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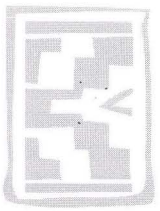

Virulence of South African isolates of *Haemophilus paragallinarum*. Part 3: experimentally produced NAD-independent isolate

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ABSTRACT

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A NAD-dependent isolate 46 (C-3) of *Haemophilus paragallinarum*, which was previously demonstrated to be of high virulence, was transformed to NAD independence using a plasmid isolated from a naturally occurring NAD-independent isolate of *H. paragallinarum*. The transformation was performed by two different methods and the identity of all of the isolates, before and after transformation was confirmed using a *H. paragallinarum*-specific PCR test. The transformed NAD-independent serovar C-3 isolate and the wild-type serovar C-3 isolate were used to experimentally infect vaccinated layer chickens. It was shown that the transformation to NAD independence significantly altered the virulence of the serovar C-3 isolate that was used in the transformation experiment. The mechanisms responsible for a decrease in virulence are not clear, but may be related to the pathology of the transformed isolate in the sinus of the chickens.

Keywords: *Haemophilus paragallinarum*, infectious coryza, NAD dependent, NAD independent, transformation

INTRODUCTION

Infectious coryza, caused by the bacterium *Haemophilus paragallinarum*, remains a problem in the layer industries in many countries of the world, despite the widespread use of vaccines. Normally, *H. paragallinarum* requires nicotinamide adenine dinucleotide (NAD) for growth (Blackall & Reid 1982) but in the 1990s, the occurrence of NAD-independent variants of *H. paragallinarum* was reported in South Africa (Horner, Bishop & Haw 1992; Horner, Bishop, Jarvis & Coetzee 1995; Mouahid, Bisgaard, Morley, Mutters & Mannhem 1992; Bragg, Coetzee & Verschoor 1993; Bragg, Greyling & Verschoor 1997).

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Bragg, Coetzee & Verschoor (1996) investigated the serogroup distribution of *H. paragallinarum* in South Africa over a 30-year period from the early 1970s to the later 1990s. They found a significant change in the incidence of the different serovars from the isolates collected in the 1970s, before the widespread use of vaccines, to the isolates obtained in the 1990s, when infectious coryza was a serious problem in the layer industry despite the use of different vaccines. Bragg *et al.* (1996) found that the incidence of serogroup A isolates and serovar C-2 isolates had decreased, while the incidence of serovar C-3 had increased substantially. They postulated that these changes in the prevalence were due to the use of vaccines not containing serovar C-3. This hypothesis was substantiated when a

serious outbreak of infectious coryza occurred in Zimbabwe (Bragg 2002a) where serovar C-3 isolates were found to be the cause of the problem, in spite of extensive vaccination efforts using vaccines not containing serovar C-3.

Bragg (2002b) investigated the virulence of NAD-dependent isolates of *H. paragallinarum* from the four known serovars which occur in South Africa (Kume, Sawata, Nakase & Matsumoto 1983; Blackall, Eaves & Rogers 1990; Bragg 1995; Bragg *et al.* 1996). From this work, Bragg (2002b) found that the serogroup C isolates were of much higher virulence when compared with the serogroup A or serogroup B isolates. Bragg (2002c) also tested the virulence of NAD-independent isolates of *H. paragallinarum* which were made from poultry in South Africa. It was found that the virulence of the NAD-dependent and NAD-independent variants of serogroup A were similar. In sharp contrast to this, it was discovered that there was a marked difference in the virulence of the NAD-dependent serovar C-3 isolate (Bragg 2002b) and the naturally occurring NAD-independent serovar C-3 isolate (Bragg 2002c). No possible reasons for this difference in virulence could be found.

Bragg *et al.* (1993) and Bragg, Purdan, Coetzee & Verschoor (1995) demonstrated that the NAD-independence trait could be transferred from naturally occurring NAD-independent isolates to reference strains of NAD-dependent *H. paragallinarum*. It was postulated that the NAD-independent trait is carried on a transferable plasmid (Bragg *et al.* 1993, 1995). Windsor, Gromkova & Koornhof (1991) demonstrated plasmid-borne NAD independence in *H. parainfluenza*, in naturally occurring NAD-independent variants of this bacterium (Gromkova & Koornhof 1990).

In order to explain the apparent lack of virulence in the NAD-independent serovar C-3 isolates, when compared to the naturally occurring NAD-dependent isolates, it was decided to transform a wild-type serovar C-3 isolate into a NAD-independent isolate and determine the effects of this transformation on the virulence of the isolate.

METHODS AND MATERIALS

Bacterial isolates

H. paragallinarum strains 46 C-3 (NAD dependent) and 1742 (NAD independent) which had been isolated previously from chickens in South Africa (Bragg *et al.* 1996; Bragg *et al.* 1997) were propagated and maintained on TM/SN medium (Reid & Blackall 1984). A broth version of this medium (TMB) was also used for propagation of the isolates (Reid & Blackall 1984).

The identity of the isolates as *H. paragallinarum* was previously confirmed using biochemical tests (Bragg *et al.* 1996; Bragg *et al.* 1997) and was confirmed as *H. paragallinarum* for this experiment using PCR according to the methods described by Chen, Miflin, Zhang & Blackall (1996) without any modifications.

Isolation of plasmid DNA from *H. paragallinarum*

Plasmid DNA was extracted from the NAD-independent strain (1742), using a modification of the alkaline lysis method. A 50 mℓ culture of *H. paragallinarum* was centrifuged at 3 000 x *g* for 10 min to recover the cells. The cells were resuspended in 200 µℓ GTE [50mM glucose, 25 mM Tris-CL (pH 8) and 1 mM EDTA] and left to stand at room temperature for 5 min. The cells were then lysed by the addition of freshly prepared 200 µℓ NaOH/SDS [0.2 M NaOH and 1 % (w/v) sodium dedocyl sulfate (SDS)] and the tube containing the lysate was placed on ice for 5 min. This was followed by the addition of 200 µℓ potassium acetate solution and the tube was again placed on ice for 5 min. The solution was centrifuged at 10 000 x *g* for 15 min and the supernatant transferred to a new tube. Thereafter 600 µℓ Tris buffered phenol (pH 8.2) was added and the solution vortexed for 1 min, after which it was centrifuged for 10 min at maximum speed to extract the DNA.

The upper phase was transferred to a new tube, 600 µℓ chloroform-isoamyl alcohol was added and the solution mixed and then centrifuged for 5 min to separate the phases. The upper phase was transferred to a new tube and DNA was precipitated by addition of 1 mℓ ice-cold absolute ethanol and incubated at –70 °C for 20 min. The DNA was pelleted by centrifugation at 10000 x *g* for 10 min and rinsed with 600 µℓ 75% (w/v) ethanol. The pellet was then dried under vacuum in a rotary evaporator (Savant, USA), resuspended in 40 µℓ TE buffer containing RNase A (pH 8) and incubated at 37 °C for 30 min. The DNA was then analyzed by gel electrophoresis on a 1 % (w/v) agarose gel in EDTA (pH 8.3) containing ethidium bromide [0.5 µℓ/mℓ in a TAE buffer (40 Mm Tris acetate, 2 mM EDTA pH 8.3)] (Sambrook, Fritsch & Maniatis 1989).

Samples of the plasmid extracts were plated out onto BTA plates to ensure that no viable NAD-independent *H. paragallinarum* isolates had survived during the plasmid extraction process.

Preparation of transformation competent *H. paragallinarum* cells

H. paragallinarum strain 46 C-3 was used as the recipient of the plasmid in the crude extract. The isolate was inoculated into 10 mℓ TMB (with NAD and chicken serum) and incubated overnight at 37 °C. A sample was removed after incubation and plated onto BTA plates to check for the purity of the culture. To establish a competent culture, the technique of Gromkova & Goodgal (1979) was followed. The technique involved inoculating 1 mℓ of the overnight culture of the recipient strain into 5 mℓ TMB in a sterile petri dish. The samples were incubated at 37 °C for 18 h. After incubation a sample was removed and plated out onto a BTA plate (with *Staphylococcus aureus*) to determine the purity of the culture (Bragg *et al.* 1993).

Transformation of NAD-dependent *H. paragallinarum*

Each 2 mℓ sample of the competent 46-C3 strain was mixed with 1 mℓ of the crude plasmid extract and incubated at 37 °C for 1 h according to the methods of Bragg *et al.* (1993). After incubation, 10 mℓ TMB was added to the mixture of crude plasmid and competent cells. The culture was then incubated at 37 °C for 6 h, after which it was plated out on BTA plates with *S. aureus*. The plates were then incubated in a candle jar (CO_2 rich environment) and any colonies which were found to grow without NAD were confirmed as transformed NAD-independent *H. paragallinarum* using PCR.

Preparation of electrocompetent *H. paragallinarum* cells

Bacterial cells competent for transformation by an electroporation procedure were prepared using a modification of the method described by Sambrook *et al.* (1989). Cultures of *H. paragallinarum* were prepared by inoculating strain 46 C-3 into five test tubes, each containing 5 mℓ TMB. The tubes were incubated at 37 °C overnight, after which the cultures were chilled in an ice-waterbath for 15 min. The cultures were then transferred to a pre-chilled 30 mℓ centrifuge bottle and the cells harvested by centrifugation at 5000 x *g* for 20 min at 4 °C (Beckman Model J2-MC, USA) The pellet was re-suspended in 30 mℓ ice-cold water, mixed and centrifuged (5000 x *g* for 20 min at 4 °C). This process was repeated once. Having poured off the supernatant, the pellet was re-suspended in the remaining liquid and the suspension transferred into a pre-chilled narrow-bottom 50 mℓ polypropylene tube. The cell pellet was obtained by centrifugation (5 000 x *g* for 10 min at 4 °C). This was followed by addition of 150 µℓ of ice-cold 10 % (w/v) glycerol. Finally aliquots of 50 µℓ of the cells in glycerol were dispensed into pre-chilled micro-centrifuge tubes and kept on ice (Sambrook *et al.* 1989).

Transformation by electroporation

Transformation by eletroporation was carried out on competent 46 C-3 *H. paragallinarum* cells using a modification of the transformation method described by Sambrook *et al.* (1989). The electro-competent cells were kept on ice where 5 µℓ plasmid DNA was mixed with 50 µℓ of the electro-competent *H. paragallinarum* (46 C-3). The mixture was transferred to a pre-chilled electroporation cuvette and a single pulse applied with the apparatus set to 2.5 kv, 25 µF and 200 ohms (Bio Rad model 1662089 pulse controller and Bio Rad model 1652077 gene pulser, Bio-Rad USA). The sample was removed immediately, and 1 mℓ SOC medium (0.5 % (w/v) yeast extract, 10 mM NaCl, 2 % (w/v) tryptone, 2.5 mM KCL, 10 mM $MgCL_2$, 20 mM glucose and 10 % (v/v) glycerol) was added. The cell suspension was transferred to a sterile Eppendorf tube. Aliquots of about 200 µℓ were streaked on BTA as well as on TM plates. The plates were incubated overnight at 37 °C, in a candle jar. Following incubation, single dew-drop *H. paragallinarum*-like colonies were picked out and streaked on BTA plates to ascertain their purity. PCR was then performed directly on the pure colonies to confirm their identification as *H. paragallinarum* (Sambrook *et al.* 1989).

Evaluation of virulence of the wild type (46 C-3) and the transformed strains

Once the virulent wild type NAD-dependent serovar C-3 isolate was converted to an NAD-independent variant, the virulence of both the wild-type isolate and the newly transformed isolate was tested.

The challenge work was performed according to the method of Bragg (2002b). A total of 63 vaccinated layers, at 25 weeks of age and in egg production was used. The layers were vaccinated on a commercial poultry operation with Onderstepoort

Biological Products Coryza vaccine at 12 weeks with revaccination at 16 weeks.

In the first battery of cages, three rows were used in this experiment. Each row consisted of five cages, with a communal water supply which consisted of a pipe from a header tank that passed through each cage, with a nipple drinker in each cage. The cages in each row also sheared a communal feeding trough which passed in front of the cages. There was no direct connection between the different rows of cages used in this experiment. Each cage in each row of cages contained two birds, therefore each row, except the middle row of cages which contained a total of 12 birds, contained ten birds per row. The number of birds per cage was acceptable according to the design of the cage and this experiment had been approved by the ethics committee.

The experiment was repeated in a second battery of chickens which was identical to the first battery. In this experiment, the middle row of cages contained 11 birds.

One chicken in the middle cage of the upper row was inoculated, via the intrasinus route with a 0.1 mℓ suspension of an 18-h-old culture of the wild-type isolate which were grown on TM/SN plates and suspended in 0.85 % saline. The suspension was adjusted to an optical density of 0.25 units in a spectrophotometer. Another chicken in the bottom row of cages was inoculated with a 0.1 mℓ suspension of an 18-h-old culture of the transformed isolate, which was treated in the same manner as the wild-type isolate. The remaining birds in these two groups were infected through natural routs (Bragg 2002b). The middle row of cages was left as a control group in which the chickens were not inoculated. The experiment was repeated in the second battery of cages.

Clinical signs of infectious coryza were evaluated on a daily basis in the population of chickens according to the methods and clinical signs described by Bragg (2001b) without any modifications.

RESULTS

The two wild-type strains (46 C-3 for the NAD-dependent recipient and 1742 and the NAD-independent plasmid donor) used in this experiment were both shown to be *H. paragallinarum* by the PCR test. Successful amplification of the DNA resulted in a single band of about 500 base pairs for the isolates 1742 and 46 C-3 as is expected for *H. paragallinarum* (data not shown). In the light of the fact that these isolates were previously biochemically identified as *H. paragallinarum* and the fact that the PCR test has been shown to be specific for *H. paragallinarum*, both NAD dependent and NAD independent (Chen *et al.* 1996), it can be concluded that both of these isolates were *H. paragallinarum*.

Plasmid DNA was isolated from the NAD-independent isolate 1742 using the alkaline lysis method out of all 17 preparations (see Fig. 1).

When the purified plasmid samples were tested for viable bacterial growth before the transformation experiment, no viable bacteria could be isolated. It can thus be concluded that no viable, NAD-independent *H. paragallinarum* isolates remained in the plasmid samples.

The NAD-dependent isolate 46 C-3 was transformed according to the method of Bragg *et al.* (1993) or through electroporation, using the plasmid isolated from the NAD-independent 1742 strain.

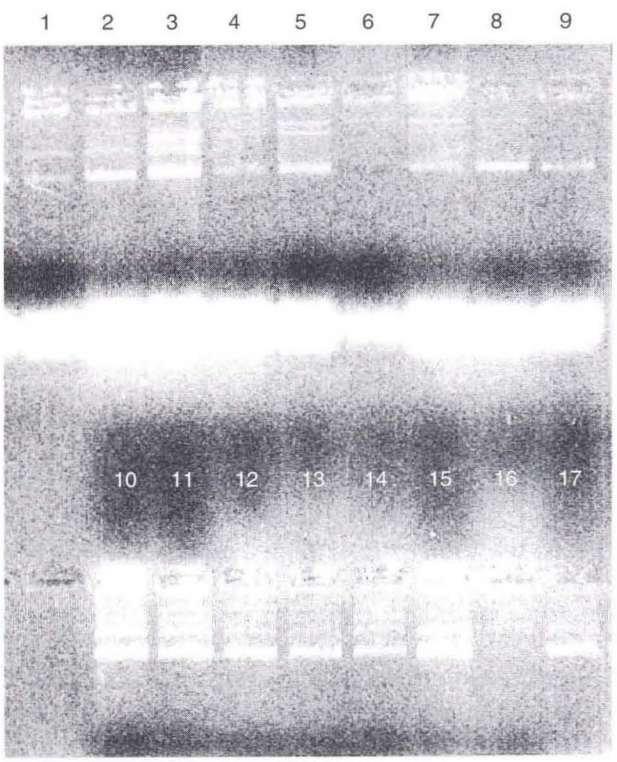

FIG. 1 Ethidium bromide stained 1 % (w/v) agarose gel of the plasmid isolated from *H. paragallinarum* strain 1742. All the lanes (1–17) were loaded with 5 µℓ plasmid extract from different tubes. The figure shows the linear, nicked and super-coiled forms of DNA in each well

Colonies that were found to grow in the absence of NAD on the plates were considered to be NAD-independent transformed strains of 46 C-3. No viable NAD-independent bacterial cells were found in the plasmid preparation or in the plasmid recipient mixture. The viable NAD-dependent competent isolates used in the transformation experiment are not capable of growing with NAD. Therefore only transformed isolates could grow on the plates without NAD. In order to rule out the possibilities that the isolates growing without NAD were contaminants, these isolates were subjected to PCR tests in order to confirm that they were in fact NAD-independent transformed *H. paragallinarum*. Isolates transformed by both methods resulted in a band of 500 bp, thus confirming their identity as transformed *H. paragallinarum*.

The challenge experiment was carried out in duplicate, and both the wild type and the strain trans-

TABLE 1 Mean daily disease scored for vaccinated layers challenged with the wild type NAD-dependent serovar C-3 isolate of *H. paragallinarum*, or the NAD-independent transformed strain of serovar C-3 for two different challenge experiments

Days post challenge	Experiment 1		Experiment 2	
	46 C-3 WT#	46 C-3 Trans*	46 C-3 WT#	46 C-3 Trans*
1	0	0	0.07	0
2	0	0	0.07	0
3	0	0	0.07	0
4	0	0	0.07	0
5	0.32	0	0.86	0.06
6	1.44	0.44	1.80	0.06
7	1.50	0.44	3.00	0.25
8	1.13	0.50	1.90	0.25
9	1.18	0.69	1.30	0.13
10	1.50	0.38	1.46	0
11	1.25	0.38	1.66	0
12	0.88	0.50	1.00	0
13	0.75	0.50	1.20	0
14	0	0.25	0.53	0
15	0	0	0.26	0
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0

\# Wild type NAD dependent serovar C-3 isolate of *H. paragallinarum*
* NAD-independent transformed variant of C-3 produced through transformation of isolate 46 C-3 of *H. paragallinarum*

TABLE 2 Total disease score, Mean disease score number and highest daily disease score obtained when vaccinated chickens (n = 10) were challenged with either the wild type serovar C-3 isolate, or with the NAD-independent transformed strain of *H. paragallinarum*

	Experiment 1		Experiment 2	
	46 C-3 WT#	46 C-3 Trans*	46 C-3 WT#	46 C-3 Trans*
Total disease score	9.92	4.06	15.23	0.75
Mean disease score	0.49	0.20	0.76	0.04
Highest daily disease score	1.4	0.68	1.8	0.125

\# Wild type NAD-dependent isolate 46 C-3
* Transformed NAD-independent variant of isolate 46 C-3

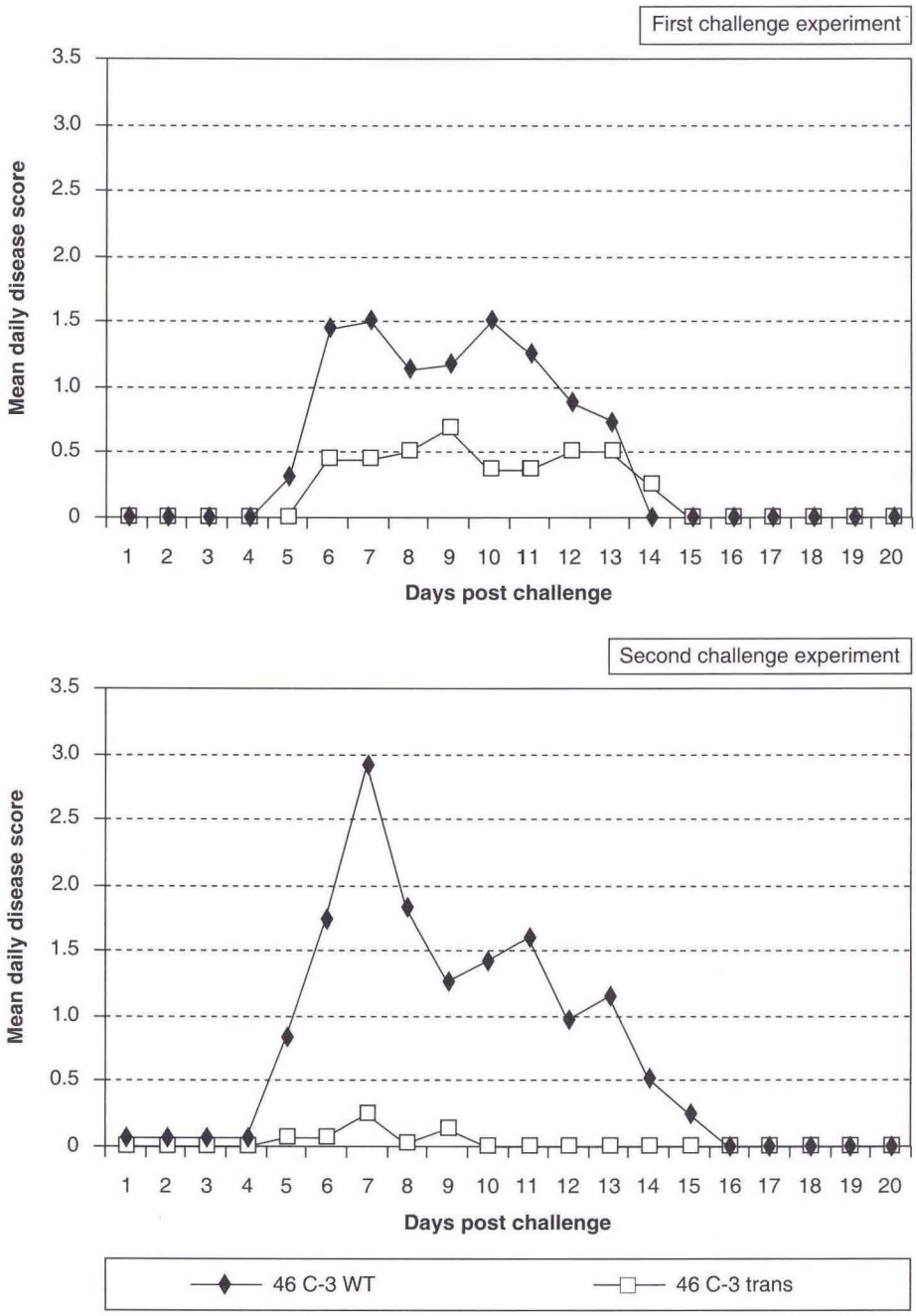

FIG. 2 Mean daily disease scores obtained when vaccinated chickens (n = 10) were challenged with the wild type isolate 46 C-3 of *H. paragallinarum*, or the transformed variant produced from this isolate through the insertion of the plasmid encoding NAD independence through electroporation

formed by electroporation were used. The daily disease scores of the observedclinical signs of infectious coryza, for the different isolates are shown in Table 1. A graphic representation of the data presented in Table 1 is given in Fig. 2.

It can be deduced from the graphs in Fig. 2, and from the data presented in Table 1, that the clinical signs in the chickens inoculated with transformed isolates were less severe than those inoculated with the wild-type isolates. The total disease score, mean disease score and maximum daily disease score obtained when chickens were challenged with the different bacterial isolates are given in Table 2. No clinical signs of infectious coryza were observed in the un-inoculated control chickens.

DISCUSSION AND CONCLUSIONS

It was demonstrated that both the NAD-recipient strain (46 C-3) and the plasmid donor were *H. paragallinarum* by performing a PCR test which had previously been demonstrated to be specific for *H. paragallinarum* (Chen *et al.* 1996; Miflin, Chen, Bragg, Welgemoed, Greyling, Horner & Blackall 1999). It was also demonstrated that there were no viable NAD-independent isolates in the plasmid extracts which were used in either transformation experiment. A screening technique was used to ensure that only the transformed organisms were obtained from the transformation experiment. Immediately after transformation, the cells were transferred to a selective growth medium, which permits growth of only the cells that have taken up the functional gene present on the plasmid. In this experiment, positive transformants were selected on their ability to grow in the absence of NAD in the media.

In both transformation methods, putative transformed colonies were obtained on both TM and BTA plates in the absence of NAD. This indicates that these colonies did loose their dependence for NAD. Confirmation that the cells were indeed *H. paragallinarum* was obtained with the resulting 500 bp PCR product. However, the isolates transformed according to the method of Bragg *et al.* (1993) could not grow directly on TM or BTA plates without NAD immediately after transformation. They had to be grown initially on media containing NAD for two generations before they were viable on media in the absence of NAD. As expected, much higher transformation efficiency was obtained through electroporation (data not shown) and in addition these cells were also viable if plated directly on media in the absence of NAD. These isolates were therefore used in subsequent challenge studies.

After transforming the wild type strain with a plasmid that allows the cells to grow in the absence of NAD, both the wild type strain and the transformed strain were used to challenge commercial poultry that had been vaccinated against infectious coryza. From the challenge experiments it was observed that the wild type strain caused infectious coryza symptoms in the bird inoculated via intrasinus injection, with a maximum disease score of 3 detected in the second challenge experiment (Fig. 2). In addition, infectious coryza was observed in the in contact birds (i.e. the birds within the same row of cages which were not directly inoculated with the bacterium) in as little as 5–6 days after the challenge experiments were started with the highest score being recorded after 7 days. This finding of the development of clinical signs in vaccinated chickens confirmed the hypothesis that current vaccination practices in commercial poultry are inadequate (Bragg *et al.* 1996; Bragg 2002b).

From the data presented in Table 1 and Fig. 2, it can be deduced that the clinical signs observed in the birds challenged with the transformed strain were less sever than those in the birds challenged with the wild type isolates. These findings correspond to those of Bragg (2002c) who demonstrated a lower virulence of the NAD-independent serovar C-3 isolates in unvaccinated chickens when compared to the severity of the clinical signs induced by the NAD-dependent forms of this serovar (Bragg 2002b). As for the birds challenged with the transformed strain, a maximum disease score of 0.70 was reached in the first challenge experiment, while a maximum disease score of only 0.25 was reached in the second challenge experiment (Fig. 2).

This is the first report of the use of electroporation for the transforming of NAD-dependent *H. paragallinarum* to NAD independence. High transformation efficacy was obtained when electroporation was used as compared to the natural transformation methods described by Bragg *et al.* (1993, 1995).

It has also been demonstrated in this work that the transformation of naturally occurring NAD-dependent isolates to NAD independence affects the virulence of the isolate. The mechanisms by which the virulence is affected are not clear at this stage and needs further investigation.

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