Research Article

Molecular Detection of *vapA* and *vapB* Genes in *Rhodococcus equi* Isolated from Human and Horses by Multiplex PCR in Baghdad

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Abstract | This study was conducted for Isolation and molecular characterization of *Rhodococcus equi* (*R. equi*) isolates from human and horse specimens. A total of 203 samples (100 human sputum and 103 horses' faecal samples) were collected from different places in Baghdad, Iraq during november 2014 to april 2015. The samples were streaked on nalidixic acid–novobiocin–actidione cycloheximide–potassium tellurite (NANAT) agar, then the suspected colonies were subcultured on nutrient and blood agars. Confirmation as *R equi* was done by assessing the colony on the basis of their morphology and through staining and biochemical tests, followed by testing for the presence of the virulence-associated protein antigen *vapA* and *VapB* genes by multiplex PCR. Culture examination of 103 horses' faeces samples revealed the isolation of 7 (6.79%) *R. equi*, all of them 14.71% (7/49) were obtained from Equestrian club in Al- Ameria location and the bacteriological examination of the 100 human sputum samples showed the recovery of 4 (4.00%) isolates of *R equi*. The results showed that only 57.14% (4/7) of the *R. equi* isolates from horses' samples were *vapA* positive. All the 11 isolates included in this study were negative for *vapB* gene. This is the first study in Iraq to confirm the presence of *vapA* gene in *R. equi* isolated from horse samples indicating the contamination of the horse stables with virulent *R. equi.* Therefore, it is very important to take care when people deal with the animals or their environment to avoid the infection with this pathogen.

Keywords | *Rhodococcus equi, vapA* gene, *VapB* gene, PCR, NANAT

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INTRODUCTION

Rhodococcus equi (*R. equi*) is a facultative intracellular, Gram positive, non-motile bacterium widely spread in the soil and the surrounding of the animals keeping like horse stables (Radositis et al., 2007; Le et al., 2015). It is an opportunistic bacterium, causing rhodococcosis, a zoonotic problem that can be confused with tuberculosis (Silva et al., 2010). Besides human and horses, infections with this pathogen has been detected in a number of animal species like cattle, pigs, deer, dogs, goats, cats, sheep, and wild animals (Prescott, 1991; Sakai et al., 2012; Cohen et al., 2014). However, members of equine family are its main target (Takai, 1997). Since the isolation of this pathogenic bacterium, the number of human cases increased (Weinstock and Brown, 2002).

The nalidixic acid–novobiocin–actidione cycloheximide–potassium tellurite (NANAT) agar has been used conventionally for isolation and identification of *R. equi* from horse faeces and mixed bacterial population collected from the environment (Woolcock et al., 1979). A significant advancement has been made towards understanding the virulence of the *R. equi* since the detection of a virulence-associated protein (Vap) (Takai et al., 1991a). The role of these genes in the pathogenesis of *R. equi* infection and its application as an epidemiological tool of bacterium virulence are well confirmed since whole strains possessing the vapA gene are fatal to foals and mice (Takai, 1997; Costa et al., 2006). The virulent strains of *R. equi* consistently contain a plasmid of 80 to 90 kb size that encoding several virulence genes, including vapA and vapB, which is associated with the pathogenicity (Takai, 1997). The virulent strain is characterized by its ability to remain, replicate inside the macrophages and kill them (Hondalus et al., 1994).

The previous studies carried out in Iraq highlighted on various aspects including, isolation, pathogenesis and immunological aspects of this pathogen (Al-Salihi, 1993; Al-Azzwa, 1996; Al-Graibawi, 1998; Al-Graibawi et al., 2014) These studies did not deal with the molecular characterization of this bacterium, therefore this study was designed for the first time in Iraq to assess the presence of the vapA and vapB genes in *R. equi* isolated from human sputum and equine faeces samples by multiplex PCR.

MATERIALS AND METHODS

Bacteriological Isolation

A total of 203 samples consist of 100 human sputum and 103 horse faecal samples were collected from different places in Baghdad city, the human sputum (88 samples from patient were admitted to chest and respiratory diseases center in Baghdad and 12 samples from stable workers), the 103 horse faecal samples were collected from different stables (49 samples from Equestrian club in Al- Amerria, 37 samples from Equestrian club in Al-Jadriah and 17 samples from Al-Zawria zoo stables) for six months (November 2014 to April 2015). One gram of each sample was diluted to a 10-fold volume of sterile saline. All samples were streaked on selective media, nalidixic acid–novobiocin–actidione cycloheximide–potassium tellurite (NANAT) medium, and incubated at 37°C for 48-72 hrs, as described previously (Woolcock et al., 1979). Several suspected colonies were transferred to nutrient and blood agars and confirmed as *R equi* by assessing the morphology of the colony, staining and through biochemical tests (Markey et al., 2014). Additionally, bacterial isolates were checked for the presence of the *vapA* and *VapB* genes by PCR. This study was approved by the ethical and research committee of Veterinary Medicine of College, University of Baghdad, Ministry of High Education and Scientific Research.

PCR Assay for Detection of *vapA* and *vapB* Genes

All steps of DNA extraction, gel electrophoresis and running of PCR were done in the PCR laboratory of Department of Internal and Preventive Veterinary Medicine / College of Veterinary Medicine / University of Baghdad.

DNA Extraction and Electrophoresis

Genomic DNA of *R. equi* isolates were extracted by using (Presto™ Mini g DNA Bacteria Kit Geneaid. USA) following the manufacture procedure. The concentration and purity of extracted DNA was quantified using a Nanodrop spectrophotometer (NuDrops) ™ [ActGene (USA)]. It was a very important step to PCR assay, which was used to check the extracted DNA by loading the eluted DNA by 1.0% agarose gel electrophoresis as follow:

- Preparation of 1.0% agarose gel in 1X TBE buffer, post cooling, (2 µL)of ethidium bromide per 50 ml gel solution was added, then the gel was poured into the tray and fixed the comb at right position and left until solidifying. Then the comb removed carefully and transferred into electrophoresis machine, which contained same 1X TBE buffer that used in preparing of agarose gel.
- The DNA samples were prepared by mixing 5µL of extracted DNA with 1µL of loading dye by drawing up and down in the micropipette.
- Then, all amounts were transferred into agarose gel wells, then running the electrophoresis power at 100 Volt and 80 Ampere for 1 hour, then the DNA bands were seen by U.V light and photographed with a digital camera.

Oligonucleotide Primers

The oligonucleotide primers for *vapA* and *vapB* primers were F 5^{-}- GACTCTTCACAAGACGGT-3^{-}, R 5^{-}-TAGGCGTTGTGCCAGCTA-3^{-} for: *vapA*gene, and F 5^{-}-TGATGAAGGCTCTTCATAA-3^{-}, R 5^{-}TTATGCAACCTCCCAGTTG-3^{-} for *vapB* gene. The product sizes were 286 Pb and 477 Pb for vapA and vapB respectively (Takai et al., 1991a).

Preparation of PCR Master Mix

All required reagents were thawed completely and put them on ice, and reagent was mixed well by inversion and spins them down prior to pipetting. The PCR master mix reaction was prepared by using GoTaq® Green Master Mix from Promega, USA.

Detection of *vapA* and *vapB* genes by multiplex PCR

The PCR amplification mixture which was used for detection the *vapA* & *vapB* genes includes master mix12. 5 µl, 3 µl of template DNA, 1.5 µl of each forwarded and reversed primers for both genes and 3.5µl of nuclease free water to complete the amplification mixture to 25µl. The PCR tubes containing an amplification mixture were transferred to thermal-cycler and started the program for amplification as shown in the Table 1.

Table 1: Thermocycler PCR program for detection *vapA* and *vapB* genes

Step	Temperature (°C)	Time	No. of cycles
Initial denaturation	94	5 min.	1
Denaturation	94	1min.	35
Annealing	48	30 sec.	
Extension	72	1 min.	
Final extension	72	10 min.	1
Hold	4	Until use	

Gel Purification Of Amplified Products

The samples containing PCR products were loaded on a broad holes in a 2.0% agarose gel. After running the gel, it was stained in freshly prepared ethidium bromide solution (2 µg/50 ml), the PCR products (bands) were visualized using a UV transilluminator [Cleaver Scientific (U.K.)] and photographed by using a digital camera.

RESULTS

Rhodococcus equi Isolation

The suspected colonies appeared as dark grey to black, sometime as dark brown in colour and mucoid and moist in consistency on NANAT medium (Figure 1), and appeared as irregularly round, smooth, semitransparent, glistening, coalescing mucoid and salmon- pink in colour on blood agar with age (Figure 2). Gram staining of suspected *R. equi* colonies showed positive, non spore forming bacterium. The biochemical tests of the isolates gave reactions of rhodococcus they were negative for oxidase and positive for catalase, nitrate reduction and CAMP tests, while urease test gave variable results.

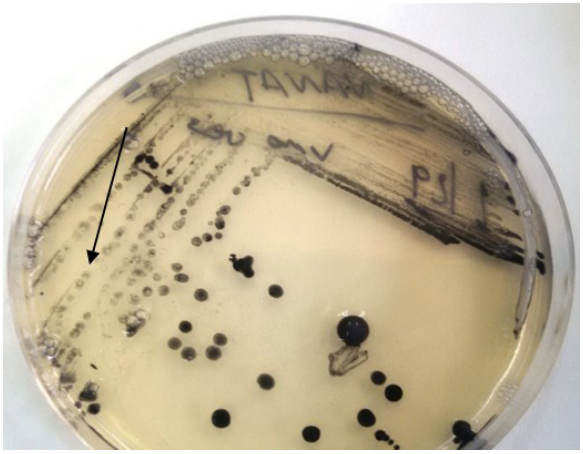

Figure 1: Growth of *R. equi* on NANAT agar after 72 hours at 37° C showed typical mucoid and grey colonies

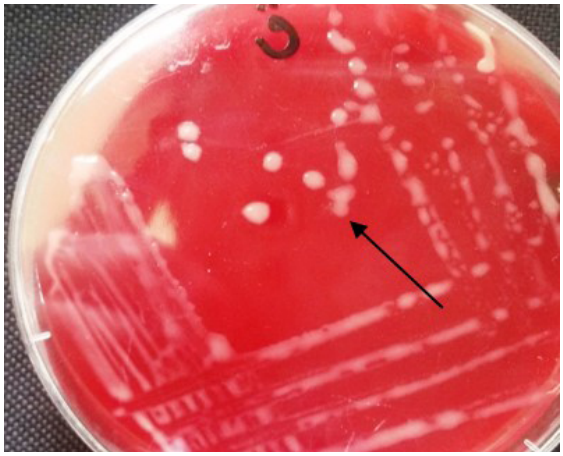

Figure 2: Growth of *R. equi* on blood agar post 48 hours at 37°C showed typical mucoid, teardrop, coalescing and glistening colonies

The result of cultural examination of 103 horses' faeces samples collected from different stables revealed the isolation of 7 (6.79%) *R. equi*, all of them 14.71% (7/49) were obtained from Al-Ameria location in Baghdad province as shown in the Table 2.

Table 2: Shows number and percent of *R. equi* isolated from horses samples with relation to location of samples

Stable location	No. of samples	*R. equi* Positive	Percent of infection%
AL- America	49	7	14.71%
AL-Jadria	37	0	0.00%
AL-Zawra'a	17	0	0.00%
Total	103	7	6.79%

The bacteriological examination of the 100 human sputum samples showed the recovery of 4 (4.00%) isolates of *R equi*, 8.33% (1/12) was isolated from the samples of stable workers, the remaining 3.40 % (3/88) from patients suspected to have pulmonary tuberculosis (Table 3).

Table 3: Shows number and percent of *R. equi* isolated from human sputum with relation to the source of the sample

Source	No. of samples	*R. equi* Positive	Percent of infections
Stable worker	12	1	8.33%
Patients suspected to have pulmonary tuberculosis	88	3	3.40%
Total	100	4	4.00%

DNA Extraction

The purity and concentration of extracted DNA were measured by a Nanodrop spectrophotometer (NuDrops)™ [ActGene(USA)] and the concentration of DNA was ranged from 60-97 ng/ml and the purity was measured from 1.84 – 2.1 in absorbance 260/280 nm. The results of 1.0 % agarose gel electrophoresis of the extracted DNA appeared as compact bands (Figure 3).

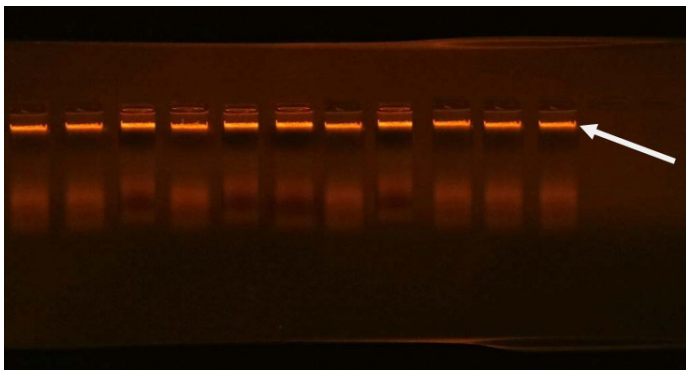

Figure 3: Gel electrophoresis of 1% agarose gel stained with ethidium bromide (2µL) showing the bands of DNA (arrow) extracted from human and horse *R. equi* isolates

Results of multiplex PCR assays of 11 isolates from human and horses samples showed that only 57.14% (4/ 7) of the *R. equi* isolates from horses samples were vapA positive, all the 4 *R. equi* isolates from human sputum were negative for both vapA and vapB gene (Figure 4).

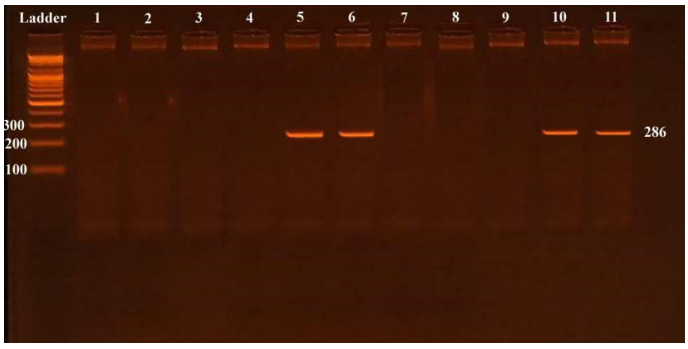

Figure 4: Agarose gel electrophoresis (2.0 %) stained with ethidium bromide (2μL) showing amplification of 286 bp fragment of *vapA* gene of *R. equi*. Lanes 4, 5, 10, 11

DISCUSSION

Rhodococcosis is a pyogranulomatous disease of domestic, wildlife animals, and humans caused by *R. equi*. The *R. equi* a member of the family *Nocardiaceae* is a Gram- positive pelomorphic zoonotic bacterium. This microorganism is widespread in the soil and the environment of the animals, particularly the horse stables (Cohen et al., 2012). The natural infection with *R. equi* has been detected in humans as well as in horse, cattle, pigs, deer, dogs, goats, cats, sheep and wild birds (Presscott, 1991; Takai, 1997; Sakai et al., 2012).

The development of the NANAT medium leads to advancement in the epidemiological and ecological studies of the *R. equi* due to its efficacy to prevent the growth of many undesirable microorganisms in the contaminated samples. Many researchers mentioned the role of antimicrobial activity which added in NANAT media as follow, Naladixic acid is effective against gram negative and gram positive bacteria, Novobiocin used against staphylococcus, Cyclohexamide act as an antifungal agent, Potassium tellurite has an inhibitory action on many anaerobic and aerobic bacteria (Sellon et al., 2001).

The results of colony morphology of *R. equi* on blood agar and nutrient agar similar to the morphological characters previously described (Markey et al., 2014; Muller et al., 1988), also the results of Gram staining and biochemical tests revealed the Characters and reactions of *R. equi* bacterium (Markey et al., 2014). The cultural examination of the 203 samples revealed that 11 isolates (7 isolates from horse samples and 4 isolates from human sputums) gave the morphology and biochemical reactions of *R. equi* (Markey et al., 2014). The high percentage of *R equi* isolation 14.71% (7/49) were in the Al-Ameria stables, this result confirmed previous studies carried out in the same stables by Al-Salihi (1993) who reported the isolation of 12 (13.18%) *R. equi* from 91 horse faecal samples. This may be due to density of animal population, widespread of *R. equi* in the environment, and poor management in this location compared to other locations involved in the study, similar findings were observed by Muscatello et al. (2006). Martens et al. (2000) noticed that the variation in the *R. equi* prevalence between the farms directly relates to the environmental factors, such as temperature, dust, farm management, soil pH as well as the number of virulent *R. equi* in the soil.

The isolation of 4 *R. equi* from 100 sputum samples indicated the importance of this bacterium in human infection. The human infection with this pathogen was increased since primary isolation (Golub et al., 1967; Silva et al., 2012), the infection with *R. equi* is thought to be occurring either via inhalation from the soil, ingestion and passing via the alimentary tract or inoculation into a wound or mucous membrane (Prescott, 1991). Exposure to domestic animals or their environment, such as horses and pigs, may play important role in several cases of infection. In Brazil, Silva et al. (2010) reported that the resemblance of the clinical course between Rhodococcosis and tuberculosis in patient makes it difficult to detect and treat *R. equi* infection. A patient infected with *R. equi* may be treated with unsuitable initial antibiotic due to misdiagnosis with *Mycobacterium* species, *Nocaredias* species and diphtheriods. Therefore, early correct diagnosis of the organism and subsequent detection of vapA by PCR must be carried out for accurate therapy and faster recovery of the patient (Silva et al., 2012).

The multiplex PCR analysis for the presence of *vap*A and vapB genes in the 11 *R. equi* isolates demonstrate that 57.14% (4/ 7) of the *R. equi* isolates from horses samples were *vapA* positive. All the 4 *vapA* positive *R. equi* were isolated from the Al-Ameria horse stables, this indicates that the environment of these stables was heavily contaminated with virulent *R. equi* as previously reported by Makrai et al. (2002). The major progress in understanding the virulence mechanisms of the *R. equi* infection was the detection of the vap family genes, which enables *R. equi* for intracellular replication in macrophages by preventing the maturation of the phagosome (Takai et al., 1991; Tan et al., 1995). *R. equi* is usually isolated from samples of healthy and diseased animals and soil, therefore, detection of the virulence genes associated with *R. equi* is of considerable importance in characterizing pathogenicity of the infection in human and animals (Monego et al., 2009). DeLa-PenaMoctezuma et al. (1996) reported that *vapA* gene was efficient techniques for the detection of virulence more than other phenotypes methods such as Congo red bind-

ing, conventional bacterial tests, resistance to antibiotics, employment of various carbon sources. Many researchers have reported the association of *vap*-A gene in *R. equi* with its virulencity and lethality to susceptible foals (Takai et al., 1995; Takai et al., 1996; Wada et al., 1997), this can be used as epidemiological tools in *R. equi* virulence in human and animals (Cohen et al., 2005; Takai et al., 1999). This study revealed that the adult horses can carry the *vapA* positive *R.equi* in their faeces and may spread them in their environment; therefore, it is very important to take care when dealing with the animals.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHORS CONTRIBUTION

Both authors contributed equally in all the details of this manuscript.

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