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DETECTION AND CHARACTERIZATION OF *ORNITHOBACTERIUM RHINOTRACHEALE* IN COMMERCIAL LAYER POULTRY OF METROPOLITAN LAHORE, PAKISTAN

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Abstract

Background: *Ornithobacterium rhinotracheale (ORT) poses a significant threat to the poultry industry, causing respiratory illnesses, growth retardation, reduced egg production, and increased chicken mortality. In Pakistan, with the poultry sector's vital role in meeting protein demands, the impact of ORT infections is substantial. This study aimed to comprehensively detect and characterize ORT infections in metropolitan Lahore's commercial layer poultry. It utilized a combination of biochemical tests, including the MacConkey agar, triple iron sugar, and oxidase tests, along with molecular techniques like 16SrRNA analysis.*

Methods: *A cross-sectional study was undertaken, involving the collection of 600 tracheal swab samples from commercial chicken establishments across Lahore, Pakistan. The samples were categorized into three groups: symptomatic flocks, deceased flocks, and physically healthy flocks, each comprising 200 samples.*

Results: *The findings of the MacConkey agar test, the triple iron sugar test, and the oxidase test (confirmed by 16SrRNA PCR-analysis) offer a more accurate image of infection diagnosis compared to other biochemical approaches. The study unveiled an ORT infection prevalence of 39% (n = 78) among symptomatic flock samples, 36% (n = 72) in deceased flock samples, and 10.5% (n = 21) in physically healthy flock samples. Additionally, the study explored the antimicrobial resistance profiles of ORT isolates against commonly used drugs. The isolates demonstrated susceptibility to tetracycline (89.20%) and florfenicol (100%) but exhibited resistance to ciprofloxacin (100%), ampicillin (91.88%), amoxicillin (78.04%), enrofloxacin (72.32%), and gentamicin (100%).*

Conclusion: *The study revealed an ORT infection rate of 28.5% (n=171) in commercial poultry, marking the first report of its kind in Lahore, Pakistan. Further research is needed to develop effective strategies for ORT infection management and antibiotic stewardship practices.*

Keywords: *Avian respiratory diseases, Commercial poultry, Gram-negative bacterium, Ornithobacterium rhinotracheale*

INTRODUCTION

Ornithobacterium rhinotracheale (ORT), is an infectious, gram-negative, anaerobic pathogen associated with respiratory diseases among avian species. It is found in commercial chicken all over the world with airsacculitis and pneumonia as the most common features of infection. *ORT* was discovered in Germany in 1981 as a new pleomorphic rod that infects hens' respiratory systems (1-3). In industrial chicken farming, *ORT* has led to severe issues. It has mostly been connected to hens and turkeys and has been responsible for deaths, stunted growth, and decreased egg production throughout the world. The organism is fastidious, which makes isolation difficult (4-6). Clinical manifestations range from moderate to severe and include inflammation of the trachea, air sacs, and pericardium, exudative unilateral pneumonia, and sinusitis with purulent lesions with large death rates, particularly affecting turkeys and chickens (1, 7). Poultry respiratory diseases can have negative sanitary and financial effects, including significant financial losses, increased mortality rates, high medical expenses, and the need to put down animals (8,9). Chicken meat accounts for about 36 percent of the world's total meat production. Therefore, respiratory infections in commercial poultry constitute a global problem rather than only an issue for the biggest chicken producers. It is

unfortunate that, despite its importance, this disease has been overlooked on chicken farms due to a lack of proper diagnostic techniques.

Treatment failures have been attributed to ineffective isolation and diagnostic approaches as well as improper antimicrobial drug administration (1, 10-12). National initiatives to prevent and control avian respiratory illnesses must be incorporated. Identifying *ORT* can be challenging due to its anaerobic nature, slow growth rate, and the requirement for specific growth conditions (11). Specific and sensitive laboratory diagnostic methods are critical to acquiring more precise results. For a broiler breed to be regarded as an excellent contribution to the food source and economy, it must be disease-free (1).

The primary objective of this study was to assess the prevalence of pathogenic *ORT* infection in poultry by utilizing a combination of biochemical and molecular techniques. Furthermore, the study investigated the emergence of antibiotic resistance, a critical factor in shaping effective treatment strategies. The outcomes of this research hold significant implications for both the economic and sanitary aspects of chicken production.

MATERIALS AND METHODS

Tracheal swab samples were taken from commercial chicken outlets in the north, west, and center of Lahore, Pakistan, between February 2019 and April 2021 for this cross-sectional analysis. Samples were gathered from commercial chicken shops in Lahore, Pakistan, which were located in the city's north, west, and center. The experiments in this study were approved by the Institutional Ethical Committee for Animal Care (Letter No./D198/FIMS).

SAMPLE COLLECTION

A total of 600 tracheal swab samples were collected from freshly sacrificed and deceased commercial broilers, both with and without symptoms of coughing, sneezing, nasal discharge, conjunctivitis, and swelling of the head. Based on the method of collection, the samples were divided into three categories (symptomatic, deceased, and physically healthy broilers). Testing for bacteria was done on samples that had been obtained using dry, sterile swabs.

CHEMICALS, CULTURE MEDIA AND ANTIBIOTICS

Nutrient Agar (Oxoid, UK) served as the culture medium for bacterial growth. Antibiotics discs includes Ampicillin, Amoxicillin, Ciprofloxacin, Tetracycline, Gentamicin, Ampicillin, Florfenicol (Epico, Egypt), and Enrofloxacin (Sigma Aldrich, USA) were added to the culture media for specific experiments. For the molecular analysis, primers targeting the 16SrRNA region were custom-synthesized by Thermo-Scientific, USA. Additionally, a Taq PCR kit was procured from Thermo Fisher Scientific to carry out polymerase chain reaction processes. The Gram staining process involved the use of Crystal Violet (Merck, Germany), Gram's Iodine Solution (Sigma-Aldrich, USA), Ethanol or Ethyl Alcohol (Fisher Scientific, USA), and Safranin (VWR, USA). The Catalase test employed 3% Hydrogen Peroxide (H_2O_2) from Sigma-Aldrich, while the Triple Iron Sugar Test (TSI) utilized TSI Agar from Thermo Fisher Scientific. The Urease test was conducted using Stuart's Urea Broth with Phenol Red, also from Sigma-Aldrich, USA.

ORT ISOLATION AND CHARACTERIZATION

The tracheal swabs were streaked on nutrient agar enriched with 5% chicken blood and 10 mg/mL gentamicin at 37 °C for 48 h under 5% carbon dioxide under microaerophilic conditions (13). The suspected *ORT* colonies were identified and characterized using microbiological, biochemical, and molecular techniques (14, 15).

BIOCHEMICAL ANALYSIS

GRAM STAINING

Gram staining followed the method described by Bartholomew and Mittwer (16).

UREASE

Stuart's urea broth containing urea and phenol red as a pH indicator was employed. Positive results were indicated by a color change from yellow to pink (17).

CATALASE

A small amount of organism from an 18-to-24-hour well-isolated colony was placed on a glass slide, and 3-4 drops of 3% H_2O_2 were added. The observation focused on the formation of bubbles, indicating catalase activity (18).

OXIDASE

A piece of filter paper was immersed in 1% Kovac's solution. A drop of overnight culture was added to the filter paper treated with the reagent. The change in the color of the filter paper indicated the oxidase status as either positive or negative (18).

MACCONKEY AGAR

A plate of MacConkey's agar was inoculated with pure *ORT* culture, and the observation was centered on a color change from red to yellow (19).

TRIPLE IRON SUGAR

An isolated colony's tip was touched with an inoculation needle and then carefully stabbed into the TSI agar slant. Afterward, the slant was streaked with a needle from the medium's center to the tube's bottom. Colonies from the primary plate were examined separately. The streaked slant was incubated at 37 degrees for 18–24 hours with loose caps, and observations were made regarding any color change in the medium (20).

ANTIMICROBIAL SUSCEPTIBILITY

The antibiotic sensitivity of *ORT* isolates to tetracycline, florfenicol, ampicillin, amoxicillin, enrofloxacin, ciprofloxacin, and gentamicin was tested using the piddock (1990) disc diffusion method (21).

MOLECULAR CHARACTERIZATION: 16SrRNA PCR ANALYSIS

The 784 bp gene product, derived from isolated genomic DNA, was successfully amplified using forward and reverse primers (Thermo-Scientific) designed for the ORT 16SrRNA sequence. These primers were originally developed and published by Van Empel and Hafez (22), as outlined in Table I. The PCR amplification (on the Bio-Rad thermal cycler) was achieved after a 5-minute denaturation stage at 94 °C, followed by 45 cycles at 94 °C for 30 seconds, 52 °C for 1 minute, and 72 °C for 7 minutes (13). Ethidium bromide (0.5 g/mL) in 2% agarose gels was used to examine the amplified product. The PCR products featuring a molecular size of 784 bp indicated the presence of *ORT*, as per our identification criteria.

Table I. Primer Sequences for 16SrRNA Amplification

Primer 16SrRNA	Primer sequences
Forward primer	5′-GAGAATTAATTTACGGATTAAG-3′
Reverse primer	5′-TTCGCTTGGTCTCCGAAGAT-3′ (22)

STATISTICAL ANALYSIS

Descriptive statistical techniques, including frequency and percentage analyses, were applied to assess the prevalence of *Ornithobacterium rhinotracheale* in commercial broiler chickens. The data were analyzed using SPSS software, version 21.0.

RESULTS

ORT INFECTION FREQUENCY IN COMMERCIAL BROILERS

The frequency of *Ornithobacterium rhinotracheale* in commercial chicken flocks from various locations of Lahore was investigated using seven different methods of *ORT* detection and characterization (Table II).

Table II. *ORT* Infection Frequency in Symptomatic, Deceased, and Healthy Flocks

Test Variables	Infection Status	Symptomatic Samples	%	Deceased Samples	%	Physically Healthy	%
Gram Staining	*ORT* +ve	62	31%	56	28%	16	8%
	ORT -ve	138	69%	144	72%	184	92%
	Total	200	100%	200	100%	200	100%
Catalase	*ORT* +ve	91	45.5%	92	46%	30	15%
	ORT -ve	109	54.5%	108	54%	170	85%
	Total	200	100%	200	100%	200	100%
Oxidase	*ORT* +ve	77	38.5%	70	35%	21	10.5%
	ORT -ve	123	61.5%	130	65%	179	89.5%
	Total	200	100%	200	100%	200	100%
Triple Iron Sugar	*ORT* +ve	71	35.5%	72	36%	21	10.5%
	ORT -ve	129	64.5%	128	64%	174	87%
	Total	200	100%	200	100%	200	100%
MacConkey Agar	*ORT* +ve	74	37%	72	36%	23	11.5%
	ORT -ve	126	63%	128	64%	177	88.5%
	Total	200	100%	200	100%	200	100%
Urease	*ORT* +ve	66	33%	69	34.5%	12	6%
	ORT -ve	134	67%	131	65.5%	188	94%
	Total	200	100%	200	100%	200	100%
PCR Analysis	*ORT* +ve	78	39%	72	36%	21	10.5%
	ORT -ve	122	61%	128	64%	179	89.5%
	Total	200	100%	200	100%	200	100%

Small pin-point colonies with a butyric odor and a diameter of 1-2 mm were deemed *ORT* positive because of their transparent, greyish-white color morphology (Fig. 1a & b). Gram-staining-based bacteriological examination of 600 samples (200 per group) revealed the presence of non-motile, pleomorphic, gram-negative rods in 62 samples (31%) from symptomatic broilers, 56 samples (28%) from deceased broilers, and 16 samples (8%) from healthy broilers (Fig. 1c). In order to determine the anaerobic state of *ORT*, the catalase enzyme activity (used to track the production of catalase in aerobic organisms) was evaluated. Out of 200 samples in each group, 91 samples (45.5%) from symptomatic broilers, 92 samples (46%) from deceased broilers and 30 samples (15%) from healthy broilers were classified as *ORT* positive (catalase negative) with no bubble formation (Fig. 1d).

The Oxidase test was used to characterize the potential *ORT* colonies for the presence of cytochrome oxidase (the enzyme indophenol oxidase converts added colorless reagent into an oxidized violet colored product). *ORT* positive (oxidase positive) results were observed in 38.5% (n = 77) of the samples from symptomatic broilers, 35% (n = 70) of the samples from deceased broilers, and 10.5% (n = 21) of the samples from healthy broilers (Fig. 1e). The findings of the Triple Iron Sugar test (TSI -ve, alkaline slant without H_2S gas generation) revealed that 71 samples (35.5%) from symptomatic broilers, 72 samples (36%) from deceased broilers and 21 (10.5%) of the positive samples from physically healthy broilers had *ORT* positive status (Fig. 2a).

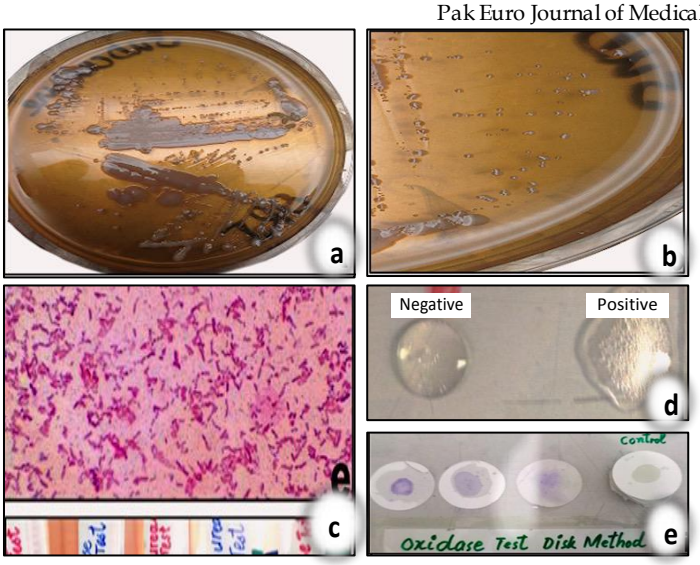

Fig. 1 *a.* The morphology of 1 to 2 mm translucent grey colonies of *Ornithobacterium rhinotracheale* isolates; b. Several tiny, isolated colonies; c. Gram staining of *ORT*; d. Catalase test of *ORT* suspected colonies (catalase negative); e. Oxidase test showing Light to dark purple color for *ORT* positive isolates (oxidase positive)

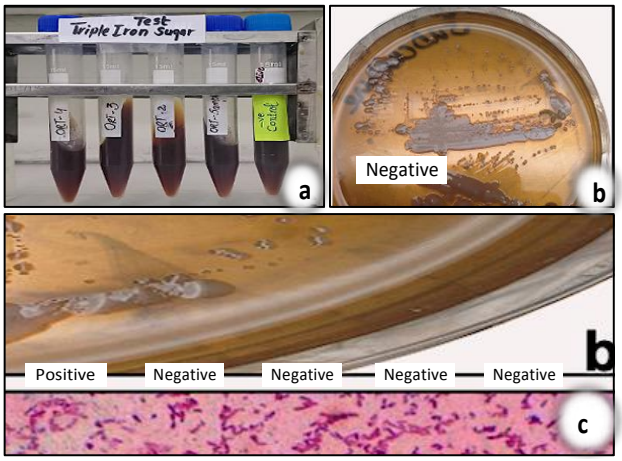

Fig. 2 *a.* No alteration in color of TSI agar (no H2S gas production); b. MacConkey agar showed no change in color; c. Urease test for ORT positive colonies showed no color change (urease negative)

The MacConkey agar test was performed to describe the suspected *ORT* colonies as gram-negative rods (*ORT* positive with no growth on MacConkey agar). The findings showed that gram-negative rods were found in 74 (37%) samples from symptomatic, 72 (36%) samples from dead, and 23 (11.5%) samples from physically healthy broilers (Fig. 2b). The Urease enzyme's activity was used to characterize the *ORT* (which regulates the conversion of urea to ammonia and carbon dioxide). In each group (600 total samples), 66 (33%) samples from symptomatic broilers, 69 (34.5%) samples from dead broilers, and 12 (6%) samples from healthy broilers were found to be *ORT* positive (Fig. 2c).

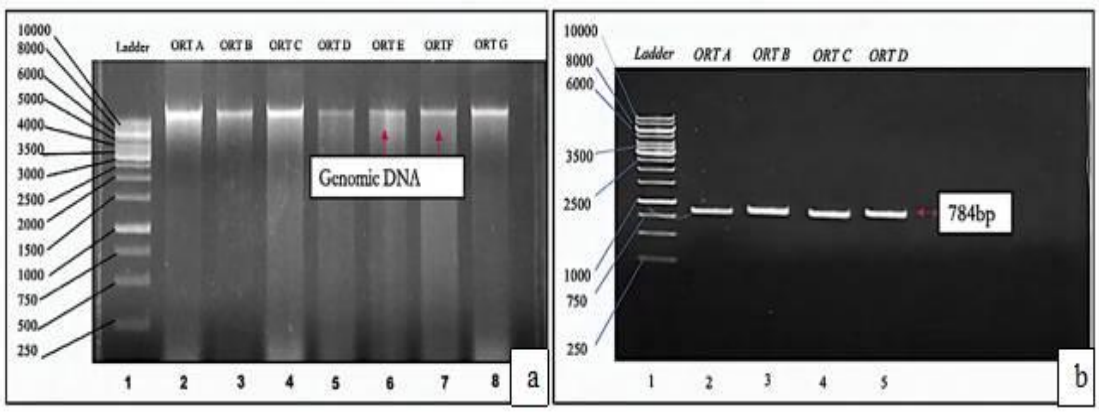

Fig. 3*a*. A 1 kb gene ruler was displayed in lane 1, and extracted genomic DNA from swab culture was shown in lanes 2 through 8; b. Lane 1 depicts a 1 kb gene ruler; lanes 2–5 display a band of the 784 base pair amplified product of 16SrRNA gene.

16srRNA based PCR analysis of molecular makeup revealed, 78 (39%), 72 (36%), and 21 (10.5%) positive ORT cases respectively in symptomatic, dead, and healthy samples from broiler flocks (Fig. 3a and b). Oxidase, MacConkey, and TSI assays were shown to be more precise when referenced with the findings of PCR analysis. Data analysis suggests that these tests are preferable to others in recognizing and categorizing the ORT infection, with outcomes comparable to PCR analysis.

PCR analysis results, as depicted in Table II, were employed to confirm the infection status across the entire study population (n=600). The cumulative infection rate for all three groups stands at 28.5%, as illustrated in Fig.4.

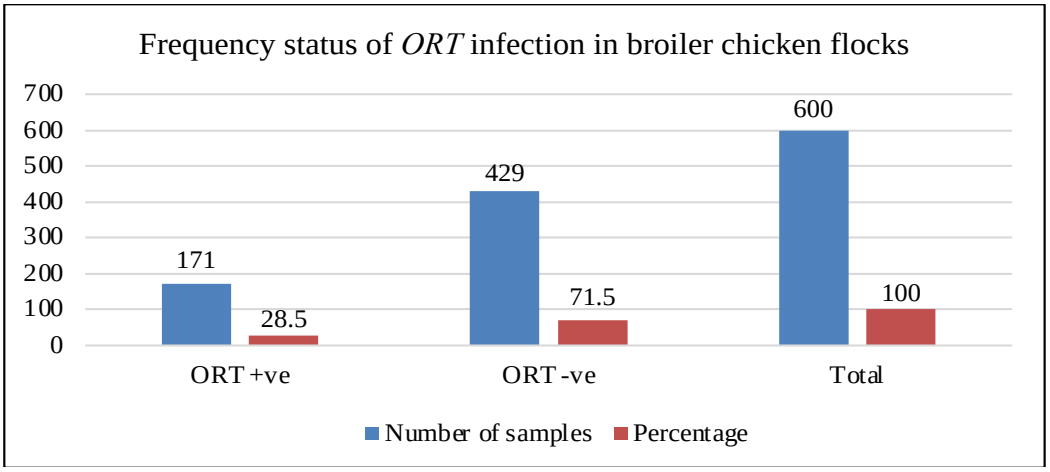

Fig. 4. *ORT* infection prevalence in broiler chicken flocks of metropolitan Lahore

ANTIMICROBIAL SUSCEPTIBILITY

The different antibiotics demonstrated considerable inhibitory effects on the susceptibility profile of the *ORT* isolates (Table III). Out of 171 positive isolates of *Ornithobacterium rhinotracheale*, 89.20% of the isolates were sensitive to tetracycline, and 100% were sensitive to florfenicol antibiotic. On the other hand, ampicillin exhibits 91.88%, enrofloxacin 72.32%, gentamicin 100%, amoxicillin 78.04%, and ciprofloxacin 100% resistance with respect to 171 positive *ORT* samples.

Table III. Antimicrobial susceptibility profile of 171 positive samples

Antibiotics	Concentration (µg)	Resistant %	Sensitive%
Amoxicillin	30	78.04	21.96
Ciprofloxacin	5	100	0.0
Tetracycline	25	10.80	89.20
Gentamicin	10	100	0.0
Enrofloxacin	5	72.32	27.68
Ampicillin	10	91.88	8.12
Florfenicol	30	0.0	100

DISCUSSION

ORT is a gram-negative rod, known for causing avian respiratory illnesses. *ORT* infections have been reported in various countries, including the United States, the Republic of Korea, Japan, Iran, and Jordan (23). In Pakistan, the poultry industry plays a pivotal role, contributing significantly to meat production, agricultural output, and the national GDP, accounting for 26.8%, 5.76%, and 1.26%, respectively. This sector is instrumental in bridging the protein supply-demand gap in the country. To be considered valuable for both food security and the economy, broiler breeds must remain disease-free (1, 24). Unfortunately, the lack of reliable identification techniques has led to the neglect of this disease within chicken farms. Consequently, issues such as improper antibiotic dosing, ineffective isolation methods, and diagnostic procedures have resulted in treatment failures (1, 10, 11). This study employed a total of seven diagnostic techniques, encompassing both biochemical and molecular approaches, to assess the prevalence of *ORT* infection in commercial layer chickens within the metropolitan region of Lahore, Pakistan. The collected samples were categorized into three groups based on their source: symptomatic flocks, deceased

flocks, and physically healthy flocks. The present study unveiled varying *ORT* infection rates across different groups, with a prevalence of 39% in symptomatic samples, 36% in deceased samples, and 10.5% in healthy flocks. The overall infection rate, encompassing all three groups (n=200/group), stands at 28.5% (n=171) based on the entire sample size (n=600). In comparison to our findings, Roussan et al. (23) reported a 14% *ORT* infection rate in Jordanian flocks, while Mayahi et al. (25) identified an *ORT* infection rate of 8.57% in Iranian chicken samples. Moreover, several studies have reported even higher infection rates (26, 27). These disparities in findings suggest the potential existence of regional variations in *ORT* infection rates, highlighting the need for further research to elucidate contributing factors. In this study, culture-based biochemical tests for *ORT* were employed, and their accuracy was validated using PCR analysis with gene-specific 16s rRNA (784 bp product) as the benchmark. The findings from the oxidase, MacConkey agar, and triple iron sugar tests closely aligned with the results obtained from the 16SrRNA PCR analysis. This consistency in outcomes mirrors previous research that utilized these biochemical tests, such as oxidase, MacConkey agar, and triple iron sugar (2, 6, 13, 22, 25, 28). These techniques have proven to be straightforward and cost-effective for identifying *ORT* infections in commercial layer poultry.

The escalating prevalence of multi-drug resistant *ORT* strains represents a significant concern in the veterinary field. Our study assessed the susceptibility profiles of *ORT* isolates to various antibiotics, yielding noteworthy results. Specifically, 89.20% of *Ornithobacterium rhinotracheale* isolates displayed susceptibility to tetracycline, while florfenicol exhibited 100% effectiveness. Conversely, amoxicillin (78.04%), enrofloxacin (72.32%), gentamicin (100%), ampicillin (91.88%), and ciprofloxacin (100%) demonstrated resistance in positive *ORT* samples. Previous research has consistently reported *ORT* isolates' resistance to enrofloxacin, amoxicillin, ciprofloxacin, and gentamicin, along with susceptibility to tetracycline and florfenicol, aligning with our current findings. However, it's worth noting that amoxicillin and tetracycline have shown varying resistance patterns in previous studies (25, 29-31). Instead, Umar and colleagues validated the sensitivity of tetracycline in Pakistani strains of *ORT* in lapwing in 2017, aligning with our current study's results (32). Nevertheless, there remains a necessity for refining the current *ORT* susceptibility classification to enhance treatment efficacy. Discrepancies between our study and others may be attributed to variations in research locations, sample genetic compositions, sample sizes, and diagnostic methodologies. These factors collectively underline the importance of region-specific and tailored approaches in tackling *ORT* infections effectively.

CONCLUSION

This study provides a conclusive assessment of *ORT* infection prevalence in commercial layer chickens within the Lahore metropolitan area of Pakistan, revealing an overall rate of 28.5%. Notably, the infection rates among distinct flock categories were observed at 10.5% for healthy flocks, 39% for deceased chickens, and 36% for symptomatic flocks. The utility of the triple iron sugar, MacConkey agar, and oxidase tests emerged as more accurate, reliable, and cost-effective methods, aligning closely with the outcomes of 16SrRNA PCR analysis. The study further underscores the importance of employing a combination of biochemical and molecular techniques for precise *ORT* isolation and identification. Additionally, the susceptibility of all *ORT*-positive samples to tetracycline and florfenicol highlights potential treatment avenues. These findings collectively emphasize the need for effective *ORT* infection control strategies, including enhanced in-situ examination and testing for *ORT* presence.

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Authors contribution:

Conceptualizations; AH (Aroosha Hussain) and ZQS (Zahoor Qadir Samra); execution of experiments and curation of data AH (Aroosha Hussain); Drafting of Manuscript AH (Aroosha Hussain); reviewing and

editing, AH (Amina Hussain); Software, AH (Amina Hussain); Supervision, ZQS. All authors have approved the final version of the manuscript.

Conflicts of Interest:

The authors declare no competing financial or non-financial interests.

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