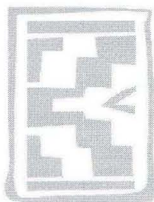

Virulence of South African isolates of *Haemophilus paragallinarum*. Part 1: NAD-dependent field isolates

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ABSTRACT

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The virulence of four South African field isolates of NAD-dependent *Haemophilus paragallinarum*, representing the four serovars known to occur in that country, was investigated.

During this study an alternative challenge model for infectious coryza was used, in which the infectivity as well the virulence of different isolates could be evaluated.

The challenge model consisted of the direct challenge, via intrasinus injection of one chicken in a row of interconnected layer cages, containing 10 chickens, which are subsequently infected by natural routes. A scoring system of the clinical signs was established in which a score is given to the ability of the isolate to produce clinical signs in the challenge birds. The mean daily disease score for the flock can be calculated and plotted on a graph to give a graphic representation of the disease profile. A mean disease score, calculated over a 20-day examination period can be calculated. Isolates can then be compared to each other, either graphically or by a comparison of the mean disease scores.

It has been demonstrated using this scoring system that the South African serogroup C isolates appear to be more virulent than the South African serogroup A or B isolates. It was further established that the serovar C-3 isolate appeared to be the most virulent.

Keywords: *Haemophilus paragallinarum*, virulence, NAD-dependence, South Africa

INTRODUCTION

Infectious coryza (IC) of domestic fowls caused by the bacterium *Haemophilus paragallinarum* remains a problem in the layers industry in many parts of the world. Bragg (1995) and Bragg, Coetzee & Verschoor (1996) reviewed the historical aspects of infectious coryza in South Africa. The disease was first recorded in South Africa in the early 1970s (Buys 1982). Vaccines were first used against this disease in South Africa in the 1970s (Buys 1982), and appeared to be effective in controlling the disease. During the mid 1980s, it became apparent that vaccines used at that stage were not providing adequate protection (Bragg *et al.* 1996). During the 1990s, IC was once again the most serious disease in layers in this country (Bragg *et al.* 1996).

It has been established by a number of workers that there are four different serovars of NAD-dependent *H. paragallinarum* currently in South Africa (Kume, Sawata, Nakase & Matsumoto 1983; Black-

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all, Eaves & Rogers 1990; Bragg *et al.* 1996). During an investigation of the incidence of the four serovars of *H. paragallinarum* in South Africa, Bragg *et al.* (1996) discovered that there had been a significant change in the prevalence of the different serovars over a 30-year period. It was shown that the prevalence of serogroup A isolates had all but disappeared in the 1990s, while the prevalence of serovar C-3 had increased significantly during this period. This lead Bragg *et al.* (1996) to postulate that these changes in the prevalence of the different isolates was possibly a result of the use of vaccines in this country not containing the correct serovars. Bragg *et al.* (1996) and Bragg, Greyling, Beer & Verschoor (1997) stated the need for vaccines containing local isolates in countries where unique serovars of *H. paragallinarum* occur. In order to further investigate the needs for local vaccines, the next step was to investigate the virulence of the different serovars.

The virulence mechanisms in *H. paragallinarum* are not clearly understood. It has been postulated that the attachment of *H. paragallinarum* to mucous membranes of chickens may be important in the virulence of these isolates (Sawata, Nakai, Kume, Yoshikawa & Yoshikawa 1985a, 1985b). Virulent isolates of *H. paragallinarum* have a thick capsule layer which is used to attach the bacterium to the host cell cilia layer (Matsumoto 1988). Serogroup B isolates have been regarded as non-virulent by a number of researchers (Kume, Sawata & Nakase 1980a). However, no direct comparison of the virulence of the different isolates could be found in the literature.

A number of researchers have conducted experiments in which chickens are challenged with *H. paragallinarum.* The first reported experimental infection with *H. paragallinarum* was by Nelson in 1933. Experimental infection of chickens has been performed for a number of different reasons, the most frequent of which being the challenging of vaccinated chickens in order to evaluate vaccines. Several authors have reported on this aspect (Matsumoto & Yamamoto 1971; 1975; Reid & Blackall 1986; Blackall 1988, 1991; Blackall, Eaves, Rogers & Firth 1992; Jacobs, Cuenen & Storm 1992; Nakamura Hoshi, Nagasawa & Ueda 1994). A common denominator in all of these experiments was the use of intrasinus injection of the challenge material. Generally between 10^6 and 10^8 colony forming units (cfu) per mℓ were used. In most cases the volume of liquid containing bacteria that was injected was 0.2 mℓ, the bacteria being suspended in a variety of liquid media. Jacobs *et al.* (1992) used *H. paragallinarum*-infected egg yolk as challenge material. Nakamura *et al.* (1994) suspended their challenge material in phosphate buffered saline (PBS). All of the other authors used the media in which the bacterium was cultured as the challenge material.

Other challenge work on *H. paragallinarum* was performed to obtain information related to the pathogenicity of the bacterium (Rimler, Davies & Page 1977; Rimler 1979; Yamaguchi, Blackall, Takigami, Iritani & Hayashi 1990). Rimler *et al.* (1977) used infected egg yolk and challenged the birds by placing the challenge material into the nasal passages of the birds. Rimler (1979) evaluated the difference in pathogenicity obtained when the challenge material was dropped onto the external nasal orifices or when it was administered by injection into the sinuses. He found that of the 35 isolates tested, eight did not produce clinical signs when the challenge material was dropped onto the nasal orifices, but did when it was injected into sinuses, thus clearly establishing that the route of challenge is an important factor in the establishment of infection.

Kume *et al.* (1980a, 1980b) used a challenge method to evaluate the immunological relationship between different isolates. They also used an intrasinus injection method of infecting the chickens, the infectious material consisting of 0.2 mℓ of a 10^8 cfu per mℓ culture of *H. paragallinarum.*

All the authors who have experimentally infected chickens with *H. paragallinarum* kept the chickens under observation after the challenge and recorded the development of clinical signs. All of them, except Matsumoto & Yamamoto (1971, 1975) scored these chickens that manifested clinical signs as positive and those that did not as negative. Matsumoto & Yamamoto (1971, 1975) on the other hand, graded the severity of the clinical signs as mild, moderate or severe. "Mild" infection was used to indicate nasal discharge with or without mild facial oedema. "Moderate" was used when there was nasal discharge on both left and right sides plus slight facial oedema. "Severe" was used in cases where there was severe bilateral oedema with or without haemorrhage and conjunctivitis. However, they did not ascribe a numerical score to these clinical signs.

In these challenge experiments the chickens were kept under observation for periods varying from 7 days to 2 weeks and in addition to the recording of clinical signs, the total number of birds which manifested evidence of disease was also noted. None of the authors reported on the number of birds

showing clinical signs on any particular day or days during the observation period.

Bragg & Greyling (1999) used a challenge system based on modifications of the challenge methods used by the above-mentioned authors. The rating system of Mustsumoto & Yamamoto (1971, 1975), in which the clinical signs were rated as mild, moderate and severe, was used. However, in the scoring system of Bragg & Greyling (1999), a score of 1 was given for chickens showing mild clinical signs, 2 for moderate clinical signs and 3 for severe clinical signs, in birds challenged by intrasinus injection of the infectious material. In this challenge model, only one bird in a group of ten birds in adjoining layer cages with a communal water supply, consisting of a water pipe passing through the cages, with nipple drinkers in each cage, was challenged by intrasinus injection of the infectious material. The remaining chickens in the group were left as "in contact" birds in the challenge model. Clinical signs caused by the bacterium in the in contact birds carried more weight that the clinical signs caused by the bacterium that has been artificially introduced into the sinus of the bird via injection and double the score for these clinical signs was given to diseases symptoms found in birds infected by the natural route. This was based on the work of Rimler (1979) who found clear differences in the virulence of bacteria when introduced by injection into the sinus or were introduced by the more natural route of dropping the bacterial challenge onto the nose of the birds. The proposed scoring system allows for the calculation of daily disease scores. The numerical values given to the disease signs were then plotted, following the course of the disease over time. This permited the construction of a disease profile which was then used to compare different isolates or efficacy of different vaccines.

In this study, a numerical scoring system was used to evaluate the virulence of four different serovars of *H. paragallinarum* found in South Africa.

MATERIALS AND METHODS

Source of chickens

A total of 40 unvaccinated Lohmann layer chickens, at an age of 11 weeks, were obtained from a suppler of point-of-lay chickens. The flock from which these birds originated had no previous history of infectious coryza. These chickens were obtained before the flock had been routinely vaccinated against infectious coryza. The chickens, on arrival at the university facilities, were housed in isolated layer facilities which had been extensively cleaned and disinfected prior to the placement of the birds in them, until they were used in the experiments. The disinfection efficacy in the experimental layer facilities was monitored and consisted of two disinfection sessions in which a 1 % dilution of the disinfectant Virukill (obtained from ICA Laboratories, Cape Town) was used at a dose rate of 600 mℓ/m^2. The resulting disinfection efficacy after the two disinfection sessions was judged to be 100 %, as no viable bacteria were detected on ten Radac plates (obtained from Sterilab Services, Johannesburg) collected after the final disinfection stage. The chickens were monitored throughout the rearing period and no clinical signs of infectious coryza were seen in any of the birds during the full period that they were in the layer cages at the university. During this time, there were no experiments underway in any of the facilities in which *H. paragallinarum* was used. There is therefore no possibility of any of these birds being exposed to the bacterium during the rearing period.

The chickens were housed in these facilities until they were 25 weeks of age, when they were moved to a separate similarly disinfected layer facility and were challenged with *H. paragallinarum.*

Bacterial isolates used for challenge

The bacterial isolates used in this experiment consisted of four South African NAD-dependent field isolates of *H. paragallinarum* that have been serotyped by the author (Bragg *et al.* 1996). The isolates were referred to as 49 (A-1), 58 (B-1), 7 (C-2) and 46 (C-3) (the number indicating the isolate number while the serovar of each isolate is presented in the brackets). The bacteria were grown in grown in TMB medium (Reid & Blackall 1984).

Challenge methods

When the birds were 25 weeks of age, a total of five smaller groups each comprising ten unvaccinated chickens were removed and placed into layer cages in disinfected isolation facilities where they were challenged. Chickens from each group were housed in separate rows of five cages which had no connecting water supply, feeders, or any other direct means of contact between the groups.

The ten chickens in each group of the five smaller groups were placed, two birds to a cage, into five adjacent layer cages in a row of cages. An adjoining water supply passed through each of the five

cages from a header tank which was common to all five cages of the group. Each cage was supplied with one nipple drinker. A communal feed trough passed in front of the five cages of the group. Therefore, each of the five cages per group was connected through a communal drinking water system and feeding trough.

One chicken in the middle cage of each of the rows of five cages was marked and challenged by intrasinus injection of 0.1 mℓ of an 18-hour-old culture, grown in TMB (Reid & Blackall 1984) of one of the four different South African serovars of *H. paragallinarum*. This was repeated until all four of the South African serovars were used to infect a group of chickens. The remaining group of chickens remained as an unchallenged control in order to determine if there was any cross contamination between the rows of cages.

Scoring of clinical signs

The clinical signs of infectious coryza were rated according to the criteria described by Matsumoto & Yamamoto (1971) with the modification that numerical scores were given to each of the categories. A score of 0 was given to a bird with no clinical signs, 1 for a bird showing mild clinical signs, 2 for moderate clinical signs and 3 for severe clinical signs. It was considered that an increased disease score should be applied to the birds infected via a natural route of infection and that it should carry a score of double that obtained via direct intersinus injection. This scoring system was used in this experiment.

The chickens were kept for 20 days after challenge. Clinical signs were evaluated and scored on a daily basis in the population of chickens. The total disease score for each group for each day was calculated and the mean daily disease score was calculated for each day by dividing the daily disease score by the total number of chickens.

The mean daily disease score was plotted graphically to present the disease profile for the flock. The mean of the daily disease scores can also be calculated, resulting in a numerical evaluation of the disease profile. The disease profiles of the different isolates were plotted graphically for the purpose of comparison. The numerical disease profiles are also compared with each other.

RESULTS

When unvaccinated chickens are challenged with a particular isolate of *H. paragallinarum* and the dis-

TABLE 1 Mean daily disease scores obtained when unvaccinated chickens (n = 10) were challenged with different serovars (A-1, B-1, C-2 and C-3) of NAD-dependent of *H. paragallinarum*

Days post challenge	Serovars used to challenge chickens				
	A-1	B-1	C-2	C-3	Control
1	0	0	0.4	0.3	0
2	0.2	0.4	0.6	0.1	0
3	0.2	0	0.6	1.5	0
4	0.2	0	1.4	2.0	0
5	0	0	1.2	4.3	0
6	0	0.2	2.6	2.8	0
7	0	0	2.2	4.8	0
8	0	0	3.4	6.2	0
9	0	0	2.6	5.3	0
10	0	0	4.2	4.0	0
11	0	0	3.0	4.0	0
12	0	0.2	3.0	4.2	0
13	0	0.2	2.6	2.4	0
14	0	0.2	4.8	3.2	0
15	0.8	0.2	2.8	0	0
16	1.2	0	0	0.4	0
17	0.6	0	0	0	0
18	0	0	0	2.2	0
19	0	0	0.4	1.2	0
20	0	0	0.2	0.2	0
Mean	0.16	0.07	1.67	2.25	0

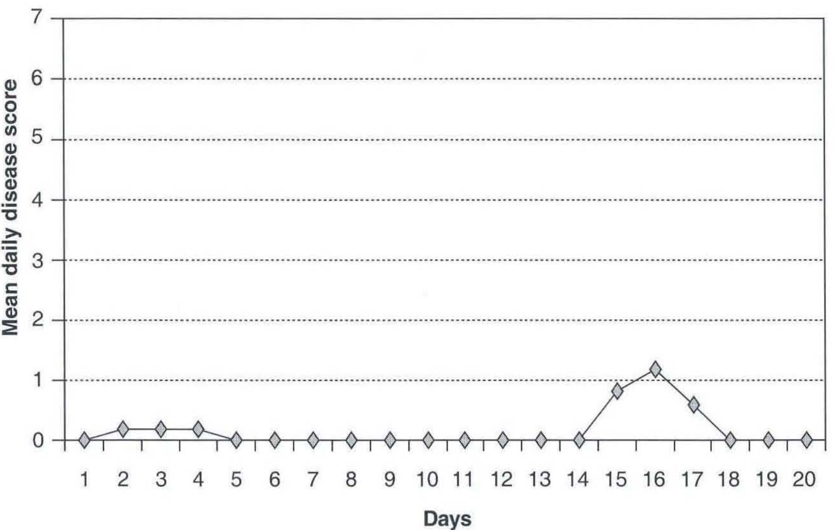

FIG. 1A Graphic representation of the disease profiles obtained when unvaccinated chickens were challenged with serovars A-1 of *Haemophilus paragallinarum*

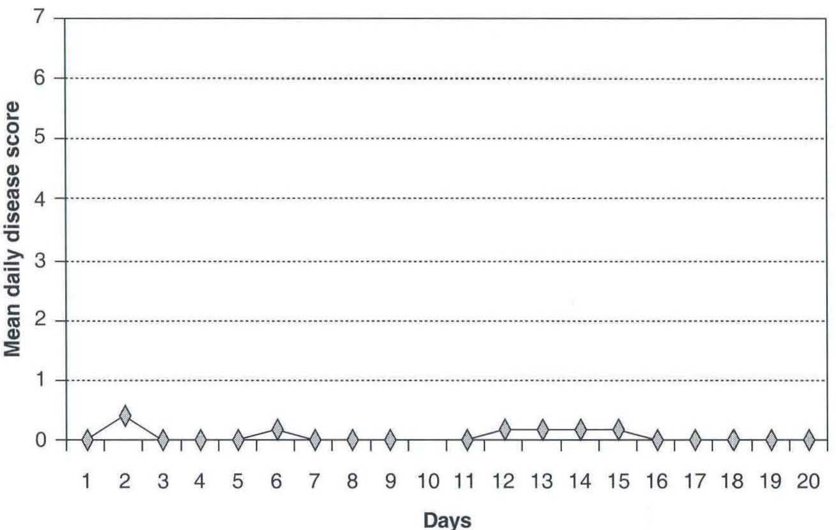

FIG. 1B Graphic representation of the disease profiles obtained when unvaccinated chickens were challenged with serovars B-1 of *Haemophilus paragallinarum*

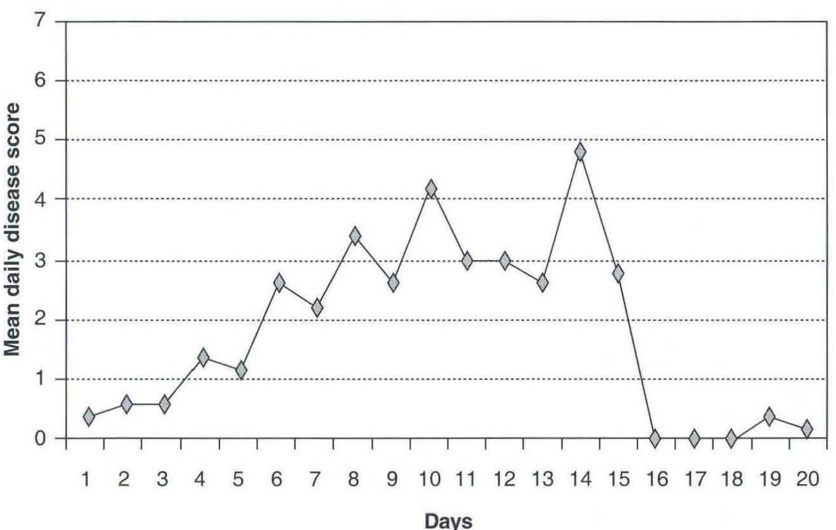

FIG. 1C Graphic representation of the disease profiles obtained when unvaccinated chickens were challenged with serovars C-2 of *Haemophilus paragallinarum*

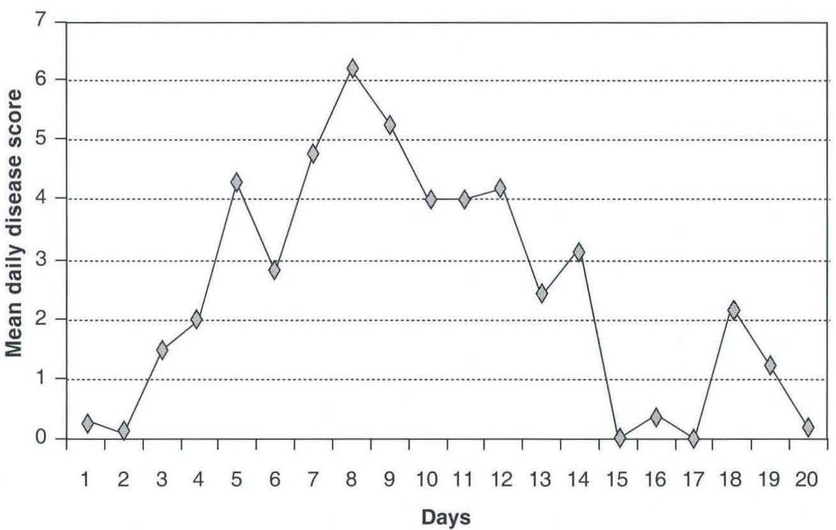

FIG. 1D Graphic representation of the disease profiles obtained when unvaccinated chickens were challenged with serovars C-3 of *Haemophilus paragallinarum*

ease profiles calculated, some indication of the invasiveness and virulence of the isolates can be obtained. The mean daily disease scores obtained in this experiment in which unvaccinated chickens were challenged with four different serovars of NAD-dependent *H. paragallinarum* are presented in Table 1. Graphic comparisons of the disease profiles of the four different NAD-dependent isolates are depicted in Fig. 1A–D. None of the challenged birds in this experiment died.

No clinical signs were recorded in the unchallenged control group, thus indicating that there was not cross contamination between the different groups of challenged birds.

The mean disease scores for the four serovars of NAD-dependent *H. paragallinarum* were calculated, thus presenting a numerical comparison of the disease profiles induced by them (Table 1).

DISCUSSION

Using the scoring system presented here, a clear indication of the ability of the four isolates to spread and induce disease in the unvaccinated chickens can be deduced from the results given in Table 1 and Fig. 1A–D. From this data, it would appear that the most virulent of the four South African NAD-dependent serovars of *H. paragallinarum* is the serovar C-3 isolate. This isolate had a mean disease profile number of 2.25 (Table 1 and Fig. 1D) as compared to the lowest mean disease profile number of 0.07 for the serovar B-1 isolate tested (Table 1 and Fig. 1B). An unexpected result was the low mean disease profile number (0.16) obtained for the serovar A-1 isolate (Table 1 and Fig.1A). From this data it would appear that this serogroup A isolate is not very virulent.

Bragg *et al.* (1996) found that there have been changes in the prevalence of the different serovars of *H. paragallinarum* in South Africa over a 30-year period. From the data presented here, it is evident that the serogroup C isolates, and serovar C-3 in particular, are more virulent then the serogroup A and B isolates. Bragg *et al.* (1996) suggested that the changing prevalence observed in the South African population of NAD-dependent *H. paragallinarum* isolates was due to the use of vaccines not containing serovar C-3 isolates. However, from this study, it can be seen that the serovar C-3 isolates are more virulent then the other serovars (serovar A-1, B-1 and C-2) found in this country. The finding of the increased virulence of this serovar could be an explanation for the increased prevalence of this serovar in South Africa as observed by Bragg *et al.* (1996). In order to fully understand the situation with regards to IC in South Africa, it is essential that this challenge model be used to investigate the protective capabilities of the currently registered vaccines in South Africa in the face of a challenge by the highly virulent serovar C-3 isolates. The results of these experiments will be presented separately.

CONCLUSIONS

From the results obtained it is considered that this challenge model system is suitable for evaluating the virulence and invasiveness of different isolates of *H. paragallinarum*. This work demonstrates that there are substantial virulence differences between the different serogroups of *H. paragallinarum* found in South Africa, with the serovar C-3 isolates being the most virulent. This high level of virulence of the serovar C-3 isolates could be an explanation for the increased incidence of IC cases caused by serovar C-3 isolates (Bragg *et al.* 1996).

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