

Antimicrobial sensitivity pattern of *Haemophilus paragallinarum* isolated from suspected cases of infectious coryza in poultry

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Abstract

Among infectious diseases of avian species Infectious coryza is one of the major problems affecting commercial poultry industry in the country. Infectious coryza is an upper respiratory disease of chickens caused by infection with *H. paragallinarum* (HPG). The disease is characterized by swollen infra-orbital sinuses, nasal discharge, and depression. The disease is seen most commonly in adult chickens and can cause a very significant reduction in the rate of egg production. Considering the economic importance of the disease, the present research pursuit was undertaken with the aim to isolate *H. paragallinarum* from the suspected cases of Infectious coryza in commercial poultry farms in Gujarat state with reference to their cultural, morphological characterization and antimicrobial drug sensitivity patterns. Further these isolates were confirmed by using specific colony PCR test. The research work aims to characterize *Haemophilus paragallinarum* field isolates of poultry origin from Infectious coryza outbreak in and around Anand, Kheda and Mahua area of Saurashtra region of Gujarat state, India.

Key words: *Haemophilus paragallinarum* Antibiogram, Colony PCR,

Introduction

Haemophilus paragallinarum causes an acute respiratory disease of chickens known as infectious coryza (IC), a disease first recognized as a distinct entity in the late 1920's. Among infectious diseases of avian species Infectious coryza is one of the major problems affecting commercial poultry industry in the country. It is an upper respiratory disease of chickens caused by infection with *H. paragallinarum* (HPG). Since the disease proved to be infectious and primarily affected nasal passages, the name "infectious coryza" was adopted (Blackall, 1989). It may occur in both growing chickens and layers. The major economic effect of the disease is an increased culling rate in meat chickens and a reduction in egg production (10 to 40%) in laying and breeding hens, particularly on multiage farms. Even in an uncomplicated outbreak in which prompt antibiotic treatment was used, a 6-week drop in egg production, averaging 15%, occurred. The most common clinical signs are nasal discharge conjunctivitis and swelling of the sinuses and face. Swelling of the wattles may develop also. The disease is limited primarily to chickens and has no public health significance (Yamamoto, 1991). Various sulfonamides and antibiotics are useful in alleviating the severity and course of infectious coryza, however, none of the therapeutic agents has been found to be bactericidal. Relapse often occurs after treatment is discontinued,

and the carrier state is not eliminated (Yamamoto, 1991). There is no specific knowledge about resistance mechanisms in *H. paragallinarum* (Blackall, 1988).

The present study was undertaken with the view to understand the antibiotic sensitivity patterns of the *H. paragallinarum* isolates from the suspected cases of Infectious coryza in commercial poultry farms in Gujarat state.

Materials and Methods

Bacteria:

The reference strains of *H. paragallinarum* (strain A, C) were procured from Ventry Biologicals, Pune, Maharashtra State (India). The strains of *L. monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135), *Escherichia coli* (MTCC 443), used in the study were obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India.

Staphylococcus spp. used as the feeder culture for Satellitism phenomenon in *H. paragallinarum* identification was procured from Poultry Disease Diagnosis and Research Centre, Pune, Maharashtra.

A total of one hundred and nine samples were collected from commercial poultry birds from suspected cases of Infectious coryza. The samples were collected from live birds showing signs of

infectious coryza and from dead birds showing post mortem lesions typical of the disease. The samples were further processed for isolation of *H. Paragallinarum*.

Separate sterile cotton swab was used to collect nasal exudates, eye exudates from live birds and sinus cavity exudates from dead birds and streaked on Haemophilus Agar and incubated at 37°C for 24 hour in 5 - 10 % CO₂ tension. The isolates which failed to grow on all other media except Haemophilus Agar and showing dew drop like small mucoid colony character were preliminary presumed to be *H. paragallinarum* and used for further characterization. Single colony of these isolates were picked up from primary culture and were re-streaked on fresh Haemophilus Agar plate and incubated at 37°C for 24 hr under reduced oxygen tension to obtain single colony of pure culture of the isolates.

Requirement for reduced oxygen tension was tested by streaking of the suspected *H. paragallinarum* colonies in duplicate on Haemophilus agar and one plate was kept directly in the incubator for overnight incubation and another plate was kept in a desiccator with a candle burnt out to reduce oxygen tension. The desiccator is then kept in incubator for incubation overnight.

Confirmation of the isolates by Colony PCR

For final confirmation of the isolates colony PCR was carried out using method described by Chen et al. (1996). The primers for the detection of suspected isolates of *H. paragallinarum* was synthesized by Bangalore Genei Ltd., India. The details of the primer sequences are F1 (TGAGGGTAGTCTTGACGCGAA T) and R1 (CAAGGTATCGATCGTCTCTCT ACT). The template DNA was prepared by suspending single colony from Haemophilus Agar plate into 200 µl phosphate buffered saline (PBS) in PCR tubes. After heating at 98°C for five min the cells were pelleted by centrifuging in a bench-top microfuge (Universal 30RF Hettich Zentrifugen, Germany) for 5 min at 13000 rpm. One of the supernatant was used in the PCR test. The colony PCR reveals an amplified product of 490 bp.

PCR reaction for HP PCR

Composition of Master Mix for PCR reaction.

1. 2X PCR Mastermix (Fermentas, Life Sciences):

- 4mM MgCl₂
- 0.4mM of each dNTPs (dATP, dCTP, dGTP, dTTP)
- 0.05 units/ml of Taq DNA polymerase
- 150 mM tris-HCl PCR buffer

To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading buffer from each tube and electrophoresed on 2.0 per cent agarose gel along with 100 bp DNA ladder (GeneRuler- Fermentas) and stained with ethidium bromide (1 per cent solution at the rate of 5

l/100 ml) at constant 80 V for 30 minutes in 0.5X TBE buffer.

Visualisation of the product

The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

Assay for Antibiotic sensitivity pattern of *Haemophilus paragallinarum*

The antibiotic discs and other antibiotics used during the present study were procured from HiMedia. The six isolates of *Haemophilus paragallinarum* were tested in vitro for sensitivity for 7 antibiotics. The isolates were subjected to in vitro antibiotic sensitivity as per Bauer et al. (1966) with minor modification. Isolates were tested against most commonly used antimicrobial/antibiotic.

The results were interpreted as per chart furnished by the manufacturer.

Procedure

Each test strain was grown in Haemophilus Agar plate overnight. The growth obtain on Haemophilus Agar plate was suspended in sterile Haemophilus broth solution. This suspension was spread over sterile Haemophilus Agar plate with the help of sterile cotton swab. The plates were allowed to dry for five minutes before placing the antimicrobial/antibiotic discs. Antimicrobial/antibiotic discs were placed on inoculated agar surface at about two cm apart. The plates were incubated at 37°C overnight in 5 – 10 % CO₂ tension and diameters of the zones of inhibition were measured on the next day. The sensitivity of the isolate to a particular antibiotic was determined as per the interpretative chart furnished by the manufacturer.

Results

In all six isolates could be obtained on culture isolation which were further analyzed for antibiogram pattern and PCR based detection.

Colony characters

The growth of the *H. paragallinarum* isolates on Haemophilus Agar revealed smooth, dew drop like moist type colonies. The colonies were tiny and faint yellowish colored. These colonies were further used for biochemical characterization and other tests.

Blood Agar with cross streaking of feeder culture of *Staphylococcus* was used to demonstrate Satellitism in the isolates. Blood Agar when supplemented with feeder culture showed growth of *H. paragallinarum* colonies just adjacent to the *Staphylococcus* culture. This finding showed the satellitic phenomenon of the organism. The colonies of *H. paragallinarum* with feeder culture on BA were observed to grow just near the *Staphylococcus* culture growth and appeared shiny silver colored.

This finding suggested the need of V factor on Blood agar for *H. paragallinarum*.

The suspected isolates were further characterized for their dependence on V factor by observing the growth on various media like Blood Agar, Nutrient Agar (NA), MacConkey agar (MCA), EMB Agar, Brilliant Green Agar (BGA) which lack NAD supplementation.

The suspected *H. paragallinarum* isolates which grew on NAD containing media showed the requirement for V factor but these isolates failed to grow on NAD deficient media. This suggested that either the isolate failed to grow on NAD deficient media or the media themselves acted as inhibitory medium for the growth of the organisms. (Table 4.2).

The purity of the culture was also determined by uniformity in the morphological characters of the isolates. The isolates were stained by Grams staining method (Annexure II) and revealed Gram negative pleomorphic organisms.

The colony PCR revealed an amplified product of 490 bp for the entire six colony extracted samples (Plate 4.10).

In vitro* antibiotic sensitivity of *H. Paragallinarum

All the six isolates of *Haemophilus paragallinarum* were tested in vitro for sensitivity for 7 antibiotics (Table 3.2). The sensitivity of individual isolates to various agents, as interpreted, according to the manufacturer's (Hi Media) instructions, are presented in Table 4.5 and Table 4.6. (Plate 4.7).

The results of in vitro antibiotic sensitivity were interpreted as per the chart furnished by the manufacturer and results are depicted in Tables 4.4.

The frequency of sensitivity and resistance of the isolates to various antibiotics is being shown in Table no.4.7.

All the isolates were sensitive to enrofloxacin, chloramphenicol, Ampicillin and kanamycin. The isolate revealed 100 % resistance for tetracycline and streptomycin. Two isolates HP III and IV have shown sensitivity to Cotrimoxazole (33%) while rest of the isolates were resistant to Cotrimoxazole (66%).

Discussion

In the present study the growth of the *H. paragallinarum* isolates on *Haemophilus* Agar revealed smooth, dew drop like moist type colonies. The colonies were tiny and faint yellowish colored.

Hinz, (1973) reported type of colonies to be smooth and correlated these colony structure with the virulence of the organism.

Blackall (1989) have shown that the colonies of *H. paragallinarum* are typically tiny (0.3mm after 24h of growth) with a dewdrop shape. Colony morphology ranging from mucoid (smooth) iridescent and rough noniridescent to other intermediate colony forms when inspected under obliquely transmitted light was reported by Rimler, (1979) and Sawata et al., (1978).

Characteristic Satellitic growth of the *H.*

paragallinarum isolate was observed on Blood Agar when the isolates were cross streaked with feeder culture of *Staphylococcus*. Kesler (1997) also reported similar character for isolation of *H. paragallinarum*. Blackall and Yammamoto (1998) have shown the comparison for Satellitic growth to distinguish NAD dependant and NAD independent organisms. Sobti et al. (2001) reported the growth of the *H. paragallinarum* colonies adjacent to feeder culture of *Staphylococcus*.

There was absence of growth of *H. paragallinarum* on any other media which lack the NAD supplementation. This finding suggests either V factor dependency of the isolates or inhibitory nature of some of the media for growth of *H. paragallinarum* isolates.

Different type of culture media containing NAD as external supplementation were used by Terzolo et al. , (1993) which has also showed the same results. The media lacking NAD failed to show growth of *H. paragallinarum* isolates.

Sameera Akhtar et al. (2001) have reported the use of blood agar, chocolate agar and tryptose agar for isolation of *H. paragallinarum* which supplies V factor for the growth of isolates.

The colony extracted samples PCR revealed an amplified product of 490 bp (Plate 4.9).

The results of the PCR tests are in accordance with the work done by Chen et al. (1996). Similar findings about the 490 bp amplification product on use of HP-2PCR was reported by Poernomo et al. (2000), Tongaonkar et al. (2003) and Jaswinder-Kaur et al. (2004).

During the present study all the isolates were found sensitive to enrofloxacin, chloramphenicol, ampicillin and kanamycin. 100 % resistance was observed by the isolates for tetracycline and streptomycin. Two isolates HP III and IV have shown sensitivity to cotrimoxazole (33%) while rest of the isolates were resistant to cotrimoxazole (66%).

Sensitivity to enrofloxacin is in agreement with the findings reported by Hinz (1980), Prasad et al., (1999), Sobti et al, (2001) and. Kurkure et al, (2001).

Sensitivity to chloramphenicol by *H. paragallinarum* isolates is in correlation with the studies by Rimler (1979), Gyurov (1984). Takahashi et al, (1990) have reported the second highest sensitivity of the *H. paragallinarum* isolates to chloramphenicol.

During the present study 100% sensitivity of *H. paragallinarum* isolates is observed for ampicillin. Sensitivity to ampicillin is in agreement with the reports by Blackall (1988). Takahashi et al, (1990), Sobti et al, (2001). Poernomo et al, (2000) reported 82% sensitivity of *H. paragallinarum* isolates for ampicillin. Sensitivity to kanamycin is not been described by any worker.

100 % resistance for tetracycline is in agreement with the report by Blackall (1988). However Lower

degree of sensitivity is shown by Rimler (1979), Lu-YS et al. (1983), Gyurov (1984) and Takahashi et al. (1990). In one study, Poernomo et al. (2000) reported least sensitivity of *H. paragallinarum* isolates to tetracycline.

Resistance for streptomycin is in correlation with the reports by Blackall (1988), Takahashi et al. (1990) and Poernomo et al. (2000). However Lu-YS et al. (1983) and Gyurov (1984) have shown lower degree of sensitivity of *H. paragallinarum* to streptomycin.

During present study cotrimoxazole is found to be 33% sensitive to *H. paragallinarum* isolates. Sensitivity to cotrimoxazole is in accordance with the reports by Lu-YS et al. (1983), Poernomo et al. (2000) and Sobti et al. (2000). However 66% resistance is shown by the isolates for cotrimoxazole. Resistance to cotrimoxazole is also reported by Reece and Coloe (1985).

The antimicrobial agents are of great value for devising curative measures against bacterial infections. Use of antimicrobials in livestock production is suspected to significantly contribute to multiple drug resistance in species of bacteria, which are common to humans and animals (Acar and Rostel, 2001).

Conclusion

All the six isolates suspected for *H. paragallinarum* obtained from various samples showed growth on *Haemophilus* agar medium kept under reduced oxygen tension but these isolates could not be recovered from the plates kept under normal incubation condition. This proved the reduced oxygen tension requirement for the *H. paragallinarum* isolates. The broth culture could be obtained when directly kept in incubator, hence broth cultures could be obtained without the requirement of reduced oxygen tension.

All the six isolates of *H. paragallinarum* were sensitive to enrofloxacin, chloramphenicol, ampicillin and kanamycin. The isolate revealed 100 % resistance for tetracycline and streptomycin. Two isolates HP III and IV have shown sensitivity to cotrimoxazole (33%) while rest of the isolates were resistant to cotrimoxazole (66%).

Variation in antibiotic sensitivity showed the necessity of in vitro antibiotic sensitivity prior to the treatment. It also emphasizes to have judicious selection of antibiotics for effective treatment. The combined use of biochemical tests and antibiotic sensitivity pattern of the isolates can prove basis for biotyping.

The HP-2PCR detection of the isolates was carried out from growth of the isolates on *Haemophilus* Agar (Colony PCR) using F1 and R1 primer pair. The colony PCR revealed an amplified product of 490 bp. *P. multocida* used as negative control, did not show any amplification by the same primer pair.

Acknowledgements

The authors are grateful to Principal, College of Veterinary Science and Animal Husbandry, Anand for providing necessary facilities to carry out the research work. We also thank Dr. S. Tongaonkar, Ventr Biologicals, Pune for providing the reference strains for the research work and his technical suggestions during the course of the study.

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Table-1. Antibiotic sensitivity of individual *H. paragallinarum* isolate

Sr. No.	Isolate No.	Antibiotics						
		EX	C	A	CO	S	K	T
1.	HP I	S	S	S	R	R	S	R
2.	HP II	S	S	S	R	R	S	R
3.	HP III	S	S	S	R	R	S	R
4.	HP IV	S	S	S	R	R	S	R
5.	HP V	S	S	S	R	R	S	R
6.	HP VI	S	S	S	R	R	S	R

EX- Enrofloxacin, C- Chloramphenicol, A- Ampicillin, Co- Cotrimoxazole, S- Streptomycin, K- Kanamycin, T- Tetracycline, R- Resistant, S- Sensitive

Table-2. Frequency of sensitivity to individual antimicrobial agent amongst *H. paragallinarum* isolates

Sr. No.	Antimicrobial agents	Isolate (n=6)			
		Sensitive		Resistant	
		Number	%	Number	%
1.	Enrofloxacin	6	100	0	0
2.	Chloramphenicol	6	100	0	0
3.	Tetracycline	0	0	6	100
4.	Ampicillin	6	100	0	0
5.	Kanamycin	6	100	0	0
6.	Cotrimoxazole	2	33	4	66
7.	Streptomycin	0	0	6	100
