Incidence, Serotyping and Antimicrobial Susceptibility Profile of Avibacterium Paragallinarum Isolated From Local Commercial Poultry of Balochistan

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Research article

Keywords: Avibacterium, Balochistan, coryza, layer chicken, paragallinarum

Posted Date: July 1st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-36937/v1

Abstract

Background

Infectious coryza (IC) caused by *Avibacterium paragallinarum*, a Gram negative, non-motile coccobacilli, is a severe upper respiratory tract disease of poultry. This study aimed to report on the isolation rate and serotyping of *Avibacterium paragallinarum* causing Infectious Coryza (IC) in commercial layer poultry in Balochistan.

Results

Total 500 samples were collected from IC-suspected or recently dead birds Results revealed that 80.62% of sample were found positive for *A. paragallinarum.* Of these, serotype B was 59.60% and serotype C was 21.02%. The isolates of *A. paragallinarum* were growing well at 35–37 ^{o}C, however, growth rate was declined at 24 ^{o}C, and 42 ^{o}C. Similarly, *A. paragallinarum* showed optimal growth between pH 5 and 9, but the superlative pH growth values were from 6 to 8 pH. Antimicrobial susceptibility test showed that all tested isolates displayed resistance against metronidazole, colistin sulphate, bacitracin, streptomycin, chloramphenicol and lincomycine, while were found susceptible to tetracycline, erythromycin, vancomycin, amoxicillin, and ciprofloxacin. Pathogenicity test performed indicated that experimental birds showed signs of dullness, fever, serous nasal discharge and facial swelling with pus inside infra-orbital sinus, severe congestion in the trachea and partial cloudiness of air sacs.

conclusions

Overall, we report, for the first time, on high incidence rate of pathogenic *A. paragallinarum* among layer birds.

Background

Infectious coryza (IC) caused by *Avibacterium paragallinarum*, a Gram negative, non-motile coccobacilli, is a severe upper respiratory tract disease of poultry [1] which could be very frustrating due to its prolonged nature [2]. IC is characterized by nasal and ocular discharge and facial edema. IC has been reported from all over the world and has been considered as one of the most economically important disease with heavy economic impact. The economic impact of the diseases is mainly associated with drop in egg production and retarded growth rate. Random outbreaks have been reported in developing countries. Regular monitoring and surviellance are considered crucial for better management of IC [3].

Generally, IC is diagnosed based on clinical signs, isolation and confirmation of the causative satellitic organism. Because, majority of the *A. paragallinarum* isolates require nicotinamide adenine dinucleotide (NAD) for their growth, hence isolation process requires availability of expensive artificial media and skill,

and is often laborious [3]. Currently, a responsive and fast real-time PCR assay has been widely used for diagnosis and identification of *A. paragallinarum*. Three major serovars, A, B, and C, of *A. paragallinarum* have been described so far, however, serotype A has been rarely reported [4].

Poultry industry in Pakistan is a fast growing sector with lion share in national economy. Various infectious diseases including IC are considered to be major constraints in profitability of poultry farmers and in expansion in poultry industry generally in Pakistan and particularly in Balochistan. Various reports from Pakistan suggest that IC is endemic in layer poultry in Pakistan [5, 6], however, there is no single report available regarding prevalence of IC in Balochistan. Furthermore, reports from Pakistan from poultry and other food animals suggest an increase in emergence of antimicrobial resistance [7, 8, 9] however, no reports is available regarding antimicrobial susceptibility of local isolates causing IC. Such reports are necessary for effective therapy and control of IC in poultry. Regular surviellance also helps better management and planning of infectious disease in poultry. The current study was designed to report on the incidence of IC caused *A. paragallinarum*, frequency of occurrence of its serotypes and drug susceptibility pattern. To the best of our knowledge, we report for the first time, on high occurrence rate of *A. paragallinarum* causing IC in local poultry farms in Balochistan.

Results

Frequency of occurrence of *A. paragallinarum* and its different serotypes

A total of 500 samples were collected from recently dead and infected birds suspected of IC. Results revealed that 402 (80.62%) of samples were found positive for *A. paragallinarum*.

In the current study, molecular diagnostic procedure based on gene specific Polymerase Chain Reaction assay was practiced to detect *Avibacterium paragallinarum*. All the isolates of *Avibacterium paragallinarum* used in current study produced the predicted size of 500 base pair amplicons of HPG2 gene as shown in Fig 1.

All these isolates were further processed for PCR based serotyping and our results indicated that all isolates were either serogroup B or C as suggested by the 1100 or 1600 bp amplicon, however, no serotype A specific 800 bp amplicon could be amplified suggesting absence of serogroup A. Overall, our results showed that serotype B was 59.60% and Serotype C was 21.02% Fig 2.

Growth and cultural characteristics

A random isolates were picked up to investigate general cultural characteristics of clinical isolates. Our results indicated that *A. paragallinarum* was growing normally between 24 ^{o}C to 42 ^{o}C with pH range 5-9 (Table-1). No growth was recorded below 24 ^{o}C and above 42 ^{o}C.

Antibiotic sensitivity test

Antimicrobial susceptibility through disc diffusion method was performed and interpreted as per CLSI guidelines. Out of 403 isolates of *A. paragallinarum,* serotype – B (298) and serotype C (105). Seventy percent (70%) of each serotype were tested for antibiotic susceptibility through disc diffusion. Our results indicated that isolates of both serotypes were highly susceptible to ciprofloxacin (100% and 97.2% for serotype B and C, respectively) followed by amoxicillin (98.1% and 93%), vancomycin (96.8% and 90.2%), erythromycin (95% and 86.1%) and tetracycline (94% and81.9). of note, highest resistance was also observed against bacitracin (96.8% and 95.8%) followed by colistin sulphate (96.3% and 91.6%), streptomycin (95.9% and 94.4%) and metromedizole (95.4% and 95.8%) (Table-2a,2b).

Pathogenicity test

The inoculated birds of group C showed dullness, anorexia, and fever after 8-10 hrs. Serous nasal discharge after 26-28hrs and facial swelling as well partial swelling of unilateral infra orbital sinus with marked edema of the surrounding tissues was observed after 48 –50 hrs of inoculation, respectively. At post mortem of these experimental birds, severe congestion of trachea and cloudiness of air sacs were observed as shown in Fig 3.

Discussion

Avibacterium paragallinarum is a fastidious bacterium and the particular requirement for unusual media has resulted its isolation and identification as costly task [17]. Moreover, *A. paragallinarum* is a comparatively slow-growing bacterium that can be simply overgrown by other polluting bacteria that commonly populate the upper respiratory and nasal passages. Furthermore, nonpathogenic haemophili, formerly identified as *Avibacterium volantium* and *Avibacterium avium*, are also found in chickens, that makes the isolation and identification procedure problematic [18]. The use of PCR technique after initial isolation as an alternative of biochemical identification can minimize the complication of the diagnostic activity [18]. Additional advantage of this technique is its speed, for the reason that the results are available within 24–48 h. In the current study, the culture-PCR method was practiced for the identification of *A. paragallinarum* and was successfully validated and utilized for prevalence of IC in Quetta Balochistan. This is important to mention that the diagnosis of IC became complicated in South Africa due to the presence of both types of *A. paragallinarum i.e.* NAD-independent and NAD-dependent as well as *Ornitho bacterium in the country;* consequently, HPG-2 PCR proved to be actually useful in such situations[19]. This study also demonstrated that growth and survival of the organism is not only affected by minimum, maximum temperature and pH of the media in addition to requirements of NAD.

We report on high occurrence of IC in poultry birds sick of respiratory distress. More than 80% of collected samples were found positive for A. paragallinarum with high prevalence of serotype B followed by C, however, no serotype A was detected. Literature regarding prevalence of IC in Balochistan is not available. In contrast to our findings, previous reports identified 15% prevalence in Lahore, Punjab and [5] 2.5% in Khushab [6], and 43.3% in Jammu and Kashmir Pakistan [14]. Our observation of high occurrence or isolation rate of *A. paragallinarum* may be due to the fact that we have considered sample collection only

from birds suspected of IC. We exclude all healthy or birds showing other signs. We speculate that the incidence rate of IC in layer chicken may be reasonably low as compared to our current observation. Finally, no serotypes based survey were found in Pakistan for *A. paragallinarum*.

Antimcirobial resistance (AMR) is a growing challenge for healthcare settings as well as livestock and poultry farmers all over the world [7]. Situation is even worse in developing countries like Pakistan partly due to excessive usage of antibiotics and unavailability of data of AMR. Particularly, antibiotics are used at low dose rate as growth promotes in livestock and poultry production. Hence, this practice is likely to select for bacteria resistant to antibiotics which are routinely used for growth performance. In line with this, high incidence rate has recently been reported from poultry and other food producing animals in countries with practice of use of high level of antibiotics [9, 20, 8]. Very limited data on antibiotic susceptibility profile of *A. paragallinarum* is available due to the factor that the organism is quite difficult to isolate. Moreover, due to absence of standards and breakpoints for definition of susceptible and resistant isolates by CLSI, comparison of local isolates and its interpretation become challenging. We tested different antibiotics which are commonly used in poultry production, and results suggested that more than half of the tested antibiotics were not effective suggesting that the bacteria has developed resistance against these compounds. In our study, for those antibiotics for which CLSI standards were not available, we define resistant and susceptible based on the manufacturer instruction or on general parameters set for Gram negative bacteria. Our results showed that maximum resistance was observed against ciprofloxacin followed by erythromycin and tetracycline. Furthermore, resistance against colistin sulphate was also observed very high. Our findings of high resistance to tetracycline corroborate with high MIC values of Dutch poultry isolates Heuvelink *et al.*,[21]. This goes along with a high level of resistance of Thai isolates against erythromycin and lincomycin [15]. More striking was the high resistance level against colistin sulphate suggesting an alarming situation. This is more likely due to persistence use of colistin drugs for prevention of enteric diseases. This is intriguing to further investigate mechanism of resistance conferred against colistin. We speculate that these isolates possibly carry colistin resistance genes (*mcr*) on conjugative plasmids resulting a clonal selection, thus further study should investigate this possibility. In conclusion, our results suggest high occurrence of *A. paragallinarum* in layer poultry in Balochistan with most of the isolates were found resistant to few of the commonly used antibiotics raising concern of further dissemination of these resistance features through horizontal transfer.

Conclusions

In conclusion, we report on high incidence rate of *A. paragallinarum* in suspecte cases of IC in layer poultry in Balochistan. Further, PCR based serotyping suggested high occurrence of serotype C followed by B while no serotype A could be detected. All strains were found resistant to few of the important antibacterials such as colistin, chloramphenicol etc. Further study is suggested to identify the accurate prevalence of IC caused by *A. paragallinarum* in all districts of Balochistan.

Material And Methods

Ethical approval

The current study was approved from the ethical committee of the University of Balochistan and all procedures were performed as per local and national ethical guidelines.

Samples collection

A total of 500 samples were collected from different regions of Balochistan from commercial layer chicken suspected of infectious coryza. Swab samples were collected from different sites including infra-orbital sinuses, nasal cavities, trachea, lungs and air sacs etc. of infected or recently dead birds with history of respiratory distress. Samples were transported in 30% G-PBS (glycerol phosphate buffer saline) to CASVAB.

Culturing and isolation

All samples were inoculated into brain heart infusion chocolate agar (BHICA), brain heart infusion blood agar (BHIBA) and brain heart infusion agar with 0.01% (w/v) NAD (nicotinamide adenine diphosphate) and incubated at 37 ^{o}C supplemented with 5% CO_2. All media were purchased from Oxoid (UK).

Identification of *A. paragallinarum*

The isolated organisms were predicted on the basis of colony appearance and gram staining as described earlier by Cheesbrough, [10]. Phenotypically and biochemically confirmed presumable colonies [11,12] were further subjected to PCR based identification as described earlier by Chen *et al*., [13]. For this purpose, genomic DNA was extracted using genomic DNA purification kit (Promega, USA) and HPG2-gene specific primers [F1 (TGAGGGTAGTCTTGCACGCGAA T) R1 (CAAGGTATCGATCGTCTCTCT ACT)] in a PCR reaction resulting a 500 bp amplicon. The PCR reaction was performed in a total of 25 µl reaction mixture with a total of 25 cycles of 94^{o}C for 1min, 65^{o}C for 1min and 72^{o}C 30 Sec followed by a final extension for 10 min as reported earlier by Chen *et al*., [13].

Effect of temperature and pH on the growth of *Avibacterium paragallinarum*

Clinical isolates of *A. paragallinarum* were grown at different temperatures and pH to determine optimal temperature and pH [14].

PCR base serotyping of *Avibacterium paragallinarum*

HTM gene specific set of primers were used to serotypes A, B and C types respectively [13]. The planning of primers was F 5’GGCTCACAGCTTTATGCAACGAA-3 common for all serotypes, R: 5’-CGCGGGATTGTTGATTTTGTT-3’, R: 5’-GGTGAATTTCACCACACCAC-3 and R: 5’TAATTTTCTTATTCCCAGCATCAATACCAT-3’ were specific for serotypes A, B and C respectively. For serotyping PCR conditions were same for all serotypes as practiced in molecular detection/confirmation

of *Avibacteriumparagallinarum* except annealing temperature which was reduced to 55 °C for 1 min [13]. All PCR products were subjected to 1.5% agarose gel electrophoresis and visualized through a BioRad gel doc system.

Antibiotic sensitivity test

Antibiotic sensitivity test (AST) was performed using Mueller Hinton agar (supplemented with NAD) following Kirby Bauer disc diffusion method. For AST, inoculum was prepared from fresh overnight culture after adjusting to 0.5 McFarland Turbidity Standard as per clinical and laboratory standard institute (CLSI). Results were interpreted as per CLSI M31-A3 (2014) recommendation. *Escherichia coli* ATCC 25922 was used as quality control strain. Interpretation of the result to classify isolates into resistant and susceptible was based either on findings reported by Chukiatsiri and his colleagues or as per manufacturer (Oxoid, UK) instructions [15]. In brief, for isolates with zone of inhibition ≤7mm were declared resistant, while, ≥17mm were declared sensitive.

Pathogenicity of isolated bacteria in healthy bird

To test the pathogenicity of clinical isolates, three (3) groups, A, B and C, each of twenty (20) birds were used in experimental trail. Group A was used as control, group B was vaccinated against coryza, while group C was inoculated with isolates of *A. paragallinarum*. A 0.5ml of the isolated *A. paragallinarum* growth suspension comprising 1×10^9 CFU/ml was inoculated into infra-orbital sinus of birds of all groups. Birds of all groups were monitored. Birds that got sick with severe respiratory distress signs were then slaughtered for post mortem examination [16].

Declarations

Acknowledgements

We cordially acknowledged centre for advanced studies in vaccinology & bacteriology (CASVAB), University of Balochistan, Pakistan

Data Availability : The data generated and analysed during the current study are available from the corresponding author.

Competing interest

The author decleared no conflict of interest.

Funding

None

Authors' contributions

Conceived and designed the experiments: G.M, M.K.T, F. A.

Performed the experiments: G. M, I. P, I. T

Analyzed the data:: G. M, M. K. T, F. A.

Wrote the paper: G. M, M. K T, Z.U.A.

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Tables

Table – 1: In vivo Growth of *Avibacterium paragallinarum* on different temperature and pH.

Temperature	Growth	pH	Growth
0°C	-	2	-
4°C	-	3	-
10°C	-	4	-
15°°C	-	5	+
20°C	-	6	+
24 °C	+	7	+
30 °C	+	8	+
35 °C	+	9	+
37 °C	+	10	-
40 °C	+	11	-
42 °C	+	12	-
43 °C	-	13	-

Table – 2a: Antibiotic susceptibility pattern of *Avibacterium paragallinarum* serotype – B

Antibiotic	No's tested	sensitive	Resistance
Tetra cycline	209	196 (94.0%)	13 (6.0%)
Erythromycin	209	199 (95.0%)	10 (5%)
Vancomycin	209	202 (96.8%)	7 (3.2%)
Amoxicillin	209	205 (98.1%)	4(1.9%)
Ciprofloxacin	209	(100%)	0 (0%)
Metromediazole	209	10 (4.6%)	199 (95.4%)
Colisten sulphate	209	8 (3.6%)	201 (96.4%)
Bacitracin	209	7 (3.2%)	202(96.8%)
Streptomycin	209	9 (4.1%)	200 (95.9%)

Table – 2b: Antibiotic susceptibility pattern of Avibacterium paragallinarum serotype - C

Antibiotic	No's tested	Sensitive	Resistance
Tetra cycline	73	60 (81.9%)	13 (18.1%)
Erythromycin	73	63 (86.0%)	10 (14.0%)
Vancomycin	73	66 (90.2%)	7 (9.8%)
Amoxicillin	73	68 (93.1%)	5 (6.9%)
Ciprofloxacin	73	71 (97.3%)	2 (2.7%)
Metromediazole	73	3 (4.2%)	70 (95.8%)
Colisten sulphate	73	6 (8.4%)	67 (91.6%)
Bacitracin	73	3 (4.2%)	70 (95.8%)
Streptomycin	73	4(5.6%)	69 (94.4%)

Figures

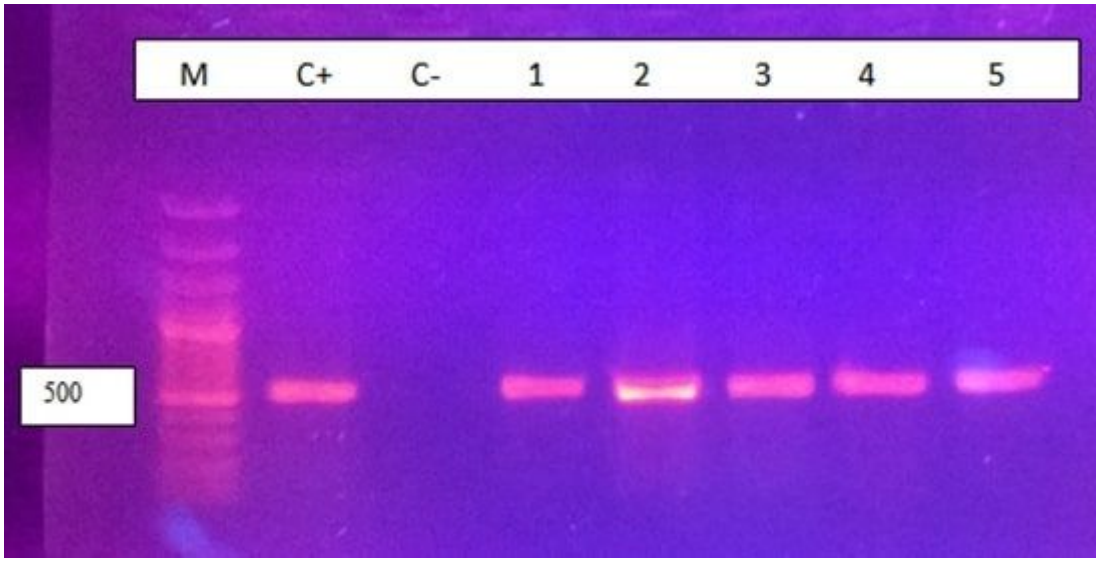

Figure 1

Molecular identification of Avibacterium paragallinarum in layer and broiler samples by direct using HPG 2 gene specific primers. Lane M: 100 bp plus DNA ladder: C+ Positive control (500bp): C- negative control: Lane 1 to 5 positive samples.

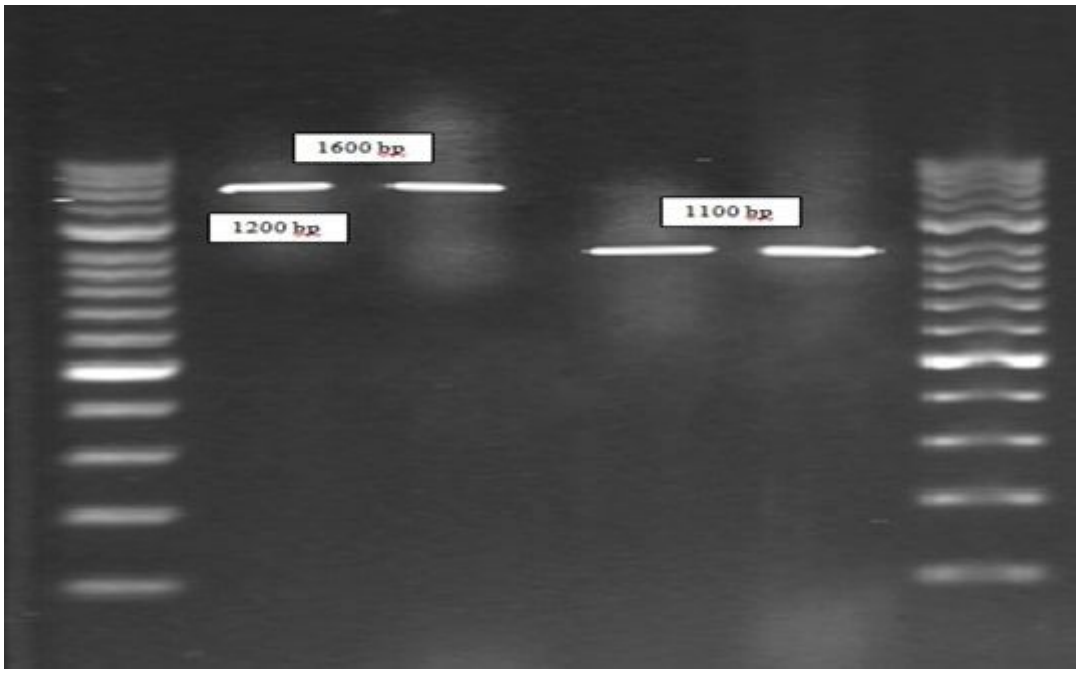

Figure 2

PCR base serotyping of Avibacterium paragallinarum in layer and broiler samples by direct using specific primers. Lane 1: 100 bp plus DNA ladder: Lane 2 and 3 C serotype: Lane 4 and 5 B serotype.

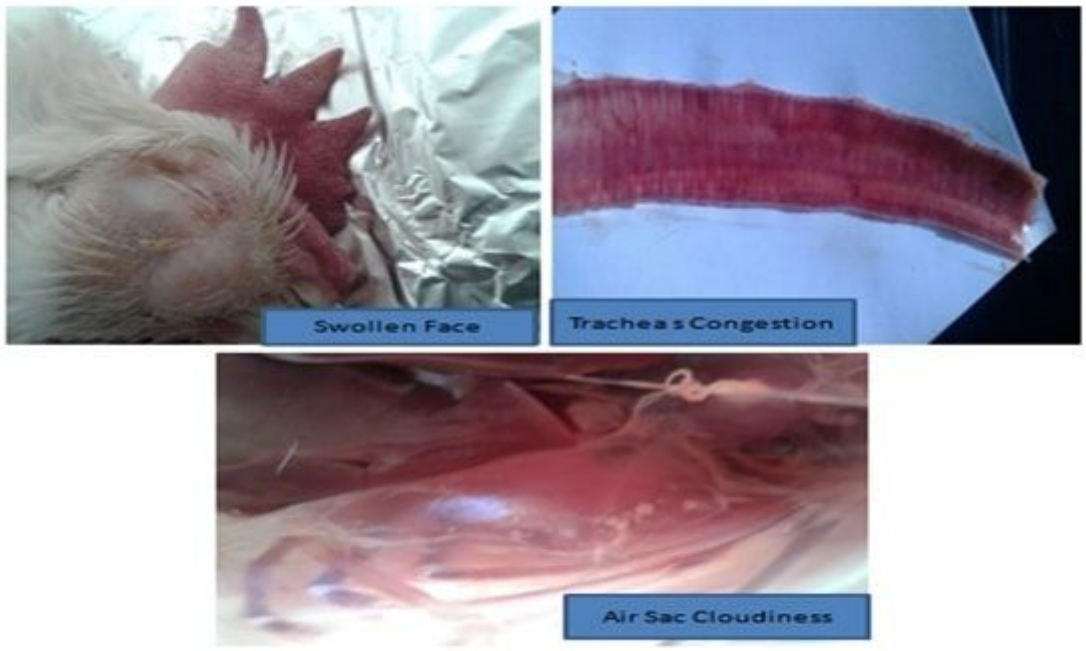

Figure 3

Head of inoculated layer bird showing swollen face as well as swelling of unilateral infra orbital sinus; Trachea showing congestion; Air sac showing partial cloudiness at postmortem.