Salmonella detection in poultry samples*

Comparison of two commercial real-time PCR systems with culture methods for the detection of Salmonella spp. in environmental and fecal samples of poultry

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1 Dedicated to Prof. Dr. H. M. Hafez to his 65th birthday.

Key words
Poultry, Salmonella detection, culture, commercial real-time PCR

Summary
Study: The efficiency of two commercial PCR methods based on real-time technology, the foodproof® Salmonella detection system and the BAX® PCR Assay Salmonella system was compared to standardized culture methods (EN ISO 6579:2002 – Annex D) for the detection of Salmonella spp. in poultry samples. Material and methods: Four sample matrices (feed, dust, boot swabs, feces) obtained directly from poultry flocks, as well as artificially spiked samples of the same matrices, were used. All samples were tested for Salmonella spp. using culture methods first as the gold standard. In addition samples spiked with Salmonella Enteritidis were tested to evaluate the sensitivity of both PCR methods. Furthermore all methods were evaluated in an annual ring-trial of the National Salmonella Reference Laboratory of Germany. Results: Salmonella detection in the matrices feed, dust and boot swabs were comparable in both PCR systems whereas the results from feces differed markedly. The quality, especially the freshness, of the fecal samples had an influence on the sensitivity of the real-time PCR and the results of the culture methods. In fresh fecal samples an initial spiking level of 100 cfu/25 g Salmonella Enteritidis was detected. Two-days-dried fecal samples allowed the detection of 14 cfu/25 g. Both real-time PCR protocols appear to be suitable for the detection of Salmonella spp. in all four matrices. The foodproof® system detected eight samples more to be positive compared to the BAX® system, but had a potential false positive result in one case. In 7-days-dried samples none of the methods was able to detect Salmonella likely through lethal cell damage. Clinical relevance: In general the advantage of PCR analyses over the culture method is the reduction of working time from 4–5 days to only 2 days. However, especially for the analysis of fecal samples official validation should be conducted according to the requirement of EN ISO 6579:2002 – Annex D.

Zusammenfassung

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Tierärzt Prax 2012; 40 (K): 383–389
Received: March 13, 2012
Accepted after revision: June 16, 2012
**Introduction**

Salmonellae are an important cause for severe human gastroenteritis after consumption of contaminated poultry products especially eggs and meat. In 2010, 99,020 confirmed cases of human salmonellosis were reported in the EU (11). The purpose of the European regulation 2160:2003 is the protection of consumers by reduction of Salmonella spp. and other specified food-borne zoonotic agents by controlling primary productions (8). A major aim is the reduction of Salmonella prevalence in poultry flocks to less than 1%. Additional measures are covered in several additional European regulations like the 1168:2006 (10). In 2010, 25 EU member states including Germany reported prevalence of Salmonella that was lower than or equal to the EU reduction target limit of 1% (11). In Germany, the regulation “For protection against specific salmonellae infections in barn fowl” became operative in 2009. Detection of salmonellae of category 1, S. Enteritidis and Typhimurium, draws legal consequences. In contrast detection of salmonellae of category 2, S. Hadar, Virchow and Infantis, is only noted statistically (3). According to German legislation three samples of every flock and one government taken sample of one flock per farm per year are required. Only eggs of Salmonella-free laying hen flocks are permitted for sale on the fresh egg market. Depending on the housing system boot swabs, dust and feces are examined (3), therefore those sample matrices were included within this study and additionally feed that may serve as a possible vector of Salmonella spp. (15, 26).

Since 1993 the culture-based isolation of Salmonella as described in the ISO 6579:2002 has been accepted as the gold standard (18). In 2007, an amendment covering the detection of Salmonella in animal feces and in samples from the primary production level was published (18). This method is very time-consuming because it consists of four stages, a) non-selective pre-enrichment, b) selective enrichment, c) plating out and identification of suspicious colonies, and d) confirmation by biochemical or serological tests. In a positive case the isolated Salmonella species is serotyped by the German Federal Institute of Risk Assessment (BfR) in accordance to the EU regulation EU 2160/2003 and EU regulation 1168/2006 as well as to the German regulation from 2009 to prevent consequences for the consumer (8, 10). However, rapid detection of Salmonella in poultry samples is critical for ensuring consumer safety. As large numbers of samples have to be investigated, an optimal method would use as little labor and time as possible. Additionally, laboratory detection of Salmonella needs to be very sensitive as well as comparable between laboratories.

Polymerase chain reaction technology has been internationally standardized and guidelines as well as standards for the validation of PCR based methods have been established (23, 25). To date, real-time PCR systems are regularly used for Salmonella detection. Real-time PCR is divided in two main categories, a) those employing intercalating double-stranded (ds)-DNA dyes and b) those using fluorescence resonance energy transfer (FRET) sequence-specific hybridization probes. By now several PCR methods for detection of Salmonella in groceries exist (2, 4). Recent studies of spiked and naturally contaminated foods have reported a good agreement between culture-based and commercial PCR methods for Salmonella detection (23). However, commercial PCR systems are currently validated only for food stuff and environmental dry swab samples (1, 24). Until now no validated methods for poultry samples like dust, boot swabs and feces have been established.

The following investigation compared two real-time PCR systems (one SYBR® Green based, one probe-based) for their efficiency and practical use for detection of Salmonella spp. in samples originating from poultry primary production.

**Materials and methods**

The foodproof® Salmonella detection kit (Merck KGaA, Darmstadt, Germany) and the BAX® PCR Assay Salmonella system (DuPont Qualicon, Wilmington, DE, USA) were evaluated by analysis of positive and negative field samples from layer farms in Germany. In order to determine sample matrix dependent effects and to get the whole spectrum of samples from layers, four different sample matrices were used: feed, dust, boot swabs and feces. A total of 30 positive field samples and 30 negative field samples (in each case 5 feed, 10 boot swabs, 10 dust, 5 feces) and altogether 90 spiked samples (45 of 14 cfu and 45 of 100 cfu, same matrices) were used (sample weight throughout 25 g). All field samples were tested first for Salmonella spp. using culture methods (MSRV medium, Merck KGaA, Darmstadt, Germany; Gassner medium, Oxoid Deutschland GmbH, Wesel, Germany; Columbia blood agar, bioMérieux Deutschland GmbH, Nürtineng, Germany) as a gold standard (EN ISO 6579:2002 – Annex D) (18). Salmonella suspicious colonies were agglutinated for confirmation with omnivalent anti-Salmonella serum (Enteroclon Anti-Salmonella A 67, omnivalent (SIFIN Institute, Berlin, Germany). Furthermore all methods were tested during the annual ring-trial of the National Salmonella Reference Laboratory of Germany. The study was performed in the Laboratory of the Clinic for Birds, Reptiles, Amphibians and Fish, which is accredited according to DIN EN ISO/IEC 17025:2005 and EG No. 882/2004 (registry number SAL-HE-G085–04–08) (9, 16).

**Artificial spiking**

Additionally each sample type collected from a controlled Salmonella negative flock was artificially spiked (14 or 100 cfu) with Salmonella Enteritidis (SE) Lenticule® discs (Salmonella Enteritidis NCTC 6676, HPACC, Porton Down, Salisbury, SP4 0JG UK) in two different doses (in each case 10 feed, 5 boot swabs, 5 dust and 5 feces samples). Spiking was performed according to the protocols of the Salmonella ring-trial of the National Salmonella Reference Laboratory of Germany (21). Lenticule discs containing the specific amount of cfu were dissolved in 225 ml BPW (sterile buffered peptone water, Merck KGaA, Darmstadt, Germany) and then incubated for 4 h at 37 ± 1 °C. Samples were added to the BPW after this incubation by stirring.
To evaluate the influence of the freshness of fecal samples to PCR and culture results, further 40 fecal samples were spiked on day of collection. Afterwards they were tested as follows: 20 samples were pre-enriched on the same day (feces fresh moist), 10 samples were incubated in a laminar flow for 2 days and 10 samples for 7 days at room temperature to dry prior pre-enrichment (20–22 °C, 30–35% humidity). Half of the samples of each group were spiked with 14 cfu/25 g and the other half with 100 cfu/25 g. Artificial spiking was performed with a Salmonella suspension that had been subjected to a colony count determination. The suspension was mixed with 25 g feces at the same time for all samples.

### Real-time PCR systems

The BAX® system assay for Screening Salmonella (DuPont Qualicon, Wilmington DE, USA) is based on principles of real-time PCR technology and analyses intercalating dye melt curve data (SYBR Green®) after all amplification has been completed. This system represents a closed system where the raw data is not accessible for the user in routine diagnostic. The software of the BAX® system is analyzing this data and determines the result as negative or positive. The protocol of the BAX® system was carried out according to the manufacturer instructions. A positive control is included in the system. Briefly, the method involved pre-enrichment in BPW for 18 ± 1 h at 37 ± 0.1 °C, a transfer of a 10 μl aliquot BPW to 500 μl BHI (Brain heart infusion broth, Merck KGaA, Darmstadt, Germany) and incubation of this broth for 3 h at 37 °C. 5 μl of the enriched sample was transferred into 200 μl lysis buffer. The samples were incubated at 37 °C for 20 min, followed by 95 °C for 10 min. These DNA preparations were stored at 4 °C until the next day. PCR was performed with the Q7-BAX® cyclertdetector (DuPont Qualicon, Wilmington DE, USA) using a pre-programmed cycling protocol (BAX® system software, instrument verification plates, DuPont Qualicon, Wilmington DE, USA).

The foodproof® Salmonella Detection Kit (Merck KGaA, Darmstadt, Germany) is appropriate for the use as hybridization probe-based system on the Mx3005P®QPCR system (5’Nuclease). DNA isolation from the samples was carried out according to the manufacturer instructions. In brief, DNA extraction (foodproof® StarPrep I Kit, Merck KGaA, Darmstadt, Germany) was performed on 1 : 10 dilution of 1 ml incubated BPW with 9 ml BHI for 5–10 min at 20–22 °C. A 500 μl aliquot of this dilution was transferred in a 1.5 ml reaction tube. After centrifugation (12,000 × g for 5 min) the supernatant was discarded. A 200 μl aliquot lysis buffer was added to the pellet which was then re-suspended. Samples were now incubated for 10 min at 100 °C in a heating block. After a short cooldown period of 60 sec, samples were vortexed and centrifuged for 2 min at 13,000 × g. The supernatant containing extracted DNA was stored at −20 °C for later PCR. A Salmonella suspension made from a SE Lenticule® disc incubated in 100 ml BPW (18 ± 1 h at 37 ± 0.1 °C) acted as a positive preparation control. Plasmid fragments as an additional internal positive control are included in the kit. Pure DNA-free water, provided in the kit, acted as negative control. PCR was performed with the Mx3005P®QPCR system (Agilent Technologies, Böblingen, Germany).

### Ring-trial of the National Salmonella Reference Laboratory of Germany

The National Salmonella Reference Laboratory of the German Federal Institute of Risk Assessment (BfR) undertakes an annual interlaboratory test for the detection of Salmonella in Salmonella-free feces of laying hens. Nineteen samples (blinded, 10 g) are spiked in the participating laboratory using Lenticule® discs which contain an unknown Salmonella quantity. Lenticule® discs are solved in 100 ml BPW and incubated for 4 h at 37 ± 1 °C. Feces are added to enrichment broth after this incubation. Supplied feces are very dry. Those samples were investigated with culture and both PCR systems.

### Results

#### Detection of Salmonella spp. in feed, boot swabs and dust samples

Salmonellae were detected with both real-time PCR systems in each of the 25 positive field samples (5 feed samples, 10 boot swabs, 10 dust samples). Representative amplification plots using the foodproof® protocol showed expected fluorescence intensities and exponential amplification plots. Each of the 25 negative field samples was confirmed as such with both PCR protocols (5 feed samples, 10 boot swabs, 10 dust samples). All samples spiked with 14 cfu and 100 cfu (in each case 10 feed samples, 5 boot swabs, 5 dust samples) were detected as Salmonella positive in the culture as well as with each PCR protocol. The use of these sample matrices demonstrated no detectable differences (Table 1). All controls displayed the expected results.

#### Detection of Salmonella spp. in feces

All 5 negative field feces samples were found to be negative with both PCR protocols. Out of 5 positive field fecal samples detected by culture the foodproof® system detected 4 and the BAX® system only 3. Out of 5 fecal samples spiked with 14 cfu the foodproof® system detected 3 and the BAX® system none. Each of 5 samples spiked with 100 cfu was detected with both the foodproof® system and the BAX® system. Salmonella isolation was possible from all of the spiked fecal samples (Table 1).

During comparison of Salmonella detection in spiked a) fresh moist, b) 2-days-dried and c) 7-days-dried fecal samples the results differed. Of the 10 fresh moist fecal samples spiked with 14 cfu, the...
same 5 out of 10 fresh moist samples were detected as positive by all methods ([Table 2]) whereas the other 5 samples were detected negative by every method. Investigating the fresh fecal samples spiked with 100 cfu, the foodproof® system and culture detected all as positive whereas the BAX® system detected only 6. In the group of 2-days-dried feces all 10 samples (5 × 14 cfu, 5 × 100 cfu) were tested positive by both PCR systems and culture ([Table 2]). With the 10 samples (5 at 14 cfu, 5 at 100 cfu) of 7-days-dried feces Salmonella was not detected by any method ([Table 2]). All controls displayed the expected results.

### Annual interlaboratory test

In 2010 the results from cultivation and from the BAX® system were identical and matched completely with the results provided by the National Salmonella Reference Laboratory.

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**Table 1**

<table>
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<tr>
<th>Sample matrix</th>
<th>Bacterial culture</th>
<th>foodproof®</th>
<th>BAX®</th>
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<td>n positive negative</td>
<td>positive negative</td>
<td>positive negative</td>
</tr>
<tr>
<td>field negative</td>
<td>5 0</td>
<td>5 0</td>
<td>5 5</td>
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<tr>
<td>field positive</td>
<td>5 0</td>
<td>5 0</td>
<td>5 0</td>
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<tr>
<td>14 cfu¹</td>
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<tr>
<td>100 cfu¹</td>
<td>10 10</td>
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**Table 2**

<table>
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<td>Feed</td>
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<td>positive negative</td>
<td>positive negative</td>
</tr>
<tr>
<td>field negative</td>
<td>5 0</td>
<td>5 0</td>
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</tr>
<tr>
<td>field positive</td>
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<tr>
<td>14 cfu¹</td>
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<tr>
<td>100 cfu¹</td>
<td>5 5</td>
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<td>5 0</td>
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</tbody>
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¹Spiked with Lenticule® discs in BPW (sterile buffered peptone water)

Red: Differing results of *Salmonella* detection in chicken feces

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**Table 3**

<table>
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<tr>
<th>Sample matrix</th>
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<th>BAX®</th>
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<tbody>
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<td>positive negative</td>
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<tr>
<td>field negative</td>
<td>5 0</td>
<td>5 0</td>
<td>5 5</td>
</tr>
<tr>
<td>field positive</td>
<td>5 0</td>
<td>5 0</td>
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</tr>
<tr>
<td>14 cfu¹</td>
<td>5 5</td>
<td>5 0</td>
<td>5 0</td>
</tr>
<tr>
<td>100 cfu¹</td>
<td>5 5</td>
<td>5 0</td>
<td>5 0</td>
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</tbody>
</table>

¹Spiked with *Salmonella* suspension after determining colony count, feces directly spiked.

²Each time same samples were detected as false negative with all detection methods.
Table 3: Results of the annual ring-trial of the National Salmonella Reference Laboratory of Germany 2010 samples using bacterial culture (EN ISO 6579:2002 – Annex D) and two real-time PCR systems foodproof®, Salmonella detection system and BAX® PCR Assay Salmonella system.

<table>
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<th>Technique</th>
<th>Sample number</th>
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<th>5</th>
<th>6</th>
<th>7</th>
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<tr>
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<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>foodproof®</td>
<td></td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>BAX®</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>CFU per sample</td>
<td>B</td>
<td>B</td>
<td>C</td>
<td>A</td>
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<td>B</td>
<td>A</td>
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<td>A</td>
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</table>

1 Results from cultivation and the BAX® system were identical and matched completely with the results provided by the German Salmonella Reference Laboratory. The foodproof® system detected one additional sample as positive (red). CFU per sample: A = blank; B = 15; C = 678; 0 = only feces.

Discussion

In the present study, two real-time PCR systems were compared with standardized cultivation methods (EN ISO 6579:2002 – Annex D) to investigate their efficiency and practical use for the detection of Salmonella spp. in poultry samples (18). Molecular techniques, such as real-time PCR, are capable of detecting Salmonella in a wide variety of matrices. The use of real-time PCR greatly reduces the time and the manpower required when compared with conventional culture methods. Cultivation normally takes 3–5 or more days, whereas the real-time PCR assays compared in this study required only about 25 hours (including overnight pre-enrichment). The BAX® PCR assay Salmonella and the foodproof® Salmonella detection kit are validated examination methods for detection of Salmonella spp. in foodstuff and environmental samples (1, 24). Both methods have been evaluated previously with food products but so far not in poultry samples (2, 4).

The results of this study demonstrate that the Salmonella detection rate by PCR depends on the sample matrix. Experiments with feed, boot swabs and dust, both native and spiked samples, led to identical results with both real-time PCR systems and no different detection limits were observed. This coincides with other studies where the detection of Salmonella in food or feed materials led to very similar results for conventional culture methods and PCR (4, 7, 19). This is probably due to a lesser and different microbial background flora compared to fecal materials. It seems the same applies for testing boot swabs, recent studies showed that by using boot swabs culture methods and the used 20 hour real-time PCR to similar results (20).

In contrast fecal samples were identified in this study to be a more problematic sample matrix. The quality of chicken feces, particularly freshness and moisture level of feces, led to detection problems and to false negative results. The quality of feces has not been considered in previous studies. Although 14 cfu/25 g samples were detected in 2-day-dried fecal samples, the same spiking level led to partial positive samples using fresh feces and total detection failure in 7-days-dried feces by real-time PCR methods and culture. However, the reason for the failure of five fresh fecal samples to be detected by any method remains unclear. The cause for detection failure in fresh moist fecal samples by PCR systems might be due to inhibition by physiological background flora (5). Furthermore it is known that feces contain large amounts of enzymes like DNases and proteases, and polysaccharides that may be inhibitory for PCR. Pre-enrichment serves to dilute these PCR inhibitors (22). By now PCR methods still require an enrichment culture step for the multiplication of cells to a level of approximately 10^2 to 10^4 cells per ml of enriched broth (19). The reason for the total detection failure in 7-days-dried feces might be a lethal damage of the bacteria cells. It is known that viability of salmonellae depends highly on water activity levels (12).

Salmonella detection problems in chicken feces have been described in previous studies. For example chicken cecal contents as a matrix resulted in a reduced sensitivity by using real-time PCR (5). Background flora like Enterobacter cloacae may act as competitor (6). Cultural methods were compared previously with the BAX® system and another PCR for the detection of Salmonella in natural and artificially contaminated fecal samples, with the best results being given by culture and both PCR assays showing false negative results (13). In contrast, several other studies found PCR methods to be a fast and secure diagnostic tool for detecting Salmonella in fecal samples compared with current diagnostic technologies (14, 21). But to date each sample detected positive re-
quires culture and isolation of the bacteria to be serotyped according to German legislation.

The method of spiking the samples may have influenced the results. In the majority of the studies spiking was performed with *Salmonella* suspensions of a certain level, but the exact procedure of spiking has rarely been described (5, 13). The current study demonstrates that imitating field conditions by direct spiking of the feces and spiking of the enrichment broth can lead to different results. The spiking method used in the annual ring-trial of the National *Salmonella* Reference Laboratory of Germany is admittedly not comparable with field conditions and does not reflect the natural conditions of *Salmonella*-containing feces. Therefore the significance of the annual ring trial needs to be discussed.

Both systems proved to be reliable for detection of *Salmonella* in feed and environmental samples. During the ring-trial the foodproof® system detected one more sample as most likely false positive. It remains unclear if this is related to the method used or a potential laboratory contamination during spiking or DNA-extraction which seems unlikely. The interpretation of foodproof® results is more flexible for the user as the raw data from each test is accessible, which is routinely not the case in the BAX® system. The use of the foodproof® *Salmonella* Detection Kit does not require any additional hardware like the BAX® system and can be used in most standard real time PCR thermocyclers. The foodproof® system uses fluorescence dyed probes (5’Nuclease or Hybridization Probes) which are conform to the ISO/FDIS 22119 standard (17). It is questionable if SYBR® Green based PCR protocols are conform to this standard in the future. However, SYBR® Green based protocols can still be used for screening purposes.

Acknowledgements

The authors thank Antoinette Huhn and Ralf Dörn for their technical assistance. They are also grateful to the National *Salmonella* Reference Laboratory of Germany for the data of the *Salmonella* ring-trail.

**Conclusion for practice**

Although culture-based methods detect viable bacterial cells and offer an epidemiological advantage over molecular techniques, they are time-consuming, particularly when large numbers of samples are involved. However, bacterial culture is still considered the gold standard in reliability for the detection of *salmonella*, at least in feces. PCR systems can only reduce the need for bacterial isolations but cannot completely replace them. A certain percentage of bacterial cultures would still have to be processed for obtaining *Salmonella* cultures for typing purposes. As time efficiency in poultry primary production is an important issue, there is a trend for commercial laboratories to move towards molecular based methods. Nevertheless according to German legislation it is necessary to isolate the bacteria from each sample detected positive via culture methods and serotype it to exclude *salmonellae* of categories 1 or 2 (8, 10).

**Conflict of interest**

The authors declare that they do not have any conflict of interest. The co-authors Holger Schönbrücher und Jörg Slaghuis are employees of the company Merck KGAA.

**References**


Rezensionen

Tiermedizinische Fachangestellte in Schule und Beruf


Zusammenfassend ist das Buch ein wichtiger und vor allem verbindlicher Leitfaden für den Berufschulunterricht und eine gute Orientierung für Auszubildende und Ausgelernte, für den Unterricht sollten jedoch weitere Quellen hinzugezogen werden.

Beatrix Klein, Gießen


Arbeitsbuch für die Tiermedizinische Fachangestellte

Band 1–3, Lernfeld 1–12


Axel Wehrend, Gießen