PATHOGENICITY STUDY OF FIELD ISOLATES OF *AVIBACTERIUM PARAGALLINARUM* IN SEVEN WEEK OLD CHICKS.

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ABSTRACT

Avibacterium paragallinarum isolated from various places in northern India were studied for their virulence by infecting the experimental chicks. An inoculum of 0.2 ml (1011 cfu/ml) was dropped in the nostrils of birds or injected in the right infraorbital sinus of three seven-week-old chickens for each isolate. Birds infected with A. paragallinarum showed typical signs of Infectious Coryza. Bacteria could be reisolated from the birds. The identified bacteria and the direct clinical samples subjected to species specific HP2-PCR for A. paragallinarum. PCR was found to be specific and it produced 500bp amplicons. In the challenge study on the experimental birds, accumulation of whitish fluid like exudates in the infraorbital sinuses was more prominent and air sacculitis and pneumonia noticed less frequently in birds.

Key words: Virulence, *Avibacterium paragallinarum*

INTRODUCTION

Avibacterium paragallinarum is an obligatory pathogen as its presence in birds is reported to be always associated with clinical infection Blackall et al., (2005) and Sobti et al., (2001) have reported the development of clinical signs in birds experimentally infected with *Avibacterium paragallinarum*. Workers like Reid and Blackall (1984) have also differentiated the virulence of *Avibacterium paragallinarum* with other avian Haemophili and reported that the catalase positive organism is not produce typical infection like *Avibacterium paragallinarum*. Hence, the present work was undertaken to determine the classical signs and pathological lesion by field isolates.

MATERIALS AND METHODS

Day old chicks were purchased from the Mattur hatcheries which are free of Infectious Coryza. For each isolate (n=3) four birds were used and one group containing four birds used as control. The isolates were obtained from in and around Palampur (H.P) area of Mandi, Unna and Cholan district during the year 2008. The birds environment was

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maintained in sterile manner by fumigating the sheds with Potassium permanganate and formalin. During the sixth to seventh week of the age the birds were inoculated with the *Avibacterium paragallinarum* suspensions.

Isolates APG1, APG2 and APG3, did not have the ability to hemagglutinate fresh and GA-fixed RBC of various animal species. Furthermore the isolates APG1, APG2 and APG3 attained the ability to hemagglutinate RBC of day-old and 60-day old chicken erythrocytes when they were treated with Potassium thiocyanate alone or when they were only sonicated or when they were subjected to both KSCN and sonication Blackall et al. (1990). The titres progressively increased with each treatment for most isolates. However the hemagglutinins lost their ability when treated with trypsin, formalin or heat. The above mentioned observations when contemplated revealed that all three isolate could classify under one serotype.

Culturel suspension of 1011 CFU of each isolate grown in Brain heart infusion broth was used as the inoculum for Pathogenicity test. An inoculum of 0.2 ml was dropped in the nostrils of birds (8 birds) or injected (8birds) in the right infraorbital sinus of eight seven week old chickens for each isolate. The birds were examined daily for 10 days for clinical signs of Infectious Coryza.

Group-I: Nasal discharge, conjunctivitis, and swelling of head.

Group-II: Nasal discharge, conjunctivitis and depression.

Group-III: Infraorbital sinuses swelling, reddened eyes, decreases in water and feed intake.

Birds having facial swellings were sacrificed and decapitated. The head was swabbed clean with 70% alcohol and allowed to dry. A spatula was heated in a Bunsen burner flame for a minute and the hot spatula was used to sear the surface skin of the infraorbital sinus. The skin over the infraorbital sinus was then cut to about 1.0 centimeter with a sterile Bard-Parker blade. The mucoid fluid from the infraorbital sinus was collected with a nicrome loop and was streaked directly onto the chocolate agar plates, and Levinthal's agar plates. The plates were kept in a carbon dioxide incubator supplying 7% CO2, at 37°C or in a Mc Intosh and Fildes jar containing a HiGas pack capable of generating carbon dioxide when 10 ml of water is added to it in the presence of a palladium catalyst, or keeping a candle in a Mc Intosh and Fildes jar and closing it, thus allowing it to burn out providing an anaerobic atmosphere.

Polymerase chain reaction of DNA extracted from *A. paragallinarum* isolates was performed according to the protocol reported by Chen et al. (1996). From an agar plate that had been incubated overnight, four or five A. paragallinarum like colonies were suspended in 30µL of deionized water in 1.5 ml of eppendorf tubes. The sample was heated at 98°C for 10 minutes and stored at -20°C. DNA was extracted from decontaminated clinical samples as per method prescribed by Chen et al. (1998). Dislodge the content of the clinical samples of Glycerin-PBS and centrifuge at 1200 rpm for 30 minutes at 4°C then the supernatant was

discarded. The 500 µL digestion buffer with 5 µL protinase K was added to the pellet and incubated for 8-12 hrs at 50°C. The mixture was extracted with equal volume of phenol/ chloroform /isoamyl alcohol in the ratio of 25:24:1 and mix it for 30 minutes and centrifuge at 10,000 rpm for 15 minutes at 4°C. Top aqueous phase was transferred to another tube. Extract was once again done with equal volume of phenol/chloroform /isoamyl alcohol in the ratio of 25:24:1 and centrifuge at 10,000 rpm for 15 minutes at 4°C. Top aqueous phase was transferred to another tube. 1/10 volume of ammonium acetate (3M) and double the volume of ethanol were added and incubated at 4°C for overnight. The mixture was centrifuged at 10,000 rpm for 15 minutes and the supernatant was discarded and the pellet rinsed with 70% chilled ethanol and ethanol discarded. The DNA pellet was air dried at room temperature and resuspended in T.E. buffer /Deionized water and stored at -20°C for further use.

The oligonucleotide primers used in this study were N1 and R1 (Chen et al.,1996). The sequence of primers were primer N1 (TGA GGG TAG TCT TGC ACC CGA AT) R1(CAA GGT ATC GAT CGT CTC TCT ACT). For polymerase chain reaction, 50µl reaction mixtures were prepared on ice , the reaction mixture contained pure sterile water 25.75µl, 10X PCR buffer 5µl, dNTPs 4µl, $Mgcl_2$ 5µl, Primers 2.5µl Template DNA 5µl and Taq DNA polymerase 2.5µl. The cycling programme was standardized as initial denaturation 94°C for 5 minutes, denaturation 94°C for five minutes, Annealing 65°C for 1 minute and ,Extensions 72°C for 2 minutes. The cycle was repeated for 25 times followed by a final cycle of 94°C for 1 min, 65°C for 1 minute, and 72°C for 10 min. The PCR products were run in a Bio-Rad Submerged gel apparatus. Agarose gels were prepared with 1.5 per cent agarose in TAE buffer. The agarose was melted in water bath at boiling temperature for until totally dissolved. The gel was allowed to cool down to 45-50°C. Gels were casted in casting tray with comb inserted. Gel lane was loaded with 10 µl of sample volume comprising 8 µl of PCR product and 2 µl of gel loading dye 6X (MBI fermentas). Marker DNA, 1 Kbp was used during electrophoresis to interpret for 500 bp of *A. paragallinarum*. After sample loading, the gel was loaded on the electrophoresis unit (Pharmacia Biotech EPS 200 with TAE (1X; P^H 8.0) or TBE (1X; P^H 8.2) and run on 58 volt for one hour. The gel was stained with ethidium bromide (Amresco) (10 l/100 ml distilled water) by placing gel in a borosil glass tray, for 30 minutes. Finally gel was visualized in Gel Doc system to analyze bands and photographed.

RESULTS AND DISCUSSION

Post infection observation and isolation

Chicks in control groups did not show any changes. In all the four groups no mortality was been observed. During the post-mortem a slight congestion of the trachea and nasal mucosa, accumulation of whitish fluid like exudates in the infraorbital sinuses was observed in 10th day. Reisolation of the organism could be done successfully from all the three groups and the isolates confirmed by the HP-2 PCR which is specific for *Avibacterium paragallinarum.*

Table:1
Clinical signs, lesions and reisolation post-challenge with *Avibacterium paragallinarum*

ISOLATES	DPI	SIGNS[a]	LESIONS[b]	CULTURE[c]
APG1	1,2	NIL	NIL	NEGATIVE
	3	4	3	4
	6	4	3	1
	10	3	4	-
APG2	1,2	NIL	NIL	NEGATIVE
	3	4	2	3
	6	3	3	1
	10	3	4	-
APG3	1,2	NIL	NIL	NEGATIVE
	3	3	3	3
	6	4	4	1
	10	3	4	-

APG- *Avibacteriumparagallinarum* : DPI-Days of post inoculation

All birds were giving positive for HP-2 PCR

a – Sinusitis, nasal discharge, conjunctivitis.

b – A slight congestion of the mucosa of the infraorbital sinus and trachea.

c – Reisolation of Haemophilus paragallinarum from the infected birds.

The clinical signs of the three isolates of *Avibacterium paragallinarum* were observed for ten days from the day of post inoculation. All the isolates were showing the symptoms of the Facial swelling, nasal discharge, infraorbital swelling of the eyes and no lesions on the wattle and combs. But Blackall and Yamamoto (1989) detected lesion in the wattle. The post-mortem revealed the accumulation of yellowish white inflammatory exudates in the trachea and congestion tracheal membrane. Similar clinical signs were also observed by Bragg (2002a) and Bragg (2002b). All the clinical case was given for positive for the HP-2 PCR which is showing the 500 bp amplicons (Chen et al., 1998). Using disease symptoms and lesions described by Bragg (2002b), there was no significant variation in the virulence between the isolates in chickens. Similarity in virulence of A. paragallinarum isolates has been reported by other worker like Byarugaba et al., (2007) in agreement with the findings of the present study.

REFERENCES

Blackall, P.J., Christensen, H., Beckenham, T., Blackall, L.L. and Bisgaard, M. (2005). Reclassification of Pasteurella gallinarum, [Haemophilus] paragallinarum, Pasteurella avium and Pasteurella volantium. International Journal of Systematic and Evolutionary Microbiology. **55:** 353–362.

Blackall, P. J., Morrow, C. J., McInnes, A., Eaves, L. E., and Rogers, D. G. (1990) Epidemiologic studies on infectious coryza outbreaks in northern New South Wales, Australia, using serotyping, biotyping, and chromosomal DNA restriction endonuclease analysis. Avian Diseases **34**: 267-276

Blackall PJ, R Yamamoto (1989). Haemophilus gallinarum- a re-examination. Journal Genenral Microbiology. **135**: 469-474.

Bragg, R.R., (2002a). Isolation and identification of NAD-independent bacteria from chickens with symptoms of infectious coryza. Onderstepoort Journal of Veterinary Research, **69**:171.

Bragg R. R., (2002b). Virulence of South African isolates of Haemophilus paragallinarum. Onderstepoort Journal of Veterinary Research. **69**: 163.

Byarugaba, D, Minga, UM, Gwakisa, PS, Katunguka-Rwakishaya, E , Bisgaard, M & Olsen, JE (2007) Virulence characterization of Avibacterium paragallinarum isolates from Uganda. Avian Pathology. **36**: 35-42.

Chen, X., P. Zhang., J. K. Miflin, and P.J. Blackall. (1996). Development and application of DNA probes and PCR test for Haemophilus paragallinarum. Avian Dis.**40**:398-497

Chen X, G Chen, P Zhang, PJ Blackall, W.Feng (1998). Evaluation of PCR test for Haemophilus paragallinarum in china. Avian Pathology. **27**: 296.

Reid, G.G., and P. J. Blackall. (1984). Pathogenicity of Australian isolates of Haemophilus paragallinarum and Haemophilus avium in chickens. Veterinary Microbiology. **9**: 77-82.

Sobti DK, Dhaneswar NS, Chaturvedi VK, Mehra,.K.N (2001).) Isolation and characterisation of Haemophilus paragallinarum and morphoculturally related organisms from cases of infectious coryza in Mahakaushal belt. Indian Veterinary Journal. **78**(11): 987