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Review on antibiotic resistance pattern and virulent genes content in avian pathogenic escherichia coli (APEC) from broiler chickens in Chitwan, Nepal

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Abstract
The aim of the study is to evaluate various pattern of Antimicrobial resistance of Eschirichia coli. The rational use of different generation and different groups of antimicrobials in poultry led the E.coli to be potentiated itself to a significant degree of resistance. The strain of concern was Avian Pathogenic E.coli (APEC) capable of establishing itself outside the intestine on immunocompromised bird. Avian colibacillosis is a complex syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis. Initial viral infections, environmental factors as well as constitution of poultry may influence appearance of avian colibacillosis. However, on terms of virulence a single APEC can have different variations and there is still to learn more about. Virulence factors of APEC known are adhesins (F1- and P-fimbriae), iron acquisition systems (siderophores), hemolysins, resistance to the bactericidal effects of serum and phagocytosis. On the basis of genetic criteria, E. coli isolates are considered pathogenic containing at least five virulence APEC genes namely iss, iucd, hlyf, ompt, iron and iuta. These virulent APEC is causing significant loss in poultry industry. Also there is chance of cross resistance of these virulent APEC with human enteric E. coli if unjudicious use of antimicrobial will go on.

Keywords: Antimicrobial, Avian Pathogenic Eschirichia coli (APEC), cross resistance, virulence

Introduction
Avian colibacillosis is a complex syndrome characterized by multiple organ lesions with airsacculitis and associated pericarditis, perihepatitis and peritonitis. Among various subgroups of extraintestinal pathogenic E.coli (ExPEC), Avian Pathogenic E.coli (APEC) is one of the major subgroup that affects poultry. It uses different routes including respiratory and genital tracts causing various extraintestinal diseases i.e. colibacillosis in poultry leading to significant loss in poultry industry (Stehling, Campos, Azevedo, Brocchi, & Silveira, 2007) [25]. Commensal E.coli have become virulent APEC due to pathogenicity of APEC and is infecting the extraintestinal niche by expressing various putative virulence factors(Dziva & Stevens, 2008) [9].

Number of virulence traits are present which can be categorized as adhesion, iron acquisition, hemolysis, protection from bactericidal factors of host and production of toxins(Johnson & Nolan, 2009) [14]. However, on terms of virulence a single APEC can have different variations and there is still to learn more about. The pathogenic ability of E.coli strain is facilitated by broad range of virulence factors which are coded by virulence – associated genes(iutA, iss, papC, iucD, tsh, irp-2, ompT, hlyF, iron, cva/cvi and astA) (Subedi, Bhattarai, Devkota, Phuyal, & Luitel, 2018) [26]. *Tsh* gene also known as temperature sensitive haemagglutinin plays a role in colonization of air sacs(Dozois *et al*., 2000) [8]; similarly *pap* i.e. P- fimbriae plays a role in adhesion to internal organs at later stage of infection (Dho-moulin *et al*., 1999) [7]. The resistance shown by pathogenic E.coli towards complement which helps them survive the phagocytosis is linked with col V (*SA. Pourbakhsh et al., 1997*) and the increased serum survival gene iss is linked with serum resistance (Barbieri *et al*., 2013) [3]. Based on genetic criteria, the pathogenicity of APEC strain is determined by presence of at least five virulence genes (De Carli *et al*., 2015) [6]. Also some of the avian and human extraintestinal pathogenic E. coli (ExPEC) has a similar phylogenic backgrounds and shares similar virulence genes

possessing zoonotic risk (Griffin, Manges, & Johnson, 2012) [11].

Antibiotics are amazing drugs to tackle microbes. These are either bactericidal or bacteriostatic drugs that acts by inhibiting the bacterial cell wall synthesis, protein synthesis, DNA/RNA synthesis by cell membrane disruption or by any other specific mechanisms. Antibiotics are like magical spears that selectively target the microorganism responsible for disease occurrence and at the same time it do not effects host. There is a huge use of antimicrobials around the world in poultry industries including country like Nepal (Subedi, Bhattarai, *et al*., 2018) [26]. These antibiotics are usually used in the poultry industries in order to combat the difficulties posed by APEC strains. Several studies have reported that antibiotics have been used in chicken as growth promoter and disease preventive measures (Osti, Bhattarai, Chaudhary, & Singh, 2017) [20]. However, imprudent use of antimicrobials in food producing animals resulted in hazards like alteration of intestinal microflora, appearance of antibiotics residues in food products, threatening impact on public by the evolution of antimicrobial resistance (Miles, McLaughlin, & Brown, 2006) [18]. The most concerning fact is that the multiple antibiotic resistance microbes has challenged in the treatment protocol of zoonotic diseases and its transmission from food animal source to human have made the situation even more threatening in health sectors (CDC, 2013).

In the developing country like Nepal where poultry industries are emerging very rapidly, there is a huge consumption of antibiotic growth promoters. In Nepal, Chitwan, Kathmandu and Kaski districts are the major areas of poultry farms (Osti *et al*., 2017) [20]. Among the diseases reported, the outbreak of colibacillosis is one of the highly reported poultry disease in Nepal (Mellata, 2013) [17]. There are very few literatures available in regarding assessment and investigation of APEC in the context of Nepal. Moreover investigation trend for the pathogenicity of APEC in Nepal is based on clinical symptoms and isolation of E. coli from fecal samples (*Recent Case Flow Pattern in Veterinary Teaching Hospital of*, 2017). Also investigation of virulent genes and molecular detection of APEC strains from broiler chicken of Nepal have not been reported till now except subedi *et al.*, 2018.

A. Methods

1. Sample collection

Liver samples can be collected from colibacillosis suspected broiler chickens

2. Isolation

Obtain the swab from liver sample aseptically and streak in MacConkey agar. Then incubate aerobically at 37^0 C for 24hrs. The pure colonies are further streaked in eosine methylene blue (EMB) agar and incubate overnight at 37^0 C.

3. Identification

If green metallic sheen is observed on EMB agar then it is suspected as E. coli strains which can be further confirmed by microbiological methods like Gram staining, study of colony morphology and biochemical tests (indole, methyl red, Voges-Proskauer, citrate, catalase, oxidase and motility indole ornithinase test) (Overdevest *et al*., 2011) [21].

B. Antibiotic susceptibility testing

It is performed by using methods like Kirby-bauer disk diffusion method(CLSI, 2015). Antibiotics that are extensively used in poultry feed are selected (Shrestha *et al*., 2017) [24].

C. Detection of virulence genes

From among the isolated strains of colibacillosis investigation procedure is put forward for the detection of virulence genes. At first genomic DNA is extracted from the pure colonies with the aid of Dneasy Blood and Tissue Kit. Then using gel electrophoresis and appropriate measuring absorbance ratios by spectrophotometer quality of genomic DNA is checked. By using conventional PCR target primers of virulence genes are amplified (Ferreira *et al*., 2015) [10]. The PCR is run in 25µl volume comprising 12.5µl Hot start Taq 2X master mix(BioLab Inc., New England), 1µl each primer, 2µl DNA template and 8.5µl nuclease free water (Subedi, Luitel, *et al*., 2018) [26]. Now PCR amplifications is done in Multigene Optimax Thermal Cycler keeping identical cycling conditions for all samples as follows: 94^0 C for 4 min; 35 cycles of 30 sec at 94^0 C, 1 min at 60^0 C and 2 min at 68^0 C and 72^0 C for 7 min and then amplicons by using agarose gel electrophoresis with 1.5% agarose gel prepared in 1× TBE buffer are analyzed (Subedi, Luitel, *et al*., 2018) [26]. Staining of all PCR products by ethidium bromide is done and after using electrophoresis, all the bands obtained are visualized and photographed under UV light (Mohamed, Shehata, & Rafeek, 2014) [19]. Under UV light photography if the amplicons that are amplified produce the band of the expected size then the amplified product is ensured to have virulence gene.

Table 1: Primer sets for detection of virulence genes (Jeong, Kim, Kim, & Kwon, 2014) [13]

Gene	Primer sequence (5`-3`)	Amplicon size
FimC	F: GGAAATAACATTCTGCTTGC R: TTTGTTGCATCAAGAATACG F: GGAAATAACATTCTGCTTGC R: TTTGTTGCATCAAGAATACG F: GGAAATAACATTCTGCTTGC R: TTTGTTGCATCAAGAATACG F: GGAAATAACATTCTGCTTGC R: TTTGTTGCATCAAGAATACG F: GGAAATAACATTCTGCTTGC R: TTTGTTGCATCAAGAATACG	288
fyuA	F: CAACATCGTCACCCAGCAG R: CGCAGTAGGCACGATGTTGTA	949
HlyF	F: GGCGATTTAGGCATTCCGATACTC R: ACGGGGTCGCTAGTTAAGGAG	599
Irp-2	F: AAGGATTCGCTGTTACCGGAC R: TCGTCGGGCAGCGTTTCTTCT	280
Iron	F: AAGTCAAAGCAGGGGTTGCCCG R: GACGCCGACATTAAGACGCAG	667
Iss	F: AGCAACCCGAACCACTTGATG R: TAATAAGCATTGCCAGAGCGG	329
iucD	F: GTGAGTTGTACCACCGTTTT R: CCATTCCAGAGTGAAGTCAT	278
Lt	F: ATGAGTACTTCGATAGAGG R: ATG GTATTCCACCTA ACGC	279
ompT	F: ATCTAGCCGAAGAAGGAGGC R: CCCGGGTCATAGTGTTCATC	559
St	F: TCTGTATTGTCTTTTTCACCTTTC R: TTAATAGCACCCGGTACAAGC	165
Stx1A	F: CAGTTAATGTGGTGGCGAAG R: CTGCTAATAGTTCTGCGCATC	895
Stx2A	F: CTTCGGTATCCTATTCCCGG R: GGATGCATCTCTGGTCATTG	482
Tsh	F: GGGAAATGACCTGAATGCTGG R: CCGCTCATCAGTCAGTACCAC	420
Vat	F: TCCTGGGACATAATGGTCAG R: GTGTCAGAACGGAATTGT	981
chuA	F: GACGAACCAACGGTCAGGAT R: TGCCGCCAGTACCAAAGACA	279
yjaA	MF: TGAAGTGTCAGGAGAYGCTG MR: ATGRAGAATGCGTTCCTCAAC	211
tspE4C2	F: GAGTAATGTCGGGGCATTCA MR: CGCGYCAACAAAGTATTRCG	152

Table 2: Primer sets for detection of target virulence gene(Subedi, Luitel, *et al*., 2018) [26]

Genes	Primer sequence (5`-3`)	Amplicon size (bp)
IutA	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	302
Iss	F: TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA	323
papC	F: TGATATCACGCAGTCAGTAGC R: CCGGCCATATTCACATAA	501
iucD	F: ACAAAAAGTTCTATCGCTTCC R: CCTGATCCAGATGATGCTC	714
Tsh	F: ACTATTCTCTGCAGGAAGTC R: CTTCCGATGTTCTGAACGT	824
Irp-2	F: AAGGATTCGCTGTTACCGGAC R: AACTCCTGATACAGGTGGC	413
ompT	F:TCATCCCGGAAGCCTCCCTCACTACTAT R: TAGCGTTTGCTGCACTGGCTTCTGATAC	496
hlyF	F: GGCCACAGTCGTTTAGGGTGCTTACC R: GGCGGTTTAGGCATTCCGATACTCAG	450
Iron	F: AATCCGGCAAAGAGACGAACCGCCT R:GTTCGGGCAACCCCTGCTTTGACTTT	553
Cva/cvi	F: TGGTAGAATGTGCCAGAGCAAG R: GAGCTGTTTGTAGCGAAGCC	1181
astA	F: TGCCATCAACACAGTATATCC R: TCAGGTCGCGAGTGACGGC	116

D. Statistical Analysis

The standard data entry and analyzing software like Microsoft Office Excel 2010 and Chi- square test can be used for statistical analysis.

Results

Table 3: Antibiogram profile revealing Antibiotic resistance to several antibiotics(Subedi, Luitel, *et al*., 2018) [26]

Antibiotics	Resistance (%)
Amikacin	16%
Nitrofurantoin	47%
Ciprofloxacin	57%
Levofloxacin	49%
Gentamicin	56%
Ampicillin	98%
Cotrimoxazole	85%
Doxycycline hydrochloride	60%
Colistin	50%

Table 4: Antimicrobial resistance of E. coli isolate(Azam & Mohsin, 2019) [2]

Antibiotic	Resistance%
Ampicillin	98.6%
Tetracycline	97.3%
Ciprofloxacin	72%
Chloramphenicol	69.3%
Sulphamethoxazole-Trimethoprim	67%
Colistin	67%
Streptomycin	62%
Cefotaxime	28%
Ceftriaxone	23%
Gentamicin	10%
Imipenem	0%

Table 5: Virulent gene content in broiler chicken in Chitwan,Nepal (Subedi, Luitel, *et al*., 2018) [26]

Genes	APEC isolates (*n* = 45) n(%)	Non-APEC isolates (*n* = 5) n(%)	Total (*n* = 50) n(%)
iutA	37(82.2)	1 (20)	38 (76)
iss	45 (100)	0 (0)	45 (90)
papC	25 (55.6)	0 (0)	25 (50)
iucD	44 (97.8)	3 (60)	47 (94)
tsh	28 (62.2)	0 (0)	28 (56)
irp-2	33 (73.3)	3 (60)	36 (72)
ompT	45 (100)	4 (80)	49 (98)
hlyF	45 (100)	3 (60)	48 (96)
iroN	45 (100)	1 (20)	46 (92)
cva/cvi	26 (57.8)	2 (40)	28 (56)
astA	43 (95.6)	2 (40)	45 (90)

Table 6: Virulent gene content in chicken in Brazil(De Carli *et al*., 2015) [6]

Genes	Frequency, n=74, n%
hlyF	43(58.1%)
sitA	48(64.9%)
ompT	42(56.8%)
Iron	39(52.7%)
Iss	38(51.4%)
Tsh	23(31.1%)
iutA	31(41.9%)
fyuA	31(41.9%)
cvaC	36(48.6%)
irp-2	27(36.5%)

Discussion

In the recent trend poultry industries are commercializing very rapidly in southeast Asian countries, including country like Nepal. With the rise in poultry farming antimicrobial use in chicken is expected to rise by 129% by 2030 in Asia-Pacific region (Boeckel, Brower, Gilbert, Grenfell, & Levin, 2015) [4]. So there is great challenge in upcoming days to fight against antimicrobial resistance. Varying degree of antimicrobial resistance is shown by APEC in different research conducted. Multiple antibiotic resistance patterns showed 94% of isolates were resistant to three or more antimicrobials which is really a threatening situation in Chitwan district in Nepal (Subedi, Bhattarai, *et al*., 2018) [27] which also reveals the face of Nepal. Most of the research showed significant degree of resistance to commonly used antimicrobial like Ampicillin, Tetracycline, Cotrimoxazole, Colistin, Ciprofloxacin, Gentamicin, etc that clearly is warning the globe. There is not the single antibiotic tested that showed 100% effectiveness,Table.3 (Subedi, Luitel, *et al*., 2018) [26] except Imipenem Table.4 (Azam & Mohsin, 2019) [2]. Also most of the research have shown that virulence genes namely iss, iucD, hlyF, ompT and iron are mostly associated with the virulence of APEC.

In the context of Nepalese condition there is an unorganized and haphazard veterinary market (Ramdam N, 2015) [22]. About USD 1 billion per year i.e. 30% of total value of medicine imports is occupied by antibiotics in Nepal(Acharya & Wilson, 2019). According to a genuine study that examined prescription behavior of several drug dispensers in Biratnagar,

Kathmandu, Chitwan, Pokhara and Surkhet district found around 46% of veterinary medicine were sold under self prescription and 12% on farmer demand (Ramdam N, 2015) [22]. There are very few research on assessment and investigation of antimicrobial resistance and virulent genes posed by APEC in Nepal. In Nepal, issue of antimicrobial resistance is often neglected as compared to various other public health toplist. Also there is no any such strict legal standards established that could monitor the way drugs are being sold in Nepalese market.

Moreover, there is high risk for the emergence of antimicrobial resistance as poultry meat is becoming popular day by day due to its low cost and palatability among public possessing the zoonotic threat. Chicken is the most commonly farmed species, with over 90 billion tons of chicken meat produced per year and Nepal`s poultry meat production in 2014 was 43360 tons (FAO, 2017). Recent studies have focused that poultry meat can also be the source of ExPEC strain transmission to humans (Manges & Johnson, 2015) [15]. Poultry source E.coli isolates have been shown to be involved in causing urinary tract infections, sepsis and meningitis in murine models that copy the human ExPEC infections (Jakobsen, Hammerum, & Frimodt-møller, 2010) [12] but exactly it is difficult to explain the human health risk posed by poultry product since there is no documentation of direct transmission of ExPEC from poultry to human(Manges & Johnson, 2015) [15]. Also one study showed that even if APEC consumed from poultry does not result in disease, cells surviving the passage of intestine will have the chance to transmit and recombine plasmids, leading to the conversion of commensal organisms into pathogenic or drug resistant APEC strains (Marshall & Levy, 2011) [16].

Conclusion

Studying the research in Nepal as compared to those of other countries, Nepal is also on serious stage on upcoming days regarding growth of antimicrobial resistance and virulence of E.coli in poultry sector. Till now antibiotics are serving as a weapons for us. There is very problematic situation rising in the form of antimicrobial resistance due to which that day is not far when we are going to be weaponless. We are heading slowly towards our extinction in near future. So a strong policy and far sighted intervention strategies is the must. A comprehensive approach is essential to tackle the risk of avian colibacillosis in Nepal. This is only possible when we bring farmers, all veterinary manpower, responsible government regulatory bodies, drug importers and distributors and hatchery workers under the mainstream. Safety first so that we can last.

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