Determination of veterinary bactericidal activity of Virkon-S against *Coxiella Burnetii* in high soiling conditions

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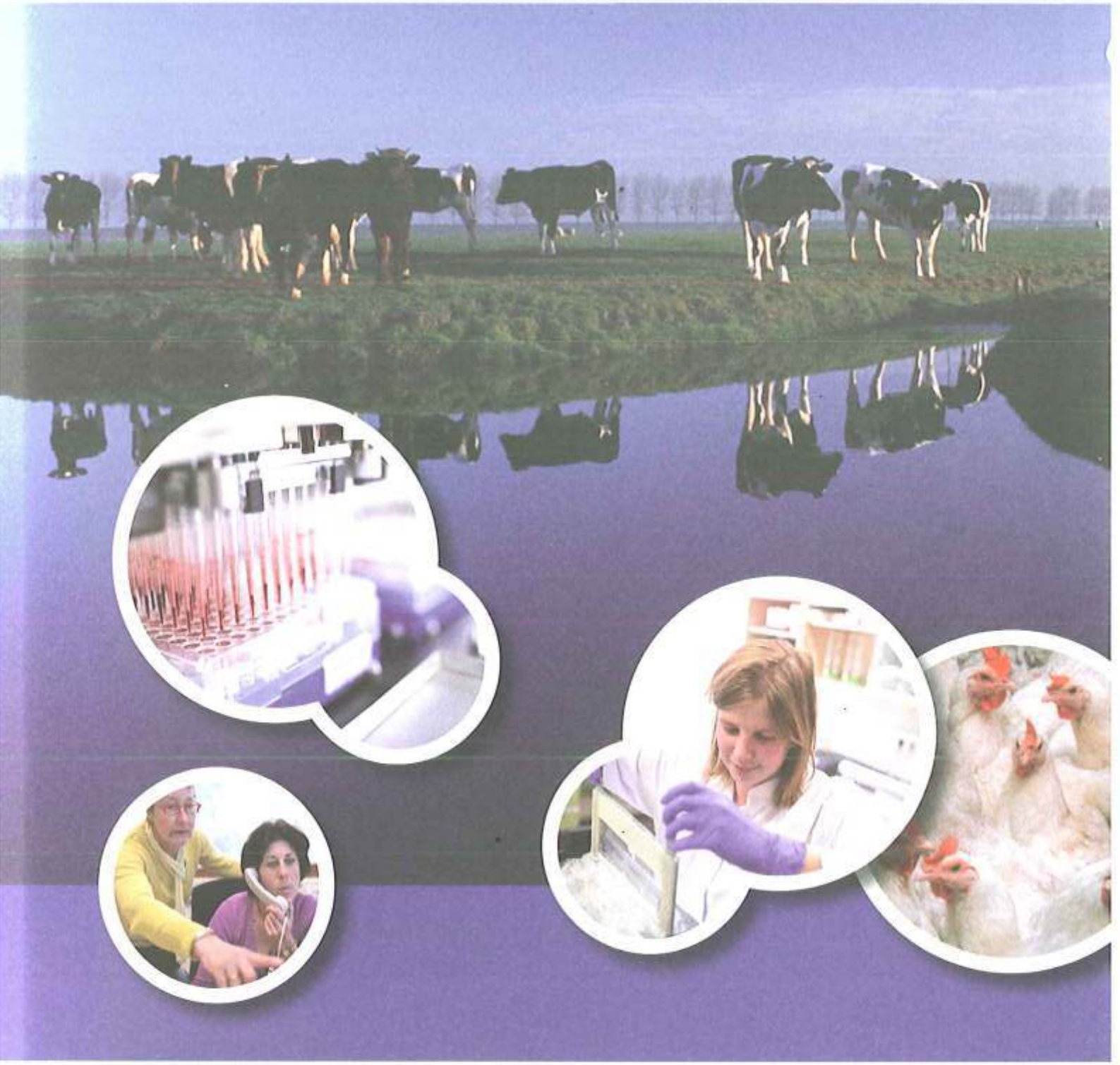

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Determination of veterinary bactericidal activity of Virkon-S against *Coxiella Burnetii* in high soiling conditions

The test method to determine the veterinary bactericidal activity of Virkon-S against Coxiella Burnetii was based on the requirements of NEN 1656:2000.

A test suspension of Coxiella Burnetii in a solution of high level soiling conditions (interfering substance) was added to a prepared sample of Virkon-S® . The mixture was maintained at 10°C ± 1°C. At a contact time of 30 min ± 10 s, the mixture was centrifuged and the disinfectant was washed away (PBS). The Virkon-S ® treated and washed *Coxiella Burnetii* bacteria were incubated in a serial 10-fold dilution on Slide Flasks with buffalo-green-monkey (BGM) cells. After two weeks, the supernatant of the cell cultures were harvested and tested by qPCR for the presence or absence of Coxiella Burnetii. All tests were performed in duplicate .

1. Test Laboratory

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All procedures with infectious *Coxiella burnetii* were conducted within biological safety cabinets under Biosafety level 3.

2. Identification of the Product Sample

Name of sample:	Virkon-S®
Batch number:	1011BA0062
Manufacturer:	Antec™ International Windham Road, Chilton Industrial Estate Sudbury, Suffolk CO102XD, UK
Date of delivery:	26 MAY 2011
Storage conditions:	laboratory storage condition at room temperature
Active substances:	Potassium Peroxomonosulphate

3. Experimental conditions

Period of analysis: May – November 2011

Appearance of the product and its dilutions: pink crystals, pink clear solution

Test product concentrations: product test solutions were prepared freshly in SHW at three different concentrations (0.5% = 50 mg in 10 ml, 1.25% = 125 mg in 10 ml and 2.5% = 250 mg in 10 ml) that were 1.25 times the required test concentrations (0.4%, 1.0% and 2%). They included two concentrations in the active range (1% and 2%) and one concentration in the non-active range (0.4%). The Product Test Solutions were prepared freshly and used within 60 min.

Stability of test mixture: no precipitation of product through test

Test temperature & contact time: 10°C ± 1°C for 30 minutes ± 10 s

Product diluent:

Standardized hard water (SHW) for dilution of test products was prepared freshly.

- Solution A was prepared by dissolving 19.84g $MgCl_2$ (Merck, $MgCL_2.6H_2O$ with M=203.30 g/mol) and 46.24g $CaCl_2$ (Sigma; 223506-500g; $CaCl_2.2H_2O$ with M=1474.01 g/mol) in Water for Injection (Fresenius Kabi Nederland B.V., 's-Hertogenbosch) and made up to 1000ml. Solution A was sterilized in the autoclave.
- Solution B was prepared by dissolving 35.02g NaHCO3 in Water for Injection, which was made up to 1000ml. Solution B was sterilized by filtration (0.22µm filter).
- 300ml Water for Injection was added to 3ml of solution A and 4ml of solution B and this solution was made up to 500ml with Water for Injection.

Interfering substance (high):

The interfering substance was prepared freshly at 10 times its final concentration in the test. 50g yeast extract powder (Fisher BioReagents®, New Yersey. EC 232-387-9) was dissolved in 150ml Water for Injection and sterilized in the autoclave. 5ml of this solution was pipetted into a 10ml volumetric flask and 2ml of Water for Injection was added. 1g Bovine Albumin (Cohn fraction V, CALBIOCHEM®, EMD Biosciences, Germany. Cat#12657) was dissolved in the yeast extract solution. This solution was made up to 10 ml with Water for Injection. The albumin/yeast mixture was sterilized by filtration (0.22µm filter). Final concentration in the test procedure was 10 g/l yeast extract and 10g/l bovine albumin.

4. Test method and its validation

Interfering substance (100 µl) and *Coxiella Burnetii* suspension (100 µl containing 10^8 Cb) were incubated at 10°C for 2 minutes in a heat block (T3 Biometra®). After incubation of the mixtures, 800 µl of the test product solutions were added and incubated at 10°C for 30 minutes in the heat block. At the end of the contact time, the mixtures were centrifuged at 16,000 x g for 10 minutes to separate the test product (supernatant) from the bacteria (as an alternative neutralisation method). The pellets were resuspended in 1ml PBS and 10-fold dilution samples were prepared in medium for Buffalo Green Monkey (BGM) cells (Earles Minimum Essential Medium (EMEM) + 10% Foetal Calf Serum (FCS) + 1% nonessential amino acids (NEAA) + 1% Glutamine).

The test for bactericidal activity was performed in duplicate for all samples. Besides the three test product concentrations, several control and validation samples were included (see section 5. Results).

Temperature of incubation:	Of each diluted sample of the test mixture, 100 µl (in positive control sample corresponding with 10^7 Cb) was transferred to slide flasks (Nunc, 9 cm^2) with a monolayer of BGM cells. The flasks were cultured at 37 °C and 5% CO_2 for 14 days and BGM medium was refreshed every three to four days.
Counting procedure:	Supernatant of the inoculated slide flasks was harvested 14 days post inoculation. The read out for viable Cb in serial 10-fold dilutions of the test mixture was the presence of Cb in the supernatant on 14 days post inoculation detected by qPCR (as described by Hendrik I.J. Roest et al., Emerging Infectious Diseases, volume 17, issue 4, 2011) . A positive result demonstrates the growth and infection of BGM cells during the incubation period.
Bacterial strain used:	*Coxiella burnetii* strain Nine Mile (RSA 493, lab reference strain) was used for this experiment. Concentration of bacterial stock was is $10^{9.7}$ DNA copies / ml determined by qPCR. Final test concentration was ~10^8 Cb / ml.

5. Results

Table 1. Results of first inactivation test

			10-fold dilution of mixtures						
no.	type of sample	content of mixture	0	-1	-2	-3	-4	-5	-6
1	validation for effect of IS on growth of cells	IS in SHW	-/-	nt	nt	nt	nt	nt	nt
6	validation for effect of IS on growth of Cb	IS + Cb in SHW	+/+	+/+	+/+	+/+	+/+	+/+	+/+
3	validation for effect of neutralisation on growth of Cb	Cb in SHW without washing	+/+	+/+	+/+	+/+	+/+	+/+	+/+
4	validation for effect of incubation (10°C) on growth of Cb	Cb in SHW without incubation	+/+	+/+	+/+	+/+	+/+	+/+	+/+
10	validation for neutralisation method of Virkon®S	Incubation Cb after washing 2% Virkon®S	+/+	+/+	+/+	+/+	+/+	+/+	+/+
2	positive control	Cb in SHW	+/+	+/+	+/+	+/+	+/+	+/+	+/+
5	negative control	SHW	+/+	nt	nt	nt	nt	nt	nt
7	test sample 0.4% Virkon®S	Cb + Virkon®S in SHW	+/+	+/+	+/+	+/+	+/+	+/+	+/-
8	test sample 1% Virkon®S	Cb + Virkon®S in SHW	+/+	+/+	D/+	-/-	-/D	-/-	-/D
9	test sample 2% Virkon®S	Cb + Virkon®S in SHW	+/+	D/D	-/D	D/-	-/-	-/-	-/-

Abbreviations and explanations:
IS Interfering Substance
Cb Coxiella Burnetii strain Nine Mile (final test concentration 10^8 DNA copies/ml)
SHW Standardized Hard Water
Ct Threshold cycle

\+ tested positive for presence of Cb by PCR (Ct value >40) in supernatant of cell culture
\- tested negative for presence of Cb by PCR (Ct value <36) in supernatant of cell culture
D tested dubious for presence of Cb by PCR (Ct value 36-40) in supernatant of cell culture
nt not tested

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Conclusions of results per sample of first inactivation test

1	no effect of IS on growth of cells
6	no effect of IS on growth of Cb
3	no effect of washing on growth of Cb
4	no effect of incubation (10° C) on growth of Cb
10	washing is a suitable method to neutralise Virkon®S
2	mixtures should be further diluted to obtain end-point titration
5	contamination occurred (during refreshment of medium?)
7	0.4% Virkon®S results in no measurable reduction
8	1% Virkon®S results in ≥ 10E4 reduction
9	2% Virkon®S results in ≥ 10E6 reduction

Note
dilutions 10E-7, 10E-8 and 10E-9 were tested as yet to obtain end-point titration

frozen material of positive tested negative control sample and positive tested dilutions with lowest concentration of Cb) were recultured to confirm that contamination of negative control had occurred during culture and to exclude that contamination had occurred in test samples

Table 2. Results of additional testing

			10-fold dilution of mixtures									
no.	type of sample	content of mixture	0	-1	-2	-3	-4	-5	-6	-7	-8	-9
1	validation for effect of IS on growth of cells	IS in SHW										
6	validation for effect of IS on growth of Cb	IS + Cb in SHW							+/+	-/-	-/-	-/-
3	validation for effect of neutralisation on growth of Cb	Cb in SHW without washing						-/+	+/-	D/-	-/-	-/-
4	validation for effect of incubation (10°C) on growth of Cb	Cb in SHW without incubation						+/+	-/-	-/-	-/-	-/-
10	validation for neutralisation method of Virkon®S	Incubation Cb after washing 2% Virkon®S						+/+	-/-	-/-	-/-	-/-
2	positive control	Cb in SHW							+/+	-/-	-/-	-/-
5	negative control	SHW	-/-									
7	test sample 0.4% Virkon®S	Cb + Virkon®S in SHW					+/+	+/-	-/-	-/-	-/-	-/-
8	test sample 1% Virkon®S	Cb + Virkon®S in SHW	+/+	+/+	-/+							
9	test sample 2% Virkon®S	Cb + Virkon®S in SHW	+/+	D/+	D/-	-/-						

Abbreviations and explanations:
IS Interfering Substance
Cb Coxiella Burnetii strain Nine Mile (final test concentration 10^8 DNA copies/ml)
SHW Standardized Hard Water
Ct Threshold cycle

\+ tested positive for presence of Cb by PCR (Ct value >40) in supernatant of cell culture
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Conclusions of results per sample of additional testing

1	no effect of IS on growth of cells
6	no effect of IS on growth of Cb
3	no effect of washing on growth of Cb
4	no effect of incubation (10° C) on growth of Cb
10	washing is a suitable method to neutralise Virkon®S
2	sensitivity of the test is sufficient to detect ≥ 10E5 reduction
5	contamination in negative control as observed in first results did not affect results of other positive tested samples
7	0.4% Virkon®S results in no measurable reduction
8	1% Virkon®S results in 10^4 reduction
9	2% Virkon®S results in 10^5reduction

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Project name: ISVS CBNM

Inactivation Study Virkon®S *Coxiella burnetti* strain Nine Mile

CRO study number: 16.300.39000

Sponsor reference number: Not applicable

Department Virology

Preliminary study results

Table 3. Final results

no.	type of sample	content of mixture	10-fold dilution of mixtures									
			0	-1	-2	-3	-4	-5	-6	-7	-8	-9
1	validation for effect of IS on growth of cells	IS in SHW	-	nt	nt	nt	nt	nt	nt	nt	nt	nt
6	validation for effect of IS on growth of Cb	IS + Cb in SHW	+	+	+	+	+	+	+	-	-	-
3	validation for effect of neutralisation on growth of Cb	Cb in SHW without washing	+	+	+	+	+	+	+	-	-	-
4	validation for effect of incubation (10°C) on growth of Cb	Cb in SHW without incubation	+	+	+	+	+	+	+	-	-	-
10	validation for neutralisation method of Virkon®S	Incubation Cb after washing 2% Virkon®S	+	+	+	+	+	+	+	-	-	-
2	positive control	Cb in SHW	+	+	+	+	+	+	+	-	-	-
5	negative control	SHW	-	nt	nt	nt	nt	nt	nt	nt	nt	nt
7	test sample 0.4% Virkon®S	Cb + Virkon®S in SHW	+	+	+	+	+	+	+	-	-	-
8	test sample 1% Virkon®S	Cb + Virkon®S in SHW	+	+	+	-	-	-	-	nt	nt	nt
9	test sample 2% Virkon®S	Cb + Virkon®S in SHW	+	+	-	-	-	-	-	nt	nt	nt

Abbreviations and explanantions:

IS	Interfering Substance
Cb	Coxiella Burnetii strain Nine Mile (final test concentration 10^8 DNA copies/ml)
SHW	Standardized Hard Water
Ct	Threshold cycle
+	tested positive for presence of Cb by PCR (Ct value >40) in supernatant of cell culture
-	tested negative for presence of Cb by PCR (Ct value <36) in supernatant of cell culture
D	tested dubious for presence of Cb by PCR (Ct value 36-40) in supernatant of cell culture
nt	not tested

NB If at least one of the duplicate samples was tested positive in the first or in the additional results, the final result was displayed as positive.

6. End Conclusions

The batch 1011BA0062 of the product Virkon-S® possesses bactericidal activity (demonstrated by 10^5 log reduction in viable concentration) for the *Coxiella Burnetii* strain Nine Mile (RSA 493, lab reference strain, 10^8 DNA copies/ml) at a concentration of 2% at 10 °C with 30 min contact time and under high soiling conditions.

The batch 1011BA0062 of the product Virkon-S® demonstrated 10^4 log reduction in viable concentration for the *Coxiella Burnetii* strain Nine Mile (RSA 493, lab reference strain, 10^8 DNA copies/ml) at a concentration of 1% at 10 °C with 30 min contact time and under high soiling conditions.

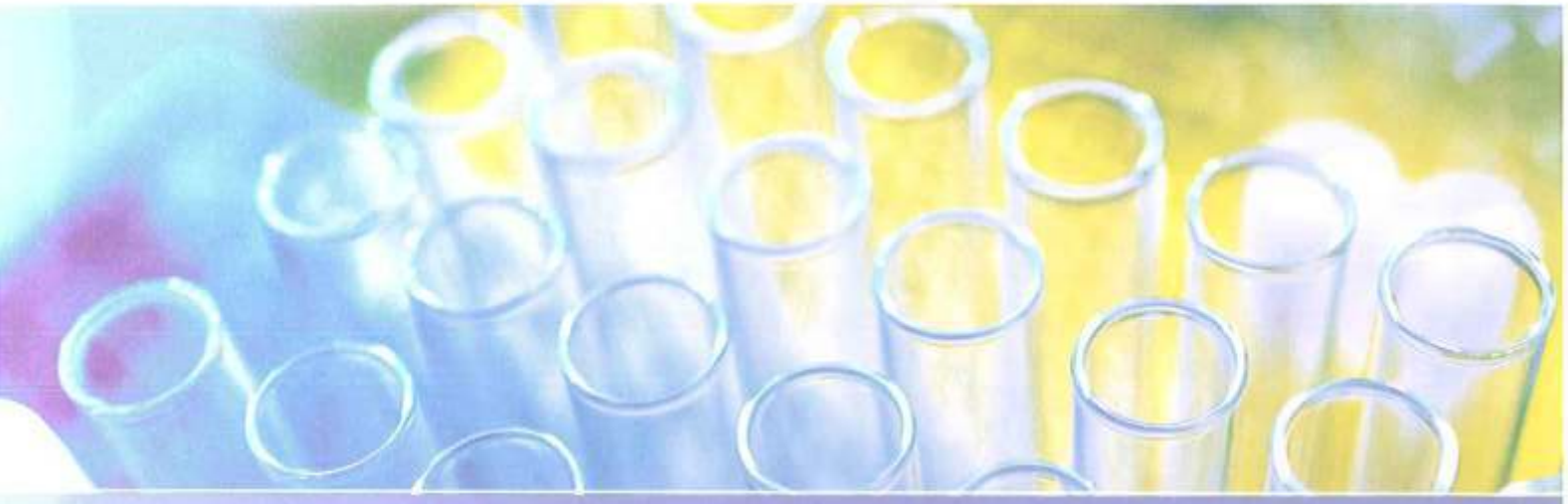
7. Approval signatures

date: 22dec11 signature:

name Rineke de Jong
title/function DVM, Project leader

date: 22-12-2011 signature:

name Riks Maas
title/function PhD, Head Division Virology



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