Genome-Wide Analysis of the Emerging Infection with *Mycobacterium avium* Subspecies *paratuberculosis* in the Arabian Camels (*Camelus dromedarius*)

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Abstract

*Mycobacterium avium* subspecies *paratuberculosis* (*M. ap*) is the causative agent of paratuberculosis or Johne’s disease (JD) in herbivores with potential involvement in cases of Crohn’s disease in humans. JD is spread worldwide and is economically important for both beef and dairy industries. Generally, pathogenic ovine strains (*M. ap-S*) are mainly found in sheep while bovine strains (*M. ap-C*) infect other ruminants (e.g. cattle, goat, deer), as well as sheep. In an effort to characterize this emerging infection in dromedary/Arabian camels, we successfully cultured *M. ap* from several samples collected from infected camels suffering from chronic, intermittent diarrhea suggestive of JD. Gene-based typing of isolates indicated that all isolates belong to sheep lineage of strains of *M. ap* (*M. ap-S*), suggesting a putative transmission from infected sheep herds. Screening sheep and goat herds associated with camels identified the circulation of this type in sheep but not goats. The current genome-wide analysis recognizes these camel isolates as a sub-lineage of the sheep strain with a significant number of single nucleotide polymorphisms (SNPs) between sheep and camel isolates (~1000 SNPs). Such polymorphism could represent geographical differences among isolates or host adaptation of *M. ap* during camel infection. To our knowledge, this is the first attempt to examine the genomic basis of this emerging infection in camels with implications on the evolution of this important pathogen. The sequenced genomes of *M. ap* isolates from camels will further assist our efforts to understand JD pathogenesis and the dynamic of disease transmission across animal species.

Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (*M. ap*), the causative agent of Johne’s disease (JD) in ruminants, continues to be a significant economic drain on the livestock population throughout the world [1]. JD, also known as paratuberculosis, is widespread and infamously complex to control, especially in dairy operations. A recent study by The National Animal Health Monitoring System revealed that 68.1% of cattle dairy operations are infected with *M. ap* in the United States alone [2]. In Australia, a similar trend of *M. ap* infection has been observed among sheep flocks, causing an average loss of $60,500 due to mortalities in 2002 [3]. Once animals are infected with *M. ap*, the disease gradually advances towards its chronic form, which is characterized by granulomatous enteritis, progressive weight loss with diarrhea, and ultimately death [4]. Clinically infected animals can shed $10^{6}–10^{8}$ CFU/g in fecal materials and as little as $10^{3}$ CFU/animal is sufficient to infect other animals through fecal oral route [5]. In addition, *M. ap* can survive in the environment for an extended periods of time [6], causing a significant risk to naïve animals from infected hosts. A number of domesticated ruminant species, e.g. cattle, buffalo, sheep, and goats are susceptible to *M. ap* infection with variable genotypes [7–9]. In camels (*Camelus dromedarius*), the nature of *M. ap* infection is uncharacterized, with increasing numbers of suspected cases in several regions of the world [10–12]. In this study we examined groups of camels suggested to contract *M. ap* infection to characterize the causative agent on the gene and genomic levels.

The epizootology of *M. ap* infection among a wide host range remains elusive and examining the interspecies transmission of JD could help in understanding the basis for such wide prevalence. Findings from earlier reports on experimental infection with *M. ap* from different host sources [13,14] could not generalized to cross species natural infections under field conditions. Fortunately, several assays for genotyping *M. ap* were developed to examine the interspecies transmission of *M. ap* among domesticated animals
Genetic variations based on IS900 [17], IS1311 [15] and
\( \text{mvb} \) sequences [18] have revealed the presence of major groups of
\( \text{M. ap} \) strains with preference to specific host source: the Type I/
Type III or sheep strain (\( \text{M. ap-S} \)) and the Type II or cattle strain
(\( \text{M. ap-C} \)). A recent report on genome-wide insertions and
deletions further confirmed the presence of the two major lineages
of \( \text{M. ap} \) [19]. Sheep strains of \( \text{M. ap} \) appear to have a substantial
host preference for sheep with fastidious growth and the prevalence of
yellow-orange colonies [9]. On the other hand, the \( \text{M. ap-C} \) strains infect a broad host range with more prevalence
in cattle while most of the isolates show non-pigmented colonies
[20]. The \( \text{M. ap-C} \) isolates are relatively easier to culture and faster
to grow than sheep strains. However, the genetic basis of such host
preferences for these variable strains is yet to be determined,
especially in exotic animals. A few reports examined the
development of JD in camels despite being probably the most
notable member among the camelidae family. This situation, not
surprisingly, is likely due to the failure to isolate the causative agent
from the diseased camels.

Camels represent an important animal species in Saudi Arabia,
where camel producers have suffered tremendously in recent years
because of the annual increase of incidence of JD [11,21]. The
present study was undertaken to isolate and characterize the
causative agent responsible for JD in dromedary camels (\( \text{Camelus dromedarius} \)) in Saudi Arabia. We were able to isolate \( \text{M. ap} \) from
infected tissues, and genetic typing indicated these were \( \text{M. ap-S} \)
strain. However, genome wide sequencing identified a significant
number of SNPs between the camel isolates and the sequenced
sheep strain. The results obtained provide interesting insights not
only into the genome of \( \text{M. ap} \) infecting camels but also to the
dynamic of disease transmission among animal species that share
the same grazing fields.

Results

Johnne’s disease detection in \( \text{Camelus dromedarius} \)

Veterinary practitioners in north-east Saudi Arabia suspected
the presence of JD in camel, sheep and goat herds based on
clinical signs and serological testing [10,11]. The local herd
shepherds called this illness “Silag”, an Arabic terminology
indicating profuse diarrhea. Our strategy for characterizing this
infection in camels was based on collecting samples from animals
suffering from chronic diarrhea suspected to have JD as well as
from healthy animals. Our strategy also included the sampling of
contact animals, mainly sheep and goat. During routine necropsy,
sera and tissue samples (intestine, lymph node, liver) were
collected. Additionally, faecal samples were collected from living
animals with chronic diarrhea, mainly from the Al-Hassa region.
While culturing results were pending, we used PCR to detect the
presence of \( \text{M. ap} \) genomic DNA in collected samples. Surpris-
ingly, tissues from all suspected cases (camels, sheep and goats)
were positive for mycobacterial IS900 (Fig. S1), 16S rRNA and
hsp65 genes (Fig. S2) suggesting an active infection with \( \text{M. ap} \)
in agreement with the clinical signs of JD, while none of the healthy
animals tested positive for any of \( \text{M. ap} \) genes. In most cases, faecal
samples were acid-fast stain and PCR positive, a further
confirmation for shedding of \( \text{M. ap} \) bacilli from infected animals.

In a second level of analysis, histopathology of sampled tissues
revealed a high level of multi-bacillary form of \( \text{M. ap} \) infections
(Fig. 1) in all infected organs (intestine, Lymph node and liver),
another confirmation of the late stage of the disease in the
examined camels. Examined sections showed diffuse granuloma-
tous lesions (mainly of lymphocytic infiltrations, macrophages and
a few giant cells) that were covered with patches of acid fast bacilli.

Tissue sections from healthy animals did not show any
granulomatous lesions or bacillary forms. When ELISA was
performed, only 2 out of 6 serum samples were found to be
positive for \( \text{M. ap} \) antigens. Unfortunately, the antibody ELISA has
not been validated for the diagnosis of \( \text{M. ap} \) infection in camels
and the sensitivity of this assay was based on testing in cattle.
Overall, PCR and histopathology approaches indicated the
presence of a multi-bacillary form of paratuberculosis in all
suspected camel cases.

Bacterial isolation from infected camels

Previous attempts to isolate \( \text{M. ap} \) from infected camels were
unsuccessful [21,22], likely due to the fastidious nature of this
pathogen. In our hands, isolates of \( \text{M. ap} \) were cultured from 3 out
of 29 clinical samples despite the extensive infection rate expected
from PCR and histological analyses. Such disparity was likely
caused by the heat-treatment step (72°C for 30 min) mandated by
our shipping protocol that yielded most of the bacteria uncultur-
able. However, colonies were observed from positive samples after
11–12 weeks of incubation and only on 7H10 medium (JQ5 camel
Lymph node, Intestine and JQ6 camel liver) despite culturing on
7H9 broth, Lowenstein-Jenson and HEYM media. Suspected
colonies were characterized by Ziehl-Neelsen (ZN) staining that
revealed their acid resistance. Failure to grow both on 7H9 liquid
broth and 7H10 plate without mycobactin J, an iron-chelating cell
wall component, indicates that these isolates are strongly
dependent on mycobactin, a distinctive feature of \( \text{M. ap} \) from the
closely related \( \text{M. avium} \). The comparative growth curve with
\( \text{M. ap-K10} \) (cattle strain), and \( \text{M. ap-S397} \) (sheep strain) indicated
that the camel isolates were much slower to grow to that of the
\( \text{M. ap-K10} \) but with similar rate to that of \( \text{M. ap-S397} \) (data not
shown).

To confirm the identity of the grown colonies, we subjected
isolates to a panel of PCR amplification and restriction enzyme
analysis in comparison to other isolates with known host origin,
namely, \( \text{M. ap-K10} \) (cattle) [23], \( \text{M. avium} \) 104 (human) [24] and
\( \text{M. ap-S397} \) (sheep) [25]. The target genes for analysis included
hsp65, 16S rRNA, IS900 and IS1311. For this analysis, a partial
sequence (938 bp) of the 16S rRNA gene was amplified from all
camel isolates, confirming their identity on the mycobacterial
genus level [26]. Also, amplicons (517 bp) of the hsp65 gene were
obtained and subjected to PsA digestion, identifying that these
isolates as \( \text{M. ap} \) and not \( \text{M. avium} \) species [27] (Fig. 2). However,
sequencing 5’ region of hsp65 gene failed to discriminate camel
isolates (\( \text{M. ap-JQ5} \) and \( \text{M. ap-JQ6} \) at strain level but when whole
gene was analyzed, we were able to differentiate the camel clinical
isolates from \( \text{M. ap-K10} \) (Fig. S3). Another confirmation of \( \text{M. ap} \)
identity came from the successful amplification of the insertion
sequence IS900 [28] that has 17 copies encoded in the \( \text{M. ap} \)
genome [23]. To further characterize the new isolates, amplicons
were obtained for the IS1311 sequences and subjected to Hinfl
restriction digestion (Fig. 3). The obtained pattern of digestion
characterized that the JQ5 and JQ6 isolates are \( \text{M. ap-S} \) strains and
not \( \text{M. ap-C} \) strains [15]. All of the obtained digestion patterns
were further supported by Sanger sequencing of amplicons
(Table 1) from each isolate and BLAST comparison to \( \text{M. ap-K} 
\)-10. Overall, the genotyping protocols applied here strongly
suggested the identification of a \( \text{M. ap-S} \) strains that caused this
multi-bacillary form of JD in examined camels.

Origin of the \( \text{M. ap} \) infection in camels

In several regions of the world, animals are raised in mixed
populations with other animal species or even with access to
wildlife animals. In searching for the origin of \( \text{M. ap} \) infection in
Figure 1. Histological analysis of camel samples collected from animals suffering from Johne’s disease. A) A representative of lymph node thin section stained with H&E showing diffuse granulomatous response (arrows). B) A lymph node section stained with Zeil-Neelsen stain showing high level of acid-fast bacilli. C) A representative of intestinal section stained with H&E showing aggregates of lymphatic infiltration (arrows). D) An intestinal section stained with Zeil-Neelsen stain showing patches of acid-fast bacilli. Size bars are included in the bottom of each section. doi:10.1371/journal.pone.0031947.g001

Figure 2. Species-level typing of mycobacterial isolates from camels. Ethidium bromide stained 2% agarose gel of PCR amplicons of the hsp65 gene, following restriction enzyme analysis (REA) with PstI. For each set, both undigested and digested products (second lane) are shown. A 100-bp Molecular size marker is shown in the first lane. doi:10.1371/journal.pone.0031947.g002
We focused our analysis on 3 house-keeping genes (gyrA, gyrB, and recF) that are polymorphic between M. ap-S strains (Type I and Type III) and M. ap-C (Type II) strains [18,30]. There were a total of 9 polymorphic sites between the sheep isolates (M. ap-JQS1 and M. ap-JQS2) that distinguished them from camel isolates (Fig. 4). The sequence between M. ap-JQS1 and Type II strains was least variable whereas, M. ap-JQS2 was grouped with sheep strains, an indication of potential sheep source for camel infections. Further, camel isolates were variable at three sites (one SNP in each gyrA, gyrB, and recF gene) when compared to Type I strains. Such variations suggest the inclusion of camel isolates in type III group, a sub-lineage of sheep isolates identified before [31]. Interestingly within these 3 loci, we found a single variable site in gyrB gene that distinguished camel isolates (M. ap-JQS5 and M. ap-JQ6) from Type III strains. This site represents camel strain of M. ap specific SNP and could be employed in future comparative epidemiological studies of Johne’s disease.

To evaluate the nature of evolutionary pressure on the camel isolates, we examined the dN/dS ratio for SNPs in each examined strain. Sequences from 11 house-keeping genes (gyrA, gyrB, dnaA, dnaK, apB, gnd1, hisG5, groEL1, pepB, recF, and sodA) were combined and concatenated to generate a 17,190-bp in-frame semantide for each strain included in the study. In total, 175 polymorphic sites (1%) were found within these variable alleles leading to the separation of these strains into individual branches (Fig. S5). In addition, there were 24 SNPs that could be used to distinguish M. ap-K-10 from sheep strains of M. ap (Table S1). In comparison to M. av 104, the average dN/dS ratio for all M. ap subtypes was ~0.07, an indication to the highly conserved nature of the selected sequences among members of the M. avium complex. In contrast, out of the 24 SNPs that segregated M. ap-S and M. ap-C strains, 12 (50%) were non-synonymous (nSNPs). The average pairwise dN/dS ratio for both camel isolates and the standard M. ap-S397 was increased to 0.5, comparable to that previously observed [30] when M. ap-S sequences were compared against M. ap-K-10 (M. ap-C). Such analysis indicated the diversifying selective pressure of camel isolates compared to cattle isolates.

### Whole genome analysis of camel isolates

Although gene-based approaches grouped the camel isolates with M. ap-S strains, they are not enough to get insights into the unidentified genomic changes (e.g. indels or SNPs) at the whole genome level in these camel isolates. Such genomic rearrangements [24] could play a role in disease surveillance and control [32] as well as provide a better understanding of the evolution of camel, samples were collected from animals co-mixed with camels to determine the possible source of camel infection. Interestingly, PCR-restriction enzyme analysis of IS1311 from sheep and goat infected samples identified the presence of mainly M. ap-C strains in the examined goats. However, both Types M. ap-S (M. ap S397) and M. ap-C (M. ap K-10) strains in addition to camel isolates JQS5 and JQ6. A 100-bp Molecular size marker is included. B) DNA sequencing electropherogram showing the C/T nucleotide polymorphism surrounding base pair 223 in IS1311.

**Table 1.** IS1311 polymorphisms in M. av and within M. ap strains.

| Polymorphism position in IS1311 sequence | M. avium GenBank: U16226 | M. avium ss. paratuberculosis |
|------------------------------------------|--------------------------|--------------------------------|
|                                          | M. ap K-10 from cattle   | M. ap 397 from sheep          | M. ap from camel               |
| 68                                       | T                        | C                             | C                              |
| 223                                      | C                        | C or T                        | C                              |
| 236                                      | T                        | C                             | C                              |
| 422                                      | T                        | C                             | C                              |
| 527                                      | G                        | A                             | A                              |
| 628                                      | T                        | C                             | C                              |

doi:10.1371/journal.pone.0031947.t001

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Figure 4. Phylogenetic analysis of *M. ap* isolates form different hosts. A dendrogram displaying the relationship between several *M. ap* isolates based on the sequence of 3 genes (gyrA, gyrB and recF). Isolates with JQ designation were analyzed in this study either from camels (JQ5, JQ6) or sheep (JQS1, JQS2). *M. ap* S397 is the standard strain for *M. ap*-S [23], *M. ap* K10 is the standard strain for *M. ap*-C [23] while the rest of the isolates were genotyped before [18]. All of the known genotyped are included in the figure. The bootstrap values (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. See Table 2 for the length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. See Table 2 for the

![Dendrogram](image)

**Table 2. A list of accession numbers for organisms and genes used in this manuscript.**

| Organisms or genes | Accession number |
|--------------------|------------------|
| *M. avium* ATCC 104 | NC_008595        |
| *M. ap* S397       | EU029111         |
| *M. ap* K-10       | EU029112         |
| *M. ap* QJ6        | EU029113         |
| *M. intracellular* ATCC 13950 | EU099890         |
| gyrA for Type I *M. ap* | EU209982       |
| gyrA for Type III *M. ap* | EU209982      |
| gyrB for Type I *M. ap* | EU209982       |
| gyrB for Type III *M. ap* | EU209982      |
| recF for Type I *M. ap* | EU209982       |
| recF for Type III *M. ap* | EU209982     |

| Parameters | JQ5 isolate | JQ6 isolate |
|-----------|-------------|-------------|
| Reference organism | *M. ap* S397 | *M. ap* S397 |
| Reference length (bp) | 4,813,711 | 4,813,711 |
| Consensus length (bp) | 4,735,471 | 4,697,010 |
| % G+C | 69.3 | 69.3 |
| % Homology to *M. ap* S397 | 98.3 | 97.6 |
| % Homology to *M. ap* K-10 | 97 | 97 |
| Average Coverage | 406 | 240 |
| Mean mapped read length | 82.7 | 80.3 |
| Mean paired read distance | 300 | 335 |
| No. of indels against *M. ap* S397 | 89 | 69 |
| No. of SNPs against *M. ap* S397 | 1026 | 929 |
| No. of indels against *M. ap* K-10 | 269 | 244 |
| No. of SNPs against *M. ap* K-10 | 3439 | 3245 |

**Table 3. Summary of next generation sequencing results.**

| Parameters | JQ5 isolate | JQ6 isolate |
|-----------|-------------|-------------|
| Reference organism | *M. ap* S397 | *M. ap* S397 |
| Reference length (bp) | 4,813,711 | 4,813,711 |
| Consensus length (bp) | 4,735,471 | 4,697,010 |
| % G+C | 69.3 | 69.3 |
| % Homology to *M. ap* S397 | 98.3 | 97.6 |
| % Homology to *M. ap* K-10 | 97 | 97 |
| Average Coverage | 406 | 240 |
| Mean mapped read length | 82.7 | 80.3 |
| Mean paired read distance | 300 | 335 |
| No. of indels against *M. ap* S397 | 89 | 69 |
| No. of SNPs against *M. ap* S397 | 1026 | 929 |
| No. of indels against *M. ap* K-10 | 269 | 244 |
| No. of SNPs against *M. ap* K-10 | 3439 | 3245 |

Evolutionary history of the camel isolates

One goal of our study was to understand the mechanisms of the emergence of JD among camels. To get a better understanding of the diversity among *M. ap* isolates circulating in independent but mixed animal populations, we compared the genome sequence of camel isolates to *M. ap* S397 (see Table 2 for accession number) as well as the genome of *M. ap* K-10 (see Table 2 for accession number). Genome-wide comparative analysis identified single nucleotide polymorphisms (SNPs) and indels in *M. ap* isolates from camels compared to *M. ap* K-10 by 3439 and 3245 SNPs in *M. ap* JQ5 and *M. ap* JQ6, respectively (Fig. 5). Also, small indels (1–8 bp) were identified (269 to 244 for *M. ap* JQ5 and *M. ap* JQ6, respectively). Interestingly, when the genome of JQ6 compared to that of JQ5, small differences in SNPs (N = 99) were found, suggesting a high sequence conservation in the genomes of isolates from sheep and cattle are detailed in Table 3.

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Table 2. A list of accession numbers for organisms and genes used in this manuscript.

This important pathogen. In an effort to resolve the whole genome of *M. ap* isolated from camels, we used Next-generation sequencing technology (Illumina, HiSeq 2000) to generate a draft sequence of two camel isolates (*M. ap* JQ5, and *M. ap* JQ6; see Table 2 for accession numbers). A total of 22,685,456 and 14,118,565 read counts (for *M. ap* JQ5 and *M. ap* JQ6, respectively) were mapped against a reference genome from a sheep isolate (*M. ap* S397) (see Table 2 for accession number). 6 sequenced by our group. An average coverage of 406 × and 240 × across the genome for *M. ap* JQ5 and *M. ap* JQ6 isolates, respectively, was achieved. Sequence statistics revealed that the camel isolate *M. ap* JQ5 has a G+C content of 69.3% and a genome size of 4,735,471 bp with 98.3% complete coverage of the genome when compared to the *M. ap* S397 genome. More comparative genomic features of the camel isolates relative to the genomes of isolates from sheep and cattle are detailed in Table 3.
hand, when the genome of the sheep strain, *M. ap* S397 was compared, the average numbers of SNPs (N = 1036 for JQ5 and 929 for JQ6 isolate) and indels were significantly less than when the *M. ap* K-10 strain was compared. Moreover, when we compared camel isolate JQ5 and *M. ap* S397 against *M. ap* K-10, a small difference in SNPs was observed between sheep strain and camel isolate (Fig. S7). Finally, our analysis indicated majority of the SNPs were synonymous (lower selective pressure) in the genome of camel isolates when compare to the *M. ap* S397 strain, suggesting a recent divergence from a common ancestor. Overall, whole-genome analysis confirmed the identified genotypes based on selected house-keeping genes.

To examine the impact of genomic changes on pathobiology of *M. ap* in camel JD, we analyzed the presence of SNPs on coding sequences. Compared to *M. ap* K-10, SNPs were detected in a 1860 gene-coding proteins in the camel isolates (Fig. S8). Most of these genes harbored one to six SNPs with single point mutations being the most common. In certain genes, there were higher numbers of SNPs per gene relative to others (Table 4). Other polymorphic genes included those coding for antigenic proteins (*e.g.* *psaL*), mammalian cell entry proteins (*e.g.* *mceA1-2*), PE/PPE family proteins, and mycobactin biosynthesis (*e.g.* *mbtB-mbtH*). These genes are suggested to be involved in JD pathogenesis and detection of SNPs in their coding sequences suggests a selective pressure for adaptation to the camels. Because of the importance of the *mce* gene family, we further analyzed some of its members (*mceA2, mce1B, mce2, mce3, mce4, and mce1F*) by pairwise alignment that confirmed the close relationship between the camel isolates with *M. ap*-S strain (Fig. S9). Unfortunately, we did not have the full gene sequence of the *mce1A*, a member of the *mce* gene family, to be included in such comparison. Finally, our analysis revealed a large number of SNPs in the mycobactin biosynthesis gene cluster (*e.g.* *mbtB-mbtH*), providing a possible explanation of delay in growth of the camel isolates in synthetic media.

**Discussion**

Several reports suggested the emerging infection with *M. ap* in camels (*Camelus dromedarius*) raised in the Arabian Peninsula [11,12]. However, the molecular basis of this infection remained elusive likely due to the unsuccessful attempts to culture the organism from the diseased camels. Camels are an economically important species in South Asia, Middle East, and North Africa, where they are utilized for transportation, sport, and the production of meat and milk. These attributes have generated our interest in investigating the causative agent of JD in camel. An important aspect of *M. ap* infection in camels demonstrated in this study was the prevalence of the multi-bacillary form observed in examined tissues. Although we focused our sampling strategy on diseased animals for bacterial isolation, we were surprised that all infected animals showed a large number of granulomas with patches of mycobacterial bacilli. This lepromatous form of JD has

![Figure 5. Circular map of the newly identified SNPs and indels in camel isolate JQ5 relative to the *M. ap* K-10 strain [51]. The outer circle shows the genomic scale. The second circle shows the location of the 4,395 ORFs in the *M. ap* K-10 genome. Genes (magenta) on the forward strand are shown outside of the baseline; genes (olivegreen) on the reverse strand are shown inside of the baseline. Inner circles show all indels (blue), synonymous SNPs (orange), and nonsynonymous SNPs (lime) identified in *M. ap* of camel origin. The figure was generated with GenVision software (DNAStar, Madison, WI).](https://www.plosone.org/doi/10.1371/journal.pone.0031947.g005)
also been reported in sheep before [4,9] but the extent of M. ap induced lesion was much higher in the examined camels. The nature of the infection transmission was further analyzed when majority of examined fecal samples were positive for the presence of M. ap. Such analysis also suggested that most of the examined animals were at the late stage of JD, another reason for the observed multi-bacillary form. Analysis of more animals with less severe clinical signs could shed the light on the pathogenesis of JD in camels.

We successfully isolated M. ap from only two diseased camels (JQ5 and JQ6) despite the detection of M. ap in all diseased animals. This low isolation rate could be attributed to the necessary heat treatment protocol for all tissue samples collected from the sampling location to inactivate potential sample contamination with Foot-Mouth Disease. Additionally, the isolates were fastidious and slow to grow, in contrast to the M. ap-C strain. Similar observations were noticed previously for the isolation of sheep strains of M. ap-C strain. Similar observations were noticed previously for the isolation of sheep strains of M. ap-C. Interestingly, members of the mce gene family from M. ap (e.g. mce1A) was shown before to be highly similar to its counterpart in M. avium subspecies hominisuis 104 suggesting an M. avium origin [36]. However, in our hands, most of this gene family (mceA2, mce1B, etc) were closely related to the M. ap-S strain, suggesting a potential origin of this gene family from M. ap-S isolates (and not M. avium) circulating in sheep. Unfortunately, the full gene sequence for mce1A was not obtained for comparative analysis. It is also possible that the mce1A is independently evolved from the rest of the gene family. Taken together, our analysis indicated that camels were infected with a member of the M. ap-S cluster, most likely originating from sheep despite the presence of M. ap-C isolates circulating in goats and sheep raised in the same geographical location. Based on our MLSA and genome-wide comparative analysis, camel isolates could represent a distinctive sub-group of M. ap-S, an idea remains to be investigated with a larger number of camel isolates.

In M. tuberculosis, a possible mechanism of antigenic drift has been proposed due to the mutations within the immune-dominant epitopes of secretory antigenic protein [37]. Members of the mce gene family, PE and PPE families have been found to be immunogenic and are implicated in virulence [38–40]. Moreover, mutations in the mce operons have been suggested to alter the functional changes in the biology of M. tuberculosis [41]. The high number of SNPs observed in the antigenic proteins encoded in the camel isolates included mceA and PE/PPE family proteins could be the result of host adaptation during camel infection. Whether these differences are due to adaptive evolution or specific geographic distribution of M. ap genotypes is the subject of ongoing investigation. Overall, our study successfully genotyped isolates causing Johne’s disease in camels and provided a better understanding of the disease transmission among animals under the desert conditions of the Arabian Peninsula.

### Materials and Methods

**Animals and study site**

A total of 45 clinical samples were tested from 20 different camels (Camelus dromedarius) suspected of having JD with signs of chronic diarrhea. Of these specimens there were 24 tissue samples (intestine, lymph node and liver), 15 fecal samples and 6 serum samples. We also analyzed a few samples from sheep and goats (N = 5) showing signs of JD (chronic diarrhea, emaciation). Camels were raised in the Arabian Desert, north east region of Saudi Arabia (Qassim and Al-Hassa), in small-sized but mixed flocks with other animals, mainly sheep and goats. Camels suffering from chronic diarrhea and severe weight loss were anaesthetized by intramuscular injection of xylazine (0.35 mg/kg) and ketamine (6 mg/kg). Animals were bled by severing the major vessels of the neck. Euthanized camels were undergone necropsy for post-mortem changes and sample collection. All serum, tissue and fecal samples were collected by teams of researchers in Saudi Arabia and were heat-treated at 72°C for 30 min before transportation to

| Table 4. List of genes with high number of SNPs in M. ap JQ5 compared to M. ap K-10. |
|---------------------------------|-----------------|----------------|
| Gene description               | Size of the gene (bp) | Number of SNPs |
| "MAPK_2348"                    | 19,155           | 55             |
| "MAPK_0436"                    | 1,389            | 17             |
| "MAPK_2538"                    | 1,221            | 17             |
| "MAPK_0308"                    | 1,386            | 15             |
| "MAPK_1273"                    | 1,419            | 14             |
| pks12                           | 12,513           | 14             |
| kasB_2                          | 1,260            | 13             |
| "MAPK_2303"                    | 1,455            | 10             |
| "MAPK_1538"                    | 11,040           | 10             |
| "MAPK_4243"                    | 3,645            | 8              |
| las                             | 9,279            | 8              |
| mbtE                            | 5,280            | 8              |
| "MAPK_0028"                    | 9,207            | 7              |
| "MAPK_1689"                    | 9,156            | 7              |
| pks8                            | 3,288            | 7              |

doi:10.1371/journal.pone.0031947.t004
the Laboratory of Bacterial Genomics, University of Wisconsin-Madison, USA, where they were kept at -80°C until further sample processing. The heat treatment was necessary to inactivate foreign pathogens as mandated by our import license issued by the APHIS services, USA.

Ethics statement
All procedures for animal euthanasia and sample collection were carried out at the veterinary teaching hospital, Qassim University according to the approved animal protocol (QU#01100) from the Qassim University Committee for Rules of Experimentation on Live Animals. Anaesthesia and euthanasia of animals were carried out in strict accordance with the recommendations by the Code of Practice for camels in Western Australia and others [42–44].

Sample preparation and culturing
Tissue samples were placed in 10 ml PBS containing 5% oxalic acid in order to remove any contaminating organisms and homogenized using a Tissue-Tearor (BioSpec Products, Inc., Bartlesville, OK) with a 7 mm probe diameter at 20,000 rpm within a BSL-II cabinet. The homogenates were centrifuged (4,000 rpm) at room temperature for 10 minutes and the resulting pellets were washed twice with PBS before final resuspension in 10 ml of warm 7H9 broth (Difco, Sparks, MD) supplemented with 0.5% glycerol, 2 μg/mL mycobactin J (Allied Monitor, Fayette, MO), and 10% ADC (2% glucose, 5% bovine serum albumin fraction V, and 0.85% NaCl) [45]. All homogenates were plated on each of the following solid media: Lowenstein-Jensen (LJ) medium, Herold egg yolk medium (HEYM; BD, Franklin Lakes, NJ), Middlebrook 7H10 agar (BD) supplemented with 0.5% glycerol, 2 μg/mL mycobactin J, and 10% ADC and plates were incubated at 37°C for 12 weeks. To improve recovery rate, homogenates were also inoculated onto the Middlebrook 7H9 broth supplemented with 0.5% glycerol, 2 μg/mL mycobactin J, 0.05% Tween 80, and 10% ADC at 37°C with shaking at 115 rpm/min. Fecal samples were decontaminated according to the protocol described before [46]. One gram of each fecal sample was treated with 10 ml of 0.9% hexadecyl pyridinium chloride (HPC) overnight (16 hr) to remove fungal and non-mycobacterial contaminants. Supernatants were centrifuged at 4,000 rpm for 15 min to harvest mycobacterial pellets. Pellets were washed with PBS and resuspended in 10 ml of warm 7H9 broth following inoculation on several medium described earlier.

Histopathology and serological analysis
All organ specimens were preserved in 10% neutral buffered formalin (NBF) before tissues embedded in paraffin and prepared for microtome sectioning (3 to 5 μm). Sections were stained with hematoxylin and eosin staining or Ziehl–Neelsen staining for microscopic evaluation as described previously [47]. Stained slides were examined carefully to score changes in the inflammatory response using a scale ranging from 0 to 5. A score of 1 is indicative of restricted inflammatory response, while a score of 5 represented multiple granulomas in more than three representative microscopic fields. All camel serum samples were sent to the Johne’s testing center, Madison, WI for the detection of antibodies against M. sp using the Parachek ELISA kit as per manufacturer’s directions (Biocheck Animal Health Inc. Omaha, NE).

Isolation of genomic DNA from cultures
To obtain high quality genomic DNA (gDNA), mycobacterial cultures at OD_{600} = 1.5 were pelleted, washed and resuspended in equal volume of TE buffer. The bacterial suspension was placed at 90°C for 20 min to kill all the mycobacteria [26]. Tubes were allowed to cool to room temperature and 10 μl of 100 mg/ml lysozyme was added to each tube followed by incubation at 37°C for 3 hr with occasional mixing. A solution of SDS (10%) and protease K (20 mg/ml) was added to each tube in a ratio of 88:12. Tubes were incubated at 65°C for 2 hr followed by addition of 100 μl of 5 M NaCl at 65°C for additional 10 min. Solution of 10% Cetyl trimethyl ammonium bromide (CTAB) (Sigma-Aldrich, St. Louis, MO) (90 μl) was added, mixed and incubated at 65°C for another 10 min. DNA was then extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1 v/v/v) and similarly with chloroform/isoamylalcohol (24:1 v/v) followed by precipitation with 0.6 volumes of ice cold isopropanol [26]. The DNA was pelleted by centrifugation (10,000 rpm) at 4°C for 15 min and pellets were washed with cold 75% ethanol and dried in Speed-Vac for 5 min. The DNA was finally resuspended in 50 μl sterile distilled water. Quality of the gDNA was verified by both NanoDrop (Thermo Scientific, Wilmington, DE) machine and electrophoresis. This gDNA was used for PCR and subsequent reactions.

Polymerase chain reaction (PCR) and Sanger DNA sequencing
PCR reactions 25 μl, containing 1 M betaine, 50 mM potassium glutamate, 10 mM Tris-HCl pH 8.8, 0.1% Triton X-100, 2 mM magnesium chloride, 0.2 mM dNTPs, 0.5 μM each primer, 0.5 μl of Taq DNA polymerase (Promega) and 25 ng of genomic DNA. In some cases, 5 μl of tissue homogenates were directly used as a template. The amplification thermocycle was subjected to an initial denaturation step of 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, and followed by a final extension at 72°C for 7 min [26]. Polymerase chain reaction amplicons were evaluated by electrophoresis in 2% agarose gels stained with ethidium bromide (0.5 μg/ml). The interested single-band products from PCR reactions were purified and extracted using gel extraction kit (Promega). A total of 250–300 ng of DNA and 1 U of the restriction enzymes Hinfl or PstI (NEB, MA, USA) were used in the digestion reactions according to the manufacturer's recommendations. Digestion reactions were assessed using 2–3% agarose gels stained with ethidium bromide. Following amplification, purified PCR fragments for genotyping and MLSA analysis were sequenced with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA) with primers listed in Table 5, according to the manufacturer’s instruction.

Phylogenetic analysis
All sequences were analyzed with BLASTn algorithm on the NCBI web portal http:// blast.ncbi.nlm.nih.gov/Blast.cgi. For genotyping camel isolates, we concatenated sequences from 11 genes (dnaA, dnaK, hsp65, apB, gud1, goE1L, gya1, gya2, gyaB, pepB, recF, and sodA), generating a 17,190-bp semantide. A rooted tree was computed using MEGA [48] with M. intracellulare ATCC 13950 (see Table 2 for accession number) serving as the out-group. Distances were calculated using the neighbor-joining (p-distance) method by pairwise analysis with gaps and performing 1,000 bootstraps replicates. Analyses for Non-synonymous (dN) to synonymous (dS) polymorphisms (dN/dS) [49] were computed for each M. sp strains against M. avium 104 using the Nei-Gojobori method with Jukes-Cantor correction [48,50]. MLSA approach was also employed to check relatedness of these camel isolates with M. sp strains identified in the sheep herds from the same region of Saudi Arabia. For this, polymorphic regions distinguishing M. sp-S
from *M. ap* were amplified from gyrA, gyrB, and recF loci with primers used before [18,30]. The accession numbers for genes used in this manuscript are listed in Table 2.

**Next-generation whole genome sequencing**

Massive parallel sequencing using the Solexa-technology (Illumina) was performed at the UW-Madison Biotech Center sequencing facility. Whole-genome was sequenced using the Illumina HiSeq 2000 platform using one flow cell lane with 100-cycle paired end chemistry (www.illumina.com). Assembly and analysis of sequencing data were performed with the CLC Genomics Workbench 4.6 (http://www.clcbio.com/). Read mapping of these camel isolates (JQ5 and JQ6) was performed against reference sheep strain (*M. ap* S397) (see Table 2 for accession number). Assemblies were performed for each camel isolate individually and later combined together to get a better and deeper coverage of the genome. Single nucleotide polymorphisms (SNPs) and indels (insertion and deletion polymorphisms) were performed against *M. ap* S397 and *M. ap* K-10 (cattle strain) (see Table 2 for accession number) using CLC Genomics Workbench 4.6. To reduce false calls, SNP analysis parameters were set as follows: average base quality filter cutoff 15, central base quality filter cutoff 20, minimum sequence coverage 10, minimum variation frequency cutoff 50%, and maximum variation 2. For the indel analysis, minimum sequence coverage was set to 10 and minimum variation frequency cutoff was 50%. The sequences for the Whole Genome Shotgun project have been deposited at DDBJ/EMBL/GenBank under the accession AHAZ00000000 for *M. ap* JQ5 and AHBA00000000 for *M. ap* JQ6. The versions described in this paper are the first version.

**Supporting Information**

**Figure S1** Confirmation of *M. ap* isolates identity using PCR. Amplification of *IS900* fragments from selected clinical samples. PCR reactions show 241 bp band from the infected tissue samples (A) (lanes: 4–9) and fecal samples (B) (lanes: 12–18). Origins of the clinical samples along with the example animal identification are listed at the top of the gel. A 100-bp Molecular size marker is included in lanes: 1 and 10. PCR controls are included in lanes: 2, 3, 11 and 12. (TIF)

**Figure S2** Species-level differentiation of mycobacterial isolates from camels using *hsp65* and 16S rRNA gene targets. Ethidium bromide stained agarose gel of PCR reactions show 517 bp band for *hsp65* (A) (lanes: 2–6) and 938 bp band for 16S rRNA (B) (lanes 8 and 9). Top of the gel shows origin of the clinical samples along with the animal identification. (TIF)

**Figure S3** Phylogenetic analysis of camel isolates based on whole *hsp65* gene. Camel isolates were distinguished as *M. ap*-S when whole *hsp65* gene was analyzed. A total of 4 polymorphic sites, shared with *M. ap* S397, was able to differentiate these clinical isolates from *M. ap* K-10. The bootstrap value (1000 replicates) is shown next to the branch. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. (TIF)

**Figure S4** PCR-REA of *IS1311* sequence from sheep and goat infected samples. Ethidium bromide stained agarose gel of PCR amplicons of the *IS1311* gene, following REA with *Hinf1*. For each set, both undigested and digested products (second lane) are shown. The PCR-REA analysis of *IS1311* shows presence of mainly *M. ap*-C strains in the examined goats (*JQG1, JQG2, and JQG3*) but both *M. ap*-S and *M. ap*-C strains are detected in sheep samples (*JGS2 and JQ1S1, respectively*). A 100-bp Molecular size marker is shown in the first lane. (TIF)

**Table 5.** Primers used in this study.

| Primer | Gene and direction | Purpose | Sequence |
|--------|--------------------|---------|----------|
| 1      | IS900, forward     | PCR, Sequencing | TACCTTCCTTGAAAGGTGTTCGCGG |
| 2      | IS900, reverse     | PCR, Sequencing | TTGGCCACACACACTCCCG |
| 3      | IS1311, forward    | PCR-REA, Sequencing | GCGTGAGGCTCTGTTGGTAA |
| 4      | IS1311, reverse    | PCR-REA, Sequencing | TCAAGATACACAGGTGAC |
| 5      | hsp65, forward     | PCR-REA, Sequencing | GAGGGCCTCCTACCGTTGGG |
| 6      | hsp65, reverse     | PCR-REA, Sequencing | CGCCGATGGCCTGGAGTAC |
| 7      | gyrA, forward      | PCR, Sequencing | ACGTCGCTGACCATCAC |
| 8      | gyrA, reverse      | PCR, Sequencing | CCTACACCGATATCATCAG |
| 9      | gyrB (region 1), forward | PCR, Sequencing | AAGAAGGCGAAGAGAATA |
| 10     | gyrB (region 1), reverse | PCR, Sequencing | AGCTCCGTCGCTGACGTA |
| 11     | gyrB (region 2), forward | PCR, Sequencing | GTACGCCAAGGACAAGAGC |
| 12     | gyrB (region 2), reverse | PCR, Sequencing | GTGGGCATCATTGGGTTTC |
| 13     | recF, forward      | PCR, Sequencing | CGAAAGACAGAATCTGGTA |
| 14     | recF, reverse      | PCR, Sequencing | GGACTGTCGTGCTGCTCAT |

doi:10.1371/journal.pone.0031947.t005
replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (pairwise deletion option) and are in the units of the number of base substitutions per site. (TIF)

Figure S6 Summary of single nucleotide polymorphisms in \(M. \) \(ap\) isolates from camel compared to \(M. \) \(ap\) K-10 strain. The number in parentheses represents the total number of SNPs in each sequenced isolate. The color bars show the percentage distributions of different SNP categories in each isolate. (TIF)

Figure S7 Venn diagram of single nucleotide polymorphisms in sheep strain and camel isolate, JQ5 compared to \(M. \) \(ap\) K-10. SNP analysis was performed using MAUVE algorithm [52]. (TIF)

Figure S8 Distribution of number of SNPs per gene. Note the large number of genes with single nucleotide polymorphism. (TIF)

Figure S9 Phylogenetic analysis of \(mce\) genes. The phylogenetic tree displaying close relationship between the camel isolates with \(M. \) \(ap\)-S strain is based on members of \(mce\) genes (\(mceA2, mceB, mce2, mce3, mce4, and mce1F\)). The percentage bootstrap values (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method using pairwise deletion option. (TIF)

Table S1 List of polymorphic sites in 11 house-keeping genes from various \(M. \) \(ap\) subtypes. (DOC)

Table S2 Complete list of SNPs in \(M. \) \(ap\) JQ5 and \(M. \) \(ap\) JQ6 isolates compared to \(M. \) \(ap\) K-10. (XLS)

Table S3 Complete list of indels in \(M. \) \(ap\) JQ5 and \(M. \) \(ap\) JQ6 isolates compared to \(M. \) \(ap\) K-10. (XLS)

Acknowledgments

The authors would like to thank Chia-wei Wu and Sarah Ward for reading the manuscript.

Author Contributions

Conceived and designed the experiments: EJA MMS AMA AMT. Performed the experiments: PG CH EJA MMS MAAA AH OM MM KB JA KA. Analyzed the data: PG MMS AMT. Contributed reagents/materials/analysis tools: MAA AA MH OM MM KB JA KA AMT. Wrote the paper: PG MMS EJA AMA AMT.

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