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Detection of *Salmonella* spp. in small ruminants using immunoassay and PCR

Nachweis von Salmonella spp. bei kleinen Wiederkäuern mittels Immunoassay und PCR

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Summary

Salmonellosis is still one of the leading foodborne zoonotic diseases. Culture-based methods for detection of *Salmonella* spp. are laborious and can take up to seven days to obtain final results. Accurate and rapid methods for *Salmonella* screening in livestock are important to improve food safety. In this study, the occurrence of *Salmonella* spp. was studied in 80 pooled samples and fecal samples collected from healthy small ruminants at slaughter using the VIDAS® SLM immunoassay and SYBRGreen® based real-time PCR. Positive samples were subcultivated onto xylose-lysine-desoxycholate (XLD) and hectoen-enteric (HE) agar plates. The detection rate of *Salmonella* spp. was significantly higher by VIDAS® than by PCR. This pathogen was detected in 85 % and 48 % of the tonsil samples by VIDAS® and PCR, respectively. In total, 70 % of the fecal samples were positive by VIDAS® but only 5 % by PCR. *Salmonella* spp. was isolated only from samples, which were highly positive (test values >3.00) by VIDAS®. High positive VIDAS® test values (> 3.00) were only obtained in tonsil samples. Most (26/28) of the VIDAS®-positive fecal samples had very low test values (0.23–1.00). These fecal samples were all culture negative and could thus not be confirmed as true positives. The detection rate of *Salmonella* spp. in sheep tonsils was high with both VIDAS® and PCR methods. *Salmonella* spp. was isolated from 55 % of the tonsil samples. The goat tonsil samples were mostly (11/14) weak positive with VIDAS® (0.23–1.00); only two samples were PCR positive and one culture positive. Further research using more effective culture methods is needed to exclude possible false-positive results obtained by VIDAS®. More information is also needed about the significance of goats as a reservoir for *Salmonella*.

Keywords: *Salmonella* spp., VIDAS®, PCR, small ruminants, tonsils, feces

Zusammenfassung

Die Salmonellose zählt weiterhin zu den wichtigsten lebensmittelbedingten Zoonosen. Kulturelle Methoden zum Nachweis von *Salmonella* spp. sind sehr aufwendig und können bis zu einem endgültigen Resultat sieben Tage in Anspruch nehmen. Zur Verbesserung der Lebensmittelsicherheit sind daher genaue und schnelle Screeningmethoden unabdingbar. In dieser Studie wurden zum Nachweis von *Salmonella* spp. 80 gepoolte Tonsillen- und Fäzesproben gesunder kleiner Schlachtwiederkäuern mittels VIDAS® SLM Immunoassay und SYBRGreen® basierter Real-Time PCR untersucht. Positive Proben wurden auf Xylose-Lysin-Desoxycholat (XLD) und Hektoen-Enteric (HE) Agar Platten subkultiviert. Die Nachweisrate von *Salmonella* spp. war mittels VIDAS® signifikant höher als mittels PCR. *Salmonella* spp. wurde zu 85 % mittels VIDAS® und zu 48 % mittels PCR in den Tonsillenproben detektiert. Insgesamt waren 70 % der Fäzesproben mittels VIDAS® positiv, aber lediglich 5 % mittels PCR. Eine Isolierung von *Salmonella* spp. gelang ausschließlich bei im VIDAS® hoch positiven Proben (Testwerte > 3,00). Dabei traten hoch positive VIDAS® Testwerte (> 3,00) nur bei Tonsillenproben auf, während die meisten (26/28) im VIDAS® positiven Fäzesproben einen sehr geringen Testwert (0,23–1,00) aufwiesen. Keine dieser im VIDAS® positiven Fäzesproben konnte kulturell und somit als richtig positiv bestätigt werden. Die Nachweisrate von *Salmonella* spp. in Schaftonsillen war sowohl mittels VIDAS® als auch mittels PCR hoch. So wurde *Salmonella* spp. aus 55 % der Tonsillenproben isoliert. Die Ziegentonsillenproben waren meistens (11/14) schwach VIDAS® positiv (0,23–1,00); nur zwei dieser Proben wiesen auch mittels PCR ein positives Ergebnis auf, wovon sich ein Ergebnis auch kulturell bestätigen ließ. Weitere Studien unter Verwendung effektiverer Kulturmethoden sind erforderlich, um mögliche falsch positive Resultate im VIDAS® ausschließen zu können. Ebenso wird mehr Information über die Bedeutung von Ziegen als Reservoir für *Salmonella* spp. benötig.

Schlüsselwörter: *Salmonella* spp., VIDAS®, PCR, kleine Wiederkäuer, Tonsillen, Fäzes

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Introduction

Salmonella is an important zoonotic bacterial pathogen of economic significance in animals and humans worldwide (Callaway et al., 2008). It is still one of the leading causes of foodborne diseases and outbreaks in Europe (EFSA, 2011). Foodborne illnesses caused by *Salmonella* are primarily due to foods of animal origin like raw and insufficiently heated meat and meat products (Callaway et al., 2008). *Salmonella* has been isolated from all livestock animals. Asymptomatic ruminants have shown to carry this pathogen in oral cavity, feces and rumen material, and on the hide (Fegan et al., 2005). *Salmonella* can easily be distributed from asymptomatic carriers and intermittent or persisting shedders into the food chain through contamination of the carcasses during slaughter.

Isolation methods for detection of *Salmonella* spp. in asymptomatic animals, food and environmental samples need usually at least one enrichment step prior to isolation on selective agar plates, which requires at least 2 days yielding a negative result and up to 7 days for a positive result. Because of the time-consuming isolation methods, several faster methods for first screening of this pathogen have been developed including enzyme-linked fluorescence assays (ELFA) (for a review see: Maciorowski et al., 2006) and polymerase chain reaction (PCR) (for a review see: Maciorowski et al., 2005) method. An advantage of using automated VIDAS® system is that the assay is rapid, less labor intensive and with the ability to handle large numbers of samples. PCR offers also a potential reduction in assay time and an improved sensitivity. However, inhibitory substances present in feces may lead to false-negative results by PCR.

Small ruminants are potential carriers and asymptomatic shedders of *Salmonella* spp. and they may pose a risk for salmonella entry into the food chain (Zweifel et al., 2004). Accurate and rapid methods for *Salmonella* screening in livestock are important to improve food safety. In this study, the presence of *Salmonella* spp. was screened in pooled tonsil and feces samples of small ruminants at slaughter using VIDAS® and PCR.

Material and Methods

Salmonella spp. was studied in tonsil and fecal samples from 100 sheep and goats each by VIDAS® and PCR. The samples were collected at slaughter (Bonke et al., 2012).

For the VIDAS® experiments, about 10 g of the tonsil sample were homogenized in 90 ml tryptic-soy broth (CASO, Merck, Darmstadt, Germany) supplemented with 1.5 g/l bile salt (Oxoid, Basingstoke, UK) (mTSB), whereas 1 g feces was homogenized in 99 ml mTSB followed by incubation for 16 to 18 h at 37 °C. After overnight enrichment, 5 samples (200 µl per sample) were pooled, heat treated at 99 °C for 10 min and then studied by VIDAS® SLM (BioMérieux, Nürtingen, Germany) according to the manufacturer's instructions. Pool samples with a test value (test value) greater than or equal to 0.23 indicated a positive result. The VIDAS®SLM is an enzyme-immunoassay for the detection of *Salmonella* antigens using the Enzyme Linked Fluorescent Assay (ELFA) on an automated VIDAS® instrument. VIDAS® SLM methods have only been validated for food and environmental samples but not for clinical samples. The validated methods are based on different enrichment steps, which all include a selective enrichment step, before studying with the automated VIDAS® analyzer.

For the detection of *Salmonella* spp. in the tonsils by real-time PCR, 100 µl of the overnight enrichment were used for DNA extraction using InstaGene (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Due to inhibitory substances in feces, PCR was not done directly from enrichment broth. The enrichment broths were first subcultured on xylose-lysine-desoxycholate (XLD, Merck) agar (Bonke et al., 2012). Colonies were washed of with saline and the DNA was released from an aliquot by heating. Two µl of the template was added to 23 µl of master mix, which contained 1X ready-to-use mix (QuantiTect™SYBRGreen PCR Kit, Qiagen, Hilden, Germany) and 200 nM of primers amplifying a 429-bp chromosomal fragment of *Salmonella* (Aabo et al., 1993). A 3-step protocol (denaturation at 95 °C for 10 s, annealing at 58 °C for 30 s and elongation at 72 °C for 10 s) with 40 cycles followed by a melting curve analysis was performed. The PCR fluorescence was detected using the iQ™ Real-Time PCR Detection system (Bio-Rad). A threshold cycle (C_t) below 38 and a specific melting temperature (T_m) of 85.5 °C ± 0.5 °C indicated a positive result.

A loopful (10 µl) of overnight enrichment form VIDAS®- and PCR-positive samples were plated onto XLD and hectoen-enteric (HE, Merck) plates (Bonke et al. 2012). After incubation of the plates at 37 °C for 18 to 20 h, all presumptive colonies were identified using API® 20E (BioMérieux) and serotyped at the Swiss National Reference Laboratory (NENT), Zurich, Switzerland.

Results and discussion

Salmonella spp. was detected in 34 (85 %) pooled tonsil samples by VIDAS® and 19 (48 %) by PCR (Tab. 1). In most (13/19) of the PCR-positive pooled samples, several subsamples were PCR positive, especially when high VIDAS® test value (> 3.00) were obtained. Surprisingly, only 19 of 34 VIDAS®-positive samples could be confirmed by PCR. Especially, the samples with low test value (0.23–1.00) were mostly (14/16) PCR negative. One reason can be that the number of *Salmonella* was too low for PCR but another reason can be that some of the VIDAS®-positive samples, especially those with low test values, were false-positive results.

Salmonella spp. was detected in 28 (70 %) pooled feces samples by VIDAS® and only in 2 (5 %) by PCR (Tab. 1). No VIDAS®-positive results with high test values were obtained. Most of the VIDAS®-positive feces samples (26/28) had very low test values (0.23–1.00) which indicate that the number of *Salmonella* in feces samples was low. However, false-positive results in these samples cannot be excluded. Using only non-selective enrichment prior to VIDAS® analysis may have an influence on the results. The validated methods for food and environmental samples include an enrichment step in selective enrichment broth prior to analysis. This step is missing in this study which may have produced false-positive results.

The VIDAS® system has been evaluated for *Salmonella* analysis of animal feces in some earlier studies (Eriksson & Aspan, 2007; Korsak et al., 2004). Korsak et al. (2004) demonstrated that VIDAS® significantly improved the recovery in naturally contaminated fecal samples compared to culturing. Eriksson and Aspan (2007), however, reported that VIDAS® gave slightly poorer results for cattle feces than culture method which is in contrary to our

results (Eriksson & Aspan, 2007; Korsak et al., 2004). One reason for the high isolation rate is probably the fact that the samples were artificially contaminated.

Salmonella spp. was confirmed by culture only from VIDAS®-positive samples with high test values and from tonsil samples. This demonstrates that the number of *Salmonella* spp. was higher in tonsils than in feces. The reason for the very low isolation rate compared to the clearly higher VIDAS®-positive results can be due to the insensitive culturing method used in this study. Adding a selective enrichment step following the overnight enrichment would have probably increased the isolation rate. Uyttendaele et al. (2003) evaluated VIDAS®, PCR and a culture method using a semi-solid medium for detection of *Salmonella* in food and reported that the semi-solid medium gave comparable results with VIDAS® and PCR (Uyttendaele et al., 2003). When naturally contaminated pork carcasses were studied, more *Salmonella*-contaminated carcasses were detected by VIDAS® than by culturing (Yeh et al., 2002).

TABLE 1: *Detection of Salmonella spp. in 80 pooled tonsil and feacal samples from small ruminants by VIDAS® SLM.*

Sample type	VIDAS® test values	Number of VIDAS®-positive pooled samples[a]	Number of culture confirmed VIDAS®-positive pooled samples	Number of PCR-positive sub-samples in the pooled sample at least 1	2–5
Tonsils (40)[b]	> 3.00	15	12	15	12
	1.00–3.00	3	0	2	0
	0.23–1.00	16	0	2	1
	≥ 0.23	34	12	19	13
Feces (40)[b]	> 3.00	0	0	0	0
	1.00–3.00	2	0	1	0
	0.23–1.00	26	0	1	0
	≥0.23	28	0	2	0

[a] One pool consists of 5 subsamples; [b] Number of pooled samples

TABLE 2: *Comparison of the detection rate of Salmonella spp. in pooled tonsil and feacal samples from sheep and goats by VIDAS® SLM, PCR and culture.*

Species	Sample type	Number of pooled samples[a]	Number of positive pooled samples by VIDAS® test values >3.0	>1.0–3.0	0.23–1.0	PCR	Culture
Sheep	Tonsil	20	14	1	5	17	11[b]
	Feces	20	0	2	16	1	0
Goats	Tonsil	20	1	2	11	2	1[b]
	Feces	20	0	0	10	1	0

[a] One pool consists of 5 subsamples; [b] Only VIDAS®-positive samples were plated onto XLD and HE agar plates

Salmonella spp. was detected in all pooled sheep tonsil samples and in most (14/20) of the pooled goat tonsil samples by VIDAS® (Tab. 2). By PCR, *Salmonella* spp. was also frequently detected in sheep tonsils whereas only 2 out of 20 goat tonsils samples were positive. Furthermore, the isolation rate of *Salmonella* spp. was high (11/20) in sheep tonsils, yet this pathogen was isolated only from one goat tonsil. However, the isolation rates could have been higher if selective enrichment would have been used prior to plating on selective agar plates. The importance of this pathogen in goat tonsils has to be further studied to exclude possible false-positive results by VIDAS®. It is necessary to include different isolation methods in future studies to increase sensitivity of culturing.

All examined *Salmonella* isolates belonged to the serotype 61:k:1,5,(7) which is an untypical serotype that is very seldom found in food and environmental samples. Sheep have shown to be an important reservoir for *S. enterica* subsp. *diarizonae* 61:k:1,5,(7) (Zweifel et al. 2004). This study shows that the VIDAS® immunoassay can also be used for detection of serotype 61:k:1,5,(7).

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