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Incidence and antimicrobial susceptibility profile of *Avibacterium Paragallinarum* isolated from commercial birds

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ABSTRACT: Infectious coryza (IC) is a severe upper respiratory tract disease of birds. This research was aimed to study different aspects of *Avibacterium paragallinarum* causing IC in commercial birds. A total of 1000 samples were collected from IC suspected or recently dead birds. Results showed that 80.40% of the samples were positive for *A. paragallinarum.* All the isolates of *A. paragallinarum* produced the predicted size of 500 bp amplicons of HPG2 gene on PCR. The percentages of positive samples infected with infectious coryza in commercial birds were: 19.2% for layers, 18.4% for broilers, 16.0% for quails, 15.8% for chukars and 11.0% for pigeons. Among positive cases, serotype A was 24%, serotype B was 29% and serotype C was 27.40%. The isolates of *A. paragallinarum* were growing well at 35-37 ºC, however, growth rate was declined at 24 ºC, and 42 ºC. 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 they were found susceptible to Tetracycline, Erythromycin, Vancomycin, Amoxicillin, and Ciprofloxacin. Investigation of IC in commercial birds will certainly help the diagnosis of the disease, which causes considerable economic loss to the farmers. 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. This study will also alert poultry professionals about the disease and help determine specific medication as well as formulate prevention and control strategies.

KEY WORDS: *Avibacterium,* Balochistan, Birds, Coryza, Commercial.

INTRODUCTION

Infectious coryza (IC) caused by *Avibacterium paragallinarum*, a Gram negative, non-motile coccobacilli, is a severe upper respiratory tract disease of poultry (Wafaa and Abd, 2011), which could be very frustrating due to its prolonged nature (Blackall *et al*., 2005). 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 diseases 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 surveillance are considered crucial for better management of IC (Blackall and Soriano, 2008). A study in Morocco reported on 10 coryza outbreaks that were associated with 14-41% drop in egg production and 0.7- 10% mortalities of (Thitisak *et al*., 1988). A study of village chicken in Thailand reported that infectious coryza was the most common cause of death in chicks less than 2 months old and those over six months old (Sanchez *et al*., 2004). It was estimated that over the three year period the disease caused a lost of about 100 million Yuan (app $ US 16.5 million at 1996 exchange rate) in China. In the US it is most prevalent in California and the southeastern US. In New England IC has occurred in Connecticut in the 80's, but has not been diagnosed in Maine during the last 20 years (Chen *et al*., 1993).

Generally, IC is diagnosed based on clinical signs, isolation and confirmation of the causative 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 (Blackall and Soriano, 2008). Currently, a responsive and fast real-time PCR assay is being 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 (Corney *et al*., 2008).

Commercial birds industry is a fast growing sector with the lion's share in national economy. Various infectious diseases including IC are considered to place major constraints on profitability of poultry farmers and expansion of poultry industry (Mustafa and Ali, 2005; Abbas *et al*., 2015). However, the reports on poultry and other food animals suggest an increase in emergence of antimicrobial resistance (Sadeeq *et al*., 2018). Furthermore, no reports are available regarding antimicrobial susceptibility of isolates causing IC in commercial birds. Such reports are necessary for effective medication and control of IC in commercial

birds. Regular surveillance also helps improve management 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 commercial birds.

MATERIALS AND METHODS

Ethical Approval

The current study was approved from the ethical committee of University of Balochistan and the animals care center CASVAB, University of Balochistan, Quetta. All procedures were performed as per the international guidelines for human animal treatment of experimental birds.

Samples Collection

A total of 1000 samples were collected all over Balochistan (Quetta, Pishin, Zhob, Sibi, Naserabad, Kalat, Uthal, Gawadar, Khuzdar and Karan) including different age groups of commercial birds (layers, broilers, pigeons, quails and chukars, 200 samples each) suspected of infectious coryza. Swab samples were collected from different sites including infra-orbital sinuses, nasal cavities, trachea, lungs and air sacs of infected or recently dead birds with a 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 agar with 0.01% (w/v) NAD (nicotinamide adenine diphosphate) and incubated at 37 ºC supplemented with 5% CO_2.

Identification of A. paragallinarum

The isolated organisms were predicted on the basis of colony appearance and gram staining as described earlier by Cheesbrough, (2009). Phenotypically and biochemically confirmed presumable colonies (Van Empel *et al*., 1997) were further subjected to PCR based identification as described earlier by Chen *et al*., (2010). For this purpose, genomic DNA was extracted using genomic DNA purification kit (Promega, USA) and HPG2-gene specific primers [F1 (TGAGGGTAGTCTTGCACGCGAAT) R1 (CAAGGTATCGATCGTCTCTCTACT)] 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 at 94ºC for 1min, 65ºC for 1min and 72ºC for 30 sec followed by a final extension for 10 min as reported earlier by Chen *et al*., (2010).

PCR Base Serotyping of Avibacterium paragallinarum

HTM gene specific set of primers were used for serotype A, B and C respectively. The planning of primers was F 5'GGCTCACAGCTTTATGCAACGAA-3 (Chen *et al*., 2010) common for all serotypes, R: 5'-CGCGGGATTGTTGATTTTGTT-3', R: 5'-GGTGAATTTCACCACACCAC-3 and R: 5'TAATTTTCTTATTCCCAGCATCAATACCAT-3' were specific for serotype A, B and C respectively. For serotyping PCR conditions were the same for all serotypes as practiced in molecular detection/confirmation of *A. paragallinarum* except annealing temperature which was reduced to 55 ºC for 1 min. All PCR products were subjected to 1.5% agarose gel electrophoresis and visualized through a BioRad gel doc system.

Effect of Temperature and pH on the Growth of A. paragallinarum

Clinical isolates of *A. paragallinarum* were grown at different temperatures and pH to determine optimal temperature and pH (Ahmad *et al*., 2012).

Antibiotic Sensitivity Test

Antibiotic sensitivity test was performed using Mueller Hinton agar (supplemented with NAD) following Kirby Bauer disc diffusion method. For AST, inoculums 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 (Chukiatsiri *et al*., 2012). In brief, for isolates with zone of inhibition ≤ 7mm were declared resistant, while those ≥ 17mm were declared sensitive.

RESULTS

Frequency of A. paragallinarum in the Birds

A total of 1000 samples were collected from recently dead and infected commercial birds suspected of IC. Results revealed that 80.4% of the samples were positive and 19.60% were negative for *A. paragallinarum* (Fig. 1).

In current study, molecular diagnostic procedure based on gene specific Polymerase Chain Reaction assay was performed to detect *A. paragallinarum*. All the isolates of *A. paragallinarum* used in current study produced the predicted size of 500 base pair amplicons of HPG2 gene (Fig. 2).

Incidence of A. paragallinarum in Different Commercial Birds

These isolates were further processed for PCR based

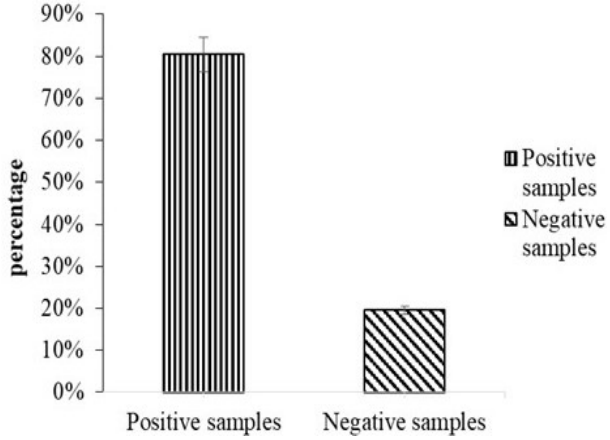

Fig 1. Frequency of ***A. paragallinarum*** in commercial birds

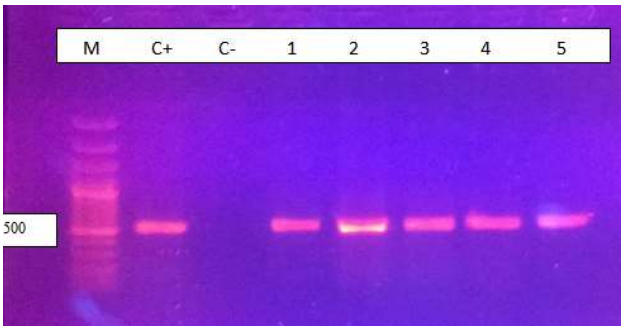

Fig 2. Molecular identification of ***Avibacterium paragallinarum*** in Commercial birds samples by direct using HPG 2 gene specific primers. Lane M: 100 bp plus DNA ladder: C+ Positive control (500bp): C- negative control: Lane 1 to 5 positive samples.

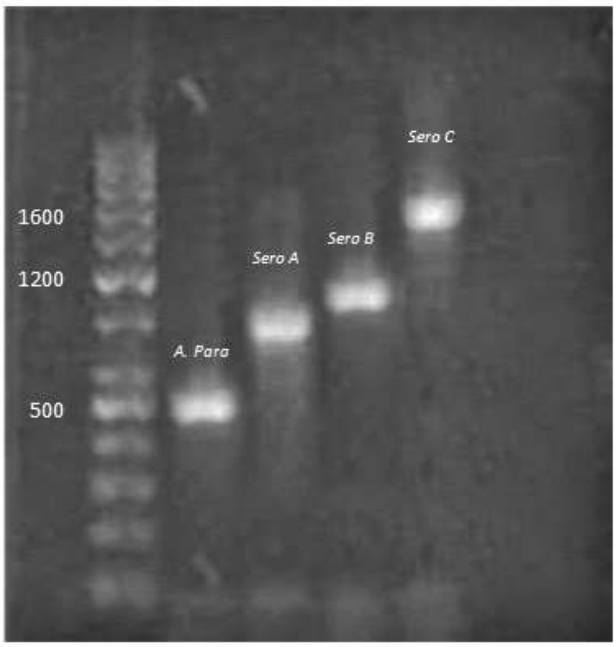

Fig 3. PCR base serotyping of ***Avibacterium paragallinarum*** in commercial birds samples by direct using specific primers.

Table 1. Serotypes percentage in different commercial birds

Commercial Birds	Serotype A	Serotype B	Serotype C	Total
Layer	56(5.6%)	67(6.7%)	69(6.9%)	19.2%
Broiler	52(5.2%)	72(7.2%)	60(6.0%)	18.4%
Pigeon	53(5.3%)	57(5.7%)	0(0%)	11.0%
Quail	0(0%)	94(9.4%)	66(6.6%)	16.0%
Chukar	79(7.9%)	0(0%)	79(7.9%)	15.8%
Total	24%	29%	27.40%	80.4%

Table 2. In vivo growth of ***Avibacterium paragallinarum*** under 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	-

serotyping and our results indicated that the serogroups A, B and C were found as suggested by the 800 bp, 1100 and 1600 bp amplicon (Fig. 3).

The results revealed that the percentage of positive samples in layers was 19.2%, followed by broilers (18.4%), quails (16.0%), chukars (15.8%) and pigeons (11.0%) (Table 1). Among positive cases, serotype A was 24%, serotype B was 29% and serotype C was 27.40%. In layer birds the prevalence of serotype A was 5.6%, serotype B was 6.7% and serotype C was 6.9%. In broiler birds the prevalence of serotype A was 5.2%, serotype B was 7.2% and serotype C was 6.0%. The pigeon samples have A and B serotypes, the quail samples have B and C serotype, and the chukar samples have A and C serotypes (Table 1).

Growth and Cultural Characteristics

Random isolates were selected to investigate general cultural characteristics of clinical isolates. Our results indicated that *A. paragallinarum* was growing normally between 24 °C to 42 °C with pH ranging 5-9 (Table 2). No growth was recorded below 24 °C and above 42 °C.

Antibiotic Sensitivity Test

Antimicrobial susceptibility through disc diffusion method was performed and interpreted as per CLSI guidelines. The 100 isolates of each serotype were tested for antibiotic susceptibility through disc diffusion. Our results indicated that isolates of all serotypes were highly susceptible to Ciprofloxacin (96%, 100% and 97% for serotype A, B and C, respectively) followed by Amoxicillin (95%, 98% and 93%), Vancomycin (94%,

Table 3. Antibiotic susceptibility pattern of ***Avibacterium paragallinarum*** serotype A, B and C.

Antibiotic	No's	Serotype Susceptibility	Resistance
Serotype A			
Tetracycline	100	91(91%)	9(9%)
Erythromycin	100	90(90%)	10(10%)
Vancomycin	100	94(94%)	6(6%)
Amoxicillin	100	95(95%)	5(5%)
Ciprofloxacin	100	96(96%)	4(4%)
Metromediazole	100	7(7%)	93(93%)
Colisten sulphate	100	6(6%)	94(94%)
Bacitracin	100	7(7%)	93(93%)
Streptomycin	100	3(3%)	97(97%)
Serotype B			
Tetracycline	100	94 (94%)	6(6.0%)
Erythromycin	100	95 (95%)	5 (5%)
Vancomycin	100	97 (97%)	3 (3%)
Amoxicillin	100	98(98%)	2(2%)
Ciprofloxacin	100	(100%)	0 (0%)
Metromediazole	100	5 (5%)	95 (95%)
Colisten sulphate	100	4(4%)	96(96%)
Bacitracin	100	3(3%)	97(97%)
Streptomycin	100	4(4%)	96(96%)
Serotype C			
Tetracycline	100	82(82%)	18(18%)
Erythromycin	100	86 (86%)	14(14%)
Vancomycin	100	90(90%)	10 (10%)
Amoxicillin	100	93(93%)	7 (7%)
Ciprofloxacin	100	97(97%)	3 (3%)
Metromediazole	100	4 (4%)	96 (96%)
Colisten sulphate	100	9 (9%)	92(92%)
Bacitracin	100	4 (4%)	96 (96%)
Streptomycin	100	6(6%)	94(94%)

97% and 90%), Erythromycin (90%, 95% and 86%) and Tetracycline (91% 94% and 82%). The *A. paragallinarum* was resistant to many antibiotics and highest resistance was observed against Bacitracin (93%, 97% and 96%) followed by Colistin sulphate (94%, 96% and 92%), Streptomycin (97%, 96% and 94%) and Metromedizole (93%, 95% and 96%) (Table 3).

DISCUSSION

Avibacterium paragallinarum is a fastidious bacterium and the particular requirement for unusual media has made its isolation and identification a costly task (Blackall, 1999). The use of PCR technique after initial isolation as an alternative of biochemical identification can minimize the complication of the diagnostic activity (Chen *et al*., 1998). Additional advantage of this technique is its speed the results are available within 24–48 h. In current study, the culture-PCR method successfully identified *A. paragallinarum* and allowed for estimates of IC prevalence.

This study also demonstrated that growth and survival of the organism is not only affected by temperature and pH of the media, but also requires NAD. We reported high occurrence of IC in commercial 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 and A. In contrast to our findings, previous reports identified 15% prevalence in Lahore, Punjab (Mustafa and Ali, 2005), 2.5% in Khushab (Abbas *et al*., 2015), and 43.3% in Jammu and Kashmir Pakistan (Ahmad *et al*., 2012). 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 excluded healthy birds 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 surveys were found in Pakistan for *A. paragallinarum*.

Antimicrobial resistance (AMR) is a growing challenge for healthcare professionals as well as livestock and poultry farmers all over the world (Ali *et al*., 2017). Situation is even worse in developing countries such as Pakistan partly due to excessive usage of antibiotics and unavailability of data on 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 using high-level antibiotics (Ali *et al*., 2016; Adnan *et al*., 2017). Very limited data on antibiotic susceptibility profile of *A. paragallinarum* is available due to the fact 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 the results indicated that more than half of the tested antibiotics were not effective suggesting that the bacteria have 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 sensitivity was observed against Ciprofloxacin followed by Erythromycin and Tetracycline. Furthermore, the antibiogram result showed that serotype A was 97% resistant to Streptomycin, serotype B was 97% resistant to Bacitracin, and serotype C was 96% resistant to Metrodiazole and Bacitracin. Our findings of high resistance to different drugs corroborated with high MIC values of Dutch poultry isolates (Heuvelink *et al*., 2018). This is also in agreement with a high level of resistance of Thai isolates against Bacitracin and Streptomycin (Chukiatsiri *et al*., 2012). More importantly, the high resistance level against Bacitracin is alarming. This is more likely due to persistent use of Bacitracin drugs for prevention of enteric diseases. There is an urgent need to further investigate possible mechanisms for the resistance against Bacitracin.

CONCLUSIONS

In conclusion, we reported high incidence rate of *A. paragallinarum* in suspected cases of IC in commercial birds. Further, PCR based serotyping indicated high occurrence of serotype B followed by C and A. All strains were found resistant to few of the important antibacterial such as Colistin, Bacitracin and Streptomycin.

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