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Current surveys on the prevalence and distribution of Dirofilaria spp. and Acanthocheilonema reconditum infections in dogs in Romania

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Corresponding Author:	Angela Monica Ionică, DVM University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca Cluj - Napoca, Cluj ROMANIA
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca
Corresponding Author's Secondary Institution:	
First Author:	Angela Monica Ionică, DVM
First Author Secondary Information:	
Order of Authors:	Angela Monica Ionică, DVM
	Ioana Adriana Matei, DVM
	Viorica Mircean, Dr.
	Mirabela Oana Dumitrache, Dr.
	Gianluca D'amico, DVM
	Adriana Győrke, Dr.
	Nikola Pantchev, Dr.
	Giada Annoscia, Dr.
	Kateřina Albrechtová, Dr.
	Domenico Otranto, Prof. Dr.

	David Modrý, Prof. Dr.
	Andrei Daniel Mihalca, Dr.
Order of Authors Secondary Information:	
Author Comments:	
Suggested Reviewers:	

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Angela Monica Ionică[1]

Ioana Adriana Matei[1]

Viorica Mircean[1]

Mirabela Oana Dumitrache[1]

Gianluca D'Amico[1]

Adriana Győrke[1]

Nikola Pantchev[2]

Giada Annoscia[3]

Kateřina Albrechtová[4]

Domenico Otranto[3]

David Modrý[4,5,6]

Andrei Daniel Mihalca[1]

[1] Department of Parasitology and Parasitic Diseases, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Calea Mănăştur 3-5, 400372, Cluj-Napoca, Romania

[2] IDEXX Vet Med Labor GmbH, 71636, Ludwigsburg, Germany

[3]Dipartimento di Medicina Veterinaria, Università degli Studi di Bari, Str. prov. per Casamassima km 3, 70010,Valenzano (Bari), Italy

[4]Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences, 612 42 Brno, Czech Republic

[5]CEITEC –VFU, University of Veterinary and Pharmaceutical Sciences, Palackého tř. 1/3, 612 42 Brno, Czech Republic

[6]Institute of Parasitology, Biology Centre of Academy of Sciences of the Czech Republic, v.v.i., České Budějovice, Czech Republic

*Corresponding author (A.M. Ionică): e-mail: ionica.angela@usamvcluj.ro; tel.: +40766628367; fax: +40354819772.

Abstract

During the last decades, *Dirofilaria* spp. infection in European dogs has rapidly spread from historically endemic areas towards Eastern and North-Eastern countries, but little or no information is available from these geographical regions. The present study provides a picture of filarial infections in dogs from Romania and compares two tests for the diagnosis of *Dirofilaria immitis*. From July 2010 to March 2011, blood samples were collected from 390 dogs from 9 counties of Romania and serological SNAP tests were performed for the detection of *D. immitis* antigen. The remaining blood clots were subsequently used for DNA extraction followed by multiplex PCR for assessing filarioid species diversity (i.e. *Dirofilaria immitis*, *D. repens* and *Acanthocheilonema reconditum*). Based on molecular detection, an overall prevalence of 6.92% (n=27; 95% CI: 4.70-10.03%) for *D. repens*, 6.15% (n=24; 95% CI: 4.07-9.14%) for *D. immitis* and 2.05% (n=8; 95% CI: 0.96-4.16%) for *A. reconditum* was recorded, with significant variations according sampling areas. Coinfections of *D. immitis* and *D. repens* were recorded in 23.91% (n=11) positive dogs. A slightly higher prevalence for *D. immitis* was detected at the SNAP test (n=28, 7.17%; 95% CI: 4.91-10.33%), but this difference was not statistically significant (p=0.66). However, only 53.57% (n=15) of antigen positive dogs were confirmed by PCR, while other dogs (n=9) PCR-positive for *D. immitis* were negative at the serology. The present study shows that *Dirofilaria* species are endemic in the Southern and South-Eastern areas of Romania, *D. repens* being the most common canine filarioid species. This article also provides, for the first time, an epidemiological picture of the distribution of *A. reconditum* in Romania.

Keywords: dogs, *Dirofilaria*, *Acanthocheilonema*, Romania, prevalence, distribution, diagnosis

Introduction

A series of vector-borne filarioids belonging to the genera *Dirofilaria*, *Acanthocheilonema*, *Onchocerca* and *Cercopithifilaria* (Spirurida, Onchocercidae) infect dogs in Europe. Among these, *Dirofilaria immitis* and *Dirofilaria repens* cause a severe cardio-pulmonary affection and a mild dermatological condition, respectively (Venco, 2007, Albanese et al., 2012). Although both species are regarded as zoonotic agents (Orihel and Eberhard, 1998), most human cases in Europe are caused by *D. repens* (Pampiglione and Rivasi, 2000, Otranto et al., 2013). During the last decades *Dirofilaria* spp. have expanded their geographical boundaries towards Eastern and North-Eastern countries, in relation to several factors, such as the availability of vector species and climate (Genchi et al., 2009, 2011). However, *D. repens* seems to be spreading more rapidly than *D. immitis* (Pantchev et al., 2009a, 2011, Genchi et al., 2011), with autochthonous cases reported in several countries previously regarded as non-endemic, such as in Czech Republic (Svobodová et al., 2006, Dobesová et al., 2007), Germany (Hermosilla et al., 2006, Pantchev et al., 2009b, Sassnau and Genchi, 2013), Hungary (Fok et al., 2007), Poland (Sapierzyński et al., 2010,

Masny et al., 2011), Slovakia (Miterpáková et al., 2010, Bocková et al., 2013), Ukraine (Hamel et al., 2013) and Austria (Silbermayr et al., 2014). Autochthonous cases of *D. immitis* infection in dogs have also been suggested based on detection of DNA in whole mosquitoes (Kronefeld et al., 2014) or reported in all the above-mentioned countries, except Austria (Svobodová et al., 2006, Miterpáková et al., 2010, Świątalska and Demiaszkiewicz, 2012, Hamel et al., 2013, Krämer et al., 2014, Tolnai et al., 2014). However, the detection of filarioid DNA in mosquitoes is not necessarily a proof of stable transmission within a region, as no identification of infectious third stage larvae was provided as yet. Furthermore, in Czech Republic and Poland, the first suggested cases of autochthonous *D. immitis* infection are questionable, as they relied only on immunological evidence of infection and no confirmation by direct (e.g. microfilariae) or molecular methods (species-specific PCR / sequencing) was provided (Svobodová et al., 2006, Świątalska and Demiaszkiewicz, 2012). A recent study seems to support this, at least in Poland, where the examination of 1588 canine blood samples (2011-2013) revealed only the presence of *D. repens*, but not that of *D. immitis* in the country (Demiaszkiewicz et al., 2014).

Other species of filarioids affecting dogs (e.g. *Acanthocheilonema reconditum*, *A. dracunculoides*, *Cercopithifilaria grassii*, *C. bainae*, *Onchocerca lupi*) have also been reported in Europe, but due to their minimal clinical importance (with the exception of *O. lupi*), they are poorly known (reviewed by Otranto et al., 2013).

There are several approaches for the diagnosis of filarial infections in dogs. Classical methods applicable for all species with blood-circulating microfilariae include morphological identification (i.e. blood smears or concentration methods, such as Knott's or filtration test) or histochemical staining of microfilariae. More recently, multiple tools for the highly specific molecular identification of various species of filarioids became available. One disadvantage of all direct methods is that their outcome depends on the presence or the number of microfilariae in the examined sample (Genchi et al., 2007, Pantchev et al., 2011, Latrofa et al., 2012). Other methods (i.e. ELISA and immunocromatographic tests) that can detect circulating antigens of adult female nematodes are currently available only for *D. immitis* and are recommended as the most sensitive by the American Heartworm Society (2014) because they are also useful in the detection of amicrofilaremic infections. However, cross-reactivity of some commercially available antigen tests for *D. immitis* with *Angiostrongylus vasorum* has been recently described and should be taken into consideration in endemic areas where parasites are sympatric (Schnyder and Deplazes, 2012, Krämer et al., 2014). Like in most of the countries from Eastern Europe, the current occurrence of filarial infections in dogs from Romania is still unclear. The first extensive epidemiological study was performed in 1933 by Popesco, who described 20 foci of canine filariasis along rivers in the south-west, south and south-east of the country, based on the visualization of microfilariae from blood samples. However, in the report above morphological details of the microfilariae were not provided. Later on, a nation-wide serological screening was performed for *D. immitis* (Mircean et al., 2012), but data regarding *D. repens* were only recorded locally, in 4

counties in the western (Ciocan et al., 2010, 2013), north-eastern (Paş ca et al., 2008) and southern (Tudor et al., 2013) regions of Romania. Furthermore, four dogs exported from Romania to Germany were positive for *D. repens*, confirmed by molecular methods (Pantchev et al., 2011). In addition, *A. reconditum* was also diagnosed in Germany in dogs exported from Romania (Hamel et al., 2012) and recently *C. bainae* has been reported in a dog from Danube Delta region (Ionică et al., 2014).

The objectives of the present study were to provide a more detailed view on filarial infections in dogs from Romania by assessing the prevalence and diversity of filarioid species infecting dogs from various areas of the country and to compare two different diagnostic tests employed for diagnosing *D. immitis*.

Materials and Methods

Study areas and sampling

From July 2010 to March 2011, 390 blood samples were collected from randomly selected owned dogs from 9 counties situated in 5 regions of Romania, presenting different ecological conditions. Most sampling sites were in rural localities, where dogs were housed outdoors and they generally did not receive regular antiparasitic treatments. With the owners' consent, samples were collected from the cephalic vein of each dog using a clotting activator S-Monovette syringe (SARSTEDT AG & Co, Germany). After separation, serum was collected into a separate, labeled tube and stored at -20˚C until further processing. The remaining blood clots were kept and stored under the same conditions.

Serological assays

Serum samples were tested for the presence of *D. immitis* antigen by using an in-clinic SNAP® 4Dx® test (IDEXX Laboratories, Inc., Westbrook, ME, USA), in accordance with the manufacturer's instructions. This is a rapid assay test system based on enzyme immunoassay technique, and has been validated for dogs, having a sensitivity of 99.2% (Chandrashekar et al., 2010). The *D. immitis* analyte is derived from antibodies specific to heartworm antigens, which are primarily produced by adult females (Weil, 1987).

Molecular assays

Genomic DNA was extracted from the blood clots using a phenol-chloroform method (Maslov et al., 1996, Albrechtová et al., 2011). Approximately 200 µl of clotted blood were dried at 56˚C for 30 min and then suspended in 1.5 ml lysis buffer (0.1 M NaCl, 0.05 M EDTA, 0.01 M Tris, 4.8% SDS; pH 8) and digested at 56˚C with 20 µl Proteinase K (Bioline, UK) for 1 hour. After proteins lysis, the mixture was extracted with a 1:1 blend of phenol and chloroform, followed by one extraction with chloroform alone. Each extraction was performed by 1 min of

shaking and a 10 min centrifugation step (13.000xg). DNA was precipitated with 96% ethanol for 15 min and the dried DNA pellet was re-suspended by adding 100 µl of PCR water.

Multiplex PCR-s amplifying partial cytochrome c oxidase subunit 1 (*cox*1) gene regions of different sizes for 3 filarioid species (*D. immitis* - 169bp, *D. repens* - 479 bp and *A. reconditum* – 589 bp) were performed using species-specific forward primers coupled with the reverse primer NTR, following reaction procedures and protocols described in literature (Latrofa et al., 2012). In each set of reactions, a positive control and a sample with no DNA were included in order to test the specificity of the reaction and to assess the presence of contaminants. The positive control sample was obtained by mixing DNA of all 3 filarioid species, isolated from blood of infected dogs and confirmed through sequencing. PCR products were visualized by electrophoresis in a 2% agarose gel stained with RedSafe™ 20000x Nucleic Acid Staining Solution (Chembio, UK) and their molecular weight was assessed by comparison to a molecular marker (O'GeneRuler™ 100 bp DNA Ladder, Thermo Fisher Scientific Inc., USA).

Statistical analysis

Data analysis was performed using EpiInfo 7 software (CDC, USA). The frequency of infection, prevalence and its 95% confidence intervals were established and the differences of prevalence between identified filarioid species and between the two *D. immitis* diagnostic tests were assessed using chi-square testing. The differences were considered significant if p values were lower than 0.05.

Serological (SNAP®) and molecular (multiplex PCR) methods used for *D. immitis* detection were evaluated in EpiTools (Sergeant, 2014). Agreement between SNAP and PCR was calculated using overall agreement measure and Cohen's Kappa statistic. As the overall agreement does not differentiate between the agreement on the positives and agreement on the negatives, positive and negative percent agreement were also calculated. A value of k <0 indicates no agreement, between 0 and 0.20 slight agreement, 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial agreement, and 0.81–1 almost perfect agreement (Landis and Koch, 1977).

Results

Molecular diagnosis

Out of 390 sampled dogs, 11.79% (n=46) were positive for DNA of at least one filarial species. The overall prevalence of each species was as follows: 6.15% (95% CI: 4.07-9.14%) for *D. immitis*, 6.92% (95% CI: 4.70-10.03%) for *D. repens* and 2.05% (95% CI: 0.96-4.16%) for *A. reconditum*, with significant local variation (Table 1). The prevalence of *A. reconditum* was significantly lower (p<0.001) compared to *Dirofilaria* spp., but its

distribution range was more extended (Fig. 1). Coinfections with *D. immitis* and *D. repens* were detected in 23.91% (n=11) of positive dogs and those with *D. repens* and *A. reconditum* in 4.34% (n=2) of the positive dogs.

Serology

Meanwhile, 7.18% (95% CI: 4.91-10.33) of dogs, deriving from 3 counties were seropositive for *Dirofilaria immitis* antigen at SNAP tests.

Method comparison

Overall, 9.48% (n=37) of dogs were positive for *D. immitis* at least in one of the performed tests. Immunoenzymatic tests have shown a slightly higher prevalence of *D. immitis* infection in dogs compared to the molecular method (Table 2), but without statistically significant differences ($p>0.5$). However, when considering each positive individual (Table 3), the positive percent agreement was in fact of 40.54%. The negative percent agreement was of 90.51% and overall agreement scored 94.36%. The *k* value was of 0.55 (0.38 - 0.72), indicating a moderate agreement between the two tests. Discordant results consisted in: (*i*) 6 samples (16.21%) were positive only for heartworm antigen; (*ii*) 7 samples (18.91%) were positive for *D. immitis* antigen but tested positive for *D. repens* at molecular methods; (*iii*) 9 (24.32%) samples were antigen negative in animals which scored positive for *D. immitis* at PCR.

Discussion

In Romania, data from the first half of the 20th Century (Popovici, 1916, Popesco, 1933, 1935) suggest that Southern regions are historically endemic for canine dirofilariasis, even if it is unclear exactly which filarial species the authors were referring to. The climate of this country is temperate-continental of transitional type, with four clearly defined seasons varying at regional level according mainly to altitude. In this context, periods when the development of both *D. immitis* and *D. repens* can occur are longest (May-October) in the south and south-east, followed by the west and south-west (May-September) and shorter (June-August or September) in the rest of the country (Genchi et al., 2011). The highest prevalence rates of both *Dirofilaria* species were recorded in the counties that include the Danube's floodplains, where the climate is suitable for the development of confirmed vector species (i.e. *Anopheles maculipennis*, *Culex pipiens*) (Nicolescu et al., 2003). Similar to the situation described by Popesco (1933), *D. immitis* was only identified in proximity of major rivers (i.e. Olt in Braş ov county and Danube in Dolj, Teleorman and Tulcea counties) whereas *D. repens* had a wider distribution range. Indeed, where both species occur in sympatry, the prevalence of *D. repens* generally exceeded that of *D. immitis*, as also recorded in other parts of Europe (Genchi et al., 2011). This might be the effect of a protective cross-immunity at individual level, as

inferred by the experimental infection of dogs initially infected with *D. repens*, in which the ability of *D. immitis* to develop was reduced (Genchi et al., 1995).

The distribution of *A. reconditum* is herein investigated for the first time in Romania and it seems to occur in a large territory, despite its relatively low prevalence. Transmission of this filarioid is via fleas or lice (Nelson, 1962) and requires proximity between the infected and non-infected dogs (Brianti et al., 2012). Compared to *Dirofilaria* spp., this species appears to be better adapted to dry areas (Constanţa county) and colder climate (Vâlcea county), but so far its development in the vector in relation to temperature or other climatic factors has not been assessed.

Three types of apparent inconsistencies between serological SNAP tests and PCR diagnosis have been identified in the diagnosis of *D. immitis*, but their frequency was significantly lower ($p<0.005$) than the level of agreement between the two tests.

The most frequently encountered situation (17.30%, n=9) was that animals negative for *D. immitis* at SNAP test were positive for *D. immitis* DNA, which suggests the presence of microfilariae or soluble genomic DNA in the blood at sampling time. This may be due to a low number of adult worms, e.g. 1-2 gravid females, previous adulticidal treatment or delayed antigenaemia based on low worm burdens and chemoprophylaxis (Courtney and Zeng 2001, Nelson et al., 2005, Pantchev et al., 2011). Furthermore, given that *D. immitis* microfilariae have a life span of up to 2.5 years (Abraham, 1988), they could persist after the natural death of adult females. Moreover, recent data has revealed that heating the serum samples before laboratory processing, renders more sensitivity to the SNAP test, suggesting the existence of inhibitors of a yet unknown nature in the serum (Little et al., 2014, Velasquez et al., 2014).

Interestingly, 13.46% (n=7) of samples were positive for *D. immitis* antigen while molecular methods identified only *D. repens*. Since the possibility of cross-reaction between the two species was excluded by Pantchev et al. (2009b, 2011), this finding may suggest the occurrence of a patent *D. repens* infection associated with an occult *D. immitis* infection, revealing an interesting pattern that deserves further investigation. Since the actual relationship between the two *Dirofilaria* species has only been partially studied (Genchi et al., 1995), these results could represent the outcome of a potential inhibition of *D. immitis* microfilarial production by the presence of *D. repens* and the host immune responsiveness. In addition, a small number of *D. immitis* microfilariae present in the peripheral blood could fall under the detection limit of the employed PCR (i.e. 26 microfilariae/ml; Latrofa et al., 2012). As microfilaremia fluctuates during the day, false negative results may emerge according to the sampling time. For both species, the periodicity of microfilariae has been assessed and it seems to vary not only with external factors like the feeding behavior of vector species (indicating the optimum sampling time is during the evening and

night), but also with internal (host-related) factors, like the blood oxygen pressure, which decreases while the animal is sleeping, determining a rise in microfilaremia (Hawking, 1956, Aoki et al., 2011, Di Cesare et al., 2013).

Some samples (11.53%, n=6) were positive only for *D. immitis* antigen and negative for all filarioid species by PCR, which may indicate occult (amicrofilaremic) infections, such as in the case of prepatency period, unisexual infection, drug-induced sterility of adults, or immune-mediated clearance of microfilariae (Rawlings et al., 1982). Another potential explanation would be a cross-reaction with antigen of the "French" heartworm *Angiostrongylus vasorum* (Schnyder and Deplazes, 2012). This parasite has not been reported in Romania so far, but models show that parts of the country may be included in its distribution range (Morgan et al., 2009). Nowadays, a revised version of the test system used for the current study, SNAP® 4Dx® Plus (IDEXX Laboratories, Inc., Westbrook, ME, USA), which does not show any cross-reactivity between *D. immitis* and *A. vasorum* (Schnyder and Deplazes, 2012) and a specific rapid *A. vasorum* device (Schnyder et al., 2014), that were not on the market when testing for the present study was performed, are commercially available.

Failure of serological tests in detecting patent infections may have serious implications in the spreading of the disease and in the clinical outcome, so they should not be used as the only screening method in epidemiological studies. Since molecular methods offer the possibility to identify more filarioid species, we regard them as a necessary additional screening tool for surveillance, also taking into consideration of the zoonotic potential of *D. repens*.

Conclusion

The present study shows that in Romania *Dirofilaria* species are commonly present in the south and south-east of the country and *D. repens* is the most common canine filarioid species. The current study is the first to provide a more extensive overview on the prevalence and geographical distribution of *A. reconditum* in dogs from Romania.

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Disclosure

There are no commercial associations for author N.P. of IDEXX Laboratories, there is no commercial conflict of interest since the information generated here is solely for scientific dissemination. There are no conflicts of interest to report by any author.

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TABLE CAPTIONS

Table 1. Molecular prevalence of filarial species for each county

Table 2. Seroprevalence and molecular prevalence of *D. immitis* for each county

Table 3. The complete filarial profile of each positive dog

FIGURE CAPTIONS

Fig.1 Distribution of identified filarioid species, established by both employed tests

Table 1
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County	*D. immitis*		*D. repens*		*A. reconditum*	
	pos/tot (%)	**95% CI**	**pos/tot (%)**	**95% CI**	**pos/tot (%)**	**95% CI**
Hunedoara	0/62	-	0/62	-	0/62	-
Alba	0/37	-	0/37	-	0/37	-
Braş ov	2/13 (15.38)	1.92-45.45	0/13	-	0/13	-
Argeş	0/46	-	1/46 (2.17)	0.06-11.53	1/46 (2.17)	0.06-11.53
Teleorman	7/51 (13.73)	5.70-26.25	5/51 (9.80)	3.26-21.41	1/51 (1.96)	0.05-10.45
Vâlcea	0/43	-	0/43	-	1/43 (2.33)	0.06-12.29
Dolj	4/51 (7.84)	2.18-18.88	7/51 (13.73)	5.70-26.26	1/51 (1.96)	0.05-10.45
Tulcea	11/69 (15.94)	8.24-26.74	13/69 (18.84)	10.43-30.06	2/69 (2.90)	0.35-10.08
Constanţ a	0/18	-	1/18 (5.56)	0.14-27.29	2/18 (11.11)	1.38-37.41
Total	24/390 (6.15)	4.07-9.14	27/390 (6.92)	4.70-10.03	8/290 (2.05)	0.96-4.16

Table 2
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County	*D. immits* Ag		*D.immitis* DNA		p
	pos/tot (%)	95% CI	pos/tot (%)	95% CI	
Hunedoara	0/62	-	0/62	-	-
Alba	0/37	-	0/37	-	-
Braş ov	0/13	-	2/13 (15.38)	1.92-45.45	0.48
Argeş	0/46	-	0/46	-	-
Teleorman	3/51 (5.88)	1.23-16.24	7/51 (13.73)	5.70-26.25	0.31
Vâlcea	0/43	-	0/43	-	-
Dolj	7/51 (13.72)	5.70-26.26	4/51 (7.84)	2.18-18.88	0.52
Tulcea	18/69 (26.08)	16.25-38.06	11/69 (15.94)	8.24-26.74	0.14
Constanţ a	0/18	-	0/18	-	-
Total	28/390 (7.18)	4.91-10.33	24/390 (6.15)	4.07-9.14	0.66

Table 3
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No.	County	Ag test	Multiplex PCR		
			D. immitis	*D. repens*	*A. reconditum*
1	Argeş	neg	neg	**pos**	neg
2	Argeş	neg	neg	neg	**pos**
3	Braş ov	neg	**pos**	neg	neg
4	Braş ov	neg	**pos**	neg	neg
5	Constanţ a	neg	neg	**pos**	**pos**
6	Constanţ a	neg	neg	neg	**pos**
7	Dolj	neg	neg	**pos**	neg
8	Dolj	neg	neg	**pos**	neg
9	Dolj	neg	neg	**pos**	neg
10	Dolj	neg	neg	neg	**pos**
11	Dolj	**pos**	neg	neg	neg
12	Dolj	**pos**	neg	neg	neg
13	Dolj	**pos**	neg	**pos**	neg
14	Dolj	**pos**	**pos**	neg	neg
15	Dolj	**pos**	**pos**	**pos**	neg
16	Dolj	**pos**	**pos**	**pos**	neg
17	Dolj	**pos**	**pos**	**pos**	neg
18	Teleorman	neg	neg	neg	**pos**
19	Teleorman	neg	**pos**	neg	neg
20	Teleorman	neg	**pos**	neg	neg
21	Teleorman	neg	**pos**	neg	neg
22	Teleorman	neg	**pos**	**pos**	neg
23	Teleorman	neg	**pos**	**pos**	neg
24	Teleorman	**pos**	neg	**pos**	neg
25	Teleorman	**pos**	**pos**	**pos**	neg
26	Teleorman	**pos**	**pos**	**pos**	neg
27	Tulcea	neg	neg	**pos**	neg
28	Tulcea	neg	neg	**pos**	neg
29	Tulcea	neg	neg	**pos**	neg
30	Tulcea	neg	neg	**pos**	neg
31	Tulcea	neg	neg	neg	**pos**
32	Tulcea	neg	**pos**	neg	neg
33	Tulcea	neg	**pos**	**pos**	neg
34	Tulcea	**pos**	neg	neg	neg
35	Tulcea	**pos**	neg	neg	neg
36	Tulcea	**pos**	neg	neg	neg
37	Tulcea	**pos**	neg	neg	neg
38	Tulcea	**pos**	neg	**pos**	neg
39	Tulcea	**pos**	neg	**pos**	neg
40	Tulcea	**pos**	neg	**pos**	neg
41	Tulcea	**pos**	neg	**pos**	neg
42	Tulcea	**pos**	neg	**pos**	**pos**
43	Tulcea	**pos**	**pos**	neg	neg
44	Tulcea	**pos**	**pos**	neg	neg
45	Tulcea	**pos**	**pos**	neg	neg
46	Tulcea	**pos**	**pos**	neg	neg
47	Tulcea	**pos**	**pos**	neg	neg
48	Tulcea	**pos**	**pos**	neg	neg
49	Tulcea	**pos**	**pos**	**pos**	neg
50	Tulcea	**pos**	**pos**	**pos**	neg
51	Tulcea	**pos**	**pos**	**pos**	neg
52	Vâlcea	neg	neg	neg	**pos**

Figure 1
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