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Experimental infection of ostriches with Crimean–Congo haemorrhagic fever virus

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SUMMARY

Following the occurrence of an outbreak of Crimean–Congo haemorrhagic fever (CCHF) among workers at an ostrich abattoir in South Africa in 1996, 9 susceptible young ostriches were infected subcutaneously with the virus in order to study the nature of the infection which they undergo. The ostriches developed viraemia which was demonstrable on days 1–4 following infection, with a maximum intensity of 4·0 $\log_{10}$ mouse intracerebral LD_{50}/ml being recorded on day 2 in 1 of the birds. Virus was detectable in visceral organs such as spleen, liver and kidney up to day 5 post-inoculation, 1 day after it could no longer be found in blood. No infective virus was detected in samples of muscle, but viral nucleic acid was detected by reverse transcription-polymerase chain reaction in muscle from a bird sacrificed on day 3 following infection. It was concluded that the occurrence of infection in ostriches at abattoirs could be prevented by keeping the birds free of ticks for 14 days before slaughter.

INTRODUCTION

Crimean–Congo haemorrhagic fever (CCHF) is a tick-borne virus of Africa, Asia and eastern Europe which causes human illness with an approximately 30% fatality rate [1–5]. Infection can be transmitted by ticks of several genera, but the world distribution of the virus coincides with the distribution of the main vectors, members of the genus *Hyalomma*. The virus causes mild infection with transient viraemia in farm animals such as sheep and cattle which serve as hosts of adult *Hyalomma* ticks. Immature *Hyalommas* feed on small wild mammals, up to the size of hares, and ground-frequenting birds. The small mammal species that have been tested also appear to undergo mild infection with viraemia, and they are thought to play an important role as a source of infection for ticks. However, little information was available on CCHF infection of birds prior to 1984 when a worker contracted the disease at an ostrich abattoir in Oudtshoorn district, South Africa: limited observations in the former Soviet Union had indicated that passerine birds and domestic chickens were refractory to the virus although a low prevalence of antibody could be detected in wild birds [5, 6].

The abattoir worker infected in 1984 reported that he had encountered numerous ticks on ostrich carcases and while he could not recall being bitten, he mentioned that the ticks caused scratch marks on his arms and hands when he pulled the skin off carcases [6, 7]. Although the implication was that he became

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infected from contact with the blood of ostriches, it was known that humans do not have to be bitten by ticks, but can become infected from merely squashing them [5]. We failed at that time to obtain ostriches which had been reared under tick-free conditions, and opted to study viraemia and antibody response in commercially bred domestic chickens and guinea fowl instead. Chickens were refractory to infection, but guinea fowl developed low-titred viraemia and transient antibody response [6]. Since we detected a much higher prevalence of antibody to CCHF virus in ostriches than in wild guinea fowl, the implication was that ostriches may undergo a more intense infection than do other birds, with a stronger and more durable antibody response [3, 6, 8]. Subsequently it was found that a few species of wild bird tested in West Africa fail to develop demonstrable viraemia following experimental infection [9].

In October 1996 there was an outbreak of 17 cases of CCHF among workers at an ostrich abattoir which employs about 400 people in Oudtshoorn (to be reported elsewhere). Once again we were prompted to investigate the nature of the infection which ostriches undergo in order to devise protective strategies for workers in the industry, and to determine the risk to consumers of ostrich products. We studied viraemia and antibody response to experimental infection in nine ostrich chicks which had been raised under tick-free conditions, and the findings are presented here.

MATERIALS AND METHODS

Materials

The South African prototype strain of CCHF virus, 4/81, isolated from the blood of a human patient with fatal disease in 1981 [10], was grown in Vero cell cultures and stocks for inoculating ostriches were stored at −70 °C. Nine 3-month old ostriches (approximately 50 cm high at the shoulder) which had been raised under tick-free conditions at the research farm of the Klein Karoo Co-operative, Oudtshoorn, were transported by road to the Onderstepoort Biological Products Institute near Pretoria where they were kept during the experiment in an isolation stable with rubber gasket sealed doors, negative air pressure with Hepa-filtered exhaust, and heat treatment of sewage effluent. The stables were cleaned daily with chlorine-based disinfectant. Personnel wore disposable protective clothing including gloves, gowns, Hepa-filtered masks, visors and overshoes. Discarded clothing and the carcases of sacrificed birds were disposed of in incinerators within the stable building.

Experimental procedure

The ostriches were fed a compound ration on which they had been reared and were given fresh drinking water daily. They were bled and found to lack antibody to CCHF virus by competition enzyme-linked immunoassay (CELISA) [8] prior to experimental infection. The birds were inoculated subcutaneously with $10^{5.5}$ fluorescent focus units (FFU) of stock virus, and bled daily for 14 days for titration of viraemia and antibody response. Individual birds were sacrificed on days 3, 4, 5, 7 and 14 post-inoculation to test various organs for the presence of virus, as indicated in the results. Sacrificed birds were stunned and bled by severance of the major blood vessels of the neck to simulate abattoir procedure. Organ samples were collected for virological and histopathological examination. An upper leg, with thigh muscles which represent the bulk of the meat that is utilized for human consumption, was taken from each sacrificed bird and hung at 4 °C. Samples of meat were taken from the leg on the day that the bird was sacrificed and daily for the following seven days to test for virus content.

Assays for virus content of serum and organ samples were performed in parallel in Vero cell cultures and infant mice as described previously [11]. Initial 10% suspensions of visceral organ and muscle samples were prepared by homogenizing weighed pieces of approximately 1 cm^3 of tissue in cell culture medium, and clarified by centrifugation at 3000 g for 15 min at 4 °C. Tenfold serial dilutions were prepared from the supernatant fluids for titration of virus infectivity and titres were expressed as $\log_{10}$ FFU/ml and mouse intracerebral 50% lethal doses/ml ($\log_{10}$ $MICLD_{50}$/ml). Suspensions prepared from meat samples collected on the day that each bird was sacrificed were also tested for presence of viral RNA by reverse transcription and polymerase chain reaction (RT-PCR) [12]. The CELISA for antibody to CCHF virus in ostrich sera was performed as described previously for livestock and wild vertebrate sera, starting at an initial dilution of 1/10 [8]. Antibody titres were recorded as the reciprocal of the highest serum dilution in which a positive result was recorded. The sera were also tested for antibody at doubling dilutions from 1/100 in a sandwich enzyme-linked immunoassay (ELISA), as described previously

Table 1. *Viraemic titres in 9 ostriches during the first 14 days following subcutaneous infection with Crimean–Congo haemorrhagic fever virus. Individual ostriches were sacrificed on each of days 3, 4, 5, 7 and 14 post-inoculation to test selected organs for the presence of virus*

	Viraemic titre on indicated day post-inoculation													
Ostrich	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	—*	2·6†	2·5											
2	—	1·9	2·6	—										
3	—	—	2·0	1·8	—	—	—	—	—	—	—	—	—	—
4	—	1·6	2·6	—	—	—	—	—	—	—	—	—	—	—
5	1·6	2·0	2·5	1·6	—	—	—	—	—	—	—	—	—	—
6	—	2·5	2·5	—	—	—	—	—	—	—	—	—	—	—
7	—	2·5	—	1·9	—									
8	—	4·0	1·8	1·6	—	—	—	—	—	—	—	—	—	—
9	—	2·0	1·6	—	—	—	—	—	—	—	—	—	—	—
n =	9	9	9	8	7	6	6	5	5	5	5	5	5	5

* No viraemia detected.
† Viraemic titre expressed as $\log_{10}$ $MICLD_{50}$/ml.
Blanks indicate that ostriches had been sacrificed and were no longer available for testing.

Table 2. *Detection of virus in selected organs of 5 ostriches sacrificed on days 3, 4, 5, 7 and 14 following subcutaneous infection with Crimean–Congo haemorrhagic virus*

	Detection of virus in organ on indicated day post-inoculation				
Organ	3	4	5	7	14
Muscle	—*	—	—	—	—
Spleen	1·8†	1·9	—	—	—
Liver	2·6	—	1·6	—	—
Kidney	1·9	—	—	—	—
Lung	—	—	—	—	—
Heart	—	—	—	—	—

* No virus detected.
† Virus titre expressed as $\log_{10}$ $MICLD_{50}$/ml.

for sheep [8], except that an anti-ostrich immunoglobulin peroxidase conjugate supplied by Mr C. H. Boshoff of the Onderstepoort Veterinary Institute was used at a dilution of 1/250.

RESULTS

No signs of illness were observed in the ostriches. The results of mouse assays for virus content of blood and other tissue samples are summarized in Tables 1 and 2, and a curve for mean viraemia titres is shown in Figure 1. Viraemia was detected during the first 4 days following infection only, with a maximum titre of 4·0 $\log_{10}$ $MICLD_{50}$/ml (Table 1). Virus was detected variously in spleen, liver and kidney of three ostriches sacrificed on days 3, 4 and 5 post-inoculation, with a maximum titre of 2·6 $\log_{10}$ $MICLD_{50}$/ml (Table 2), but not from organs of birds sacrificed on days 7 and 14. No infective virus could be isolated from samples of thigh muscle taken from any of the 5 ostriches on the days on which they were sacrificed, nor from repeat samples collected over the following 7 days while the meat hung at 4 °C. Viral nucleic acid was, however, detected by RT-PCR in the first meat sample collected from the bird sacrificed on day 3 post-inoculation. Infective virus was detected in the spleen, liver and kidney of the same ostrich (Table 2), and it had a viraemia titre of 2·5 $\log_{10}$ $MICLD_{50}$/ml on the day that it was sacrificed (ostrich number 1 in Table 1). As observed previously [11], cell cultures proved to be less sensitive than mouse inoculation for detection and titration of infective virus (data not shown). No overt histopathological lesions were observed, but immunostaining was not performed to detect infected cells.

Antibody activity first became detectable by CELISA in the serum of a single ostrich on day 5 after infection, and by day 13 all ostriches had seroconverted (Fig. 1). A maximum CELISA titre of 1280 was recorded in 1 bird on day 9 post-inoculation. In the ELISA using anti-ostrich immunoglobulin conjugate, antibody activity became demonstrable day 6 post-inoculation, and all ostriches had seroconverted by day 12, with maximum titres of 1600 being recorded in individual birds (Fig. 1).

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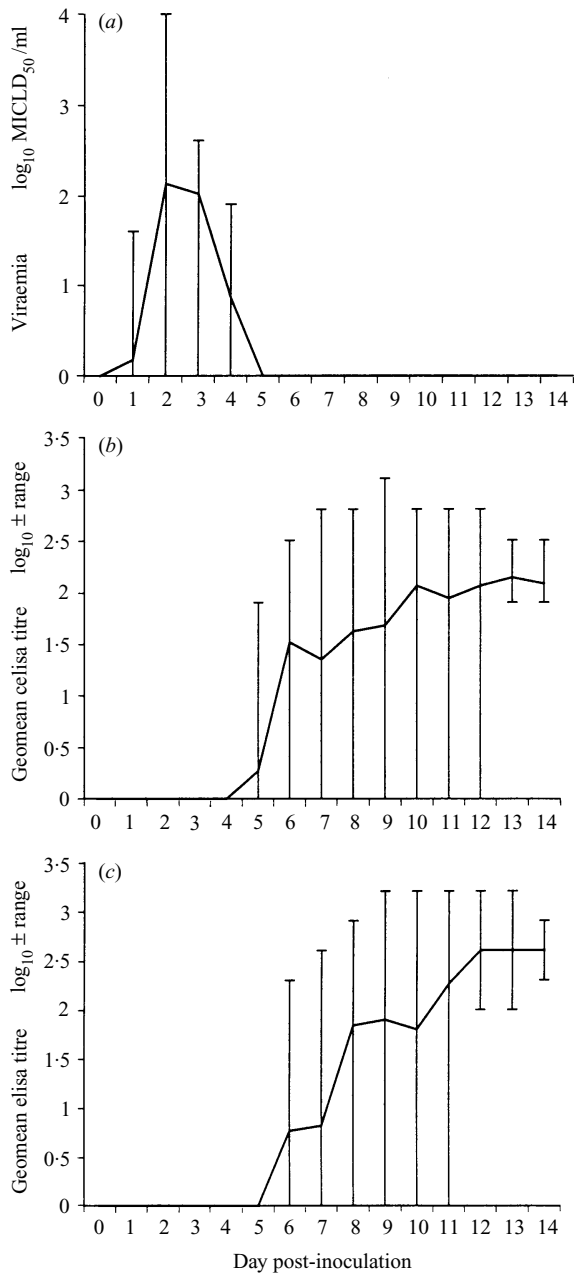

Fig. 1. Curves showing (*a*) mean viraemic titre in $\log_{10}$ $MICLD_{50}$/ml, ± range; (*b*) geometric mean antibody titre as determined by CELISA, ± range, and (*c*) geometric mean antibody titre as determined by ELISA, ± range, during the first 14 days following subcutaneous infection of ostriches with CCHF virus.

DISCUSSION

In brief, the present findings were that ostriches developed viraemia which was demonstrable on days 1–4 following subcutaneous infection with CCHF virus, with a maximum intensity of 4·0 $\log_{10}$ $MICLD_{50}$/ml recorded on day 2 in 1 of the birds. Furthermore, virus was detectable in visceral organs such as spleen, liver and kidney up to day 5, which was at least 1 day after it could no longer be found in blood. No infective virus was detected in samples of muscle, which is normally utilized as meat for human consumption, but it must be conceded that low concentrations of infectivity may be present in muscle during viraemia, even after routine exsanguination of carcases at abattoirs. This is supported by the fact that viral nucleic acid was detected by RT-PCR in muscle from a bird sacrificed on day 3 following infection. It is even possible that infectivity may have been detected if muscle had been examined earlier, on days 1 and 2 post-inoculation, since mean viraemia was maximal on day 2 (Table 1, Fig. 1). The viral nucleic detected in muscle may have been present in residual blood in the tissue sample, or in blood vessel walls since it has been shown by immunohistochemistry that endothelial cells, mononuclear phagocytes and hepatocytes are the main targets of CCHF virus infection in humans [13].

The results of the CELISA and ELISA tests were in close agreement, and confirm that ostriches have a strong antibody response to infection with CCHF virus. Either technique could be used in routine antibody tests.

The viraemic titres recorded in ostriches are in marked contrast to the total lack of detectable viraemia found in domestic chickens and wild birds following experimental infection, with the exception of guinea fowl in which a maximum titre of 2·5 $\log_{10}$ $MICLD_{50}$/ml was observed [5, 6, 9]. It is notable that sheep and cattle develop viraemia of similar intensity, up to 5·0 $\log_{10}$ $MICLD_{50}$/ml, to that observed in ostriches, but with a maximum recorded duration of up to 8 days [1–5, 14]. Antibody to CCHF virus was found in 22/92 ostrich sera from 6/12 farms tested in South Africa [2, 6], and in a much lower proportion of thousands of sera tested from Namibia and Zimbabwe [unpublished laboratory records]. There are many accounts of humans becoming infected from contact with fresh blood of domestic ruminants, including farmers and abattoir workers in South Africa 1–5, 7, 15, unpublished laboratory records]. In contrast, there has been no indication that urban consumers anywhere encounter CCHF infection from meat derived from either ostriches or ruminants, and processed according to normal health regulations [1–5]. Apart from nosocomial infections, the disease has never been recorded in a town dweller who did not have a history of exposure to ticks or to fresh blood and other tissues of livestock in a rural setting or

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abattoir over the past 17 years of observation in southern Africa, during which more than 2000 individual cases or outbreaks of suspected viral haemorrhagic fever were investigated, and during which 141 cases of CCHF were diagnosed [1–3, 7, 10, 15, unpublished laboratory records].

Possible explanations for the lack of reported disease in urban consumers include the speculation that the fall in pH which occurs during the maturation of meat in abattoirs may be deleterious to the virus [1]. Furthermore, the viraemia observed in sheep, cattle and ostriches is of moderate intensity as compared to that which occurs in other zoonotic diseases such as Rift Valley fever of ruminants [16], and hence meat derived from an infected carcase that has been properly exsanguinated and hung to mature would contain a low concentration of CCHF virus, and should be a relatively dry and less infectious product to handle than fresh blood. This is supported by the fact that the risk of infection in ruminant and ostrich abattoirs appears to be greatest for workers involved at the beginning of the slaughter process where bleeding and handling of fresh carcases occurs (unpublished information to be presented elsewhere). Finally, it is pertinent that most consumers probably only encounter meat after it has been cooked, and little or nothing appears to be known about the infectivity of the virus by the oral route.

Despite the apparent lack of evidence of disease in urban consumers of meat, it remains unacceptable that CCHF infected animals should reach abattoirs to pose a potential threat to workers and the public. Tick infested animals pose an additional threat to abattoir workers since partially engorged ticks tend to detach from their hosts after slaughter, or from the hides and skins of the hosts, and may then attach to any humans in the vicinity. These problems can be solved by ensuring that animals remain free of ticks for at least a set minimum period before slaughter. In calculating the required length of this period for ostriches, the duration of the incubation period following natural exposure to infection by ticks must be added to the potential duration of viraemia. In the absence of specific information for ostriches, it is necessary to extrapolate from observations on other animals. We found that 3 sheep infested with experimentally infected ticks had a 3-day incubation period up to the onset of demonstrable viraemia, and 53 human patients with confirmed CCHF became ill within 1–3 days after exposure to tick bite [1, 7, unpublished laboratory records]. (A further 53 patients who had contact with infected blood of livestock or humans had incubation periods of 5–6 days or longer, and incubation periods could not be fixed accurately for the remaining 35 patients that we have studied.) Therefore, a theoretical incubation period of 3 days following tick infestation can be added to the observed 4 days duration of viraemia in ostriches, and doubling of this sum to allow a margin of safety, yields a period of 14 days during which ostriches should be kept free of ticks before slaughter. An equivalent period for sheep and cattle would probably be 21 days since viraemia has been recorded up to day 8 post-infection [1–5, 14].

Ostriches could be kept free of ticks for the required period by treating them with an acaricide which has low toxicity for the birds and for humans, high efficiency and rapid lethality for ticks, and adequate residual efficacy on feathers without accumulation of residues in internal tissues. Modern pyrethroid preparations meet these requirements, and some are suitable for use on the bare, trampled soil in ostrich feedlots as an added precaution. Ostriches are either run in extensive paddocks or confined in feedlots. In either type of husbandry, holding pens should be constructed, or feedlots modified, so that the birds can be treated with acaricide and kept in quarantine, if necessary under veterinary supervision, for 14 days prior to translocation to slaughterhouses. Holding pens should be enclosed by a fence, preferably double, of suitable construction to exclude feral terrestrial hosts of immature ticks. Equivalent measures should be adopted for sheep and cattle.

Furthermore, it is advisable that the effects of management practices on host–parasite relationships on ostrich farms should be investigated in order to optimize tick control measures. For instance, it was observed prior to the outbreak of CCHF at the abattoir in Oudtshoorn in 1996 that certain batches of ostriches which had been run in extensive camps with natural vegetation were heavily infested with ticks, whereas ticks were rarely seen on birds from feedlots. The timing of slaughter operations in relation to peak seasons of tick activity should also be investigated. Ultimately, the development and use of CCHF vaccine in ostriches, and domestic ruminants, may prove to be an important and effective public health measure.

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