Evaluation of the environmental persistency of *Devriesea agamarum* and the development of an indirect enzyme-linked immunosorbent assay for the assessment of antibody response in lizards against *Devriesea agamarum.*

Spiny tailed lizard (*Uromastyx acanthinura*) with *Devriesea agamarum* associated cheilitis presented as chronic hyperkeratosis. (Courtesy of Division of Poultry, Exotic Companion and Laboratory animals, Faculty of Veterinary Medicine, Ghent University)

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Introduction

Skin diseases in reptiles

In recent years reptiles have become increasingly popular as pets. At first, almost all captive reptiles were wild-caught and little was known about the appropriate husbandry of most species. These individuals rarely thrived or lived for prolonged periods of time in captivity. Based on increasing knowledge of reptile husbandry and reptile medicine, many reptile species are being kept and bred successfully. Still, there are frequently observed health problems in captive reptiles. Dermal disease is a common reason for veterinary intervention in reptile medicine (Hoppman and Wilson Barron, 2007; Jacobson, 1991, 1992). Especially in desert-dwelling lizard species belonging to the genera *Agama*, *Laudakia*, *Pogona* and *Uromastyx* bacterial skin disease caused by *Devriesea agamarum* is commonly observed.

Infectious dermatitis in reptiles can be associated with viral (Herbst et al,. 1999), bacterial (Chineme and Addo, 1980; Pasmans et al., 2008, Hellebuyck et al*.,* 2009), mycotic (Jacobson et al*.,* 2000; Bowman et al*.*, 2007) and parasitic (Goldberg and Bursey, 1991; Hoppmann and Wilson Barron, 2007) pathogens. Although bacteria are frequently isolated from skin lesions, it often remains unclear if they act as primary etiological agents. Many predisposing factors such as suboptimal husbandry (humidity, temperature, social stress) and underlying diseases (gastrointestinal, respiratory, ectoparasites) can play a major role in the development of skin diseases of reptiles.

Devriesea agamarum, a novel Actinobacterium

Actinobacteria are Gram-positive bacteria with a high G+C content in their DNA. The different *Actinobacteria* show a wide variety in morphology and in physiological and metabolic properties (Ventura et al., 2007). Bacteria belonging to the group of Actinobacteria can be pathogens (for example *Mycobacteria* spp., *Nocardia* spp. and *Corynebacterium* spp.), soil inhabitants (*Streptomyces* spp.), gastrointestinal microbiota (*Bifidobacterium* spp.) and plant commensals (*Leifsonia* spp.) (Ventura et al., 2007).
A novel *Actinobacterium, Devriesea agamarum* isolated from lizards with chronic proliferative dermatitis and septicaemia, has been described by Martel et al. (2008).
D. agamarum are Gram-positive, short (1-2 µm) rod-shaped bacteria, that can occur singly, in pairs or in short chains. They are non-motile. After incubation on Colombia agar with 5 % sheep blood, colonies are small, smooth and mucoid, whitish in colour and show a small zone of haemolysis (Martel et al., 2008).

The role of this bacterium as a causative agent of dermatitis in desert dwelling lizards has been demonstrated (Hellebuyck et al*.,* 2009). First, the bacterium was isolated from several lizards (mainly desert-dwelling species) showing proliferative dermatitis and/or septicaemia. After inoculating intact and abraded skin of healthy bearded dragons (*Pogona vitticeps*) with a *D. Agamarum* bacterial suspension, all animals developed dermatitis at the inoculated abraded skin region. *D. agamarum* was re-isolated from these dermal lesions. Hence the postulates of Koch were fulfilled (Hellebuyck et al., 2009). Furthermore, *D. Agamarum* was isolated from the oral cavity of clinically healthy *P.*

Vitticeps (Hellebuyck et al*.,* 2009). Hence, *P. vitticeps* appears to be an asymptomatic reservoir for *D. agamarum.*

Based on these results, *D. agamarum* proved to be a facultative pathogenic bacterium, able to cause dermatitis in agamid lizards when the integrity of the skin is breached (Hellebuyck et al., 2009).

A successful antimicrobial therapy for the elimination of *D. agamarum* infections from lizards was designed following the determination of susceptibility patterns of this bacterium for several antimicrobial agents. Treatment with ceftiofur (5mg/kg B.W., I.M., q24h) resulted in clearance of infection in experimentally inoculated *P. vitticeps* and in clinically infected and naturally infected *Uromastyx* lizards, in contrast to the use of enrofloxacin (5mg/kg B.W., I.M., q24h) (Hellebuyck et al., 2009).

Serological assay for *Devriesea agamarum*

The basis of serological tests is that foreign molecules elicit a distinct humoral immune response in vertebrates. This response can be assayed. Serology allows animals to be screened for exposure to an almost infinite array of foreign proteins. The information provided can be used to evaluate individual animals and their exposure status and, for free-ranging wildlife, the serologic status of a population (Jacobson and Origgi, 2002).

Like in mammals, the immune system of reptiles is complex and involves innate, cell-mediated and humoral compartments, but overall there is considerably less known about reptile immune function. Differences between mammal and reptilian immune function require a different approach in case of using and interpreting serology. For example differences in humoral response between mammals and reptiles after immunization are that reptiles show a slower response, a lower increase in antibody titer, often no increase in titer upon a second exposure and no production of antibodies with a increased binding affinity (Zimmerman et al 2009).

A serological assay that is able to detect antibodies against *Devriesea agamarum* would possibly be able to give information about the exposure status of an animal. When paired sera can be obtained, seroconversion could be detected for example after auto-vaccination of lizards against *D. agamarum*.

Aims of the study

Evaluation of the environmental persistency of *D. agamarum*

The first the aim of this study was to evaluate the persistency of *D. Agamarum* in the environment. If prolonged environmental persistency could be demonstrated, efficient disinfection procedures are required for disinfection of environmental surfaces and equipment to control *D. agamarum* associated disease in lizard collections

Development of an indirect enzyme-linked immunosorbent assay

As a second part of this study, an indirect enzyme-linked immunosorbent assay (ELISA) was developed for the assessment of antibody response in lizards against *D. agamarum* after immunisation. The development of this assay could contribute to evaluate the effect of auto-vaccination of lizards against *Devriesea agamarum*.

Materials and methods

Evaluation of the environmental persistency of *Devriesea agamarum*

Devriesea agamarum strain and preparation of the test suspension

The bacterial suspension for the evaluation of environmental persistency under different environmental conditions was prepared by growing the *D. agamarum* type strain (=LMG 24257^T=IMP 2) on Colombia agar plates with 5% sheep blood (COL, Oxoid GmbH, Wesel, Germany) for 24 hours at 37 °C. The *D. Agamarum* colonies were transferred from the agar to blood heart Infusion (BHI) broth and incubated for 24 hours at 37 °C. The bacterial suspension was centrifuged (10 minutes, 3000 rpm) at 4 °C. The supernatant was discarded, and the pellet was resuspended in distilled water.

Determining the amount of Colony Forming Units (cfu) of the bacterial suspension

For determination of the cfu/ml of the bacterial suspension the following procedure was carried out. Dilutions of $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{5}$, $\frac{1}{7}$, $\frac{1}{10}$ and $\frac{1}{20}$ were made. From each of these dilutions 10-fold serial dilutions up till 10^{-8} were made. A selection of these dilutions was inoculated on Colombia agar plates with 5 % sheep blood (COL, Oxoid GmbH, Wesel, Germany) and incubated for 24 hours at 37 °C.
The optical densities (O.D.) of the main dilution series were determined with a spectrophotometer, which was calibrated with distilled water at 450 nm. For the results, see Table 1 and Figure 1.
The experiment was carried out with an $\frac{1}{5}$ dilution corresponding with approximately 2.16 x 10^8 cfu/ml. The O.D. of this dilution is 0,664.
After transferring the bacterial suspension to the test tubes, the cfu/ml of the used suspension was determined by growing the dilutions on Colombia agar plates with 5 % sheep blood (COL, Oxoid GmbH, Wesel, Germany) for 24 hours at 37 °C.

Dilution	**Optical Density$_{450}$**	**cfu/ml**
undiluted	*1,786*	*1,83 x 10^9*
$\frac{1}{2}$	*1,325*	*9,33 x 10^8*
$\frac{1}{3}$	*1,006*	*4,50 x 10^8*
$\frac{1}{5}$	*0,672*	*2,67 x 10^8*
$\frac{1}{7}$	*0,490*	*1,88 x 10^8*
$\frac{1}{10}$	*0,346*	*7,83 x 10^7*
$\frac{1}{20}$	*0,172*	*4,00 x 10^7*

Table 1. The optical densities and the corresponding cfu/ml of the main dilutions of the Devriesea agamarum suspension prepared for the evaluation of the environmental persistency.

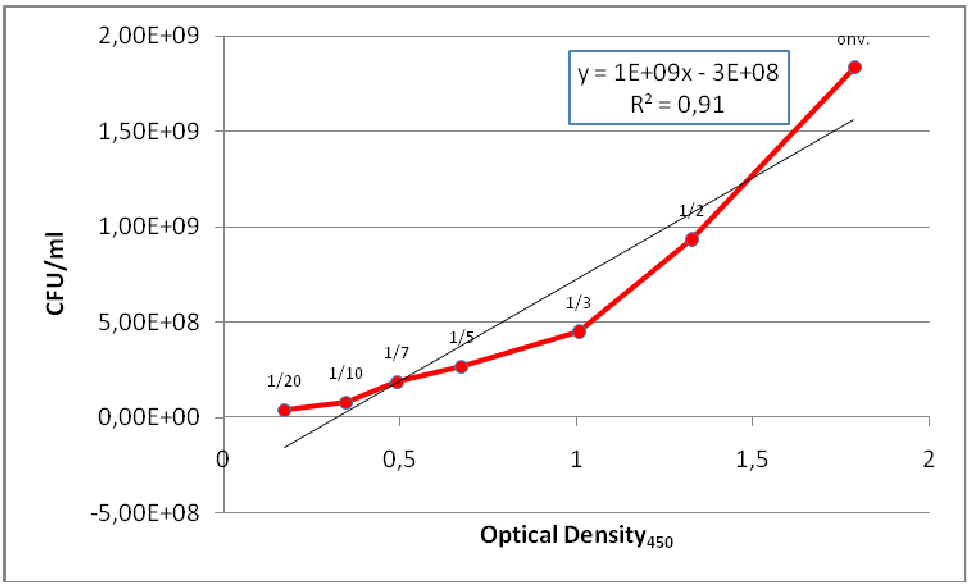

Figure 1. The correlation of the optical density and the cfu/ml of the dilutions of the Devriesea agamarum suspension ….

Determining the bacterial survival of D. agamarum under different experimental 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.

Survival of *D. Agamarum* under dry conditions

For the evaluation of survival of *D. agamarum* under dry conditions, 10 µl of the bacterial suspension was added to microtubes which were left in the vertical flow chamber until all fluid evaporated. The microtubes were then sealed with microfilm.

Survival of *D. Agamarum* in distilled water

For the evaluation of survival of *D. agamarum* in distilled water, 200 µl of the bacterial suspension was added to microtubes which were closed immediately, and sealed with microfilm.

Survival of *D. agamarum* in moist sand

For the evaluation of survival of *D. agamarum* in moist conditions, 50 µl of the bacterial suspension was mixed into 500 milligrams of filtered, rinsed and autoclaved white sand and than closed immediately, and sealed with microfilm.

The survival was determined at different testing points in time (as depicted in figure 3, 4 and 5). The samples were plated onto Colombia agar with 5 % sheep blood and incubated for 24 hours at 37 °C. One microtube per condition and per temperature was used for each testing point in time. The cfu/ml was determined zero when growth of *D. agamarum* could no longer be observed for two consecutive samplings.

Survival of *D. Agamarum* under dry conditions

In order to estimate the number of viable bacteria still present at the different testing points two hundred µl of distilled water was first added to the microtube previously prepared for the assessment of survival under dry conditions. A 100 µl aliquot from the test tube and from serial tenfold dilutions made from the test tube were plated onto Colombia agar plates with 5 % sheep blood. The colonies were counted after 24 and 48 hours incubation at 37 °C.

Survival of *D. Agamarum* in distilled water

In order to estimate the number of viable bacteria at the different testing points a 100 µl aliquot from the test tube and serial tenfold dilutions were plated onto Colombia agar plates with 5 % sheep blood. The colonies were counted after 24 and 48 hours incubation at 37 °C.

Survival of *D. agamarum* in moist sand

In order to estimate the number of viable bacteria still present at the different testing points 200 µl of distilled water was first added to the microtube previously prepared for the assessment of survival under dry conditions. A 100 µl aliquot from the test tube and from serial tenfold dilutions were plated onto Colombia agar plates with 5 % sheep blood. The colonies were counted after 24 and 48 hours incubation at 37 °C.

Development of an indirect enzyme-linked immunosorbent assay

Preparation of anti-lizard antibodies

Animals

Rabbits

For this experiment 6 rabbits (SPF New Zealand White, female, 3 months old) were used. They were housed as a group in a stable with a surface of 2 x 2 meters. Before taking the stable into use, it was disinfected by vaporizing H_2O_2. Wood shavings were used as bedding. The bedding was replaced regularly. Cardboard boxes were used as shelters. The room temperature was kept constant at 20 °C. The lights were on for 12 hours a day. The rabbits were ad libitum fed hay and pellets. They received ad libitum fresh water.

The animals were housed in the experimental facility two weeks prior to the experiment so that the animals could get used to the environment and the researchers.

Bearded dragons (Pogona vitticeps)

For this experiment 8 bearded dragons (*Pogona vitticeps,* male and female, adult) were used. The lizards were housed in groups in boxes of 1 x 1 meter. River sand was used as bedding. Roof tiles were used as shelters. The room temperature was kept constant at 20 °C. In the boxes a temperature gradient ranging from 20 – 35 °C was created with heat lamps. An UV-lamp was installed above each box.

The animals were fed vegetables daily and insects every other day. The food was supplemented with vitamins and minerals.

Spiny-tailed lizards (Uromastyx species)

For this experiment 8 spiny-tailed lizards (*Uromastyx species,* male and female, adult) were used. The lizards were housed in groups in boxes of 1 x 1 meter. River sand was used as bedding on the floor. Roof tiles were used as shelters. The room temperature was kept constant at 20 °C. In the boxes temperature gradients of 20 – 35 °C were created with heat lamps. An UV-lamp was attached above each box.

The animals were fed vegetables and seeds daily and insects every other day. The food was supplemented with vitamins and minerals.

All experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University.

Blood collection

To obtain lizard antibodies, blood was taken from the *Pogona vitticeps* and from the *Uromastyx* species. The samples were centrifuged (5000 g, 3 minutes) to obtain plasma.

The plasma samples were than pooled together so that 2 groups remained (*Pogona, Uromastyx*). 10 ml of plasma was obtained for each group.

Purification of lizard antibodies

To purify the protein fraction in the samples, the ammonium sulphate precipitation method was used. Solid ammonium sulphate was added to the pooled *Pogona* and *Uromastyx* plasma samples until a saturation of 50 % was reached. The samples were then stirred for 24 hours at a temperature of 4 °C, followed by centrifuging (10000 rpm, 120 minutes, 4 °C). The supernatant was discarded, and the pellet was resuspended in PBS. To remove the ammonium sulphate from the solution, a dialysis (buffer 10 mM Tris-HCl pH 8.5) was carried out. To this end the sample solution was transferred inside a membrane (Molecular porous membrane tubing MWCO 3500) which was clamped shut. The membrane with the sample solution was than placed in 4 litre of the buffer, and stirred for 24 hours at a temperature of 4 °C. The following day the contents of the membrane were transferred to sterile microtubes.

Determining the protein concentration of the precipitated solutions

For determination of the protein concentration of the precipitated solution, a protein assay kit was used (RC DC™ protein assay, Bio-Rad Laboratories inc.). For the protocol of the protein assay, see appendix 1. The reference solutions were made by dissolving bovine serum albumin (BSA, Sigma-Aldrich) in distilled water.

The results are shown in Table 2. The optical density of the 10^{-2} dilution of the test samples is comparable with the optical density of the 0,200 mg protein/ml reference solution. Hence, the test samples have a protein concentration of approximately 20 – 25 mg of protein/ml.

Solution	Optical Density$_{750}$ for *Pogona* sample	Optical Density$_{750}$ for *Uromastyx* sample
0,200 mg protein/ml	*0,085*	*0,075*
0,400 mg protein/ml	*0,113*	*0,121*
0,600 mg protein/ml	*0,155*	*0,141*
0,800 mg protein/ml	*0,197*	*0,181*
1,000 mg protein/ml	*0,240*	*0,212*
1,200 mg protein/ml	*0,247*	*0,246*
1,400 mg protein/ml	*0,277*	*0,260*
Undiluted sample	*1,047*	*1,179*
10^{-1} diluted sample	*0,397*	*0,389*
10^{-2} diluted sample	*0,081*	*0,084*

Table 2. Protein concentration determination of precipitated lizard antibody samples with the RC DC™ protein assay from Biorad Laboratories Inc.

Immunisation of the rabbits

The purified protein solution was filtered through 0,45 µm filters. Filtering did not affect the protein concentration of the solution.

The rabbits were randomly assigned numbers for recognition (numbers 1, 2, 3, 4, 5, 6). Rabbits 1 – 3 were subcutaneously immunised with 0,1 ml of *Pogona* purified protein solution and 0,9 ml incomplete Freund's adjuvant. Rabbits 4 – 6 were subcutaneously immunised with 0,1 ml of *Uromastyx* purified protein solution and 0,9 ml incomplete Freund's adjuvant.

Each rabbit was immunised 3 times, with 14 days between each immunisation. 14 days after the third and last immunisation the rabbits were exsanguinated under isoflurane anesthesia (see figure 2 for a time schedule). The collected sera were stored at -80 °C. The rabbit sera served as source for the secondary antibody in the developed indirect ELISA.

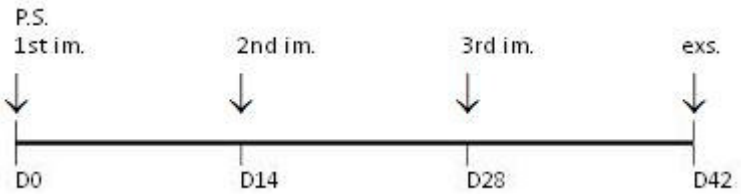

Figure 2 .Time schedule for the immunisation of the rabbits.
P.S. = Pre-immune serum
im. = Immunisation
Exs. = Exsanguination

Coating 96-well microtitre plates

For coating of 96-well microtitre plates (NUNC, Maxisorb) the *D. agamarum* type strain (=LMG 24257^{T}=IMP 2) was used. This strain was grown in Luria broth (LB, Sigma) and inactivated with formaldehyde. Bacterial growth was not observed when the inactivated broth was plated on Colombia agar plates with 5 % sheep blood.
After centrifuging (4300 rpm, 30 minutes, 25 °C) the broth, the pellet was resuspended in PBS supplemented with formaldehyde, and washed three times (1500 rpm, 30 minutes, 25 °C).
Before coating the microtitre plates, the obtained solution was centrifuged (5000 rpm, 10 minutes, 5 °C), and the pellet was resuspended and washed with coating buffer (washing by centrifugation at 5000 rpm, 10 minutes, 5 °C). The pellet was finally resuspended and diluted in coating buffer until an optical density of 0,3 was reached, measured in a spectrophotometer at 660 nm. One hundred and fifty µl of this solution was transferred to each well of 96-well microtitre plates (NUNC, Maxisorb). The plates were then sealed with adhesive foil and incubated for 24 hours at 4 °C. The plates were washed 4 times with washing buffer and were left to dry for 24 hours at a temperature of 4 °C. The following day the plates were sealed with adhesive foil and stored at 4 °C until used for the ELISA.

Protocol for the indirect ELISA

1. The wells were washed with 100 µl washing buffer, which was supplemented with 2,2% skim milk powder.

2. The lizard plasma was diluted with washing buffer containing 2,2% skim milk powder (the different dilutions are represented in table 4 and 5). One hundred µl of each lizard sample was transferred to the wells and the plates were then sealed with adhesive foil. Next, the plates were incubated for 2 hours at 37 °C. The wells were emptied and washed 5 times with washing buffer.

3. The rabbit sera were diluted with washing buffer containing 2,2% skim milk powder (the different dilutions are represented in table 4 and 5). One hundred µl the diluted rabbit sera was transferred to the wells used in the previous step, and the wells were then sealed with adhesive foil. Next, the plates were incubated for 2 hours at 37 °C. The wells were emptied and washed 5 times with washing buffer.

4. A tenfold dilution serie of goat anti-rabbit antibody labelled with horseradish-peroxidase was made in washing buffer containing 2,2% skim milk powder. 100 µl of the dilution was transferred to the wells used in the previous steps and the wells were then sealed with adhesive foil. The plates were incubated for 30 minutes at 37 °C. The wells were emptied and washed 5 times with washing buffer.

5. One hundred µl of a citric acid-phosphate buffer (pH 5.0), containing 0,07% *o*-phenylenediamine and 0.22% hydrogenperoxide, was transferred to each well used in the previous steps, and left to incubate for 10 minutes at room temperature. After the 10 minutes, 50 µl of 2.5 M hydrochloric acid was added to each used well.

6. The color change in the wells was determined with an ELISA reader measuring at 492 nm.

Two preliminary tests with the ELISA's were done. The goal of the first ELISA was to see whether samples would give a measurable result, and to determine possible differences in results from different samples.
The first goal of the second ELISA was to determine the effect of using higher dilutions of the primary and secondary antibodies. The second goal was to determine if the results from a lizard with a *D. agamarum* associated dermatitis would differ from the results from a lizard with no history of *D. agamarum* associated disease.

Results

Prolonged environmental persistency of *Devriesea agamarum*

Figures 3, 4 and 5 show the survival of *D. Agamarum* under the three different conditions and at the four different temperatures.

Under the dry condition no growth of *D. agamarum* could be observed after 16 days and 4 days at 40 °C and 50 °C respectively. At 20 °C and 30 °C however, bacterial growth was observed until the 27th and 20th day respectively.

No survival of *Devriesea agamarum* could be demonstrated after the first day at 50 °C in moist sand and in distilled water. At 40 °C survival was observed until the 8th day and the 4th day for moist sand and distilled water respectively.

At 20 °C and 30 °C *D. agamarum* persisted for more than 3 months in moist sand and in distilled water (see Figure 4 and 5).

At 30 °C, the number of remaining cfu/ml remained high until approximately the 50th day in moist sand, and started to decrease gradually from that point on. In distilled water the number of viable cfu/ml remained high up to the last testing point.

At 20 °C a minor decrease in the number of viable cfu was observed for both moist sand and distilled water during the complete test period.

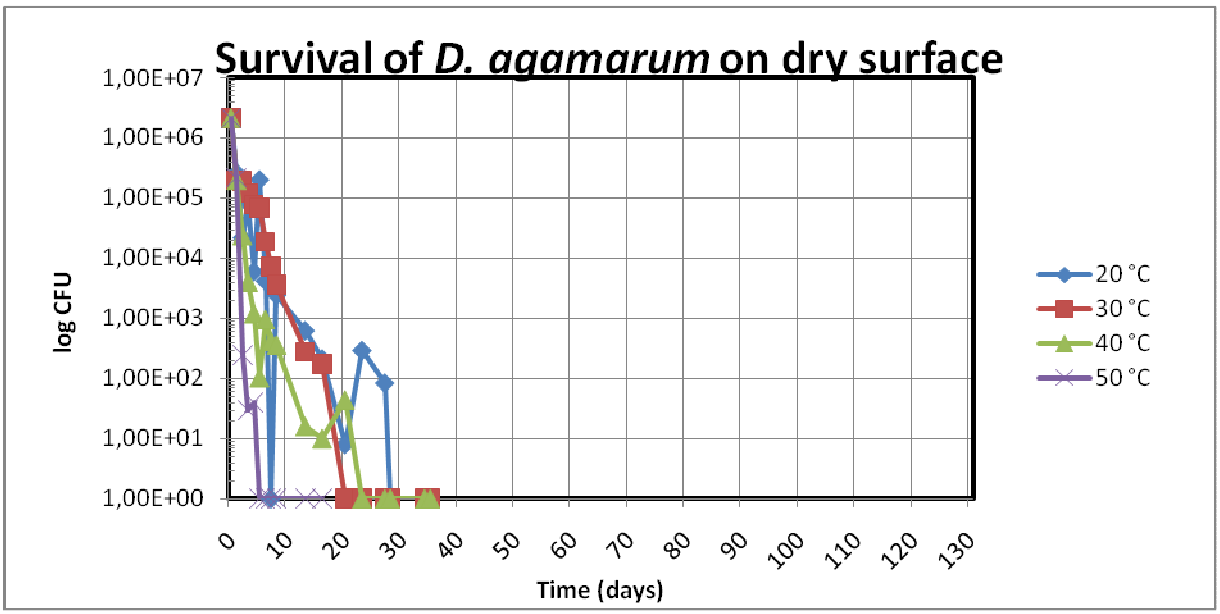

Figure 3. The survival of D. agamarum on dry surface. The horizontal axis shows the time in days. The vertical axis shows the logartitmic value of the remaning colony forming units (cfu).

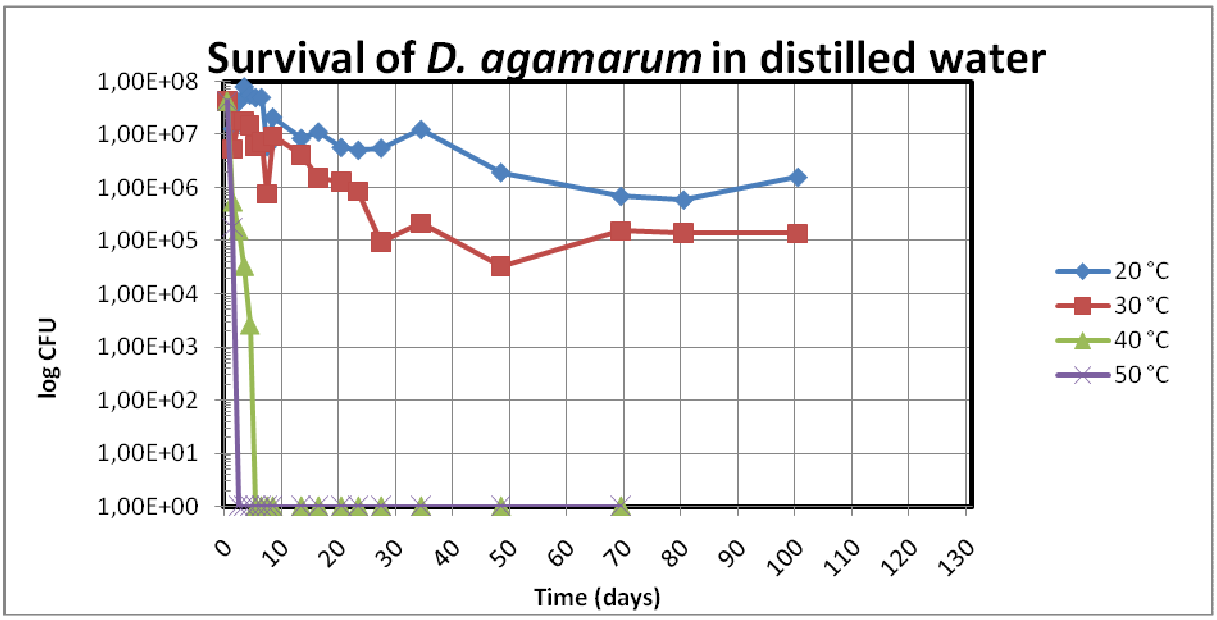

Figure 4. The survival of D. agamarum in distilled water. The vertical axis shows the logartitmic value of the remaning colony forming units (cfu).

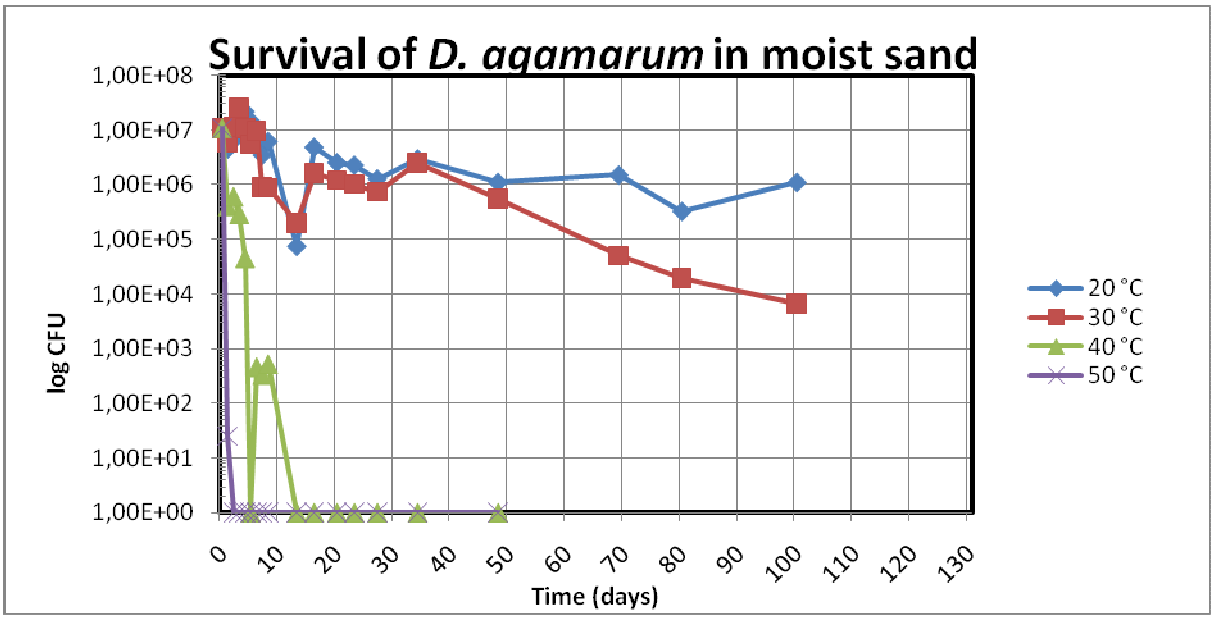

Figure 5. The survival of D. agamarum in moist sand. The vertical axis shows the logartitmic value of the remaning colony forming units (cfu).

Based on the results of an indirect enzyme-linked immunosorbent assay antibodies against *Devriesea agamarum* can be demonstrated in lizards

First experiment

In the first experiment the dilution used for all the lizard plasma was $\frac{1}{200}$. The dilution used for all the rabbit anti-lizard sera was $\frac{1}{500}$.

No negative and positive controls for this ELISA are available. Different combinations of primary, secondary and tertiary antibodies were used to determine the amount of aspecific binding and to determine if false positive results occurred.
Plasma from a clinically healthy bearded dragon with no history of *D. agamarum* associated disease was used in this ELISA (wells A1 – F1) so that the results could be compared with samples obtained from 3 lizards, previously infected with *D. agamarum* but clinically healthy at time of sampling (Wells A5 – F5/A6 – F6/A7 – F7). It was suspected that plasma from the animal with no history of *D. agamarum* associated disease would generate lower signals.
Plasma from a clinically and naturally with *D. agamarum* infected *Uromastyx dispar* was used in this ELISA chosen to serve as a positive control in this ELISA because it was suspected that it would generate relatively higher signals.
Colum 11 and 12 show the results of different combinations of primary, secondary and tertiary antibodies. These values are all lower in comparison to the other included samples.
There is little difference in the values in one column (apart from column 11).
The values generated with the plasma from a clinically healthy bearded dragon with no history of *D. agamarum* associated disease are lower than the values from the other included samples, and higher than the values in column 11 and 12.
There is little difference between the values obtained from the clinically *D. agamarum* infected *Uromastyx dispar* and the 3 clinically healthy lizards with a history of *D. agamarum* infection.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1,448		3,471		3,429	3,357	3,516				0,044	0,245
B	0,888		3,491		3,261	3,377	3,212				0,084	0,251
C	1,245		3,458		3,269	3,295	3,323				0,102	0,627
D	2,916		3,255		3,181	3,229	3,159				0,362	0,329
E	2,366		3,445		3,407	3,207	3,445					0,255
F	2,857		3,218		3,147	3,244	2,986					0,354

Table 3. Results of first experiment with the developed indirect ELISA .In this ELISA several samples were tested and the results compared.

1. A – F = Wells with plasma from a healthy *Uromastyx dispar* + serum from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies.

3. A - F= Wells with plasma taken from an *Uromastyx dispar* infected with *D. agamarum* + serum from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies.

5. A – F = Wells with plasma taken from healthy *Pogona vitticeps* with a history of a *D. agamarum* + serum from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies.

6. A – F = Wells with plasma taken from healthy *Pogona vitticeps* with a history of a *D. agamarum* + serum from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies.

7. A – F = Wells with plasma taken from healthy *Pogona vitticeps* with a history of a *D. agamarum* + serum from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies.

11. Wells with different combinations of primary, secondary and tertiary antibodies
    A = Coating only (empty well)
    B = Coating + enzyme-labeled goat anti-rabbit antibodies
    C = Plasma taken from an *Uromastyx dispar* infected with *D. agamarum* + enzyme-labeled goat anti-rabbit antibodies
    D = Plasma taken from an *Uromastyx dispar infected* with *D. agamarum* + pre-immune serum taken from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies

12. A - F = Wells with serum from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies

<u>Second experiment</u>

Dilutions were prepared from the plasma of a clinically healthy *Pogona vitticeps* that previously suffered from *D. agamarum* associated dermatitis, as well as from a clinically healthy *Pogona vitticeps* with no history of a *D. agamarum* associated disease (the different dilutions are represented in Table 4 and 5).
The rabbit serum used in this experiment was the same serum as used in the first experiment in row A (see first experiment).
Column 6 shows the results of different combinations of primary, secondary and tertiary antibodies. These values are all lower in comparison to the other included samples.
At higher dilutions of both the primary and secondary antibodies the optical density starts to decline with both samples.
When comparing the results from the higher dilutions from both samples, lower values are detected for the plasma taken from the lizard with no history of a *D. agamarum* associated disease.

Reptile → Rabbit ↓	**1 (1/5)**	**2 (1/10)**	**3 (1/50)**	**4 (1/100)**	**5 (1/500)**	**6**
A (1/500)	3,464	3,26	3,423	3,423	3,464	0,101
B (1/500)	3,281	3,105	3,371	3,485	3,371	0,214
C (1/1000)	3,263	3,452	3,317	3,347	3,113	0,306
D (1/1000)	3,249	3,111	3,339	3,223	3,223	
E (1/5000)	3,304	3,439	3,401	3,304	2,206	
F (1/5000)	3,441	3,265	3,327	3,163	2,035	
G (1/10000)	3,033	3,26	3,163	2,268	1,211	
H (1/10000)	3,028	3,003	3,267	2,255	1,189	

Table 4. Results of second experiment with the developed indirect ELISA .In this ELISA the serum of a clinically healthy Pogona vitticeps with a history of D. agamarum associated disease was used. The optical density of the different samples was measured.

Column 6 = Wells with different combinations of primary, secondary and tertiary antibodies

A = Coating + enzyme-labeled goat anti-rabbit antibodies

B = Coating + pre-immune serum taken from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies

C = Coating + serum from rabbits immunized against reptile proteins + enzyme-linked goat anti-rabbit antibodies

Reptile→ Rabbit ↓	**1 (1/5)**	**2 (1/10)**	**3 (1/50)**	**4 (1/100)**	**5 (1/500)**	**6**
A (1/500)	3,228	3,345	3,379	3,283	3,312	0,101
B (1/500)	3,224	3,275	3,28	3,438	3,178	0,23
C (1/1000)	3,311	3,408	3,487	3,208	2,636	0,298
D (1/1000)	3,217	3,244	3,068	3,334	2,804	
E (1/5000)	3,298	3,244	3,132	2,381	1,504	
F (1/5000)	3,205	3,205	3,205	2,371	1,461	
G (1/10000)	2,778	2,718	2,283	1,433	0,865	
H (1/10000)	2,803	2,726	2,021	1,457	0,879	

Table 5. Results of second experiment with the developed indirect ELISA. In this ELISA the serum of a clinically healthy Pogona vitticeps with no history of D. agamarum associated disease was used. The optical density of the different samples was measured.

Column 6 = Wells with different combinations of primary, secondary and tertiary antibodies

A = Coating + enzyme-labeled goat anti-rabbit antibodies

B = Coating + pre-immune serum taken from rabbits immunized against reptile proteins + enzyme-labeled goat anti-rabbit antibodies

C = Coating + serum from rabbits immunized against reptile proteins + enzyme-linked goat anti-rabbit antibodies

Discussion

This study demonstrates that *Devriesea agamarum* is able to persist and remain viable for more than 3 months in moist sand and distilled water and survival on dry surfaces is limited. Older literature frequently proposes that enclosures of desert-dwelling lizards partly should have a relatively high humidity, for example to facilitate shedding (Divers 1996, Johnson 2006). Based on the results of this study however, the need for a dry environment for desert lizards is suggested.

The prolonged persistency of *D. agamarum* in the environment emphasises the need for efficient disinfection procedures to eliminate *D. agamarum* from infected lizard collections next to effective antimicrobial treatment against *D. agamarum* as described by Hellebuyck et al. (2009).

The described indirect enzyme-linked immunosorbent assay (ELISA) was developed to asses antibody response in lizards against *D. agamarum*. Since no positive controls are available for this ELISA aspecific binding of the different antibodies was evaluated. When either one of the primary, secondary or tertiary antibody was lacking the resulting signal was significantly lower in comparison to signals obtained when all antibodies were present. Although some aspecific binding and background signal was observed, the detected O.D. values suggest that the different antibodies mainly bind to the desired target. In conclusion, antibodies against *D. agamarum* can be demonstrated in lizard plasma samples when analysed with the described indirect ELISA.

Signals that were detected when using low dilutions of the primary and secondary antibodies in the second ELISA showed little variation.

How specific for the measured antibodies are for *D. agamarum* is not known. Although the first preliminary ELISA detected, in comparison to the other samples, a relatively lower signal for the plasma obtained from a clinically healthy bearded dragon with no history of *D. agamarum* associated disease, no conclusions can be made so far concerning seroconversion. The second preliminary ELISA detected lower signals for a plasma sample collected from a clinically healthy lizard with no history of *D. agamarum* associated disease, mainly at the higher dilutions of both the primary and secondary antibodies, in comparison to a sample from a lizard that had been previously infected with *D. agamarum*. As mentioned before the increases in antibody titer can be less distinctive in comparison to antibody responses in mammals (Zimmerman et al., 2009).

Conclusively the preliminary tests of the developed ELISA demonstrates that antibodies present in reptile plasma bind to *D. agamarum*. In order to demonstrate seroconversion in lizards after natural infection with *D. agamarum* or auto-vaccination against *D. agamarum* optimalisation of the described assay should be preformed.

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Appendix A . Protein content determination protocol

Materials and equipment:

- Test sample(s)
- Sterile 200 ml microtubes
- Phospate buffered saline (PBS)
- Bovine Serum Albumin (BSA)
- Spectophotometer set at 750 nm.
- RC/DC™ protein assay, Bio-Rad Laboratories inc., which contains:
    - Solution A :used for making the solution more basic.
    - Solution B: contains Cu^{2+}-ions which form complexes with the peptide-links in the proteins.
    - Solution C: A buffer used for resuspending the formed pellet
    - Solution D: Contains Folin's reagent, which is used to make proteins which have Cu^{2+} attached to them color blue.

Protocol:

1. Use BSA and PBS to make reference solutions with which the test sample can be compared at the end of the protein determination.

Final concentration	Dilution	µl BSA stock	µl PBS
0,200 mg/ml BSA	50 x	20	980
0,400 mg/ml BSA	25 x	40	960
0,600 mg/ml BSA	16,66 x	60	940
0,800 mg/ml BSA	12,5 x	80	920
1,000 mg/ml BSA	10 x	100	900
1,200 mg/ml BSA	8,33 x	120	880
1,400 mg/ml BSA	7,14 x	140	860

2. Transfer 25 µl of each sample (including the reference solutions) + 125 µ solution A in separate microtubes, and vortex them.
3. Incubate for 1 minute at room temperature.
4. Add 125 µl of solution B, and centrifuge the microtubes (5 minutes, 16000 G)
5. Pour the supernatant off, resuspend the pellet in 127 µl of solution C, and vortex the microtubes.
6. Add 1 ml of solution D.
7. Incubate for 15 minutes at room temperature.
8. Measure the samples in the spectrophotometer at 750 nm.