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Original article

Experimental infection of honeybees by *Pseudomonas aeruginosa*

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Summary — Honeybees kept in cages were experimentally infected by dipping in a bacterial suspension of *Pseudomonas aeruginosa* ATCC 27014 *(P apiseptica)* known to cause septicaemia. The concentration of the bacterial suspension was *ca* 5 x 10^9 CFU per ml saline. The highest mortality rate (66.8%) was observed 10–50 h after infection. The number of bacteria isolated in the haemolymph of diseased honeybees was *ca* 10^6–10^9 CFU per ml haemolymph. Between the 10th and the 50th h, it was found that the mean concentration of viable bacteria in the haemolymph in bees which showed clinical symptoms of infection varied significantly at different 10-h-intervals after infection.

***Pseudomonas aeruginosa* / septicaemia / experimental infection / haemolymph / individual variability**

INTRODUCTION

Septicaemia, a bacterial disease in adult bees, is probably a secondary but fatal infection (Bailey, 1981). *P apiseptica* is one of the most frequent pathogens of this disease, favoured by factors such as high moisture level, adverse weather, unbalanced feeding, massive artificial feeding, comb building in a swarm, or existence of other diseases (Burnside, 1928; Landerkin and Katznelson, 1959; Wille and Pinter, 1961; Langridge, 1963; Wille, 1965; Otte, 1966; Wagener, 1968; Horn and Eberspächer, 1986). The present investigation examines the experimental infection of bees by the bacterium *P aeruginosa*, and provides data on enumeration of viable bacteria present in the haemolymph of diseased bees at certain times following infection. An examination of the *in vivo* growth of bacteria in a diseased bee might provide insight into the significance in bacterial resistance of the various honeybee defences.

MATERIALS AND METHODS

Insects

Adult honeybees (*Apis mellifera macedonica)* < 24 h old emerged in the incubator at 35 °C from sealed brood taken from a healthy colony. They were kept 50 to a cage (Jacobs, 1977; Van Steenkiste, 1988) for nine days at 30 °C. The insects were anesthetized by CO_2 and infected by dipping in 20 ml of a bacterial suspension with a concentration of *ca* 5 x 10^9 CFU (colony forming units) per ml saline. Control bees were either anesthetized by CO_2 (control A) or dipped into normal saline after they had been anesthetized by CO_2 (control B).

Bacteria

P aeruginosa ATCC 27014 *(P apiseptica)* was obtained from the American Type Culture Collection, and grown aerobically at 37 °C on a blood–agar base (Starr *et al,* 1981). After 15 h incubation, bacteria from a broth culture were plated on blood–agar base in Roux bottles. After 48 h incubation they were suspended in saline, centrifuged for 15 min at 3 500 rpm and washed three times. The number of viable cells was determined by dilution plating on blood agar base.

Haemolymph was withdrawn by puncturing the integument under the third abdominal tergite after the surface of the bees had been treated with 70% v/v ethanol. This method was similar to that of Wille and Vecchi (1966) and Van Steenkiste (1988). 0.5 µl haemolymph was taken from each insect and diluted in sterile saline. The concentration of viable bacteria was estimated by dilution-plating on a blood–agar base.

Mortality rate

Ten experiments were performed using 1 500 insects to determine the mortality rate of bees after infection by *P aeruginosa.* Out of these, 500 honeybees were infected, 500 were used as controls B and the remainder as controls A.

Concentration of viable bacteria in haemolymph of diseased bees

The concentration of viable bacteria in the circulation was estimated between the 10th and the 50th h after infection in 139 bees. To sample a reliable number of diseased bees at each 10-h period, the experiment was repeated several times. In these experiments 2 050 honeybees were used: out of these, 1 450 were infected, while 600 were used as controls.

Statistics

Differences between means were analysed by using 1-way analysis of variance. In cases in which variances were not equal even after transformation to square root, non-parametric tests were used.

RESULTS

Clinical manifestation of septicaemia

The symptoms associated with septicaemia appeared suddenly and were similar to those of natural infection as described by Burnside (1928) and Wille and Pinter (1961). Infected bees died 15–30 min following clinical manifestation of the disease.

Mortality rate

Honeybee mortality was noted every 10 h; the results are given in table I. The highest death rate (66.8%) took place 10–50 h after infection. There was a significant ($P \leq 0.05$) increase in the number of bees which died 10–50 h and 60–70 h after infection in treated bees compared with controls. However, there was not any significant difference between control A and B in the number of dead bees.

Table I. No of dead bees at times following infection by *Pseudomonas aeruginosa* ATCC 27014.

Time after infection (h)	*Infected dead bees**		*Control A dead bees**		*Control B dead bees**	
	No	*%*	*No*	*%*	*No*	*%*
1–10	16	3.2	2	0.4	7	1.4
10–20	47	9.4	2	0.4	4	0.8
20–30	167	33.4	0	0.0	4	0.8
30–40	88	17.6	1	0.2	0	0.0
40–50	32	6.4	1	0.2	0	0.0
50–60	10	2.0	4	0.8	3	0.6
60–70	9	1.8	2	0.4	1	0.2
70–80	2	0.4	2	0.4	0	0.0
80–90	1	0.2	1	0.2	5	1.0
90–100	10	2.0	4	0.8	11	2.2
1–100	382	76.4	19	3.8	35	7.0

* *N*: 500 total number of experimental bees in each treatment.

Concentration of viable bacteria in the haemolymph of diseased honeybees

The concentration of viable bacteria in circulation 10–50 h after infection was *ca* 10^6–10^9 CFU per ml haemolymph, with some extreme values of 10^4 and 10^{10} (table II). The mean concentration of circulating bacteria varied significantly between bees which showed clinical symptoms of infection at different 10-h intervals after infection. No bacteria were isolated from the controls.

Table II. No of viable bacteria per ml haemolymph of diseased bees at various times following infection.

Time after infection (h)		
10–20	*n*	43
	$\bar{x}$	2.458×10^8
	SE	1.718×10^8
20–30	*n*	38
	$\bar{x}$	6.872×10^8
	SE	$1,568 \times 10^8$
30–40	*n*	35
	$\bar{x}$	1.761×10^9
	SE	0.563×10^9
40–50	*n*	23
	$\bar{x}$	5.831×10^9
	SE	1.722×10^9

n: No of bees; x: mean of the No of viable bacteria; SE: standard error.

DISCUSSION

Nine-d-old bees were selected, as studying the immunity of young bees is of greater importance than that of older insects. A 5×10^9 CFU per ml saline dose was used as it caused high mortality (70%) a short time after infection (50 h) and because the

mortality rate was graduated. As a method of infection dipping was preferred to injection, because in natural infections the bacterium enters from the front spiracle and reaches the blood through the trachea or tracheole wall (Burnside, 1930; Wille, 1964). Oral admini-stration was not utilized as it resulted in low insect mortality, neither was spraying with the bacterial suspension as it resulted in high mortality a long time after infection (Burnside, 1928; Wille and Pinter, 1961). The results showed that CO_2 did not affect bee mortality. The use of control A was not therefore necessary.

A statistically significant increase in the number of dead bees was observed from the 10th to the 50th h after infection (table I). Enumeration of bacteria was therefore performed during this period of time. *P apiseptica* resulted in bee mortality when its concentration in the haemolymph reached *ca* 10^6–10^9 CFU per ml. The mean concentration of bacteria was significantly different in bees which showed clinical symptoms of infection at different 10-h intervals. It seems that there is a varying sensitivity of bees to *P apiseptica*. Additionally, the great variance in the number of viable bacteria in the haemolymph of diseased bees sampled at the same 10-h period (table II), shows that individuality plays a significant role in honeybee defense. We are of the opinion that it would be of interest to study in detail the defense mechanisms of bees in bacterial diseases including infection by *P apiseptica*.

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Résumé — Infection expérimentale d'abeilles (*Apis mellifera* L) par *Pseudomonas aeruginosa*. La bactérie *Pseudomonas aeruginosa* ATCC 27014 *(P apiseptica)* est responsable de la septicémie des abeilles. L'étude porte sur le nombre de bactéries vivantes présentes dans l'hémolymphe des abeilles malades à diverses périodes après déclenchement expérimental de l'infection. Des abeilles, âgées de 9 j et maintenues en cagette depuis leur émergence, ont été infectées en étant plongées dans 20 ml d'une suspension de bactéries dans une solution saline normale à la concentration d'environ 5 x 10^9 CFU par ml. Durant les 10 premières h après l'infection le taux de mortalité a été de 3,2%. Le taux le plus élevé a été observé entre 10 h et 50 h après l'infection (tableau I). La concentration en bactéries vivantes par ml d'hémolymphe a été estimée à cette période là par placage dilué sur de l'agar au sang. *P apiseptica* a causé la mort lorsque sa concentration dans l'hémolymphe a atteint environ 10^6–10^9 CFU par ml. La concentration moyenne en bactéries a été significativement différente entre les abeilles, qui montraient des signes cliniques d'infection, examinées à intervalles de 10 h après l'infection. Il semble qu'il existe des différences dans la sensibilité des abeilles à *P apiseptica*. La forte variabilité dans le nombre de bactéries vivantes dans l'hémolymphe d'abeilles malades à une période donnée prouve que l'individualité joue un rôle important dans le mécanisme de défense des abeilles.

***Pseudomonas aeruginosa* / septicémie / infection expérimentale / variabilité individuelle / concentration en bactéries**

Zusammenfassung — Experimentelle Infektion von Honigbienen mit *Pseudomonas aeruginosa*. Diese Arbeit befaßt sich mit der experimentellen Infektion von Bienen mit dem Bakterium *Pseudomonas aeruginosa* ATCC 27014 (P apiseptica)

und bringt Zahlenangaben über lebende Bakterien in der Hämolymphe (= im Blut) erkrankter Bienen in der Zeit nach der Infektion. Es wurden Honigbienen verwendet, die nach dem Schlüpfen für 9 Tage in Käfigen gehalten worden waren. Die Tiere wurden durch Eintauchen in 20 ml einer Bakterien-aufschwemmung mit einer Konzentration von etwa 5 x 10^9 CFU pro ml normaler Kochsalzlösung infiziert.

In den ersten 10 Stunden nach der Infektion betrug die Sterberate 3,2%. Die höchste Sterberate war 10 bis 50 Stunden nach der Infektion zu beobachten (Tabelle I). Die Konzentration lebensfähiger Bakterien pro ml Hämolymphe wurde zu dieser Zeit durch verdünnte Ausstriche auf Blutagar bestimmt. *P apiseptica* verursachte den Tod sobald eine Konzentration in der Hämolymphe von 10^6 bis 10^9 CFU pro ml erreicht war. Die mittlere Konzentration der Bakterien bei Bienen mit klinischen Symptomen der Infektion war zu verschiedenen Zeitintervallen nach der Infektion signifikant verschieden. Es scheint Unterschiede in der Empfindlichkeit der Bienen gegenüber *P apiseptica* zu geben. Die große Variabilität in der Zahl lebensfähiger Bakterien in der Hämolymphe kranker Bienen zum selben 10-Stundigen Zeitperiode zeigt ebenfalls, daß die Individualität bei der Abwehr durch die Bienen eine bedeutende Rolle spielt.

***Pseudomonas aeruginosa* (= *P apiseptica*) / experimentelle Infektion / Bakterienzahl / individuelle Variabilität / bakterielle Septikämie**

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