbreak strain showing relationship to Zimbabwe dog type virus.[8]

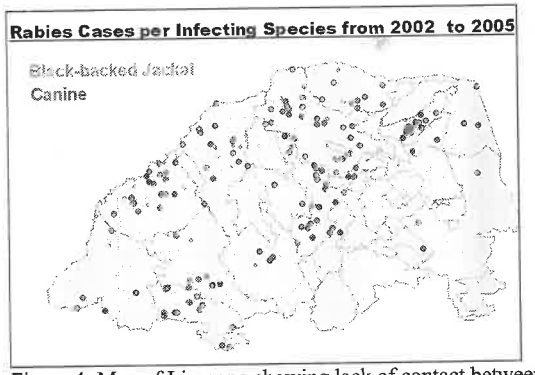

Figure 4: Map of Limpopo showing lack of contact between western jackal cycle and north-eastern dog outbreak.

HUMANS

Initially there was no contact with veterinary and health authorities to alert the health authorities to the presence of the zoonotic disease in the area. As can be seen from the graph below in Figure 5, the health authorities started experiencing cases of a fatal encephalitis whose aetiology could not be determined. It was only in February of 2006 that there was an outbreak investigation and rabies was suggested as a possible cause. A child that died at that time was sampled and confirmed to be rabies. At the same time the veterinary authorities were contacted and a link between this outbreak was established. To date there have been a total of 26 human fatalities (20 confirmed, 4 probable and 2 possible). 24 cases were in children between 3 – 12 years of age and 2 in adults.[8]

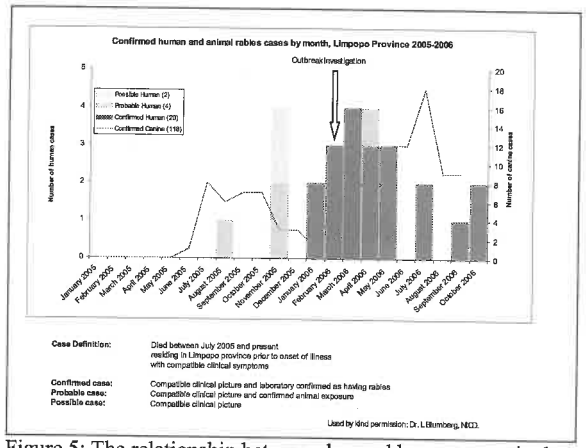

Figure 5: The relationship between dog and human cases in the outbreak.[8]

INTERVENTION

The outbreak in the Thulamela Municipality in July 2005 elicited an immediate response from the veterinary authorities; the most important are listed below:

- In order to put a stop to the spread of the virus, intensified vaccinations of dogs and cats in the area were instituted.

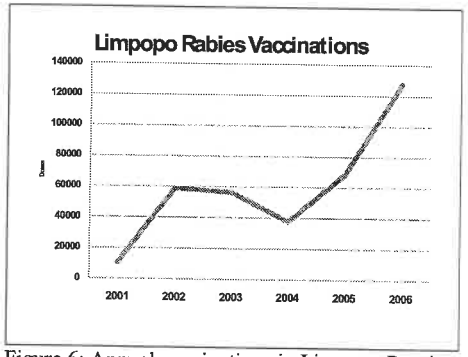

Figure 6: Annual vaccinations in Limpopo Province.

- In November 2006, the veterinary management took a decision to form a Provincial Rabies Advisory Group (PRAG) under the umbrella of the national Rabies Advisory Group (RAG). This body forms a link with the expertise and practical implementation of rabies control strategies nationwide.
- The Limpopo Provincial Department of Health and Social Welfare has been co-opted onto the PRAG in order to facilitate co-ordination between health and veterinary.
- On the local level joint operational committees were established between veterinary, health, SAPS, SPCAs and disaster management.
- Feral cats and dogs are being controlled with co-ordination between veterinary, SAPS and SPCA.
- Road shows and awareness drives have been held in each and every area. Radio stations were used to warn people of the dangers and to get their animals vaccinated.

DISCUSSION

Vaccinations

There is a need to explain the apparent lack of an adequately protected population in the area. For there to be adequate protection of a population there needs to be at least 70% of the animals with a protective titre against rabies. [2] Therefore the success or otherwise of the provincial effort in this regard needs to be studied in greater depth.

As can be seen from the provincial vaccination statistics in Figure 6 above, there was a drastic decrease in the vaccinations carried out in 2001 and in 2004. This is a result of the veterinary section having to cope with large epidemics of Foot and Mouth Disease in these specific years. Technical staff are withdrawn from their wards to combat the spread during these outbreaks and the routine vaccinations are not done adequately.

Vaccination spots are generally used to vaccinate rabies during campaigns. Here a school or cool tree is chosen where the vaccinators wait for the dogs to be brought to them. In a large village this may mean that pet owners need to walk long distances to a vaccination point (sometimes over 8 kilometers).This has led to the practice of having multiple spots within a village. This has to some extent alleviated the situation. However, consider the situation in a typical village (Motupa-Moleketla complex) in Figure 7 below. Each circle represents a 200 m walk to the centre point where a vaccination spot is held. In a village complex like this you can clearly see that the 21 vaccination points did not adequately address the problem of a long distance to take the owner's pets to a vaccination point.

Kwa-Zulu-Natal has been using house to house vaccinations in order to address this problem with great success over a number of years (Personal communication-Kevin le Roux, KZN veterinary). A technician in the Greater Tzaneen Municipality has implemented this method in Ward T1. In 2001 he just had a spot or two in each village. In 2002-03 he conducted a survey to estimate dog numbers and announced the upcoming vaccination. This resulted in a moderate increase in vaccinations (See Figure 9 below). However in 2004-07 house-to-house vaccinations have been carried out. In 2007 the vaccination team was strengthened to include 4 teams. A 71% increase (1,112 in 2001 to 1,903 in 2007) over the 2001 numbers was seen in the 12 villages of this ward (Some of this increase may be due to an increase in the dog population which was not measured).

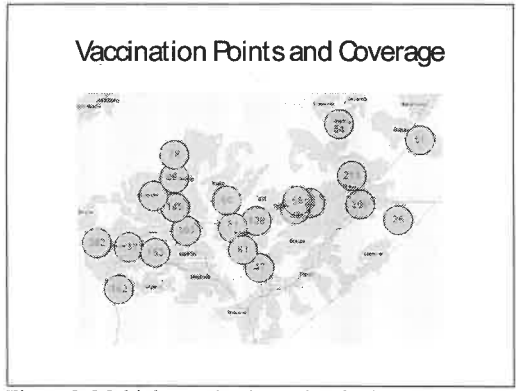

Figure 8: Multiple vaccination points in the Greater Tzaneen Municipality to address long walking distances. Each circle represents a 200 m walk to the vaccination spot in the centre.

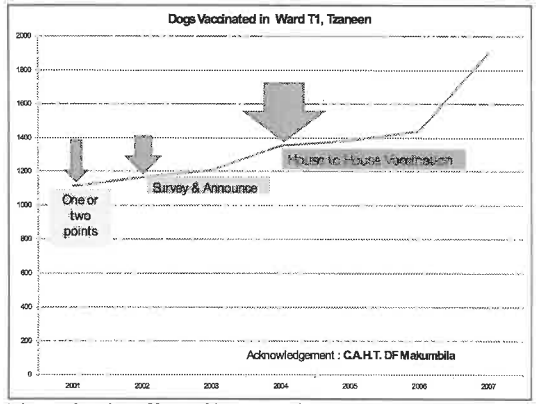

Figure 9: The effect of house-to-house vaccinations in the Greater Tzaneen Municipality from 2001 to 2007.

If an increase like this is possible in a Ward then what can be expected in the province. 30 informal surveys were conducted in the Greater Tzaneen and Ba-Phalaborwa Municipalities. 20 households were surveyed in each survey in different villages (a mix of semi-rural and very rural villages was chosen). The results showed that in these households there is an average of 5.1 people to one dog (1,475 people had 363 dogs). This was an informal survey and the results may not reflect the true situation in the province as a whole. KZN has done better controlled studies and found the ratio to be 7 : 1. . Using the provincial population of 5.27 million this then relates to 750, 000 dogs (at 7:1). In 2006 the province as a whole only vaccinated 127,000 dogs. This amounts to 16.9% of the estimated population.

CONCLUSION

Census figures for dogs can be misleading. Each area needs to have a thorough census done in order that meaningful management decisions can be taken, especially when organising a vaccination campaign. Currently it is at best a thumb suck figure that is used.

Watch tendencies over time and respond rapidly to unusual increases in incidence of disease in an area. Be aware of what the normal incidence of a disease in an area is and monitor the rate of incidence using this *criterium*. If a disease is seen which is exotic to the area, be mindful of adequate protection through immunisation figures of the past.

Report new cases of rabies in an area to the health authorities. If there is a grouping of medical doctors, inform them also. If this is not possible, at least place an article in the local press informing everyone of the situation.

Be aware that rabies infection can spread rapidly over vast distances. Infected but not yet rabid dogs can easily be transported, bought or sold and land up in a distant area. It was also noted that rabid animals can travel large distances until they succumb. A case in point is the case seen near Pafuri in the Kruger national park. Motorists had seen the dog along the tarred road when it entered the KNP at Punda Maria, more than 45 kilometers away. Therefore do not assume that a history of absence of the disease necessarily means it will not become a problem.

House to house vaccination for an effective coverage of an area is the desired method for effective disease containment. If this cannot be applied annually due to a shortage of resources, then planning should be in place to do it every three years at least in order to ensure an adequately protected population. In the event of an outbreak, this method should be used in the vaccination zone to ensure adequate boosting of immunity.

ACKNOWLEDGEMENTS

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DIAGNOSIS AND EPIDEMIOLOGY OF RABIES AND OTHER AFRICAN LYSSAVIRUSES

Markotter, W and Nel, L.H.

University of Pretoria, Department of Microbiology and Plant Pathology, 0002 Pretoria, South Africa. Dr. Wanda Markotter, Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria, 0002. South Africa
Tel: +27 (0)12 420 4602, Fax: +27 (0)12 420 3266, E mail: wanda.markotter@up.ac.za

ABSTRACT

Lyssaviruses belong to the family *Rhabdoviridae*, a group of bullet-shaped viruses that can all cause rabies encephalitis in mammals. The lyssavirus genus consists of seven genotypes of which four are present in Africa. These are rabies virus (gt 1) and three rabies-related viruses; Duvenhage virus (gt 4), Mokola virus (gt 3), Lagos bat virus (gt 2). Two virus biotypes of rabies virus occur in Africa – respectively circulating in herpestid species (mongooses) and in canid species (mostly domestic dogs, jackals and bat-eared foxes). The gold standard for lyssavirus diagnostics is a fluorescent antibody test that detects all lyssaviruses, but does not distinguish between different lyssavirus genotypes. To identify the lyssavirus genotype, additional methods such as monoclonal antibody typing or PCR/nucleotide sequencing can be used. Analyses of the changes in the genome sequence of lyssavirus isolates over time, in different geographical locations and different host species, also allows for a better understanding of the epidemiology of rabies.

During the past five years several new isolates of rabies-related lyssaviruses have been obtained from South Africa. These isolates have been characterized using monoclonal antibody typing and molecular techniques and have contributed to a better understanding of the prevalence and epidemiology of these rabies-related lyssaviruses in general, but of LBV in particular. Rabies vaccines do not protect against gt 2 and 3 viruses (LBV and MOKV) and to be able to make decisions on control strategies as well as the public and veterinary health risk of these African lyssaviruses it is important to identify lyssavirus infections in animal and humans and to accurately determine the lyssavirus genotype involved.

INTRODUCTION

Rabies is caused by all members of the lyssavirus genus in the family *Rhabdoviridae,* a group of single stranded negative sense RNA viruses, currently consisting of seven genotypes (gts). Classical rabies viruses (including the rabies vaccine strains) are grouped in the lyssavirus genotype 1 (Rabies virus (RABV)) and this group occurs almost worldwide, whereas the remaining six genotypes (2 to 7) are known as the rabies-related lyssaviruses (Tordo *et al.*, 2004). Two biotypes of gt 1 (RABV) occur in Africa; the mongoose biotype and the canine biotype (Nel *et al.*, 2005). Apart from the above mentioned gt 1 lyssaviruses; gt 2 (Lagos bat virus (LBV)), gt 3 (Mokola virus (MOKV)) and gt 4 (Duvenhage virus (DUVV)) have been reported exclusively from the African continent (Nel and Markotter, 2007).

Commercial vaccine strains belong to gt 1 (RABV) and it is believed that the corresponding vaccines will be effective against gt 1, and 4-7 but not against gt 2 and gt 3 (Nel, 2005). The history of rabies in Africa, prior to the 20th century is poorly recorded (Swanepoel, 2004; Nel and Rupprecht, 2007) but it appears that canine rabies in most of Africa and in particular in sub-Saharan Africa, is a disease of modern times. The 'cosmopolitan' canine variant of RABV, is thought to have originated from the Palearctic region (Badrane and Tordo, 2001) and introduced from European territories into Africa during the years of colonization. RABV became well established in dogs but also in wildlife reservoirs such as bat-eared foxes and black-backed jackals. Although specific statistics for the incidence of rabies in Africa is not available due to poor surveillance, it is believed that rabies is an emerging disease throughout the African continent (WHO, 2005). Although all mammals are considered susceptible to rabies infection, only a few can serve as a successful reservoir that by itself sustains the infection in a given geographical area (Rupprecht *et al.*, 2002). For RABV (gt 1), the domestic dog (canine) is the major reservoir and vector and responsible for the majority of animal and human rabies cases on the African continent. In humans, rabies only occurs as spill-over dead-end infections. In Africa mongooses and related species are important reservoirs of the mongoose biotype of RABV. LBV appears to primarily circulate in frugivorous bats in Africa (Markotter *et al.*, 2006a) and DUVV in insectivorous bats in Africa (Paweska *et al.*, 2007). MOKV has been isolated from shrews, cats and dogs but the reservoir species have not been identified and bats cannot be excluded (Nel and Rupprecht, 2007).

Most frequently, lyssavirus diagnosis is performed post-mortem on animals suspected of being rabid and those that have been involved in potential human exposures. In the latter case, it is important that the diagnosis should be quick and reliable in order to ensure correct post-exposure prophylaxis (PEP) decisions and prevent fatal infections in exposed individuals (Nel and Markotter, 2007). It is also important to use reliable diagnostics in epidemiological studies to be able to make informed decisions about prevention and control of the disease. The gold standard for lyssavirus diagnostics approved by both the World Organisation for Animal Health (OIE) and the World Health Organisation (WHO), the fluorescent antibody test (FAT), is performed on brain tissue. These preparations are treated with anti-lyssavirus serum (conjugate) that is labeled with fluorescein isothiocyanate (FITC) and viewed under a fluorescence microscope (Dean *et al.*, 1996). This test can detect all lyssavirus genotypes but cannot distinguish between them. To characterize the lyssavirus genotype involved additional tests such as antigenic and molecular methods can be used. Different panels of monoclonal antibodies were developed and can be used to identify the gt of lyssaviruses involved or to identify the lyssavirus biotypes present after positive identification with the FAT (Markotter *et al.*, 2006a and b). The lyssavirus nucleoprotein gene is the most conserved and therefore targeted in diagnostic techniques. DNA sequences generated from this region can then be compared with known lyssavirus sequences and the genotype involved can be identified. For molecular epidemiology studies a more variable region of the genome such as the G-L intergenic region can be targeted (Nel *et al.*, 2005).

In the past five years seven new LBV isolations have been reported from South Africa, one new DUVV isolate and two more MOKV isolates. The isolates were identified using monoclonal antibody typing as well as DNA sequencing and phylogenetic analyses. In addition several molecular epidemiology studies on rabies virus isolates (gt 1) from South Africa were performed, indicating the spread of the disease. The molecular epidemiology of isolates from the Kwazulu Natal province will be discussed in more detail.

MATERIALS AND METHODS

In the past five years various samples suspected to be infected with a lyssavirus were analyzed using the following methods; Samples were first tested using the standard fluorescent antibody test (FAT), with modifications, using a polyclonal fluorescein isothiocyanate conjugated immunoglobulin (Onderstepoort Veterinary Institute, Rabies Unit) (Dean *et al.*, 1996). Samples that tested positive using the FAT test were then further analyzed by monoclonal antibody typing (Markotter *et al.*, 2006b). Antigenic typing was performed by using the indirect fluorescent antibody test with a panel of 16 antinucleocapsid monoclonal antibodies (N-MAbs) (Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada). Genetic characterization was based on sequencing of the nucleoprotein (N) gene as described by Markotter *et al.*, 2006b. To genetically compare gt 1 isolates from KwaZulu Natal the method as described by Coetzee and Nel, 2007 was used. The G-L intergenic region was amplified by RT-PCR, the DNA sequence generated and the phylogenetic analysis performed.

RESULTS

Isolations of rabies-related lyssaviruses

During the past five years (2003-2008) ten new isolations of rabies-related viruses were made from South Africa (Table 1). These isolates were characterized using monoclonal antibody typing as well as partial DNA sequencing of the nucleoprotein gene.

Table 1: New isolations of rabies-related lyssaviruses in South Africa in the past five years (2003-2008).

Gt	Geographical location	Year	Origin	Reference
2	Durban, KwaZulu Natal Province, South Africa	2003	Frugivorous bat *(Epomophorus wahlbergi)*	Markotter *et al.* 2006a
2	Durban, KwaZulu Natal Province, South Africa	2003	Canine	Markotter *et al.*, 2008
2	Durban, KwaZulu Natal Province, South Africa	2004	Frugivorous bat *(E. wahlbergi)*	Markotter *et al.* 2006a
2	Durban, KwaZulu Natal Province, South Africa	2004	Water mongoose (*Atilax paludinosus*)	Markotter *et al.* 2006b
3	East Londen, Eastern Cape Province, South Africa	2004	Feline	Sabeta *et al.*, 2007
2	Durban, KwaZulu Natal Province, South Africa	2005	Frugivorous bat *(E. wahlbergi)*	Markotter *et al.*, 2008
3	Nkomazi, Mpumalanga Province, South Africa	2005	Canine	Sabeta *et al.*, 2007
4	Sun City, North West Province, South Africa	2006	Human	Paweska *et al.*, 2006
2	Durban, KwaZulu Natal Province, South Africa	2006	Frugivorous bat *(E. wahlbergi)*	Markotter *et al.* 2006a
2	Durban, KwaZulu Natal Province, South Africa	2008	Frugivorous bat *(E. wahlbergi)*	Unpublished

Molecular epidemiology of rabies virus in the KwaZulu Natal province, South Africa

In recent years the amount of rabies cases in South Africa in animals and humans increased. In an extensive analysis of RABV from KwaZulu Natal and surrounding regions, viruses from the KwaZulu Natal and Eastern Cape provinces were found to belong to a unique lineage, circulating as two independent and expanding epidemiological cycles (Figure 1). The first presented as closely related dog cycles along the eastern coastal regions of the two provinces, while the second, in northern KwaZulu Natal, has entered into at least one wildlife reservoir, the black backed jackal. All the viruses were found to belong to the canine RABV biotype. The fact that the majority of viruses from the KZN province clustered into subfamily A, led us to believe that this lineage was representative of the present day core of the epidemic. The order of the divergence events suggested that the EC cycles became established at an earlier time period than the present-day cycles of subfamily A, that were identified from the coastal regions of the KZN province (KZN group of subfamily A viruses). Viruses in the second major group (subfamily B) were isolated from the northern regions of KZN and presented as two clusters. Perhaps not surprisingly, this lineage was found to be related to viruses (dog and jackal) from a northerly bordering province (Mpumalanga). It implicated that jackals may play an important role in the epidemiology of the disease within northern KZN.

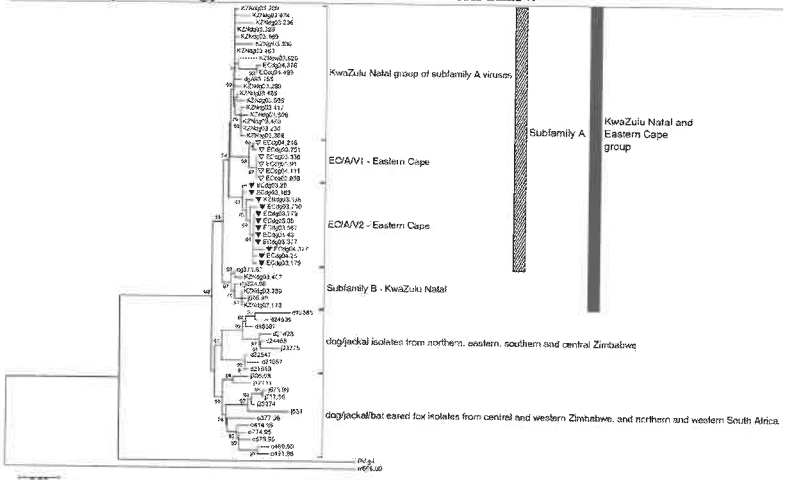

Figure 1: Neighbourhood-joining tree of 64 nucleotide sequences of the cytoplasmic domain, and G-L intergenic region, for canine, domestic livestock and wildlife rabies viruses from KwaZulu Natal ($n = 20$), the Eastern Cape ($n = 19$), and rabies endemic regions from elsewhere in South Africa and Zimbabwe ($n = 25$). Isolate numbers are preceded by prefixes indicating the geographic region (KZN, KwaZulu Natal/EC, Eastern Cape) as well as host species of isolation (dg (d), dog/cw, bovine/gt, caprine/j, jackal/o, bat eared fox) (Coetzee and Nel, 2007).

CONCLUSIONS

Although rabies-related viruses appear to be rare and have only been sporadically identified from South Africa in the past, ten new isolations were made through passive surveillance in the past five years. Before these new isolations LBV has not been reported from South Africa for 13 years, DUVV for more than 36 years and MOKV for 7 years. This re-emphasizes our lack of understanding of the true prevalence of lyssaviruses throughout Africa, due to poor surveillance throughout the continent. In the case of LBV considered here, it is worth re-iterating that human cases have not been documented to date. At the same time, this virus has however been reported in cats and dogs as well as from terrestrial wildlife (mongoose spp) (Markotter *et al.*, 2006b). MOKV has also been reported from domestic cats and dogs. The recent 2005 report from MOKV from Mpumalanga is the first report from this area (Sabeta *et al.*, 2007). Previous reports of MOKV have been from the Eastern Cape or KwaZulu Natal provinces. The recent 2006 DUVV isolate followed contact with a bat in a well known upmarket resort in the North West Province, which led to fatal rabies in an elderly tourist (Paweska *et al.*, 2007). This again emphasized the inadequate surveillance for DUVV since two of the three DUVV cases reported in South Africa have been spill-over infections in humans. It is clear from cross-neutralization data obtained in rodent models that rabies pre- and post-exposure prophylaxis is unlikely to be effective against LBV and MOKV (Nel, 2005). It is not possible to provide informed advice on the control and prevention of any disease where basic epidemiological data are as scarce as for the African lyssaviruses. Even for gt 1 (RABV), general surveillance is inadequate and the amount of cases occurring is greatly underestimated. Surveillance should be maintained as part of a strategy towards better understanding of the epidemiology of rabies and rabies-related lyssaviruses the FAT should be used in combination with other tests to be able to discriminate between different lyssavirus genotypes.

Furthermore, molecular epidemiology allowed us to track and describe the emergence and continued expansion of rabies in geographical regions. This will allow the more accurate prediction of rabies into new geographical areas, involvement of wildlife in the spread of the disease and applying this information in deciding on future control programs.

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HUMAN RABIES IN SOUTH AFRICA

Weyer, J.[1], Blumberg, L.H.[2], Leman, P.A.[1], Swanepoel, R.[1], Paweska, J.T[1]
[1]Special Pathogens Unit and [2]Epidemiology Unit

National Institute for Communicable Diseases, National Health Laboratory Service, South Africa.
Dr Jacqueline Weyer, Special Pathogens Unit, National Institute for Communicable Diseases, Private Bag X4, Sandringham 2131. Tel: +27 113866376, Cell: +27 829039131, Fax: +27118823741, Email:jacquelinew@nicd.ac.za

BACKGROUND

Rabies is an incurable, fatal encephalitis of possibly all mammalian species. In fact it is the infectious disease with the highest case-fatality ratio. Humans are exposed to the rabies virus through interactions with rabid animals. Most often humans are infected through animal bites, but scratches or licks on broken skin or mucosa are also possible routes of infection. The incubation period of rabies virus infection is very variable, but usually 4-6 weeks. During this incubation period the virus replicates locally at the site of the wound and then progresses to the peripheral nervous system and ultimately the central nervous system. Before the virus reaches the nervous system a window period is available for post exposure prophylaxis. Rabies vaccine and anti-rabies immunoglobulin (RIG) should be administered to all animal bite patients considered to be at risk to rabies virus exposure according to the guidelines depicted by the World Health Organization.

THE RABIES SITUATION IN SOUTH AFRICA

Rabies is endemic in South Africa (and the entire African Continent). In Southern Africa the Black-backed jackal (*Canis mesomelas*), Bat-eared fox (*Otocyon megalotis*), Yellow mongoose (*Cynictis penicillata*) and domestic dog is the primary reservoirs of the disease, although possibly any mammalian species (particularly carnivores) may become infected and therefore act as vectors of the disease. The majority of human cases in South Africa is attributed to dog exposures, although some cases related to domestic cats, mongoose and other wildlife are also reported. Six to 31 cases (mean number of cases: 15.3 cases per annum) of human rabies have been confirmed annually since the early 1980s. The majority of human rabies cases in South Africa are reported from the KwaZulu-Natal (more than 80% of laboratory confirmed human cases over the past 20 years) and Eastern Cape Provinces (Table 1). These provinces are characterized by abundant rural populations where domestic dogs are often free-roaming and/or community owned. Vaccination of these animals, an indirect mode of protection for humans, is problematic, with these animals often being unapproachable and without responsible ownership. Some communities may also have alternate beliefs regarding the vaccination of dogs, believing that the vaccine may render the animal sterile or less aggressive, a concern when these animals are kept for protection purposes. High and continuous vaccination coverage of 80 % of a dog population is required to break the cycle of transmission in a given setting. Stray dog population control is also a critical factor in rabies control.

Table 1: Summary of the laboratory confirmed human rabies cases for South Africa from 1986 to 2007 per province. The grand totals per province is provided at the bottom of the table (Figures from National Institute for Communicable Disease, National Health Laboratory Service)

Year	KZN	EC	MP	NC	FS	NW	LPP	WC	GP	Total per year
1986	7	0	1	0	0	1	0	0	0	9
1987	16	1	0	1	0	0	0	0	0	18
1988	25	1	0	0	2	0	0	0	0	28
1989	10	0	0	0	0	0	0	0	0	10
1990	11	0	0	1	0	0	0	0	0	12
1991	20	0	0	0	0	0	0	0	0	20
1992	22	2	0	0	0	0	0	0	1	25
1993	21	2	0	0	1	0	0	0	0	24
1994	18	1	0	0	0	0	0	0	0	19
1995	29	0	0	0	0	0	0	0	0	29
1996	12	2	0	0	0	0	0	0	0	14
1997	3	2	0	0	0	1	0	0	0	6
1998	5	1	0	0	0	0	0	0	0	6
1999	4	2	0	1	0	0	0	0	0	7
2000	7	1	0	0	0	1	0	0	0	9
2001	6	0	1	0	0	0	0	0	0	7
2002	8	0	0	1	0	0	0	0	0	9
2003	9	1	0	0	0	1	0	0	0	11
2004	7	0	1	0	0	0	0	0	0	8
2005	3	3	0	0	1	0	0	0	0	7
2006	4	4	0	0	0	1	22	0	0	31
2007	8	5	0	0	0	0	1	0	0	14
Total	**255**	**28**	**3**	**4**	**4**	**4**	**23**	**0**	**1**	**321**

Rabies biologicals (vaccine and RIG) for human administration are often not available due to local and global shortages. The RIG available in South Africa is produced locally from rabies vaccinated human donors. The stock of RIG is very limited and prohibitively expensive due to limited bank of donors and the intensive screening of adventitious agents required for this type of product. Stocks have been completely depleted on various occasions, and provincial depots are often required to shuffle stock around to deal with demand.

CURRENT CONCERNS

Rabies statistics

The number of laboratory confirmed human rabies cases were relatively high in the early and mid 1990s, with an average of more than ten cases per year. This figure dropped below ten confirmed cases from 1998 to 2006 (with the exception of 2003, when 11 cases were confirmed) possibly reflecting the increased vaccination of dogs particularly in KwaZulu Natal. The number of confirmed cases reached an unprecedented high in 2006 with 31 cases. 23 of these cases were related to the outbreak of rabies in Limpopo Province, a province where no cases have been reported for the two preceding decades. In 2007, 14 cases were reported with involvement in KwaZulu Natal, Eastern Cape and a single case from Limpopo Province. Dog vaccination and control programs instituted in Limpopo Province in 2005/2006 led to a coinciding drop in the number of human cases in 2007. For the first 5 months of 2008, 12 cases of human rabies have already been confirmed (KwaZulu Natal: 5; Eastern Cape: 4 and Limpopo Province: 3). The rise in the number of cases from the Limpopo Province again in 2008 places a question mark on the sustainability of dog vaccination and population control programs in this area.

The public health burden of rabies is greatly underestimated. Many probable rabies cases (due to clinical presentation and case history) are not confirmed. Examples include two probable cases from Mpumalanga Province to date for 2008– a province that as reflected through the statistics (Table 1) appears not to have a great human rabies concern (NICD Communique vol 7 no 3, March 2008).

Laboratory confirmation of human rabies cases (as with animal cases) requires a post mortem brain specimen to be tested. Families often refuse invasive necropsy of the demised patients and morticians may be apprehensive to perform necropsy on patients suspected to have died of rabies. Ante mortem confirmation of human rabies cases is playing an important role in the recognition of cases, with roughly a third of the cases reported for 2008 being confirmed by PCR on saliva specimens. Unfortunately awareness of the availability of this testing is relatively low and often this testing is not considered.

Serology is of limited importance in the confirmation of rabies virus infection although blood is most often submitted to the laboratory for testing.

Rabies case histories

Investigation of rabies post exposure prophylaxis and treatment details of the 12 confirmed cases for the first 5 months of 2008 highlights some of the common problems in the effective delivery of prophylaxis (Figure 1). In the 59 % of the cases (7/12) apparently no treatment was sought by the patient after the animal bite. In two of the cases the patients visited local health care facilities and only received tetanus toxoid, wound cleaning and dressing with apparently no consideration for the requirement of rabies post exposure prophylaxis. In one case the patient visited a local health care facility and the necessity of rabies post exposure prophylaxis was realized. The facility was out of stock for rabies vaccine and immunoglobulin when the patient visited the facility. The patient was requested to return to the facility after a weekend to provide the staff with time to source the biologicals from elsewhere. After returning to the health care facility the vaccine stock was not replenished. The patient was referred to another health care facility, situated more than 50 km away and due to financial constraints the patient was not able to visit this facility. The patient passed away due to rabies encephalitis about a month afterwards.

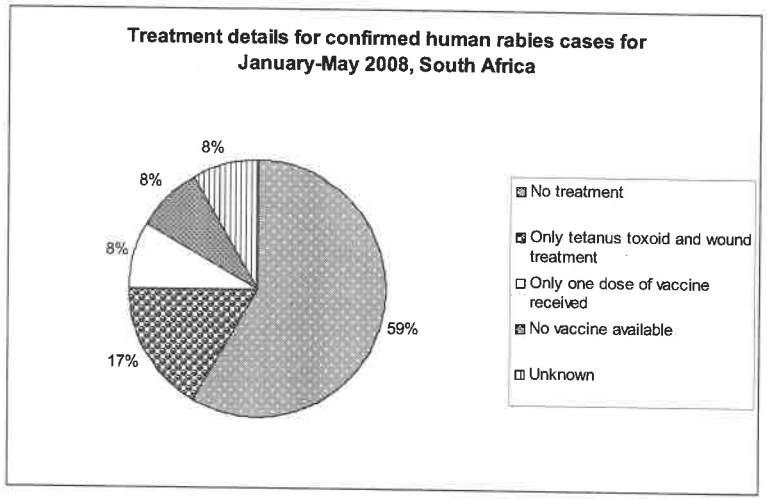

Figure 1: A summary of the post exposure prophylaxis and treatment that laboratory confirmed human rabies cases received after being bitten (Figures from National Institute for Communicable Disease, National Health Laboratory Service).

CONCLUSION

The rise in the number of laboratory confirmed human rabies cases over the past 3 years is worrisome. Although rabies is a 100 % fatal upon onset of clinical disease, it is also completely preventable when rabies biologicals are administered according to the World Health Organization guidelines.

The **public awareness** of the necessity for rabies post exposure prophylaxis is low, with more than half of the laboratory confirmed cases of human rabies for 2008 apparently not seeking medical attention after an animal bite. Compounding this problem is the fact that young children are often involved in these cases and are likely not to report scratches or small bites to their guardians. Secondly the **awareness of health care workers** of the requirement for assessing the risk of rabies exposure for all animal bites and the administration of rabies biologicals in probable cases is not adequately illustrated by the cases where patients that did present to a health care facility were not considered for rabies prophylaxis. Thirdly the **availability of rabies biologicals** (vaccine and RIG) is problematic, with stock outs in critical areas often reported.

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COULD THE TRANSFER OF ANTIMICROBIAL RESISTANCE BE CONSIDERED A NEW FORM OF ZOONOSIS?

McCrindle, C.M.E.[1] ,Cenci-Goga, B.

[1] Prof CME McCrindle, Section VPH, Dept Paraclinical Sciences, Veterinary Faculty, University of Pretoria, Pvte Bag X04, Onderstepoort 0110, South Africa. Tel +27 12 529 8181, Fax +27 12 5298311, E-mail: Cheryl.mccrindle@up.ac.za

ABSTRACT

Both the environment and food of animal origin can contribute to the spread of antimicrobial resistance. It can even be considered that certain bacteria, belonging to *Enterococcus* spp., *Staphylococcus* spp. and the lactic acid bacteria group, which are used as starters or adjunct cultures in dairy products could show antimicrobial resistance. It is known that antimicrobial resistance can be transferred to pathogenic bacteria and the World Health Organisation has suggested that the use of antimicrobials in animals, thus increasing antimicrobial resistance, could result in human pathogens becoming resistant. A direct method to detect antimicrobial resistance in raw cows milk has been developed to enable rapid estimation of risk without the need for bacterial culture. The risk of such transfer will be discussed in the light of antimicrobial resistance being considered a new form of zoonosis.

INTRODUCTION

Antimicrobial resistance (AMR) in bacteria is a concrete threat to human health. It has emerged in the last decades as a consequence of the large scale use of antibiotics as growth promoters and in human and veterinarian curative medicine. AMR results in the failure of therapeutic actions against bacterial infections, which causes an increase in morbidity and mortality in both animals and people. The misuse of antimicrobials is today considered the most important factor for the emergence, selection and dissemination of AMR (Bonfiglio *et al.,* 2002; van de Bogaard, 1997). The extent of the environmental contribution to diffusion of AMR and transmission through food of animal origin has not been thoroughly investigated (Davidson, 1998; Mariano *et al.,* 2007). AMR bacteria, belonging to *Enterococcus* spp., *Staphylococcus* spp. and to the lactic acid bacteria group, also used as starters or adjunct cultures in dairy products, are being isolated from types of food (particularly yoghurt and cheese) that forms a large proportion of human diets . Rapid estimation of risk, particularly in perishable dairy products, could be one of the main risk management options. Over the last years, methicillin resistant Staphylococci (MRSA), vancomycin resistant enterococci (VRE), tetracycline resistant enterococci and high-level gentamicin resistant enterococci (HLGR) have increasingly become responsible for serious nosocomial infections, ranging from urinary tract and wound infections to endocarditis, bacteremia and neonatal sepsis (Cenci Goga et. al., 2004, Corpet, 1998, Steinfeld, *et al.,* 2006). This increased incidence of these infections is mainly due to their remarkable ability to rapidly develop high-level resistance to antimicrobial agents (Tueber, 1999).

Bacteria make use of a variety of mechanisms to avoid the effect of antimicrobials controlled by specific genes, these mechanisms include: enzymatic inactivation of

antibiotics, impermeability of the bacteria membrane, active expulsion of the drug by the cell efflux pump and alteration in target receptors (Chopra *et al.,* 2001). The acquisition of new genes, encoding for different kinds of AMR, can often be explained by horizontal transfer of genes between bacteria, rather than by the sequential modification of gene functions by accumulation of point mutations (Davidson, 1999, Linch *et al.,* 1999). Antimicrobial resistance is mainly gained though various mobile elements, such as plasmids, transposons, and integrons, which result in mutation in genes responsible for antimicrobial agent uptake or binding sites, or activation of bacterial chromosomes (Sayah *et al.,* 2005). There are three identified mechanisms for gene transfer in bacteria (Davidson, 1999):

1. Transformation, involving the uptake and incorporation of naked DNA.
2. Conjugation, a cell contact-dependent DNA transfer mechanism found to be very common in bacteria.
3. Transduction, where host DNA is encorporated into a bacteriophage, which acts as the vector for its injection into the recipient cell.

There are many studies that confirm that AMR can be exchanged between bacteria of different classes and different species (Kruse *et al.,* 1994; Van de Braak *et al.,* 1998; Aarestrup *et al.*, 2000; Sayah *et al.,* 2005). In addition, the continuous ingestion of sub-therapeutic levels of antimicrobials, used for performance enhancement in animals, has lead to resistance in enteric bacteria. For example, in a prospective study, it was determined that tetracycline resistance among *E. coli* in faecal samples from a chicken farm increased within a week of the introduction of tetracycline-supplemented feed to the flock (Levi *et al.,* 1976). In the USA, where virginiamycin is widely used as a performance enhancer, resistance is common in *Enterococcus faecium* of animal origin, whereas for avoparcin, that has not been used, the acquired resistance to glycopeptides is virtually non-existent in enterococci isolated from animals (Phillips *et al.,* 2004). In the European Union (EU), it has been shown that the resistance levels of *Enterococcus* species to vancomycin in broilers reduced from 72.7% in 1995 to 5.8% in 2000, after avoparacin was banned for use in food animals (Kaszanytzky, 2006). It has been estimated that discontinuing the use of antimicrobials as performance enhancers would reduce the total amount of the usage of antibiotics used in production animals by at least 50% (Kaszanytzky, 2006). However after the growth promoter ban in the EU, it was documented that, in countries like Denmark and Sweden, there was a slight increase in the total amount of antibiotics used for therapeutic applications (Phillips *et al.*, 2004). The Swedish monitoring report indicated an increased use of fluoroquinolones, cephalosporins, sulphonamides-trimethoprim as well as tetracyclines for treatment of individual animals (SWARM, 2004). This increased use of antibiotics for therapeutic interventions was not followed by an increase of AMR in production animals, but instead by a general decrease in the amount of AMR, perhaps because the total amount of antibiotic sold decreased (SWARM, 2004). The decreased use of performance enhancers did not lead to an appreciable decrease in production or in animal welfare problems (van de Bogaard *et al.,* 1997).

It is generally accepted that the greatest driver of multi-resistance in bacteria of human origin, is the treatment of humans with antimicrobials. However, there are examples that show linkages between the usage of antimicrobials in food animals and development of resistant infections in humans, indicating that transfer of resistant bacteria from animal to human could occur (Chopra *et al.,* 2001, You *et al.,* 2005).

Trends in antimicrobial resistance in S*almonella* isolates and *Campylobacter jejuni* in animals, tend to be similar in humans (Swartz, 2002). Persons working with livestock have been found to carry a higher level of resistant bacteria. A good example is that of methicillin resistant *Staphylococcus aureus,* which is highly prevalent in the pharynx of pigs as well as in the throats of farm workers. Although there is epidemiological evidence of transfer of these agents to humans, there is no experimental proof of direct transmission between food animals and humans (Phillips *et al.,* 2004).

The public health threat resulting from the use of antimicrobials as performance enhancers has been the subject of an intense debate since 1960, when the Swann Committee, in the UK, concluded that antimicrobials used in human chemotherapy, or those that promote cross-resistance to other therapeutic agents, should not be used as performance enhancers in animals (Chopra *et al.,* 2001; WHO, 2001; Turnidge, 2004). The concern about this issue caused the EU to ban most antibiotics, with the exception of coccidiostats, as performance enhancers in animals for precautionary reasons in 1996. Recently the World Health Organisation (WHO), has supported this decision (Steinfeld *et al.*, 2006). This study describes a method for the rapid detection of AMR resistant bacterial DNA directly from cows milk, without the need for prior culture.

METHODS

Bacteria with AMR genes : *E. faecalis* (*van*(A), *E. faecium* (*aac(6')-Ie-aph(2'')-Ia*) *S.aureus (mecA)* were cultured in nutrient broth and added to raw milk. Following plating of each dilution on nutrient agar , the numbers of CFU/ml were in accordance with expected values based on the decimal dilution factor (Cenci Goga *et al.*, 2004)

Samples were centrifuged at 2200g at room temperature for 5 min. Supernatant, including the hardened fat layer, was aspirated and discarded, except for the bottom 1 ml, which included all the cells and the casein pellet. To dissolve casein, 300 µl 0.5M disodium ethylenediamine-tetracetate (EDTA) pH 8.0 and 200 µl 10 mM Tris-HCl-1 mM EDTA, pH 7.6 (TE) were added to the supernatant. DNA from these bacteria was successfully extracted from adulterated raw milk, without the need for prior culture, by the preliminary purification of milk samples with TE and EDTA followed by a DNA extraction method using the QIAamp DNA Blood Mini Kit[1]. All samples were tested to detect the presence of the *mecA*, *van*(A) and *aac(6')-Ie-aph(2'')-Ia* genes by means of Polymerase chain reaction (PCR).

RESULTS

In the first PCR, the rapid and simple method of DNA extraction, provided a detection limit of 10^4 CFU/ml for *mecA* and *vanA* genes, while 10^5 CFU/ml for *aac(6')-Ie-aph(2'')-Ia* gene (Figure 1). The second PCR with nested primers allowed a 10^5-fold improvement in the detection limit, with visible amplicons obtained at concentration levels of 10 CFU/ml. (Figure 2). No visible amplicons were obtained from control samples where no *E. faecalis* (*van*(A), *E. faecium* (*aac(6')-Ie-aph(2'')-Ia*) and *S.aureus (mecA)* had been added.

[1] (QIAamp DNA Blood Mini Kit, Qiagen, 52306, Milan, Italy).

DISCUSSION AND CONCLUSIONS

Risk assessment for AMR in perishable foodstuffs of animal origin, particularly milk is made difficult because of the lag time of 36-72 hours resulting from antimicrobial culture for identification of bacteria present in the sample, followed by further culture to demonstrate the level of AMR. The PCR method developed in this study enables rapid detection of AMR genes without the need for culture. It also opens the possibility of determining AMR in cultured milk products such as yoghurt.

It is known that antimicrobial resistance can be transferred to pathogenic bacteria and the WHO has suggested that the use of antimicrobials in animals could result in human pathogens becoming resistant. If the nature of transfer of resistance between bacteria is taken into account, i.e. transformation, conjugation and transduction, as described earlier in this article, then antimicrobial resistance can be considered an infective particle. The question is, therefore, whether transfer of the genetic component that codes for antimicrobial resistance can in essence be regarded as a form of zoonotic disease, in line with the OIE definition:
"*A zoonosis is a disease transferred between vertebrate animals and humans*".
In this study, there is a definite indication that animal products such as milk are a medium for transmission of antimicrobial resistance factors to humans.

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