

Pathogenic properties of a *Corynebacterium diphtheriae* strain isolated from a case of osteomyelitis

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Corynebacterium diphtheriae is typically recognized as a colonizer of the upper respiratory tract (respiratory diphtheria) and the skin (cutaneous diphtheria). However, different strains of *Corynebacterium diphtheriae* can also cause invasive infections. In this study, the characterization of a non-toxigenic *Corynebacterium diphtheriae* strain (designated BR-INCA5015) isolated from osteomyelitis in the frontal bone of a patient with adenoid cystic carcinoma was performed. Pathogenic properties of the strain BR-INCA5015 were tested in a *Caenorhabditis elegans* survival assay showing strong colonization and killing by this strain. Survival rates of $3.8 \pm 2.7\%$, $33.6 \pm 7.3\%$ and 0% were observed for strains ATCC 27010^T, ATCC 27012 and BR-INCA5015, respectively, at day 7. BR-INCA5015 was able to colonize epithelial cells, showing elevated capacity to adhere to and survive within HeLa cells compared to other *Corynebacterium diphtheriae* isolates. Intracellular survival in macrophages (THP-1 and RAW 264.7) was significantly higher compared to control strains ATCC 27010^T (non-toxigenic) and ATCC 27012 (toxigenic). Furthermore, the ability of BR-INCA5015 to induce osteomyelitis was confirmed by *in vivo* assay using Swiss Webster mice.

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INTRODUCTION

Corynebacterium diphtheriae is the classical aetiological agent of diphtheria (Hadfield *et al.*, 2000; Burkovski, 2013a, b). The infection causes localized inflammatory lesions of the upper respiratory tract, often with associated necrosis at distant sites, i.e. myocarditis and neuritis, attributable to the dissemination of the diphtheria toxin (Pennie *et al.*, 1996). Cases of cutaneous diphtheria, caused by toxigenic strains of *Corynebacterium*, occur when the organism is associated

with wounds and lesions (often ulcerative) and are mainly found in tropical areas. The effects of diphtheria toxin are typically less detrimental in this form compared to respiratory diphtheria (Hadfield *et al.*, 2000; Burkovski, 2013a).

The introduction of mass immunization against diphtheria in the 1950s resulted in a lower incidence of this disease in most industrialized countries (Saragea *et al.*, 1979; Galazka, 2000; von Hunolstein *et al.*, 2003). Although the circulation of toxigenic strains declined in all countries with good vaccination coverage, non-toxigenic *Corynebacterium diphtheriae* strains have been increasingly related to cases of invasive infections, including septicaemia (Isaac-Renton *et al.*, 1981; Huber-

Abbreviations: ECM, extracellular matrix; FBS, foetal bovine serum.

Schneider *et al.*, 1994; Zasada *et al.*, 2005; Farfour *et al.*, 2012), endocarditis (Tiley *et al.*, 1993; Mortier *et al.*, 1994; Lehnert *et al.*, 1995; Mattos-Guaraldi & Formiga, 1998; Belko *et al.*, 2000; Zasada *et al.*, 2005; Hirata *et al.*, 2008; Muttaiyah *et al.*, 2011), splenic abscesses (Alexander, 1984), arthritis (Guran *et al.*, 1979; Afghani & Stutman, 1993; Mortier *et al.*, 1994; Puliti *et al.*, 2006; Dias *et al.*, 2011; Peixoto *et al.*, 2014) and osteomyelitis (Poilane *et al.*, 1995; Stavrakakis-Peixoto *et al.*, 2011). A poor health status may predispose individuals to become infected by different *Corynebacterium* species (Mattos-Guaraldi *et al.*, 2001, 2003; Martins *et al.*, 2009; Rizvi *et al.*, 2011, 2013).

Cases of arthritis/osteomyelitis due to infections by other corynebacterial species have also been reported (Morrey *et al.*, 1977; von Graevenitz *et al.*, 1998; Wong *et al.*, 2010; Rizvi *et al.*, 2011; Verma & Kravitz, 2016). Patients with cancer are more susceptible to the appearance of cases of osteomyelitis obtained by haematogenic contamination, an infectious focus or lesions adjacent to the bone (Brady *et al.*, 2008). A case of osteomyelitis due to a non-toxigenic *Corynebacterium diphtheriae* biovar mitis (BR-INCA5015) strain in the frontal bone of a patient with adenoid cystic carcinoma was reported (Stavrakakis-Peixoto *et al.*, 2011).

Since there are still only a low number of investigations of pathogenic potential of non-toxigenic strains of *Corynebacterium diphtheriae*, BR-INCA5015 strain obtained after the microsurgical removal of the frontal bone due to osteomyelitis was characterized in more detail in this study.

METHODS

Case presentation. A 41-year-old woman residing in a poor area of the city of Rio de Janeiro, Brazil, was admitted to the National Cancer Institute (Instituto Nacional do Cancer – INCA) in 2006, with a tumour in the right nostril. The patient underwent surgery, and to correct a surgical dehiscence, the patient was again operated on, in 2008. *Corynebacterium diphtheriae* BR-INCA5015 and methicillin-sensitive *Staphylococcus aureus* strains were found during primary culture obtained after the microsurgical removal of the frontal bone due to osteomyelitis (Stavrakakis-Peixoto *et al.*, 2011). The corresponding strain was characterized in more detail in this study.

Bacterial strains and culture conditions. *Corynebacterium diphtheriae* and *Escherichia coli* strains used in this study are listed in Table 1. *Corynebacterium diphtheriae* strains were grown in heart infusion (HI) (Becton and Dickinson) broth at 37 °C and stored in HI medium with 20 % glycerol at –80 °C (Antunes *et al.*, 2015). *E. coli* OP50 was grown in Luria-Bertani (Becton and Dickinson) medium at 37 °C (Sambrook *et al.*, 1989; Antunes *et al.*, 2015; Santos *et al.*, 2015a).

Nematode infection model. The assays were performed as previously described with some adaptations for *Corynebacterium diphtheriae* and analysed by the Kaplan–Meier survival method (de Bono & Bargmann, 1998; Browning *et al.*, 2013; Antunes *et al.*, 2015; Santos *et al.*, 2015a). Briefly, *Caenorhabditis elegans* N2 was maintained on plates containing nematode growth medium agar for approximately 6 to 7 days at 20 °C and used in infection assays with *Corynebacterium diphtheriae* strains. Twenty L4 stage larval worms were infected with 20 µl of each bacterial strain (OD₆₀₀ 1.0) on nematode growth medium plates at 20 °C for 24 h. The worms were assessed daily following infection, and the dead nematodes were counted and removed every 24 h for 7 days. For each strain,

approximately 60 nematodes were used, and the assays were performed three times. *E. coli* OP50 was used as a control in these experiments.

Extracellular matrix (ECM) binding assays. The ECM protein binding assay was performed based on a modified protocol previously described (Gomes *et al.*, 2009; Birkenhauer *et al.*, 2014). Briefly, standard 96-well polystyrene microtitre plates (Greiner CELLSTAR) were coated with collagen types I and IV (Sigma) dissolved in PBS (50 µg ml^{–1}). To each well, a total of 200 µl of solution was added. Plates were then covered and allowed to incubate at 4 °C for 24 h. Coating solutions were gently removed after this time, and the wells were rinsed twice with PBS. The density of the inoculum was adjusted to an OD₆₀₀ of 0.2, and 200 µl was inoculated in triplicate into the coated wells. Uninfected wells were used as control. Microtitre plates were incubated for 24 h at 37 °C under static conditions in order to allow biofilm formation. For quantification of biofilms, after 24 h incubation, microbial solutions were discarded, and the wells were washed three times with 200 µl PBS in order to remove unattached cells. Biofilms were heat fixed at 65 °C for 60 min and subsequently stained with 0.1 % (w/v) crystal violet (Sigma-Aldrich) for 15 min at room temperature. Crystal violet solution was removed, and the plates were rinsed by submersion in a container of tap water. Plates were then allowed to dry for 60 min at 35 °C. After this, crystal violet from stained biofilms was re-solubilized in 200 µl of 95 % ethanol, and absorbance was measured at 620 nm.

Human epithelial carcinoma (HeLa) cell interaction assays. The cellular interaction assays were performed using epithelial cells derived from human cervical carcinoma according to previously described protocols (Antunes *et al.*, 2015; Hacker *et al.*, 2015). Briefly, HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM), high glucose with L-glutamine (PAA Laboratories) supplemented with 100 µg ml^{–1} gentamicin and 12 µg ml^{–1} ciprofloxacin and 10 % heat-inactivated foetal bovine serum (FBS) (Life Technologies) in a 5 % CO₂ incubator at 37 °C. Cells were passaged at a ratio of 1 : 10 twice per week. HeLa cells were seeded in 24-well plates without antibiotics (Nunc) at a density of 1 × 10⁵ cells per well 24 h prior to infection. Bacteria were harvested by centrifugation, and cell density was adjusted to OD₆₀₀ 1.0. A master mix of the inoculum with an m.o.i. of 50 was prepared in DMEM, and 500 µl per well was used to infect the cells. The plates were centrifuged for 5 min at 300 g to synchronize infection and subsequently incubated for 90 min. The cells were washed with PBS three times, detached with 500 µl Accutase per well (5 min, 37 °C, 5 % CO₂, 95 % humidity) and lysed by addition of 12.5 µl 10 % Tween 20 under the same conditions. Serial dilutions were made in pre-chilled 1 × PBS and plated on Columbia agar with sheep blood (Oxoid) to determine the number of c.f.u.

For analysis of internalization, the cells were washed three times with PBS to remove planktonic and loosely attached bacteria. Subsequently, the cells were incubated for 2 h in DMEM (500 µl per well) containing 100 µg ml^{–1} gentamicin to kill the remaining extracellular bacteria. After incubation, the cell layers were washed three times with PBS, detached by adding 500 µl Accutase per well (5 min, 37 °C, 5 % CO₂, 95 % humidity) and lysed by addition of 12.5 µl 10 % Tween 20 to liberate the intracellular bacteria. Serial dilutions of the inocula and the lysates were plated out on Columbia agar with sheep blood (Oxoid) to determine the number of c.f.u. The percentage adhesion/invasion efficiency was calculated by the ratio of bacteria used for infection (c.f.u. inoculum plate) and bacteria in the lysate (c.f.u. lysate plate) multiplied by 100.

Human (THP-1) and murine (RAW 264.7) macrophage interaction assays. The macrophage interactions were performed based on protocols previously described (Hacker *et al.*, 2016). Human THP-1 cells were cultured in 10 % FBS-supplemented Roswell Park Memorial Institute medium 1640 (RPMI 1640; containing 100 U ml^{–1} penicillin and 100 mg ml^{–1} streptomycin) and 10 % FBS. Murine RAW 264.7 cells were cultivated in DMEM, high glucose with L-glutamine and sodium pyruvate (PAA Laboratories), supplemented with 120 mg ml^{–1} penicillin,

Table 1. Micro-organisms, human/murine cell lines and plasmids used in this study

Micro-organism	Description	Reference/source
<i>Corynebacterium diphtheriae</i>		
BR-INCA5015	*Biovar mitis, <i>tox</i> gene negative, previously isolated from the infected frontal bone of a cancer patient	Stavracakis-Peixoto <i>et al.</i> (2011)
ATCC 27010 ^T	*Biovar mitis, <i>tox</i> gene negative, type strain of the species <i>Corynebacterium diphtheriae</i> from the American Type Culture Collection	Dias <i>et al.</i> (2011)
ATCC 27012	*Biovar mitis, <i>tox</i> gene positive (positive diphtheria toxin expression), a well-used strain as reference. This strain is often used to demonstrate (significant amounts of) toxin production and can be used as a positive control for the ELEK test	Dias <i>et al.</i> (2011)
<i>E. coli</i>		
OP50	Uracil-auxotrophic <i>E. coli</i> B	Brenner (1974)
Cell line		
THP-1	Human leukaemia monocytic cells	Tsuchiya <i>et al.</i> (1980)
RAW 264.7	Murine leukaemic macrophages	Raschke <i>et al.</i> (1978)
HeLa	Human epithelial cervical carcinoma cells	Gey <i>et al.</i> (1952), Scherer <i>et al.</i> (1953)
Plasmid		
pEPRI-p45gfp	<i>P45</i> , <i>gfpuv</i> , <i>rep</i> , <i>per</i> , T1, T2, KM ^R	Knoppová <i>et al.</i> (2007)

*A multiplex PCR for detection of *Corynebacterium diphtheriae* infection and differentiation between toxigenic and non-toxigenic strains was performed based on previously described techniques (Pimenta *et al.*, 2008). According to the API-Coryne System (API code 1010324; bioMérieux), BR-INCA5015 was *Corynebacterium diphtheriae* biovar mitis, as also observed by conventional phenotypic tests (Murray *et al.*, 2002; Stavracakis-Peixoto *et al.*, 2011).

120 mg ml⁻¹ streptomycin and 10% FBS. Before infection, THP-1 and RAW 264.7 cells were cultured in antibiotic-free medium containing 10% FBS for 24 h, and THP-1 cells were differentiated into macrophage-like cells using 10 ng ml⁻¹ phorbol 12-myristate 13-acetate. Bacteria were harvested by centrifugation, and cell density was adjusted to OD₆₀₀ 1.0. A master mix of the inoculum with an m.o.i. of 25 was prepared in the respective medium, and the cells were infected with the inoculum or left uninfected. The plates were centrifuged for 5 min at 500 r.p.m. to synchronize infection. After incubation for 30 min, the medium was aspirated, and cells were treated first with medium containing 100 µg ml⁻¹ gentamicin for 1 h. Then, medium with a lower gentamicin concentration (10 µg ml⁻¹) was added, and cells were incubated for 1, 7 or 23 h. At each time point of incubation, the supernatant was removed, and cells were lysed with 500 µl 0.1% (v/v) Triton X-100. Serial dilutions of the inoculum and the lysates were plated out on Columbia agar with sheep blood (Oxoid) to determine the number of c.f.u. The intracellular viability was calculated by the ratio of bacteria used for infection (c.f.u. inoculum plate) and bacteria in the lysate (c.f.u. lysate plate) multiplied by 100.

Fluorescence microscopy. Fluorescence microscopy assays were performed by methods previously described (dos Santos *et al.*, 2010; Hacker *et al.*, 2016). Briefly, 1 day prior to infection, HeLa and RAW 264.7 cells were seeded at a density of 0.5 × 10⁵ and 1 × 10⁵ cells, respectively, on sterile coverslips in 24-well plates. *Corynebacterium diphtheriae* strains transformed with plasmids encoding *gfp* cultivated in HI medium with kanamycin were harvested by centrifugation, and cell density was adjusted in PBS to an OD₆₀₀ 1.0 to infect macrophages as described above. After different time points, the medium was aspirated, and cells were fixed by addition of 500 µl 4% paraformaldehyde in PBS and incubated for 20 min at 37 °C. Until further staining, cells were stored in PBS at 4 °C. For subsequent analysis by microscopy, coverslips were incubated with 30 µl of Alexa Fluor 647 phalloidin diluted 1:200 in Image-iT FX Signal Enhancer (Molecular Probes, Life Technologies) for 45 min in the dark to stain the cytoskeleton of the cells. After washing twice

with PBS, the coverslips were dried and embedded on glass slides in ProLong Gold antifade mountant with DAPI (Molecular Probes, Life Technologies), and samples were stored in the dark at 4 °C. Micrographs were taken with the confocal laser scanning microscope Leica SP5 II (Leica Microsystems) and analysed with the LAS software suite.

Statistical analysis. Each experiment was conducted at least in triplicate (independent biological replicates), and statistical analyses were performed with the appropriate tests using GraphPad Prism 5.0 (GraphPad). *P* < 0.05 was considered significant.

Animal experiments using mice model. Conventional Swiss Webster mice, sex matched, 18 to 22 g, from Ceval/Fiocruz were used. The study was performed in compliance with guidelines outlined in the International Guiding Principles for Biomedical Research Involving Animals as issued by the Council for the International Organizations of Medical Sciences and with the Brazilian government's ethical guidelines for research involving animals (Fiocruz Ethic Committee for Animal Experiments – CEUA/FIOCRUZ – L-034/07).

The possibility of developing osteomyelitis was analysed by the methods described previously (Dias *et al.*, 2011). Mice were infected intravenously through a tail vein with 0.5 ml of bacterial suspension prepared in sterile saline at OD₅₇₀ 0.2. Control mice were injected with 0.5 ml of sterile saline. The experiments were performed in triplicate, and the mice were examined daily for 30 days by independent observers to assess the presence of joint inflammation and scarring macroscopically.

Histopathological studies. Histopathological procedures to evaluate the features of the disease were based on methods previously described (Dias *et al.*, 2011). Briefly, mice were inoculated intravenously with *Corynebacterium diphtheriae* BR-INCA5015 or sterile saline as control. Mice were sacrificed after 15 days. Paws (one per mouse) were removed aseptically, fixed in 10% v/v formalin for 24 h and then decalcified using 10% EDTA in PBS (0.1 M, pH 7.2) for 7 days. Subsequently, the

specimens were dehydrated, embedded in paraffin, sectioned at 5 to 7 µm and stained with haematoxylin and eosin. At this point, the specimens were decalcified in 5 % trichloroacetic acid (v/v) for 7 days and then dehydrated, embedded in paraffin, sectioned at 3 to 4 µm and stained with haematoxylin and eosin. Joints were examined for synovitis (defined as synovial membrane thickness of more than two cell layers), extent of infiltrate (presence of inflammatory cells in the subcutaneous and/or periarticular tissues), exudate (presence of inflammatory cells in the articular cavity), cartilage damage, bone erosion and loss of joint architecture.

RESULTS

Interaction with *Caenorhabditis elegans*

The nematode experiments indicated a high ability of nematode colonization and killing by the isolated *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain. Most (90 %) of the nematodes were already killed by the BR-INCA5015 strain after 3 days. However, the survival rates during infection of nematodes were much higher for the homologous strains toxigenic ATCC 27012 when comparing to the non-toxigenic ATCC 27010^T. Therefore, the BR-INCA5015 strain was more harmful to *Caenorhabditis elegans* compared to the homologous toxigenic ATCC 27012 and non-toxigenic ATCC 27010^T strains (Fig. 1).

Collagen binding activities of *Corynebacterium diphtheriae* strains

Compared to the control strains ATCC 27010^T (A_{620} 0.53 ± 0.01) and ATCC 27012 (A_{620} 0.72 ± 0.03), the binding level of the *Corynebacterium diphtheriae* BR-INCA5015 to type I collagen was significantly higher (A_{620} 1.57 ± 0.04) (Fig. 2a). In the case of type IV collagen, only slight differences were observed between the strains: BR-INCA5015 (A_{620} 0.77 ± 0.03), ATCC 27012 (A_{620} 0.87 ± 0.06) and ATCC 27010^T (A_{620} 0.61 ± 0.01) (Fig. 2b).

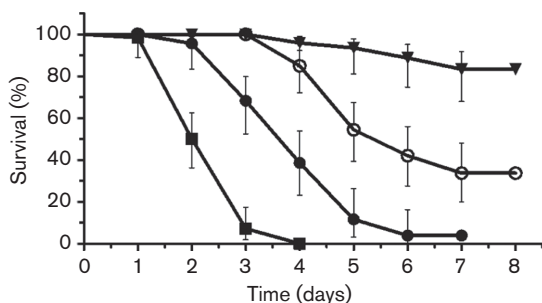


Fig. 1. *Caenorhabditis elegans* N2 nematode survival assay: infection with *E. coli* OP50 (▼), *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain (■), non-toxigenic ATCC 27010^T (●) and toxigenic ATCC 27012 (○) strains. Data shown represent the mean of three independent experiments; error bars represent SD.

Adhesion and invasion properties to HeLa epithelial cells

Although all three *Corynebacterium diphtheriae* strains tested were able to adhere to the HeLa epithelial cell line, after 90 min of infection, the *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain reached almost double the adhesion rate (9.59 ± 0.91 %), compared to ATCC 27010^T and ATCC 27012 (5.64 ± 0.61 % and 4.07 ± 0.45 %, respectively) strains (Fig. 3).

Data of intracellular viability of *Corynebacterium diphtheriae* strains in HeLa cells using gentamicin protection assays (Fig. 4) showed that BR-INCA5015 expressed the highest internalization rates (0.46 ± 0.06 %) 2 h post-infection, while invasion rates of 0.1 ± 0.01 % and 0.03 ± 0.007 % were observed for ATCC 27010^T and ATCC 27012, respectively (Fig. 4a). The ability to survive in the HeLa intracellular environment was also higher for strain BR-INCA5015 (0.23 ± 0.05 %) compared to the controls ATCC 27010^T (0.002 ± 0.0005 %) and ATCC 27012 (0.0003 ± 0.0001 %) when evaluated 24 h after infection (Fig. 4b). Obviously, the *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain is highly active in colonization of HeLa cells.

Intracellular survival of *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain within human THP-1 and murine RAW 264.7 macrophage cell lines

In THP-1 macrophages, an internalization rate of 0.03 ± 0.005 % was observed for *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain at 2 h post-infection, while ATCC 27010^T and ATCC 27012 reached only 0.01 ± 0.002 % and 0.0003 ± 0.0001 %, respectively. Analysis at 8 h post-infection indicated 0.06 ± 0.02 % of viable BR-INCA5015 strain and 0.002 ± 0.0006 % and 0.0005 ± 0.0002 % for the ATCC 27010^T and ATCC 27012 strains, respectively. Viable bacteria were also recovered 24 h post-infection for all the three strains tested, and the percentages were 0.00013 ± 0.00004 % for the BR-INCA5015 strain, 0.00001 ± 0.000004 % for the ATCC 27010^T strain and 0.00002 ± 0.00001 % for the ATCC 27012 strain (Fig. 5a–c).

In order to exclude cytotoxic effects due to diphtheria toxin, experiments using murine RAW 264.7 macrophage line lacking the toxin receptor were done. An uptake rate of 0.4 ± 0.08 % was detected for BR-INCA5015 strain, while ATCC 27010^T and ATCC 27012 strains reached only 0.12 ± 0.03 % and 0.15 ± 0.04 % at 2 h post-infection. The number of viable bacteria decreased over time for all strains. After 8 h infection, the percentage of viable bacteria recovered for BR-INCA5015 strain was 0.25 ± 0.07 % and, for the controls ATCC 27010^T and ATCC 27012, was 0.05 ± 0.01 % and 0.07 ± 0.03 %, respectively. Viable bacteria were recovered at 24 h post-infection for all the strains tested, and significant differences in the percentage of c.f.u were observed between BR-INCA5015 strain (0.01 ± 0.002 %) and the controls

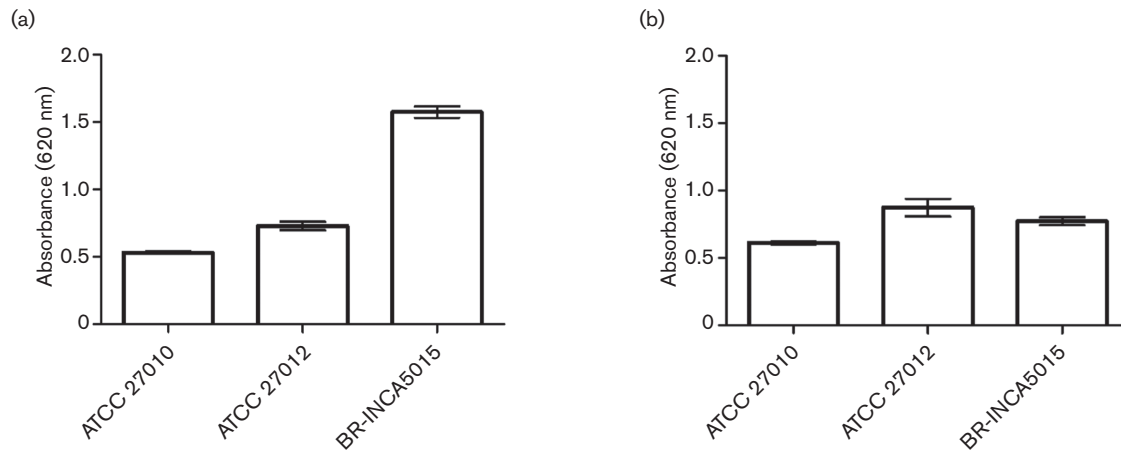


Fig. 2. Semi-quantitative analysis of *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain binding properties to I (a) and IV (b) collagen types. Data shown are mean \pm SD values of at least three independent biological replicates.

ATCC 27012 ($0.004\pm0.002\%$) and ATCC 27010^T ($0.003\pm0.0007\%$) (Fig. 5d–f).

Fluorescence microscopy assays

Fluorescence microscopy was used as an additional method to examine the interaction of *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain and control strains with RAW 264.7 macrophages and HeLa epithelial cell lines. All the strains were marked by GFP expression, nuclei were stained with DAPI and cytoskeleton was stained with Alexa Fluor 647 phalloidin. Z-stack micrographs were taken using

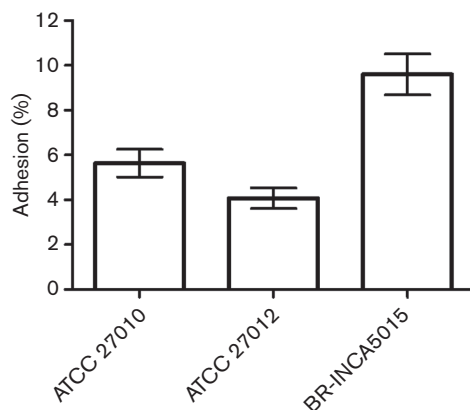


Fig. 3. Quantitative analysis of *Corynebacterium diphtheriae* adhesion rates to HeLa cells. Epithelial cells were infected with *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain, non-toxicogenic ATCC 27010^T and toxigenic ATCC 27012 at m.o.i. of 50 for 90 min. After incubation, the cells were washed three times, detached and lysed, and dilutions of the lysate were plated on agar plates to determine the bacteria adherence percentage of the inoculum. Data shown are mean \pm SD values of at least three independent biological replicates.

the confocal laser scanning microscope Leica SP5 II and analysed with the LAS software suite to prove that the bacteria were located inside the cell (Fig. 6). In experiments using RAW 264.7 macrophages, the number of fluorescent bacteria declined within 24 h post-infection. However, a higher number of bacteria located inside the macrophage were observed in the BR-INCA5015 strain in all time points (Fig. 6a–c) compared to both ATCC 27010^T (Fig. 6d–f) and ATCC 27012 (Fig. 6g–i) strains. Similar results were obtained with THP-1 macrophages indicating that these findings are not limited to a single macrophage cell line or exclusively to murine macrophages (data not shown). Fluorescence microscopy images revealed adhesion to the surface of HeLa cells and internalized bacteria for all *Corynebacterium diphtheriae* strains tested. A higher number of bacterial cells associated and/or internalized within HeLa cells were observed in the *Corynebacterium diphtheriae* BR-INCA5015 strain (Fig. 6j, m) compared to those in the ATCC 27010^T (Fig. 6k, n) and ATCC 27012 (Fig. 6l, o) strains.

Arthritogenic activity and histopathological findings

The ability to cause haematogenic and bone infection of *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain was demonstrated by an *in vivo* model system using mice. Upon injection of the bacteria into the tail vein and haematogenic spread, clinical signs of arthritis were evident starting 5 days post-infection (Fig. 7). The most frequently affected joints were ankle and wrist (Fig. 7a). Nodes on the tail and testicles were also observed as illustrated in Fig. 7(b, c). Histopathological studies of joints of mice infected with *Corynebacterium diphtheriae* BR-INCA5015 confirmed the clinical signs of arthritis and osteomyelitis. Three affected paws were examined. Micrographs in Fig. 7 (d, e) illustrate the histopathological features of arthritis and osteomyelitis in mice caused by *Corynebacterium*

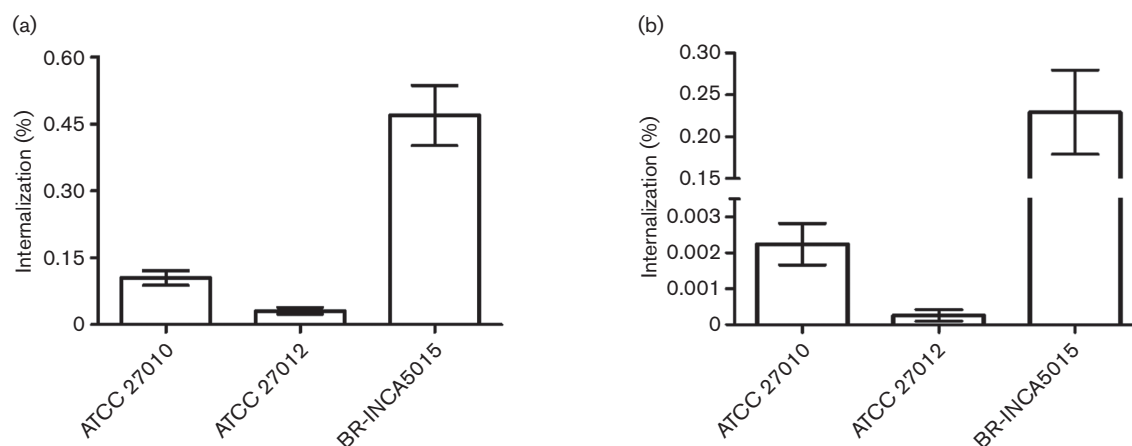


Fig. 4. Invasion of epithelial cells. HeLa cells were infected for 90 min with *Corynebacterium diphtheriae* strains, treated for 2 h with $100 \mu\text{g ml}^{-1}$ gentamicin in DMEM, washed with PBS, detached with trypsin solution, lysed with Tween 20 and plated on agar plates to determine the bacteria invasion percentage of the inoculum; (a) 2 h post-infection and (b) 24 h post-infection. Data shown are mean \pm SD values of at least three independent biological replicates.

diphtheriae BR-INCA5015, while control paws were completely symptom free (Fig. 7f).

DISCUSSION

The occurrence of diphtheria among immunized persons in addition to the increasing frequency of cases of invasive infections caused by *Corynebacterium diphtheriae* points to the importance of other virulence factors besides diphtheria toxin production (Hirata *et al.*, 2002; Mattos-Guaraldi *et al.*, 2003; Sabbadini *et al.*, 2010; Burkovski, 2013a; Santos *et al.*, 2015b). Non-toxicogenic *Corynebacterium diphtheriae* strains, formerly considered as extracellular colonizers, have also been associated with cases of bacteraemia, catheter-related infection, endocarditis, septic arthritis and osteomyelitis (Guran *et al.*, 1979; Poilane *et al.*, 1995; Wilson, 1995; Mattos-Guaraldi & Formiga, 1998; Mattos-Guaraldi *et al.*, 2003; Puliti *et al.*, 2006; Hirata *et al.*, 2008; Gomes *et al.*, 2009; Dias *et al.*, 2011; Stavrakakis-Peixoto *et al.*, 2011; Peixoto *et al.*, 2014).

Previous studies indicated a great variability in the pathogenicity islands of *Corynebacterium diphtheriae*, which may reflect bacterial lifestyle and physiological versatility (D'Afonseca *et al.*, 2011). Common adhesive properties and potential adhesins including pili (Gaspar & Ton-That, 2006; Ott *et al.*, 2010), haemagglutinins, hydrophobins, exposed sugar residues and enzymes with *trans*-sialidase activity to *Corynebacterium diphtheriae* strains were described (Mattos-Guaraldi *et al.*, 2000).

Adherence to and internalization by epithelial and endothelial cells were also highlighted as critical steps during blood barrier disruption and systemic dissemination of both non-toxicogenic and toxicogenic *Corynebacterium diphtheriae* strains (Hirata *et al.*, 2002; Peixoto *et al.*, 2014). Lately, the non-

fimbrial DIP0733 protein was described as a multi-functional virulence factor involved in adhesion, invasion of epithelial cells and induction of apoptosis of a *Corynebacterium diphtheriae* strain. Furthermore, based on its fibrinogen-binding activity, DIP0733 seemed to play a role in avoiding recognition of *Corynebacterium diphtheriae* by the immune system (Colombo *et al.*, 2001; Sabbadini *et al.*, 2010, 2012; Antunes *et al.*, 2015). However, *Corynebacterium diphtheriae* isolates may behave differently when they meet host surfaces, with regard to the cells that they efficiently infect and the kind of inflammatory response that they trigger.

In this study, virulence properties that contribute to the ability of *Corynebacterium diphtheriae* strain BR-INCA5015 to cause bone diseases and disorders were verified.

Besides guinea pigs already employed by Loeffler, wax moth larvae (*Galleria mellonella*) and *Caenorhabditis elegans* nematodes were established as model systems for the investigation of *Corynebacterium diphtheriae* pathogenicity and virulence factors (Ott *et al.*, 2012; Broadway *et al.*, 2013; Santos *et al.*, 2015a; Antunes *et al.*, 2015, 2016). *Caenorhabditis elegans* is one of the major invertebrate model systems in biology based on advantageous properties such as short lifespan, transparency, genetic tractability and ease of culture using an *E. coli* diet. In its natural habitat, compost and rotting plant material, this nematode lives on bacteria. However, while *Caenorhabditis elegans* is a predator of bacteria, it can also be infected by nematopathogenic coryneform bacteria such *Microbacterium* and *Leucobacter* species. Depending on the nematode pathogen, aggregates of worms, termed wormstars, can be formed, or severe rectal swelling, so-called Dar formation, can be induced. *Caenorhabditis elegans* can also be infected by *Corynebacterium diphtheriae*. Earlier studies showed that *Corynebacterium*

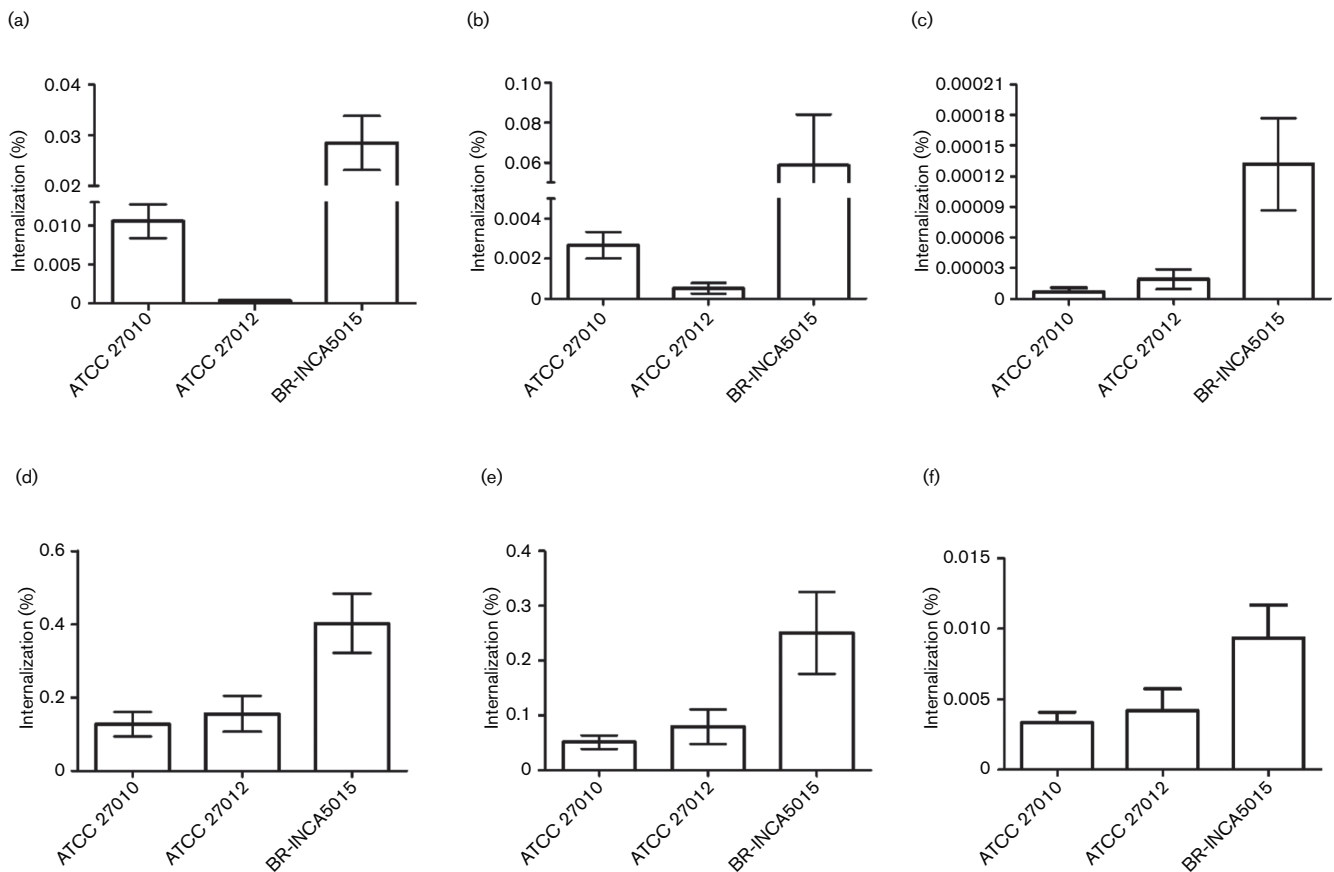


Fig. 5. Intracellular survival within differentiated human THP-1 cells using phorbol 12-myristate 13-acetate (a–c) and murine RAW 264.7 macrophage cells (d–f) of *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain, homologous non-toxicogenic ATCC 27010^T control strain and toxigenic ATCC 27012 control strain. Percentage of viable bacteria at (a and d) 2 h, (b and e) 8 h and (c and f) 24 h post-infection was determined based on the inoculum. Data shown are mean \pm SD values of at least three independent biological replicates.

diphtheriae were found to induce star formation slowly in worms, as well as a severe tail swelling phenotype (Santos *et al.*, 2015a; Antunes *et al.*, 2015, 2016). In the present study, results of nematode survival assays indicated that the *Corynebacterium diphtheriae* BR-INCA5015 strain, isolated from osteomyelitis in the frontal bone of a patient with adenoid cystic carcinoma, expressed a high ability of host colonization and killing of *Caenorhabditis elegans*, as previously demonstrated for the toxigenic *Corynebacterium diphtheriae* CDC-E8392 strain (Santos *et al.*, 2015a; Antunes *et al.*, 2015, 2016). Interestingly, the osteomyelitis-related BR-INCA5015 strain was more harmful to *Caenorhabditis elegans* compared to the homologous toxigenic ATCC 27012 and non-toxicogenic ATCC 27010^T strains tested.

When the first case of isolation of a non-toxicogenic *Corynebacterium diphtheriae* biovar mitis of the non-sucrose-fermenting biotype (BR-INCA5015 strain) from osteomyelitis in the frontal bone of a patient with adenoid cystic carcinoma was reported, DNase, catalase and nitrate-reductase activities which possibly contribute to the invasive potential

and survival of BR-INCA5015 strain within host tissues were observed (Peixoto *et al.*, 2011).

Similar to many other micro-organisms, *Corynebacterium diphtheriae* was formerly found to exploit the ECM and/or plasma proteins to colonize human tissues or to evade immune mechanism for clearance of bacteria (dos Santos *et al.*, 2010; Sabaddini *et al.*, 2010; Antunes *et al.*, 2015; Santos *et al.*, 2015a). The present data showed the ability of *Corynebacterium diphtheriae* strains to bind to collagen types I and IV. Type I collagen is the organic matrix molecule of mammalian bone and the most abundant in the human body (Balzer *et al.*, 1997; Maynes, 2012). Interestingly, the binding levels of the *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain were significantly higher for the type I collagen compared to those of the ATCC 27010^T and ATCC 27012 control strains.

The adhesion to and intracellular survival of *Corynebacterium diphtheriae* in epithelial cells have been demonstrated for different human cells, including epithelial cell lines like HeLa, HEp-2 (Hirata *et al.*, 2002; Stavracakis Peixoto *et al.*, 2014;

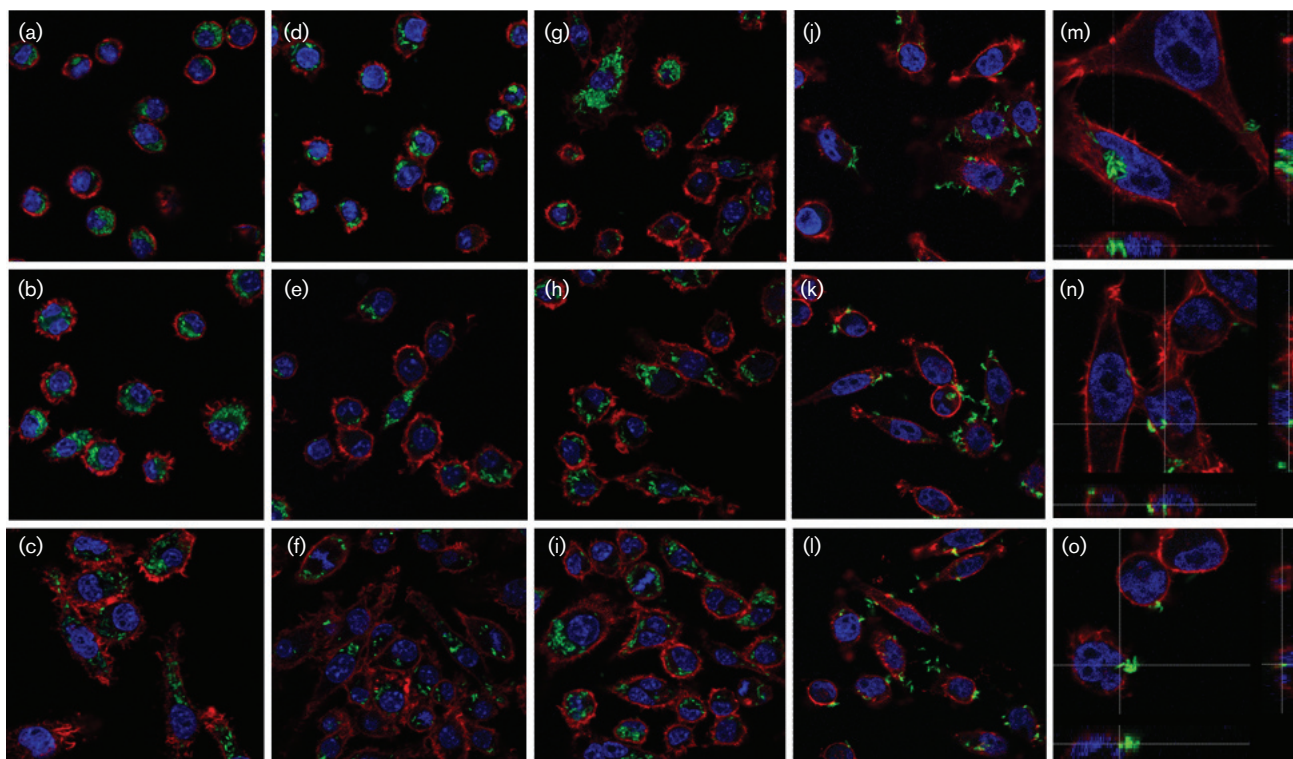


Fig. 6. Micrographic data of fluorescence microscopy assays of RAW 264.7 macrophage (30 min) and HeLa (90 min) cell line interaction with *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain prEPRIp45gfp, ATCC 27010^T prEPRIp45gfp strain and ATCC 27012 prEPRIp45gfp strain (m.o.i. of 25). Extracellular bacteria were killed by the addition of gentamicin and fixed at different periods of incubation. Nuclei were stained with DAPI, and cytoskeleton was stained with Alexa Fluor 647 phalloidin. Z-stack micrographs were taken using the confocal laser scanning microscope Leica SP5 II and analysed with the LAS software suite to prove that bacteria are located inside of the cell. Fluorescence microscopy images of RAW 264.7 cells 2, 8 and 24 h post-infection: BR-INCA5015 (a–c); ATCC 27010^T (d–f) and ATCC 27012 (g–i), respectively. Adhesion of *Corynebacterium diphtheriae* to epithelial cells (HeLa): BR-INCA5015 (j), ATCC 27010^T (k) and ATCC 27012 (l). Invasion of *Corynebacterium diphtheriae* into epithelial cells (HeLa): BR-INCA5015 (m), ATCC 27010^T (n) and ATCC 27012 (o).

Antunes *et al.*, 2015; Santos *et al.*, 2015a) and Detroit 562 (Ott *et al.*, 2012; Bertuccini *et al.*, 2004), human umbilical vein endothelial cells (Peixoto *et al.*, 2014) and U-937 and THP-1 macrophages (dos Santos *et al.*, 2010; Antunes *et al.*, 2015).

Corynebacterium diphtheriae BR-INCA5015 osteomyelitis-related strain expressed a higher ability to adhere to and survive within human (HeLa) epithelial cells compared to the other *Corynebacterium diphtheriae* strains currently tested.

For a successful dissemination in the case of a systemic infection, micro-organisms have also to cope successfully with macrophages. Human macrophages in the absence of opsonins may not be promptly effective at killing *Corynebacterium diphtheriae*. Those strains exhibit strategies to survive within macrophages and to exert apoptosis and necrosis in human phagocytic cells, independent of the *tox* gene (dos Santos *et al.*, 2010). Endorsing the aforementioned, intracellular survival of the non-toxicogenic *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain within both human (THP-1) and murine (RAW

264.7) macrophages was significantly higher compared to both non-toxicogenic ATCC 27010^T and toxicogenic ATCC 27012 control strains. Interaction of the BR-INCA5015 strain with macrophages and epithelial cells was morphologically observed by fluorescence microscopy.

In an attempt to validate the ability of *Corynebacterium diphtheriae* BR-INCA5015 to promote haematogenic and bone infections *in vivo*, a murine experimental model was used. Histopathological studies of joints of mice infected with *Corynebacterium diphtheriae* BR-INCA5015 confirmed the clinical signs of arthritis and osteomyelitis. Interestingly, Dias *et al.* (2011) showed that the non-toxicogenic ATCC 27010^T induced low arthritis index rates and the toxicogenic strain ATCC 27012 had no ability to induce signs of arthritis in mice.

In conclusion, non-toxicogenic *Corynebacterium diphtheriae* should not be merely regarded as contaminants, since they can be directly or indirectly related to the establishment and/or maintenance of invasive infectious processes. Data also endorsed the ability of some *Corynebacterium*

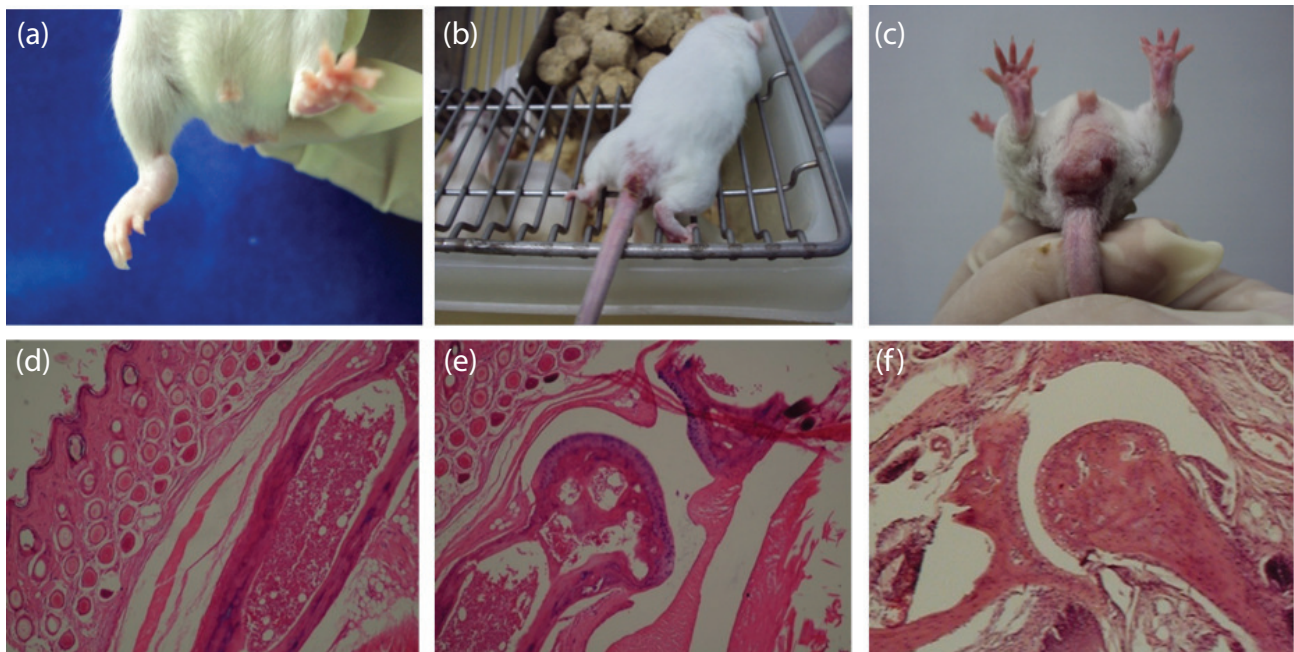


Fig. 7. Haematogenic and bone infection by *Corynebacterium diphtheriae* BR-INCA5015 osteomyelitis-related strain demonstrated by an *in vivo* model system using mice. Clinical signs of arthritis were present in mice on day 15 after intravenous infection with BR-INCA5015 strain. Photographs showing arthritic hind paws and tail with nodose. Arthritis on the right forepaws (a), excoriation in the tail (region of insertion) (b) and abrasions in the testicles, with increased volume indicating orchitis (c). Joint with arthritis of mice infected with BR-INCA5015 exhibiting capsular distension. (d, e) Intense leukocyte inflammatory infiltrate and osteochondral deformation. (f) Histopathological features of a normal joint. Magnification $\times 100$.

diphtheriae strains, such as BR-INCA5015, to induce osteomyelitis. In all aspects evaluated in this study, BR-INCA5015 osteomyelitis-related strain expressed more severe virulent properties than the *Corynebacterium diphtheriae* control strains, clearly independently of the presence of *tox* gene or diphtheria toxin production.

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