JJBS

Volume 18, Number 4,December 2025
ISSN 1995-6673
Pages 657 – 662
https://doi.org/10.54319/jjbs/180413

Jordan Journal of Biological Sciences

# Detection and Prevalence of Pathogenic *Arcobacter* Species (*A. butzleri* and *A. cryaerophilus/A. skirrowii*) in Chicken Samples Using Multiplex Real-Time PCR

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*Received: March 28, 2025; Revised: June 12, 2025; Accepted: June 27, 2025*

**Abstract**

*Arcobacter* species are Gram-negative, spiral-shaped bacteria that belong to the *Campylobacteraceae* family. Earlier, they were misclassified as 'aerotolerant *Campylobacters'*. Conventional cultural methods for their identification are time-consuming and technically demanding, urging the need for a rapid and practical approach to distinguish the most common pathogenic *Arcobacter* species, particularly *A. butzleri*, from other *Arcobacter* species and closely related pathogens, such as *C. coli* and *C. jejuni*. Differentiation among *Arcobacter* species was achieved using melting points, which showed distinct values of 63°C for *A. butzleri*, 65°C for *A. cryaerophilus* or *A. skirrowii*, and 67°C for *A. nitrofigilis* type strains, respectively. The specificity of this method was validated using pure cultures of 150 chicken samples, identified through phenotypic methods and a 16S rRNA adjacent hybridization RT-PCR assay. The assay detected *Arcobacter* in 22% of samples (14.66% *A. butzleri*, 7.33% *A. cryaerophilus / A. skirrowii*, with *A. butzleri* (14.6%) being the most frequently detected. The method demonstrated high sensitivity, detecting as few as 27 CFU of *A. butzleri*, with reaction efficiencies ranging between 90% and 110%. The described real-time PCR method offers a reliable solution for the rapid detection and differentiation of *Arcobacter* species in chicken samples.

**Keywords**: *Arcobacter, A. butzleri*, RT-PCR, melting temperature, sensitivity, specificity

## 1. Introduction

*Arcobacter* species are part of the *Campylobacteraceae* family and are Gram-negative and spiral in shape. At first, these campylobacters were called "*aerotolerant*" because they grew both aerobically and anaerobically. Later, it was discovered that they are able to live and reproduce aerobically within a temperature range of 15 to 30°C (Collado and Figueras, 2011; Figueras *et al.*, 2012). At present, there are 36 known species of *Arcobacter* in different types of environments (On *et al.*, 2021; Parte *et al.*, 2020). Of these, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* have been linked to diarrhea, nausea, stomach pain and occasionally infection of the blood in humans (Chieffi *et al.*, 2020; Shah *et al.*, 2011). Animal diseases such as abortions and problems of the digestive system have also been reported previously (Chieffi *et al.*, 2020; Ramees *et al.*, 2017). After an eight-year study, Vandenberg et al. (2004) revealed that around 3.5% of the *Campylobacter* isolates taken from human stool were in fact *A. butzleri*. Most types of *Arcobacter* are found in the environment and they are linked to diseases in people or animals. For example, *A. nitrofigilis* is usually found together with *Spartina alterniflora*, a salt marsh plant (McClung *et al.*, 1983).

Emerging foodborne zoonotic pathogens, including pathogenic *Arcobacter* species, are frequently found on food when it is harvested, processed, or prepared. They are found in meats such as beef, pork, poultry, rabbit, or lamb, and poultry is where they are most often detected (Aydin *et al.*, 2020; Kandeil, 2023; Ma *et al.*, 2022; Schönknecht *et al.*, 2020; Suelam, 2013). Studies on flocks show that birds can carry *Arcobacter* at rates ranging from 3% to 100%, which confirms their role as a major factor in spreading the disease (Acik, 2016; Piva *et al.*, 2013).

Although *Arcobacter* infections are self-limiting, developing fast and accurate tests for finding these pathogens is necessary to prevent them from spreading. It has been common to use microbiological, biochemical and immunological methods to identify pathogens in foods. However, these methods tend to give inconsistent outcomes because *Arcobacter* requires special care for growth; it is closely related to *Campylobacter*, and identification schemes have not been fully developed (Atabay *et al.*, 2006; Hausdorf *et al.*, 2013). For this reason, *Arcobacter* could be present in food products more often than what is currently measured.

Because of these challenges, techniques that look for specific regions of DNA for each species and genus have been made. Some of these are based on DNA hybridization (Wesley *et al.*, 1995) and PCR-based methods focusing on ribosomal genes, for example, 16S rRNA (Figueras *et al.*, 2012; Raut *et al.*, 2007) and 23S rRNA genes (Çelik and Otlu, 2020). Recently, researchers began using real-time PCR (RT-PCR) because it offers high sensitivity,

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accuracy, the ability to detect a particular species and quantitative features. For this reason, adjacent hybridization probes have been applied to test both clinical stool samples (Abdelbaqi *et al.,* 2007) and chicken meat (Abu-Halaweh, 2005). Several studies using TaqMan probe-based assays have been conducted to test chicken and environmental samples worldwide, (Carson *et al.,* 2024; Shrestha *et al.,* 2018).

Since conventional methods are not ideal for detecting *A. butzleri* fast and accurately, this study aimed to evaluate a multiplex RT-PCR assay for the rapid identification and differentiation of *A. butzleri* from related *Arcobacter* species in chicken obtained from retail markets in Jordan. This study utilizes the advantages of RT-PCR to enhance food safety and public health outcomes.

## 2. Material and Methods

### *2.1. Sample preparation and enrichment*

A convenience sampling approach was used to collect 150 raw chicken carcasses obtained from the local market in the Municipality of Amman, Jordan, that were either organically or conventionally raised. This sampling occurred between 2021 and 2022.

### *2.2. Isolation of Arcobacter from chicken samples.*

Liquid samples from each package were collected and refrigerated at 4°C for 30 minutes. To stimulate the growth of potential *Arcobacter* species, an enrichment culture was prepared by combining 5 mL of the liquid sample or 1 g of fecal material with 45 mL of *Arcobacter* broth (Oxoid, UK), supplemented with 5% lysed horse blood and antimicrobial agents (Sigma-Aldrich, Germany): 5´-fluorouracil (100 mg/L), amphotericin B (10 mg/L), novobiocin (32 mg/L), cefoperazone (16 mg/L), and trimethoprim (64 mg/L) (Oxoid, UK). The mixture was incubated for 48–72 hours at 30°C under microaerobic conditions with gentle agitation.

Following incubation, 50 µL of the broth was streaked onto modified charcoal-cefoperazone-deoxycholate agar (mCCDA, Oxoid). Colonies exhibiting characteristics consistent with *Arcobacter* spp. including small, colorless to beige or off-white, translucent morphology were isolated and purified through repeated streaking. Presumptive *Arcobacter* isolates were phenotypically confirmed via Gram staining, oxidase activity testing (Oxidase, UK), and catalase activity assays (Mottola *et al*., 2016). Confirmed *Arcobacter* spp. colonies were inoculated into fresh Arcobacter broth (Oxoid, UK) and incubated at 30°C for 48 hours.

### *2.3. DNA extraction*

Genomic DNA extraction was performed according to the heat lysis protocol as described by Abu-Halaweh and Al-Bsoul (2024). Whereas this is a cost-effective method, it may produce lower DNA purity compared to commercial kits, its performance was validated for RT-PCR sensitivity, as shown in this study. In Brief, 1 mL of pre-enriched bacterial culture was centrifuged, and the supernatant was discarded. The resulting pellet was then resuspended in 200 µL of DNase- and RNase-free distilled water using vortex mixing. After that the suspension was subjected to thermal lysis by incubation at 100°C for 15 minutes, followed by immediate chilling on ice. Cellular debris was removed by centrifugation at 14,000 × *g* for 5 minutes at 4°C. The supernatant was transferred to a fresh microcentrifuge tube, followed by reheating to 100°C for 10 minutes to ensure complete DNA release, and rapidly cooled on ice. A final centrifugation step (14,000 rpm, 5 minutes, 4°C) was applied to eliminate residual particulates. Finally, 2 µL of the purified supernatant served as the DNA template for RT-PCR amplification.

### *2.4. Real-time PCR assay*

#### *2.4.1. PCR Primer and Probe Design*

This study used primers and probe sets previously described by Abu-Halaweh (2005), which target the 16S rDNA gene. In this assay, universal 16S rRNA forward (F2) and reverse (R5) primers, in addition to two hybridization probes (a universal region probe and a hypervariable region probe), enable rapid detection and differentiation of *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus*, the most common *Arcobacter* pathogens from the other *Arcobacter* species. In this assay, a 20 µL PCR reaction mixture consisted of 10 µL of 2X PCR master mix (NEB, UK), 0.3 µM each primer, 0.1 µM fluorescently labeled probes (FAM and Cy5), 2 µL of chromosomal DNA template, and 6 µL of nuclease-free water. Amplification was performed using a Rotorgene thermocycler (Australia) with the following parameters: initial denaturation at 95°C for 3 minutes; 40 cycles of denaturation (95°C, 15 seconds), annealing (55°C, 25 seconds), and extension (72°C, 30 seconds), with fluorescence measured during the annealing step. Following amplification, a melting curve analysis was conducted by gradually increasing the temperature from 45°C to 95°C at a rate of 1°C per second to assess probe-specific melting profiles.

**Table 1** provides a list of the American Type Culture Collection (ATCC) reference strains utilized in the study for the identification of *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. nitrofigilis* using a RT-PCR assay.

| *Arcobacter* Reference isolates | Strain |
|---|---|
| *A. butzleri* | ATCC 49616 |
| *A. cryaerophilus* | ATCC 43158 |
| *A. skirrowii* | ATCC 51132 |
| *A. nitrofigilis* | ATCC 33309 |

ATCC: American Type Culture Collection Number

**Table 2** provides a list of the non-*Arcobacter* bacterial isolates included in the study to evaluate the specificity of the RT-PCR assay, along with their respective sources of isolation.

| Bacterial spp. | Source | Number of isolates |
|---|---|---|
| *Campylobacter jejuni* | Chicken pieces | 2 |
| *C. coli* | Chicken pieces | 2 |
| *C. fetus* subsp. *fetus* | Chicken pieces | 2 |
| *Escherichia* coli | Raw milk | 3 |
| *Salmonella* spp. | Chicken pieces | 3 |
| *Shigella* spp. | Chicken pieces | 3 |

#### *2.4.2. Standard curve and sensitivity analysis*

To establish the relationship between the cycle threshold (Ct) values and the logarithmic colony forming units (log CFU) in a sample, standard curves were

produced using *A. butzleri* the reference strain ATCC-49616. The strain was subculture in *Arcobacter*-specific media (mCCDA) and incubated at 30°C for 24 hours. A single colony was picked up, suspended in sterile water, and subjected to serial dilutions. The colony-forming units (CFU/mL) for each dilution were determined by culturing onto *Arcobacter* agar plates, which were then incubated under anaerobic conditions at 30°C for 48 hours. DNA was extracted from the dilution series using a rapid boiling method, and each DNA extract was evaluated in duplicate using the RT-PCR protocol described in this study. This method enabled the precise quantification of *A. butzleri* and the establishment of a consistent relationship between Ct values and bacterial concentration.

## 3. Results

### *3.1. Multiplex RT-PCR assay development*

Optimized RT-PCR conditions were achieved using 2.5 mM MgCl2, 0.3 µM of universal 16S rRNA primers, and 0.1 µM of adjacent probe (Butz probe, Ski probe, and Nitro probe). An annealing temperature of 55°C was identified as optimal for efficient hybridization of primers and probes. The FRET signal observed was obtained only for *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. nitrofigilis.* They were not observed in *Campylobacter spp.* or the other tested enteropathogens that could be present in chicken or human stool samples. These results confirm the discriminatory power of the hybridization probe assay. Furthermore, Melting-curve analysis of the amplicons revealed distinct Tm values for *A. butzleri* (63°C), *A. cryaerophilus* or *A. skirrowii* (65°C), and *A. nitrofigilis* (67°C), allowing for clear differentiation among these species (Fig. 1). This study was unable to differentiate between *A. skirrowii* and *A. cryaerophilus* due to lower levels of 16S rDNA variations between those two species.

### *3.2. Specificity and Detection of Naturally Contaminated Chicken Samples*

To evaluate the specificity of the RT-PCR assay under the optimized conditions, type strains of *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. nitrofigilis* were amplified (Table 1), along with fifteen other bacterial strains commonly associated with chicken contamination, including *E. coli*, *C. jejuni*, *C. coli*, *Shigella spp.*, and *Salmonella spp* (Table 2). This assay specifically detected the four *Arcobacter* strains without cross-reactivity with the other bacterial strains, including the closely related *Campylobacter* species. This confirms the high specificity of the assay for *Arcobacter* spp. detection.

The assay was subsequently applied to analyze 150 enriched chicken samples, successfully identifying 17 isolates of *A. butzleri* and 5 isolates of *A. skirrowii* or *A. cryaerophilus* (Table 3). These results highlight the assay's accuracy and efficiency in detecting target pathogens within complex sample matrices. The results from RT-PCR and traditional phenotyping methods are very similar, and the RT-PCR assay stands out by being able to distinguish different species when that is not possible with the other method. More evidence that the assay is specific and can be used to separate *Arcobacter* species from others. Just the ATCC reference strain of *A. nitrofigilis* was available for the study. The assay showed specific results when tested with the ATCC strain and the *A. nitrofigilis* specific probe. This research reveals that RT-PCR is a reliable and dependable test for detecting and identifying *Arcobacter* in a variety of samples.

**Table 3**. Detection limit of *Arcobacter* spp. in chicken meat using RT-PCR assay and conventional culture method

| Arcobacter Species | Phenotypic and Biochemical results (n=150) | The RT-PCR results (n=150) |
|---|---|---|
| Arcobacter Species | 33 | 33 |
| A butzleri | ND* | 22 (14.66%) |
| A cryaerophilus | ND* | 11 (7.33%) |
| A skirrowii | | |

ND* not determined: (unable to differentiate to species level by culture methods)

### *3.3. Assay sensitivity, linearity, and efficiency*

The RT-PCR assays were conducted using DNA extracted from *A. butzleri* reference ATCC strains, with colony counts serially diluted from $5.0 \times 10^6$ to $5.0 \times 10^1$ CFU. This essay demonstrates high detection efficiency and analytical sensitivity, with the primer and probe specifically targeting *A. butzleri*. A sensitivity as low as 27 CFU was achieved. The Rotorgene software generated the standard curves derived from these assays, exhibiting an average slope of −3.427, accompanied by correlation coefficients ($R^2$ values) of 0.98,

reflecting strong linearity. Furthermore, the amplification efficiency (E) was determined to be 96%, confirming the assay's high precision and reliability in quantifying *A. butzleri* (Fig. 2). These findings indicate the ability of the RT-PCR assay in detecting *A. butzleri* at low concentrations.

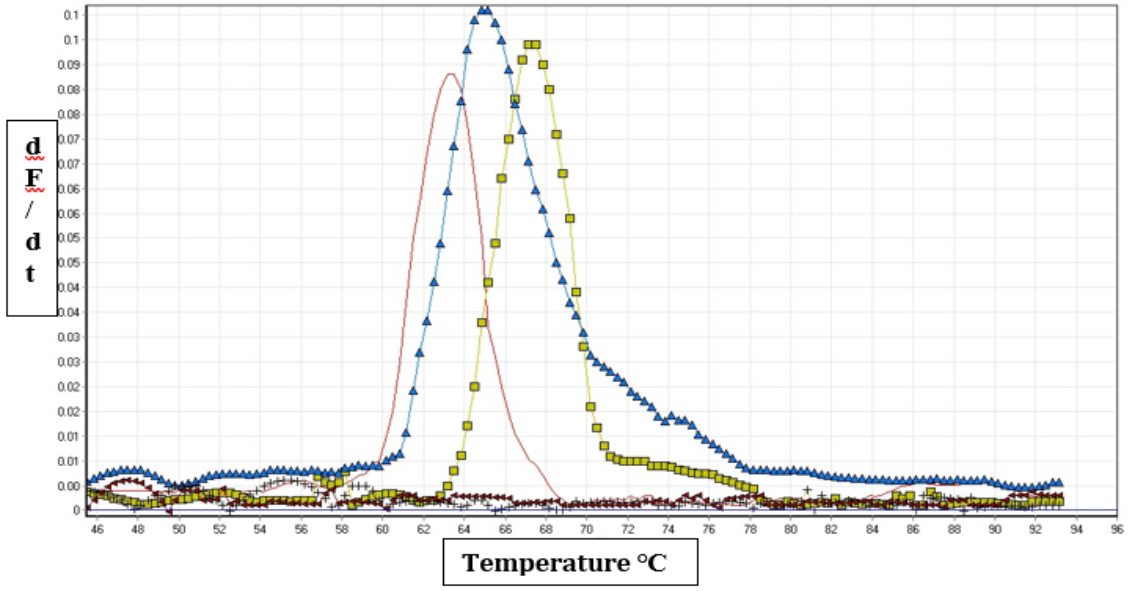


**Figure 1** presents a melt peak chart depicting the melting profiles of RT-PCR amplicons derived from the 16S rRNA gene for *A. butzleri* 63°C (−), A. *cryaerophilus* or A. *skirrowii* 65°C (▲), *A. nitrofigilis* 67°C (■), and *C. jejuni* (►) or -ve control (without DNA template) (+). Representative samples for each species are incorporated in the figure to improve clarity and facilitate interpretation.

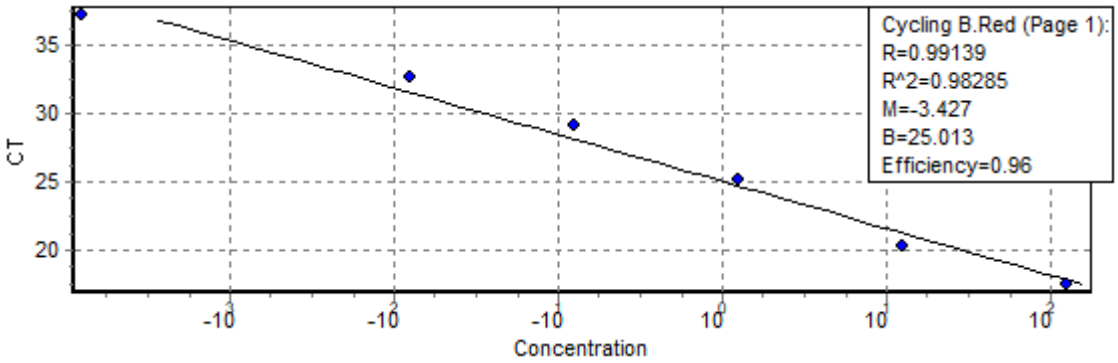


**Figure 2** illustrates the detection limit of RT-PCR targeting the 16S rRNA gene in *Arcobacter* spp. A standard curve was generated using (CT) values obtained from a 10-fold serial dilution series ranging from 5.0 × $10^6$ to 5.0 × $10^1$ CFU per reaction.

## 4. Discussion

Recent advances in research on the incidence and pathogenic potential of *A. butzleri* and *A. cryaerophilus* have encouraged the International Commission on Microbiological Specifications for Foods ((ICMSF), 2002) to recognize these species as a major human health concern. In spite of this recognition, the lack of standardized protocols for isolation and identification has left gaps in understanding the worldwide prevalence of *Arcobacter* spp. This study was developed to investigate the occurrence of pathogenic *Arcobacter* species, including *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus*, in chicken samples using a multiplex RT-PCR assay, as previously reported by Abu-Halaweh, (2005), combined with conventional culture methods. The findings, shown in Table 3, indicate that both RT-PCR and culture techniques were effective in detecting and identifying *Arcobacter* species. However, while the sensitivity of RT-PCR using DNA isolated from enrichment media was comparable to that of conventional culturing on mCCDA media, the RT-PCR assay offered distinct advantages. Specifically, it enabled species-level differentiation of *Arcobacter* isolates, a capability lacking in conventional culturing-based methods.

Besides, RT-PCR is much faster, with results often ready in 3 days, while other methods require 5–6 days. This is mentioned by Engberg et al. (2000) and Ramees et al. (2014). Pre-enrichment was very important for lowering the effect of inhibitors found in chicken and stool samples. Because antibacterial supplements were shown in the media, it helped to get rid of unwanted bacteria and favored the growth of *Arcobacter*.

The FRET signal was found only for *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis*. The analysis of the melting curves gave unique temperatures: 63°C for *A. butzleri*, 65°C for *A. cryaerophilus* and *A. skirrowii* and 67°C for *A. nitrofigilis*. The used RT-PCR essay was highly accurate and could easily separate important pathogenic *Arcobacter* species from non-pathogenic strains, related *Campylobacter* species and other poultry enteropathogens, according to a previous study by Abu-Halaweh (2005). Moreover, the assay showed excellent efficiency, since the $R^2$ value was approximately 98% while the efficiency was 96%, within the acceptable validation range of 90–110%. The results prove that RT-PCR is accurate and dependable for spotting and differentiating Arcobacter species. Although the assay

does not identify *A. cryaerophilus* from A. *skirrowii* (which was not the study's focus), additional research could use other genetic markers such as *rpoB* or *gyrB*, to make this identification.

In this study, *Arcobacter* was detected in 22% of chicken samples, a prevalence rate consistent with findings from other studies, such as 25.5% in Egypt and 18% in India (Hassan, 2017; Verma *et al.,* 2015). However, this rate is lower than those reported in Chile 39.4%, Lithuania 39%, Malaysia 41% and Northern Ireland 62% (Amare *et al.*, 2011; Fernandez *et al.,* 2015; Scullion *et al.,* 2006; Uljanovas *et al.,* 2023). These findings, combined with former studies, demonstrated that *Arcobacter* spp. is relatively prevalent in chicken samples and poses probable food safety threats. This emphasizes the need for region-specific risk assessments and tailored intervention strategies. Factors including slaughterhouse hygiene, refrigeration requirements, and the use of antimicrobials during poultry farming have a great influence on *Arcobacter* spp. contamination rates. The predominance of *A. butzleri* (14.66%) confirms its major role as the principal human pathogen within the genus and highlights the importance of poultry as a transmission vector. Integrating this RT-PCR assay into routine food safety monitoring could improve early detection, enable targeted interventions and reduce the risk of outbreaks.

Finally, the results demonstrate the ability of RT-PCR reported in this study as a rapid, sensitive, and specific assay for rapid detection and discrimination of *Arcobacter* species, providing significant advantages over traditional culture methods. The prevalence of *Arcobacter* spp. in chicken samples reported in this study recommended the need for high standard of food safety measures to eliminate the risk of foodborne transmission.

## 5. Conclusion

In brief, this study has shown that the developed RT-PCR assay works very well for detecting and counting A. *butzleri* and (A. *skirrowii / A. cryaerophilus*), the two most important *Arcobacter* species for human health. The assay showed that it was highly sensitive, with a lowest detectable amount of 27 CFU and was also straight forward, shown by its slope of −3.427, a good $R^2$ value of 0.98 and an accurate amplification percentage of 96%. The current study confirmed that RT-PCR can quickly detect and identify harmful *Arcobacter spp.*, so it is an important tool for food safety and monitoring public health.

## 6. Funding

The Deanship of Scientific Research and Graduate Studies at Philadelphia University, Jordan, funded this study entirely.

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