Praca oryginalna *Original paper*

Occurrence of Yersinia enterocolitica O:9 in feces from cows seropositive for brucellosis

KRZYSZTOF SZULOWSKI, MARCIN WEINER, WOJCIECH IWANIAK

Zakład Mikrobiologii, Państwowy Instytut Weterynaryjny – Państwowy Instytut Badawczy w Puławach,
Al. Partyzantów 57, 24-100 Puławy

Szulowski K., Weiner M., Iwaniak W.

Occurrence of Yersinia enterocolitica O:9 in feces from cows seropositive for brucellosis

Summary

The diagnosis of brucellosis is mainly based on serological tests. All animals classified as serologically positive are obligatorily slaughtered and subjected to bacteriological examination. B. abortus has not been reported in Poland since the eradication of bovine brucellosis in 1980. On the other hand, B. suis biovar 2 is sporadically isolated from cattle. In accordance with the instructions of the Chief Veterinary Officer, samples of feces from animals slaughtered following positive serological results for brucellosis have been examined for the presence of Yersinia enterocolitica O:9 since 2011. Because of the similarity of its O-polysaccharide component of S-LPS with that of Brucella, this microorganism is considered to be a major contributory factor of false positive serological reactions (FPSR). The paper presents the results of the bacteriological and molecular examination of feces from 26 cows seropositive for brucellosis and 30 healthy cows, negative for brucellosis, for the presence of Y. enterocolitica O:9. Y. enterocolitica O:9 was found in 7 of the positive cows, whereas all samples from cows negative for brucellosis were free from this bacteria. These results indicate that Y. enterocolitica O:9 may be responsible for some of the positive results for brucellosis in cattle.

Keywords: cattle, brucellosis, FPSR, Yersinia enterocolitica O:9

Bovine brucellosis is an infectious disease of worldwide importance, usually caused by *Brucella abortus*, less frequently by *B. melitensis*, and occasionally by *B. suis*. Several countries in northern and central Europe, Canada, Japan, Australia, and New Zealand are believed to be free from the agent (10). According to Directive 64/432/EEC, Poland, like most other countries of the European Union, is recognized as "officially free from bovine brucellosis", and it obtained this status in 2009 (3, 5). Nevertheless, cattle herds have to be monitored for potential re-emergence of the disease. The main role in these examinations is played by serological tests. All animals monitored for brucellosis are tested by the Rose Bengal test, and all positives undergo a further examination with the use of the serum agglutination test (SAT) and the complement fixation test (CFT). Further examination also involves the 2-mercaptoethanol test (2-Me), the Coombs antiglobulin test (Coombs), and the indirect ELISA. All animals classified as serologically positive by the National Reference Laboratory for Brucellosis (NRL) are obligatorily slaughtered and subjected to bacteriological examination. In addition, this kind of examination is performed in cases of abortions. The results of surveys show that *B. abortus* has not been reported in Poland since the eradication of bovine brucellosis in 1980. On the other hand *B. suis* is sporadically isolated from cattle (13). When specimens from 176 seropositive cows slaughtered in 2002-2011 were cultured for the isolation of *Brucella*, in 5 cases *B. suis* biovar 2 was isolated. In cattle, the infection caused by this biovar appears to be a noncontagious disease with limited induced pathology and no induction of abortion, but antibody response cannot be readily differentiated from that caused by an infection with *B. abortus* (4, 6, 11). However, the origin of the vast majority of positive serological results for brucellosis in cows is still unknown.

Since some of these results can be regarded as false positive serological reactions (FPSR) related to *Yersinia enterocolitica* O:9, the Chief Veterinary Officer ordered that feces samples from slaughtered animals be examined for the presence of this microorganism in order to assess the scale of this phenomenon (1). Since 2011 these examinations have been performed by the NRL.

The paper presents the results of bacteriological and molecular research for the presence of *Y. enterocolitica* O:9 in feces and in supramammary lymph nodes from cows slaughtered because of positive serological results for brucellosis.

Material and methods

Material from cows. Fecal samples from 26 animals slaughtered following positive serological reactions for brucellosis were examined. In parallel, supramammary lymph nodes from the same animals were tested. All samples were cultured for the presence of brucellae with the use of Farrell's medium, serum dextrose agar, and a liquid medium (serum dextrose broth), as described previously (13), with negative results. For comparison, 30 samples of feces from healthy cows, negative in serological examinations for the presence of anti-*Brucella* antibodies, were examined.

Bacteriological examination. The isolation of *Y. enterocolitica* O:9 was performed according to ISO 10273:2003 by applying two enriched media: phosphate buffered saline with sorbitol bile salts (PSB broth) and a modified ITC broth containing irgasan, ticarcillin, a reduced amount (80%) of magnesium chloride, malachite green, and no potassium chlorate (ITC II). The homogenates of samples (0.5 ml) were transferred to 25 ml of each broth. After 48 h incubation at 25°C for ITC II and 14 days at 2-8°C for PSB, the cultures were treated in a proportion of 10 µl to 100 µl with 0.25% KOH in 0.5% saline for 10 s to enhance the isolation of *Y. enterocolitica*, and then transferred onto cefsulodin-irgasan-novobiocin agar (CIN) plates and incubated at 29-30°C for 24 or 48 hours, depending on the growth of bacterial colonies. Typical *Yersinia*-suspected "bull-eye" colonies were selected for further analysis with API-20E and two PCR assays. The *Y. eneterocolitica* positive colonies in API-20E were tested for the presence of the universal *Y. enterocolitica* 16S rRNA gene (14), and then, if positive, for the presence of the per gene (9), characteristic of *Y. enterocolitica* O:9 serotype only. The same PCR assays were used for the examination of feces and lymph nodes.

DNA extraction. One individual colony of each isolate was suspended in 50 µl of sterile, DNase, RNase-free deionized water (ICN Biomedicals). The suspensions were heated at 99°C for 5 min, chilled on ice, and then centrifuged at 13,000 × g for 1 min to pellet the cellular debris. The supernatant (5 µl) was subsequently used as a source of DNA template. When the specimens of feces and lymph nodes had been examined, they were stored at 3-8°C for 24 h before DNA extraction. Next, the samples were added to 9 ml of saline and homogenised in a stomacher. The saline suspensions (500 µl) of the samples were heated at 99°C for 5 min (Thermomixer Comfort, Eppendorf), then chilled on ice, and centrifuged at 2,500 × g for 2 min. The supernatant was used for the DNA extraction according to a commercial protocol (DNeasy Blood&Tissue Kit, Qiagen).

PCR assays. Each DNA amplification was performed in a total volume of 50 µl of reaction mixture consisting of 5 µl of DNA template, 1X PCR buffer (Fermentas), 200 µM of dNTPs, 4 mM of $MgCl_2$, 1 U of Taq DNA polymerase (Fermentas), nucleotide primers at a concentration of 80 nM, and water. Sequences, characteristics, and concentration of the primers were used in accordance with Wannet et al. (14) for the 16S rRNA gene and in accordance with Lubeck et al. (9) for the per gene. PCRs were run in a thermocycler (T3, Biometra) under the following conditions: initial DNA denaturation at 94°C for 5 min, followed by 36 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min (16S rRNA gene), and 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min (per gene). The final extension step was done at 72°C for 5 min. After staining with ethidium bromide (50 mg/ml) for 0.5 min and washing in distilled water, the gels were visualised by UV light and documented. The sizes of PCR amplicons were compared with a 100 bp DNA marker (Fermentas).

Results and discussion

In the bacteriological examination of cows positive in serological examination for brucellosis, typical *Yersinia* "bull-eye" colonies were observed in feces from 9 animals (Tab. 1). In all these cases, affiliation to *Y. enterocolitica* was confirmed by using API-20E and PCR for the amplification of the universal *Y. enterocolitica* 16S rRNA gene. Seven of these isolates were identified as *Y. enterocolitica* O:9 serotype by confirming the presence of the per gene in the second PCR. Identical results were obtained when supramammary lymph nodes were cultured. Also, when DNA extracted directly from feces and lymph nodes was tested in PCR, positive samples originated from the same cows as the samples found positive in the bacteriological examination.

As shown in Table 1, *Y. enterocolitica* O:9 was isolated from 4 animals in whose sera anti-*Brucella* antibodies were detected in all tests (samples no. 5, 8, 12, 22) and from 3 animals reacting only in the RBT, SAT, and CFT (sample no. 4, 7, 11). On the other hand, bacteria and genetic material of *Y. enterocolitica* were detected in 3 feces samples originating from 30 animals negative in serological examination for brucellosis, but none was identified as *Y. enterocolitica* O:9.

As the diagnosis of brucellosis is primarily based on the results of serological examinations, one should always bear in mind that the classification of animals as "positive" may not reflect the actual status of the animal. This is a particularly important issue in countries in which the disease has become very rare, since the misinterpretation of observed reactions results in the imposition of unnecessary restrictions and waste of resources. The absence of *Brucella* in the tissues of slaughtered animals means that one needs to search for other causative agents of positive reactions in serological tests. Some of these reactions may be due to nonspecific antibodies contained in bovine sera, thought to be mainly of the IgM isotype, which reduces the specificity of conventional tests. Another potential cause are cross-reactions demonstrated between smooth *Brucella* species and some other microorganisms, such as, most importantly, *Y. enterocolitica* O:9. It is often found in pigs in different countries (17, 19), as well as in cattle (7, 8, 16), and is known to cause false positive serological reactions (FPSR). Because of the similarity of the O-polysaccharide component of S-LPS of *Brucella* and *Y. enterocolitica* O:9, routinely used serological tests do not distinguish between antibodies raised to these two infections (2). The results of

Tab. 1. Detailed results of tests for the presence of *Y. enterocolitica* in cows positive in serological examination for brucellosis

No. of animal	Bacteriological examination		PCR				Serology					
			feces		lymph nodes							
	feces	lymph nodes	16S rRNA	per	16S rRNA	per	RBT	SAT (iu/ml*)	CFT (icftu/ml**)	Coombs	2-Me	I-ELISA
1.	–	–	–	–	–	–	+	+(287)	+(134)	–	–	–
2.	–	–	–	–	–	–	+	+(102.5)	+(33.5)	–	–	–
3.	–	–	–	–	–	–	+	+(36)	–	+	–	–
4.	+	+	+	+	+	+	+	+(246.0)	+(93.0)	–	–	–
5.	+	+	+	+	+	+	+	+(51.5)	+(33.5)	+	+/–	–
6.	–	–	–	–	–	–	+	+(61.5)	+(33.5)	–	–	–
7.	+	+	+	+	+	+	+	+(205)	+(67)	–	–	–
8.	+	+	+	+	+	+	+	+(102.5)	+(46.5)	+	+/–	+
9.	–	–	–	–	–	–	–	+(205)	+(160)	–	+/–	–
10.	–	–	–	–	–	–	–	+(51.5)	+(40)	–	–	–
11.	+	+	+	+	+	+	+	+(123)	+(20)	–	–	–
12.	+	+	+	+	+	+	+	+(31)	+(46.5)	+	+/–	+
13.	–	–	–	–	–	–	+	+(143.5)	+(23.3)	–	–	–
14.	–	–	–	–	–	–	+	+(123)	+(124)	–	–	–
15.	–	–	–	–	–	–	+	+(33.5)	+(205)	–	–	–
16.	–	–	–	–	–	–	+	+(410.5)	+(80)	–	–	–
17.	–	–	–	–	–	–	+	+(51,5)	–	+	–	–
18.	–	–	–	–	–	–	+	+(123)	+(106)	+	+/–	+
19.	–	–	–	–	–	–	+	+(51.5)	+/–(11.6)	+	+/–	–
20.	–	–	–	–	–	–	+	+(123)	+(33.5)	–	–	–
21.	+	+	+	–	+	–	+	+(246)	+(40)	–	–	–
22.	+	+	+	+	+	+	+	+(36)	+(46.5)	+	+/–	+
23.	+	+	+	–	+	–	+	+(123)	+(23.3)	–	+/–	–
24.	–	–	–	–	–	–	+	+(492.5)	+(134)	–	+/–	–
25.	–	–	–	–	–	–	+	+(82)	+(40)	+	+/–	–
26.	–	–	–	–	–	–	+	+(123)	+(20)	–	–	–

Explanations: * – international units per ml; ** – international complement fixation test units per ml

our investigations show that *Y. enterocolitica* O:9 was detected in specimens from 7 out of 26 animals slaughtered because of positive results for brucellosis and, with a high degree of probability, could be regarded as responsible for these reactions. On the other hand, no isolates were found in material from 30 serologically negative animals.

Examinations for the presence of *Y. enterocolitica* O:9 of animals positive in serological examination for brucellosis and the isolation of this bacteria are very important for accurate interpretation of the results. They are particularly important in the case of cattle, in which we usually have isolated reactions in the herd and no information on other animals, whereas it is easier to interpret results obtained on a serological basis in the case of pigs, in which reactions are usually observed in more animals and information is more complete (12). In our previous studies we showed that lymph nodes are good material for the isolation of *Y. enterocolitica* O:9 (16). The results of the present examinations indicate that similar results can be obtained when feces are examined. These are only preliminary findings, and we intend to continue our studies of samples from animals slaughtered because of positive results for brucellosis and from herds with no history of serological reactions for brucellosis.

References

1. Annex no. 1 to Instruction no. GIWz.401/BB-16/2010 of the Chief Veterinary Officer, Warsaw 2011.
2. *Caroff M., Bundle D. R., Perry M. B.*: Structure of the O-chain of the phenol-phase soluble cellular lipopolysaccharide of Yersinia enterocolitica serotype O:9. Eur. J. Biochem. 1984, 139, 195-200.
3. Commission Decision 2009/600/EC of 5 August 2009 amending Decision 2003/467/EC as regards the declaration that certain Member States and regions thereof are officially free of bovine brucellosis (OJ L204, 6.8.2009, pp. 39-42).
4. *Cook D. R., Noble J. W.*: Isolation of Brucella suis from cattle. Aus. Vet. J. 1984, 61, 263-264.
5. Council Directive 64/432/EEC of 26 June 1964 on animal health problems affecting intra-Community trade in bovine animals and swine (OJ No. 121, 29.7.1964, pp. 1977/64).

6. *Ewalt D. R., Payeur J. P., Rhyan J. C., Geer P. L.*: Brucella suis biovar 1 in naturally infected cattle: a bacteriological, serological and histological study. J. Vet. Diagn. Invest. 1997, 9, 417-420.
7. *Garin-Bastuji B., Hummel N., Gerbier G., Cau C., Pouillot R., Da Costa M., Fontaine J. J.*: Nonspecific serological reactions in the diagnosis of bovine brucellosis: experimental oral infection of cattle with repeated doses of Yersinia enterocolitica O:9. Vet. Microbiol. 1999, 66, 223-233.
8. *Gerbier G., Garin-Bastuji B., Pouillot R., Very P., Cau C., Berr V., Dufour B., Moutou F.*: False positive serological reactions in bovine brucellosis: evidence of the role of Yersinia enterocolitica serotype O:9 in a field trial. Vet. Res. 1997, 28, 375-383.
9. *Lubeck P. S., Skurnik M., Akrens P., Hoorfar J.*: A Multiplex PCR-detection assay for Yersinia enterocolitica serotype O:9 and Brucella spp. based on perosamine syntethase gene. Adv. Exp. Med. Biol. 2003, 529, 451-455.
10. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, OIE, Paris, France 2010.
11. *Olsen S. C., Hennager S. G.*: Immune response and Protection against Experimental Brucella suis Biovar 1 Challenge in Nonvaccinated or B. abortus Strain RB51-Vaccinated Cattle. Clin. Vaccine Immunol. 2010, 17, 1891--1895.
12. *Szulowski K., Iwaniak W., Pilaszek J.*: Porcine brucellosis in Poland: problems accompanying serological surveys conducted in 1995-2000. Bull. Vet. Inst. Pulawy 2001, 45, 153-161.
13. *Szulowski K., Iwaniak W., Weiner M., Złotnicka J.*: Brucella suis biovar 2 isolations from cattle in Poland. Ann. Agric. Environ. Med. 2013, vol. 20, no 4, 677-680.
14. *Wannet W. J. B., Reessink M., Brunings H. A., Maas H. M. E.*: Detection of pathogenic Yersinia enterocolitica by a rapid and sensitive Duplex PCR. J. Clin. Microbiol. 2001, 39, 4483-4486.
15. *Weiner M., Iwaniak W., Złotnicka J., Szulowski K.*: Diagnosis of bovine brucellosis using traditional serological techniques and fluorescence polarization assay. Bull. Vet. Inst. Pulawy 2010, 54, 485-488.
16. *Weiner M., Szulowski K., Iwaniak W.*: Isolation of Yersinia enterocolitica O:9 from cows positive in serological examination of bovine brucellosis. Bull. Vet. Inst. Pulawy 2012, 56, 473-477.
17. *Weiner M., Szulowski K., Iwaniak W.*: The porcine brucellosis – evidence of the role of Yersinia enterocolitica O:9 in occurence of false positive serological reactions. Polish J. Vet. Sci. 2013, 16, 129-130.
18. *Weynants V., Tibor A., Denoel P. A., Saegerman C., Godfroid J., Thiange P., Letesson J. J.*: Infection of cattle with Yersinia enterocolitica O:9 a cause of the false positive serological reactions in bovine brucellosis diagnostic tests. Vet. Microbiol. 1996, 48, 101-112.
19. *Wrathall A. E., Broughton E. S., Gill K. P., Goldsmith G. P.*: Serological reactions to Brucella species in British pigs. Vet. Rec. 1993, 132, 449-454.

Corresponding author: dr hab. Krzysztof Szulowski prof. nadzw., Al. Partyzantów 57, 24-100 Puławy; e-mail: kszjanow@piwet.pulawy.pl