

Characterisation of isolates of *Haemophilus paragallinarum* from Indonesia

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Objective To characterise 18 isolates of *Haemophilus paragallinarum* isolated from chickens in Indonesia.

Procedure The isolates were identified to species level by traditional phenotypic methods. Six of the isolates were also identified by a species-specific polymerase chain reaction. Fourteen of the isolates were examined for resistance to a panel of seven antimicrobial agents using a disc diffusion method. All 18 isolates were serotyped according to the Page scheme using reference antisera in a haemagglutination inhibition test.

Results Four of the 18 isolates were obtained from indigenous (kampung) chickens, with the remainder being from typical intensive poultry production systems. The 18 isolates were obtained from 11 outbreaks that showed the typical clinical signs of infectious coryza and 11 of the isolates were obtained from chickens that had been vaccinated with infectious coryza vaccines. All 18 isolates were confirmed as *H paragallinarum* by biochemical testing and six isolates were also identified as *H paragallinarum* by the polymerase chain reaction test. Eleven isolates were resistant to erythromycin and streptomycin, 10 to neomycin, eight to oxytetracycline, five isolates to doxycycline, three to sulphamethoxazole-trimethoprim but only one to ampicillin. Seven isolates were Page serovar A, four were Page serovar B and seven were Page serovar C.

Conclusion The presence of all three Page serovars (A, B and C) has been confirmed for the first time in Indonesian chickens. As the majority of the infectious coryza vaccines in use in Indonesia contain only serovar A and C, the presence of serovar B in chickens indicates that the protection by these bivalent vaccines would be reduced. The use of trivalent infectious coryza vaccines that contain serovars A, B and C is recommended for use in Indonesia.

Aust Vet J 2000;78:759-762

Key words: Poultry, *Haemophilus paragallinarum*, serological characterisation, Page scheme

Haemophilus paragallinarum is the causative agent of infectious coryza, a disease of the upper respiratory tract of chickens.¹ The clinical signs of the disease include nasal discharge, facial swelling and a reduction in feed and water consumption.¹ Infectious coryza in poultry is a disease of economic significance in many parts of the world with the greatest economic losses resulting from an increased number of culls and marked reduction (10 to 40 %) in egg production.¹

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The first serological classification of *H paragallinarum* was performed in 1962 by Page who recognised three different serovars, termed A, B and C.² The Page scheme has been widely used in many parts of the world.¹ However, other than studies performed in Japan, there have been only a few, limited serological characterisation studies performed in Asia. There have been studies performed in China, with all 29 isolates shown to be serovar A,^{3,4,5} Malaysia, with all 10 isolates being serovar A,⁶ the Philippines, with one isolate each of serovar A, B and C⁷ and Taiwan, with both isolates being serovar A.⁸

In Indonesia, the isolation of *H paragallinarum* has been reported in 1975,⁹ 1978¹⁰ and 1987.¹¹ Unfortunately, none of the isolates from these studies were maintained and, therefore, there is no knowledge of the serovars to which they belonged. Takagi et al¹² reported the isolation of three *H paragallinarum* strains in Indonesia, two of which were identified as serovar A and the other as serovar C.

In Indonesia, infectious coryza has become a major problem with affected chickens, including indigenous (kampung) chickens, being of all ages. Imported commercial inactivated vaccines are widely used for the prevention of the disease. However, there is considerable field evidence that suggests that vaccine failure might be occurring.

In this paper, we present the results of a characterisation study of 18 *H paragallinarum* isolates obtained from a number of outbreaks of infectious coryza in several parts of Indonesia between 1991 and 1999.

Materials and methods

Bacteria

A total of 18 isolates of *H paragallinarum* were examined. All of the isolates were obtained from outbreaks of infectious coryza in broilers, layers and kampung chickens, between 1991 and 1999 from West Java (thirteen isolates), Central Java (two isolates) and Lampung in Sumatera (three isolates). As well, reference strains, 0083 (Page serovar A), 0222 (Page serovar B) and Modesto (Page serovar C) were used. These reference strains were supplied from the culture collection held at the Animal Research Institute, Australia. Following initial isolation, all of the isolates were inoculated into yolk sac of 6- to 7-day-old embryonating chicken eggs (obtained from flocks known to be free of *H paragallinarum*). After 24 h, the infected yolk sac was harvested and dispensed in 0.5 mL volumes in ampoules for freeze drying.

Media

Blood agar with 5% sheep's blood cells was used for both isolation and for the examination of the satellite phenomenon with a feeder culture of *Staphylococcus hyicus*.¹³ Test medium

agar supplemented with 5% (v/v) oleic albumin complex, 1% (v/v) chicken serum, 0.01% (w/v) nicotinamide adenine dinucleotide (NAD) was used for growth of the cultures and was prepared as previously described.¹⁴ This medium, called TM/SN, was used for the preparation of inocula for biochemical and other tests. Liquid media were incubated aerobically, while agar plates were incubated under increased CO₂ tension (approximately 10%). All incubation was at 37°C.

Fermentation of carbohydrates

A plate fermentation method¹⁵ was used for the detection of acid production from the following carbohydrates: galactose, glucose, lactose, maltose, mannitol, sorbitol, sucrose, trehalose and xylose. A 48 h culture of each isolate was suspended in phosphate buffered saline (PBS, pH 7.5) and the fermentation plates were inoculated with these suspensions. The fermentation patterns of all 18 field isolates and the three reference strains of *H paragallinarum* were determined.

Other biochemical tests

The field isolates that were tested for carbohydrate fermentation were also tested for a range of other properties. Indole production was tested according to Cowan¹⁶ with the modification that the basal medium was supplemented with 1% (v/v) chicken serum and 0.01% (w/v) NAD. The presence of catalase, the requirement for NADH and pigment production were performed as described previously¹³ while the requirement for haemin was determined according to Schalm and Beach.¹⁷

Polymerase chain reaction test

Six field isolates were tested using a polymerase chain reaction (PCR) test known to be specific for *H paragallinarum*.¹⁸ The test was performed using colony preparations as previously described.¹⁸

Antimicrobial drug resistance

The sensitivity of 14 field isolates and the three *H paragallinarum* reference strains for seven antimicrobial drugs (ampicillin 10 mg per disc, doxycycline 30 mg, erythromycin 15 mg, neomycin 30 mg, oxytetracycline 30 mg, streptomycin 10 mg and sulfamethoxazol-trimethoprim 25 mg) were tested using a standardised disc-diffusion method.¹⁹ Mueller Hinton agar supplemented with 5% (v/v) oleic albumin complex, 1% (v/v) chicken serum and 0.01% (w/v) nicotinamide adenine dinucleotide was used. The inoculum was prepared from fresh overnight cultures and adjusted to a density of equivalent to that of a 0.5 MacFarland Standard. The suspension was then swab inoculated on the modified Mueller Hinton agar, the antimicrobial discs (Oxoid Unipath Ltd, Basingstoke, UK) placed on the agar surface and the plate incubated at 37°C for 24 h under 5% CO₂. The diameter of the zone of inhibition was measured. A reference strain of *Escherichia coli* (ATCC 25922) was used as described above, as well as being tested on unsupplemented Mueller Hinton agar. All tests were performed twice. The interpretive criteria used for determining if isolates were sensitive or resistant to the various antimicrobial agents were those recommended by the National Consultative Committee on Laboratory Standards.²⁰

Serological characterisation

The 18 isolates and three of the reference strains of *H paragallinarum* (0083, 0222 and Modesto, serovar A, B and C respectively) were serotyped, with antisera produced at the Animal Research Institute,²¹ according to the Page scheme² using a haemagglutination-inhibition test.²²

Results

The available field information on the 18 isolates examined in this study is presented in Table 1.

Table 1. Field information and serotyping results for the 18 *Haemophilus paragallinarum* isolates used in this study.

Code	Year	Farm	Flock size	Chicken Type	Age (wks)	Vaccinated ^a	Region	Serovar
18/2	1991	A	70,000	Broiler Multi-age	5	No	Bogor	A
SH ₂	1992	B	12,000	Layer Multi-age	35	Yes (2)	Lampung	A
SH ₉	1992	B	6,000	Layer Multi-age	8	Yes (1)	Lampung	A
SH ₁₀	1992	B	6,000	Layer Multi-age	20	Yes (2)	Lampung	A
16CMG1 ^b	1993	C	1,200	Kampung	24	No	Ciamis	A
CP	1993	D	12,000	Broiler Multi-age	6	No	Bandung	A
A ₁	1997	E	5,000	Broiler Breeder	21	Yes (2)	Tangerang	C
A ₂	1997	E	5,000	Broiler Breeder	24	Yes (2)	Tangerang	C
A ₃	1997	E	5,000	Broiler Breeder	34	Yes (2)	Tangerang	C
A ₄	1997	E	5,000	Broiler Breeder	38	Yes (2)	Tangerang	C
AD ₁	1997	F	5,000	Broiler Breeder	24	Yes (2)	Sukabumi	C
AD ₂	1997	F	5,000	Broiler Breeder	24	Yes (2)	Sukabumi	C
AD ₃	1997	F	5,000	Broiler Breeder	24	Yes (2)	Sukabumi	C
B11.1 ^b	1998	G	60,000	Broiler	7	No	Bogor	A
C1.1 ^b	1998	H	70,000	Layer Multi-age	15	Yes (1)	Cianjur	B
AK ₂ ^b	1999	I	19	Kampung Multi-age	4	No	Bogor	B
AkBur ₄ ^b	1999	J	25	Kampung Multi-age	8	No	Bogor	B
AkPar ₄ ^b	1999	K	35	Kampung Multi-age	8	No	Bogor	B

^aVaccinated = Vaccinated with a commercial infectious coryza vaccine containing only serovars A and C. Number in brackets is the number of doses.

^bIndicates that the isolate was identified by both conventional and PCR methods and also serotyped. All the other isolates were identified by conventional methods only as well as being serotyped.

All the field isolates required NAD but not haemin for growth and showed satellitic growth on blood agar. All 18 isolates lacked catalase activity, did not produce a yellow pigment and were Gram negative rods. The isolates were all nonhaemolytic on blood agar and did not produce indole. All 18 isolates produced acid from glucose but not from galactose, lactose, trehalose or xylose. Of the 18 isolates tested, eight did not ferment maltose, one did not ferment mannitol and one did not ferment mannitol, sorbitol or sucrose. As all 18 field isolates were similar to the reference strains of *H paragallinarum*, all 18 were identified as *H paragallinarum*. The three reference strains of *H paragallinarum* gave the same carbohydrate fermentation patterns as reported previously.¹³

Six of the isolates yielded a DNA product of the expected size (0.5 kb), in the HP-2 PCR for *H paragallinarum*. On the basis of this result, all six isolates were identified as *H paragallinarum*.

In the antimicrobial sensitivity testing, *E coli* strain ATCC 25922 gave the expected results, on both modified and normal Mueller-Hinton agar. Table 2 shows the results of testing the 14 field isolates of *H paragallinarum* with all seven antimicrobial agents.

The three reference strains of *H paragallinarum*, 0083, 0222 and Modesto, were correctly serotyped as serovar A, B and C respectively. The overall serotyping results, with 12 isolates being serotyped in Indonesia and six in Australia, were as follows: seven isolates were Page serovar A, four isolates were Page serovar B and seven isolates were Page serovar C.

Discussion

The Indonesian isolates of *H paragallinarum* tested by phenotypic methods in this study showed, in general, the typical properties of *H paragallinarum*. We found variation in the ability of the field isolates to produce acid from four carbohydrates – maltose, mannitol, sorbitol and sucrose. Similar variation has been reported previously.²³ It would appear that there is little variation in the biochemical properties of *H paragallinarum* as isolates from such diverse parts of the world as Argentina²⁴, Australia¹³, China⁴ and the United States of America²⁵ all show a similar carbohydrate fermentation pattern.

As *H paragallinarum* isolates do not grow on the standard media used in antimicrobial sensitivity testing, we used supplemented Mueller Hinton agar, which is capable of supporting the growth of *H paragallinarum*. The use of supplemented Mueller Hinton agar had no major impact on the results of the reference *E coli* strain (data not shown) so we regard the results obtained for the six *H paragallinarum* isolates as valid. We found a frequent occurrence of resistance to erythromycin, neomycin and streptomycin. Others have also reported that *H paragallinarum* isolates can be resistant to streptomycin.^{12,25,26,27}

We found four Indonesian isolates to be serovar B, the first time this serovar has been recognised in this country. The only previous serological characterisation of Indonesian isolates of *H paragallinarum* reported the presence of serovars A and C.¹² Our finding of serovar B has several important practical implications. Although there has been some confusion in the earlier literature, there is now compelling evidence that serovar B is a true serovar.²⁸ Further, Page serovar B isolates show little cross-immunity with serovars A and B.²⁹ This means that vaccines based on serovar A or C have little chance of providing protection against a Page serovar B challenge. Additionally, there is evidence, within Page serovar B, of limited cross-protection

Table 2. Sensitivity to seven antimicrobial drugs of 14 field isolates of *Haemophilus paragallinarum* from 1991 to 1999.

Strain	Year	Sensitivity to antimicrobial drugs ^a						
		AMP (10 µg)	DO (30 µg)	ERY (15 µg)	NEO (30 µg)	OT (30 µg)	S (10 µg)	SXT (25 µg)
18 ₂	1991	S ^b	S	R	R	S	R	R
16CMG ₁	1993	S	S	S	S	S	S	S
SH ₂	1992	S	S	S	S	S	S	S
SH ₉	1992	S	S	S	S	S	S	S
SH ₁₀	1992	S	S	R	R	S	R	S
AD ₁	1997	S	R	R	R	R	R	S
AD ₂	1997	S	R	R	R	R	R	S
A ₁	1997	S	R	R	R	R	R	S
A ₃	1997	S	R	R	R	R	R	S
B11.1	1998	S	S	R	S	S	R	S
C.1	1998	S	S	R	R	R	R	S
AK ²	1999	R	R	R	R	R	R	R
AKBur ₅	1999	S	I	R	R	R	R	S
AKPar ₄	1999	S	I	R	R	R	R	R

^aAmp = Ampicillin, DO = Doxycycline, ERY = Erythromycin, NEO = Neomycin, OT = Oxtetracycline, S = Streptomycin, SXT = Sulphamethoxazole-trimethoprim. The amount of antimicrobial agent in the disc is indicated in parenthesis.

between some isolates.²⁹ Hence, our finding of serovar B in both commercial and kampung chickens indicates that there is a need for careful selection of the seed strains used to produce infectious coryza vaccines for use in Indonesia. In particular, unless there is specific knowledge of the serovars present in the target population, the use of bivalent vaccines that contain only Page serovars A and C cannot be recommended in Indonesia.

The finding of Page serovar B *H paragallinarum* in Indonesian chickens has important implications for the Australian poultry industry. Isolates of Page serovar B have never been found in any of the serological characterisation studies performed on Australian isolates of *H paragallinarum*.^{21,30-32} Hence, Page serovar B *H paragallinarum* can be regarded as an exotic agent. As well, all the infectious coryza vaccines used in Australia contain only Page serovar A and C, meaning any entry of Page serovar B would result in vaccine failures. Hence, the presence of Page serovar B in a near neighbour is an important reminder of the need for tight biosecurity.

Infectious coryza is often regarded as a disease that has the greatest impact in intensively raised chickens. Indeed, the disease is not often considered when working with village chickens. Hence, our finding of confirmed isolates of *H paragallinarum* in kampung chickens is a reminder that the disease can be present in less intensive production systems. Our finding of isolates of *H paragallinarum* in kampung chickens supports the earlier serological work of Takagi et al.¹² who found a high prevalence of antibodies to serovar A and C in kampung chickens. The potential importance of coryza in less intensive systems is supported by the work of Thitisak et al.³³ who found that infectious coryza killed more chickens than any other disease, including Newcastle disease, in Thai village chickens less than 2 months old or more than 6 months old. It was only in the 2 to 6 months age group that other diseases such as Newcastle Disease and pasteurellosis killed more chickens.³³

Fourteen of the isolates in this study were from outbreaks of infectious coryza in seven different commercial flocks. Of these seven outbreaks, four occurred despite the use of infectious coryza vaccines. In one of these outbreaks, the isolate was serovar B while the vaccine used was a serovar A/serovar C bivalent product. This mis-match of challenge serovar with vaccine is the most likely explanation for this case of vaccine failure. Other explanations such as improper vaccine handling or improper vaccination technique may also have played a role in some of these apparent vaccine failures. Overall, there is a need for the active investigation of suspect infectious coryza vaccine failures, including the isolation and serotyping of suspect *H paragallinarum* isolates.

Acknowledgments

The provision of funds by Mr. Yapto Nazarudin from Kepuhardjo, Karangploso, Malang East Java to support this study is gratefully acknowledged.

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(Accepted for publication 2 June 2000)