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# Efficient Identification and Characterization of Pathogenic Bacteria in Veterinary Diagnostics

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**Abstract**: Isolates of Shiga toxin-producing *Escherichia coli* (STEC), *Campylobacter* sp., *Clostridium perfringens*, *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Mycobacterium avium* were successfully identified using the QIAxcel® System for separation and analysis of either single or multiplex PCR products. Furthermore, the associated ScreenGel Software enabled automated analysis and identification of pathogens, thereby minimizing manual intervention and yielding reproducible and highly accurate results. Results demonstrate the utility of PCR product analysis using the QIAxcel as a method for the efficient and reliable identification of pathogenic bacteria significant in both veterinary and human medicine.

## Introduction

Rapid and accurate identification of pathogens in veterinary medicine is cornerstone for a successful battle against animal infectious diseases, which can also endanger human populations. The use of antibiotics has led to the development of an increasing number of antibiotic-resistant bacteria that may cause significant health problems in humans and animals. The latter are a significant source of resistant strains. Thus, the definition of an approach to rapidly and efficiently identify pathogens is of great clinical and commercial interest in order to take appropriate preventative measures to the benefit of animal and public health.

## Material and methods

Isolates of Shiga toxin-producing *Escherichia coli* (STEC), *Campylobacter* sp., *Clostridium perfringens*, *Clostridium difficile*, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium avium* were collected in the scope of routine and research work performed at the home research institute. DNA for PCR assays was extracted from cultures with a simple lysis: the colonies were suspended in PCR-grade water, boiled at 100°C for 15 minutes and centrifuged at 12,000 rpm for 2 min. The supernatant was used for PCR without further purification. Some of the pathogens, such as STEC and thermotolerant campylobacters, were detected also directly from enrichment media. In those cases, DNA was extracted using a commercial DNA extraction kit adapted to food samples.

For each pathogen, references related to individual PCR assays are cited in the respective sections. The subsequent electrophoretic analysis of PCR products was identical for all bacterial isolates. Using the QIAxcel capillary electrophoresis system, the amplicons were separated with the QX DNA Screening Kit employing the AM420 method with the following electrophoresis parameters: alignment marker injection at 4kV for 20 s, sample injection ▶

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at 5 kV for 10 s, and separation at 5 kV for 420 s. The alignment markers 15 bp–1kb or 15 bp–3 kb and the DNA size markers 50–800 bp or 100 bp–2.5 kb were run simultaneously.

The selected genes listed in Table 1 were targeted in pathogen-specific PCR assays and subsequently identified by the QIAxcel ScreenGel Software. The established PCR protocols followed by an optimized analysis procedure on the QIAxcel Advanced System yielded clear results, as seen in Figures 1–6.

**Table 1. Overview of genes targeted in specific PCR assays.**

| Bacterium | Gene | Amplicon size (bp) |
|---|---|---|
| ***Escherichia coli* (STEC)** | *stx1* | 180 |
| | *stx2* | 255 |
| | *eae* | 384 |
| | *stx2f* | 428 |
| | *hlyA* | 534 |
| ***Campylobacter coli*** | *glyA* | 126 |
| ***Campylobacter upsaliensis*** | *glyA* | 204 |
| ***Campylobacter lari*** | *glyA* | 251 |
| ***Campylobacter jejuni*** | *hipO* | 323 |
| ***Campylobacter fetus* subsp. *fetus*** | *sapB2* | 435 |
| ***Campylobacter* sp.** | 23S rRNA | 650 |
| ***Clostridium perfringens*** | *etx* | 655 |
| | *cpb2* | 567 |
| | *iA* | 446 |
| | *cpa* | 324 |
| | *cpe* | 233 |
| | *cpb* | 190 |
| ***Clostridium perfringens*** | *netB* | 384 |
| ***Clostridium difficile*** | *tcdA* | 369 |
| | *tpi* | 230 |
| | *tcdB* | 160 |
| | *tcdA* deleted | 110 |
| **Methicillin-resistant *Staphylococcus aureus* (MRSA)** | *nuc* | 255 |
| | *mecA* | 527 |
| | 16S rRNA | 886 |
| ***Mycobacterium avium* subsp. *avium*** | IS*901* | 1108 |
| ***Mycobacterium avium* subsp. *avium*/ *Mycobacterium avium* subsp. *hominissuis*** | IS*901*/ FR*300* | 1742/300 |
| ***Mycobacterium avium*** | IS*1245* | 427 |

## Results and discussion

### *Campylobacter* sp.

Thermotolerant campylobacters are the causative agents of intestinal campylobacteriosis, the leading bacterial zoonosis in Europe and worldwide (1). *Campylobacter jejuni* and *C. coli* are the two most important species among thermotolerant campylobacters and responsible for the vast majority of cases of this food-borne illness. Another two species, *C. lari* and *C. upsaliensis*, seem to be isolated only sporadically. *C. jejuni* and *C. coli* are commensals in the intestinal tract of a wide range of birds and mammals, including domestic animals used for food production (especially poultry), and are therefore widespread in the environment. Contaminated poultry meat is considered a major source of infection for humans. Consumption and handling of undercooked contaminated poultry meat was identified as an important risk factor (2). While the usual manifestation is an acute self-limiting gastroenteritis, severe infections might also occur.

*C. fetus* subsp. *fetus* is usually found in the intestinal tract of cattle and sheep, and may cause sporadic abortion in both these animals (3). The pathogen is also involved in human infections.

Members of the genus *Campylobacter* have fastidious growth requirements, making conventional detection and identification procedures problematic. As a consequence, rapid and reliable detection procedures are essential. A colony multiplex PCR has been developed and optimized to simultaneously identify the 23S rRNA from *Campylobacter* sp., the *hipO* gene (hippuricase) from *C. jejuni*, the *glyA* gene (serine hydroxymethyltransferase) from *C. coli*, *C. lari*, and *C. upsaliensis*, and the *sapB2* gene (surface layer protein) from *C. fetus* subsp. *fetus*. The multiplex PCR protocol is capable of detecting the type strains and clinical isolates from all 5 species with a high degree of specificity (4). In our hands, this PCR assay proved to be a useful tool for differentiating campylobacters isolated from animal clinical samples, food, and environmental samples (Figure 1). Accurate and simple to perform, the assay offers an effective alternative to traditional biochemical methods for the identification and differentiation of *C. jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. fetus* subsp. *fetus*.

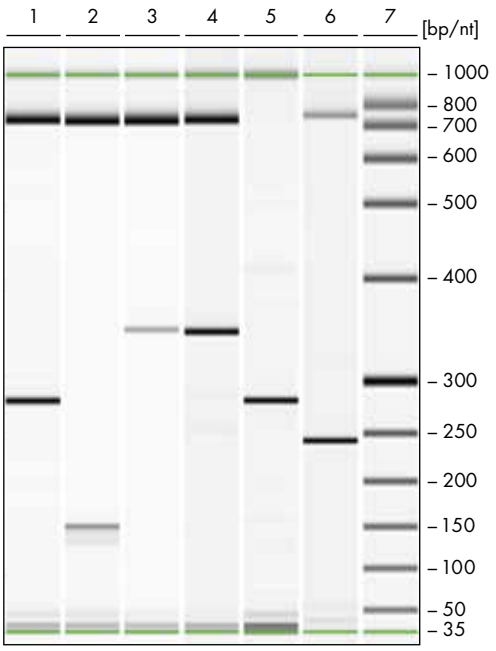


**Figure 1. PCR product analysis of thermotolerant campylobacters on QIAxcel. Lanes 1 and 5**: *C. lari*, **lane 2**: *C. coli*, **lanes 3 and 4**: *C. jejuni*, **lane 6**: *C. upsaliensis*, **lane 7**: DNA size marker 50–800 bp. Amplicon of the 23S rRNA gene amplified as an internal control is visible on all lanes with the exception of lane 5.

### *Clostridium difficile*

This anaerobic sporogenic Gram-positive bacterium is known as an important cause of hospital-acquired diarrhea in humans. Recent reports have indicated an increased frequency and severity of community-acquired *C. difficile*-associated disease. Three toxins, toxin A (TcdA), toxin B (TcdB) and binary toxin, are known to be primarily responsible for the pathogenicity of this organism. Apart from toxigenic strains, non-toxigenic strains lacking all toxin genes (*tcdA*, *tcdB*, and *cdtA/B*) are known. These strains do not induce any of the symptoms specific for *C. difficile*-associated diseases. Toxigenic strains of *C. difficile* have also been recognized as a cause of disease in different animal species. On the other hand, a high percentage of subclinical animal carriers are reported. The overlap of animal strains with those from some human cases and isolation of the organism from food and the environment suggests that animals may be a reservoir and *C. difficile* a zoonosis (5).

Since toxigenic culture is a method of choice for the detection of *C. difficile* (providing isolates suitable for epidemiological analysis), a PCR or real-time PCR-toxigenic culture scheme may be proposed as a diagnostic approach for animal *C. difficile* intestinal infections/subclinical colonization, offering combined species identification and toxin type characterization of isolate (6).

In our laboratory, a conventional multiplex PCR described by Lemee et al. (7) is used for the identification of isolates and toxin type characterization. Differentiation between the three main toxin types (A+B+, A–B+, and A–B–) of *C. difficile* can be performed with this multiplex PCR assay (Figure 2).

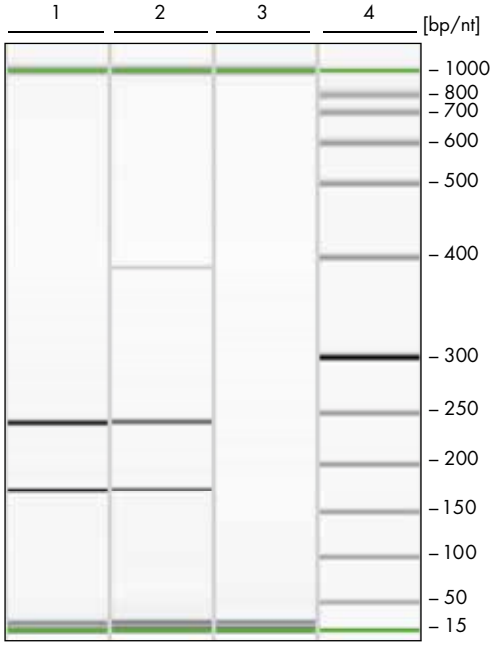


**Figure 2. PCR product analysis of *Clostridium difficile* strains on QIAxcel.** The genes identified are *tcdB* and *tpi* in **lane 1** and *tcdB*, *tpi* and *tcdA* in **lane 2**. **Lane 3** contains the negative control and **lane 4** displays the DNA size marker 50–800 bp.

### *Clostridium perfringens*

This Gram-positive, rod-shaped, anaerobic, spore-forming, non-motile bacterium is widely distributed in the environment and in the gut of animals and humans. *C. perfringens* is one of the most common causes of food poisoning. *C. perfringens* strains are classified into 5 types (A, B, C, D, and E) based on the production of 4 major toxins and on their fatal effect in mouse lethality tests. Type A is mediated by alpha-toxin encoded by the *cpa* gene and causes disease in both humans and animals. In humans, it is related to food poisoning and strains often produce enterotoxin encoded by the *cpe* gene. *C. perfringens* types B, C, D, and E are associated with animal diseases. Types B, C, and D are mediated by beta-toxin (encoded by *cpb*) and epsilon-toxin (encoded by *etx*). Beta-toxin in combination with epsilon-toxin gives rise to type B, but when expressed ▶

individually each gives rise to types C and D, respectively (8). The iota-toxin is encoded by the *iA* gene and associated with type E. In addition, a novel toxin, beta2-toxin, has been found to be encoded by *cpb2*, but its association with a disease in animals remains unclear (9, 10). In poultry, *C. perfringens* type A can cause necrotic enteritis by expressing the virulence factor *netB*, which encodes a toxin (11). Outbreaks are most common in broiler chickens aged 2–6 weeks and have devastating economic effects on producers because the disease causes high mortality and considerable loss of productivity. Therefore, it is of commercial interest to rapidly identify *C. perfringens* infection in the poultry industry (12).

The results of a PCR assay used to genotype *C. perfringens* isolates are presented in Figure 3. The assay identifies genes *cpa*, *cpb*, *etx*, *iA*, *cpb2* and *cpe* encoding the alpha-, beta-, epsilon-, iota-, and beta2-toxins, and enterotoxin (13, 14). A previously described PCR protocol (11) was used to detect the *netB* gene.

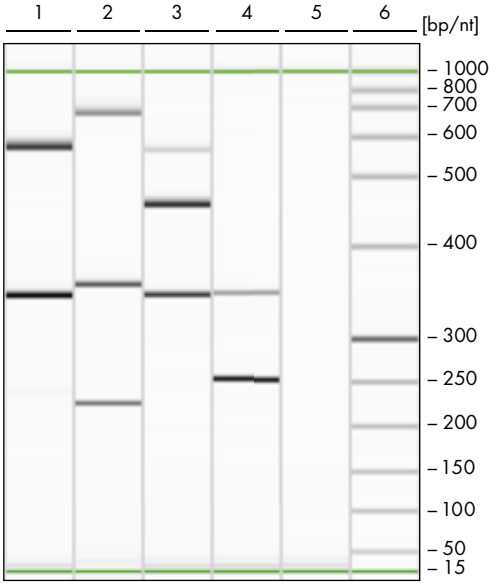


**Figure 3. PCR product analysis of *Clostridium perfringens* strains on QIAxcel.** The genes identified are *cpb2* and *cpa* in **lane 1**, *etx*, *cpa*, and *cpb* in **lane 2**, *cpb2*, *iA*, and *cpa* in **lane 3**, and *cpa* and *cpe* in **lane 4**. **Lane 5** contains the negative control and **lane 6** displays the DNA size marker 50–800 bp.

## Shiga toxin-producing *Escherichia coli* (STEC)

A group of pathogenic *E. coli*, STEC (also called verotoxin-producing) can cause bloody diarrhea and more severe diseases in humans, such as hemolytic-uremic syndrome (HUS). The infection occurs after consumption of contaminated food (including ground beef, leafy greens, and sprouts) and water. STEC possess unique virulence factors that facilitate effective colonization of the human gastrointestinal tract and subsequent release of Shiga toxin and are often associated with foodborne outbreaks. The major virulence genes of STEC are *stx1*, *stx2*, and *eae*, which encode toxins Stx1, Stx2 and intimin, respectively (15, 16). Toxin Stx1 is almost identical to the toxin produced by *Shigella dysenteriae*, though the second is immunologically distinct (17). There is an increasing demand for improved diagnostic procedures for the detection of STEC not only in fecal samples (the organism is present in the gastrointestinal tract of healthy ruminants) and food (e.g., meat and dairy products), but also in drinking water. The organism propagates in the animal gut and is shed in feces, which represents the major source of food contamination (18). A large number of serogroups are Shiga toxin producers; however, human infections are associated with a minor number of them (19).

In our laboratory, detection and genetic characterization of STEC from enrichment cultures is performed by multiplex PCR targeting *stx1*, *stx2*, *eae*, *hlyA* and the *stx2f* subtype of *stx2* gene (20, 21; Figure 4).

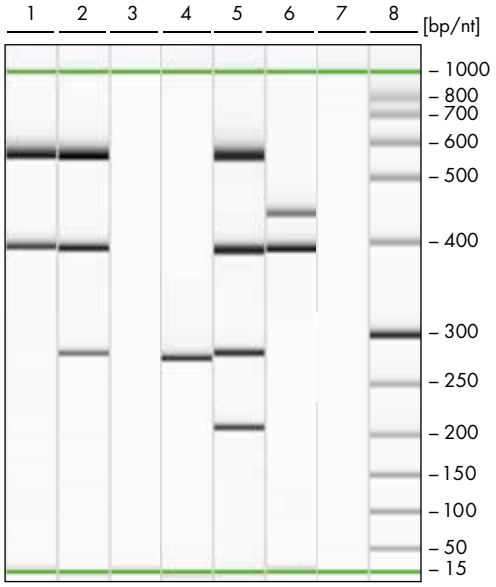


**Figure 4. PCR product analysis of STEC strains on QIAxcel.** The genes identified are *hly* and *eae* in **lane 1**, *hly*, *eae*, and *stx2* in **lane 2**, *stx2* in **lane 4**, *hly*, *eae*, *stx2*, and *stx1* in **lane 5**, and *stx2f* and *eae* in **lane 6**. The sample in **lane 3** was negative. **Lane 7** contains the negative control and **lane 8** displays the DNA size marker 50–800 bp.

***Mycobacterium avium***

This bacterial species comprises organisms that range from ubiquitous mycobacteria causing opportunistic infections in a variety of hosts to obligate pathogens of birds and ruminants. On the basis of biochemical and DNA analysis, pathogenicity, and host-range characteristics, this species is currently divided into three subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (22) while a fourth, *M. avium* subsp. *hominissuis* (23), has not been formally proposed yet (24; www.bacterio.net).

*M. avium* subsp. *avium* (MAA) is the causative agent of avian tuberculosis. The bacterium may infect many animal species but birds (especially poultry) are particularly susceptible to infection which often leads to fatal outcome. In farm animals, particularly pigs and cattle, MAA causes avian tuberculosis with tuberculous lesions mostly localized in the head and mesenteric lymph nodes (25). *M. avium* subsp. *hominissuis* (MAH) was proposed to distinguish organisms found in humans and pigs from those isolated from birds. Predominantly found in the environment, MAH isolates are weakly virulent for birds but are frequently encountered in tuberculous lesions in different animals (especially pigs, rarely in cattle, deer, and other animals) and in humans (23). From the veterinary laboratory and disease investigation standpoint, *M. avium* subsp. *paratuberculosis* (MAP) which causes chronic enteritis in ruminants (paratuberculosis) is often referred to separately from other subspecies of *M. avium*.

The genome of *M. avium* contains several insertion sequences, short DNA fragments which are capable of transposition. These insertion sequences can be species-specific, strain-specific, or specific for a certain group of strains and represent a suitable tool in epidemiological research as genetic markers (26). The insertion sequence IS*901* is present in all MAA isolates that are virulent for mice (27) and in all field MAA isolates that are fully virulent for birds (25), while IS*1245* is found in the majority of *M. avium* isolates (28, 29) except in MAP. IS*901* is used routinely in our laboratory to differentiate between MAA (IS*1245+*, IS*901+*) and MAH (IS*1245+*, IS*901-*), following the protocol of Kunze et al. (27). IS*901* and its flanking regions are targeted, generating PCR products of 1108 bp or 1742 bp in case of MAA isolates (depending on amplification of IS*901* itself or its flanking regions, respectively) and a PCR product of 300 bp in MAH isolates from amplifying flanking regions (Figure 5). However, a protocol for detection of IS*1245* (28) is usually applied to first identify isolates to the species level (Figure 6).

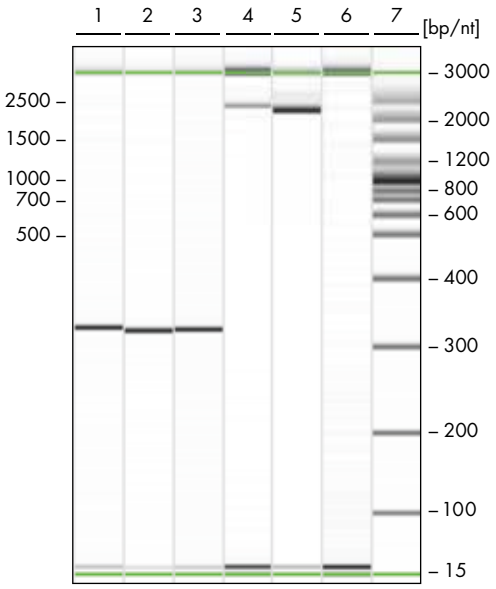


**Figure 5. PCR product analysis of *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* isolates on QIAxcel. Lanes 1–3**: *M. avium* subsp. *hominissuis* (IS*901-*), **lanes 4–5**: *M. avium* subsp. *avium* (IS*901+*), **lane 6**: negative control, **lane 7**: DNA size marker 100 bp–2.5 kb.

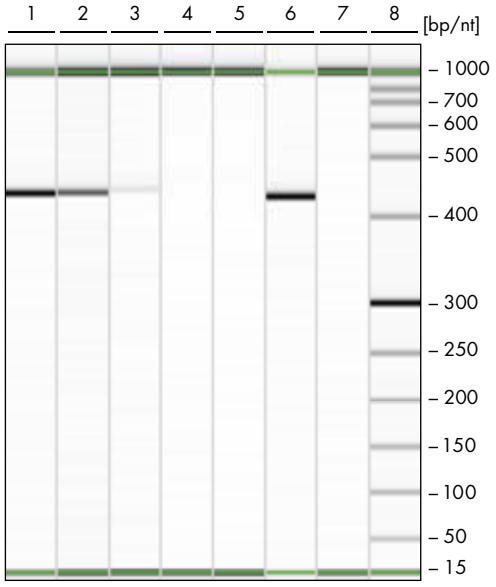


**Figure 6. PCR product analysis of *M. avium* isolates on QIAxcel. Lanes 1–3** show positive samples (IS*1245+*) while **lanes 4 and 5** show negative samples. **Lane 6** is a positive control, **lane 7** contains the negative control, and **lane 8** displays the DNA size marker 50–800 bp.

**Methicillin-resistant *Staphylococcus aureus* (MRSA)**

First reported in 1961, MRSA is the most common multidrug-resistant pathogen causing nosocomial infections in Europe (30). Hospitals and other healthcare facilities have been the traditional locations for MRSA infections for many decades. Since the 1990s, an increasing number of community-acquired MRSA infections have been documented in some countries. In recent years, studies have concentrated on livestock and the term livestock-associated MRSA is often used to describe these isolates (31). This pathogen has been isolated from both healthy and diseased animals including dogs, cats, horses, cows, pigs, and poultry.

Until recently, MRSA was generally considered a human problem, with most efforts devoted to controlling MRSA within the human population (32). However, reports of a porcine-associated MRSA strain causing infections in humans in close contact with pigs (33) have highlighted the risk that animals, particularly food-producing animals, might pose to public health. In addition, the recent discovery of a novel bovine-associated MRSA strain infecting cattle and humans (34) has heightened concerns about livestock acting as a potential reservoir of resistant pathogens. Rapid detection and accurate identification of MRSA are therefore crucial for the effective management of infections caused by this organism.

In our laboratory, a multiplex PCR assay using primer sets previously described (35, 36) is implemented to identify MRSA isolates. The assay targets *nuc*, which encodes the thermostable nuclease specific for *S. aureus*, *mecA*, which encodes the low-affinity penicillin-binding protein PBP 2A, and 16S rRNA genes. Detailed instructions to perform this assay are available in the protocol established by the DTU National Food Institute of the Technical University of Denmark (37). The results of the PCR product analysis on QIAxcel are shown in Figure 7.

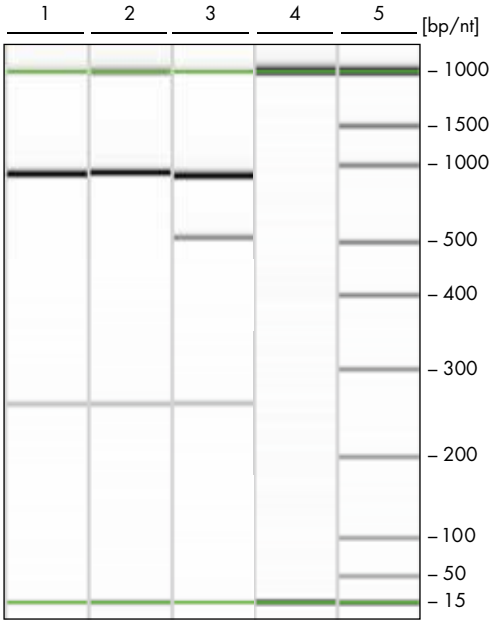


**Figure 7. QIAxcel analysis of *Staphylococcus aureus* isolates. Lanes 1and 2**: *S. aureus* (*nuc*+), **lane 3**: MRSA (nuc+, mecA+), **lane 4**: negative control, **lane 5**: DNA size marker 100 bp–2.5 kb. Amplicon of the 16S rRNA gene amplified as an internal control is visible in **lanes 1–3**.

## Conclusion

With a growing need for fast and accurate identification of pathogenic bacteria, the QIAxcel System for analysis of PCR products can make an important contribution to improving veterinary diagnostics.

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| QIAxcel Advanced Instrument | Capillary electrophoresis device: includes computer, QIAxcel ScreenGel software, and 1-year warranty on parts and labor | 9001941 |
| QIAxcel DNA High Resolution Kit (1200) | QIAxcel DNA High Resolution Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips | 929002 |
| QIAxcel DNA Screening Kit (2400) | QIAxcel DNA Screening Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips | 929004 |
| QX Alignment Marker 15 bp/3 kb (1.5 ml) | Alignment marker with 15 bp and 3 kb fragments | 929522 |
| QX Alignment Marker 15 bp/1 kb (1.5 ml) | Alignment marker with 15 bp and 1 kb fragments | 929521 |
| QX DNA Size Marker 50–800 bp (50 µl) | DNA size marker with fragments of 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, and 800 bp; concentration 100 ng/µl | 929561 |
| QX DNA Size Marker 100 bp–2.5 kb (50 µl) | DNA size marker with fragments of 100, 200, 300, 400, 500, 600, 700, 800, 1000, 1200, 1500, 2000, and 2500 bp; concentration 100 ng/µl | 959559 |

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