Isolation of serovar C-3 *Haemophilus paragallinarum* from Zimbabwe: A further indication of the need for the production of vaccines against infectious coryza containing local isolates of *H. paragallinarum*

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ABSTRACT

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Various isolates of *Haemophilus paragallinarum*, collected from a severe outbreak of infectious coryza in poultry from Zimbabwe, were serotyped and were found to belong to serovar C-3. Previously, isolates were serotyped using polyclonal antiserum produced against serogroup reference strains (0083 for serogroup A, 0222 for serogroup B and Modesto, or H-18 for serogroup C) of *H. paragallinarum*. In this case, polyclonal antiserum produced against these reference isolates were used, as well as polyclonal antiserum that has been raised specifically against the serovar C-3 isolate 46 C-3. When using the latter serum at a 1 in 50 dilution, no cross-reaction with other members of serogroup C were found.

The severity of the disease outbreak in Zimbabwe, the vaccination history of the infected flocks on the sites and the isolation of the uniquely southern African serovar C-3, further highlights the need for vaccines composed of local isolates to control infectious coryza in regions where vaccination failures occur.

Keywords: *Haemophilus paragallinarum*, infectious coryza, vaccines, Zimbabwe

INTRODUCTION

Infectious coryza (IC) of poultry, caused by *Haemophilus paragallinarum*, remains a serious problem in many parts of the world, despite the widespread use of vaccines against the disease (Bragg, Coetzee & Verschoor 1996).

Bragg *et al.* (1996) established that a change in the prevalence of the serovars of *H. paragallinarum* had occurred in South Africa over a 30-year period. They speculated that this change in serovar distribution could be correlated to the use of vaccines against IC in South Africa that did not contain the serovar C-3 strain and hence the significant increase in the prevalence of serovar C-3 over the period of their study. These findings prompted them to suggest that there is a need for vaccines against IC which contain local strains of *H. paragallinarum*.

The author has investigated a number of isolates of *H. paragallinarum* from countries in South America where vaccination failures have been reported (R.R. Bragg, unpublished data 2001). It has been found that a number of serovars which appear to

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differ from the nine known serovars (Blackall, Eavers & Rogers 1990a) have been found in South America (R.R. Bragg, unpublished data 2001). These findings further indicate that there is a need for vaccines containing local isolates in South America.

The current techniques used for the serological classification of *H. paragallinarum* are based on the haemagglutination (HA) and haemagglutination inhibition (HI) tests derived by Kume, Sawata, Nakase & Matsumoto (1983) and modified by Blackall *et al.* (1990a) and Blackall, Eaves & Aus (1990b). It has previously been reported (Sawata, Kume & Nakase 1980) that serogroup C isolates do not spontaneously agglutinate with glutaralderhyde fixed red blood cells (GA fixed RBC), but require additional treatment, such as hyaluronidase treatment (Yamaguchi, Iritani & Hayashi 1989) or according to the methods of Blackall *et al.* (1990b), who suggested that the bacteria can be treated by washing with phosphate buffered saline (PBS) followed by storage of the washed bacterial sample at 4 °C for 3 days. Bragg, Purdan, Coetzee & Verschoor (1995) and Bragg *et al.* (1996) have successfully used the methods of Blackall *et al.* (1990b) for the serotyping of *H. paragallinarum*.

In this study, isolates of *H. paragallinarum* were obtained from Zimbabwe from a serious disease outbreak of infectious coryza on an integrated poultry facility. These isolates were serotyped, using antiserum raised against the reference strains of *H. paragallinarum*. In addition, antiserum produced against serovar C-3 was also used for the serotyping of these isolates.

MATERIALS AND METHODS

Bacterial isolates

Reference isolates of *H. paragallinarum* (strains 0083, 0222 and Modesto) were obtained from the Department of Poultry Health, Faculty of Veterinary Science, University of Pretoria, Onderstepoort. Strain 46 C-3 is a South African isolate of nicotinamide adenine dinucleotide (NAD) dependent serogroup C isolate which has been serotyped previously as serovar C-3 (Bragg *et al.* 1996). These isolates were obtained in a freeze-dried state and after reconstituting the bacteria and growing them on a defined medium for *H. paragallinarum,* developed by Reid & Blackall (1987) who called the medium TN medium, they were stored in MicroBank (Davies Diagnostics) and were freeze-dried using standard methods.

Samples of *H. paragallinarum* isolated from cases of infectious coryza in the field in Zimbabwe were obtained on two different occasions from the same integrated production site. The first set of samples was sent from Zimbabwe via courier. These isolates were transported on blood tryptose agar (BTA) plates and were passaged onto fresh BTA plates once received in the laboratory. They were labeled as follows: B3, B6, D1/2, UI and 1629 and were stored in MicroBank (Davies Diagnostics) and by freeze-drying, using standard methods.

Another set of three isolates, on Amies transport swabs, was collected directly from the laboratory in Zimbabwe and was flown to Bloemfontein. Upon arrival at the laboratory, these isolates were passaged onto BTA plates and were stored in MicroBank and by freeze-drying. They were labelled BTY 2, BTY 10 and BTY 14 respectively.

Confirmation of the identity of bacterial isolates

The identity of the bacterial isolates as *H. paragallinarum* was confirmed by observing satellite growth on BTA plates (De Blieck 1932) and by performing of the *H. paragallinarum* specific PCR test, termed HPG-2 PCR, of Chen, Miflin, Zhang & Blackall (1996) and Miflin, Chen, Bragg, Welgemoed, Greyling, Horner & Blackall (1999) without any modifications.

Preparation of serogroup specific antisera

Serogroup specific antisera were prepared in New Zealand white rabbits according to the methods used by Bragg (1995) without modifications. In short, the rabbits were injected with a bacterial suspension that had been incubated for 24 h at 4 °C in a 0.5 % formal saline to inactivate the bacteria. The bacterial suspension was adjusted to 10 international opacity units and was then suspended in 5 mg/mℓ Quill-A adjuvant (supplied by the South African Institute of Medical Research). The rabbits were re-inoculated after a 4-week interval with an identical suspension of bacteria plus adjuvant. A week later, they were bled to determine antibody titre levels in their serum. The rabbits were inoculated after another 2-week period with 1 mℓ bacterial suspension without adjuvant. Finally they were exsanguinated after another week. The serum of each animal was collected and stored at –70 °C. Antisera were prepared against strains 0083 (serogroup A), 0222 (serogroup B), Modesto (serogroup C) as well as isolate 46 C-3, the latter having previously been shown to belong to serovar C-3.

The antisera so produced were tested for specificity against the reference strains of *H. paragallinarum* prior to their use in this experiment.

Serotyping of the isolates

Serotyping was performed using the unmodified haemagglutination (HA) and haemagglutination inhibition (HI) tests according to the methods establish by Kume *et al.* (1985) and modified by Blackall *et al.* (1990b).

Bacteria were inoculated into TN broth medium (Reid & Blackall 1987) and incubated for 24 h. The bacteria were washed with sterile PBS and reconstituted to a final optical density (OD) reading of 0.1 at 540 nm. Thereafter the bacterial suspensions were incubated at 4 °C for 4 days prior to performing the HA and HI tests.

Isolates which proved to be untypable after the first attempt, were re-inoculated into TN broth media and processed for a second time according to the methods described above.

RESULTS

All of the isolates obtained from Zimbabwe were found to be *H. paragallinarum* by virtue of the production of PCR product after amplification of approximately 500 base pairs (Miflin *et al.* 1999). These isolates were found to be NAD dependent, as could be seen by the occurrence of satellism on BTA plates (De Blieck 1932).

Three out of the five isolates received from Zimbabwe were found to be serovar C-3 by virtue of the fact that HI occurred when using antiserum against isolate 46 C-3, which belongs to serovar C-3. HI was not seen with antiserum against the reference strains of *H. paragallinarum*. The specificity of these serum samples had been confirmed before the commencement of this experiment using reference strains of *H. paragallinarum* (data not shown). Thus no cross reactions with any of the reference serum samples used were obtained with these Zimbabwe isolates; they only reacted with the serovar C-3 specific antibodies. The remaining two isolates of this batch could not be typed even after three attempts. These isolates did not agglutinate the red blood cells, thus it was impossible to perform HI tests on these isolates.

Of the other three isolates, which had been collected directly in Zimbabwe, all were found to be serovar C-3 and showed reactions identical to those described above.

The antiserum produced against serovar C-3 is specific for serovar C-3 and showed no cross reaction with any of the reference bacteria (0083, 0222 and Modesto). These bacterial isolates also did not show any reaction with the antiserum raised against 46 C-3, which has previously been identified at a serovar C-3 isolate. The Zimbabwean isolates were found to only react with the serovar C-3 antibodies, thus clearly indicating that these isolates are serogroup C serovar C-3 isolates.

DISCUSSION

Bragg *et al.* (1996) have demonstrated that there has been a significant alteration in the distribution of the different serovars of *H. paragallinarum* in South Africa over a period of 30 years and have shown that serovars A-1, B-1, C-2 and C-3 are found in South Africa. As a result of this alteration in serovar distribution, they have suggested that these changes could have been the result of the widespread use of vaccines not containing the serovar C-3 strain. This led them to suggest that there is a need for a vaccine containing South African serovars of *H. paragallinarum* in South Africa.

Before the investigation described here, the serovar C-3 isolate of *H. paragallinarum* had only been isolated in South Africa and this is the first report of this serovar from another country. However, bearing in mind the close proximity of Zimbabwe to South Africa, it is not surprising that serovar C-3 has been isolated from the former country.

When investigating the disease outbreak in Zimbabwe, it was determined that the birds had been vaccinated using commercially available vaccines not containing serovar C-3. The vaccine used did contain serogroup C isolates, and according to Blackall (1991, 1995) and Kume, Sawata & Nakase (1980a, b), there should be cross protection between the serogroup C isolates used in the vaccine and any serogroup C field isolates. However, from the disease situation, it was obvious that the vaccines used on the farm were not providing protection against challenge by serovar C-3. These findings substantiate the hypothesis of Bragg *et al.* (1996) that there is only limited cross protection between other serogroup C strains and serovar C-3, and that there is a need for a vaccine containing local isolates of the C-3 serovar of *H. paragallinarum.*

This is the first report on the production and testing of serovar C-3 specific antibodies that can be used to clearly serotype serovar C-3 isolates.

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