OCCURRENCE OF *CAMPYLOBACTER* SPP. IN CHICKEN EGG PRODUCTION CHAIN

JOBY ELIZABETH JOHN
(14-MVM-26)

THESIS
Submitted in partial fulfilment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE
(Veterinary Public Health)
2016

Faculty of Veterinary and Animal Sciences
Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY PUBLIC HEALTH
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR – 680 651
KERALA, INDIA

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ABSTRACT OF THE THESIS

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DECLARATION

I hereby declare that this thesis, entitled **"Occurrence of *Campylobacter* spp. in chicken egg production chain"** is a bonfide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Occurrence of *Campylobacter* spp. in chicken egg production chain"** is a record of research work done independently by Joby Elizabeth John (14-MVM-26)**,** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Mannuthy

Dr. C. Sethulekshmi
Chairman
Advisory Committee

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Joby Elizabeth John (14-MVM-26),** a candidate for the degree of Master of Veterinary Science in Veterinary Public Health, agree that this thesis entitled **"Occurrence of *Campylobacter* spp. in chicken egg production chain"** may be submitted by **Joby Elizabeth John (14-MVM-26)** in partial fulfilment of the requirement for the degree.

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DGEMENTS
strengthens me" Philippians 4:13

ghty Lord *for the infinite blessings*
providing me stren

EXTERNAL EXAMINER

I consider myself greatly privileged and fortunate to have worked under the supervision and guidance of ***Dr. C. Sethulekshmi****, Assistant Professor, Department of Veterinary Public Health and Chairperson of Advisory Committee, for her kind affection, inspiring words, invaluable guidance, whole-hearted encouragement, generous support, consideration and for all the trust she has in me. She has been a strong and supportive advisor to me from the commencement of this research and has always given me great freedom in my work.*

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Joby Elizabeth John

Dedicated To My

Beloved Husband

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1. INTRODUCTION

Foodborne diseases are an important cause of morbidity, mortality and an obstacle to socio-economic development worldwide. The global burden of foodborne diseases has resulted in a loss of 33 million healthy life years and 4.2 lakh deaths in 2010 (WHO, 2015). In developing countries, foodborne and water-borne diseases together account for 1.8 million deaths mainly of infants and children. Among this, Campylobacteriosis is the second leading zoonotic enteric infection in both developing and developed countries.

Eggs are considered as a natural source of high quality protein, containing all essential amino acids, fats, vitamins A, D, E, choline, folate, iron, calcium, magnesium and zinc. India is the fifth largest producer of eggs and ranks 17th in world poultry production. Over the past decade, the poultry industry in India has contributed approximately US$229 million to the Gross National Product. It is estimated that the egg consumption will rise from 34 billion in 2000 to 106 billion in 2020.

Campylobacter spp. is one of the major foodborne pathogen encountered in poultry. Most of the cases are caused by *Campylobacter jejuni*, and to a lesser extent by *Campylobacter coli*. These microaerophilic organisms prefer three to 15 per cent oxygen and five to 10 per cent carbon dioxide concentration. The preferable temperature for the growth of this thermophilic *Campylobacter* spp. is 34 to 44^{o}C, with an optimum temperature of 42^{o}C. The organism gets destroyed above 48^{o}C and is capable of surviving for a shorter time at refrigeration temperature. Optimum pH and minimum water activity required for the growth of the organism is 6.5 to 7.5 and 0.987, respectively. *Campylobacter* spp. is extremely sensitive to acidic conditions, desiccation and high relative humidity. These fastidious organisms require complex growth media and are unable to ferment carbohydrates.

When colonizing the intestines, enteric *Campylobacter* spp. is anticipated to express several putative virulence factors. The cork screw forms of the *Campylobacter* spp. along with the flagella will help in colonization in the intestine. The organism is capable of crossing, adhering and subsequently invading the epithelial cells resulting in mucosal damage and inflammation. They produce cytotoxin, enterotoxin or both of these. The important virulence factor associated with serum resistance, endotoxicity and adhesion are the lipo-oligosaccharide and lipopolysaccharide of the outer membrane. The super oxide dismutase protein is the main component of stress defence by *Campylobacter* spp. Expression of heat shock protein will help the organism in thermal stress response.

Campylobacteriosis in humans are caused by the consumption of contaminated food or water. The most common vehicle for the spread of infection is the foods of poultry origin. Direct contact with infected animals and person-to-person transmission can also occur. *Campylobacter* infection was significantly higher in children under the age of five years. According to the estimates of CDC in 2010, *Campylobacter* spp. is responsible for 76 deaths in United States each year. HIV-positive individuals are highly predisposed to *Campylobacter* infection than the general healthy population.

Campylobacter spp. require low infectious dose of about 500 organisms to cause human infection. Despite this low infectious dose and ubiquity of *organism* in the environment, most of the infections occur as isolated or sporadic events. But very large outbreaks have been documented as a result of consumption of contaminated milk or unchlorinated water supplies.

Incubation period of *Campylobacteriosis* in humans ranges from one to 10 days and the infection will *lead to abdominal pain, severe diarrhoea and lethargy.* Although most cases are self-limiting, up to 20 per cent have a prolonged illness or a relapse; and two to 10 per cent may be followed by chronic sequelae, resulting in autoimmune conditions known as Guillain-Barré syndrome (GBS) and Miller Fisher

syndrome. An estimated one in 1000 patients with *Campylobacter* infection develop Guillain-Barré syndrome. According to 2010 estimates of CDC, about 40 per cent cases of GBS in United States may be associated with *Campylobacter* infection. Another chronic condition associated with *Campylobacter*iosis is Reiter's syndrome, a form of reactive arthritis.

Poultry which are considered as the main reservoir host for *Campylobacter* spp. are capable of shedding these organisms asymptomatically in their faeces. The pathogen may even enter the egg production chain or can contaminate foods of poultry origin. Identification of critical control points at which a control can be applied to eliminate this organism in the final product is essential to prevent epidemics of infections.

The colonization of the organism in the animals or birds at the farm level should be reduced by adopting strict biosecurity measures and hygienic practices. The infection control measures at all stages of food processing such as pasteurization of milk, chlorination or ozonation of water, proper refrigeration and adequate cooking of meat and poultry products may help to reduce the occurrence of *Campylobacteriosis.*

There is dearth of information regarding the occurrence of *Campylobacter* spp. and critical control points of this organism in egg production chain. Also there is only limited data available on the occurrence of *Campylobacter* spp. in eggs from retail market. By considering all these factors the present study was undertaken with the following objectives.

1. Identification of critical control points (CCPs) of *Campylobacter* spp. in chicken egg production chain
2. Detection of *Campylobacter* spp. in chicken eggs from the retail markets
3. Molecular confirmation and antibiotic sensitivity of the isolates

2. REVIEW OF LITERATURE

Campylobacter has become the leading cause of enteritis among the food-borne zoonotic pathogens. Consumption of undercooked chicken or other foods cross-contaminated with raw chicken meat and egg during food preparation results in sporadic occurrence. It is associated with watery or bloody diarrhoea, abdominal cramps and nausea. Post-infection complications include Guillain-Barré syndrome, an acute demyelinating disorder of peripheral nerves and Miller Fisher syndrome, the non-paralytic variant. The main objective of the study was to determine its occurrence in poultry, egg production chain, egg and other environmental samples and to study the efficiency of different antibiotics on *Campylobacter* spp.

This chapter aims to provide an overview of the various methods for the detection of *Campylobacter* spp., its possible sources of contamination in egg production chain, microbiological quality of eggs and finally the antibiotic sensitivity pattern.

2.1. DETECTION METHODS FOR *CAMPYLOBACTER*

In 1886 Escherich discovered *Campylobacter* like organisms from stool samples of children with diarrhoea. Since then a wide variety of medias as such or with some modifications have been used for isolation and identification of *Campylobacter* spp. These methods are briefly described under the below mentioned headings.

2.1.1. Conventional culture techniques

Skirrow (1977) developed the selective culture medium for isolating *Campylobacter* from faecal samples using blood agar supplemented with vancomycin (10 mg/l), polymyxin B (2-5 IU/ml) and trimethoprim (5 mg/l). The incubation was done under microaerophilic conditions with five per cent oxygen, 10 per cent carbon dioxide and 85 per cent hydrogen at 43^{o}C overnight. *Campylobacter* spp. was recovered from 57 out of the 803 patients with diarrhoea.

Bolton and Robertson (1982) designed Preston medium for isolating *Campylobacter jejuni* and *Campylobacter coli*, a blood based medium by incorporating the antibiotics polymyxin, rifampicin, trimethoprim and actidione. Microaerophilic conditions were provided at 43^{o}C under six per cent oxygen, 10 per cent carbon dioxide and 84 per cent hydrogen for 48 h. The Preston medium was found to be more selective and superior than Skirrow's medium for *Campylobacter* isolation from all kind of samples.

Bolton and Coates (1983) investigated on the carbon dioxide requirement by different thermophilic *Campylobacter* spp. grown on Columbia agar with five per cent horse blood. Anaerobic jar was used for creating the microaerophilic conditions for 24 h at 42^{o}C. Five to 10 per cent oxygen and one to 10 per cent carbon dioxide was found to be the optimum concentration of gases required for the growth of organism.

Bolton *et al.* (1984) developed the first blood-free selective medium for the isolation of *Campylobacter*, containing charcoal known as charcoal-cefazolin-sodium deoxycholate agar (CCD agar). Selectivity of the medium was due to the presence of cephazolin (10 mg/L) and 0.1 per cent sodium deoxycholate. The temperature studies revealed that the isolation rate of *Campylobacter jejuni* from human faecal sample was maximum at 42^{o}C for 48 h. Efficiency of CCD agar was compared with Preston agar, a blood containing medium and found similar isolation rates on both medias, but less selectivity for CCD agar.

Hutchinson and Bolton (1984) found that by replacing the cephazolin with cefoperazone (32mg/L), selectivity of CCD agar can be increased. Maximum isolation rate was obtained at 48 h incubation period for both the modified CCD agar and Preston agar but the isolation rate of modified CCD agar was greater at 42^{o}C and 37^{o}C.

Karmali *et al.* (1986) designed a charcoal based selective medium for the isolation of *Campylobacter jejuni* and *Campylobacter coli* from faecal samples by

incorporating activated charcoal, hematin, sodium pyruvate, cefoperazone, vancomycin and cycloheximide in Columbia agar base. Incubation was done in anaerobic jars at 43^{o}C under seven per cent oxygen, 10 per cent carbon dioxide, 25 per cent nitrogen and 58 per cent hydrogen. The medium was more selective than Skirrow medium and charcoal was found to be the best substitute for blood in medias.

Jeffrey *et al.* (2000) designed an aerobic enrichment medium by incorporating the antibiotics rifampin, trimethoprim, cephalothin and polymyxin B and the antifungal compound amphotericin B for isolating *Campylobacter jejuni*.

Martin *et al.* (2002) evaluated the efficiency of amphotericin B (10mg/L) as the antifungal agent in *Campylobacter* selective media by replacing cycloheximide. Both these agents were able to inhibit fungal growth adequately but not at all able to eliminate all non-*Campylobacter* organisms.

Oyarzabal *et al*. (2005) reported that modified Campy-Cefex (mCC) agar and modified charcoal cefoperazone deoxycholate agar (mCCDA) appeared to be more selective and less costly for direct isolation of *Campylobacter* spp. from poultry carcass rinses. Modified Campy-Cefex agar was prepared by replacing the antifungal agent cycloheximide with amphotericin B and laked horse blood with lysed horse blood of Campy-Cefex agar.

Adzitey *et al.* (2011) swabbed the large intestine of Pekin ducks on mCCDA supplemented with mCCDA supplement and mCCDA supplemented with *Campylobacter* growth supplement (Ferrous sulphate, sodium metabisulphite and sodium pyruvate, FBP) and microaerophilic incubation was done at 42^{o}C for 48 h. The isolation rate of *Campylobacter* was relatively better in mCCDA supplemented with mCCDA supplement.

Chon *et al.* (2012) modified the mCCDA by supplementing two vials of polymyxin B (50,000 IU/vial) to one litre of mCCDA (P-mCCDA). The efficiency of P-mCCDA was compared with Campy-Cefex agar and mCCDA by streaking 10^{4}

cells of each of 44 *C. jejuni* and 88 *C. coli* strains from chicken carcass rinses and incubated the plates at 42^{o}C for 48 h under microaerobic conditions. The medium showed higher isolation rate and reduced growth rate of competing organisms, indicating the superior selectivity.

2.1.2. Polymerase Chain Reaction (PCR)

Giesendorf *et al*. (1992) first reported a PCR assay for the rapid detection and identification of *Campylobacter* spp. in chicken products from Netherlands. The samples were subjected to a short enrichment at 42.5^{o}C for 18 h under microaerobic conditions prior to PCR technique, based on *16S rRNA* gene amplification. At an annealing temperature of 52^{o}C, only *C. jejuni*, *C.coli* and *C. lari* produced 426-bp amplified products. Out of the 45 chicken samples, 80 per cent were positive with both the conventional and molecular methods.

Oyofo and Rollins (1993) described the first application of PCR for the specific detection of *C. jejuni* and *C. coli* from environmental water samples in United States. The assay targeted *fla*A gene and was demonstrated to be specific and sensitive for identification of these two species. Out of the 23 water samples from poultry farm, 52 per cent were positive for *Campylobacter* spp.

Blom *et al*. (1995) identified *C. fetus* by PCR-based DNA-probe method targeting a fragment of gene coding for *16S rRNA*. *Campylobacter fetus* specific oligonucleotide probe used in dot blot hybridization was labeled with digoxigenin, which was specific for *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*.

Linton *et al.* (1996) designed genus specific PCR assay using *16S rRNA* gene sequence to amplify an 816-bp amplicon from *Campylobacter* species. The concentrations of nucleotides and primers were 200 and 0.4 µ*M*, respectively and 0.625U *Taq* DNA polymerase was used per reaction. The 25 µl reaction volume contained 25 ng genomic DNA, 20 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl and 2.5 m*M* $MgCl_2$. The PCR conditions were 25 cycles of amplification involving denaturation at

94^{o}C for one minute, annealing temperature ranges from 55-65^{o}C depending on primer pair for one minute and extension at 72^{o}C for one minute.

Denis *et al.* (1999) developed a multiplex PCR for the genes *16S rRNA*, *map*A and *ceu*E in a 30 µl reaction volume containing 100 µ*M* of each nucleotides, 0.11 µ*M* of each of the primers for *16S rRNA* gene, 0.42 µ*M* of each of the primers for *map*A and *ceu*E gene and 0.6U *Taq* polymerase. The PCR conditions were initial denaturation at 95^{o}C for 10 min followed by 35 cycles of amplification involving denaturation at 95^{o}C for 30 sec, annealing at 59^{o}C for 1.5 min, extension at 72^{o}C for one minute and a final extension step at 72^{o}C for 10 min. Corresponding to the genus *Campylobacter*, the species *jejuni* and the species *coli*, amplicons were produced at 857-bp, 589-bp and 462-bp, respectively.

Konkel *et al.* (1999) developed a PCR assay for the conserved virulene gene *cad*F in a 100 µl reaction volume containing 10X PCR buffer, 50 m*M* $MgCl_2$, 100 p*M* each of the forward and reverse primer, each of the four deoxynucleoside triphosphates at a concentration of 2 m*M*, and 2.5U *Taq* DNA polymerase. Thirty cycles of PCR consisted of denaturation at 94^{o}C for one minute, annealing at 45^{o}C for one minute and extension at 72^{o}C for three minutes. A 400-bp amplicon was produced in 93.5 per cent of the *C. jejuni* and *C. coli* isolates tested with the *cad*F primers.

Bang *et al.* (2003) investigated the prevalence of seven virulence and toxic genes namely *cad*F, *ceu*E, *virB11*, *fla*A, *cdt*A, *cdt*B and *cdt*C from pigs and cattle of Denmark. Among the 40 *C. jejuni* and *C. coli* isolates, 100 per cent were positive for *cad*F, *fla*A, *ceu*E and *cdt*B genes and 95, 90 and 7.5 per cent were positive for *cdt*A, *cdt*C and *virB11*, respectively.

Lund *et al.* (2004) performed real-time PCR assay and selective enrichment culture directly on 111 chicken faecal swab samples for detecting *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*. The *16S rRNA* gene sequences of *Campylobacter* was used and the PCR amplification steps involved one cycle at 95^{o}C for 10 min, 40 or 50

cycles at 95^{o}C for 15 sec, 58^{o}C for 30 sec and 72^{o}C for 30 sec, with a final cycle at 72^{o}C for 5 min. There was no statistically significant difference in the performance between the two methods.

Rozynek *et al.* (2005) found that out of 53 *C. jejuni* and 39 *C. coli* isolates from chicken carcasses, 100 per cent of the isolates carried *cad*F virulence gene.

Selwet and Galbas (2012) analysed *fla*A, *cad*F, *cdt*B, and *iam* genes for the simultaneous detection of *C. jejuni* and *C. coli* using multiplex polymerase chain reaction. Out of the 100 samples, cent per cent of the *Campylobacter* isolates had *cad*F and *fla*A gene.

2.2. OCCURRENCE OF *CAMPYLOBACTER* IN POULTRY

Pillai (1989) investigated the presence of *C. jejuni* from apparently healthy market chicken in Bangalore. The 67 isolates consisting of biotype I (32), biotype II (26) and biotype III (9) which showed highest colonization in the caecum followed by large and small intestines.

Hald *et al.* (2000) tested 88 randomly selected Danish broiler flocks and reported 52 per cent overall prevalence of *Campylobacter* from cloacal swab samples and 24 per cent from neck skin samples after slaughtering. *C. jejuni* was found in 87 per cent samples, while eight and five per cent carried *C. coli* and *C. lari*, respectively.

Aydin *et al.* (2001) found 100 per cent prevalence of *C. jejuni* of the 40 cloacal swabs examined from domestic geese in Turkey.

Chattopadhyay *et al*. (2001) examined 160 intestinal contents from chicken and ducks in and around Calcutta, of which 63 samples were found to be contaminated with *C. jejuni* (88.9 per cent) and *C. lari* (11.1 per cent).

Bandekar *et al*. (2005) screened 40 hot dressed poultry carcasses from Pune and Mumbai and reported that 95 per cent of the samples were contaminated with *C. jejuni, C. coli, C. fetus* and *C. lari* with MPN range 10^2 to 10^4 CFU/kg of meat.

Vashin and Stoyanchev (2005) investigated the occurrence of *Campylobacter* spp. in Japanese quail in Bulgaria and presence of the organism was found in 80 per cent of the 30 caecum samples and 16.7 per cent of the 30 liver samples. The isolation rate of *C. jejuni* and *C. coli* was 89.7 and 10.3 per cent, respectively.

Baserisalehi *et al.* (2007) analyzed 126 faecal samples from domestic animals and poultry in India and 120 from Iran. Seventy and thirty seven isolates were obtained from India and Iran, respectively.

Stojanov *et al.* (2007) examined 50 cloacal swabs, 35 caecum and 35 magnum samples for the presence of *Campylobacter* spp. Seventy one per cent of the organism were isolated from caecum comprising of 19 (76 per cent) isolates of *C. jejuni* and six (24 per cent) of *C. coli*. Of the 19 (38 per cent) positive isolates obtained from cloacal swabs, 68.42 per cent were *C. jejuni* and 31.58 per cent were *C. coli*. Out of the 8.57 per cent positive magnum samples, 66.66 and 33.33 per cent of samples were contaminated with *C. jejuni* and *C. coli*, respectively.

A study was carried out by Rahimi and Tajbakhsh (2008) involving 800 poultry meat samples to determine the prevalence of *Campylobacter* spp. in Iran. The quail meat (68.4 per cent) was most contaminated with *Campylobacter* spp. compared to chicken (56.1 per cent), turkey (27.4 per cent) and ostrich (11.7 per cent) meat.

Feng *et al.* (2009) collected 120 cloacal samples from healthy red-crowned cranes in China and *Campylobacter* spp. were detected in 26 (21.67 per cent) samples.

In a prevalence study by Rajkumar *et al*. (2010), out of the 300 poultry breast skin samples in small scale poultry dressing units of Bareilly region of Northern India, 71.42 and 28.47 per cent were tested positive for *C. jejuni* and *C. coli*, respectively.

Adzitey *et al.* (2011) collected 40 large intestinal contents from domestic Pekin ducks in Malaysia. The overall prevalence of *Campylobacter* spp. was 67.5 per cent, among which 55.6 and 44.4 per cent were *C. jejuni* and *C. coli*, respectively.

Kumar (2011) collected 50 each of chicken caecum, quail caecum and chicken meat from Bareilly region of Uttar Pradesh, India and recorded an overall prevalence rate of 18, 18 and 12 per cent, respectively. Out of the 18 per cent positive chicken and quail caecum samples, six and eight isolates were positive for *C. jejuni* and three and one isolates were positive for *C. coli*, respectively. All the positive chicken meat samples were contaminated by *C. jejuni*.

2.3. OCCURRENCE OF *CAMPYLOBACTER* IN EGG PRODUCTION CHAIN

Miflin *et al.* (2001) conducted a longitudinal study to determine the source and transmission of *Campylobacter* spp. in broiler flock in Queensland. *Campylobacter* spp. was not detected in drinkers and litter on day 31 when two per cent of the faecal samples were positive, suggesting that drinkers and litters are not the source of contamination. On 34th day, 85 per cent of faecal samples (n=100), 83.33 per cent of the drinkers (n=12) and 33.33 per cent of the litter (n=3) samples were positive for *Campylobacter* spp.

Saleha (2004) studied the occurrence of *Campylobacter* spp. in 113 feed, 34 wood shavings, 30 flies and 114 house environmental samples consisting of wall, floor and dust swabs from broiler farms in Malaysia. All the samples tested were negative for this organism.

Byrd *et al.* (2007) made the first report of *Campylobacter* spp. recovery from tray liners used in hatchery in Mississippi state. Out of the 2,000 chick paper pad tray liners tested, 15 were positive for this organism.

Vandeplas *et al.* (2009) investigated three successive broiler flocks in six Belgian farms comprising of 4,800 birds each. Out of the 13 *Campylobacter* positive flocks, the potential sources of infection were incriminated to entrance premises (30.8

per cent), delivery tray (15.4 per cent), litter (7.7 per cent) and exit trap door (7.7 per cent).

Jones *et al.* (2012) determined the presence of *Campylobacter* spp. in different egg production systems in Salisbury. Environmental swabs (n=32) were positive for *Campylobacter* spp. in 25 per cent of samples from free-range nest box and three per cent of environmental grass samples (n=33) from free-range floor.

Vaz *et al.* (2012) conducted a longitudinal study in three consecutive broiler flocks consisted of 180 *Campylobacter* free chicks reared on reused litter in Southern Brazil. *Campylobacter* contaminated cloacal swabs were obtained at a faster rate from second flock (17.9 day) than first and third flocks (28.4 day). Litter samples were contaminated by *C. jejuni* at a faster rate in second and third flock (21 day) than first (35 day).

In a study conducted by Berghaus *et al.* (2013), 110 boot sock, 109 drag swab and 110 litter samples were analyzed from 55 broiler chicken flocks in North Georgia. *Campylobacter* spp. was found in 51.8 per cent of boot sock, 60.6 per cent of drag swab and 23.6 per cent of litter samples.

Thakur *et al.* (2013) collected 400 faecal samples, 100 indoor samples each of feed, litter and inside swab and 100 outdoor samples each of outside swab and grass from 10 commercial broiler houses in North Carolina. The prevalence of *Campylobacter* in faecal samples, inside swabs, outside swabs and grass samples was found to be 29.5, two, one and one per cent, respectively. The litter and feed samples was not contaminated with *Campylobacter* spp.

Schroeder *et al.* (2014) analyzed 10 litter samples and 10 sponge samples of feed pans and water dispensers from each chicken grown out house of four farms in western Virginia. *Campylobacter* spp. was isolated from 20 and 45 per cent of the litter and sponge samples, respectively.

Prachantasena *et al.* (2016) investigated the prevalence of *Campylobacter* species in Thailand poultry production chain. Out of the 131 egg tray swabs, egg incubator swabs, tap water and egg shells collected from hatchery and 50 paper tray and 124 environmental samples before chick rearing from broiler house tested, none of them was found to be positive for this organism.

2.4. OCCURRENCE OF *CAMPYLOBACTER* IN ENVIRONMENT

Carter *et al.* (1987) detected *Campylobacter* from the natural water sources in Washington. Out of the 99 water samples tested, 41 were contaminated with *Campylobacter* spp.

Obiri-Danso and Jones (1999) investigated the presence of *Campylobacter* species in two freshwater bathing sites in England. Out of the 144 water samples collected from each site, 84 per cent and 82 per cent were positive from Crook O'Lune and University Boathouse, respectively.

Saleha (2004) studied the occurrence of *Campylobacter* spp. in 206 water samples from broiler farms in Malaysia and found that 1.5 per cent were positive for this organism.

Vandeplas *et al.* (2009) investigated three successive broiler flocks in six Belgian farms comprising of 4,800 birds each. In 69.2 and 15.4 per cent of the *Campylobacter* positive flocks, the potential sources of infection were incriminated to open-air range soil and water-lines.

Pérez-Boto *et al.* (2010) analyzed water samples from two heavy breeder rearing farms in Spain. Out of the 20 positive isolates obtained 65 per cent were *C. jejuni* and 35 per cent were *C. coli*.

Johnson (2010) collected 135 air samples and 29 water samples from commercial broiler slaughter facility in Virginia. *Campylobacter* was detected in 10 per cent of the air and 28 per cent of the water samples tested.

Hu and Kuo (2011) studied the distribution of *Campylobacter* spp. in river water of Taiwan. Out of the 75 water samples tested, two (2.7 per cent) were positive for *Campylobacter jejuni*.

El Metwaly Ahmed *et al.* (2013) compared the efficiency of air sampling using all-glass impingers (AGI-30) and a wet cyclone technology (Coriolis®μ Air Sampler) in a laying hen flock in Germany with 70 to 93 per cent *C. jejuni* prevalence. Coriolis®μ air sampler ($2x10^5$ to $4x10^6$ CFU/m^{-3}) showed higher concentration of airborne mesophilic bacteria than AGI-30 sampler ($8x10^4$ to $2x10^6$ CFU/m^{-3}) in 17 out of the 18 air samples tested. Of the 18 airborne dust samples, 83 per cent showed the presence of *C. jejuni* specific DNA. But both of these methods failed to detect culturable *C. jejuni* by direct plating or by enrichment method.

A study was carried out by Trimble *et al.* (2013) involving 42 soil, 39 compost and 46 processing waste water samples to determine the prevalence of *Campylobacter* from broiler farms in United States. The prevalence was found to be 64.3, 64.3 and 45.7 per cent, respectively. The concentration (mean $\log_{10}$ CFU) of the organism was lower in waste water (2.19) than soil (3.08) and compost (3.83).

Schroeder *et al.* (2014) analyzed 10 air samples from each chicken grown out house of four farms in western Virginia. *Campylobacter* spp. was isolated from 15 per cent of the samples.

2.5. OCCURRENCE OF *CAMPYLOBACTER* IN HUMANS

The first documented human *Campylobacter* infection occurred in Illinois in May 1938 (Levy, 1946). The milk-borne acute outbreak involving 357 people was characterized by fever, nausea, vomiting, abdominal cramps, diarrhoea, headache, backache, profuse perspiration and general prostration. *Campylobacter* was first isolated by King (1957) from blood samples of children with diarrhoea in United States and found that they grew at an optimal temperature of 42^{o}C.

Pillai (1989) determined the presence of *Campylobacter* antibodies in human serum samples. About 10 per cent of the serum samples showed the presence of antibodies to *C. jejuni* of chicken origin.

Kirk *et al.* (1997) reported a prolonged outbreak caused by *Campylobacter* spp. at a training facility in Australia. Out of the 78 cases reported, 16 were positive for the organism with attack rate ranging from 19 to 67 per cent. Of the five food handlers tested, one was positive for *C. jejuni*. The consumption of cucumber contaminated with *Campylobacter* spp. was determined as the source of infection.

Chattopadhyay *et al*. (2001) analyzed 140 human faecal samples in and around Calcutta, of which 10 samples were found to be positive for *C. jejuni* (nine) and *C. coli* (one).

Olsen *et al.* (2001) reported an outbreak caused by *C. jejuni* among 27 persons attending school luncheon (n=161) in Kansas. A cafeteria worker with diarrhoeal illness was the likely source of infection.

Prasad *et al.* (2001) determined the occurrence of *Campylobacter* spp. in patients with diarrhea from a tertiary care centre at Lucknow for a period of 12 years. Of the 59 patients tested, 62 strains of *Campylobacter* spp. were isolated consisting of 82.3 per cent *C. jejuni*, 12.9 per cent *C. coli*, 3.2 per cent *C. lari* and 1.6 per cent *C. upsaliensis*. Children less than five years of age (57.6 per cent) were highly susceptible to infection followed by patients with 15-30 years of age (20.3 per cent). Two of the patients infected with *C. jejuni* and *C. upsaliensis* developed Guillain-Barré syndrome within two weeks of onset of diarrhoea. *Campylobacter* infection recurred in 5.1 per cent of the patients.

In the year 2004, Hennessy investigated an outbreak of gastrointestinal illness affecting 105 soldiers at Infantry Training Centre, Brecon. The illness was characterized by nausea, fever, vomiting, abdominal pain and diarrhoea. Out of the 36 stool samples tested, nine were positive for *Campylobacter* spp.

Kumar (2011) investigated the presence of *Campylobacter* spp. in humans from Bareilly region of Uttar Pradesh, India. Out of the 50 human stool samples, four per cent was found to be positive for *Campylobacter jejuni*.

Ridley *et al.* (2011) investigated the sources of *Campylobacter* spp. contamination of chicken farms in United Kingdom. Out of the 93 catchers' hand swabs and 33 foot wears tested, 18 and 39 per cent were positive for *Campylobacter* spp. Even after cleaning, 10 and 19 per cent of hands (n=59) and foot wears (n=21) showed the presence of organism. Sixty per cent of the interior and exterior of the catchers' lunch bags were positive for *Campylobacter* spp.

2.6. OCCURRENCE OF *CAMPYLOBACTER* IN EGG

Doyle (1984) found that out of 226 eggs from hens excreting *C. jejuni*, two egg shell surfaces showed the presence of organism. The inner content of the eggs were not contaminated with *C. jejuni*.

Baker *et al.* (1987) studied the prevalence of *C. jejuni* in eggs from spent layers, Peking ducks and turkeys of 23 egg farms in New York State. Out of the 276 eggs tested, all the samples were negative for the organism.

Sahin *et al.* (2003) reported that *Campylobacter* spp. was not isolated from the 1000 eggs from broiler breeders procured from hatchery and 500 eggs from broiler breeder flocks shedding *Campylobacter* spp. in faeces.

Fonseca *et al.* (2006) examined 140 cloacal swab samples from breeder hens in Brazil. The carriage of *Campylobacter* spp. was found to be 17.8 per cent by conventional method. Out of the 244 eggs collected from the *Campylobacter* shedding hens, none of them were found to be positive for the organism.

Sulonen *et al.* (2007) isolated *Campylobacter jejuni* from one egg shell sample out of the 360 table eggs collected from organic egg farms in Finland. All egg yolk samples were found to be negative for this organism.

Vashin *et al.* (2008) collected bulk faecal samples, 30 cloacal samples and 90 eggs each on the day of laying and after five days of storage at room temperature from different batches of Japanese quail in Bulgaria. *Campylobacter* spp. was detected in 76.7 per cent of the cloacal samples and 63.3 per cent of the faecal samples from the cages, but neither the egg shells nor the contents showed the contamination with the organism.

Jones *et al.* (2012) investigated the presence of *Campylobacter* spp. in different egg production systems in Salisbury. Shell emulsion pools were positive for *Campylobacter* spp. in 12.5 per cent of spring samples from free-range nest box and 2.5 per cent of fall samples from conventional cage. Out of the 249 egg contents tested, none of the samples were found to be positive for the organism.

Messelhausser *et al.* (2011) determined the presence of *Campylobacter* spp. in egg samples from Germany. Of the 2,710 tested samples, 4.1 per cent egg shell samples were positive whereas all the egg yolk samples were negative for the organism. Eight isolates were of *C. coli* and three were of *C. jejuni*.

Safaei *et al.* (2011) reported that contamination by *C. jejuni* was not detected in any of the 100 table eggs purchased in spring and summer from supermarkets of Iran.

Hedawey and Youssef (2014) analysed 100 each of cloacal swab and egg samples from poultry farm in Sohag Governorate. A total of 38 isolates detected in cloacal swabs comprised of 65.8 per cent of *C. jejuni* and 34.2 per cent of *C. coli*. The only one isolate obtained from egg shell was found to be *C. jejuni*. Inner content of the egg was proved to be not contaminated by the organism.

2.7. SEASONAL OCCURRENCE OF *CAMPYLOBACTER*

Wallace *et al.* (1997) monitored the seasonal variation of *Campylobacter* from chicken intestine from UK for a period of 12 months and found that the carriage rate

measured by the presence of organism in small intestine and caeca was 100 per cent for 11 out of the 12 months.

Willis and Murray (1997) investigated the seasonal trends of *C. jejuni* from broiler carcasses in North Carolina. Highest recovery rate observed during June and July (96.7 per cent) and lowest during December (6.7 per cent).

Prasad *et al.* (2001) studied the seasonal occurrence of *Campylobacter* in patients with diarrhea from a tertiary care centre at Lucknow for a period of 12 years. Isolation rate was higher during summer (53.2 per cent) than rainy (25.8 per cent) and winter (21 per cent) seasons.

Bandekar *et al*. (2005) screened hot dressed poultry carcasses from Pune and Mumbai and found that isolation rate of *Campylobacter* spp. increased during early December, summer and early monsoon season.

Sulonen *et al*. (2007) found that out of the 642 faecal samples and 360 table eggs collected from 19 farms in autumn and 17 farms in spring, 84 and 76 per cent of the organic laying hen farms in Finland were positive for *Campylobacter* spp. in autumn and spring, respectively.

Rahimi and Tajbakhsh (2008) determined that the highest incidence of *Campylobacter* spp. in poultry meat in Iran occurred during summer months with 66.5 per cent followed by spring (51.5 per cent), autumn (46 per cent) and winter (25 per cent).

Nather *et al.* (2009) tested 146 German broiler flocks and found that highest prevalence of *Campylobacter* occurred during summer months from May to October (53 per cent) followed by winter months from November to April (34 per cent).

Vandeplas *et al.* (2009) investigated the seasonal occurrence of *Campylobacter* in three successive Belgian broiler flocks from six farms. Cent per cent of the flocks were contaminated during summer followed by 66.7 per cent during spring and 50 per cent during winter.

Jorgensen *et al.* (2011) studied the seasonal occurrence of *Campylobacter* in 797 broiler flocks reared in Great Britain. Prevalence of *Campylobacter* was higher during July (54 per cent), August (55 per cent) and September (60 per cent) than the rest of the year (14 to 48 per cent).

Out of the 150 cloacal samples tested by Renu *et al*. (2011), six per cent were found positive for *Campylobacter* spp. The prevalence rate was found to be highest in rainy season (34 per cent), followed by summer (18 per cent) and lowest in winter (12 per cent).

2.8. MICROBIOLOGICAL QUALITY OF EGGS

2.8.1. Aerobic Plate Count

According to ICMSF (1986) Aerobic Plate Count (APC) is regarded as the indicator of raw material quality before processing. The recommended limit of APC for egg products is between 5.0×10^4 and 1.0×10^6 CFU/g.

Jones *et al.* (2004) determined the variations in microbial quality during extended storage of washed and unwashed shell eggs and reported that significant difference ($P<0.0001$) exist between the aerobic plate count during each week of storage of eggs. Washed eggs showed significantly fewer bacteria in all cases. Aerobic plate count of washed eggs reduced from 2.5 $\log_{10}$ CFU/ml on the day of collection to one $\log_{10}$ CFU/ml over the course of storage period. Aerobic plate count of unwashed eggs remained at four $\log_{10}$ CFU/ml for most of the storage period, but showed an increase at eight weeks to 5.3 $\log_{10}$ CFU/ml. Concentration of aerobic bacteria in washed egg contents remained constant during the 10 weeks of storage period. Average bacterial concentration was less than one $\log_{10}$ CFU/ml for washed and unwashed eggs.

Musgrove *et al.* (2005) studied the microbial quality on the shell surface of 990 eggs collected from 12 points in processing line of three commercial shell egg processing plants in Southeastern United States. Aerobic microorganisms showed

similar pattern of fluctuation in all the three plants. Shell rinses of eggs collected from accumulator (4.61 $\log_{10}$ CFU/ml) showed highest number of organism and the lowest in final packed eggs (1.21 $\log_{10}$ CFU/ml). At the end of processing, 30 per cent reduction in the prevalence of aerobic microorganisms were observed. Average prevalence of these organisms reduced from 100 to 70.6 per cent from preprocessing to post processing stages.

Suba *et al.* (2005) investigated the microbial quality of table eggs from Chennai and found that the bacterial contamination was highest in eggs collected from retail shops ($4x10^5$ CFU/g) followed by wholesale market ($2x10^5$ CFU/g) and layer farms ($1x10^5$ CFU/g).

Jones and Musgrove (2007) analyzed the microbial quality of restricted eggs collected from three shell egg processing plants in Georgia. Average aerobic plate count in the shells and contents of the eggs were 4.3 $\log_{10}$ CFU/ml and 2 $\log_{10}$ CFU/ml, respectively.

Wall *et al.* (2008) compared the microbial quality of shell eggs collected from 480 hens of 28 to 62 weeks of age reared in conventional and furnished cages in Sweden. Comparatively higher aerobic count was noticed in eggs produced from furnished cages at 28 (3.04 ± 0.09 $\log_{10}$ CFU/cm^2) and 62 (2.90 ± 0.10 $\log_{10}$ CFU/cm^2) weeks than the eggs from conventional cages at 28 (2.70 ± 0.20 $\log_{10}$ CFU/cm^2) and 62 (2.61 ± 0.20 $\log_{10}$ CFU/cm^2) weeks of age, but the microbial level in both the housing system can be considered low.

Ansah *et al.* (2009) monitored the bacterial contamination on 300 table eggs collected from four selected markets in Tamale metropolis, Ghana. The mean aerobic count from contents and shells of eggs from Kukuo, Lamashegu, Aboabo and Tamale Central ranged from 7.26 to 7.56, 6.54 to 6.93, 7.18 to 7.36 and 6.9 to 7.3 respectively, which was higher than the ICMSF recommended limits.

Obi and Igbokwe (2009) evaluated the microbial quality of eggs collected from four different poultry farms in Nigeria. Highest microbial contamination was found in the egg shell ($1.37x10^7$ CFU/ml), followed by albumen ($1.5x10^7$ CFU/ml), chalazae ($1.23x10^7$ CFU/ml) and the lowest in the yolk ($1.2x10^7$ CFU/ml).

Irene (2011) assessed the microbial quality of 480 duck eggs from six different points in production chain in University poultry and duck farm, Mannuthy during season A (June to November) and season B (December to March). Aerobic plate count on the egg shell showed significant difference ($P<0.05$) during two seasons and from one point of collection to another. A higher count was observed on egg shell from the point of lay in season B (8.93 ± 0.18 $\log_{10}$ CFU/egg shell) compared to season A (6.63 ± 0.21 $\log_{10}$ CFU/egg shell). After washing, APC reduced significantly on season A (4.61 ± 0.14 $\log_{10}$ CFU/egg shell) and season B (7.17 ± 0.21 $\log_{10}$ CFU/egg shell). There was not much difference in the APC of egg shell after washing, till it reached sales counter on season A (4.36 ± 0.09 $\log_{10}$ CFU/egg shell). Significantly higher ($P<0.05$) APC of egg content was observed during season B than season A except at sales counter. At point of lay, significantly higher APC of content was observed on season B (5.48 ± 0.27 $\log_{10}$ CFU/g of egg contents) compared to season A (3.64 ± 0.15 $\log_{10}$ CFU/g of egg contents). After washing, APC of contents reduced significantly on season A (2.89 ± 0.13 $\log_{10}$ CFU/g of egg contents) and season B (4.30 ± 0.32 $\log_{10}$ CFU/g of egg contents).

Bahobail *et al.* (2012) collected a total of 135 processed and unwashed chicken eggs from different shops in Taif city, Saudi Arabia. The average total plate count associated with the shells and contents of processed eggs were 3.1 $\log_{10}$ CFU/ml and 1.1 $\log_{10}$ CFU/ml and from the unwashed eggs were 5.9 $\log_{10}$ CFU/ml and 2.0 $\log_{10}$ CFU/ml, respectively.

Mahdavi *et al.* (2012) studied the microbial quality of 525 chicken eggs collected from supermarkets in Isfahan, Iran. The mean aerobic plate count in egg shell and contents were $4.31x10^4$ CFU/g and $3.95x10^4$ CFU/g, respectively.

El-Kholy *et al.* (2014) determined the microbial quality of 170 table eggs from poultry farms in Beni-Suef city, Egypt. Mean aerobic count of $8x10^3 \pm 3.8x10^3$ and $1.1x10^3 \pm 3x10^2$ CFU/shell and ml was found in 58.82 per cent of shells and 50 per cent of the contents, respectively.

2.8.2. Yeast and Mould Count

Jones *et al.* (2004) determined the variations in microbial quality during extended storage of washed and unwashed shell eggs and reported that significant difference ($P<0.0001$) existed between the yeast and mould count during each week of storage of eggs. For the washed eggs, yeast and mould count remained low throughout the study period and the highest concentration (0.7 $\log_{10}$ CFU/ml) detected during eight weeks of storage. Unwashed eggs showed lowest concentration at two weeks (1.3 $\log_{10}$ CFU/ml) and highest at eight and 10 weeks (2.9 and 2.6 $\log_{10}$ CFU/ml, respectively) of storage. Egg contents of the unwashed and washed eggs did not showed significant difference ($P > 0.05$) for all the weeks except in week eight. At week eight, 0.2 $\log_{10}$ CFU/ml was found in washed eggs compared with highest contamination of 0.4 $\log_{10}$ CFU/ml for unwashed eggs.

Musgrove *et al.* (2005) studied the microbial quality on the shell surface of 990 eggs collected from 12 points in processing line of three commercial shell egg processing plants in Southeastern United States. Yeast and mould count showed similar pattern of fluctuation in all the three plants. Shell rinses of eggs collected from rewash exit showed highest number of organism (2.01 $\log_{10}$ CFU/ml) and the lowest in wash two samples of eggs (0.51 $\log_{10}$ CFU/ml). At the end of processing, 20 per cent reduction in the prevalence of yeasts and moulds were observed. Average prevalence of these organisms reduced from 80 to 62 per cent from preprocessing to post processing stages.

Suba *et al*. (2005) investigated the microbial quality of table eggs from Chennai and found that the yeast and mould contamination was below $1x10^5$ CFU/g in the eggs collected from each of layer farms, retail shops and wholesale market.

Salem *et al.* (2009) conducted mycological examination on 50 table eggs from markets in El-Beida, Libya. Clean and soiled egg shell showed a mean total YMC of $2.7\times10^4 \pm 2.2\times10^4$ CFU/g and $3.7\times10^5 \pm 3.2\times10^5$ CFU/g and the contents showed $6.6\times10^3 \pm 3.1\times10^3$ CFU/g and $2.5\times10^4 \pm 1.1\times10^4$ CFU/g, respectively.

Neamatallah (2009) evaluated fungal contamination in 100 hen eggs from El-Behera Governorate, Egypt and found that 38 per cent of the eggs were positive for moulds with a mean value of $3.4x10^3$ CFU/g.

Irene (2011) assessed the microbial quality of 480 duck eggs from six different points in production chain in University poultry and duck farm, Mannuthy during season A (June to November) and season B (December to March). Yeast and mould count on egg shell from point of lay showed significantly higher ($P<0.05$) difference during season B (6.39 ± 0.12 $\log_{10}$ CFU/egg shell) compared to season A (4.98 ± 0.31 $\log_{10}$ CFU/egg shell). As the eggs reached sales counter significantly higher YMC was observed during season A (5.11 ± 0.07 $\log_{10}$ CFU/egg shell), but lower count was observed during season B (3.57 ± 0.09 $\log_{10}$ CFU/egg shell). Significantly higher ($p<0.05$) YMC of egg contents was observed during season B at the point of lay to point of candling than season A. As the eggs reached sales counter, significant reduction in count was observed during season A (2.86 ± 0.19 $\log_{10}$ CFU/g of egg contents) and season B (3.19 ± 0.10 $\log_{10}$ CFU/g of egg contents).

Al-Obaidi *et al.* (2011) evaluated the fungal count on a total of 1680 chicken eggs and found that eggs produced locally in Baghdad had a count of $69x10^2$ CFU/egg and Syrian, Iranian, Ukrainian and Turkish imported eggs each had fungal count of $15x10^2$ CFU/egg.

Bahobail *et al.* (2012) collected a total of 135 processed and unwashed chicken eggs from different shops in Taif city, Saudi Arabia. The average mould count associated with the shells and contents of processed eggs were 1.3 $\log_{10}$ CFU/ml and zero $\log_{10}$ CFU/ml and from the unwashed eggs were 3.4 $\log_{10}$ CFU/ml and 1.1 $\log_{10}$ CFU/ml, respectively.

Cader *et al.* (2014) compared the microbial quality of hen eggs procured from breeders soon after laying and from supermarkets in Mauritius. The mean YMC on egg shells and contents soon after laying were 2.8 $\log_{10}$ CFU/g and less than one $\log_{10}$ CFU/g and from supermarkets were 3.5 $\log_{10}$ CFU/g and less than one $\log_{10}$ CFU/g, respectively.

El-Kholy *et al.* (2014) determined the microbial quality of 170 table eggs from poultry farms in Beni-Suef city, Egypt. Mean yeast and mould count of $5.9x10^2 \pm 2.5x10^2$ and $2.6x10^2 \pm 1.7x10^2$ CFU/shell and ml was found in 44.12 per cent of shells and 23.53 per cent of the contents, respectively.

2.9. ANTIBIOTIC RESISTANCE PROFILE STUDIES

Antibiotic resistance of *C. jejuni* isolated from cloacal swabs of domestic geese were studied by Aydin *et al.* (2001). All the strains were highly susceptible to nitrofurantoin, nalidixic acid, erythromycin, streptomycin, gentamicin, amoxycillin-clavulanic acid, chloramphenicol and enrofloxacin and resistant to penicillin G and cephalothin (100 per cent), sodium cefuroxime (92 per cent), cloxacillin, ampicillin and colistin sulphate (67 per cent), tetracycline (25 per cent), sulfamethoxazole/trimethoprim and kanamycin (8.4 per cent).

Eighty two *Campylobacter* spp. isolates from humans in Berlin, during 2001-2002 period were subjected to antimicrobial susceptibility testing (Luber *et al.*, 2003). All isolated strains were sensitive to gentamicin. Most of them were resistant to trimethoprim-sulfamethoxazole (50 per cent) followed by ciprofloxacin (45.1 per cent), tetracycline (37.8 per cent), ampicillin (12.8 per cent) and erythromycin (6.1 per cent). When compared with the values from 1991, resistance rate among strains from humans to ciprofloxacin (4.9 per cent), ampicillin (7.3 per cent) and tetracycline (19.5 per cent) significantly increased during the 10 years.

Baserisalehi *et al.* (2007) carried out antibiotic susceptibility testing of *Campylobacter* spp. from faecal samples of domestic animals and poultry in India

and Iran. All the pathogenic isolates were sensitive to ciprofloxacin. Lowest minimal inhibitory concentration value was found for ciprofloxacin and highest for ampicillin and chloramphenicol.

Sulonen *et al.* (2007) evaluated antimicrobial susceptibility of *Campylobacter* spp. using five antibiotics. Majority of the isolates (96 per cent) were susceptible to ampicillin, erythromycin, nalidixic acid, ciprofloxacin and tetracycline.

Feng *et al.* (2009) evaluated the antibiotic resistance profile of *Campylobacter* spp. obtained from cloacal samples of red-crowned cranes in China and found that 70 per cent strains were sensitive to ampicillin, cephalexin and cefoperazone and 96.15 per cent were resistant to co-trimoxazole.

Parkar *et al.* (2013) done antibiotic sensitivity test with 112 *C. jejuni* and 31 *C. coli* isolates from caecum and carcasses of poultry from Pune. *Campylobacter jejuni* showed resistance to gentamicin (43.1 per cent), ciprofloxacin (28.3 per cent), erythromycin (26.6 per cent), norfloxacin (26.5 per cent), nalidixic acid (25.4 per cent), tetracycline (23.7 per cent) and ampicillin (19.6 per cent) and *C. coli* to ampicillin (48 per cent), gentamicin (36.5 per cent), tetracycline (30.8 per cent), nalidixic acid (19.2 per cent) and 15.4 per cent for chloramphenicol and ciprofloxacin.

The antibiotic susceptibility of *Campylobacter* spp. from 27 chicken and 24 dog faecal samples was determined by Begum *et al.* (2014). The isolates from dogs were found to be resistant to cephalexin (100 per cent), co-trimoxazole (87.5 per cent), tetracycline (83.33 per cent), amoxicillin (66.67 per cent) and ciprofloxacin (58.33 per cent) and sensitive to gentamicin (66.67 per cent). The isolates from poultry was found to be resistant to amoxicillin (100 per cent), co-trimoxazole (100 per cent), cephalexin (96.29 per cent) and tetracycline (55.56 per cent) and sensitive to gentamicin (100 per cent) and ciprofloxacin (48.15 per cent).

Girgis *et al*. (2014) obtained an overall prevalence of 5.8 per cent when 327 stool samples from Egyptian patients with diarrhea were tested. The antibiotic sensitivity of all the *Campylobacter* spp. isolated were examined and it was found that all the 19 isolates were resistant to ampicillin and nalidixic acid (100 per cent) followed by ciprofloxacin (57.9 per cent), erythromycin and azithromycin (5.3 per cent each). Multi-drug resistance to all the tested antibiotics were shown by 5.3 per cent of the *C. coli* isolate.

Hedawey and Youssef (2014) determined the antibiotic resistance profile of 38 *Campylobacter* isolates from cloacal swabs and eggs using 12 antibiotics. Most of the isolates showed high degree of susceptibility to spiramycin, spectinomycin, clindamycin, gentamicin and colistin sulphate, but showed resistance to amoxicillin, ampicillin and penicillin.

Noormohamed and Fakhr (2014) subjected *Campylobacter* spp. isolated from 130 chickens and 19 turkeys from Oklahoma to antimicrobial susceptibility test using 16 antimicrobials. Most of the chicken isolates were resistant to amoxicillin (99.2 per cent), cephalothin (97.7 per cent), oxytetracycline (96.2 per cent) and doxycycline (81.5 per cent) and 100 per cent of the turkey isolates were resistant to tetracycline, amoxicillin, doxycycline and cephalothin.

3. MATERIALS AND METHODS

The present study was undertaken to determine the critical control points of *Campylobacter* spp. in chicken egg production chain, its occurrence in retail market, microbiological quality of the egg shell, molecular confirmation and antibiotic resistance profile of the isolates. The study was conducted for a period of 12 months from June 2015 to May 2016. A total of 570 samples, consisting of 450 samples from University Poultry and Duck Farm (UPDF), Mannuthy, which included cloacal swab, feed, litter, wash water, wash cloth, plastic tray, filler flat, air, water, hand wash and egg; and 120 chicken egg samples from retail market in and around

Thrissur, forms the materials for the study. All the samples collected were subjected to isolation and identification of *Campylobacter* spp. by conventional culture technique. In order to determine the microbiological quality of eggs, aerobic plate count and yeast and mould count of the egg shell was evaluated. Polymerase chain reaction was carried out for species identification and to detect the presence of virulence gene in the isolates. All the positive isolates were subjected to antibiotic resistance profiling. Details of the study and the experimental methods adopted are described in this chapter.

3.1. BIOLOGICALS, CHEMICALS AND REAGENTS

3.1.1. Bacterial Culture

The standard culture of *Campylobacter jejuni subsp. jejuni* (ATCC® 33560™) and *Campylobacter coli* (ATCC® 33559™) was procured during the beginning of the study from American Type Culture Collection, USA.

3.1.1.1. Maintenance, preservation and propagation of culture

The freeze dried culture was rehydrated using six millilitres of Brucella broth (HiMedia). A loopful of this suspension was used to inoculate Tryptone Soya Agar (HiMedia) plates with five per cent defibrinated sheep blood at 37°C for 24 to 48 h under microaerophilic conditions (3-5 per cent O_2, 10 per cent CO_2) in a Steri-cycle CO_2 incubator (Thermo Scientific). The colonies on this media were colourless, transparent, circular, entire, smooth, low convex, nonhemolytic, watery and dew drops like swarming ones (Fig.1). The standard culture was also inoculated on to the selective media Blood Free *Campylobacter* Selectivity (modified Charcoal Cefoperazone Deoxycholate, mCCD) agar media supplemented with CAT selective supplement (FD 145), *Campylobacter* supplement V (FD 067) and the cultural characteristics were studied. The cultural examination revealed the growth of characteristic cream to grey colour spreading type, shiny mucoid colonies with metallic sheen, with a tendency to stick and extend when they are touched with loop

(Fig. 2). The isolate was maintained in Brucella broth with 30 per cent glycerol by sub culturing at regular intervals. Glycerol broth tubes inoculated with the organism were stored at -80^{o}C. The cultures were periodically tested for their purity, morphological and biochemical characteristics at the Biosafety level II laboratory facility available in the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

3.1.2. Chemicals and Reagents

The chemicals and reagents used in the present study were of analytical and molecular grade. The composition of buffers and reagents used in the study are appended in Annexure I. Microbiological media used for the isolation of the organism and various reagents for biochemical tests were procured from HiMedia, India. The composition of the media and specific procedure followed in its preparation are appended in Annexure II. Molecular reagents were procured from Bangalore GeNei, India Limited and from Fermentas, Bangalore.

3.2. COLLECTION OF SAMPLES

3.2.1. Sampling plan

The study period ranged from June 2015 to May 2016 which was divided into two seasons: Monsoon season (designated as season A) from June to November and the Post monsoon season (designated as season B) from December to May. Out of the total 570 samples, half of the samples were procured during season A and the remaining during season B.

3.2.2. Farm Facility

To determine the critical control points in egg production chain, samples were collected from University Poultry and Duck Farm (UPDF), Mannuthy. Following details were also collected during sample collection.

1. Facilities available in the farm

2. Sanitary practices followed during poultry rearing

3. General routine of the farm

4. Disinfection procedure

3.2.2.1. Cloacal Swabs

From the UPDF, 30 cloacal swabs were collected randomly from Gramasree chicken (*Gallus domesticus*). Cloacal swab samples from the birds were collected using sterile cotton swabs (HiMedia, India) dipped in seven millilitres of Cary Blair medium with three per cent charcoal in a screw capped tube (Fig 3).

3.2.2.2. Feed

Fifteen feed samples were collected randomly from the point of lay and examined in each season. The collection was spread over the entire length of the season. Approximately 250g each of feed samples were collected in sterile bottles.

3.2.2.3. Litter

Fifteen litter samples of 250g each were collected randomly from the point of lay during each season. All the samples were taken in sterile bottles.

3.2.2.4. Wash water

A total of 30 egg wash water samples were taken from the point of washing during two seasons. The water used for washing eggs contained the disinfectant kohrsolin at the rate of 0.1 per cent. Approximately 250 ml each of wash water samples were collected in sterile polythene bags.

3.2.2.5. Wash cloth

Cloth used for wiping the egg shell surfaces was strained and the residual water coming out was collected in to sterile polythene bags. A total of 30 wash cloth

samples of 250 ml each were collected from the point of washing during the two seasons.

3.2.2.6. Plastic trays

The plastic trays were used for collection of eggs from the point of lay. The area taken by a single egg in the tray was swabbed using sterile cotton swab dipped in seven millilitres of Cary Blair medium with three per cent charcoal in a screw capped tube. Altogether 30 plastic tray swab samples were collected during two seasons.

3.2.2.7. Filler flats

The filler flats were used for transporting eggs from the hatchery to the sales counter and for storage at the sales counter till sales. From the sales counter, 30 filler flat swab samples were collected during two seasons. Sterile cotton swab dipped in seven millilitres of Cary Blair medium with three per cent charcoal in a screw capped tube was used for rubbing over the area occupied by an egg in the filler flat.

3.2.2.8. Air

Six air samples were collected from each of the five different points in the farm namely point of lay, after washing, after candling, at the storage room and at the sales counter during two seasons. To estimate the count, duplicate P-mCCDA (Polymyxin B supplemented modified Charcoal Cefoperazone Deoxycholate Agar) plates were exposed for 30 min (Evancho *et al.*, 2001).

3.2.2.9. Water

Water for bacteriological examination was collected following the procedures described by Indian Standards (1978). All together 30 water samples were collected from the point of lay during two seasons.

3.2.2.10. Hand wash of candling personnel

Hand wash of the personnel involved in candling were collected by washing the hands of each worker in 100 ml of 0.9 per cent sterile normal saline in sterile polythene bags. A total of 30 hand washings were collected from the point of candling in two different seasons.

3.2.2.11. Hand wash of sales personnel

Thirty hand washings of sales personnel were collected during two seasons. Hundred millilitres of 0.9 per cent sterile normal saline in sterile polythene bags were used for collecting the hand washings.

3.2.2.12. Eggs

A total of 120 chicken eggs were randomly collected from Gramasree birds at UPDF, wherein eggs were used for hatching and a portion goes for sales as table eggs. Based on the external shell quality with respect to shell cracks upon candling, the eggs were separated out for sales and hatching (Fig. 4). The chicken eggs in the Table Egg Production Chain (TEPC) and Hatching Egg Production Chain (HEPC) were simultaneously subjected to isolation and identification of *Campylobacter* spp. and also for microbiological quality analysis.

3.2.2.12.1. Table Egg Production Chain (TEPC)

Four points in the TEPC were considered as sampling points for egg collection. These included the point of lay, after washing, after candling and at the sales counter.

3.2.2.12.2. Hatching Egg Production Chain (HEPC)

Five points in the HEPC were considered for egg collection during sampling. These included the point of lay, after washing, after candling, the storage room after six days of storage of eggs and the incubator after six days of setting.

3.2.2.12.3. Sampling plan

A total of 60 eggs were collected during each season. Ten eggs were randomly collected each from the point of lay, washing, candling, sales counter, storage room and incubator within a season. From each point, two eggs were procured yielding a total of 12 per day and a total of five collections were done in each season.

3.2.2.12.4. Egg collecting procedure

Eggs were picked up with fingertips and placed into individual airtight polythene covers sterilized by UV light. The fingertips were disinfected by dipping in 70 per cent alcohol solution, between each sampling point.

3.2.3. Eggs in the retail market

Eggs were transported to the retail shops in filler flats under ambient conditions and reach market within a week of production. In order to gain insight into the occurrence of *Campylobacter* spp. and microbiological quality of eggs, the eggs from different retail market in and around Thrissur were collected during two seasons and analyzed.

3.2.3.1. Sampling plan

A total of 120 eggs were collected randomly from different retail market in and around Thrissur during season A and season B. Five collections of 12 eggs each were taken during a season and eggs were brought to the laboratory in individual UV sterilized polythene covers.

3.3. TRANSPORTATION OF SAMPLES

In order to protect the swab samples from drying and the lethal effects of atmospheric oxygen, Cary-Blair transport medium with three per cent charcoal was used for transportation. The aseptically collected samples were immediately

transported at 4^{o}C to the laboratory and were processed within four hours of collection to ensure that the organisms remain viable and culturable.

3.4. PROCESSING OF SAMPLES

The collected samples were processed in the Biosafety level - II laboratory facility available in the Department of Veterinary Public Health, College of Veterinary and Animal Sciences, Mannuthy. All the samples collected from the egg production chain were subjected to isolation and identification of *Campylobacter* spp. The egg surface swab and internal contents were screened for the presence of *Campylobacter* spp. Enumeration of the microbial counts like total viable count and yeast and mould count were done for each egg surface swab samples. The samples remaining after the tests and negative samples were discarded as per standard methods including proper decontamination.

3.4.1. Egg swabbing procedure

Swabbing of intact eggs was done according to the procedure described by Evancho *et al.* (2001). A sterile cotton swab (HiMedia) was taken aseptically by grasping the end of the stick and moistened with Phosphate buffered saline (HiMedia) and the excess diluent in swab was removed by gentle pressing of the swab on the inside of test tube containing 30 ml sterile diluent. The swab handle was holded at 30^{o} angle contact with the egg surface. Rubbed the swab head slowly and thoroughly over the surface three times, reversing direction between strokes. The swab head was then returned to the diluent tube and shaked vigorously, making 50 complete cycles in 10 sec, striking the palm of the other hand at the end of each cycle. This formed the triturate of the egg surface swab.

3.4.2. Sampling of egg contents

Preparation of the sample was done according to the procedure described by Ricke *et al.* (2001). Each egg was washed with brush, using soap and water. Excess

moisture was drained off and egg was immersed in 70 per cent alcohol for 10 min, after which it was removed from alcohol, drained and flamed under a spirit lamp. The egg was then cracked using a sterile breaking knife and the egg contents were aseptically poured into a sterile beaker and homogenized using an egg beater. The homogenous sample was used for the enrichment of *Campylobacter* spp.

3.5. ISOLATION AND IDENTIFICATION OF *CAMPYLOBACTER*

Isolation and identification of *Campylobacter* from collected samples were carried out by selective enrichment followed by selective plating as recommended by Stern *et al*. (2001) and OIE Terrestrial Manual (2008) with necessary modifications (Fig 5).

3.5.1. Selective Enrichment

The selective enrichment of the samples was carried out in Blood Free *Campylobacter* (modified Charcoal Cefoperazone Deoxycholate, mCCD) broth (HiMedia, India) with CCDA selective supplement (FD 135) under microaerophilic conditions at 42^{o}C for 48 h.

All the samples except cloacal swabs and air samples were subjected to enrichment at the ratio of 1:9. A 25 gram portion of each sample collected from egg production chain was aseptically transferred to 225 ml of mCCD broth.

Twenty five millilitres of the egg surface swab triturate was enriched in 225 ml mCCD broth. For the inner egg content, five millilitre of the homogenized sample was aseptically transferred to 45 ml of mCCD broth.

The filler flat and plastic tray swab samples were enriched in 25 ml of mCCD broth.

Hundred millilitres of wash water, wash cloth, water and hand wash samples were subjected to membrane filtration through cellulose ester filters (MF-Millipore membrane filter) of 0.22 µm pore size and 47 mm diameter. Then the filters were completely immersed in 225 ml of mCCD enrichment broth.

3.5.2. Selective Plating

Cloacal swab samples were streaked directly onto P-mCCDA (Polymyxin B supplemented modified Charcoal Cefoperazone Deoxycholate Agar) plates and incubated under microaerophilic conditions at 42^{o}C for 48 h.

Air sampling P-mCCDA plates were brought to the laboratory and incubated under microaerophilic conditions at 42^{o}C for 48 h.

Loopful of the samples in mCCD broth were selectively plated on to Blood Free *Campylobacter* Selectivity (modified Charcoal Cefoperazone Deoxycholate, mCCD) agar media supplemented with CAT selective supplement (FD 145), *Campylobacter* supplement V (FD 067) and Polymyxin B selective supplement (FD 003) as per the procedure described by Chon *et al*., 2012. It was then incubated under microaerophilic conditions consisting of 10 per cent CO_2 at 42^{o}C for 48 h. Greyish, flat, spreading type, shiny, mucoid and moistened colonies with tendency to spread, and with or without metallic sheen were selected for further characterization.

3.6. CHARACTERIZATION AND IDENTIFICATION OF ISOLATES

The suspected colonies of *Campylobacter* spp. were subjected to various tests and identified based on the cultural, morphological and biochemical characteristics described by Tenover and Fennell (1992). The individual colonies from P-mCCD agar were transferred to Brucella broth (HiMedia) and incubated at 42^{o}C for 48 h under microaerophilic conditions. A brief description employed in various biochemical tests to identify *Campylobacter* spp. is described in Annexure III.

Table 1. Typical biochemical reactions of commonly isolated thermotolerant *Campylobacter* spp.

Tests	**Biochemical reaction**			
	C. jejuni	***C. coli***	***C. lari***	***C. upsaliensis***
Gram staining	-	-	-	-

Oxidase	+	+	+	+
Catalase	+	+	+	-
Motility	+	+	+	+
Aerobic growth test at 37^{o}C	-	-	-	-
Growth at 25^{o}C under microaerophilic conditions	-	-	-	-
Growth at 42^{o}C under microaerophilic conditions	+	+	+	+

Table 1. Typical biochemical reactions of commonly isolated thermotolerant *Campylobacter* spp. (Continued)

Tests	**Biochemical reaction**			
	C. jejuni	***C. coli***	***C. lari***	***C. upsaliensis***
Indoxyl Acetate hydrolysis	+	+	-	+
H_2S on TSI agar medium	-	D	-	-
Sensitivity to Nalidixic acid	S	S	R	S
Resistance to Cephalothin	R	R	R	S

+ - 90% or more of strains are positive; - - 90% or more of strains are negative; D - 11-89% of strains are positive; R - Resistant; S - Susceptible.

3.7. MICROBIAL COUNTS

3.7.1. Preparation of samples

In order to estimate the bacterial load on the surface of the egg, one millilitre of the egg surface swab triturate was transferred to nine millilitre of 0.9 per cent normal saline so as to form one in 10 dilution of the sample. Serial dilution of sample was made.

3.7.2. Aerobic Plate Count

Aerobic plate count (APC) of each sample was estimated by pour plate technique, as described by Morton (2001). From the selected tenfold dilution of each sample, one millilitre of the inoculum was transferred on to duplicate Petri-dish of uniform size. To each of the inoculated plates about 10 to 15 millilitre sterile molten standard plate count agar (HiMedia) (Annexure II) maintained at 45ºC was poured and mixed with the inoculum by gentle rotary movement i.e., clockwise, anticlockwise, forward and backward. The inoculated plates were left at room temperature and allowed to solidify, and incubated at 37^{o}C for 24 h. At the end of incubation, plates showing between 30 and 300 colonies were selected and counts taken with the help of a colony counter. The number of colony forming units (CFU) per egg shell was calculated by multiplying the mean count in duplicate plates with the dilution factor and was expressed as $\log_{10}$ CFU/egg shell.

3.7.3. Yeast and Mould Count

The method described by Beuchat and Cousin (2001) was followed for estimation of yeast and mould count. Potato dextrose agar (HiMedia) (Annexure II) was used and count estimated by spread plate technique. From the selected dilution of each sample 0.1 ml of inoculum was transferred on to duplicate plates containing the media and the inoculum was evenly distributed on the media with a sterile 'L' shaped glass rod. The plates were incubated at 25^{o}C for three to five days. After the period of incubation the colonies in the plates were counted with the help of colony counter and mean count was multiplied with the dilution factor and expressed as $\log_{10}$ CFU/egg shell.

3.8. COMPARATIVE ASSESSMENT OF THE SEASONAL OCCURRENCE OF *CAMPYLOBACTER* AND MICROBIOLOGICAL QUALITY OF EGGS FROM UPDF AND RETAIL MARKET

Occurrence of *Campylobacter* and microbiological qualities of eggs available from the UPDF, Mannuthy and those in the retail market, which are produced under two systems of rearing, were compared during the two seasons of the study period.

3.9. STANDARDISATION OF POLYMERASE CHAIN REACTION (PCR) PROTOCOL FOR *CAMPYLOBACTER* SPP.

Standardisation of PCR for *Campylobacter* spp. was carried out as per the procedure described by Denis *et al.* (1999) with slight modifications.

3.9.1. Reagents and Chemicals for PCR

Molecular reagents and chemicals used in the study were procured from Sigma, Bangalore, Fermentas, Bangalore, Thermo Scientific (EU) and Sigma-Aldrich (USA). The reagents and chemicals used for the polymerase chain reaction were PCR reaction buffer, Taq DNA polymerase, dNTP mix, $MgCl_2$, forward and reverse primer set and sterilized milliQ water. The materials used for submarine agarose gel electrophoresis were Tris Boric acid EDTA (TBE), Electrophoresis buffer, agarose gel, gel loading buffer, ethidium bromide and molecular weight marker (50 bp ladder).

3.9.2. Oligonucleotide Primers

The oligonucleotide primers were custom synthesized commercially and obtained in lyophilized form. The primers (forward and reverse) used were procured from Sigma–Aldrich, St. Louis, MO. The target gene that was detected by PCR and the primer sequences used in the study is shown in table 2.

Table 2. Primers used for the identification of *Campylobacter* spp.

Gene	Primer	Length	Primer sequence	Size (bp)	Ref.
16S rRNA	F	19	5'-GGATGACAC TTTTCGGAGC-3'	816	Linton *et al.* (1996)
	R	18	5'-CATTGTAGC ACGTGTGTC-3'		
*cad*F	F	20	5'-TTGAAGGTAA TTTAGATATG-3'	400	Rozynek *et al.*

	R	20	5’-CTAATACCTA AAGTTGAAAC-3’		(2005)
*map*A	F	24	5’-CTATTTTATTTT TGAGTGCTTGTG-3’	589	Denis *et al.* (1999)
	R	25	5’-GCTTTATTTGCC ATTTGTTTTATTA-3’		
*ceu*E	F	23	5’-AATTGAAAATT GCTCCAACTATG-3’	462	Denis *et al.* (1999)
	R	23	5’-TGATTTTATTA TTTGTAGCAGCG-3’		

3.9.3. Reconstitution of Primers

The primers obtained in lyophilized form were reconstituted in sterile milliQ water to a concentration of 100 p*M*/µL. The tubes with primers were kept at room temperature with occasional shaking for one hour. They were spun to pellet down the insoluble particles, if any and the stock solution was distributed into 10µL aliquots and stored at -20^{o}C. At the time of use, the aliquots were thawed and further diluted to obtain a concentration of 10 p*M* /µL of *16S rRNA*, *map*A and *ceu*E primers and 20 p*M* /µL of *cad*F primer before using for PCR.

3.9.4. Preparation of DNA Template

The boiling and snap chilling technique was used for the preparation of DNA template (Englen and Kelley, 2000). A loopful of the standard strain ATCC® 33560™ of *Campylobacter jejuni subsp. jejuni* and ATCC® 33559™ of *Campylobacter coli* was transferred to Brucella broth and incubated under microaerophilic conditions at 42^{o}C for 48 h. Isolates obtained in this study were also processed in the same way. From this 1.5 ml of culture was taken into an eppendorf tube and centrifuged in a cooling microcentrifuge at 8000 rpm for 10 min at 4^{o}C. The supernatant was discarded and the pellet obtained at the bottom was washed in 0.5 ml of sterilized DNAse and RNAse- free Milli-Q-water by re-centrifugation at 8000 rpm for 10 min at 4^{o}C. The pelleted cells obtained finally were resuspended in 100 µl of

sterilized DNAse and RNAse- free Milli-Q-water and then kept in a boiling water bath for 15 min and immediately snap-chilled on crushed ice at 4^{o}C for 30 min. Then the samples were centrifuged at 10000 rpm for 10 min and the supernatant was aliquoted into new micro centrifuge tubes. Samples were then stored at -20^{o}C until used as template for PCR (Fig. 6). From this 2.5 µl of template DNA were used directly for the PCR.

3.9.5. Standardisation and Setting of PCR

Polymerase chain reaction was performed in a total volume of 25 µl reaction mixture using 2.5 µl of extracted DNA as template. A master mix was prepared before setting up of the PCR reaction by combining the following reagents except template DNA in 22.5 µl volume. The components of reaction mixture for one reaction are shown in table 3.

Table 3. Components of PCR reaction mixture

Sl. No	Name of the reagent	Stock Concentration	Quantity (µl)
1	Template DNA	50ng/µl	2.50
2	10X PCR buffer	200m*M*	2.50
3	$MgCl_2$	25m*M*	2.00
4	*Taq* DNA polymerase	5Units/µl	0.25
5	dNTP Mix	2m*M* each	1.00
6	Forward primer	10p*M*/µl	1.00
7	Reverse primer	10p*M*/µl	1.00
8	Nuclease free water		14.75
Total			25.00

3.9.5.1. *Standardisation of $MgCl_2$ Concentration*

Different concentrations of $MgCl_2$ *viz.*, 1.5 m*M*, 2 m*M*, 2.5 m*M* and 3 m*M*. was used to carry out PCR, in order to find out the optimum concentration for the reaction.

3.9.5.2. *Standardisation of Annealing Temperature for PCR*

Gradient PCR was carried out at different annealing temperatures *viz.*, 47.8, 48.9, 50.2, 51.8, 53.5 and 55.1^{o}C in order to find out the optimum temperature for the reaction.

3.9.5.3. *PCR Setting*

To each PCR tube 22.5 µl of master mix and 2.5 µl of template DNA were added. The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) according to the following programme. The annealing temperatures for each of the primers *16S rRNA*, *cad*F, *map*A and *ceu*E genes were optimized as 51.8^{o}C (Table 4).

Table 4. Cyclic conditions used for PCR of *16S rRNA*, *cad*F, *map*A and *ceu*E genes

Sl. No	Step	Temperature	Time (min)	No. of Cycles
1	Initial denaturation	95^{o}C	10	1
2	Final denaturation	94^{o}C	1	30
3	Annealing	51.8^{o}C	1	
4	Extension	72^{o}C	1	
5	Final extension	72^{o}C	10	1
6	Hold	4^{o}C	10	

3.9.6. Submarine agarose gel electrophoresis

The PCR products were detected by electrophoresis in 1.5 per cent agarose gel in TBE electrophoresis buffer (1X). Tris Boric acid EDTA (TBE) electrophoresis buffer 1X was prepared by diluting TBE 10X buffer with milliQ water. Agarose was dissolved in 1X TBE buffer by heating. Then, the mixture was cooled to around 50^{o}C and ethidium bromide was added to a final concentration of 0.5µg/ml. The clean, dry gel platform edges were sealed with adhesive tape and the comb was kept in proper position before pouring melted agarose. Once the gel was set, the comb and adhesive tape were removed gently and placed the gel tray in buffer tank with wells kept towards cathode. Buffer (TBE 1X) was poured till it covered the top of the gel completely.

Amplified PCR product (five microlitre) was mixed with gel loading dye (three microlitre) and the samples were loaded in the wells. The 50 bp DNA ladder was also loaded in one of the wells as DNA molecular size marker in order to compare the size of the amplified product.

The gel was visualized under UV transilluminator (Hoefer, USA) and the images were documented on gel documentation system (Bio-Rad Laboratories, USA).

3.10. STANDARDISATION OF MULTIPLEX PCR

A multiplex PCR assay was standardised for detecting *16S rRNA*, *cad*F, *map*A and *ceu*E genes simultaneously in a single reaction tube containing all the four primer sets for *Campylobacter*. The optimum annealing temperature and $MgCl_2$ concentration used for individual PCR was employed for the standardisation of multiplex PCR. The components used in the multiplex PCR mixture are shown in table 5.

Table 5. Components of multiplex PCR mixture

Sl. No	Name of the reagent	Stock Concentration	Quantity (µl)
1	Template DNA	50ng/µl	5.00
2	10X PCR buffer	200m*M*	3.00
3	$MgCl_2$	25m*M*	2.00
4	*Taq* DNA polymerase	5Units/µl	0.75
5	dNTP Mix	2m*M* each	2.50
6	Forward primer of *16S rRNA* gene	10p*M*/µl	1.00
7	Reverse primer of *16S rRNA* gene	10p*M*/µl	1.00
8	Forward primer of *cad*F gene	20p*M*/µl	1.00
9	Reverse primer of *cad*F gene	20p*M*/µl	1.00
10	Forward primer of *map*A gene	10p*M*/µl	1.00
11	Reverse primer of *map*A gene	10p*M*/µl	1.00
12	Forward primer of *ceu*E gene	10p*M*/µl	1.00
13	Reverse primer of *ceu*E gene	10p*M*/µl	1.00
14	Nuclease free water		8.75
Total			30.00

3.11. ANTIBIOTIC RESISTANCE PROFILING

All *Campylobacter* isolates were examined for their antimicrobial drug susceptibility/resistance pattern by disc diffusion method, as per the procedure described by Baserisalehi *et al.* (2007) against 29 different antibiotics. The different antibiotics used in the test are shown in table 6.

3.11.1. Preparation of Mac Farland Standard

The turbidity standard solution was prepared by adding 0.5 ml of 0.048 M $BaCl_2$ to 99.5 ml of 0.36 N H_2SO_4 (one per cent w/v). This solution was equal to half the density of No.1 Mac Farland standard solution. This solution was taken into glass tube, sealed tightly and kept in the dark, at room temperature for further use. The tube was vigorously agitated just before each use.

3.11.2. Preparation and Standardisation of Inoculum

Three to four isolated colonies were selected from a pure culture and transferred into sterile Mueller Hinton broth and incubated at 42^{o}C, overnight under microaerophilic conditions. The turbidity of culture was adjusted to 0.5 Mac Farland standard solution. When the broth culture was found to be more turbid, it was diluted with nutrient broth and when the turbidity was found to be less, culture was incubated for more time to achieve the required turbidity.

3.11.3. Inoculation

The swab was dipped into standardised inoculum and excess inoculum was removed from the swab by rotating it several times with a firm pressure on the inside wall of the test tube, above the fluid level. The Mueller Hinton agar (HiMedia) plates with five per cent sheep blood were inoculated with the inoculums by swabbing over its entire surface, within 15 min after adjusting the density of inoculum. The swabbing procedure was repeated two more times, rotating the plates approximately 60^{o} at each time, to ensure an even distribution of inoculums. The inoculum was allowed to dry for 15 min.

Sl. No	Antibiotics	Concentration (µg)
15	Imipenem	10
16	Kanamycin	30
17	Levofloxacin	5
18	Meropenem	10
19	Moxifloxacin	5

Table 6. Antibiotics used and their concentration

Sl. No	Antibiotics	Concentration (µg)
1	Amikacin	30
2	Ampicillin	10
3	Aztreonam	30
4	Carbenicillin	100
5	Cefotaxime	30
6	Ceftazidime	30
7	Cephalothin	30
8	Chloramphenicol	25
9	Ciprofloxacin	5
10	Clindamycin	2
11	Co-trimoxazole	25
12	Doxycycline	10
13	Erythromycin	15
14	Gatifloxacin	5
20	Neomycin	10
21	Netillin	30
22	Nitrofurantoin	300
23	Norfloxacin	10
24	Ofloxacin	5
25	Oxacillin	1
26	Sparfloxacin	5
27	Streptomycin	25
28	Tetracycline	30
29	Tobramycin	10

3.11.4. Application of Antibiotic Discs

The inoculated plate was left for not more than 15 min at room temperature to absorb any excess surface moisture before applying the drug impregnated discs (HiMedia). The discs were applied to the surface of the inoculated agar with a

sterile forceps. With the tip of the forceps, each disc was gently pressed down to ensure complete contact with the agar surface. During the application of discs care was taken not to place it closer than 15 mm from the edge of the plate and the distance between the centre's of two such discs was not less than 24 mm. The inoculated plate was inverted and incubated at 42^{o}C for 24 h under microaerophilic conditions, within 15 min after the application of the discs.

3.11.5. Reading and Interpretation

At the end of the incubation period, the plates were examined and the diameter of the zones of complete inhibition was measured to the nearest whole millimetre with a scale held on the back of the Petri-plate, which was illuminated with a reflected light.

The zone of inhibition of each disc was measured in three different directions keeping the midpoint of the disc as the centre of the zone. The mean of the measurement of inhibition was used for the interpretation of the results. The interpretation of the result was made by comparing diameter of the zone of inhibition with standard zone of inhibition chart provided by the disc manufacturing company (Fig 7). The clinical breakpoints for *Campylobacter* spp. susceptibility testing was defined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2016) and the isolates were grouped as sensitive, intermediary sensitive and resistant, against each antibiotic.

3.12. STATISTICAL ANALYSIS

The data obtained were subjected to statistical analysis following procedure described by Snedecor and Cochran (1994) using the SPSS version 21.0.

The significance of occurrence of *Campylobacter* spp. from different sources during two seasons were analysed using Fisher's exact test.

The evaluation of conventional culture technique and PCR was done by calculation of sensitivity and specificity (Martin, 1977). The definitions and

calculations of these values are shown in table. 7. As the true *C. jejuni* status of naturally contaminated samples was not known, the method of calculation assumed that the PCR technique as the true value. The efficiency of two tests was compared by calculation of the k coefficient.

The significance of microbial quality of farm and retail eggs between different sources as well as between different seasons were assessed using Analysis of variance (ANOVA).

The antibiotic sensitivity test results were subjected to hierarchial cluster analysis with dendrogram using average linkage (between groups) by rescaled distance cluster combine method.

Table 7. Definition and calculation of sensitivity, specificity, predictive value and k coefficient

Test method studied		**PCR**	
		+	**-**
	+	**a**	**b**
	-	**c**	**d**

a- True positive, positive test with confirmed *Campylobacter*

b- False positive, positive test without confirmed *Campylobacter*

c- False negative, negative test with confirmed *Campylobacter*

d- True negative, negative test without confirmed *Campylobacter*

- Sensitivity (the ability to detect positive samples)- a/(a+c)
- Specificity (the ability to detect negative sample)- d/(b+d)

- Predictive value of:
  - Positive test (the probability of a positive result being correct)- a/(a+b)
  - Negative test (the probability of a negative result being correct)- d/(c+d)
- Kappa coefficient- (Efficiency-X)/(1-X)
  - Efficiency- (a+d)/(a+b+c+d)
  - X- [(a+b)/n][(a+c)/n)]+[(c+d)/n][(b+d)/n)]

4. RESULTS

The present investigation was undertaken to study the occurrence of *Campylobacter* spp. and microbiological quality of chicken eggs in University Poultry and Duck Farm (UPDF), Mannuthy and retail outlets in and around Thrissur for a period of 12 months from June 2015 to May 2016. The probable critical control points in the egg production chain to curtail the occurrence of organism in eggs were also investigated. The samples were aseptically processed and isolation of *Campylobacter* spp. was carried out by conventional cultural technique. Aerobic plate count (APC) and yeast and mould count (YMC) were used for assessing microbiological quality of eggs produced from the farm and retail market. The seasonal effect on the occurrence of *Campylobacter* spp. and microbiological quality of eggs during monsoon and post monsoon seasons were analysed. Species and virulence gene identification of *Campylobacter* spp. was carried out by PCR. The antibiotic sensitivity test of the positive isolates were carried out to determine antibiotic of choice in treatment using standard disc diffusion method.

4.1. COLLECTION OF SAMPLES

A total of 570 samples, comprising of 30 samples each of cloacal swab, feed, litter, wash water, wash cloth, plastic tray, filler flat, air, water, hand wash of candling personnel, hand wash of sales personnel and 120 eggs from University Poultry and Duck Farm (UPDF), Mannuthy and 120 samples of egg from retail market in and

around Thrissur were collected. Half of the samples were collected during monsoon season (season A) from June to November and the remaining during post monsoon season (season B) from December to May.

4.2. OCCURRENCE OF *CAMPYLOBACTER* SPP. IN CHICKEN EGG PRODUCTION CHAIN

All the samples collected were subjected to isolation and identification of *Campylobacter* spp. by conventional culture technique. Species level differentiation was carried out by using multiplex polymerase chain reaction.

The *Campylobacter* spp. on P-mCCDA plate after microaerophilic incubation at 42˚C for 48 h appeared as 1 – 2 mm diameter, greyish, round, flat to slightly raised, spreading type, shiny, moistened colonies with or without metallic sheen. When left for prolonged period on the agar plates, the colonies turned greyish and sticky (Fig. 8). Further characterization of *Campylobacter* colonies were carried out by biochemical tests (Fig. 9).

4.2.1. Cloacal swabs

Out of the 30 cloacal swab samples screened from the point of lay during two seasons, 13 samples had colonies with characteristics of *Campylobacter* spp. Higher occurrence of *Campylobacter* spp. from cloacal swab was noticed in season A with 66.67 per cent than season B with 20 per cent (Table 8).

Fisher's exact test showed a significant difference ($p<0.05$) in the occurrence of *Campylobacter* spp. in cloacal swab samples during season A as compared to season B.

4.2.2. Feed

A total of 30 feed samples were collected from the point of lay in UPDF, Mannuthy in two seasons. Of the 15 feed samples screened, each during season A and B, none of the samples were found to be positive for the presence of *Campylobacter* spp. (Table 8)

4.2.3. Litter

Fifteen litter samples were collected from the point of lay in UPDF, Mannuthy each during season A and B. All the 30 litter samples examined, were found to be negative for *Campylobacter* spp. (Table 8)

4.2.4. Wash water

From UPDF, 30 wash water samples were collected from the point of washing in two seasons. The 15 wash water samples analysed each during season A and B were not contaminated with *Campylobacter* spp. (Table 8)

Table 8. Occurrence of *Campylobacter* spp. in UPDF, Mannuthy

Sl. No	Sources	Season A			Season B		
		Total no. of samples	*Campylobacter* positive samples		Total no. of samples	*Campylobacter* positive samples	
			No	%		No	%
1	Cloacal swab	15	10	66.67 [a]	15	3	20 [b]
2	Feed	15	0	0.00	15	0	0.00
3	Litter	15	0	0.00	15	0	0.00
4	Wash water	15	0	0.00	15	0	0.00
5	Wash cloth	15	0	0.00	15	0	0.00
6	Plastic tray	15	0	0.00	15	0	0.00
7	Filler flat	15	0	0.00	15	0	0.00
8	Air	15	0	0.00	15	0	0.00

9	Water	15	0	0.00	15	0	0.00
10	Hand wash-candling person	15	0	0.00	15	0	0.00
11	Hand wash-sales person	15	0	0.00	15	0	0.00
12	Eggs	60	0	0.00	60	0	0.00
Total		**225**	**10**	**4.44**	**225**	**3**	**1.33**

Figures bearing same superscripts do not differ significantly ($p<0.05$)

4.2.5. Wash cloth

Altogether 30 wash cloth samples were procured from the point of washing during season A and B. *Campylobacter* spp. was not isolated from the 15 wash cloth samples tested each during season A and season B (Table 8).

4.2.6. Plastic trays

From the point of lay in UPDF, 15 plastic tray swab samples were collected each during season A and B. All the 30 plastic tray samples were found to be negative for *Campylobacter* spp. (Table 8)

4.2.7. Filler flats

Thirty filler flat swab samples were collected from sales counter of UPDF Mannuthy in two seasons. *Campylobacter* spp. was not detected in any of the 30 filler flat samples studied during season A and B (Table 8).

4.2.8. Air

Six air samples were collected from each of the five different points in the farm namely point of lay, after washing, after candling, at the storage room and at the sales counter during two seasons. All the 30 air samples were not contaminated with *Campylobacter* spp. (Table 8)

4.2.9. Water

A total of 30 water samples were collected from the point of lay during two seasons. Out of the 15 samples evaluated each during season A and B, all the water samples were found to be negative for the presence of *Campylobacter* spp. (Table 8)

4.2.10. Hand wash of candling personnel

Thirty hand wash samples of personnel involved in candling were collected during season A and B. *Campylobacter* spp. could not be isolated from any of the hand wash samples analysed during both seasons (Table 8).

4.2.11. Hand wash of sales personnel

Thirty hand washings of sales personnel were collected from sales counter during two seasons. The 15 hand wash samples screened each during season A and B did not showed the presence of organism (Table 8).

4.2.12. Farm eggs

A total of 120 chicken eggs were collected from UPDF, Mannuthy during two seasons. Twenty eggs each were collected from the point of lay, point of washing, point of candling, storage room, incubator and sales counter during the two seasons.

The presence of the organism could not be detected in both the outer shell and inner content of the 120 egg samples collected from different points in UPDF, Mannuthy. Eggs from the table egg production chain and hatching egg production

chain did not showed the presence of organism during season A and season B (Table 9).

Table 9. Occurrence of *Campylobacter* spp. in farm eggs

Points of collection	Egg					
	Season A			Season B		
	Total no. of samples	*Campylobacter* positive samples		Total no. of samples	*Campylobacter* positive samples	
		No.	%		No.	%
Point of lay	10	0	0	10	0	0
After washing	10	0	0	10	0	0
After candling	10	0	0	10	0	0
Storage room (6^{th} day)	10	0	0	10	0	0
Incubator (6^{th} day)	10	0	0	10	0	0
Sales counter	10	0	0	10	0	0
Total	**60**	**0**	**0**	**60**	**0**	**0**

4.2.13. Overall occurrence of *Campylobacter* spp. in UPDF, Mannuthy

Out of the 450 samples from UPDF, Mannuthy, 13 cloacal swab samples were found to be positive for *Campylobacter* spp. Among the 225 samples tested each during season A and season B, 10 and three cloacal swab samples produced characteristic *Campylobacter* colonies, respectively. Highest occurrence of *Campylobacter* spp. was found in season A (4.44 per cent) followed by season B (1.33 per cent). The organism could not be detected from any other samples. There was an overall occurrence of 2.89 per cent of *Campylobacter* spp. in UPDF, Mannuthy (Table 8). The overall occurrence of *Campylobacter* spp. in UPDF, Mannuthy during season A and season B is represented in Fig. 10.

4.3. CRITICAL CONTROL POINTS OF *CAMPYLOBACTER* SPP. IN EGG PRODUCTION CHAIN

Out of the 330 samples screened from various points of chicken egg production chain of UPDF, Mannuthy, 13 cloacal swab samples (3.94 per cent) were positive for *Campylobacter* spp. as shown in table 10. Occurrence of *Campylobacter* spp. in cloacal swab samples from the point of lay at a level of 43.33 per cent makes it a critical control point for *Campylobacter* species in the egg production chain (Fig. 11). All other samples were found to be negative for the organism. Hence cloaca of the bird was the one and only critical point of contamination, which is a major source of contamination in the egg production chain and point of lay was the critical control point where only partial control can be achieved on the occurrence of the organism (CCP2) (Fig. 12).

Table 10. Overall occurrence of *Campylobacter* spp. in critical control points in egg production chain

Sl. No	Sources	Total no. of samples	*Campylobacter* positive samples	
			No.	%
1	Cloacal swab	30	13	43.33
2	Feed	30	0	0
3	Litter	30	0	0

4	Wash water	30	0	0
5	Wash cloth	30	0	0
6	Plastic tray	30	0	0
7	Filler flat	30	0	0
8	Air	30	0	0
9	Water	30	0	0
10	Hand wash of candling person	30	0	0
11	Hand wash of sales person	30	0	0
Total		**330**	**13**	**3.94**

4.4. OCCURRENCE OF *CAMPYLOBACTER* SPP. IN EGGS FROM RETAIL MARKET

A total of 120 chicken eggs were collected from retail market in and around Thrissur during two seasons. Out of the 60 egg samples collected during season A, the characteristic colonies of *Campylobacter* spp. could be identified in four egg shell samples. But none of the inner contents examined during season A were positive for the organism. The occurrence of *Campylobacter* spp. in eggs from retail market was found to be 6.67 per cent during season A (Table 11). Both the outer shell and inner content of the 60 egg samples collected during season B were negative for *Campylobacter* spp.

Table 11. Occurrence of *Campylobacter* spp. in eggs from retail market

Source	**Season A**			**Season B**		
	Total no. of samples	***Campylobacter* positive samples**		**Total no. of samples**	***Campylobacter* positive samples**	
		No.	**%**		**No.**	**%**

Retail market eggs	60	4	6.67 [a]	60	0	0 [a]

Figures bearing same superscripts do not differ significantly ($p<0.05$)

On statistical analysis by Fisher's exact test, the occurrence of *Campylobacter* spp. in eggs from retail market during season A and season B was found to have no significant difference ($p<0.05$). The overall occurrence of *Campylobacter* spp. in retail market eggs in and around Thrissur during season A and season B is shown in Fig. 13.

4.5. COMPARATIVE ASSESSMENT ON THE OCCURRENCE OF *CAMPYLOBACTER* SPP. IN EGGS FROM UPDF, MANNUTHY AND RETAIL MARKET

A total of 240 eggs including 120 each of farm eggs and eggs from retail market were collected during two seasons. Both the outer shell and inner content of the farm eggs did not showed the presence of *Campylobacter* spp. during both the seasons. The overall occurrence of *Campylobacter* spp. in UPDF, Mannuthy was found to be 2.89 per cent. Occurrence of *Campylobacter* spp. during monsoon season in egg shell samples from retail market was found to be 6.67 per cent. All the 60 eggs from retail market screened during post monsoon season were found to be negative for *Campylobacter* spp. The overall occurrence of *Campylobacter* spp. in eggs from retail market was found to be 3.33 per cent.

4.6. MICROBIOLOGICAL QUALITY OF CHICKEN EGGS IN THE PRODUCTION CHAIN

A total of 120 eggs were collected from UPDF, Mannuthy during season A and B. Ten eggs were procured each from the point of lay, washing, candling, sales counter, storage room and incubator during each season. The chicken eggs in the Table Egg Production Chain (TEPC) and Hatching Egg Production Chain (HEPC) were simultaneously subjected to microbiological quality analysis which included aerobic plate count (APC) and yeast and mould count (YMC).

4.6.1. Table Egg Production Chain (TEPC)

A total of 80 eggs were collected from four sampling points in TEPC which includes the point of lay, after washing, after candling and at the sales counter in two seasons. Forty eggs each were collected from TEPC during season A and season B.

4.6.1.1. Aerobic Plate Count (APC)

The mean APC on egg shell of 40 eggs each collected from the production chain in the two seasons is given in table 12.

Table 12. Mean aerobic plate count on egg shell in TEPC during two seasons

Sl. No	Points of collection	Mean aerobic plate count (log_{10} CFU/egg shell)	
		Season A	Season B
1	Point of lay	7.56 ± 0.37^{ab}	7.06 ± 0.42^{a}
2	After washing	6.72 ± 0.10^{b}	6.54 ± 0.38^{ab}
3	After candling	8.31 ± 0.26^{aA}	5.78 ± 0.27^{bB}
4	Sales counter	7.56 ± 0.38^{ab}	6.94 ± 0.36^{a}

[a-b] Figures in a column bearing same superscripts do not differ significantly;
[A-B] Figures in a row bearing same superscripts do not differ significantly ($p<0.05$);
N=10 in each group

Higher mean APC count was observed at all the points of collection during season A than season B. At the point of lay, mean aerobic plate count of 7.56 ± 0.37 log_{10} CFU/egg shell was observed in season A and 7.06 ± 0.42 log_{10} CFU/egg shell in season B. After washing, the counts reduced to 6.72 ± 0.10 log_{10} CFU/egg shell and 6.54 ± 0.38 log_{10} CFU/egg shell during season A and season B, respectively. The mean APC increased after candling in season A, but reduced in season B. At the sales counter the mean counts reduced to 7.56 ± 0.38 log_{10} CFU/egg shell during season A and increased to 6.94 ± 0.36 log_{10} CFU/egg shell during season B.

On statistical analysis by analysis of variance, no significant reduction in the mean count was observed after washing in season A and B which was found to be 6.72 ± 0.10 log_{10} CFU/egg shell and 6.54 ± 0.38 log_{10} CFU/egg shell, respectively. Significantly higher ($p<0.05$) mean APC was observed after candling with 8.31 ± 0.26 log_{10} CFU/egg shell in season A compared to 5.78 ± 0.27 log_{10} CFU/egg shell noticed in season B. However, no significant difference in the mean counts was observed during both seasons at the point of lay and as the egg reaches the sales counter (Fig. 14 and 15).

4.6.1.2. Yeast and Mould Count (YMC)

The mean YMC on shell of 40 eggs each collected from the production chain in the two seasons is given in table 13.

Table 13. Mean yeast and mould count on egg shell in TEPC during two seasons

Sl. No	Points of collection	Mean yeast and mould count (log_{10} CFU/egg shell)	
		Season A	Season B
1	Point of lay	5.73 ± 0.38	5.27 ± 0.22
2	After washing	5.52 ± 0.28	5.44 ± 0.24
3	After candling	6.18 ± 0.32 ^{A}	5.35 ± 0.17 ^{B}
4	Sales counter	6.01 ± 0.31	5.37 ± 0.19

$^{A-B}$ Figures in a row bearing same superscripts do not differ significantly ($p<0.05$);
N=10 in each group

The mean yeast and mould count observed at all the points of collection during season A was higher than that during season B. Mean YMC of 5.73 ± 0.38 log_{10} CFU/egg shell was observed in season A at the point of lay and 5.27 ± 0.22 log_{10} CFU/egg shell in season B. The mean count reduced after washing in season A but increased in season B. The highest count observed in season A was at point of

candling and at season B was after washing. Higher mean count of 6.01 ± 0.31 log_{10} CFU/egg shell and 5.37 ± 0.19 log_{10} CFU/egg shell was observed at sales counter during season A and B, respectively than at the point of lay.

Analysis of variance showed no significant reduction in the mean count after washing in season A and B which was found to be 5.52 ± 0.28 log_{10} CFU/egg shell and 5.44 ± 0.24 log_{10} CFU/egg shell, respectively. Significantly higher ($p<0.05$) mean YMC of 6.18 ± 0.32 log_{10} CFU/egg shell was observed during season A at the point of candling compared to 5.35 ± 0.17 log_{10} CFU/egg shell in season B. There was no significant difference in the mean count during both seasons from the point of lay to the sales counter (Fig. 14 and 15).

4.6.2. Hatching Egg Production Chain (HEPC)

A total of 100 eggs were collected from five sampling points in HEPC which included point of lay, after washing, after candling, storage room after six days of storage of eggs and the incubator after six days of setting in two seasons. Fifty eggs each were collected from HEPC during season A and season B.

4.6.2.1. Aerobic Plate Count (APC)

The mean APC on shell of 50 eggs each collected from the HEPC in the two seasons is given in table 14.

All the five different points in HEPC during season A showed a higher mean aerobic plate count than that of season B. At the point of lay, mean APC of 7.56 ± 0.37 log_{10} CFU/egg shell was observed in season A and 7.06 ± 0.42 log_{10} CFU/egg shell in season B. The mean count reduced after washing in both seasons, but increased after candling in season A and reduced in season B. At the storage room mean APC of 7.09 ± 0.52 log_{10} CFU/egg shell and 6.38 ± 0.11 log_{10} CFU/egg shell was observed in season A and B. The mean count at the incubator was lower than that at the point of lay in both the seasons.

Table 14. Mean aerobic plate count on egg shell in HEPC during two seasons

Sl. No	Points of collection	Mean aerobic plate count (log_{10} CFU/egg shell)	
		Season A	Season B
1	Point of lay	7.56 ± 0.37^{ab}	7.06 ± 0.42^{a}
2	After washing	6.72 ± 0.10^{b}	6.54 ± 0.38^{ab}
3	After candling	8.31 ± 0.26^{aA}	5.78 ± 0.27^{bB}
4	Storage room (6^{th} day)	7.09 ± 0.52^{b}	6.38 ± 0.11^{ab}
5	Incubator (6^{th} day)	7.46 ± 0.50^{abA}	6.31 ± 0.38^{abB}

[a-b] Figures in a column bearing same superscripts do not differ significantly;
[A-B] Figures in a row bearing same superscripts do not differ significantly ($p<0.05$);
N=10 in each group

On statistical analysis by analysis of variance, a significant difference ($p<0.05$) in APC of egg shell was observed between the two seasons at the point of candling and in the incubator with a higher mean count seen during season A as shown in table 14. There was a significantly higher ($p<0.05$) mean count of 8.31 ± 0.26 log_{10} CFU/egg shell at the point of candling than after washing (6.72 ± 0.10 log_{10} CFU/egg shell) and at the storage room (7.09 ± 0.52 log_{10} CFU/egg shell). Lowest mean count of 5.78 ± 0.27 log_{10} CFU/egg shell was observed after candling in season B. However, no significant difference in the mean counts were observed during both seasons at the point of lay and as the egg reached the incubator (Fig. 16 and 17).

4.6.2.2. Yeast and Mould Count (YMC)

The mean YMC on shell of 50 eggs collected from the HEPC in two seasons is given in table 15.

Table 15. Mean yeast and mould count on egg shell in HEPC during two seasons

Sl. No	Points of collection	Mean yeast and mould count (log_{10} CFU/egg shell)	
		Season A	Season B
1	Point of lay	5.73 ± 0.38	5.27 ± 0.22
2	After washing	5.52 ± 0.28	5.44 ± 0.24
3	After candling	6.18 ± 0.32 ^{A}	5.35 ± 0.17 ^{B}
4	Storage room (6th day)	6.00 ± 0.08	5.60 ± 0.07
5	Incubator (6th day)	6.18 ± 0.17 ^{A}	5.47 ± 0.10 ^{B}

$^{A-B}$ Figures in a row bearing same superscripts do not differ significantly ($p<0.05$); N=10 in each group

Mean yeast and mould count of 5.73 ± 0.38 log_{10} CFU/egg shell was observed in season A at the point of lay and 5.27 ± 0.22 log_{10} CFU/egg shell in season B. Highest mean count of 6.18 ± 0.32 log_{10} CFU/egg shell was observed at the point of candling in season A and lowest mean count of 5.27 ± 0.22 log_{10} CFU/egg shell was observed at the point of lay in season B. Mean YMC increased when the eggs reaches incubator in both the seasons.

Analysis of variance showed a significantly higher ($p<0.05$) mean YMC of 6.18 ± 0.32 log_{10} CFU/egg shell in season A at the point of candling compared to 5.35 ± 0.17 log_{10} CFU/egg shell in season B. In season A, there was significantly high ($p<0.05$) mean YMC of 6.18 ± 0.17 log_{10} CFU/egg shell at the incubator as against 5.47 ± 0.10 log_{10} CFU/egg shell in season B. There was no significant difference in the mean count during both seasons from the point of lay to the incubator as shown in table 15 (Fig. 16 and 17).

4.7. MICROBIOLOGICAL QUALITY OF CHICKEN EGGS IN THE RETAIL MARKET

A total of 120 eggs were collected from retail outlets in and around Thrissur during two seasons.

The mean values of APC and YMC of egg shell of 120 eggs from the retail market are given in table 16.

Table 16. Mean microbiological counts in egg shell from retail market during two seasons

Sl. No	Counts	Egg shell (log_{10} CFU/egg shell)	
		Season A	**Season B**
1	APC	7.44 ± 0.08^{A}	6.90 ± 0.08^{B}
2	YMC	5.87 ± 0.11^{A}	4.45 ± 0.15^{B}

$^{A-B}$ Figures in a row bearing same superscripts do not differ significantly ($p<0.05$);
N=60 in each group

The mean aerobic plate count and yeast and mould count in the eggs from retail market was higher during monsoon season than post monsoon season.

Statistical analysis by analysis of variance revealed a significant difference in mean egg shell counts in season A and B as shown in table 16. Significantly higher ($p<0.05$) mean APC was obtained on shell during season A than in season B. The mean YMC observed in season A (5.87 ± 0.11 log_{10} CFU/egg shell) was significantly higher ($p<0.05$) than in season B (4.45 ± 0.15 log_{10} CFU/egg shell) (Fig. 18).

4.8. COMPARATIVE ASSESSMENT OF MICROBIOLOGICAL QUALITY OF EGGS FROM UPDF, MANNUTHY AND RETAIL MARKET

A total of 240 eggs including 120 each of farm eggs and retail egg samples were collected during two seasons.

4.8.1. Season A

During season A, 60 each of farm eggs and retail eggs were collected.

The mean counts on egg shell of 60 eggs collected from UPDF, Mannuthy and retail market during season A is given in the table 17.

Table 17. Mean microbiological counts in egg shell from UPDF, Mannuthy and retail market in season A

Sl. No	Counts in egg shell ($\log_{10}$ CFU/egg shell)	Source of egg	
		UPDF	Market
1	APC	7.56 ± 0.38	7.44 ± 0.08
2	YMC	6.01 ± 0.31	5.87 ± 0.11

Analysis of variance showed no significant difference in the mean APC and YMC in shell of eggs from UPDF, Mannuthy and retail market (Fig. 19). Mean APC of 7.56 ± 0.38 $\log_{10}$ CFU/egg shell was observed in UPDF eggs and 7.44 ± 0.08 $\log_{10}$ CFU/egg shell in retail eggs. Farm eggs showed comparatively higher mean YMC of 6.01 ± 0.31 $\log_{10}$ CFU/egg shell than retail eggs (5.87 ± 0.11 $\log_{10}$ CFU/egg shell).

4.8.2. Season B

Sixty each of farm eggs and retail eggs were collected during season B.

The mean counts on egg shell of 60 eggs collected from the UPDF, Mannuthy and retail market during season B is given in table 18.

Table 18. Mean microbiological counts in egg shell from UPDF, Mannuthy and retail market in season B

Sl. No	Counts in egg shell ($\log_{10}$ CFU/egg shell)	Source of egg	
		UPDF	Market

1	APC	6.94 ± 0.36	6.90 ± 0.08
2	YMC	5.37 ± 0.19 ^{A}	4.45 ± 0.15 ^{B}

$^{A-B}$ Figures in a row bearing same superscripts do not differ significantly ($p<0.05$)

Mean aerobic plate count and yeast and mould count in eggs from UPDF, Mannuthy during season B was higher than that of eggs from retail market.

On statistical analysis by analysis of variance, a significant difference ($p<0.05$) was observed in mean YMC during season B in eggs collected from UPDF, Mannuthy and retail market. The mean YMC in UPDF eggs was significantly higher (5.37 ± 0.19 $\log_{10}$ CFU/egg shell) than market eggs (4.45 ± 0.15 $\log_{10}$ CFU/egg shell). No significant difference was observed in the mean APC in egg shell from both sources as shown in figure 20.

4.9. STANDARDISATION OF POLYMERASE CHAIN REACTION (PCR) PROTOCOL FOR *CAMPYLOBACTER* SPP.

The polymerase chain reaction protocol was standardised for *C. jejuni* and *C. coli* separately. Different concentrations of $MgCl_2$ *viz.*, 1.5, 2, 2.5 and 3 m*M* were used for PCR and 2 m*M* concentration was found to be optimum. After evaluating different annealing temperatures *viz.*, 47.8, 48.9, 50.2, 51.8, 53.5 and 55.1˚C, the optimum temperature was found to be at 51.8˚C. Primers targeting *16S rRNA*, *map*A, *ceu*E and *cad*F gene were used. A single band at 816, 589, 462 and 400 bp region of DNA marker ladder represented the PCR product of *Campylobacter* genus specific (*16S rRNA* gene), *C. jejuni* specific (*map*A gene), *C. coli* specific (*ceu*E gene) and virulence gene, *cad*F respectively.

4.10. STANDARDISATION OF MULTIPLEX PCR

Multiplex PCR was standardised in a single reaction containing all the four primer sets *i.e., 16S rRNA*, *map*A, *ceu*E and *cad*F for *Campylobacter* spp. Standardised multiplex PCR protocol allowed the simultaneous amplification of genus specific *16S rRNA*, *C. jejuni* specific *map*A, *C. coli* specific *ceu*E and virulence

gene *cad*F to their respective amplicon size 816, 589, 462 and 400 bp, respectively. A single band at 816, 589 and 400 bp region of DNA marker ladder represented the PCR product of *C. jejuni* and single band at 816, 462 and 400 bp region of DNA marker ladder represented the PCR product of *C. coli* (Fig. 21).

4.11. MOLECULAR CONFIRMATION OF THE ISOLATES

All the 17 isolates, culturally positive for *Campylobacter* spp. were subjected to multiplex polymerase chain reaction. Of the 17 isolates, 13 were from cloacal swab samples and four were from eggs in the retail market. All the 17 isolates were found to carry the conserved *16S rRNA* gene, which was specific for *Campylobacter* genus and *cad*F virulence gene (Table 19).

4.11.1. Occurrence of *Campylobacter* spp. in egg production chain

A total of 13 isolates were obtained from cloacal swab samples during two seasons. Out of the 10 and three isolates obtained from cloacal swab in UPDF, Mannuthy during season A and B respectively, eight and two isolates were found to carry the *map*A gene specific for *C. jejuni* (Fig. 22). Two and one isolates obtained from cloacal swab in UPDF, Mannuthy during season A and B respectively, were found to carry both *map*A specific for *C. jejuni* and *ceu*E specific for *C. coli* as shown in Fig. 23 and Table 20. The distribution of genes in the isolates obtained from cloacal swab samples is shown in table 19.

4.11.2. Occurrence of *Campylobacter* spp. in farm eggs

Of the 120 farm eggs screened during season A and B, both the egg shell and inner content samples were found to be negative for the presence of *Campylobacter* spp. culturally.

4.11.3. Occurrence of *Campylobacter* spp. in eggs from retail market

The four isolates obtained from egg shell samples in the retail market during monsoon season were found to carry the three genes, *16S rRNA*, *cad*F and *map*A

specific for *C. jejuni* (Fig. 24). The distribution of genes in the isolates from eggs in the retail market is shown in table 19.

Table 19. Distribution of genes in the *Campylobacter* spp. isolates

Sl. No	Samples	Positive samples	Distribution of genes in the *Campylobacter* isolates			
			16S rRNA	*map*A	*ceu*E	*cad*F
1	Cloacal swab	13	13	13	3	13
2	Retail market eggs	4	4	4	0	4
Total		**17**	**17**	**17**	**3**	**17**

4.11.4. Overall occurrence of *Campylobacter* spp.

The overall occurrence of *C. jejuni* during season A and season B was 4.21 and 0.7 per cent, respectively. Mixed infection caused by *C. jejuni* and *C. coli* was 0.7 and 0.35 per cent during season A and season B respectively (Table 20 and Fig. 25).

Table 20. *Campylobacter* spp. isolated from different samples during season A and season B by PCR

Sl. No	Sources	Season A			Season B		
		No. of samples	Details of isolates		No. of samples	Details of isolates	
			C. jejuni	*C. jejuni + C. coli*		*C. jejuni*	*C. jejuni +C. coli*
1	Cloacal swab	15	8	2	15	2	1
2	Feed	15	0	0	15	0	0
3	Litter	15	0	0	15	0	0
4	Wash water	15	0	0	15	0	0
5	Wash cloth	15	0	0	15	0	0

6	Plastic tray	15	0	0	15	0	0
7	Filler flat	15	0	0	15	0	0
8	Air	15	0	0	15	0	0
9	Water	15	0	0	15	0	0
10	Hand wash-candling person	15	0	0	15	0	0
11	Hand wash-sales person	15	0	0	15	0	0
12	Farm eggs	60	0	0	60	0	0
13	Retail market egg	60	4	0	60	0	0
Total		**285**	**12**	**2**	**285**	**2**	**1**
%			**4.21**	**0.7**		**0.7**	**0.35**

Of the 17 isolates, 14 were identified as *C. jejuni* and three as mixed infection caused by both *C. jejuni* and *C. coli.* Out of the 570 samples screened, occurrence of *C. jejuni* alone was found to be 2.46 per cent. The combined occurrence of *C. jejuni* and *C. coli* was found to be 0.53 per cent. The overall occurrence of *Campylobacter* spp. was 2.98 per cent (Fig. 26). The number of isolates and species are listed in table 21.

Table 21. Occurrence of *Campylobacter* spp. in different samples by PCR

Sl. No	**Sources**	**No. of samples**	**Details of isolates**		**%**
			C. jejuni	***C. jejuni + C. coli***	
1	Cloacal swab	30	10	3	43.33
2	Feed	30	0	0	0
3	Litter	30	0	0	0

4	Wash water	30	0	0	0
5	Wash cloth	30	0	0	0
6	Plastic tray	30	0	0	0
7	Filler flat	30	0	0	0
8	Air	30	0	0	0
9	Water	30	0	0	0
10	Hand wash of candling person	30	0	0	0
11	Hand wash of sales person	30	0	0	0
12	Farm eggs	120	0	0	0
13	Retail market eggs	120	4	0	3.33
Total		**570**	**14 (2.46%)**	**3 (0.53%)**	**2.98%**

4.12. COMPARISON OF CONVENTIONAL CULTURE TECHNIQUE WITH MULTIPLEX PCR RESULT

Evaluation of conventional culture technique was carried out by calculation of sensitivity and specificity. As the true *C. jejuni* status of naturally contaminated samples was not known, the method of calculation assumed that the PCR technique as the true value. The results are given in table 22.

Table 22. Statistical evaluation of conventional culture technique with multiplex PCR assay for the detection of *Campylobacter* spp.

Culture method	**PCR**		**Total**	**Sensitivity (%)**	**Specificity (%)**
	+	**-**			

+	17	0	17	100	100
-	0	553	553		
Total	17	553	570		

The comparative study showed that the conventional culture technique has 100 per cent sensitivity and specificity. The sensitivity denotes the ability to detect positive samples and the specificity is the ability to detect negative samples.

4.13. ANTIBIOTIC RESISTANCE PROFILING

All the positive isolates of *Campylobacter* spp. obtained from different type of samples were subjected to antibiotic sensitivity test by standard disc diffusion method. The sensitivity pattern was assessed using the disc diffusion assay, according to the criteria defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015) (Fig 27).

4.13.1. Antibiotic resistance pattern of *Campylobacter* spp. isolates from different sources

A total of 17 isolates were subjected to antibiotic sensitivity testing against 29 antibiotics specified against Gram negative organisms. The results of antibiotic sensitivity profiling are summarized in table 23.

All the *Campylobacter* spp. isolates were sensitive to Amikacin, Chloramphenicol, Imipenem, Kanamycin, Moxifloxacin, Neomycin, Netillin, Nitrofurantoin, Sparfloxacin, Streptomycin and Tobramycin. Cent per cent of the isolates were showing resistance against Ceftazidime, 94.12 per cent against Cephalothin, 88.24 per cent against Carbenicillin, Ciprofloxacin, Co-Trimoxazole,

Ofloxacin and Oxacillin, 64.71 per cent against Ampicillin and Aztreonam and 58.82 per cent against Doxycycline and Tetracycline (Fig. 28).

The dendrogram of antibiotic sensitivity pattern of isolates of *Campylobacter* spp. is depicted in the figure 29. The procedure used was 'between group linkage' based on rescaled distance cluster combine. The accessions were divided into five clusters. At 25 units there was a group with maximum dissimilarity and the cluster was formed by Gatifloxacin, Meropenem, Levofloxacin, Norfloxacin, Doxycycline, Tetracycline, Cefotaxime and Ampicillin. The variability in the sensitivity patterns of the isolates against the other antibiotics was less than five per cent as shown in the dendrogram.

Table 23. Antibiotic resistance profile of *Campylobacter* spp.

Sl. No	Antibiotic	Concentration (µg/disc)	*Campylobacter* (17 isolates)		
			R*	I*	S*
1	Amikacin	30	-	-	100
2	Ampicillin	10	64.71	23.53	11.76
3	Aztreonam	30	64.71	23.53	11.76
4	Carbenicillin	100	88.24	-	11.76
5	Cefotaxime	30	47.06	5.88	47.06

6	Ceftazidime	30	100	-	-
7	Cephalothin	30	94.12	-	5.88
8	Chloramphenicol	25	-	-	100
9	Ciprofloxacin	5	88.24	-	11.76
10	Clindamycin	2	5.88	-	94.12
11	Co-trimoxazole	25	88.24	-	11.76
12	Doxycycline	10	58.82	-	41.18
13	Erythromycin	15	5.88	-	94.12
14	Gatifloxacin	5	-	17.65	82.35
15	Imipenem	10	-	-	100
16	Kanamycin	30	-	-	100
17	Levofloxacin	5	-	29.41	70.59
18	Meropenem	10	-	11.76	88.24
19	Moxifloxacin	5	-	-	100
20	Neomycin	10	-	-	100
21	Netillin	30	-	-	100
22	Nitrofurantoin	300	-	-	100
23	Norfloxacin	10	-	35.29	64.71
24	Ofloxacin	5	88.24	-	11.76
25	Oxacillin	1	88.24	-	11.76
26	Sparfloxacin	5	-	-	100
27	Streptomycin	25	-	-	100
28	Tetracycline	30	58.82	-	41.18
29	Tobramycin	10	-	-	100

R- Resistant I- Intermediate S- Sensitive *All values in percentage

5. DISCUSSION

The prevalence of Campylobacteriosis has been reported worldwide and it is one of the most widespread zoonotic gastroenteric infectious disease of the last century. Pathogenicity of *Campylobacter* spp. is mainly attributed by its low

infectious dose, toxin production, carrier status in poultry and coccoid dormant stage. These organisms became the major pathogens of foodborne illness because of its severe consequences of infection like Guillain-Barre syndrome, reactive arthritis and irritable bowel syndrome.

The foodborne infection caused by *Campylobacter* spp. is primarily associated with the consumption of contaminated or undercooked foods, particularly of poultry origin and also by environmental exposure. Chicken is the major reservoir of *Campylobacter* spp. which harbour the organisms mainly in their caecum and shed them in faeces resulting in further contamination of environment and humans (Hermans *et al.*, 2012). Since these organisms are the normal commensal in the intestinal tract of birds, these may even contaminate the foods of poultry origin. Inorder to estimate the contamination level of eggs caused by *Campylobacter* spp., the present study was undertaken with the primary objectives of identification of critical control points of *Campylobacter* spp. in egg production chain.

The present investigation was carried out to find out the occurrence of *Campylobacter* spp. of public health significance in egg production chain. A total of 570 samples from University Poultry and Duck Farm (UPDF), Mannuthy and retail outlets in and around Thrissur were examined throughout a period of 12 months. The genus specific, species specific and virulence gene present in *Campylobacter* spp. was identified by molecular method. The antibiotic sensitivity tests were performed to find out the antibiotic resistance pattern of all *Campylobacter* spp. isolates. The occurrence of the organism in egg production chain, environment and in the human beings along with molecular methods of confirmation and antibiotic resistance profile of the isolates are discussed under various titles in this chapter.

5.1. ISOLATION AND IDENTIFICATION OF ORGANISMS FROM EGG PRODUCTION CHAIN

All the samples collected from egg production chain and retail eggs were screened to detect the occurrence of *Campylobacter* spp. Isolation and identification of *Campylobacter* spp. was carried out by cultural techniques.

The cultural techniques adopted in the study utilized the ability of *Campylobacter* spp. to grow in blood-free medium. In the present study modified charcoal cefoperazone deoxycholate broth (mCCD broth) was used as enrichment broth for *Campylobacter* organisms. Kim *et al.* (2009) reported that the mCCD broth had a high enrichment efficiency for the isolation of *C. jejuni*. The selectivity of the mCCD medium was achieved through the presence of sodium deoxycholate present in the medium base and Cefoperazone and Amphotericin B added to the media. Deoxycholate effectively inhibit the growth of undesirable microflora in the samples. Cefoperazone inhibited the growth of *Pseudomonas* and *Enterobacteriaceae* and the contamination caused by yeast and mould can be inhibited by Amphotericin B. According to Oyarzabal *et al*. (2005) the modified charcoal cefoperazone deoxycholate agar (mCCDA) was more selective and highly efficient in isolating *Campylobacter* spp. The mCCDA was modified to P-mCCDA (Polymyxin B supplemented modified Charcoal Cefoperazone Deoxycholate Agar) by supplementing with polymyxin B, which resulted in higher isolation rate and superior selectivity (Chon *et al.*, 2012). Polymyxin B effectively eliminates the Gram negative competing background microflora in the samples by disrupting the cell membrane integrity. In the present study, P-mCCDA was used successfully which effectively eliminated the contaminating microflora. As recommended by the OIE Terrestrial Manual (2008), an incubation temperature of 42ºC for 48 h was used in the study. This temperature and time combination provided satisfactory isolation rate of *Campylobacter* spp. and reduced the growth of contaminants.

In the present study, all the *Campylobacter* isolates obtained from various sources showed characteristic colonies on P-mCCD agar. All these isolates produced greyish, round, flat to slightly raised, spreading type, shiny, moistened colonies with

or without metallic sheen. This was in agreement with the report by Bolton *et al.* (1984).

5.1.1. Occurrence of *Campylobacter* spp. in cloacal swab

Chickens are the natural reservoirs of *Campylobacter* organisms and the potential source of human infection. Once the organism gets colonized in the caecum of birds, they will act as asymptomatic carriers and shed *Campylobacter* in their faeces which aid in horizontal transmission (Hendrixson and DiRita 2004). Miflin *et al.* in 2001 reported that after detecting one *Campylobacter* positive sample in broiler flock in Queensland, the whole flock become infected within four to six days. In order to find out the carriage of organism by the birds in UPDF, Mannuthy, cloacal swab samples were collected from 30 birds in the farm.

In the present study 43.33 per cent of the chicken was found to be the faecal carriers of *Campylobacter* spp. The occurrence in cloacal swab sample which was found in the present study was higher than that reported by Jones *et al.* (1991) and Stojanov *et al.* (2007) where they found 20 and 38 per cent of *Campylobacter* spp. in cloacal swab samples, respectively. The present finding was contrary to the results of Pearson *et al.* (1993), Hald *et al.* (2000) and Kumar and Shenbagam (2015) where they found a higher occurrence of *Campylobacter* spp. *i.e.*, 64, 52 and 75 per cent in cloacal swab samples of poultry, respectively.

The high occurrence of *Campylobacter* spp. in poultry in the present study might be due to the low dose required for establishing colonization, the horizontal transmission of the organism within the flock and the absence of anti-*Campylobacter* therapy due to the asymptomatic carriage in chicken.

5.1.2. Occurrence of *Campylobacter* spp. in feed

In the present study, 30 feed samples were collected from UPDF, Mannuthy during monsoon and post monsoon season and investigated for the presence of *Campylobacter* spp.

None of the feed samples examined in the present study revealed the presence of *Campylobacter* spp. This finding was in agreement with some previous studies of Smitherman *et al.* (1984), Genigeorgis *et a1*. (1986), Jones *et al.* (1991), Humphrey *et al.* (1993), Jacobs-Reitsma *et al.* (1995), Van de Giessen *et al.* (1998), Saleha (2004) and Thakur *et al.* (2013) where they failed to isolate *Campylobacter* spp. from feed at the farm level. However, the result was not in accordance with the findings by Pearson *et al.* (1993) where *Campylobacter* spp. were isolated from 67 per cent of feed samples using Indirect Fluorescent Antibody test. The feed can be contaminated with *Campylobacter* spp. during storage and transport which will result in passive transmission of the organism in the flock. Feed required for the poultry in UPDF, Mannuthy was produced in the feed mill as per need and stored properly. The absence of the organism in feed could be attributed to the poor survivability at low water activity, desiccated condition of the sample and exposure to air.

5.1.3. Occurrence of *Campylobacter* spp. in litter

Contaminated litter will accelerate the horizontal transmission of *Campylobacter* spp. within a flock. To investigate whether litter is a source of *Campylobacter* spp. contamination to egg, 30 litter samples were collected from UPDF, Mannuthy during two seasons.

All the 30 litter samples examined in the present study were found to be negative for *Campylobacter* spp. This result was in accordance with the studies by Smitherman *et al.* (1984), Jacobs-Reitsma *et al.* (1995), and Thakur *et al.* (2013) where no *Campylobacter* isolates were obtained from litter samples. The findings of the present study was not in agreement with the results of Pearson *et al.* (1993) where they obtained 93 per cent occurrence of *Campylobacter* spp. from used litter samples using Indirect Fluorescent Antibody test. Berghaus *et al.* (2013) reported a higher prevalence (23.6 per cent) of *Campylobacter* spp. in litter samples from broiler farm in North Georgia than the present study. Jones *et al.* (1991) isolated *Campylobacter* spp. from 5.9 per cent of nest litter and not from any of the floor litter from the

breeder and broiler house environment, which is partially in agreement with the present study. Proper litter management might be the reasons for low occurrence of *Campylobacter* spp. in the samples. Moreover the dry condition of the litter might have adversely affected the survival of organism.

5.1.4. Occurrence of *Campylobacter* spp. in wash water

Thirty wash water samples were collected from the point of washing in UPDF, Mannuthy during two seasons and examined for the presence of *Campylobacter* spp. The 30 wash water samples analysed were not contaminated with *Campylobacter* spp. A result similar to that obtained in the present study was reported by Jones *et al.* (1991), where he could not isolate any *Campylobacter* spp. from the water samples collected from broiler house environment in North Carolina. The low occurrence of the organism in the present study might be due to the efficiency of the disinfectant kohrsolin (0.1 per cent), excess exposure of the samples to air and the presence of viable but non culturable form.

5.1.5. Occurrence of *Campylobacter* spp. in wash cloth

The presence of *Campylobacter* spp. was investigated in 30 wash cloth samples collected during two seasons from the point of washing in UPDF, Mannuthy. *Campylobacter* spp. was not isolated from the wash cloth samples tested during two seasons. Previous study of Hilton and Austin (2000) was in agreement with the results of the present study, where they failed to isolate *Campylobacter* spp. from the kitchen dishcloth. Appropriate cleaning of the cloth and the exposure of samples to an aerobic environment might be the reason for low occurrence of *Campylobacter* spp.

5.1.6. Occurrence of *Campylobacter* spp. in plastic tray

The quality of the plastic tray is essential for preventing contamination. In the present study, 30 swab samples were collected to determine the occurrence of *Campylobacter* spp.

All the 30 plastic tray swab samples were found to be negative for *Campylobacter* spp. This confirms to the observation made by Prachantasena *et al.* (2016) who had reported *Campylobacter* spp. was absent in the egg trays and faeces soiled lining papers. Absence of *Campylobacter spp.* in plastic tray swab samples might be due to the proper disinfection, exposure to air and dry state of the plastic trays.

5.1.7. Occurrence of *Campylobacter* spp. in filler flat

Thirty filler flat swab samples were collected from sales counter of UPDF Mannuthy in two seasons. *Campylobacter* spp. was not detected in any of the 30 filler flat samples studied during season A and B. Similar results were reported by Jacobs-Reitsma *et al.* (1995). However, Byrd *et al.* (2007) reported a higher recovery rate of 0.75 per cent from paper pad tray liners than the present study. Exposure to aerobic environment and proper disinfection might have contributed to the low occurrence of *Campylobacter* spp. in filler flat samples.

5.1.8. Occurrence of *Campylobacter* spp. in air

In 2006 Posch *et al.* reported aerosol as a potential vector for horizontal transmission of *Campylobacter* in poultry slaughter house. In the present study none of the air samples collected from UPDF, Mannuthy were having *Campylobacter* spp. The observation of the present investigation was in tune with the study by Pearson *et al.* (1993). Higher prevalence of *Campylobacter* spp. was reported in air samples by Johnson (2010), El Metwaly Ahmed *et al.* (2013) and Schroeder *et al.* (2014) who reported an occurrence of 10, 83 and 15 per cent, respectively. The poor survivability of *Campylobacter* spp. in air in the present study might be due to the proper disinfection of the shed and other points of collection and extreme sensitivity of the organism to drying. Moreover the direct exposure method had a lower efficiency for *Campylobacter* spp. isolation from air samples.

5.1.9. Occurrence of *Campylobacter* spp. in water

The present study investigated water as the source of contamination for *Campylobacter* spp. A total of 30 water samples were collected for the analysis.

All the water samples collected from the farm were found to be negative for the presence of *Campylobacter* spp. This occurrence was comparable with the study of Jones *et al.* (1991), Jacobs-Reitsma *et al.* (1995) and Van de Giessen *et al.* (1998). The findings of the present study did not correlate with the results of Pearson *et al.* (1993) where *Campylobacter* spp. were isolated from 62 per cent of water samples by Indirect Fluorescent Antibody test but not by culture technique. Smitherman *et al.* (1984) reported a higher prevalence (15 per cent) of *Campylobacter* spp. in water samples from chicken ranches in California than the present study. The drinking water used in UPDF, Mannuthy was properly chlorinated (0.5 ppm) for destructing the pathogenic microorganisms. Moreover the exposure of the samples to an aerobic environment and viable but non culturable form of *Campylobacter* spp. might be the reasons for the results of the present study.

5.1.10. Occurrence of *Campylobacter* spp. in hand wash

Thirty hand wash samples each from the personnel involved in candling and sales were collected from UPDF, Mannuthy during two seasons. *Campylobacter* spp. could not be isolated from any of the hand wash samples analysed during the two seasons. Dawkins *et al.* (1984) found that 4.44 per cent of the hand wash samples were contaminated with *Campylobacter* spp., which was conflicting with the observations made in the present study. The eggs are handled by workers during candling and sales. In the present investigation it was observed that the workers used to wash their hands with soap and water after candling and sales. The result of the present study might be due to the proper sanitary and personnel hygienic measures followed by the workers in UPDF, Mannuthy.

5.1.11. Occurrence of *Campylobacter* spp. in egg

One hundred and twenty eggs each were collected from UPDF, Mannuthy and retail market in and around Thrissur.

None of the egg samples collected from UPDF, Mannuthy were found to be contaminated with *Campylobacter* spp. The finding of the present study was similar to the previous studies of Sahin *et al.* (2003) and Fonseca *et al.* (2006). Higher occurrence of *Campylobacter* spp. in farm eggs than the present study was reported by Doyle (1984), Sulonen *et al.* (2007) and Hedawey and Youssef (2014) who reported 0.88, 0.28 and one per cent, respectively. In UPDF, Mannuthy, eggs were collected from the point of lay at frequent intervals and they were subjected to washing with disinfectant kohrsolin. Moreover the disinfection practices followed in the shed, fumigation of setter, hatcher and hatching eggs might have resulted in *Campylobacter* free eggs.

The present investigation revealed an overall prevalence rate of 3.33 per cent of *Campylobacter* spp. in egg samples collected from retail outlets in and around Thrissur. Messelhausser *et al.* (2011) reported a higher occurrence of 4.1 per cent than the present study in eggs collected from super market. The report of Safaei *et al.* (2011) did not correlate with the findings of present study, where they could not isolate *Campylobacter* spp. in eggs purchased from supermarkets. High occurrence of *Campylobacter* spp. in retail eggs might be due to faecal contamination, environmental contamination or from the handling personnels. Eggs reaching the retail market should be properly stored to prevent further contamination.

5.1.12. Critical control points of *Campylobacter* spp. in egg production chain

The present study evaluated 11 different possible sources of contamination for determining critical control points of *Campylobacter* spp. in egg production chain.

The samples studied included cloacal swab, feed, litter, wash water, wash cloth, plastic tray, filler flat, air, water, hand wash of candling and sales personnel.

In UPDF, Mannuthy, 43.33 per cent of the cloacal swab were found to be contaminated with *Campylobacter* spp. which contributed to the major source of contamination. However the organism could not be detected from any other sources in the present study. So it can be inferred that cloaca of the bird could be considered as the critical point of contamination in UPDF, Mannuthy. Hence the point of lay in the egg production process can be considered as the critical control point (CCP2) where the point of contamination cannot be completely eliminated, but can be reduced to a considerable limit. The following measures can be adopted for the control of *Campylobacter* spp. at CCP2.

1. Strict biosecurity measures should be adopted in the farm to reduce environmental exposure
2. Increase the resistance of poultry against *Campylobacter* spp. by means of vaccination to reduce the carriage of organism in the gastro-intestinal tract
3. An antimicrobial intervention to reduce *Campylobacter* spp. in colonized chicken

5.2. MICROBIOLOGICAL QUALITY OF EGGS

5.2.1. Eggs from UPDF, Mannuthy

5.2.1.1. Aerobic Plate Count

A total of 80 eggs were collected from four sampling points in TEPC. Significantly highest ($p<0.05$) mean APC was observed after candling in season A compared to that in season B. No significant reduction in the mean count was observed after washing in season A and B which was found to be 6.72 ± 0.10 $\log_{10}$ CFU/egg shell and 6.54 ± 0.38 $\log_{10}$ CFU/egg shell, respectively. However, no

significant difference in the mean counts were observed during both seasons at the point of lay and as the egg reaches the sales counter.

A total of 100 eggs were collected from five sampling points in HEPC. A significant difference ($p<0.05$) in APC of egg shell was observed between the two seasons at the point of candling and in the incubator with a higher mean count seen during season A. There was a significantly higher ($p<0.05$) mean count of 8.31 ± 0.26 $\log_{10}$ CFU/egg shell at the point of candling than after washing (6.72 ± 0.10 $\log_{10}$ CFU/egg shell) and at the storage room (7.09 ± 0.52 $\log_{10}$ CFU/egg shell). Lowest mean count of 5.78 ± 0.27 $\log_{10}$ CFU/egg shell was observed after candling in season B. However, no significant difference in the mean counts were observed during both seasons at the point of lay and as the egg reaches the incubator.

The result of the present study was different from that reported by Irene (2011), who had reported a higher count at the point of lay and the count reduced as the eggs move through the production chain. As per ICMSF (1986) recommendations, the APC for egg products should be between 5.0×10^4 and 1.0×10^6 CFU/g. The mean aerobic plate count of the UPDF eggs exceeded than that of the recommended limits. After washing of the eggs, there was only a slight reduction in mean counts. This could be due to the inefficiency of the disinfectant used for the washing of eggs. The high bacterial load on egg after candling might have resulted from contamination by bacteria present in the environment. Furthermore, the wash water had to be changed at frequent intervals, so as to reduce the spread of contaminants from water to other eggs. At the sales counter when the eggs are stored in open racks, as ambient conditions prevailed, with adequate temperature and relative humidity, the microbes on the egg could proliferate. The temperature provided during setting of eggs in the incubator was between 99.50 to 99.75˚F, which supported growth of the microorganisms.

5.2.1.2. Yeast and Mould Count

A total of 80 eggs were collected from four sampling points in TEPC. Significant reduction in the mean count was not noticed after washing in season A and B which was found to be 5.52 ± 0.28 $\log_{10}$ CFU/egg shell and 5.44 ± 0.24 $\log_{10}$ CFU/egg shell, respectively. Significantly higher ($p<0.05$) mean YMC of 6.18 ± 0.32 $\log_{10}$ CFU/egg shell was observed during season A at the point of candling compared to 5.35 ± 0.17 $\log_{10}$ CFU/egg shell in season B. There was no significant difference in the mean count during both seasons from the point of lay to the sales counter.

A total of 100 eggs were collected from five sampling points in HEPC. Significantly higher ($p<0.05$) mean YMC of 6.18 ± 0.32 $\log_{10}$ CFU/egg shell in season A at the point of candling compared to 5.35 ± 0.17 $\log_{10}$ CFU/egg shell in season B. In season A, there was significantly high ($p<0.05$) mean YMC of 6.18 ± 0.17 $\log_{10}$ CFU/egg shell at the incubator as against 5.47 ± 0.10 $\log_{10}$ CFU/egg shell in season B. There was no significant difference in the mean count during both seasons from the point of lay to the incubator.

As per the recommendations of FDA, the YMC on egg products should be five CFU/g, but the eggs from UPDF, Mannuthy exceeded the recommended limits. A high YMC was recorded at all points of collection than the recommended value. Beuchat and Cousin (2001) stated that yeast and mould are hardier than bacteria under many circumstances. The result of the present study did not agree with the findings of Irene (2011). The increased moisture on egg shell along with high ambient temperature and humidity could have caused proliferation of yeast and mould on shell surface. High mean YMC observed during the monsoon season may be due to high humidity present during the season. Strict hygienic measures should be followed at each point of collection in UPDF, Mannuthy to reduce the microbial population.

5.2.2. Eggs from retail market

5.2.2.1. Aerobic Plate Count

A total of 120 chicken eggs were collected randomly from different retail shops in and around Thrissur. The mean aerobic plate count in chicken eggs during monsoon and post monsoon season was 7.44 ± 0.08 and 6.90 ± 0.08 $\log_{10}$ CFU/egg shell, respectively. The mean aerobic plate count of the eggs from retail market exceeded than that of the recommendations of ICMSF (1986). The mean APC of monsoon season was significantly higher ($p<0.05$) when compared to post monsoon season. The result confirms to the findings made by Irene (2011). The results obtained in the present study were higher from that reported by Suba *et al.* (2005) who reported APC of $4x10^5$ CFU/g in eggs from retail shops. Ansah *et al.* (2009) reported higher mean APC of 7.56 from egg shell collected from market in Kukuo, Ghana than the present study. The increased ambient temperature and relative humidity in season A might have promoted the growth of microbes in the egg shell in the present investigation.

5.2.2.2. Yeast and Mould Count

A total of 120 chicken eggs were collected randomly from different retail shops in and around Thrissur. The mean YMC in chicken eggs during monsoon and post monsoon season was 5.87 ± 0.11 and 4.45 ± 0.15 $\log_{10}$ CFU/egg shell, respectively. The mean YMC on eggs from retail market exceeded the recommended limits of FDA. A significantly higher ($p<0.05$) yeast and mould count was observed in season A compared to season B.

A higher value than the present study was obtained by Irene (2011) who reported mean yeast and mould count of 4.65 ± 0.04 $\log_{10}$ CFU/egg shell in the shells of duck eggs collected from retail market in Mannuthy during post monsoon season. However the author reported a lesser value of YMC of 3.64 ± 0.29 $\log_{10}$ CFU/egg shell during monsoon season than the present study. The results obtained in the present study were higher from that reported by Bahobail *et al.* (2012) who reported average mould count of 1.3 $\log_{10}$ CFU/ml and 3.4 $\log_{10}$ CFU/ml from the shells of processed and unwashed eggs collected from different shops in Taif city, Saudi

Arabia. Cader *et al.* (2014) also reported mean YMC of 3.5 $\log_{10}$ CFU/g in the shells of eggs collected from supermarkets in Mauritius, which is lower than the present study. Higher yeast and mould count during monsoon season in the present study could be due to excess humidity in the environment. It could be concluded that strict hygienic measures should be implemented during production, processing, transportation and storage to safeguard the quality of eggs.

5.3. POLYMERASE CHAIN REACTION

All the *Campylobacter* spp. isolates obtained in the present study were subjected to multiplex PCR for the identification of *Campylobacter* genus specific *16S rRNA* gene, *C. jejuni* specific *map*A gene, *C. coli* specific *ceu*E gene and virulence gene, *cad*F. Simultaneous identification of both *C. jejuni* and *C. coli* was possible with the help of multiplex PCR. This had helped in the rapid analysis of samples than that done by cultural method. Stucki *et al.* (1995) found that the sensitivity and specificity of the PCR assay was 100 per cent for the detection of *C. jejuni* using *map*A gene. Richardson and Park (1995) detected *ceu*E gene as *Campylobacter coli* specific and encode a periplasmic substrate binding protein.

All the *Campylobacter* spp. isolates obtained by culture technique were found to be positive by molecular method, PCR. All the 17 isolates were found to carry the conserved *16S rRNA* gene, virulence gene *cad*F and *C. jejuni* specific *map*A gene. Three isolates which carried *map*A gene also showed the presence of *ceu*E gene specific for *C. coli.* Of the 17 isolates, 14 were identified as *C. jejuni* and three as mixed infection caused by both *C. jejuni* and *C. coli.* Three isolates showing the presence of both *map*A and *ceu*E gene in the present study correlate with the findings of Best *et al.* (2003). Bang *et al.* (2003), Rozynek *et al.* (2005) and Selwet and Galbas (2012) reported that 100 per cent of the *Campylobacter* spp. isolates carried *cad*F virulence gene, which was in tune with the present study.

5.4. SEASONAL OCCURRENCE OF *CAMPYLOBACTER*

All the samples in the present investigation were collected and analysed during monsoon and post monsoon seasons. Higher occurrence of *Campylobacter* spp. in cloacal swab samples was noticed in the monsoon season (66.67 per cent) than post monsoon season (20 per cent). Eggs collected from retail market also showed a higher occurrence during monsoon season (6.67 per cent) than post monsoon season (zero per cent). Results similar to that obtained in the present study was reported by Genigeorgis *et a1*. (1986) and Renu *et al.* (2011). Higher rate in the occurrence of *Campylobacter* spp. during summer season was reported by Prasad *et al.* (2001), Rahimi and Tajbakhsh (2008), Nather *et al.* (2009) and Vandeplas *et al.* (2009) than the present study. Line (2006) observed a higher colonization rate of *Campylobacter jejuni* in broiler chickens during high relative humidity. The average relative humidity of 86 per cent occurred during monsoon season in Thrissur might have contributed to the increased occurrence of *Campylobacter* spp. in the present study.

5.5. ANTIBIOTIC RESISTANCE PROFILING

The excessive use of antibiotics in food animals result in the development of resistant bacteria and may eventually reach humans through food chain. All the 17 positive isolates of *Campylobacter* spp. obtained from different type of samples were subjected to antibiotic sensitivity test by standard disc diffusion method. All the *Campylobacter* spp. isolates were sensitive to Amikacin, Chloramphenicol, Imipenem, Kanamycin, Moxifloxacin, Neomycin, Netillin, Nitrofurantoin, Sparfloxacin, Streptomycin and Tobramycin. The sensitivity pattern of all the antibiotics except Nitrofurantoin was in harmony with the findings of Kumar (2011). Susceptibility of the isolates to Nitrofurantoin was in agreement with the study of Aydin *et al.* (2001).

A high resistance to Ceftazidime and Cephalothin antibiotics observed in the present study was in accordance with the findings of Hamidian *et al.* (2011). The resistance shown by isolates to Carbenicillin, Oxacillin, Ampicillin, Aztreonam, Tetracyclin and Doxycycline was in accordance with the results of Ge *et al.* (2002),

Luber *et al.* (2003) and Senok *et al.,* (2007) and Kumar (2011). The sensitivity to fluoroquinolone class of antibiotic is higher except for Ciprofloxacin and Ofloxacin, to which 88.24 per cent of the isolates showed resistance. Ge *et al.* (2002) and Hamidian *et al.* (2010) reported quinolone resistance in *Campylobacter* spp. which clearly confirms the findings of the present study. The result of the present study was in agreement with the findings of Tribble *et al.* (2007), who reported frequent occurrence of Ciprofloxacin resistant strains associated with traveller's diarrhoea. Also, high degree of resistance was seen to co-trimoxazole, an observation which supported the findings of Rajagunalan (2010).

Campylobacter spp. are most widely distributed and found mostly in warm blooded animals. The study revealed a high occurrence of *Campylobacter* spp. in poultry and eggs from retail market in and around Thrissur. As the egg consumption is increasing every year, the risk of foodborne infection caused by *Campylobacter* spp. is also high. In order to reduce the risk of contamination of food and infections caused by *Campylobacter* spp., it is important to adopt appropriate practices for reducing *Campylobacter* colonization in poultry and hygienic practices throughout the processing, transportation and storage of eggs. The pathogenic potential of the organism is evident due to the presence of the virulence gene of *Campylobacter* spp. The results of antibiotic resistance profiling were not alarming, but the development of resistance to more antibiotics cannot be ruled out. Based on the results of present study, future research concentrating highly contaminated areas by *Campylobacter* spp. including retail eggs and foods of poultry origin will throw light on epidemiology of the organism.

6. SUMMARY

Poultry are considered as the main reservoir host for *Campylobacter* spp. organism. The present study was undertaken with the primary objective of determining critical control points of *Campylobacter* spp. in chicken egg production chain and the presence of the organism in retail eggs. A total of 570 samples,

comprising of 30 samples each of cloacal swab, feed, litter, wash water, wash cloth, plastic tray, filler flat, air, water, hand wash of candling person, hand wash of sales personnel and 120 eggs from University Poultry and Duck Farm (UPDF), Mannuthy and 120 samples of egg from retail market in and around Thrissur were collected. Half of the samples were collected during monsoon season (season A) from June to November and the remaining during post monsoon season (season B) from December to May. All the samples collected were subjected to isolation and identification of *Campylobacter* spp. by culture technique. Microbiological examination of chicken eggs was also conducted during the study. All the isolates were subjected to molecular confirmation by PCR using the genus specific *16S rRNA* gene, *C. jejuni* specific *map*A gene, *C. coli* specific *ceu*E gene and virulence gene *cad*F. The positive isolates obtained were subjected to antibiotic resistance profiling.

In UPDF, Mannuthy highest occurrence of *Campylobacter* spp. was found in season A (4.44 per cent) followed by season B (1.33 per cent). Cloacal swab samples were the single most contaminating source with 66.67 per cent during season A and 20 per cent during season B. The organism could not be detected from any other samples collected from egg production chain. Chicken eggs in the Table Egg Production Chain (TEPC) and Hatching Egg Production Chain (HEPC) in the UPDF, Mannuthy, were not contaminated with *Campylobacter* spp. The overall occurrence of *Campylobacter* spp. in UPDF, Mannuthy was found to be 2.89 per cent.

In UPDF, Mannuthy, 43.33 per cent of the cloacal swab samples were found to be contaminated with *Campylobacter* spp. The critical point of contamination for *Campylobacter* spp. in egg was found to be the cloaca of the bird. The point of lay can be considered as the critical control point (CCP2) where the point of contamination cannot be completely eliminated, but can be reduced to a considerable limit.

The present study revealed 3.33 per cent overall occurrence of *Campylobacter* spp. in eggs from retail market. Higher occurrence was noticed during monsoon season (6.67 per cent) than post monsoon season (zero per cent).

Microbiological analysis of the egg shell from TEPC in farm revealed that there was no significant difference in the mean aerobic plate count and mean yeast and mould count in both seasons at the point of lay and as the egg reaches the sales counter. Significantly highest ($p<0.05$) mean APC was observed after candling with 8.31 ± 0.26 $\log_{10}$ CFU/egg shell in season A compared to 5.78 ± 0.27 $\log_{10}$ CFU/egg shell noticed in season B. Microbiological analysis of the egg shell from HEPC in farm revealed that there was no significant difference in the mean aerobic plate count and mean yeast and mould count in both seasons at the point of lay and as the egg reaches the incubator. Significantly highest ($p<0.05$) mean APC was observed after candling and at the incubator in season A compared to season B.

Eggs from the retail market showed significantly higher ($p<0.05$) mean aerobic plate count and mean yeast and mould count during monsoon than post monsoon season.

There was no significant difference in the mean aerobic plate count and mean yeast and mould count in egg shell from UPDF and market during season A. The mean YMC in UPDF eggs was significantly higher (5.37 ± 0.19 $\log_{10}$ CFU/egg shell) than market eggs (4.45 ± 0.15 $\log_{10}$ CFU/egg shell) during post monsoon season.

All the *Campylobacter* spp. isolates obtained by culture technique were found to be positive by PCR. Out of the 10 and three isolates obtained from cloacal swab in UPDF, Mannuthy during season A and B respectively, eight and two isolates were *C. jejuni*. Two and one isolates obtained from cloacal swab during season A and B respectively, were showing mixed infection caused by *C. jejuni* and *C. coli*. The four isolates obtained from eggs in the retail market during monsoon season were found to be positive for *C. jejuni*. The overall occurrence of *C. jejuni* during season A and

season B was 4.21 and 0.7 per cent, respectively. Mixed infection caused by *C. jejuni* and *C. coli* was 0.7 and 0.35 per cent during season A and season B respectively

Confirmed isolates of *Campylobacter* spp. were 100 per cent sensitive to Amikacin, Chloramphenicol, Imipenem, Kanamycin, Moxifloxacin, Neomycin, Netillin, Nitrofurantoin, Sparfloxacin, Streptomycin and Tobramycin. All the isolates were showing 100 per cent resistance to Ceftazidime.

From the study it was clear that the poultry is the source of contamination of *Campylobacter* spp. and eggs in the retail market was also contaminated with the organism. Strict biosecurity measures should be implemented in the farm to reduce the colonization of organism in chicken. The infection control measures at all stages of food processing, transportation and storage will help to reduce the incidence of *Campylobacteriosis*. The hygienic practices followed in the production of egg at the farm and retail market needs improvement for reducing the microbial load.

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ANNEXURE I

Composition of Reagents

1. Sodium Hippurate Solution

Sodium hippurate (anhydrous)	1 g
Sterile distilled water	100 ml

One gram of sodium hippurate (HiMedia) was dissolved in 100 ml of sterile distilled water and dispensed in one millilitre amount into test tubes and used immediately. Storage of solution if and when required was made at -20^{o}C.

2. Ninhydrin Solution

Ninhydrin	3.5 gm
Acetone	50 ml
Butanol	50 ml

Ninhydrin (HiMedia) was dissolved in 100 ml mixture of 1:1 (v/v) acetone and butanol (HiMedia). It was dispensed in 10 ml amounts in to screw capped test tubes and wrapped in aluminium foil. The solution was stored at 4^{o}C and used within 2 weeks.

3. Indoxyl Acetate Solution

3-Acetoxyindole	500 mg
Acetone	5 ml

Five hundred milligram of the 3-Acetoxyindole (HiMedia) was mixed with 5 ml of acetone (HiMedia) to prepare a 10% (w/v) solution and 50 µl of the solution was added to each blank sterile paper disc (diameter of 6 mm) (HiMedia). After drying at room temperature, the discs were stored at 4^{o}C in a brown tube with a desicant, away from direct sunlight and moisture.

4. Taq DNA Polymerase

The enzyme with a concentration of five units per microlitre

5. PCR reaction buffer E (10X)

Composition: 100 mM Tris (pH 9.0)

500 mM KCl

15 mM $MgCl_2$

1% TritonX – 100

Storage: -20^{o}C

6. dNTP mix

Composition: dNTP mix is a premixed solution containing sodium salts of dATP, dCTP, dGTP and dTTP, each at 2 m*M* in water.

Storage: -20^{o}C

7. $MgCl_2$ of 25 mM

Final concentration of 2 m*M*

8. Forward and reverse primer set

Each of 10 p*M* /µL of 16S rRNA, *mapA* and *ceuE* primers and 20 p*M* /µL of *cadF* primer.

9. Tris Borate EDTA (TBE) Buffer (10X)

Composition: 89 mM Tris base

89 mM Boric acid

2 mM EDTA

TBE Buffer (1X): The 10X buffer was diluted 10 times with triple distilled water to prepare working 1X TBE buffer.

10. Agarose gel (1.5 per cent)

Weighed 1.5 g of agarose powder and mixed with 100 ml of 1X TBE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

11. Gel loading buffer

Composition:	Sucrose 40% (w/v)	4 g
	Bromophenol blue 0.25% (w/v)	25 mg
	Xylene cyanol 0.25% (w/v)	25 mg
	Distilled water	10 ml

12. Ethidium Bromide (10 mg/ml)

Dissolved 100 mg ethidium bromide in 10 ml of triple distilled water. Solution stored at 4^{o}C in amber coloured bottle.

ANNEXURE II

Composition and reconstitution of media used

1. Blood Free Campylobacter Broth Base (HiMedia)

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	10.000
Beef extract	10.000
Casein enzymic hydrolysate	3.000
Sodium chloride	5.000
Sodium deoxycholate	1.000
Ferrous sulphate	0.250

Sodium pyruvate	0.250
Charcoal, bacteriological	4.000

Final pH (at 25°C) 7.4 ± 0.2

Suspend 16.75 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Cool to 50^{o}C and aseptically add rehydrated contents of one vial of CCDA Selective Supplement (FD 135). Mix well and dispense into sterile tubes.

2. CCDA Selective Supplement (HiMedia)

Ingredients	**Concentration**
Cefoperazone	16 mg
Amphotericin B	5 mg

Rehydrate the contents of one vial aseptically with two millilitres of sterile distilled water. Mix well and aseptically add it to 500 ml of sterile, cooled (45-50^{o}C) Blood Free Campylobacter Broth Base.

3. Blood Free Campylobacter Selectivity Agar Base (HiMedia)

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	10.000
Beef extract	10.000
Casein enzymic hydrolysate	3.000
Sodium chloride	5.000
Sodium deoxycholate	1.000
Ferrous sulphate	0.250
Sodium pyruvate	0.250

Charcoal, bacteriological	4.000
Agar	12.000

Final pH (at 25°C) 7.4 ± 0.2

Suspend 22.75 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Cool to 50^{o}C and aseptically add rehydrated contents of one vial of Campylobacter Supplement V (FD 067). Alternatively to increase the selectivity of the medium, rehydrated contents of one vial of CAT Selective Supplement (FD 145) may be added to 500 ml sterile molten base. Mix well and pour into sterile Petri-plates.

4. Campylobacter Supplement V (HiMedia)

Ingredients	**Concentration**
Cefoperazone	16 mg

Rehydrate the contents of one vial aseptically with two millilitres of sterile distilled water. Mix well and aseptically add the rehydrated contents to 500 ml sterile, molten, cooled (45-50^{o}C) Blood Free Campylobacter Selectivity Agar Base. Mix well and pour into sterile Petri-plates.

5. CAT Selective Supplement (HiMedia)

Ingredients	**Concentration**
Cefoperazone	4 mg
Teicoplanin	2 mg
Amphotericin B	5 mg

Rehydrate the contents of one vial aseptically with two millilitres of sterile distilled water. Mix well and add aseptically to 500 ml of sterile, molten, cooled

(45-50^{o}C) Blood Free Campylobacter Selectivity Agar Base. Mix well and pour into sterile Petri-plates.

6. Polymyxin B Selective Supplement (HiMedia)

Ingredients	**Concentration**
Polymyxin B sulphate	50,000 Units

Rehydrate the contents of one vial aseptically with 2 ml sterile distilled water. Mix well and aseptically add it to 500 ml of sterile medium.

7. Cary-Blair Medium Base (HiMedia)

Ingredients	**Gms / Litre**
Disodium phosphate	1.100
Sodium thioglycollate	1.500
Sodium chloride	5.000
Agar	5.000
Final pH (at 25^{o}C) 8.4 ± 0.2	

Suspend 12.6 grams in 991 ml distilled water. Heat to boiling to dissolve the medium completely. Cool to 50^{o}C and aseptically add nine millilitres of one per cent aqueous calcium chloride solution. Adjust pH to 8.4 if necessary. Distribute in seven millilitres amounts in screw-capped tubes. Steam for 15 min. Cool and tighten the caps. Store the medium in the dark in either a refrigerator or at room temperature for up to 19 months.

8. Brucella HiVeg^{TM} Broth Base (HiMedia)

Ingredients	**Gms / Litre**
HiVeg peptone	10.000

HiVeg hydrolysate	10.000
Yeast extract	2.000
Dextrose	1.000
Sodium chloride	5.000
Sodium bisulphite	0.100

Final pH (at 25^{o}C) 7.0 ± 0.2

Suspend 14.05 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Cool to 45-50^{o}C and aseptically add sterile rehydrated contents of one vial of Campylobacter Growth Supplement (FD 009). Mix well before pouring into sterile tubes.

9. Campylobacter Growth Supplement (HiMedia)

Ingredients	**Concentration**
Sodium pyruvate	0.125 g
Sodium metabisulphite	0.125 g
Ferrous sulphate	0.125 g

Rehydrate the contents of one vial aseptically with two millilitres of sterile distilled water and mix well to dissolve. Avoid frothing of the solution. Aseptically add the contents to 500 ml of sterile, cooled (45-50^{o}C) medium. Mix well and dispense into sterile tubes.

10. Phosphate Buffered Saline, pH 7.2 (HiMedia)

Ingredients	Gms / Litre
Sodium chloride	8.500
Disodium hydrogen phosphate	1.910
Potassium dihydrogen phosphate	0.380

Final pH (at 25^{o}C) 7.2 ± 0.2

Suspend 10.79 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 10 lbs pressure (115^{o}C) for 10 min.

11. Triple Sugar Iron Agar (HiMedia)

Ingredients	Gms/ Litre
Peptic digest of animal tissue	10.000
Casein enzymic hydrolysate	10.000
Beef extract	3.000
Yeast extract	3.000
Lactose	10.000
Sucrose	10.000
Dextrose	1.000
Sodium chloride	5.000
Ferrous sulphate	0.200
Sodium thiosulphate	0.300
Phenol red	0.024
Agar	12.000

Final pH (at 25^{o}C) 7.4 ± 0.2

Suspend 64.52 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes. Sterilize by

autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Allow the medium to set in sloped form with a butt about 1 inch long.

12. Plate Count Agar (HiMedia)

Ingredients	**Gms/ Litre**
Casein enzymic hydrolysate	5.000
Yeast extract	2.500
Dextrose	1.000
Agar	15.000
Final pH (at 25^{o}C) 7.0 ± 0.2	

Suspend 23.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Mix well and pour into sterile Petri-plates.

13. Potato Dextrose Agar (HiMedia)

Ingredients	**Gms/ Litre**
Potatoes, infusion from	200.000
Dextrose	20.000
Agar	15.000
Final pH (at 25^{o}C) 5.6 ± 0.2	

Suspend 39.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Mix well before dispensing. In specific work, when pH 3.5 is required, acidify the medium with sterile 10 per cent tartaric acid. The amount of acid

required for 100 ml of sterile, cooled medium is approximately one millilitre. Do not heat the medium after addition of the acid.

14. Tryptone Soya Agar with 5 per cent Defibrinated Sheep Blood

Ingredients	**Composition**
Tryptone Soya Agar	40 g
Sheep Blood (Defibrinated)	50 ml
Distilled Water	950 ml
Final pH (at 25^{o}C) 7.3 ± 0.2	

Autoclave at 121^{o}C. Cool sterilized medium to ~47^{o}C. Aseptically add 50 ml of room temperature defibrinated sheep blood. Gently mix and dispense as required.

15. Soyabean Casein Digest Agar (Tryptone Soya Agar) (Casein Soyabean Digest Agar) (HiMedia)

Ingredients	**Gms/ litre**
Pancreatic digest of casein	15.000
Papaic digest of soyabean meal	5.000
Sodium chloride	5.000
Agar	15.000
Final pH (at 25^{o}C) 7.3 ± 0.2	

Suspend 40 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. If desired, aseptically add five per cent v/v defibrinated blood in previously cooled medium to 45-50^{o}C for cultivation. Mix well and pour into sterile Petri-plates.

16. Mueller Hinton Agar (HiMedia)

Ingredients	**Gms/ litre**
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000

Final pH (at 25^{o}C) 7.3 ± 0.2

Suspend 38.0 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Mix well before pouring.

17. Medium for DNA Hydrolysis Test

Ingredients	**Composition**
DNAase test agar with Toluidine blue (Hi-media)	42.10 g
TRIZMA (Sigma Chemicals Co.)	6.23 g
0.01 M Calcium chloride	1.00 ml
Distilled water	1000.00 ml

DNAase agar with toluidine blue and TRIZMA were dissolved in distilled water. To this was added one millilitre of 0.01 M calcium chloride. The mixture was sterilized by autoclaving for 15 min under 15 lbs pressure. It was then cooled to 45^{o}C and dispensed into Petri-plates in 25 ml amounts in each plate. The plates were overnight dried in a 37^{o}C incubator and kept at 4^{o}C in refrigerator till use.

18. DNAase test agar with Toluidine blue (HiMedia)

Ingredients	**Gms/ Litre**
HiVeg hydrolysate No. 1	20.0
Deoxyribonucleic acid (DNA)	2.0
Sodium chloride	5.0
Toluidine blue	0.1
Agar	15.0
Final pH (at 25^{o}C) 7.3 ± 0.2	

Suspend 42.1 grams in 1000 ml distilled water. Heat to boiling with frequent agitation to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121^{o}C) for 15 min. Cool to 45^{o}C and pour into sterile Petri-plates.

ANNEXURE III

Biochemical tests for identification of *Campylobacter* spp.

A brief description employed in various biochemical tests to identify *Campylobacter* spp. is described below.

1. PRIMARY IDENTIFICATION TESTS

1.1. Gram Staining

The colonies showing typical morphology were selected for Gram's staining. A thin smear of each isolate was made on a clean, grease free glass slide. The smear was air dried and then heat fixed by passing over a flame. The smear was then flooded with 0.5 per cent crystal violet in water and allowed to act for one minute. The stain was poured off and the smear was washed with tap water. Then it was flooded with Gram's iodine solution (one per cent iodine and two per cent potassium iodide in water) for 30 sec. The solution was poured off and the smear was decolourised with a few drops of acetone for about 30 sec by gently agitating the slide till no colour came out of the smear. Smear was washed and counter stained with carbol fuchsin for 30 sec. The stain was poured off from the slide, washed with tap water, air dried and examined under oil immersion objective of the microscope. Colonies showing Gram negative reaction and typical morphology like curved (comma or S-shaped) rods, long helical, spiral or mixed forms were presumptively considered as positive for genus *Campylobacter*.

1.2. Oxidase Test

Oxidase discs (HiMedia) were used for testing oxidase producing capacity of the test organism. Using a sterile glass rod removed a colony of the organism and smeared it on the disc. The appearance of a dark purple colour within 30 sec indicated a positive reaction.

1.3. Catalase Test

A loopful of test culture was mixed with a drop of three per cent hydrogen peroxide on a clean, grease free, glass slide and examined for the release of nascent oxygen in the form of gas bubbles. A positive reaction was indicated by the appearance of effervescence within a few seconds.

1.4. Motility Test

A drop of overnight broth culture of suspected colony was tested by hanging drop method for motility. Samples showing cork-screw darting type motility, typical of genus *Campylobacter* were presumptively considered positive.

2. SECONDARY IDENTIFICATION TESTS

2.1. Aerobic growth test

Suspected isolates were streaked on duplicate plates and incubated under aerobic conditions at 37ºC. Any isolate showing growth was discarded. This test differentiates *Campylobacter* spp. from *Arcobacter* spp., wherein the latter organisms are reported to show growth.

2.2. Growth at 25°C and 42°C

A loopful of growth was inoculated on to duplicate *Campylobacter* selective agar plates and incubated one plate at 25˚C and 42˚C for upto three days in a microaerophilic atmosphere. A positive reaction was indicated by the development of typical *Campylobacter* colonies after incubation.

2.3. Hippurate Hydrolysis test

In one millilitre of one per cent hippuric acid (one per cent hippurate, pH 8.0) (HiMedia) a loopful of 48 h old culture growth was inoculated. The suspension was mixed and incubated at 42˚C for 4h under microaerophilic condition. To this tube 0.5 ml of freshly prepared ninhydrin solution (HiMedia) was added from the sides and

reincubated further for 10 min at 42˚C. Appearance of a deep purple coloured ring at the top or colouration of the entire tube confirmed positive result.

2.4. Indoxyl Acetate hydrolysis

An absorbent filter paper disc was soaked with indoxyl acetate (10 per cent w/v) (HiMedia) and air dried. Loopful growth of colonies from agar medium was applied on the test disc and a drop of sterile distilled water was added. Appearance of a blue green-colour within five to 10 min indicated positive result.

2.5. Hydrogen Sulphide production (H_2S) on Triple Sugar Iron (TSI) agar medium

The Triple Sugar Iron agar (HiMedia), with butt stabbed and the slant streaked with the culture, was incubated at 42°C in microaerophilic conditions and observed upto seven days. A positive reaction was indicated by blackening of the media at the site of inoculation due to hydrogen sulphide production.

2.6. Rapid H_2S test

A large ball-like inoculum, enough to fill a five millimetre diameter loop was gently suspended without mixing in the upper third of medium. The inoculated tubes were incubated at 37^{o}C in a water bath for four hours. Positive result was indicated by blackening reaction around the mass of inoculum.

2.7. Sensitivity to Nalidixic acid and resistance to Cephalothin

Disc diffusion method was employed for susceptibility testing on Mueller-Hinton agar supplemented with five per cent sheep blood. A sterile swab was charged with the culture suspension and was dispersed over the surface of agar. Sterile tweezers were used to place the nalidixic acid (30µg) and cephalothin (30µg) (HiMedia) on the surface of the agar plate ensuring that the discs were widely spaced. The plates were incubated under microaerophilic atmosphere at 42°C for 48 h. The

bacterial growth zones of inhibition were examined around the antibiotic discs and sensitivity and resistance parameters were recorded.

2.8. DNA hydrolysis test

A loopful of 48 h old culture grown at 42°C under microaerophilic condition was used to inoculate heavily a circular area of about five millimetre in diameter. The plates were examined after 1, 2 and 3 days of incubation at 42°C, under microaerophilic conditions. A clear colourless or pinkish zone around the inoculum was considered positive. No change around the inoculum was taken as negative.

ABSTRACT

The present investigation was undertaken to determine the critical control points of *Campylobacter* spp. in chicken egg production chain and the presence of the organism in retail eggs. The study also assessed the antibiotic resistance profile of the isolates which were confirmed by PCR. A total of 450 samples comprising of cloacal swab, feed, litter, wash water, wash cloth, plastic tray, filler flat, air, water, hand wash of candling and sales personnel and eggs were collected from University Poultry and Duck Farm (UPDF), Mannuthy, Thrissur. These samples from egg production chain together with 120 chicken eggs from the retail markets collected during monsoon and post monsoon season formed the materials for the study. All the samples collected were subjected to isolation and identification of *Campylobacter* spp. by conventional culture technique. Microbiological quality of the egg samples collected were also analyzed. All the isolates were subjected to molecular confirmation by PCR using the genus specific *16S rRNA* gene, *C. jejuni* specific *map*A gene, *C. coli* specific *ceu*E gene and virulence gene *cad*F. The positive isolates obtained were subjected to antibiotic resistance profiling.

A high occurrence of *Campylobacter* spp. was found in cloacal swab samples during monsoon (66.67 per cent) than post monsoon season (20 per cent). The organism could not be detected from any other sample collected from egg production chain. None of the egg samples collected from the UPDF, Mannuthy were contaminated with *Campylobacter* spp. The overall occurrence of *Campylobacter* spp. in UPDF, Mannuthy was found to be 2.89 per cent. The critical

point of contamination in egg production chain was identified as the cloaca of the birds. The point of lay can be considered as the critical control point (CCP2) where the point of contamination cannot be completely eliminated, but can be reduced to a considerable level. The occurrence of *Campylobacter* spp. in retail eggs was found to be 6.67 per cent during monsoon season. The yeast and mould count in UPDF egg shell was significantly higher (5.37 ± 0.19 log_{10}CFU/egg shell) than retail eggs (4.45 ± 0.15 log_{10}CFU/egg shell) during post monsoon season, but no significant difference was observed in the aerobic plate count in egg shell from both the sources. Of the 17 isolates, 14 were identified as *C. jejuni* and three as mixed infection caused by both *C. jejuni* and *C. coli.* The confirmed isolates of *Campylobacter* spp. were 100 per cent sensitive to Amikacin, Chloramphenicol, Imipenem, Kanamycin, Moxifloxacin, Neomycin, Netillin, Nitrofurantoin, Sparfloxacin, Streptomycin and Tobramycin. The study revealed the presence of *Campylobacter* spp. in poultry and eggs from retail market. Effective pre-harvest intervention strategies should be implemented at the farm level to prevent *Campylobacter* colonization in the flock and to reduce product contamination. Hygienic practices throughout the processing, transportation and storage of eggs should be adopted to eliminate contamination of eggs in the retail market.

CURRICULUM VITAE

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8. Professional experience : Nil
9. Publications made : Nil
10. Membership in professional bodies :

a) Kerala State Veterinary Council, Reg. No: 3953

b) Indian Veterinary Association, Kerala

Fig. 1 Standard strain ATCC® 33560™ of *Campylobacter jejuni subsp. jejuni* on Tryptone Soya agar plates with five per cent defibrinated sheep blood

Fig. 2 Standard strain ATCC® 33560™ of *Campylobacter jejuni subsp. jejuni* on modified Charcoal Cefoperazone Deoxycholate agar plate

Sample (25g)

↓

Enrichment in 225 ml mCCD broth

↓ **Microaerophilic incubation at 42°C for 48 h**

Inoculate on mCCD agar supplemented with Polymyxin B selective supplement

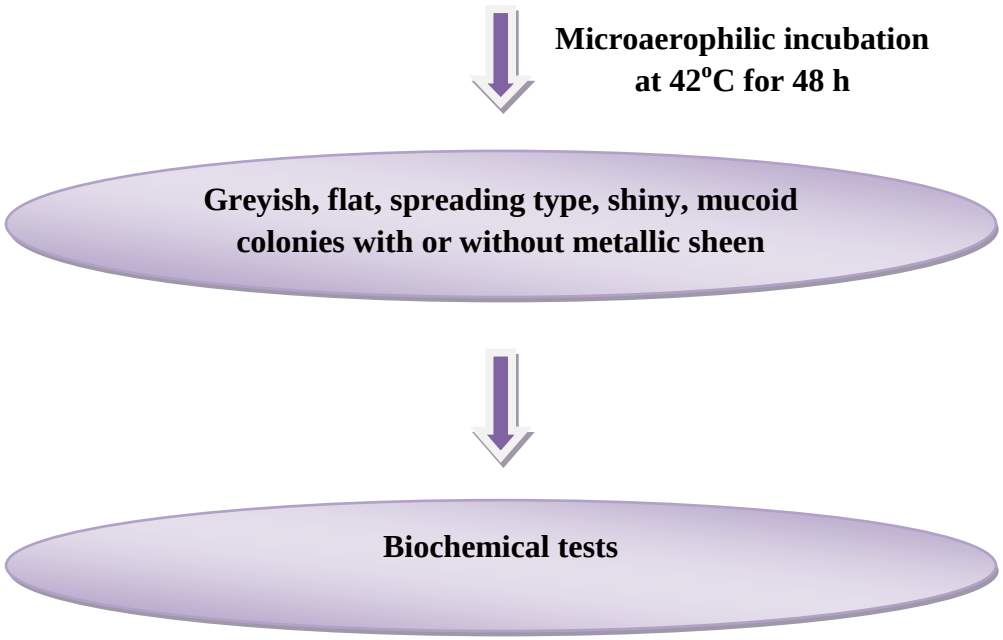


Fig. 5 Isolation of *Campylobacter* spp.

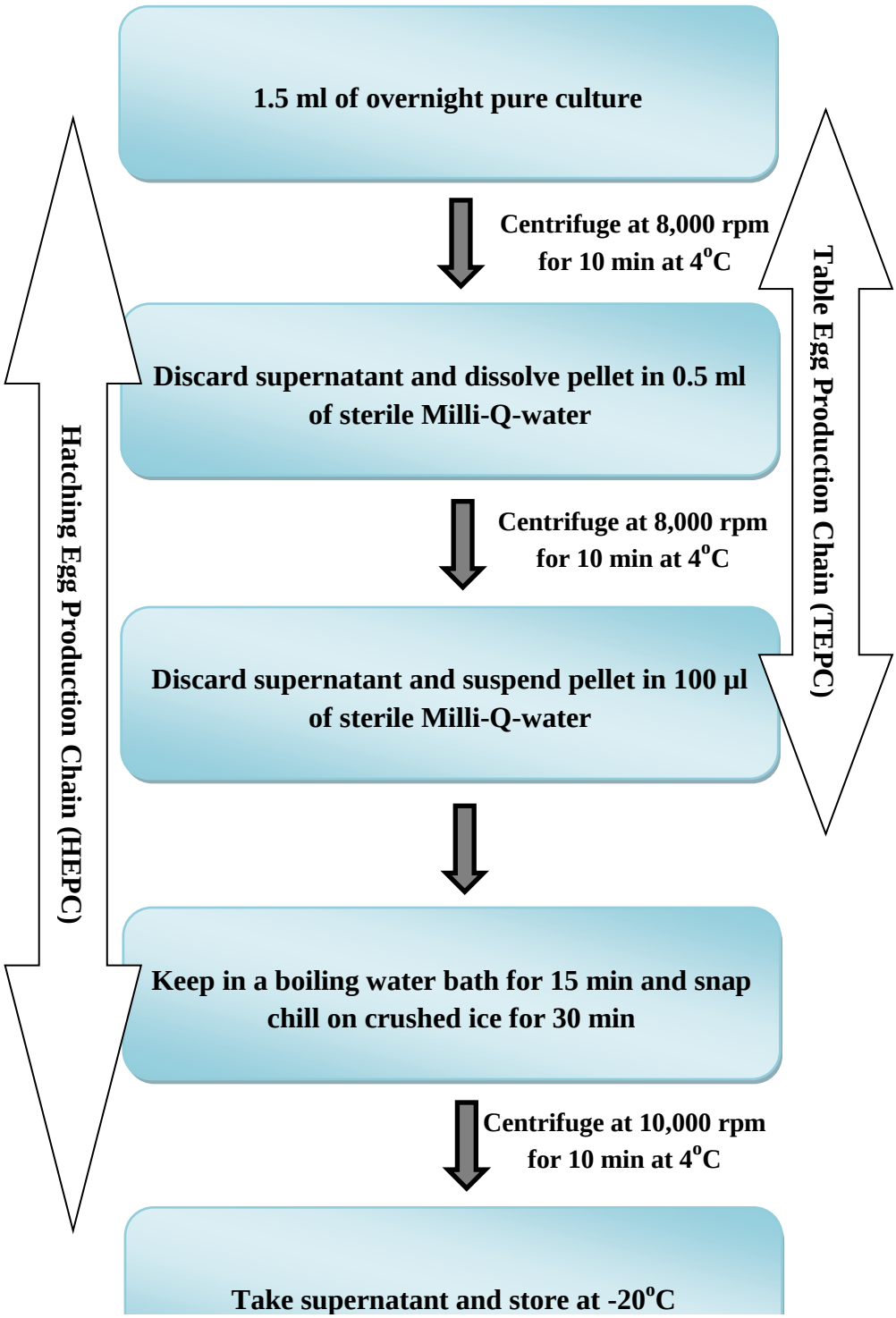


Fig. 4 Points of collection for study of the critical contamination points in egg production chain

Fig. 6 Preparation of template DNA from culture

(Boiling and snap chilling method)


Point of lay
After washing
After candling
Storage room
Sales counter
SALES SECTION
Incubator

Fig. 3 Collection of cloacal swab

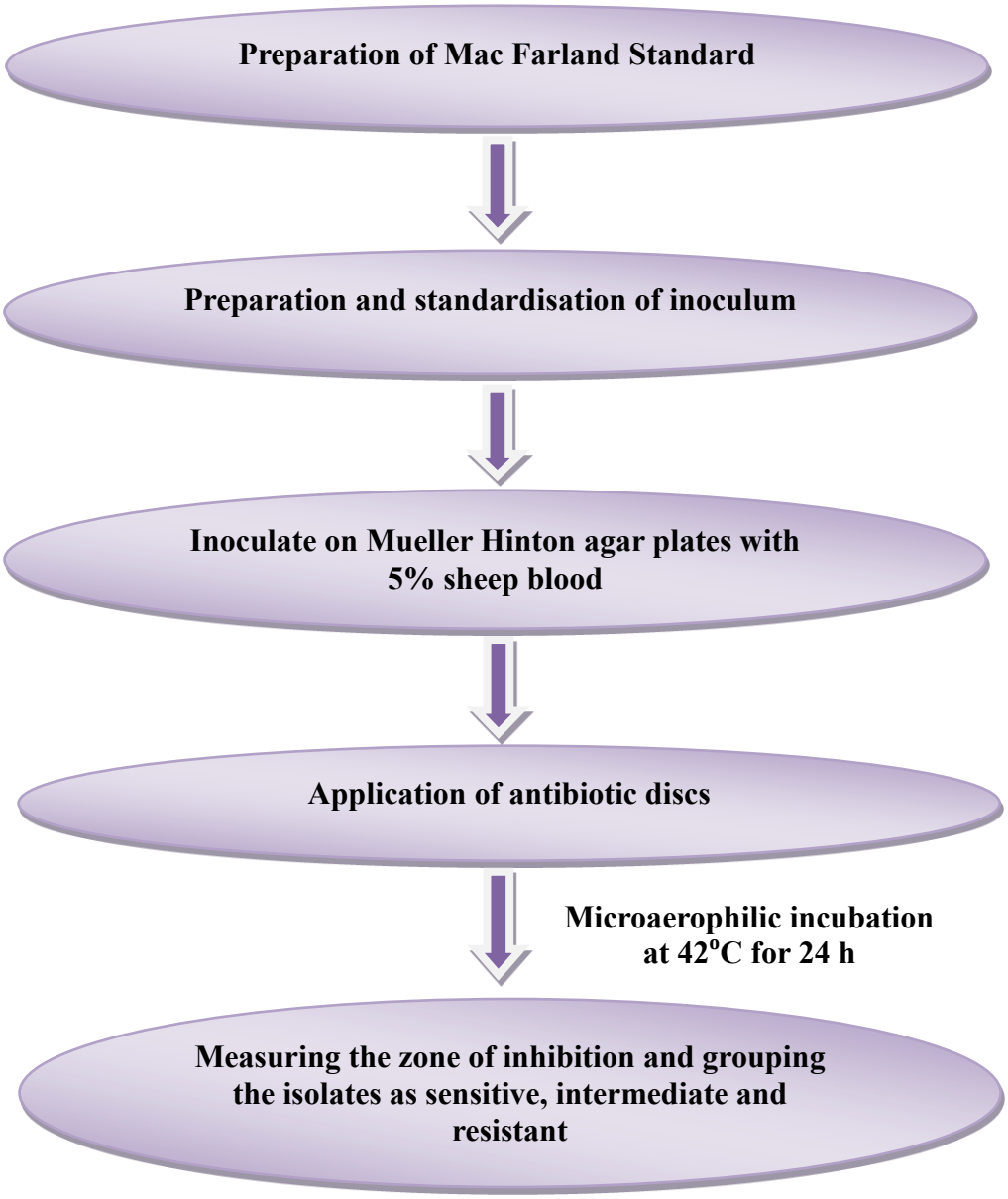


Fig. 7 Antibiotic resistance profiling of *Campylobacter* spp.

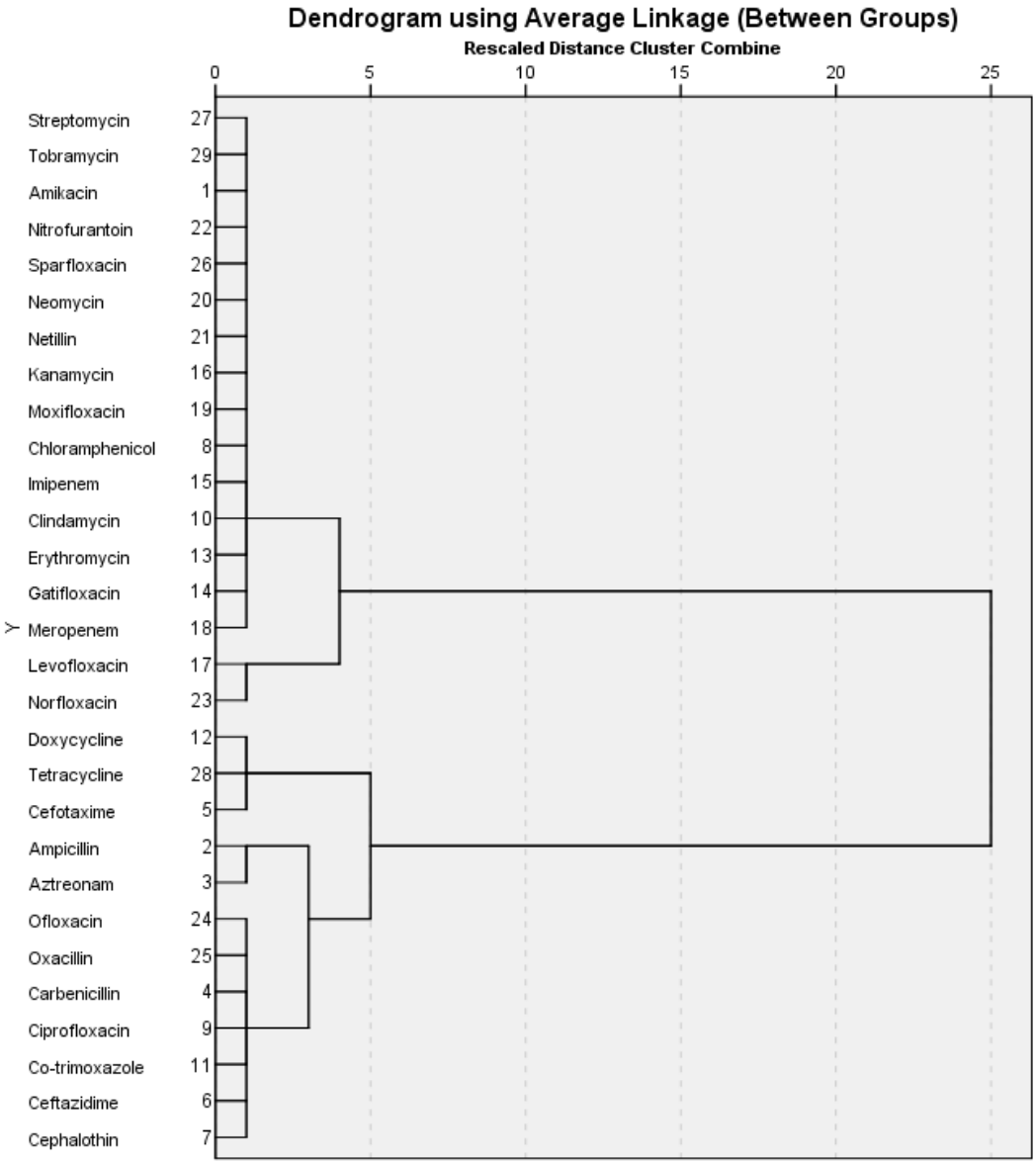


Fig. 29 Dendrogram using Average Linkage (Between Groups) for *Campylobacter* spp. isolates

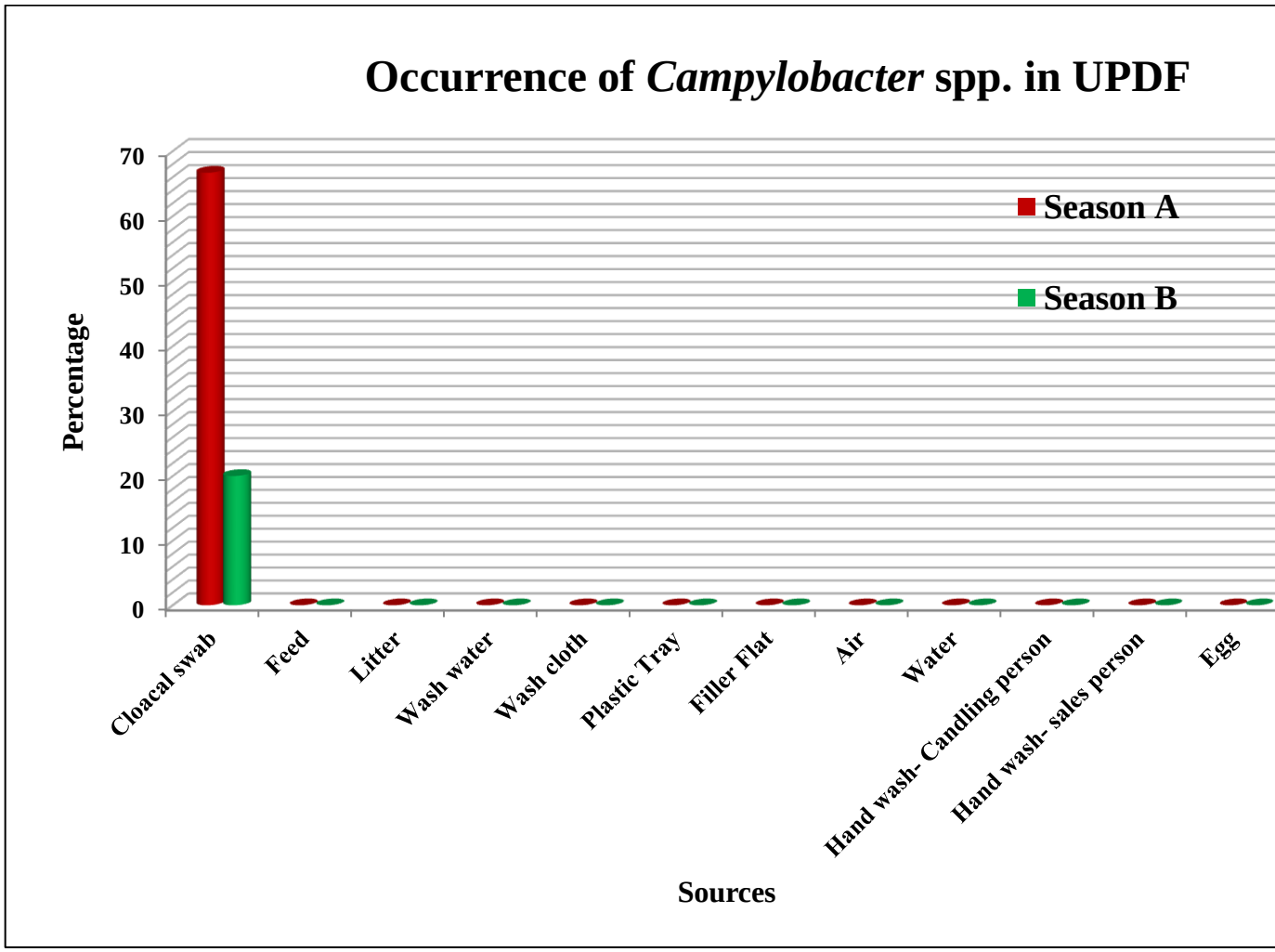


Fig. 10 Occurrence of *Campylobacter* spp. in UPDF

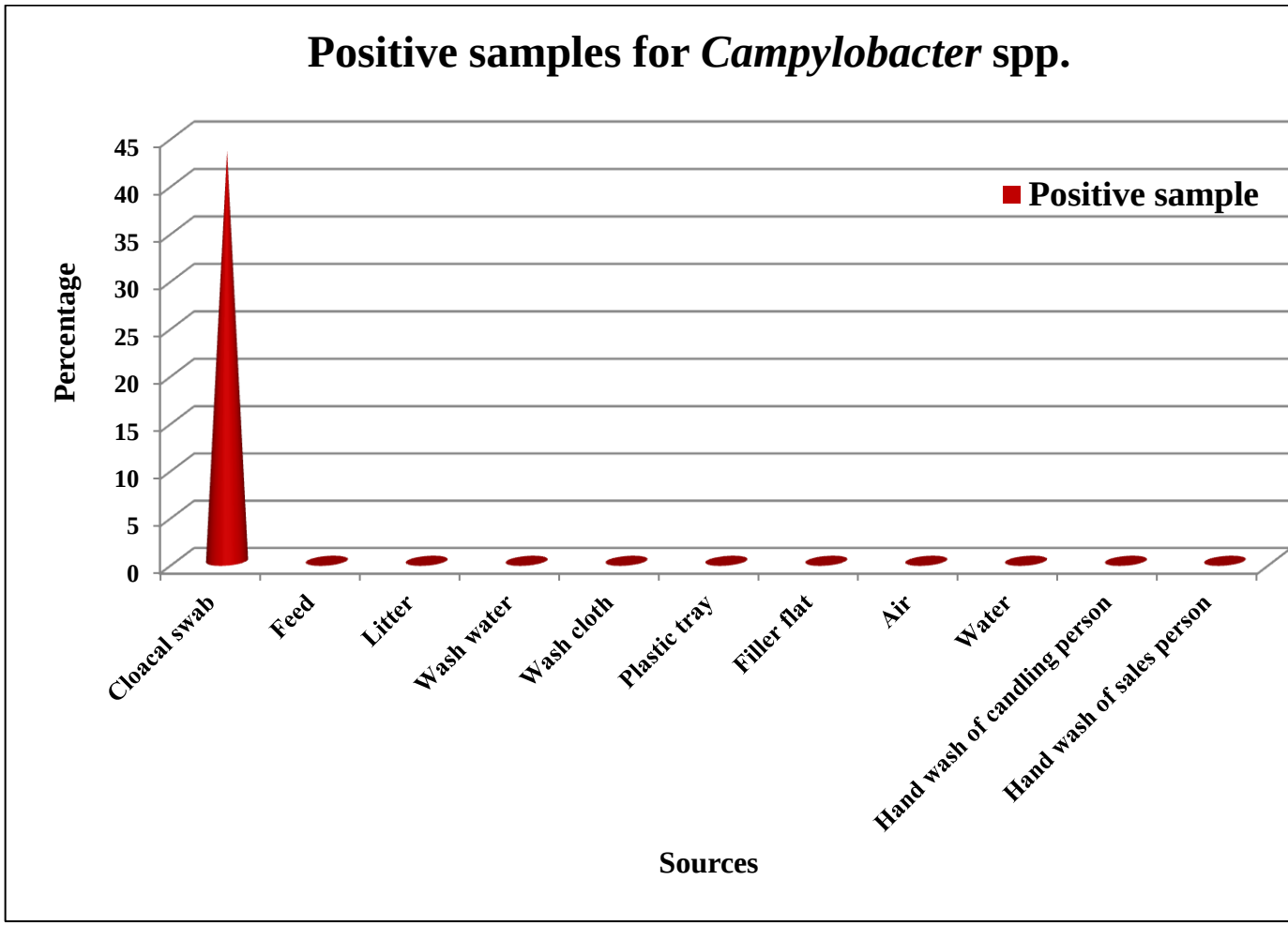


Fig. 11 Positive samples for *Campylobacter* spp.

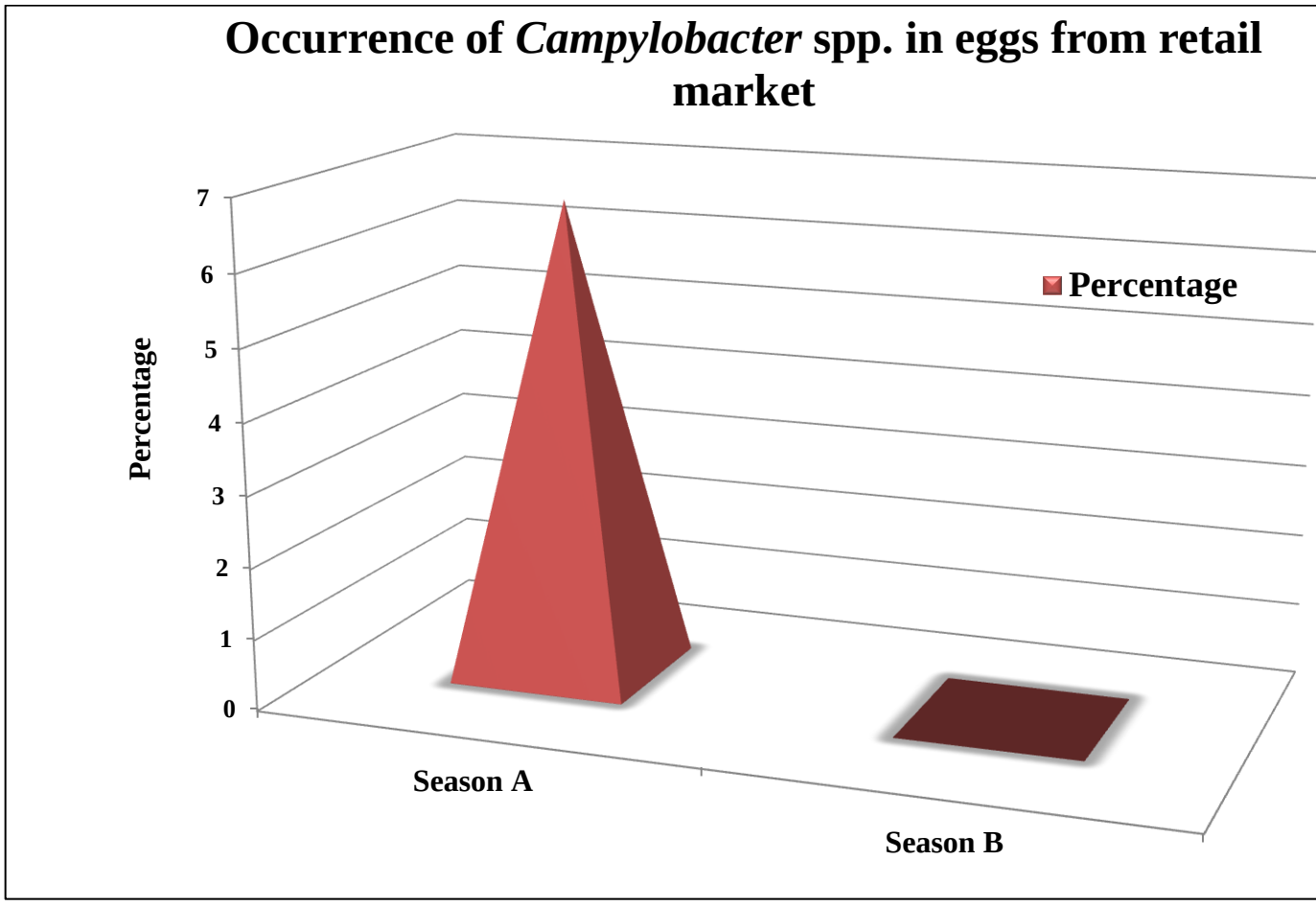
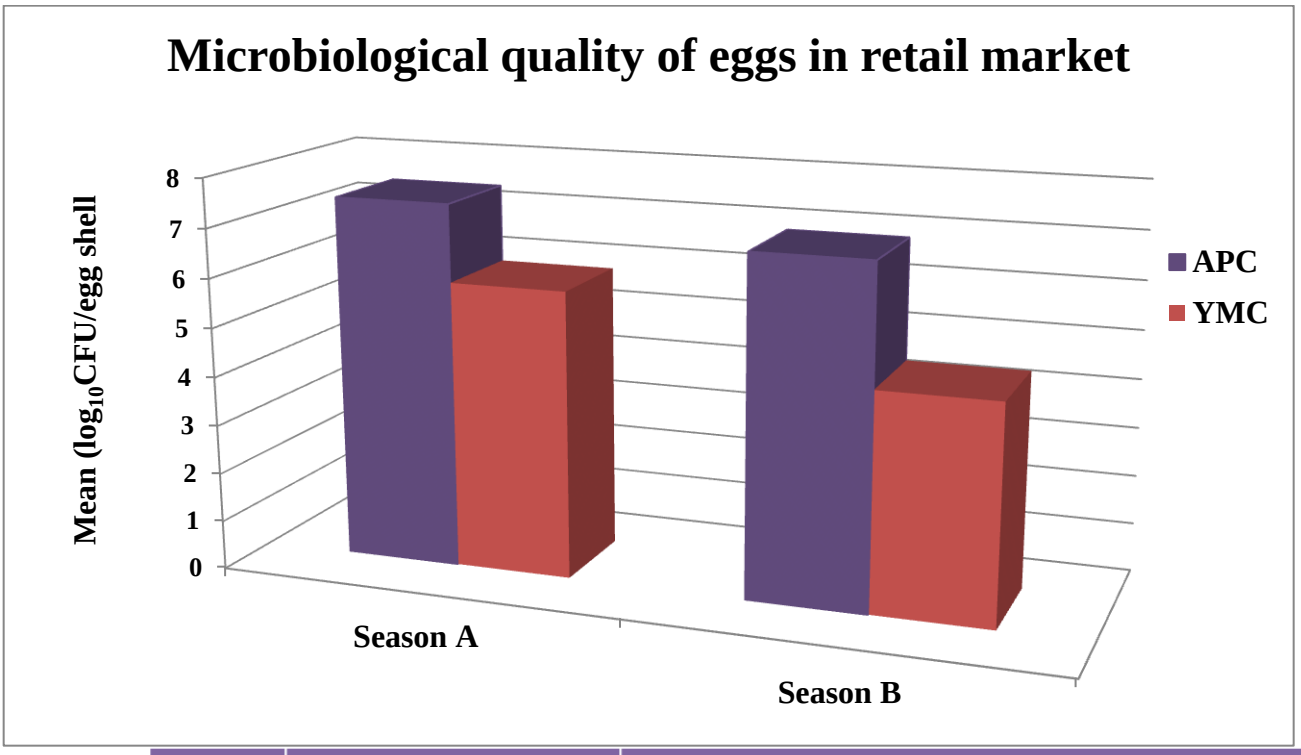


Fig. 13 Occurrence of *Campylobacter* spp. in eggs from retail market



Sl. No	Counts	Egg shell (log_{10} CFU/egg shell)	
		Season A	Season B
1	APC	7.44 ± 0.08^{A}	6.90 ± 0.08^{B}
2	YMC	5.87 ± 0.11^{A}	4.45 ± 0.15^{B}

Fig. 18 Microbiological quality of eggs in retail market

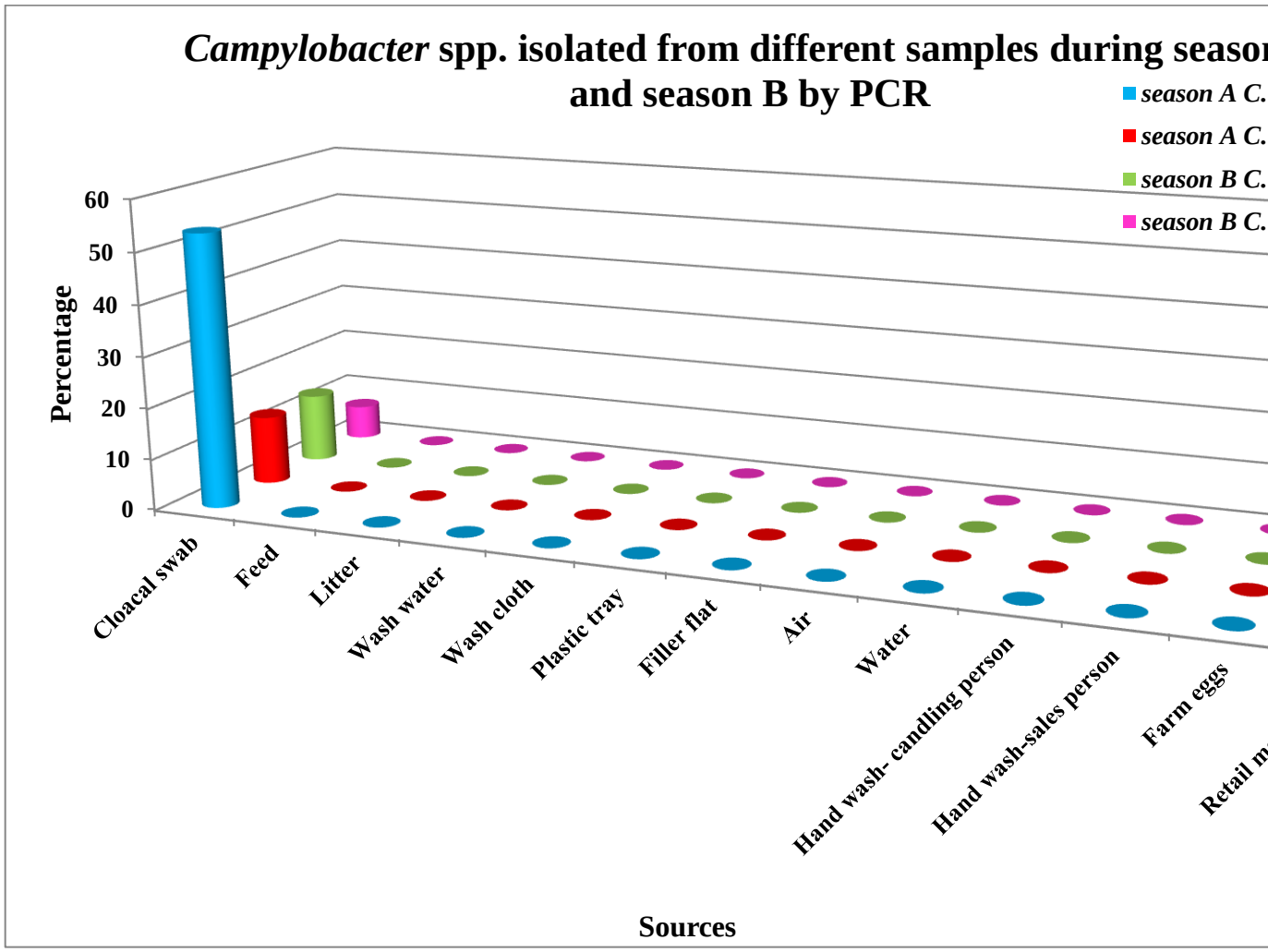


Fig. 25 *Campylobacter* spp. isolated from different samples during season A and season B by

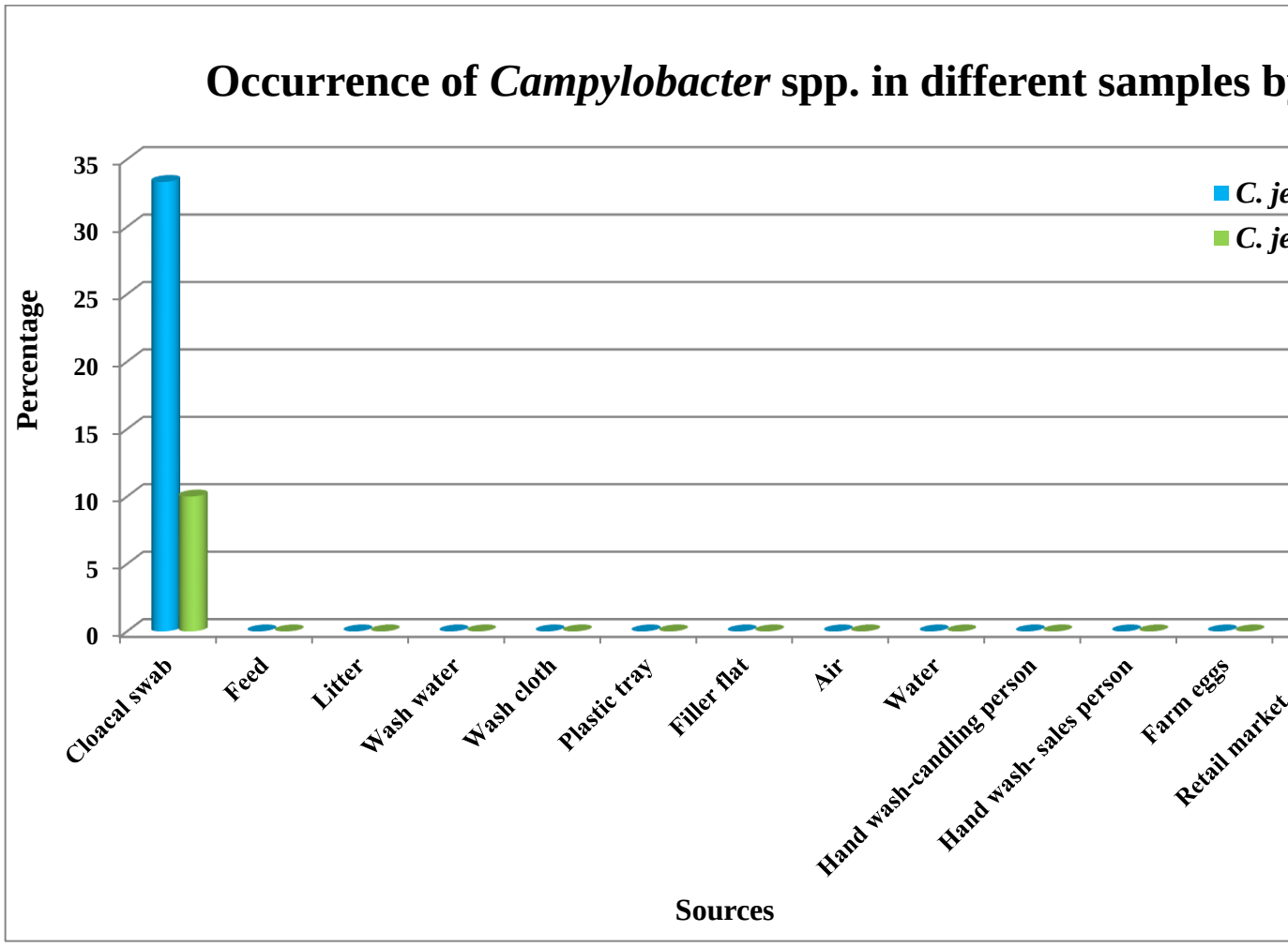


Fig. 26 Occurrence of *Campylobacter* spp. in different samples by PCR

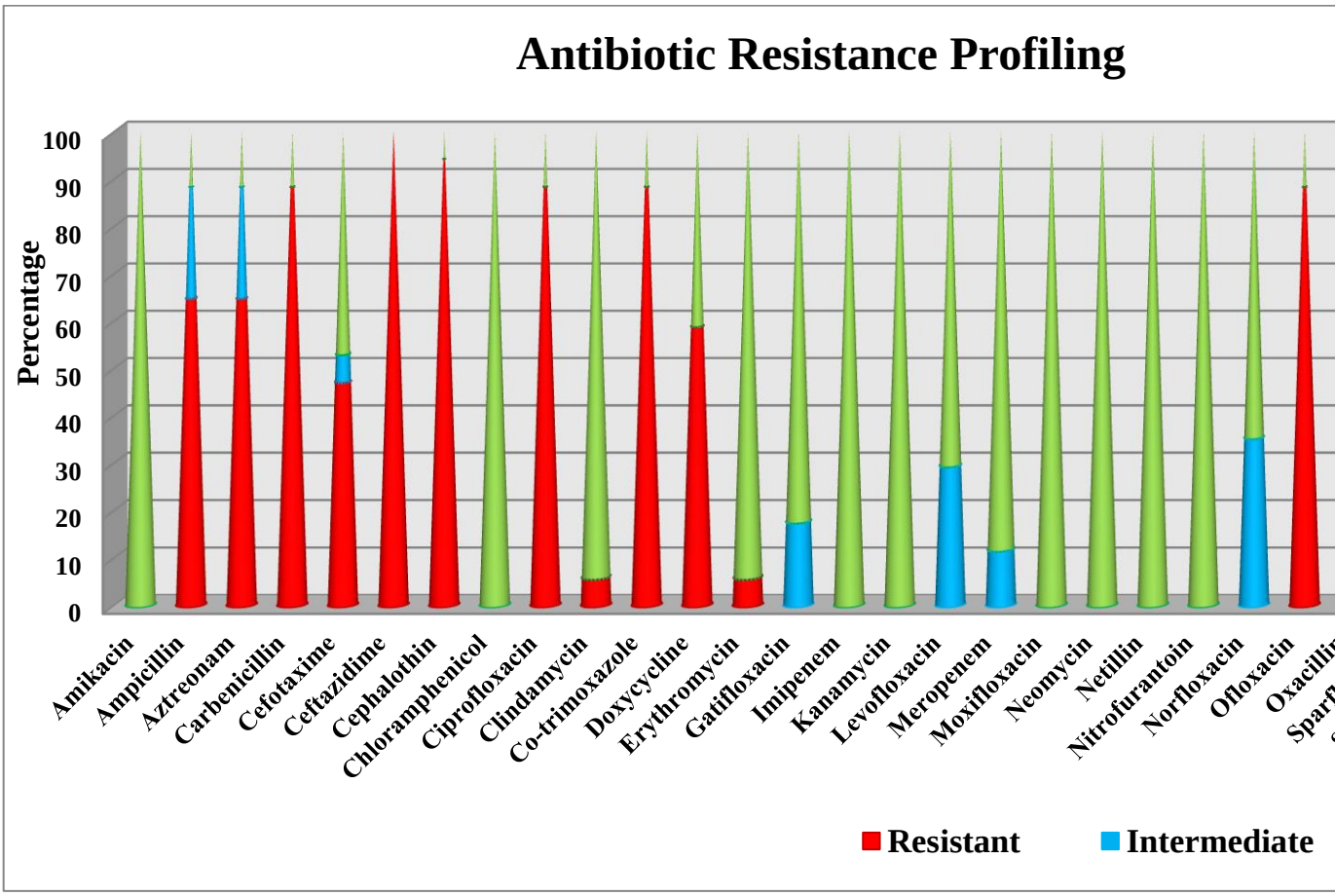


Fig. 28 Antibiotic Resistance Profiling

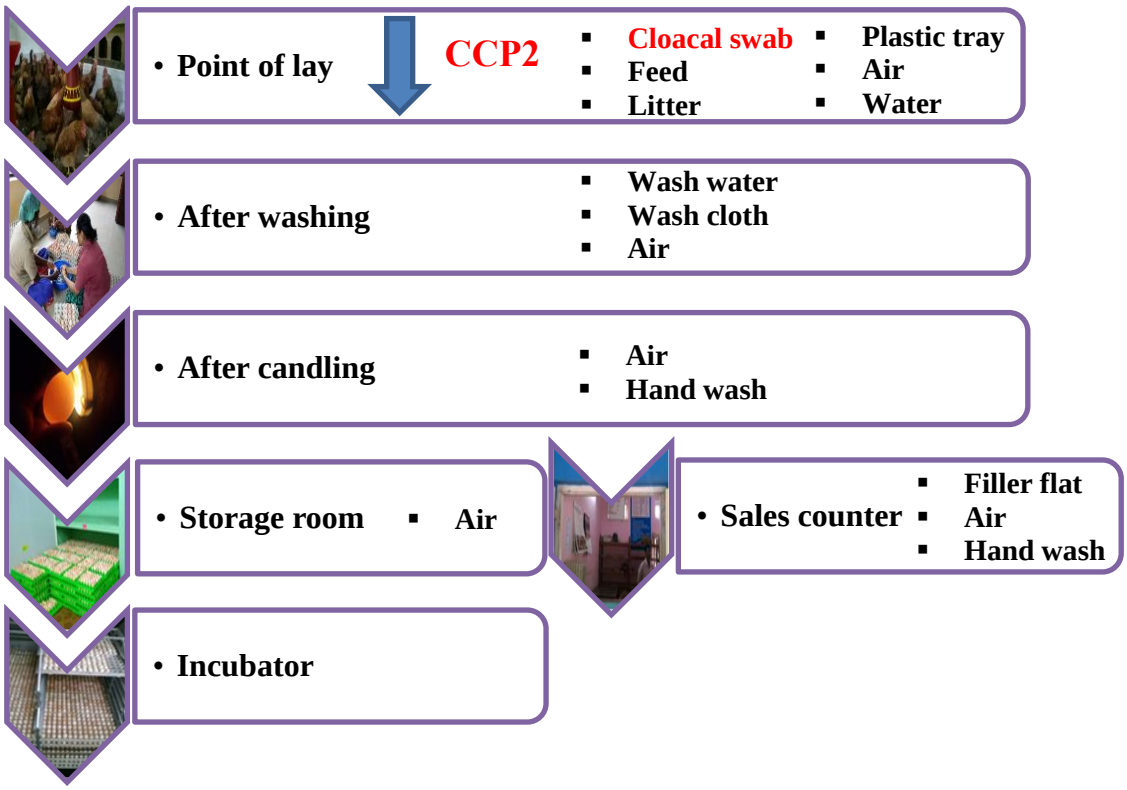


Fig. 12 CCP of *Campylobacter* spp. in egg production chain

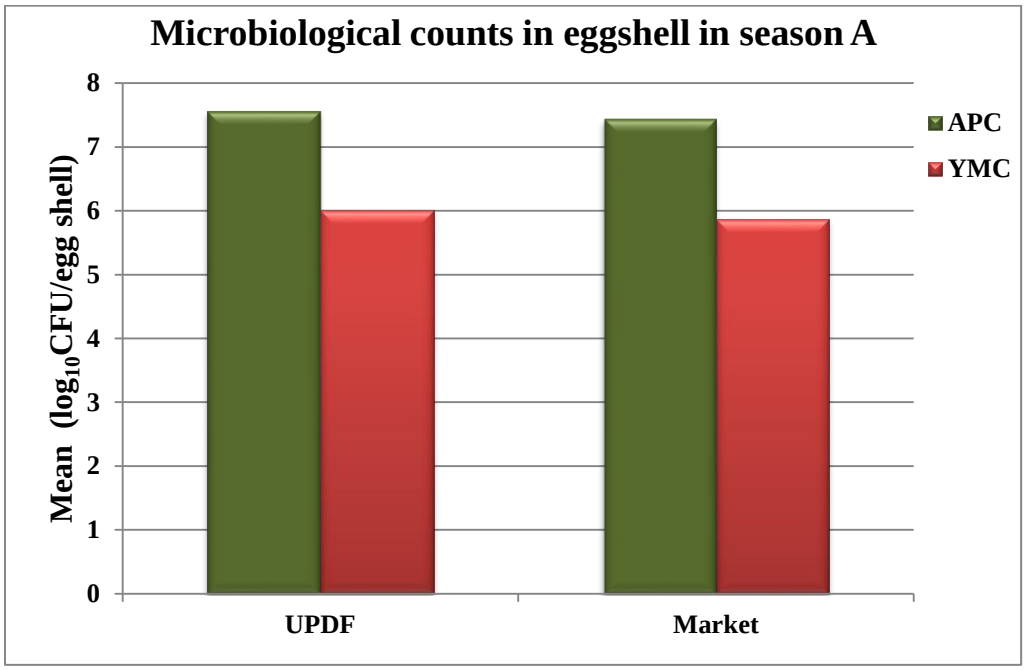


Fig. 19 Comparative assessment of microbiological quality of eggs from farm and retail market – Season A

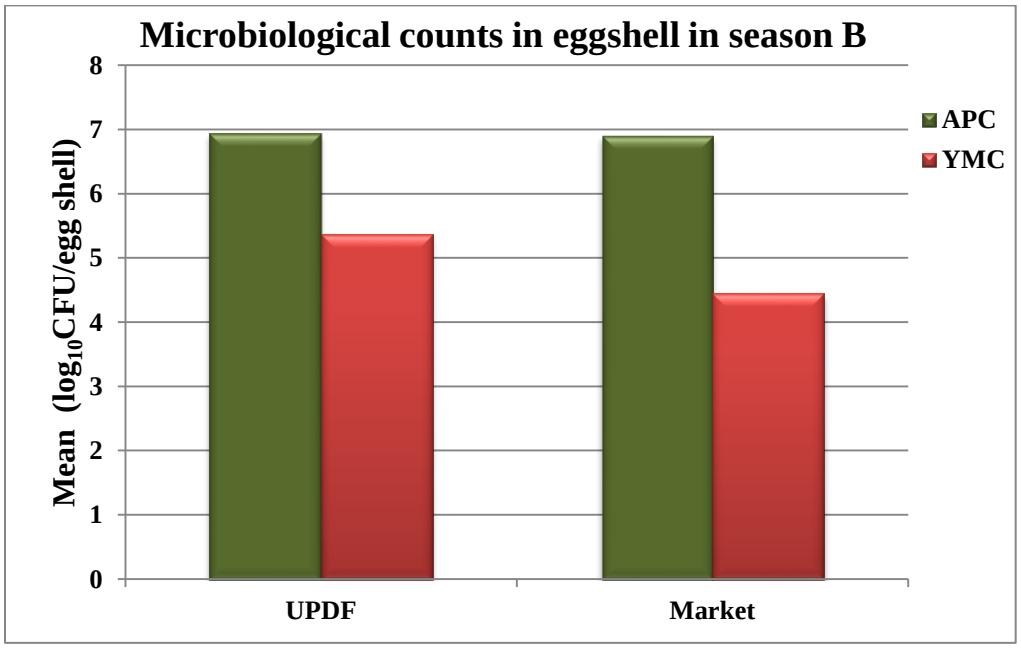
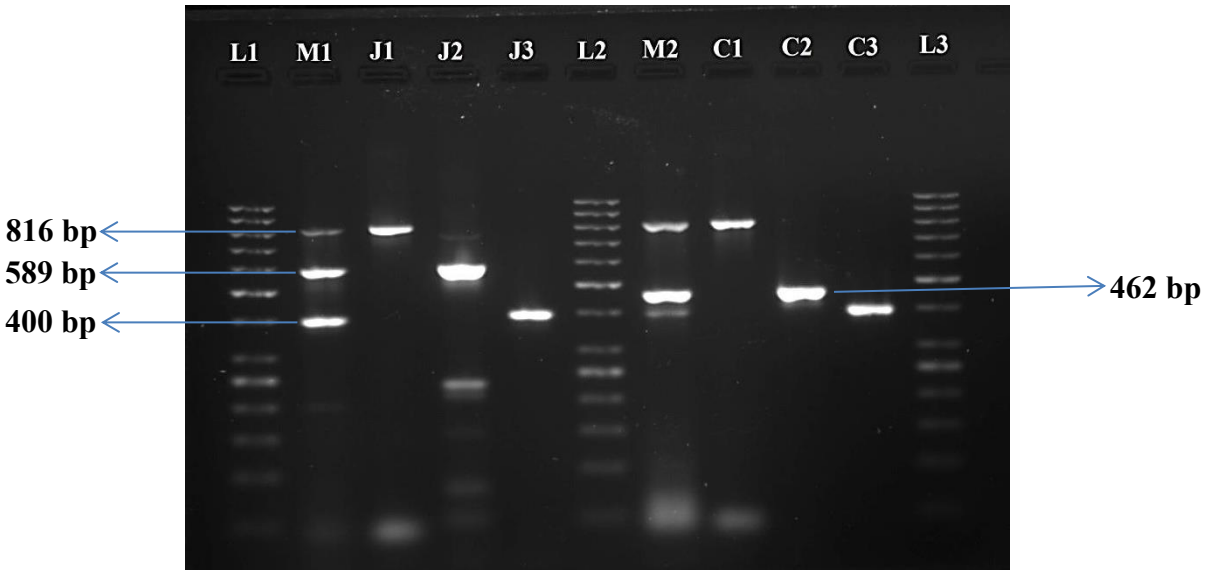


Fig. 20 Comparative assessment of microbiological quality of eggs from farm and retail market – Season B



L1, L2, L3 – 50bp ladder
M1 – Multiplex *C. jejuni*
J1 – *16S rRNA* gene of *C. jejuni*
J2 – *map*A gene of *C. jejuni*
J3 – *cad*F gene of *C. jejuni*

M2 – Multiplex C. coli
C1 – 16S rRNA gene of C. coli
*C2 – map*A *gene of C. coli*
C3 – cadF gene of C. coli

Fig. 21 Standardisation of PCR for *Campylobacter* spp.

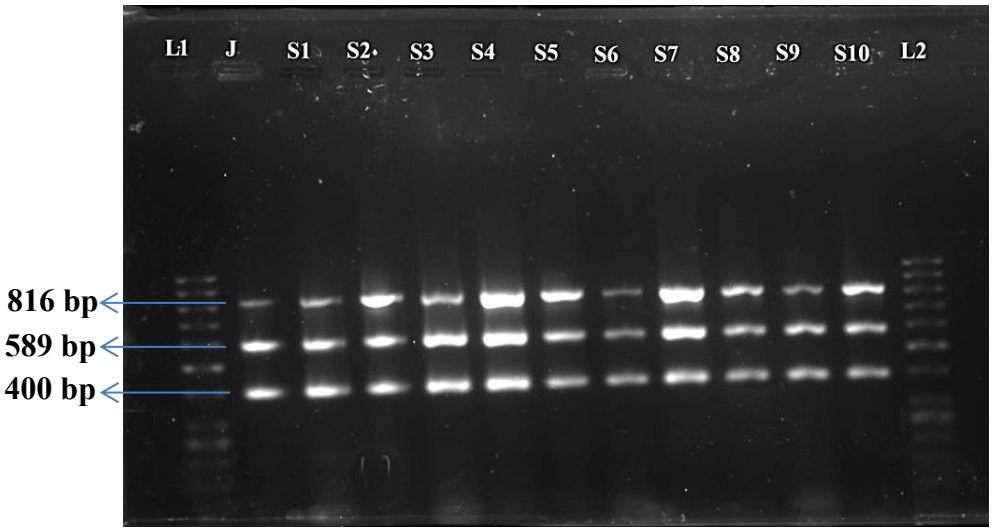


Fig. 22 Detection of *Campylobacter jejuni* by multiplex PCR in cloacal swab samples

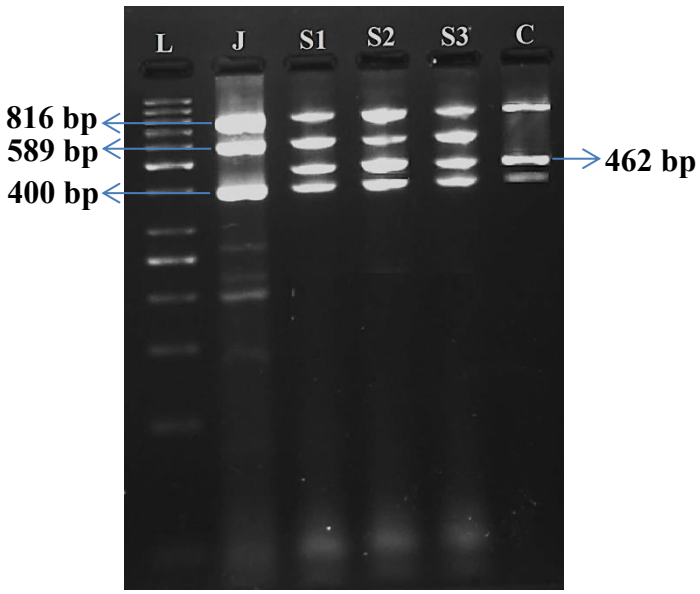


Fig. 23 Detection of *Campylobacter jejuni* and *Campylobacter coli* by multiplex PCR in cloacal swab samples

L, L1, L2 – 50 bp ladder
J – Multiplex *C. jejuni* control
C- Multiplex *C. coli* control
S1-S10 - Samples

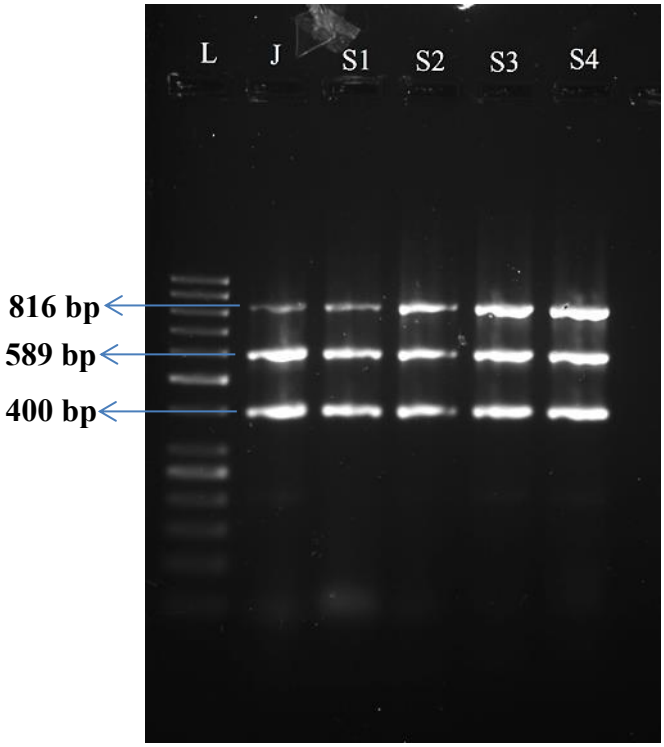


Fig. 24 Detection of *Campylobacter jejuni* by multiplex PCR in eggs from retail market

L – 50 bp ladder
J – Multiplex *C. jejuni* control
S1-S4 - Samples

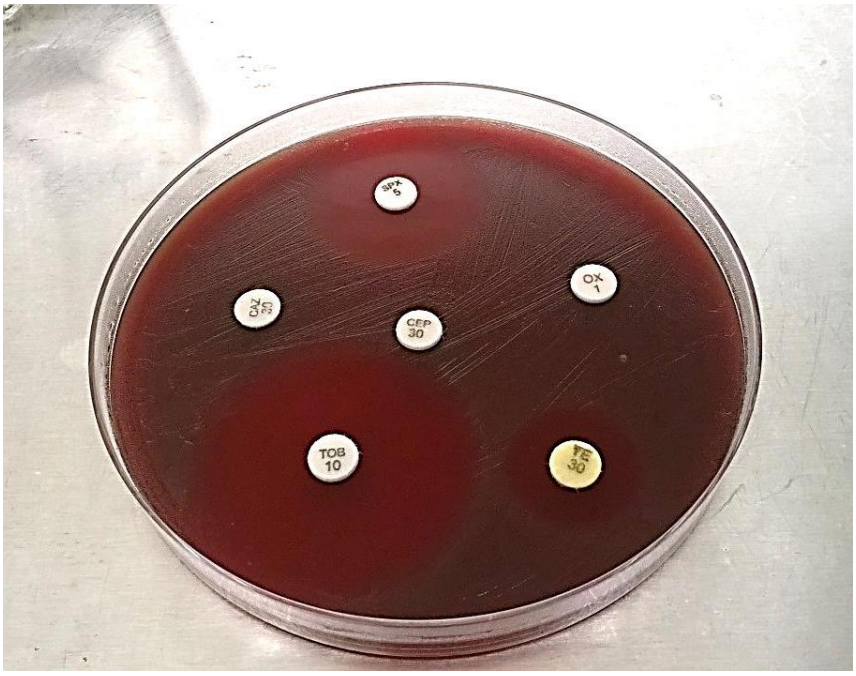
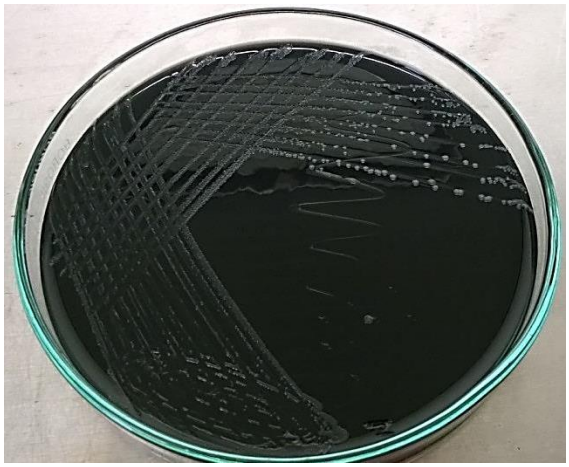


Fig. 27 Antibiogram of *Campylobacter* spp. isolates



Greyish, round, spreading type, shiny moistened colonies of *Campylobacter* spp.

Fig. 8 *Campylobacter* spp. colonies on mCCD agar supplemented with Polymyxin B selective supplement

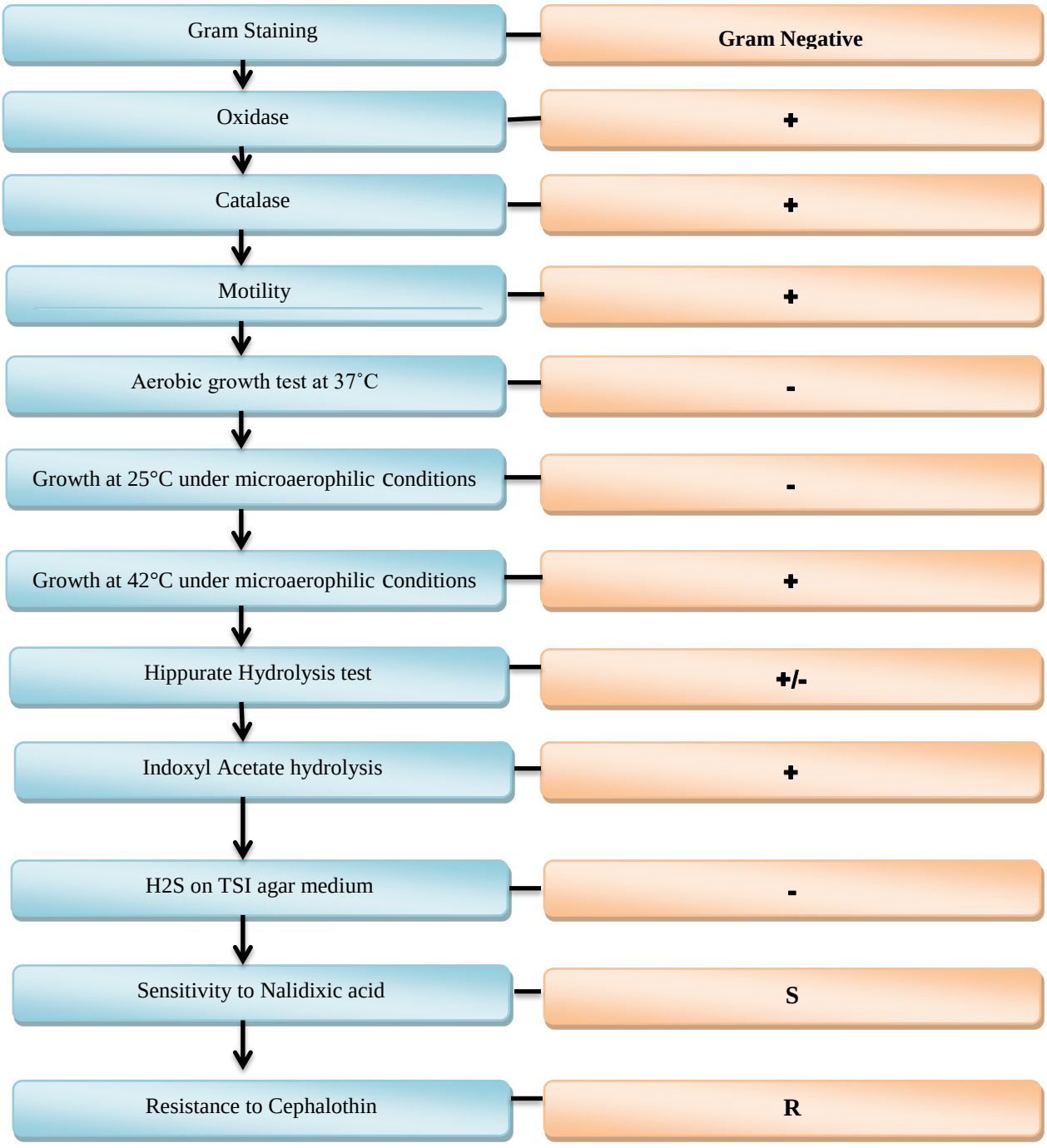


Fig. 9 Identification of *Campylobacter* spp.

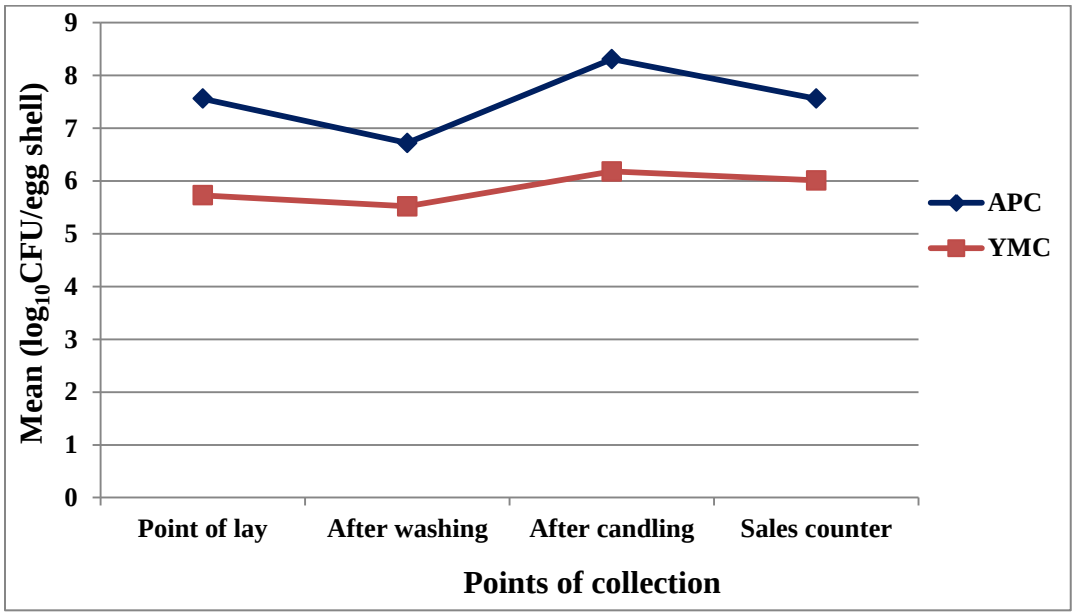


Fig. 14 Microbiological quality of eggs in table egg production chain-Season A

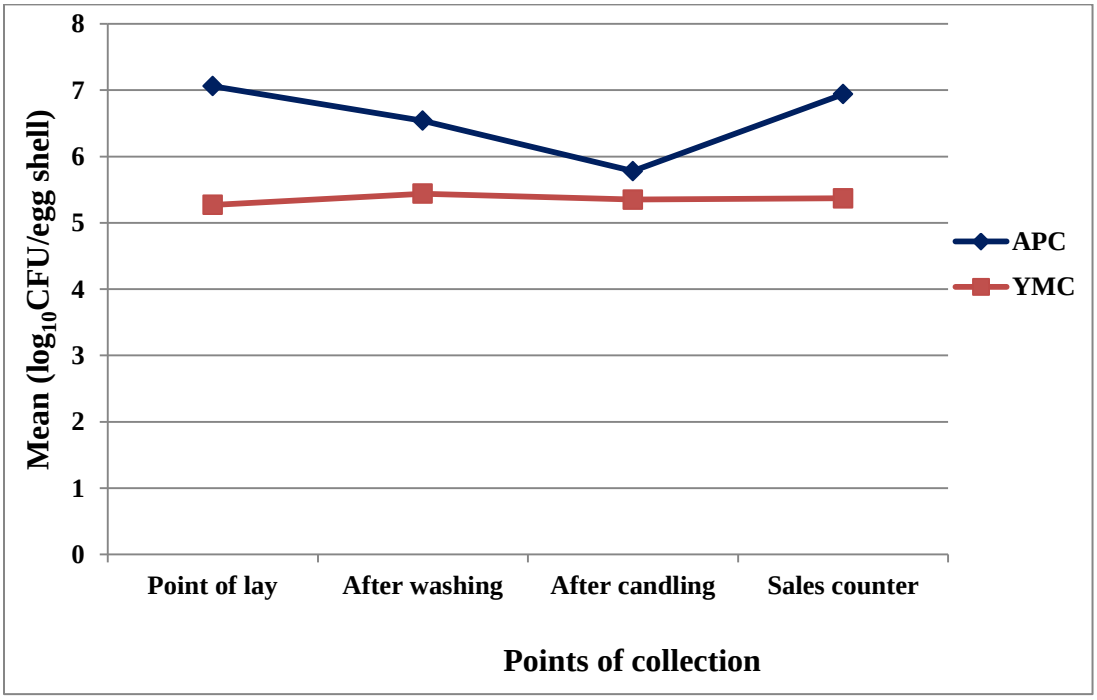


Fig. 15 Microbiological quality of eggs in table egg production chain-Season B

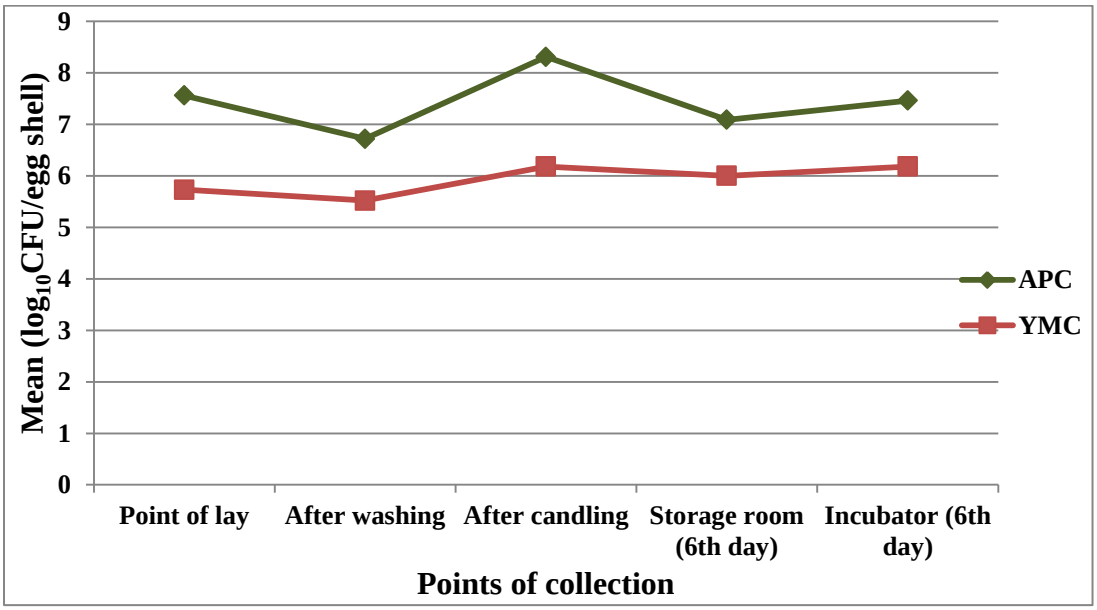


Fig. 16 Microbiological quality of eggs in hatching egg production chain- Season A

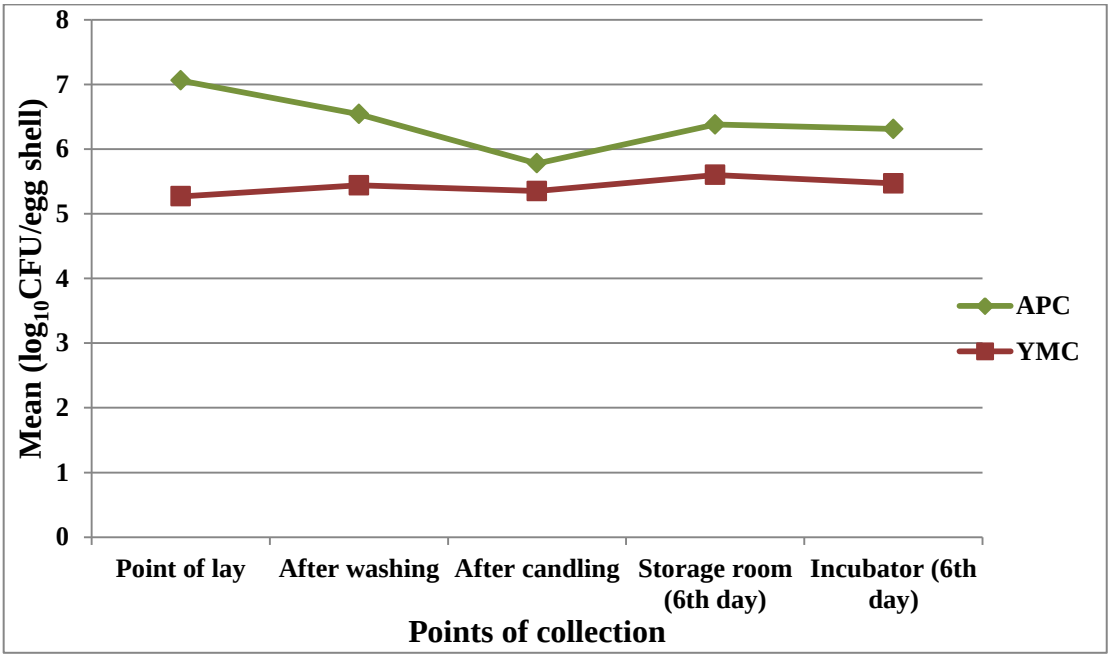


Fig. 17 Microbiological quality of eggs in hatching egg production chain- Season B