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Isolation of a Multidrug-Resistant *vanA*-Positive *Enterococcus faecium* Strain from a Canine Clinical Sample in Greece

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Abstract: An *Enterococcus faecium* strain was obtained from a paraprostatic cyst of a 17-year-old dog in Greece. Antibiotic susceptibility testing (AST) was accomplished by disc diffusion and MIC methods, and the isolate demonstrated a multidrug-resistant (MDR) phenotype against a great variety of antibiotics, such as β-Lactams, Quinolones, Macrolides, Tetracyclines, Rifampin, Nitrofurantoin, and surprisingly, Glycopeptides, Fosfomycin and Gentamicin (high-level). Molecular screening for Vancomycin resistance genes was carried out, and a *vanA* gene cluster was identified. To our knowledge, this is the first report of a *vanA*-positive *E. faecium* strain isolated from a companion animal in Greece. Importantly, this strain was related with the presence of paraprostatic cysts, a pathological condition requiring treatment. The presence of a highly resistant isolate in a canine clinical sample and the consequent need for treatment constitutes a new challenge for veterinarians due to the lack of available treatment options. Our findings indicate the occurrence of respective bacteria in companion animals, which could act as a reservoir of epidemic MDR strains or relevant mobile genetic elements (MGE) in the community, constituting a threat for public health.

Keywords: *Enterococcus*; dog; resistance; vancomycin; teicoplanin; fosfomycin; *vanA*

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1. Introduction

Enterococci are Gram-positive facultative anaerobic cocci that were classified as group D Streptococci until the 1980s [1]. They can be easily obtained from a wide variety of hosts [2]. There are at least 58 recognized species so far, with *E. faecium* and *E. faecalis* being more regularly associated with clinical infections [1]. These species are included in ESKAPE organisms (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) which, notably, have been demonstrated by World Health Organization (WHO) as a rising threat for public health due to multidrug resistance and the challenging nosocomial infections they cause [3].

Enterococci are commonly isolated from dogs, especially *E. faecium* and *E. faecalis* which are usually reported as the predominant species [4–9]. They have been associated with cases of canine pathological conditions, mainly urinary tract infections (UTIs) at a notable rate [10]. Furthermore, a matter of concern arises as multidrug-resistant strains are frequently isolated [8–14].

A variety of factors contribute to the acquisition of resistance in Enterococci. Concerning the antibiotics used in veterinary medicine, the most important aspects are described in Table 1.

Table 1. Main mechanisms of resistance in Enterococci.

Antibiotic Class	Main Mechanisms of Resistance [1]	References
B-lactams	• Production of PBPs [2] that demonstrate a lower binding affinity to agents of this class, such as Ampicillin and Cephalosporins. • Overproduction or mutations of PBPs.	[15–20]
Glycopeptides	Amino-acid substitutions in specific precursors of peptidoglycan, decreasing the binding affinity of glycopeptides to them by 7- to 1000-fold. A variety of respective gene clusters has been identified, such as *vanA*, *vanB*, *vanC*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM* and *vanN*.	[20–23]
Aminoglycosides	Enzymatic inactivation of Gentamicin, Streptomycin, or both of them, mediated by acquired ARGs, confers high-level aminoglycoside resistance (HLAR) to Enterococci, while they are intrinsically resistant against the other agents of this class, escaping their bactericidal action by variable procedures.	[20,24,25]
Tetracyclines	• Ribosomal protection encoded by genes tet(M), tet(O) and tet(S) results in resistance against all the available agents of this class in veterinary medicine (Tetracycline, Doxycycline and Minocycline). • Efflux proteins encoded by specific genes, such as *tet(K)* and *tet (L)* confer resistance against Tetracycline.	[26]
Quinolones	Mutations of the target genes of the antibiotics, *gyrA* and *parC*, confer high-level acquired resistance, while Enterococci express low levels of resistance to Quinolones intrinsically.	[20,27]
Rifampin	Mutations of the *rpoB* gene and consequently substitutions in the β-subunit of the RNA polymerase, which is the target of this agent.	[28]
Macrolides	Production of a methyltransferase that alternates the 23S rRNA subunit, inhibiting the binding of the antibiotic, and is mediated by *erm* genes (and specifically *ermB*).	[29]

[1] The mechanisms described here are the more frequently encountered. More resistance mechanisms have been described in the literature. [2] Penicillin binding proteins, membrane proteins essential for the peptidoglycan biosynthesis. B-lactam antibiotics act by a covalent binding to them.

Limited data exist on the detection of vancomycin-resistant Enterococci strains of canine origin worldwide. In this study, we report the first case of a vanA-positive *E. faecium* strain with MDR phenotype obtained from the paraprostatic cyst of a 17-year-old dog in Greece, and we discuss the challenges faced by veterinarians when dealing with MDR strains and rising public health concerns.

2. Materials and Methods

2.1. Origin of the Isolate

A 17-year-old, male mongrel dog was admitted to a veterinary clinic in Thessaloniki, Greece, in January 2023. The dog had an open fracture in the radius as a result of a car accident, which had occurred 25 days earlier (Supplementary File, Figure S1a). In the intervening period, the dog received an antibiotic treatment consisting of marbofloxacin and clindamycin for about 20 days. During the clinical examination, paraprostatic cysts were also detected and demonstrated using diagnostic imaging (Supplementary File, Figure S1b). Drainage of the cysts was carried out, samples were received and sent for investigation. Aerobic and anaerobic cultures were accomplished after inoculation on sheep blood agar and MacConkey agar and a 24 h incubation at 37 °C; *Enterococcus* spp. were isolated. The strain was initially identified by phenotypic and biochemical tests: Gram-positive cocci with characteristic colonial appearance (small colonies of approximately 1 mm with gamma-hemolysis), oxidase- and catalase-negative, aesculin-hydrolysis positive, no growth on MacConkey agar, and no sorbitol fermentation. Results were confirmed using the VITEK 2 biochemical identification system (Biomerieux, Supplementary File, Figure S2).

2.2. Antibiotic Susceptibility Testing

The disk diffusion method was used to evaluate susceptibility or resistance to a variety of antibiotics routinely tested in clinical samples of companion animals. Briefly, a colony of the strain was added to saline, and the resulting suspension was compared to a

McFarland standard tube in order to achieve a 0.5 McFarland turbidity. The suspension was vortexed and, subsequently, a sterile swab was used to inoculate a quantity of it on the surface of Mueller–Hinton agar plates. Susceptibility discs were added, and the plates were incubated at 35 °C for 16–18 h. For the evaluation of Vancomycin zone diameter, a 24 h incubation period was essential. Due to the multidrug-resistant phenotype of the *Enterococcus* isolate, additional antibacterial agents were added to the antibiotic susceptibility test (AST). Consequently, the minimum inhibitory concentration (MIC) method was also evaluated (VITEK2, Biomerieux), including some antibacterial agents strictly used in human medicine, to confirm the previous results and to identify the isolate's resistance profile. The contents of the disks, the zone diameter, and the MIC breakpoints, as specified by the CLSI documents [30,31], are available in Table 2.

Table 2. Antibiotics, disc contents and breakpoints used in this study.

Antibacterial Agent	Disk Content (μg)	Zone Diameter Breakpoints (mm)	MIC Breakpoints (μg/mL)
Ampicillin	10	S: ≥17, R: ≤16	S: ≤8, R ≥ 16
Amoxicillin + Clavulanate	20 + 10	NA1	NT
Ampicillin + Sulbactam	10 + 10	NA1	NT
Imipenem	10	NA1	NT
Gentamicin [1]	120	S: ≥10, I:7–9, R: ≤6	500 [2]
Streptomycin [1]	300	S: ≥10, I:7–9, R: ≤6	1000 [2]
Ciprofloxacin	5	S: ≥21 I:16–20, R: ≤15	S: ≤1, I:2, R ≥ 4
Tetracycline	30	S: ≥19 I:15–18, R: ≤14	NT
Doxycycline	30	S: ≥16 I:13–15, R: ≤12	NT
Minocycline	30	S: ≥19 I:15–18, R: ≤14	NT
Florfenicol	30	NA2	NT
Chloramphenicol	30	S: ≥18 I:13–17, R: ≤12	NT
Fosfomycin	200	S: ≥16 I:13–15, R: ≤12 [3]	NT
Nitrofurantoin	300	S: ≥17 I:15–16, R: ≤14	NT
Rifampin	5	S: ≥20 I:17–19, R: ≤16	NT
Erythromycin	15	S: ≥23 I:14–22, R: ≤13	NT
Vancomycin	30	S: ≥17 I:15–16, R: ≤14	S: ≤4, I: 8–16, R ≥ 32
Teicoplanin	30	S: ≥14 I:11–13, R: ≤10	S: ≤8, I:16, R ≥ 32
Daptomycin	-	NT	SDD: ≤4, R ≥ 8
Quinupristin/Dalfopristin	-	NT	S: ≤1, I:2, R ≥ 4
Linezolid	30	S: ≥23 I:21–22, R: ≤20	S: ≤2, I:4, R ≥ 8

S: Susceptible, I: intermediate, R: resistant. NA1: Breakpoints not available, susceptibility was evaluated based on absence of inhibition zone. NA2: breakpoints not available, susceptibility was evaluated based on Chloramphenicol breakpoints. NT: not tested. SDD: susceptible-dose dependent, as defined by the related CLSI document [30]. [1] Test for detection of high-level aminoglycoside resistance. [2] Any Growth = resistant. [3] Breakpoints for *E. faecalis* were used due to lack of respective breakpoints for *E. faecium*.

2.3. Molecular Screening for Vancomycin-Resistance Genes

Whole genomic DNA extraction from the presumptive strain exhibiting Vancomycin resistance was performed using a commercial spin-column kit (NucleoSpin; Macherey-Nagel) according to the manufacturer's instructions. Multiplex PCR analyses was performed by amplification with primers specific for the *vanA*, *vanB*, *vanC1-C2*, *vanD*, *vanE*, *vanG*, *ddl-Enterococcus faecium* and *ddl-Enterococcus faecalis* genes, as previously described (Table 3) [32,33]. Briefly, for the reaction, a 50 μL mix was used containing 5 μL 10× PCR buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl], 1.5 μL $MgCl_2$ (50 mM), 2 μL dNTPs (10 mM, Nucleotide Mix), 2 μL of each of the primer pairs (10 μM), 0.3 μL (5 U/μL) Taq DNA Polymerase (Invitrogen, Carlsbad, CA 92008, USA), 2 μL of sample DNA and 7.2 μL nuclease-free water. For positive controls, Vancomycin-resistant *Enterococcus* reference strains were used (Institute Pasteur, France). Amplification was carried out in a T100 Thermal Cycler (Biorad, Hercules, CA, USA) under the following thermal cycling conditions: initial denaturation for 3 min at 94 °C and 30 cycles of amplification consisting of 1 min at 94 °C (denaturation), 1 min at 54 °C (annealing), and 1 min at 72 °C (elongation), with

7 min at 72 °C for the final extension. DNA products were identified by electrophoresis in 0.5 Tris-borate-EDTA on a 1.5% agarose gel stained with ethidium bromide solution.

Table 3. Primers used in this study [32,33].

Primer	Sequence (5′→3′)	Size of PCR Product (bp)
vanA(+)	GGGAAAACGACAATTGC	732
vanA(−)	GTACAATGCGGCCGTTA	
vanB(+)	ACGGAATGGGAAGCCGA	647
vanB(−)	TGCACCCGATTTCGTTC	
vanC1/2(+)	ATGGATTGGTAYTKGTAT	815/827
vanC1/2(−)	TAGCGGGAGTGMCYMGTAA	
vanD(+)	TGTGGGATGCGATATTCAA	500
vanD(−)	TGCAGCCAAGTATCCGGTAA	
vanE(+)	TGTGGTATCGGAGCTGCAG	430
vanE(−)	ATAGTTTAGCTGGTAAC	
vanG(+)	CGGCATCCGCTGTTTTTGA	941
vanG(−)	GAACGATAGACCAATGCCTT	
ddl *E. faecalis*(+)	CACCTGAAGAAACAGGC	475
ddl *E. faecalis*(−)	ATGGCTACTTCAATTTCACG	
ddl *E. faecium*(+)	GAGTAAATCACTGAACGA	1091
ddl *E. faecium*(−)	CGCTGATGGTATCGATTCAT	

3. Results

3.1. Antibiotic Susceptibility Testing

Results of the AST are presented in Table 4. Relevant images and reports are included in the Supplementary File (Figures S3 and S4). The isolate was multidrug-resistant (MDR). More specifically, it expressed a resistant phenotype against all the β-Lactams tested (Ampicillin, Amoxicillin–Clavulanate, Ampicillin–Sulbactam, Imipenem), Ciprofloxacin, Tetracycline, Doxycycline, Minocycline, Erythromycin, Rifampin, Fosfomycin, Nitrofurantoin, Vancomycin, Teicoplanin and Gentamicin (high-level).

Table 4. Results of the AST for the *E. faecium* isolate.

Antibacterial Agent	Result of AST
Ampicillin	R [1,2]
Amoxicillin + Clavulanate	R [1]
Ampicillin + Sulbactam	R [1]
Imipenem	R [1]
Gentamicin (HL)	R [1,2]
Streptomycin (HL)	S [1,2]
Ciprofloxacin	R [1,2]
Doxycycline	R [1]
Minocycline	R [1]
Tetracycline	R [1]
Florfenicol	S [1]
Chloramphenicol	S [1]
Fosfomycin	R [1]
Nitrofurantoin	R [1]
Rifampin	R [1]
Erythromycin	R [1]
Vancomycin	R [1,2]
Teicoplanin	R [1,2]
Quinupristin/Dalfopristin	S [2]
Daptomycin	SDD [2]
Linezolid	S [1,2]

[1] AST result by disc diffusion method. [2] AST result by MIC method.

Limited agents were effective in vitro against the *E. faecium*, such as Phenicols, Linezolid, Streptomycin (high-level), Daptomycin and Quinupristin/Dalfopristin.

3.2. Multiplex PCR

The isolate was identified as *E. faecium.* Moreover, the *vanA* gene cluster was detected, confirming the Glycopeptide-resistant phenotype (Figure 1). None of the other antibiotic resistance genes (ARGs) included in the test were identified (*vanB*, *vanC1-C2*, *vanD*, *vanE*, *vanG*).

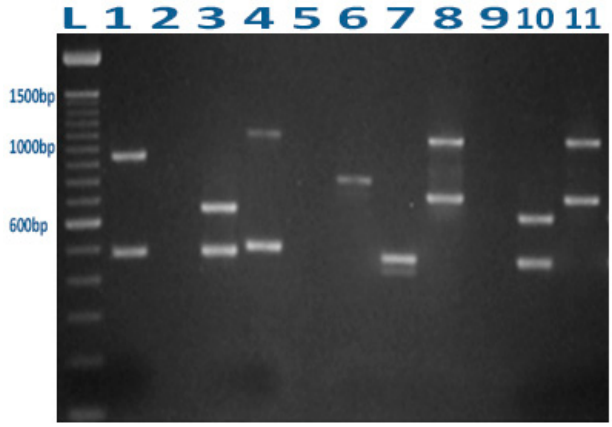

Figure 1. Multiplex PCR gel electrophoresis image with positive controls (PC), negative controls (NC) and canine positive sample. L: Ladder; Line 1: *E. faecalis* vanG PC; Line 2: NC; Line 3: *E. faecalis* vanB PC; Line 4: *E. faecium* vanD PC; Line 5: NC; Line 6: *E. gallinarum* vanC PC; Line 7: *E. faecalis* vanE PC; Line 8: *E. faecium* vanA PC; Line 9: NC; Line 10: *E. faecalis* vanB PC; Line 11: canine positive sample.

4. Discussion

4.1. The Importance of Glycopeptide Resistance in a Canine Clinical Isolate

There are limited data about Vancomycin-resistant Enterococci in companion animals worldwide. To our knowledge, in Greece, this is:

- The first report of a *VREf* isolate from a companion animal.
- The first report of a *VREf* isolate causing an infection in any animal.

Additionally, this was the first *Enterococcus* spp. strain detected by the research team, among approximately 1072 isolates from clinical samples of companion animals, during the last five years, demonstrating Glycopeptide resistance, when tested by disc diffusion method.

Furthermore, the *vanA*-mediated high-level Glycopeptide-resistance of the strain, requires greater attention due to the co-current phenotypic resistances which were detected. This MDR profile is of major significance for two reasons.

Initially, there was a lack of available agents routinely used in veterinary practice for an effective treatment. For example, the respective CLSI document for veterinary isolates [31], in the breakpoints tables for *Enterococcus* spp., includes agents against which this isolate is phenotypically resistant (Penicillin, Ampicillin, Erythromycin, Rifampin, Vancomycin, Tetracycline, Doxycycline, Minocycline, Nitrofurantoin), with the exception of Chloramphenicol. Regarding Phenicols, even though they have been used in the past against Vancomycin-resistant Enterococci [34,35], their use is not regular nowadays (especially in human medicine) due to side effects (myellosupression, aplastic anaemia) and emerging resistance [36,37]. The identified high-level Gentamicin resistance is an additional notable aspect, as it is not usually observed in high rates among Enterococci of canine origin, even MDR strains or *VRE* [6,8,38–41].

Moreover, the colonization of companion animals with respective MDR strains creates concerns regarding the transmission of these bacteria to their owners due to their accommodation in household environments.

Regarding the current literature, *VRE* were isolated from canine samples in a number of studies worldwide [4,6,8,11–14,38,42–45], but in the majority of these cases, screening of normal faecal samples using specific media was performed in order to obtain the relevant strains, and the references of bacteria originated from clinical samples are undoubtetly limited [11,14,43,44].

These things considered, resistance to Vancomycin was not identified in several other studies including Enterococci populations of canine origin [10,39–41,46–51]. Even in cases of phenotypic resistance, the relevant genes were not always detected [52]. Furthermore, in some instances, the acquired mechanisms of resistance were not identified among *VRE* [53,54], as intrinsic resistance (low-level vanC1-mediated resistance) exists in specific species of Enterococci.

4.2. Possible Factors Enhancing the Prevalence of VRE in a Companion Animal

Several causes related to the generic prevalence of such stains in the community, host affecting factors and bacterial adjustment properties could provoke the colonization of a dog by *VRE*.

The use of the glycopeptide Avoparcin as a growth promoter in food-producing animals, until its prohibition (1997 in EU), was related with the emergence of *VRE* in animals worldwide [55]. Since more than 25 years have passed though, the effect of its use is hopefully not a significant current factor for the *VREf* prevalence.

Prior exposure to several antibiotics has been described to provoke VRE colonization of human patients in several studies. Vancomycin, Cephalosporins, Aminoglycosides, Carbapenems, and Antianaerobic Agents, such as clindamycin and metronidazole, are some of the main associated agents [56–60]. Moreover, co-selection of resistance and a genetic linkage between Vancomycin and Macrolides has been identified for *E. faecium* in livestock animals [61,62]. As Cephalosporines, Aminoglycosides, Macrolides, Metronidazole, and Clindamycin are agents widely used in companion animals, the danger of *VRE* colonization of dogs through a co-selection reinforced by other antibiotics is significant.

Horizontal transfer of MGE and spread of epidemic clones enhance the prevalence of MDR Enterococci worldwide. The identification of a variety of unique strains is supported by the hypothesis that Vancomycin resistance could have initially emerged more by horizontal spread of MGE carrying the *vanA* and, perhaps, *vanB* gene cluster, among enterococci, rather than by transmission of a few major clones [63–65]. However, the spread of specific related clones with nosocomial infections has occurred in the last few years, and many of them are well characterized [66–68]. Companion animals could become a factor in a circulation of such strains in a community, as is in some cases *VRE* isolates from dogs demonstrating similar genetic lineages to hospital-acquired infections in humans [11,38,43].

Moreover, Enterococci possess the ability to develop resistance by facilitating survival in the environment of the gastrointestinal track; therefore, through intestinal colonization, the rise and spread of a multidrug-resistant clone among different hosts becomes possible, indicating a serious challenge for public health [69,70].

In accordance with all these, the gastrointestinal colonization of the dog by the *VREf* isolate in this study is possible, as Enterococci are species commonly detected in canine flora [6,7,10] and the host's own flora is usually the source of infection in prostatic and paraprostatic tissues [71,72]. The prior long-lasting antibiotic treatment could be a reinforcing factor, as the isolate is resistant to both Quinolones and Clindamycin and, therefore, its prevalence had been possibly enhanced by these agents. Finally, the presence of a mobile genetic element that could mediate an MDR phenotype to additional strains or the spreading of a specific hospital-associated MDR strain, as the animal is colonized, is definitely a matter of concern, indicating the need of surveillance in case of similar events.

4.3. Previous Research and Relevant Data in Greece

In the literature regarding *VRE* in Greece, data are mainly associated with human medicine and food-producing animals. In studies related to hospital environment, Vancomycin-resistant

isolates were mostly identified as *E. faecium* with *vanA*-type resistance [73–76]. Furthermore, a link has been detected between *VRE* colonization and exposure to agents such as Vancomycin, Piperacillin–Tazobactam, Carbapenems, Antianaerobic Agents and Quinolones [75,77], while the duration of treatment with the respective antibiotics was an additional factor [77].

In reference to livestock animals, a 21.1% resistance rate to Vancomycin was detected in Enterococci isolated from raw pork meat from 2004 to 2007 [78]. Pigs, hospital and urban wastewater were screened for *VRE* in 2005–2006. *VanA*-positive *E. faecium* was dominant among the isolates, and a genetic diversity between Enterococci of different origins was identified [79]. In another study, samples from broilers and poultry slaughterers were collected during 2005–2008, and 130 *VRE* were recovered. The majority of these isolates were *E. faecium* harboring *vanA* gene, whilst no relationship was identified between poultry and the respective human-*VREf* clinical isolates originated from two hospitals in Greece [33].

Concluding, *vanA*-positive *E. faecium* seems to be the prevalent glycopeptide-resistant *Enterococcus* spp. encountered in the country and the main threat for public health. Mobile genetic elements are, rather, the cause of spreading of resistance, as genetic diversity is present among hospital-acquired and community strains. Finally, the induction of *VREf* colonization of hosts, is possibly related with prolonged usage of specific antibiotics.

The findings of our study are in accordance with these data, as a *vanA*-type *VREf* was isolated, the presence of a plasmid-mediated resistance is suspected, and a prior long-lasting treatment with clindamycin and marbofloxacin had occurred.

A noteworthy fact is that in the studies that referred to both human and animal samples, HLGR (which is identified in the isolate from this study) was related with hospital/human-associated strains, as it was rarely observed in samples of animal origin, and a significant statistical difference was detected [33,79].

4.4. Fosfomycin Resistance

A specific mention should be carried out for the Fosfomycin-resistant phenotype of this isolate, as it is an agent infrequently used in dogs, and, to our knowledge, the dog from this study had never received the antibiotic.

Fosfomycin has potentialities as an alternative agent against *VRE*, alone or combined with other agents [80–84]. In previous studies searching Fosfomycin resistance in *VRE*, it was mediated by the *fosB* gene (one or multiple copies) located in transferable plasmids, in all the isolates tested. A physical link between the *fosB* and Vancomycin ARGs (*vanA* or *vanM)* was detected, emphasizing the need of Fosfomycin-resistance surveillance in *VRE* [85–88]. Moreover, an amino acid substitution on the agent's active site of MurA protein has been detected in *VREf* expressing high-level Fosfomycin resistance [89]. The Fosfomycin-resistant phenotype in this isolate indicates a possible occurrence of one of the previously described mechanisms. The presence of a plasmid co-conferring Fosfomycin and glycopeptide resistance would definitely be a more significant issue and should be further investigated.

5. Conclusions

The isolation of a *VREf* co-expressing resistance to a wide spectrum of antibiotics from a canine sample is undoubtedly a matter of concern. This highly resistant phenotype is rarely encountered in community strains, whereas it is more common in hospital-associated ones. Moreover, the site of the sample, a contaminated paraprostatic cyst, is indicative of the isolate's origin from the host's own flora. A possible colonization of companion animals by similar strains raises an issue for public health, enhancing the prevalence and the circulation of MDR epidemic strains and respective MGEs between pets, owners, and their environment. Variable factors could contribute to this spreading, such as the prolonged and excessive usage of antibacterial agents in human and veterinary medicine. Surveillance

measures are essential for the accomplishment of a comprehensive investigation of these factors, which could provide us the appropriate preventive actions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microbiolres14020042/s1, Figure S1: X-ray image from the dog; Figure S2. Vitek 2 Biochemical Identification Report; Figure S3. Petri dishes of Disc Diffusion Test; Figure S4. Vitek 2 MIC Report.

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