Prevalence of Hepatitis E virus specific antibodies in sera of German domestic pigs estimated by using different assays

Christine Baechlein, Anika Schielke, Reimar Johne, Rainer G. Ulrich, Wolfgang Baumgaertner, Beatrice Grummer

Title: Prevalence of Hepatitis E virus specific antibodies in sera of German domestic pigs estimated by using different assays

Authors: Christine Baechlein, Anika Schielke, Reimar Johne, Rainer G. Ulrich, Wolfgang Baumgaertner, Beatrice Grummer

PII: S0378-1135(09)00602-6
DOI: doi:10.1016/j.vetmic.2009.12.011
Reference: VETMIC 4708

To appear in: *VETMIC*

Received date: 22-9-2009
Revised date: 1-12-2009
Accepted date: 3-12-2009

Please cite this article as: Baechlein, C., Schielke, A., Johne, R., Ulrich, R.G., Baumgaertner, W., Grummer, B., Prevalence of Hepatitis E virus specific antibodies in sera of German domestic pigs estimated by using different assays, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.12.011

Prevalence of Hepatitis E virus-specific antibodies in sera of German domestic pigs estimated by using different assays

Christine Baechlein[1], Anika Schielke[2], Reimar Johne[2], Rainer G. Ulrich[3], Wolfgang Baumgaertner[4], Beatrice Grummer[1*]

[1]Institute of Virology, Department of Infectious Diseases, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany

[2]Federal Institute for Risk Assessment, Diedersdorfer Weg 1, 12277 Berlin, Germany

[3]Friedrich-Loeffler-Institut, Institute for Novel and Emerging Infectious Diseases, Suedufer 10, 17493 Greifswald - Insel Riems, Germany

[4]Department of Pathology, University of Veterinary Medicine Hannover, Buenteweg 17, 30559 Hannover, Germany

*corresponding author:

Tel.: +49-511-953-8845

fax: +49-511-953-8898

beatrice.grummer@tiho-hannover.de

Abstract

Hepatitis E virus is the causative agent of an acute hepatitis in humans. In industrialized countries, autochthonous hepatitis E cases in the past were mainly of undetermined origin, whereupon nowadays some cases may be linked to zoonotic transmission of HEV from pigs and wild boars. In contrast to several European countries the HEV status of German domestic pigs and a possible risk of transmission are unknown so far. Here, a novel peptide-based ELISA was used to detect HEV-specific antibodies in 1072 sera from German domestic pigs resulting in an average seroprevalence of 49.8% indicating widespread HEV infections in these animals. A comparative testing of 321 randomly selected sera revealed a seroprevalence of 64.8% when using a commercially available ELISA and 43.9% for the novel peptide-based ELISA but concordant results were obtained in both tests only for 56.1% of the sera. Additional re-testing of 23 randomly selected sera with a modified commercially available immunoblot revealed discordant results also. The use of different antigens and the measurement of different immunoglobulin classes are considered to be responsible for the observed variations of the results. Though the present study revealed a high seroprevalence of HEV in the German domestic pig population and a potential risk of transmission to humans, the differing results of the tests highlight the necessity of a standardization of serological assays for comparative seroprevalence and longitudinal studies.

Keywords: Hepatitis E virus, pigs, antibody assays

Introduction

Hepatitis E virus (HEV) is a non-enveloped, single-stranded RNA virus mostly causing a mild to moderate self-limiting hepatitis in humans. The positively orientated RNA genome possesses three open reading frames (ORFs), which code for the non-structural proteins (ORF1), the capsid protein (ORF2) and a phosphoprotein that is associated with the cytoskeleton (ORF3) (Zafrullah et al., 1997).

In developing countries, where HEV is endemic, the virus is transmitted to humans via contaminated drinking water and mostly affects adults. In Europe, numerous symptomatic hepatitis E cases without a travelling history to HEV-endemic regions were reported in the previous years (Buti et al., 2004; Mansuy et al., 2004; Wichmann et al., 2008). The assumption of domestic pigs and wild boar representing HEV reservoirs in industrial countries was confirmed by the detection of HEV-specific RNA and antibodies in these animals (Rutjes et al., 2007; de Deus et al., 2008; Di Bartolo et al., 2008; McCreary et al., 2008).

HEV is representing a unique serotype (Anderson et al., 1999), but is subgrouped into at least four different genotypes. Genotype 1 and 2 occur in humans only and can be found in Southeast Asia, Mexico and Central Africa, respectively (Schlauder and Mushahwar, 2001). HEV genotype 3 and 4 can be found in humans and pigs (Purcell and Emerson, 2008).

Although HEV obviously circulates in the German wild boar population at least for the last fourteen years (Kaci et al., 2008; Adlhoch et al., 2009; Schielke et al., 2009), studies on domestic pigs were still pending.

Here, we report on the application of a novel peptide-based ELISA for a seroprevalence study in German domestic pigs. The absolute values of the generally high HEV seroprevalences differed between the novel ELISA and two commercially

available tests. We discuss the possible reasons for the variation of results and suggest a standardization of test systems for future seroepidemiological studies.

Materials and Methods

Serum samples. A total of 1,072 sera were randomly collected from 142 farms of ten federal states of Germany during 2007/2008. For 264 sera data on the age of the pigs were available. Positive and negative reference field sera from domestic pigs from Spain had been previously characterized using an HEV genotype 1 ELISA (Martin et al., 2007; sera were kindly provided by M. Casas, CReSA, Barcelona, Spain).

Novel peptide-based ELISA (TiHo-ELISA). The newly developed ELISA (TiHo-ELISA) is based on a synthetic peptide representing a composite of carboxy-terminal 30 amino acid (aa) residues of ORF 2 protein and carboxy-terminal 29 aa residues of ORF 3 protein, both originating from the Burmese HEV genotype 1 strain (Tam et al., 1991). Ninety-six well plates were coated with these HEV ORF2/ORF3 peptides purchased from Acris Antibodies GmbH (Herford, Germany). Sera were applied in a dilution of 1:250 in phosphate buffered saline (PBS) with 1% horse serum. After a stringent wash with 3M urea, polyclonal rabbit anti-pig IgG conjugated with horseradish peroxidase (Sigma-Aldrich, Saint Louis, USA) was used in a dilution of 1:10,000. As substrate, tetramethylbenzidine (TMB) was added for 10 minutes followed by stopping of the enzymatic reaction with 1M hydrochloric acid. The optical density (OD) was automatically scored in an ELISA reader (Tecan Sunrise, Tecan, Crailsheim, Germany).

Commercial recombinant protein-based ELISA (Axiom-ELISA). The commercially available HEV Ab-ELISA kit (Axiom, Bürstadt, Germany) is a double-antigen sandwich ELISA based on a recombinant Burmese HEV genotype 1 capsid protein derivative covering the carboxy-terminal aa residues 394 to 606. Due to its test principle, it can detect HEV-specific antibodies independently of the host species and immunoglobulin class. The assay was performed by strictly following the manufacturer's instructions including the recommended thresholds for definition of a positive serum.

Commercial immunoblot test. The *recom*Blot test (Mikrogen, Neuried, Germany), primarily developed for the detection of anti-HEV antibodies in human serum, bases on four recombinant proteins of HEV genotype 1: three overlapping ORF2 derived polypeptides that completely cover the capsid protein and the entire ORF3 protein. This test was performed following the recommendations of the manufacturer with the following modifications: sera at a dilution of 1:200 were incubated with the blot strips for two hours. After washing, peroxidase-conjugated polyclonal rabbit anti-pig IgG (Sigma-Aldrich, Saint Louis, USA) was used to detect specific antibodies. TMB was used as substrate for up to 15 minutes. The results were defined as positive (strong bands visible), equivocal (weak bands visible) or negative (no band visible).

Results

Testing of domestic pig sera with the TiHo-ELISA. For the new ELISA, a cut-off value was defined using four negative control sera as defined by Martin et al., 2007. These sera also reacted negative in the two commercial tests used in our study. The cut-off value resulted from the average ODs of up to 27 testings of these negative

controls plus a threefold standard deviation. Two positive reference sera, which have also been confirmed by the immunoblot test and the Axiom-ELISA to contain anti-HEV antibodies, showed OD values well above the calculated cut-off value in the TiHo-ELISA in multiple investigations. For all subsequent investigations one of the negative and both positive controls were applied on each plate (Fig. 1). To reduce background reactions and to identify antibodies with high avidity an additional incubation step with 3M urea was applied.

In total, 1,072 porcine serum samples have been screened and about half of the sera (534 = 49.8%) were tested positive in the TiHo-ELISA. The seroprevalence in the different federal states ranged from 15.6% in Mecklenburg-Western Pomerania to 70.7% in Bavaria. In 111 of 142 investigated farms (78.2%) at least one anti-HEV antibody-positive animal was identified with all farms in Bavaria and Baden-Wuerttemberg being affected (Table 1). Additional information about gender or age of the pigs was available for 264 samples. The prevalence in the adult females and males was 50% or close to 50%, respectively, but lower in the younger animals, whereas the average prevalence in the whole investigated panel was about 30% (Table 2).

Comparison of the TiHo-ELISA with the Axiom-ELISA. To verify the results of the TiHo-ELISA, 321 randomly chosen field sera, representing the minimum sample number for comparison as calculated for a 95% confidence level and a 5% confidence interval (http://www.surveysystem.com/sscalc.htm), were analysed in parallel with the Axiom-ELISA. This test classified 208 (64.8%) of the samples as positive. Using the same set of samples, the TiHo-ELISA rated 141 (43.9%) of the sample as positive (Table 3). A closer analysis of the results revealed that only 180

(56.1%) of the analysed sera showed identical results in both tests. The kappa-value was calculated as 0.182 showing only slight concordance between both ELISA tests.

Comparison of ELISA results with immunoblot data. Further investigations of 23 randomly selected sera were done with the modified *recom*Blot test. The sera were chosen because of their reactivity in the TiHo-ELISA: 12 anti-HEV positive and 11 anti-HEV negative sera of differing origin were included. In this test, five sera reacted positive, six sera were classified as equivocal and 12 sera didn't show any reactivity with one of the recombinant proteins. In conclusion, seven (30.4%) of the sera revealed identical results in all three tests (Table 4). Unfortunately, we couldn't test a larger number of sera, because the manufacturer discontinued the production of the test.

Discussion

There is increasing evidence that hepatitis E is a zoonosis. Pigs, either domestic or feral, can be infected with HEV and phylogenetic analyses revealed a geographical clustering of porcine and human HEV strains indicating a molecular epidemiological evidence for pig to human HEV transmission. In this study, we show that HEV is widespread in the German domestic pig population as previously reported also for HEV in wild boars (Kaci et al., 2008; Adlhoch et al., 2009; Schielke et al., 2009). These data are in line with high seroprevalences observed in domestic pigs from Spain and France (Seminati et al., 2008; Casas et al., 2009; Kaba et al., 2009). In Europe, Germany plays a major role in the pig meat production, and it is known that sporadic cases or limited outbreaks of hepatitis E can be related to the consumption of undercooked HEV containing wild boar meat (Matsuda et al., 2003). Besides demonstrating a high HEV seroprevalence, the TiHo-ELISA also revealed that HEV-

171 specific antibodies are common in all age groups of pigs but with only a small portion
172 of piglets exhibiting anti-HEV antibodies. Most of the serum samples from piglets
173 originated from Lower Saxony where the general seroprevalence is relatively low.
174 Therefore further analyses in different federal states seem to be necessary.
175 Additionally, the farming structure may contribute to the recording of differing
176 seroprevalences between the federal states. In Baden-Wuerttemberg and Bavaria,
177 where the highest anti-HEV prevalences were recorded, the piggeries are generally
178 smaller with less structuring of pig production, which is probably also related to
179 inefficient disinfection of stables or to potential contact to wild boars.
180 Analyses with another ELISA and with a commercial immunoblot confirmed the high
181 seroprevalence of HEV in German pigs; however a closer examination of the test
182 results revealed that both ELISAs and the immunoblot test disaccorded strongly.
183 Several conceivable explanations are possible for this observation. All three tests
184 base on polypeptides of HEV genotype 1, but different regions of the immunogenic
185 proteins were presented as antigens. The TiHo-ELISA and the Axiom-ELISA use
186 antigens corresponding to the carboxy-terminal region of the capsid protein but of
187 different size. The immunoblot test uses polypeptides completely covering ORF2 and
188 ORF3 proteins, but in a denatured form. The presence of antibodies with different
189 binding specificities to the HEV polypeptides may therefore influence the test result.
190 Furthermore, the Axiom-ELISA was developed for the simultaneous detection of
191 antibodies of all classes. In contrast, the TiHo-ELISA and the modified immunoblot
192 test detect porcine IgG antibodies only. The presence of IgM in sera from acutely
193 infected pigs and IgG in sera from reconvalescent pigs may therefore also explain
194 contradictory results using the different test systems.
195 Besides, the inclusion of an incubation step with 3M urea in the TiHo-ELISA protocol
196 dropped background reactions remarkably resulting in the detection of exclusively

antibodies with high avidity to the antigen. This phenomenon was revealed by Allmang et al., 2001, who showed that in horses naturally infected with Borna disease virus, IgG with high avidity to the viral nucleoprotein could be selected by treatment with urea. Varying results using different assays to determine the HEV seroprevalence in pigs have also been reported previously (Peralta et al., 2009) showing that there is an urgent need for standardized serological assays for the detection of HEV-specific antibodies in pigs in general. Comparability of assays may for example be achieved by the use of homologous porcine sequences of HEV genotype 3 as it has been proposed by others (Jimenez de Oya et al., 2009; Peralta et al., 2009). Such assays will be needed to enable comparison of results of surveillance studies conducted by independent research groups in different countries and also for reliable testing results in order to define a distinct pig farm as free from HEV.

Acknowledgments:

For providing us with field sera we would like to thank Ursula Biesenbach (Neumünster, Germany), Jens Böttcher (Poing, Germany), Claudia Bunzenthal (Krefeld, Germany), Klaus Dräger (Koblenz, Germany), Frerk Feldhusen and Marlis Klopries (Rostock, Germany), Wolgang Gaede (Stendal, Germany), Andreas Hlinak (Frankfurt/Oder, Germany), Thomas Miller (Aulendorf, Germany), Eva Nerbas (Hannover, Germany), Katja Sachs (Bad Langensalza, Germany) and Bernd-Andreas Schwarz (Leipzig, Germany). R. G. Ulrich kindly acknowledges Paul Dremsek (Riems, Germany) for helpful comments.

References:

Adlhoch, C., Wolf, A., Meisel, H., Kaiser, M., Ellerbrok, H., Pauli, G., 2009. High HEV presence in four different wild boar populations in East and West Germany. Vet Microbiol. 139, 270-278.

Allmang, U., Hofer, M., Herzog, S., Bechter, K., Staeheli, P., 2001. Low avidity of human serum antibodies for Borna disease virus antigens questions their diagnostic value. Mol Psychiatry. 6, 329-333.

Anderson, D.A., Li, F., Riddell, M., Howard, T., Seow, H.F., Torresi, J., Perry, G., Sumarsidi, D., Shrestha, S.M., Shrestha, I.L., 1999. ELISA for IgG-class antibody to hepatitis E virus based on a highly conserved, conformational epitope expressed in *Escherichia coli*. J Virol Methods. 81, 131-142.

Buti, M., Clemente-Casares, P., Jardi, R., Formiga-Cruz, M., Schaper, M., Valdes, A., Rodriguez-Frias, F., Esteban, R., Girones, R., 2004. Sporadic cases of acute autochthonous hepatitis E in Spain. J Hepatol. 41, 126-131.

Casas, M., Pujols, J., Rosell, R., de Deus, N., Peralta, B., Pina, S., Casal, J., Martin, M., 2009. Retrospective serological study on hepatitis E infection in pigs from 1985 to 1997 in Spain. Vet Microbiol. 135, 248-252.

de Deus, N., Peralta, B., Pina, S., Allepuz, A., Mateu, E., Vidal, D., Ruiz-Fons, F., Martin, M., Gortazar, C., Segales, J., 2008. Epidemiological study of hepatitis E virus infection in European wild boars (*Sus scrofa*) in Spain. Vet Microbiol. 129, 163-170.

Di Bartolo, I., Martelli, F., Inglese, N., Pourshaban, M., Caprioli, A., Ostanello, F., Ruggeri, F.M., 2008. Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy. Vet Microbiol. 132, 47-55.

Jimenez de Oya, N., Galindo, I., Girones, O., Duizer, E., Escribano, J.M., Saiz, J.C., 2009. Serological immunoassay for detection of hepatitis E virus on the basis

of genotype 3 open reading frame 2 recombinant proteins produced in *Trichoplusia ni* larvae. J Clin Microbiol. 47, 3276-3282.

Kaba, M., Davoust, B., Marie, J.L., Barthet, M., Henry, M., Tamalet, C., Raoult, D., Colson, P., 2009. Frequent transmission of hepatitis E virus among piglets in farms in Southern France. J Med Virol. 81, 1750-1759.

Kaci, S., Nockler, K., Johne, R., 2008. Detection of hepatitis E virus in archived German wild boar serum samples. Vet Microbiol. 128, 380-385.

Mansuy, J.M., Peron, J.M., Abravanel, F., Poirson, H., Dubois, M., Miedouge, M., Vischi, F., Alric, L., Vinel, J.P., Izopet, J., 2004. Hepatitis E in the south west of France in individuals who have never visited an endemic area. J Med Virol. 74, 419-424.

Martin, M., Segales, J., Huang, F.F., Guenette, D.K., Mateu, E., de Deus, N., Meng, X.J., 2007. Association of hepatitis E virus (HEV) and postweaning multisystemic wasting syndrome (PMWS) with lesions of hepatitis in pigs. Vet Microbiol. 122, 16-24.

Matsuda, H., Okada, K., Takahashi, K., Mishiro, S., 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J Infect Dis. 188, 944.

McCreary, C., Martelli, F., Grierson, S., Ostanello, F., Nevel, A., Banks, M., 2008. Excretion of hepatitis E virus by pigs of different ages and its presence in slurry stores in the United Kingdom. Vet Rec. 163, 261-265.

Peralta, B., Casas, M., de Deus, N., Martin, M., Ortuno, A., Perez-Martin, E., Pina, S., Mateu, E., 2009. Anti-HEV antibodies in domestic animal species and rodents from Spain using a genotype 3-based ELISA. Vet Microbiol. 137, 66-73.

Purcell, R.H., Emerson, S.U., 2008. Hepatitis E: an emerging awareness of an old disease. J Hepatol. 48, 494-503.

Rutjes, S.A., Lodder, W.J., Bouwknegt, M., de Roda Husman, A.M., 2007. Increased hepatitis E virus prevalence on Dutch pig farms from 33 to 55% by using appropriate internal quality controls for RT-PCR. J Virol Methods. 143, 112-116.

Schielke, A., Sachs, K., Lierz, M., Appel, B., Jansen, A., Johne, R., 2009. Detection of hepatitis E virus in wild boars of rural and urban regions in Germany and whole genome characterization of an endemic strain. Virol J. 6, 58.

Schlauder, G.G., Mushahwar, I.K., 2001. Genetic heterogeneity of hepatitis E virus. J Med Virol. 65, 282-292.

Seminati, C., Mateu, E., Peralta, B., de Deus, N., Martin, M., 2008. Distribution of hepatitis E virus infection and its prevalence in pigs on commercial farms in Spain. Vet J. 175, 130-132.

Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology. 185, 120-131.

Wichmann, O., Schimanski, S., Koch, J., Kohler, M., Rothe, C., Plentz, A., Jilg, W., Stark, K., 2008. Phylogenetic and case-control study on hepatitis E virus infection in Germany. J Infect Dis. 198, 1732-1741.

Zafrullah, M., Ozdener, M.H., Panda, S.K., Jameel, S., 1997. The ORF3 protein of hepatitis E virus is a phosphoprotein that associates with the cytoskeleton. J Virol. 71, 9045-9053.

Fig. 1: Distribution of optical density (OD) values for the negative and positive controls as determined by the TiHo-ELISA and definition of the cut-off value. Given are the cumulated values of the four negative controls and the values of positive control A and positive control B which have been tested up to 27 times in duplicate; the dot represents an outlier; dotted line: cut-off value 0.386.

Table 1: Overview of reactivity of the pig sera in the TiHo-ELISA from the different federal states of Germany. Results comprise the total number of positive sera as well as the total number of infected farms (n. a.: not available).

Table 2: Overview of the different age groups of animals tested positively for HEV-specific antibodies in the TiHo-ELISA.

Table 3: Comparison of results for 321 randomly selected pig sera from Germany obtained by two different antibody ELISA tests.

Table 4: Comparison of results for 23 randomly selected sera from German pigs obtained by three different HEV-antibody assays.

Table 1: Overview of reactivity of the pig sera in the TiHo-ELISA from the different federal states of Germany. Results comprise the total number of positive sera as well as the total number of infected farms (n. a.: not available).

Federal state	Schleswig-Holstein	Mecklenburg-Western Pomerania	Lower Saxony	Brandenburg	Saxony-Anhalt	North Rhine-Westphalia	Thuringia	Saxony	Rhineland-Palatinate	Bavaria	Baden-Württemberg	**total**
Total number of investigated sera	151	45	159	59	53	174	50	50	88	123	120	**1072**
Number of anti-HEV-antibody positive sera	106	7	25	24	30	74	26	28	49	87	78	**534**
%	70.2	15.6	15.7	40.7	56.6	42.5	52.0	56.0	55.7	70.7	65.0	**49.8**
Total number of investigated farms	18	11	10	n. a.	11	17	10	21	21	10	13	**142**
Number of farms with anti-HEV-antibody positive pigs	13	3	6	n. a.	10	13	7	18	18	10	13	**111**
%	72.2	27.3	60.0	n. a.	90.9	76.5	70.0	85.7	85.7	100.0	100.0	**78.2**

Table 2: Overview of the different age groups of animals tested positively for HEV-specific antibodies in the TiHo-ELISA.

	North Rhine-Westphalia	Number of anti-HEV positive animals	**%**	**Brandenburg**	Number of anti-HEV positive animals	**%**	**Lower Saxony**	Number of anti-HEV positive animals	**%**	Total number of investigated sera	Total number of anti-HEV positive animals	%
piglets	27	13	48.1	-	-		74	4	5.4	**101**	**17**	**16.8**
fatteners	27	15	55.6	15	5	33.3	54	10	18.5	**96**	**30**	**31.3**
sows	-	-		15	12	80.0	31	11	35.5	**46**	**23**	**50.0**
boars	6	4	66.7	15	6	40.0	-	-		**21**	**10**	**47.6**
total	**60**	**32**	**53.3**	**45**	**23**	**51.1**	**159**	**25**	**15.7**	**264**	**80**	**30.3**

Table 3: Comparison of results for 321 randomly selected pig sera from Germany obtained by two different antibody ELISA tests.

		TiHo-ELISA		
		positive	negative	
Axiom-ELISA	positive	104	104	**Σ 208**
	negative	37	76	**Σ 113**
		Σ 141	**Σ 180**	

Table 4: Comparison of results for 23 randomly selected sera from German pigs obtained by three different HEV-antibody assays.

no. of serum	origin	TiHo-ELISA	Axiom-ELISA	*recom*Blot
1	Schleswig-Holstein	positive	positive	positive
2	Rhineland-Palatinate	positive	positive	positive
3	Rhineland-Palatinate	positive	positive	positive
4	Baden-Wuerttemberg	positive	positive	equivocal
5	North Rhine-Westphalia	positive	positive	equivocal
6	Rhineland-Palatinate	positive	positive	equivocal
7	Brandenburg	positive	negative	negative
8	Saxony-Anhalt	positive	negative	negative
9	Rhineland-Palatinate	positive	negative	positive
10	Rhineland-Palatinate	positive	negative	equivocal
11	Bavaria	positive	negative	equivocal
12	Rhineland-Palatinate	positive	negative	equivocal
13	Rhineland-Palatinate	negative	negative	negative
14	Rhineland-Palatinate	negative	negative	negative
15	Rhineland-Palatinate	negative	negative	negative
16	Rhineland-Palatinate	negative	negative	negative
17	Saxony	negative	positive	positive
18	Saxony	negative	positive	negative
19	Thuringia	negative	positive	negative
20	Brandenburg	negative	positive	negative
21	Saxony-Anhalt	negative	positive	negative
22	Rhineland-Palatinate	negative	positive	negative
23	Rhineland-Palatinate	negative	positive	negative

Figure#1
Click here to download high resolution image