Prolonged environmental persistence requires efficient disinfection procedures to control *Devriesea agamarum* associated disease in lizards

Tom HELLEBUYCK*, Frank PASMANS, Mark BLOOI, Freddy HAESEBROUCK, An MARTEL

Department of Pathology, Bacteriology and Avian diseases, Faculty of Veterinary Medicine, Ghent University. Salisburylaan 133, B-9820 Merelbeke, Belgium

Running headline: Environmental control of devrieseasis

*Corresponding author: Tom.Hellebuyck@UGent.be

Tel.: +32 9 264 74 42

Fax: +32 9 264 74 90

Department of Pathology, Bacteriology and Poultry diseases,

Faculty of Veterinary Medicine, Ghent University.

Salisburylaan 133, B-9820 Merelbeke, Belgium

Abstract

Aims: *Devriesea agamarum* infection causes chronic proliferative dermatitis, especially in desert dwelling lizards. The present study was concerned with evaluating persistency of *D. agamarum* in the environment and the evaluation of the efficacy of various disinfection procedures. **Methods and Results:** First, the survival of *D. agamarum* was assessed both in dermal crusts obtained from clinically and naturally infected lizards, and during periods of prolonged nutrient starvation on dry surface, in moist sand and in distilled water. Secondly, a modified European Suspension Test was performed to determine the efficacy of eight procedures for the disinfection of equipment, environmental surfaces and the topical treatment of *D. agamarum* associated dermal lesions. The bacterium proved to persist and remain viable for up to 57 days in dermal crusts and for more than five months in moist sand and distilled water. In contrast, survival on dry surfaces was limited. The results of the described dilution-neutralization method demonstrated that most of the tested disinfection procedures were sufficient in achieving a 5 decimal logaritmic reduction in the number of *D. agamarum* colony forming units. The use of relatively low concentrations of hydrogen peroxide and a boric and peracetic acid solution on the other hand resulted in insufficient reduction of viable counts. **Conclusions:** *D. agamarum* can persist for long periods of time in the environment, especially under moist conditions, making the use of suitable disinfection procedures necessary. **Significance and impact of the study:** This study demonstrates the need for a dry environment for most desert lizards and the use of effective disinfection procedures next to antimicrobial treatment in order to eliminate *D. agamarum* associated disease from captive saurian collections.

Key words: *Devriesea agamarum*, disinfection, environmental survival, lizards

INTRODUCTION

Chronic proliferative dermatitis caused by *Devriesea agamarum* is one of the most frequently diagnosed diseases in dab (genus *Uromastyx*) and other desert dwelling lizards, compromising successful captive maintenance of certain saurian taxa (Martel et al., 2008; Hellebuyck et al., 2009a and b). *D. agamarum* related disease appears to be highly contagious and may affect a complete lizard collection within several months (Pasmans et al., 2008).

Clinical *D. agamarum* infections seem to reoccur readily within captive lizard collections. If *D. agamarum* would be able to persist outside the host, the environment might represent an important reservoir. Indeed, various members of the phylum *Actinobacterium* are widely distributed in both terrestrial and aquatic ecosystems, especially in soil (Ventura et al., 2007). While actinomycetes form spores or other structures, which could be considered as environmentally resistant resting structures, this has not been demonstrated in other Actinobacteria, such as coryneform bacteria (Boylen and Mulks, 1978; Chen et al., 2004; Martel et al., 2008). Nevertheless, long term survival of coryneforms during prolonged periods of nutrient starvation has been documented (Boylen and Mulks, 1978).

Since it is unknown to what extent the environment may harbor *D. agamarum* and if relapses could at least be partly assigned to environmental survival of the bacterium, the present study was concerned with evaluating persistence of *D. agamarum* in the environment. Therefore, the survival of *D. agamarum* was investigated both in dermal crusts obtained from naturally *D. agamarum* infected lizards, and during periods of prolonged nutrient starvation on dry surface, in moist sand and in distilled water. Each condition was evaluated at 4 different temperatures. If *D. agamarum* would be able to persist, efficient disinfection procedures would contribute to control the disease in captive lizard collections. Therefore, the efficacy of eight procedures for the disinfection of equipment, environmental surfaces and the topical treatment of *D. agamarum* associated dermal lesions, was assessed.

MATERIALS AND METHODS

Devriesea agamarum strain and growth conditions

The test suspension for the evaluation of survival under the different environmental conditions was prepared by growing the *D. agamarum* type strain (=LMG 24257^T=IMP 2) on tryptone soya agar (TSA, Oxoid GmbH, Wesel, Germany) during 24 hours at 37°C and 5 % CO_2. Colonies were harvested and transferred to water of standard hardness (WSH) and the bacterial suspension was diluted to obtain approximately 6 x 10^7 colony forming units (cfu) ml^{-1}.

Test suspensions for the assesment of the different disinfection procedures were prepared by growing the type strain on tryptone soya agar (TSA, Oxoid GmbH, Wesel, Germany) for 24 hours at 37°C and 5% CO_2 and transferring loopfuls of cells to tryptone sodium chloride solution with five g of glass beads for each 10 ml of solution. Next, the cells were suspended by using a mechanical shaker for three minutes, followed by aspiration of the bacterial suspension from the glass beads and transferring the suspensions to another tube. The suspension to be used for the different disinfection procedures was diluted with tryptone sodium chloride solution to an optic density of 0.309, which equalled approximately 2 x 10^8 cfu ml^{-1}.

Survival of *Devriesea agamarum* in dermal crusts of naturally infected lizards

Dermal crusts were obtained from two captive dab lizards (*Uromastyx acanthinura*) suffering from *D. agamarum* related cheilitis and severe dermatitis. *D. agamarum* was isolated from the collected crusts and both isolates were identified as *D. agamarum* based on morphological aspects, API® Coryne, API® 20 STREP, API® 50 CH (bioMérieux, Marcy l'etoile, France) and 16 S rRNA gene sequencing (Martel et al., 2008). The dermal crusts from both lizards were crushed, distributed in sealed 96-well microtiter plates and kept at 20 °C or 30 °C. Once

every week 250 mg of the dermal crusts from each lizard were suspended in two ml of WSH followed by inoculation of both suspensions in duplicate onto TSA. Growth of *D. agamarum* was evaluated after 24 and 48 hours of incubation at 37 °C and 5 % CO_2. Sampling of the dermal crusts was performed until the bacterium could no longer be isolated for two consecutive testings.

Survival of *Devriesea agamarum* under various environmental conditions

The survival of the *D. agamarum* type strain was evaluated under dry and moist conditions and in distilled water at four different temperatures (20 °C, 30 °C, 40 °C and 50 °C) for each test condition. The number of viable bacteria was determined daily during the first 8 days, q 4 days from day 9 to day 32, q 14 days from day 33 to day 60 and q 21 days from day 61 to day 208, until growth of *D. agamarum* could no longer be observed for two consecutive samplings. All samples were plated onto TSA at 37 °C and 5 % CO_2.

For the evaluation of survival under dry conditions, 100 µl of the bacterial suspension was added to microtubes which were left in the vertical flow chamber until all fluid evaporated and were then sealed with microfilm. In order to assess survival in distilled water, 200 µl of the bacterial suspension was transferred to microtubes, which were closed immediately and sealed with microfilm.

Sealed microtubes containing 50 µl of the *D. agamarum* suspension mixed into 500 mg of rinsed and autoclaved white sand were prepared for the evaluation of survival of *D. agamarum* under moist conditions.

In order to estimate the number of viable bacteria at the different testing points in time, 200 µl of distilled water was first added to the tubes previously prepared for the assessment of survival under dry conditions and in moist sand. Next, a 100 µl aliquot and serial tenfold dilution from both latter tubes and the tubes prepared for the evaluation of survival in distilled

water were plated onto TSA. Colonies were counted after 24 and 48 hours of incubation at 37°C and 5 % CO_2.

Efficacy of disinfection procedures against *Devriesea agamarum*

To evaluate the effect of disinfectants and hot water on the survival of *D. agamarum*, a European Suspension Test (EST) was used (Anonymous, 1997). The efficacy of all disinfection procedures was evaluated at various concentrations and after various contact times. All tests were performed in duplicate.

The following disinfection procedures were tested: Dettol (Reckitt Benckiser, Brussel, Belgium) (0.05, 0.5 and 1.0 %; 5, 15 and 30 min), ethanol (Certa, Braine-l'Alleud, Belgium) (70 % [v/v]; 2, 5 and 10 min), hospital antiseptic concentrate (HAC, Regent Medical, Manchester, UK) (0.05, 0,1 and 0.5 %; 5 and 15 min), hydrogen peroxide (Fagron, Waregem, Belgium) (0.3, 0.5, 1.0 and 3.0 %; 5, 15 and 30 min), chlorhexidine (Regent Medical, Irlam, UK) (0.05, 0.1 and 0.5 %; 5 and 15 min), sodium hypochlorite (Forever, Courcelles, Belgium) (0.05, 0.1, 0.3, and 0.5 %; 5 min), acetic acid and boric acid (Malacetic Otic, Eurovet N.V./S.A., Heusden-Zolder, Belgium) (0.015, 0.05, 0.1, 0.5 and 1.0 %; 5 and 15 min). The main disinfecting components of HAC are cetrimide (15 %) and chlorhexidine (1.5 %). Dettol contains chloroxylenol as disinfecting constituent. All disinfectant solutions were prepared using distilled water and were filter sterilized prior to use (Puradisk FP 30; Whatman, Middlesex, UK). Next to the different disinfectants, the effect of hot water on the survival of *D. agamarum* was tested (70°C; 15, 30 and 60 min).

The inactivating neutralizer prescribed in the EST was replaced by a commercially available neutralizer (Dey-Engley Neutralizing Broth; DENB; BD, Sparks, MD, USA) or cold WSH (4 °C) for the testing of hot water. First, the efficacy of the DENB and its non-toxicity against *D. agamarum* was validated as prescribed in the EST. Three different concentrations of DENB

were assessed for the neutralization of the various disinfectants after the selected contact time. Additionally, the dilution-neutralization method and the selected experimental conditions were validated and the absence of any lethal effect in the test conditions was verified as described in the EST protocol.

For each test condition 1.0 ml of the test suspension was added to a tube containing 1.0 ml of WSH, mixed and placed in a water bath at 20 °C for 2 min. Next, 8.0 ml of one of the product test solutions or hot water was added to the tubes and placed in the water bath at 20 °C for the disinfectants or 70 °C for the hot water for the chosen contact time. At the end of the chosen contact times the samples were mixed and 1.0 ml of each sample and 1.0 ml WSH were transferred into a tube containing 8.0 ml of DENB or cold WSH (4 °C). Immediately after the chosen neutralization time, 1.0 ml of the samples was inoculated in duplicate onto TSB using the spread plate technique. The number of cfu was counted after 24 hours and 48 hours of incubation at 37 °C.

RESULTS

Devriesea agamarum survives up to 57 days in crusts from naturally infected dab lizards

From the dermal crusts obtained from the first dab lizard the presence of *D. agamarum* could be demonstrated until the 35th day and the 57th day of sampling at 20 °C and 30 °C, respectively. By sampling the dermal crusts from the second lizard, growth of the bacterium was observed until the 49th and 35th day at 20 °C and 30°C, respectively.

Moisture promotes environmental survival of *Devriesea agamarum*

Survival of *D. agamarum* under dry conditions could no longer be observed after 6 days and 4 days at 40 °C and 50 °C, respectively. At 20 °C and 30 °C on the other hand, viability was demonstrated until the 27th and 20th day of sampling, respectively.

The presence of *D. agamarum* could no longer be demonstrated after the first day of incubation at 50 °C in both the moist sand and the distilled water. At 40 °C, survival was observed for eight days in the moist sand and for four days in the distilled water. At 20 °C and 30 °C *D. agamarum* persisted and remained viable for more than five months in the moist sand and the distilled water (Figure 1). At 30 °C a progressive decline in the number of cfu/ml was observed from the 50^{th} day of incubation in moist sand, while viability remained high until the 80^{th} day of sampling in the distilled water at 30 °C. In the moist sand as well as in the distilled water the number of viable cfu showed a minor decrease over a period of more than six months at 20 °C.

Effect of disinfection procedures on the viability of *D. agamarum*

The use of DENB at a concentration as intended for use was effective in neutralizing ethanol. For NaOCl, H_2O_2 and dettol 2.5x-concentrated DENB had to be applied in order to establish neutralization of the product test solutions. For the neutralization of chlorhexidine, HAC and Malacetic Otic after the chosen contact times, 4.5x-concentrated DENB had to be applied. The evaluated disinfection procedures were deemed to establish a sufficient decrease in viable counts if at least a 5 decimal logaritmic reduction in the number of *D. agamarum* cfu was demonstrated. Overall, most of the treatments resulted in sufficient reduction in the number of cfu after the prescribed contact times (Table 1). Treatments with low concentrations of H_2O_2 (0.3, 0.5 and 1.0 %; 5 min, 15 min and 30 min), 3.0 % of H_2O_2 (5 min and 15 min), 0.05 % dettol (5, 15 and 30 min), low concentrations of Malacetic Otic (0.015, 0.05, 0.1, 0.5 %; 5 and 15 min) and low concentrations of HAC applied during short contact times (0.05 and 0.1 %; 5 min) however, revealed insufficient reduction in the number of cfu of *D. agamarum*. Treatment with 3.0 % H_2O_2 during a contact time of 30 min, 1.0 % of Malacetic Otic (5 and

15 min) and HAC (0.05, 0.1 and 0.5 %; 15 min) on the other hand, demonstrated sufficient reduction.

DISCUSSION

It can be concluded from this study that the non-sporeforming bacterium *D. agamarum* is able to persist and remain viable for up to 57 days in dermal crusts and for more than 5 months in moist sand and distilled water. In contrast, survival on dry surfaces is limited. These findings emphasise the need for a dry environment for most desert lizards. Moist retreats as proposed in older literature for desert dwelling lizards (Divers, 1996) are, moreover, not necessary for the wellbeing of at least dab lizards (personal observation).

Previously, relatively long survival limits were demonstrated for different species of coryneform bacteria (Boylen and Mulks, 1978), indicating the evolvement of adaptive mechanisms to natural conditions where low nutrient supplies are present. Stringent control of endogenous metabolism as observed in other coryneform bacteria (Boylen and Mulks, 1978) might be suggested as such an adaptation in *D. agamarum*.

Besides antimicrobial treatment, cleaning and disinfection of equipment and environmental surfaces are strongly recommended during *D. agamarum* associated disease in lizard collections, based on the long term survival of the bacterium demonstrated in the current study. The results of the described dilution-neutralization method demonstrate that most of the tested disinfection procedures were sufficient in achieving a decimal five logaritmic reduction in the number of *D. agamarum* cfu. In practice, however, the effectiveness of the tested products for the disinfection of environmental surfaces and equipment may be strongly influenced by the presence of organic residues, variable contacttimes and temperature differences (Taylor et al., 1999).

The observed low susceptibility of *D. agamarum* to H_2O_2 can be related to the high level of catalase/peroxidase activity in the cells as described by Martel et al. (2008) and as reported for other genera of aerobic or facultative anaerobic bacteria (Ohwada et al., 1999).

In conclusion, it was demonstrated that *D. agamarum* can persist for long periods of time in the environment even in the absence of nutrient supplies, especially under moist conditions. Consequently, the use of effective disinfection procedures next to antimicrobial treatment should be stressed in order to eliminate *D. agamarum* associated disease from captive saurian collections.

REFERENCES

Anonymous (1997) Chemical disinfectants and antiseptics - basic bactericidal activity - test method and requirement (phase 1) EN-1040. Nederlands Normalisatie Instituut, Delft, The Netherlands.

Boylen, C.W. and Mulks, M.H. (1978) The survival of coryneform bacteria during periods of prolonged nutrient starvation. *J Gen Microbiol* **105**, 323-334.

Chen, H.H., Li, W.J., Tang, S.K., Kroppenstedt, R.M., Stackebrandt, E., Xu, L.H. and Jiang, C.L. (2004) *Corynebacterium halotolerans* sp. nov., isolated from saline soil in the west of China. *Int J Syst Evol Microbiol* **54**, 779-782.

Divers, S. (1996) Basic reptile husbandry, history taking and clinical examination. *In Practice* **18,** 51-65.

Hellebuyck, T., Martel, A., Chiers, K., Haesebrouck, F. and Pasmans, F. (2009a) *Devriesea agamarum* causes dermatitis in bearded dragons (*Pogona vitticeps*). *Vet Microbiol* **134,** 267-271.

Hellebuyck, T., Haesebrouck, F., Pasmans, F. and Martel, A. (2009b) Designing a successful antimicrobial treatment against *Devriesea agamarum* infections in lizards. *Vet Microbiol* **139,** 189-192.

Martel, A., Pasmans, F., Hellebuyck, T., Haesebrouck, F. and Vandamme, P. (2008) *Devriesea agamarum* gen. nov., sp. nov., a novel actinobacterium associated with dermatitis and septicaemia in agamid lizards. *Int J Syst Evol Microbiol* **58,** 2206-2209.

Ohwada, T., Shirakawa, Y, Kusumoto, M., Masuda, H. and Sato, T. (1999) Susceptibility to hydrogen peroxide and catalase activity of root nodule bacteria. *Biosci Biotechnol Biochem* **63,** 457-462.

Pasmans, F., Blahak, S., Martel, A. and Pantchev, N. (2008) Introducing reptiles into a captive collection: The role of the veterinarian. *Vet J* **175,** 53-68.

Taylor, J.H., Rogers, S.J. and Holah, J.T. (1999) A comparison of the bactericidal efficacy of 18 disinfectants used in the food indystry against *Escherichia coli* O157:H7 and *Pseudomonas aeruginosa* at 10 and 20 degrees C. *J Appl Microbiol* **87,** 718-725.

Ventura, M., Canchaya, C., Tauch, A., Chandra, G., Fitzgerald, G.F., Chater, K.F. and van Sinderen, D. (2007) Genomics of Actinobacteria : tracing the evolutionary history of an ancient phylum. *Microbiol Mol Biol Rev* **71,** 495-548.

Table 1 Results of different disinfection procedures against *Devriesea agamarum* based on a dilution-neutralization method as described by the European Suspension Test. **P** (pass)**,** 5-log reduction or greater in viable counts; **F** (fail), less than 5-log reduction in viable counts

Product	**Concentration**	**Contact time**	**P / F**
Malacetic Otic (boric acid and peracetic acid)	0.015 % 0.05 % 0.1 % 0.5 % 1.0 %	5 min 15 min	F, except 1.0 %
Dettol C_8H_9ClO	0.05 % 0.5 % 1.0 %	5 min 15 min 30 min	P, except 0.05 % during 5, 15 and 30 min
Ethanol	70% (v/v)	2 min 5 min 10 min	P
Hot water	70°	15 min 30 min 60 min	P
H_2O_2	0.3% 0.5% 1.0% 3.0%	5 min 15 min 30 min	F, except 3.0 % during 30 min
HAC (cetrimide and chlorhexidine)	0.05 % 0.1 % 0.5 %	5 min 15 min	P, except 0.1 % and 0.05 % during 5 min
Chlorhexidine	0.05 % 0.1 % 0.5 %	5 min 15 min	P
NaOCl (sodium hypochlorite)	0.05% 0.1% 0.3% 0.5 %	5 min	P

323 **Fig. 1** Survival of *Devriesea agamarum* in distilled water (DW) and moist sand (MS) at

324 20 °C or 30 °C . Viability data are expressed as colony-forming units (cfu) ml^{-1}.

325 Conditions: (■)DW 20 °C, (□) DW 30 °C, (▲) MS 20 °C, (△)MS 30 °C

326