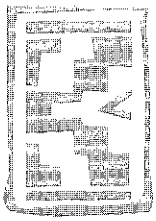

RESEARCH COMMUNICATION

Confirmation that PCR can be used to identify NAD-dependent and NAD-independent *Haemophilus paragallinarum* isolates

J.K. MIFLIN[1], X. CHEN[2], R.R. BRAGG[3], J.M. WELGEMOED[3], J.M. GREYLING[3] R.F. HORNER[4] and P.J. BLACKALL[1]

ABSTRACT

MIFLIN, J.K., CHEN, X., BRAGG, R.R., WELGEMOED, J.M., GREYLING, J.M., HORNER, R.F. & BLACKALL, P.J. 1999. Confirmation that PCR can be used to identify NAD-dependent and NAD-independent *Haemophilus paragallinarum* isolates. *Onderstepoort Journal of Veterinary Research*, 66:55–57

Seventy five bacteria tentatively identified as *Haemophilus paragallinarum* (the causative agent of infectious coryza), eight identified as *Ornithobacterium rhinotracheale* and 13 identified as NAD-independent *Pasteurella* species were isolated from chickens with respiratory infection in various provinces in South Africa. The isolates were characterized by conventional biochemical and serological methods. A polymerase chain reaction (PCR) assay specific for *H. paragallinarum* was used to identify the cultures directly from colonies. The PCR assay gave positive results for all isolates that were identified by conventional methods as *H. paragallinarum*, irrespective of whether they were nicotinamide adenine dinucleotide (NAD)-dependent (43 isolates) or NAD-independent (32 isolates). The eight isolates that were identified by conventional methods as *O. rhinotracheale* and the 13 isolates identified as various *Pasteurella* species gave negative results in the PCR assay. This study has demonstrated that colony PCR is a rapid method for uniquely identifying both NAD-dependent and NAD-independent strains of *H. paragallinarum* and distinguishing them from other bacteria, such as *O. rhinotracheale* and *Pasteurella* species.

Keywords: *Haemophilus paragallinarum*, polymerase chain reaction

Infectious coryza, caused by *Haemophilus paragallinarum*, remains a serious problem in South Africa. Bragg, Greyling & Verschoor (1997), highlighted the difficulties faced by diagnostic laboratories in South Africa as a result of a number of closely related organisms which can be isolated from the sinuses of chickens. Many of these isolates are regarded as non-pathogenic, thus there is a need for a quick diagnostic test which can unequiviable differentiate between *H. paragallinarum* (both NAD dependent and independent) and other closely related isolates.

The aim of the present study was to determine whether the PCR technique that has recently been developed for the identification of *H. paragallinarum* (Chen, Miflin, Zhang & Blackall 1996) could be used to simplify the differentiation of both NAD-dependent and NAD-independent *H. paragallinarum* from other respiratory tract pathogens of poultry, such as *Ornithobacterium rhinotracheale* and *Pasteurella* spp.

Ninety six bacterial isolates from commercial chicken flocks from Gauteng, Mpumalanga, KwaZulu Natal, North West and Free State provinces in South Africa were studied (Table 1). Isolates were grown on test medium agar supplemented with chicken serum and NAD (TM/SN) (Reid & Blackall 1987) in Australia and on blood tryptose agar (BTA) with a nurse culture of

[1] Animal Research Institute, Locked Bag 4, Moorooka, 4105 Australia

[2] Institute for Animal Husbandry and Veterinary Science, Beijing Municipal Academy of Agriculture, P.O. Box 2449-21, Beijing, 100081 People's Republic of China

[3] Department of Poultry Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag X4, Onderstepoort, 0110 South Africa

[4] Allerton Provincial Veterinary Laboratory, Private Bag X2, Cascades, 3202 South Africa

Accepted for publication 12 January 1999—Editor

S. aureus in South Africa. All solid media were incubated at 37°C under 5% CO_2. Conventional identification and serotyping was performed as previously described (Chen, Zhang, Blackall & Feng 1993; Bragg, Greyling & Verschoor 1997). The HPG-2 PCR based on a unique randomly cloned fragment of *H. paragallinarum* (Chen *et al.* 1996) was used without modification directly on colonies grown on the agars specified above.

All isolates were identified to the genus and species level by biochemical characterisation, regardless of growth factor requirement, by the criteria of Blackall & Reid (1982) for *H. paragallinarum*, Vandamme, Segers, Vancanneyt, Van Hove, Mutters, Hommez, Dewhirst, Paster, Hersters, Falsen, Devriese, Bisgaard, Hinz & Mannheim (1994) for *O. rhinotracheale* and Mutters, Piechulla, Hinz & Mannheim (1985) for the *Pasteurella* species. Of the 96 field isolates, 43 were classical (NAD-dependent) *H. paragallinarum*, 32 were NAD-independent *H. paragallinarum*, eight were *O. rhinotracheale* and 13 were *Pasteurella* species. The Page serovar of all *H. paragallinarum* isolates is given in Table 1.

In the HPG-2 PCR all 75 *H. paragallinarum* isolates gave a single amplified product of 516 base pairs (bp). The eight *O. rhinotracheale* strains, and the 13 *Pasteurella* species all gave negative results in the HPG-2 PCR.

A number of phenotypically similar bacteria can be isolated from chickens with respiratory tract infections. To date, three recognized species of NAD-dependent bacteria (*H. paragallinarum*, *Pasteurella avium* and *Pasteurella volantium*) and one unnamed taxon (*Pasteurella* species A) have been isolated from poultry in South Africa (Bragg, Coetzee & Verschoor 1996). Of these, only *H. paragallinarum* is regarded as being pathogenic.

Until the recognition of NAD-independent *H. paragallinarum*, the separation of *H. paragallinarum* from

TABLE 1 Details of isolates used in the study

Species	NAD requirement	Source	Serovar	Number
Tested in Australia				
H. paragallinarum	No	Broilers	A	3
H. paragallinarum	No	Layers	A	2
H. paragallinarum	No	Broiler Breeders	A	5
H. paragallinarum	No	Layers	C	1
H. paragallinarum	No	Broiler Breeders	C	3
H. paragallinarum	No	Unknown	C	1
H. paragallinarum	Yes	Layers	B	1
H. paragallinarum	Yes	Layers	C	13
H. paragallinarum	Yes	Layers	NT[a]	1
H. paragallinarum	Yes	Broiler Breeders	C	1
O. rhinotracheale	No	Broilers	NA[b]	4
O. rhinotracheale	No	Layers	NA	2
Tested in Australia and South Africa				
H. paragallinarum	No	Layers	A	1
H. paragallinarum	No	Layers	C	1
H. paragallinarum	Yes	Layers	B	1
H. paragallinarum	Yes	Layers	C	3
Tested in South Africa				
H. paragallinarum	No	Layers	A	2
H. paragallinarum	No	Layers	C	5
H. paragallinarum	No	Layers	NT	2
H. paragallinarum	No	Broilers	A	4
H. paragallinarum	No	Broilers	NT	2
H. paragallinarum	Yes	Layers	A	7
H. paragallinarum	Yes	Layers	B	3
H. paragallinarum	Yes	Layers	C	10
H. paragallinarum	Yes	Layers	NT	3
O. rhinotracheale	No	Broilers	NA	2
Pasteurella spp.	No	Layers	NA	13

[a] Non-typable
[b] Not applicable

other pathogenic bacteria found in the upper respiratory tract of poultry posed no major problem for diagnostic microbiologists—*H. paragallinarum* was growth-factor dependent whereas the others (such as *O. rhinotracheale)* were not. However, a number of studies have shown that NAD-independent strains of *H. paragallinarum* are now widespread in South African poultry (Horner, Bishop & Haw 1992; Bragg, Coetzee & Verschoor 1993; Horner, Bishop, Jarvis & Coetzer 1995). Respiratory disease diagnosis is further complicated as *O. rhinotracheale* does occur in commercial chicken flocks in South Africa (Travers 1996; Bragg *et al.* 1997).

O. rhinotracheale and the different *Pasteurella* spp. can be differentiated from *H. paragallinarum* using the standard biochemical tests recommended by Blackall, Matsumoto & Yamamoto (1997) for the differentiation of avian haemophili. However, carbohydrate fermentation tests are sometimes difficult to read and individual strain variation can make interpretation of results difficult unless a larger panel of substrates is used. The advantage of colony PCR is that results can be obtained in one day whereas biochemical tests take several days to complete.

The HPG-2 PCR assay used in this study has been shown previously to be specific for *H. paragallinarum* but it had not been validated on a diverse range of NAD-independent isolates. The 15 NAD-independent isolates previously tested by this PCR by Chen *et al.* (1996) all originated from KwaZulu Natal province and all were Page serovar A. In the present study, we examined a variety of NAD-independent strains from other provinces and included the newly recognized serovar C variants (Bragg *et al.* 1997).

This study has clearly demonstrated that the HPG-2 PCR test for *H. paragallinarum* is a reliable diagnostic tool that is particularly useful in South Africa, where NAD-independent *H. paragallinarum* and *O. rhinotracheale* occur. The test is capable of rapidly and accurately distinguishing between NAD-independent *H. paragallinarum* and *O. rhinotracheale.* The use of this PCR test in South Africa, where all three Page serovars of NAD-dependent and serovar A and C NAD-independent isolates of *H. paragallinarum* occur, clearly establishes the usefulness of this PCR test for field investigations of infectious coryza outbreaks. However, until a serovar specific PCR for *H. paragallinarum* has been developed, there will still be a need to isolate *H. paragallinarum* from field cases for serotyping of the isolates.

ACKNOWLEDGEMENTS

This work was partially funded by the Australian Centre for International Agricultural Research. Aspects of this work were also funded by the Foundation for Research Development in South Africa. The authors thank Dr M. Henton, Onderstepoort Veterinary Institute, South Africa, for supplying four of the isolates examined.

REFERENCES

BLACKALL, P.J. & REID, G.G. 1982. Further characterization of *Haemophilus paragallinarum* and *Haemophilus avium. Veterinary Microbiology*, 7:359–367.

BLACKALL, P.J., MATSUMOTO, M. & YAMAMOTO, R. 1997. Infectious coryza, in *Diseases of poultry* 10th ed., edited by B.W. Calneck, H.J. Barnes, C.W. Beard, L.R. McDougald & Y.M. Saif. Iowa: Iowa State University Press: 179–190.

BRAGG, R.R., COETZEE, L. & VERSCHOOR, J.A. 1993. Plasmid-encoded NAD independence in some South African isolates of *Haemophilus paragallinarum. Onderstepoort Journal of Veterinary Research*, 60:147–152.

BRAGG, R.R., COETZEE, L. & VERSCHOOR, J.A. 1996. Changes in the incidence of the different serovars of *Haemophilus paragallinarum* in South Africa: a possible explanation for vaccine failures. *Onderstepoort Journal of Veterinary Research*, 62:261–270.

BRAGG, R R., GREYLING, J.M. & J. A. VERSCHOOR, J.A. 1997. Isolation and identification of NAD-independent bacteria from chickens with symptoms of infectious coryza. *Avian Pathology*, 26:595–606.

CHEN, X., MIFLIN, J.K., ZHANG, P. & BLACKALL, P.J. 1996. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum. Avian Diseases*, 40:398–407.

CHEN, X., ZHANG, P., BLACKALL, P.J. & FENG, W. 1993. Characterization of *Haemophilus paragallinarum* isolates from China. *Avian Diseases*, 37:574–576.

HORNER, R.F., BISHOP, G.C. & HAW, C. 1992. An upper respiratory disease of commercial chickens resembling infectious coryza, but caused by a V factor-independent bacterium. *Avian Pathology*, 21:421–427.

HORNER, R.F., BISHOP, G.C., JARVIS, C.J. & COETZER, T.H.T. 1995. NAD (V-factor)- independent and typical *Haemophilus paragallinarum* infection in commercial chickens: a five year field study. *Avian Pathology*, 24:453–463.

MUTTERS, R., PIECHULLA, K., HINZ, K-H. & MANNHEIM, W., 1985. *Pasteurella avium* (Hinz & Kunjara 1977) comb. nov. and *Pasteurella volantium* sp. nov. *International Journal of Systematic Bacteriology*, 35:5–9.

REID, G.G., & BLACKALL, P.J. 1987. Comparison of adjuvants for an inactivated infectious coryza vaccine. *Avian Diseases*, 31:59–63.

TRAVERS, A.F. 1996. Concomitant *Ornithobacterium rhinotracheale* and Newcastle Disease infection in broilers in South Africa. *Avian Diseases*, 40:488–490.

VANDAMME, P., SEGERS, P., VANCANNEYT, M., VAN HOVE, K., MUTTERS, R., HOMMEZ, J., DEWHIRST, F., PASTER, B., HERSTERS, K., FALSEN, E., DEVRIESE, L.A., BISGAARD, M., HINZ, K.-H. & MANNHEIM, W. 1994. *Ornithobacterium rhinotracheale* gen. nov., sp. nov., isolated from the avian respiratory tract. *International Journal of Systematic Bacteriology*, 44:24–37.