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Tese

Partículas magnéticas: síntese e aplicações em ensaios de imunoseparação

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RESUMO

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O desenvolvimento de insumos para o diagnóstico e prevenção de doenças infecciosas é estratégico para o avanço científico e tecnológico do país. Resultados promissores vêm sendo obtidos com o uso de nanocompósitos magnéticos (NCM) aplicados à área da saúde para as mais diversas finalidades, como na separação, purificação e detecção de células ou biomoléculas. Nesse trabalho, um método de imunoseparação magnética (IMS) utilizando NCM a base de carbono e cobalto (NCMc) sintetizados, e outro de poliestireno e ferro (NCMp) disponível comercialmente, foi utilizado para capturar e detectar leptospiras patogênicas. Os NCMc foram sintetizados através do método dos precursores poliméricos e caracterizados por microscopia eletrônica de varredura/transmissão (MEV/MET), difratometria de raio-X (DRX), espectrometria no infravermelho (FTIR) e imunofluorescência (IF). A superfície dos NCMc foi funcionalizada com grupos carboxílicos mediante incorporação de ácido acrílico. Os NCMc adsorvidos com anticorpos monoclonais (MAbs) contra leptospiras patogênicas, quando utilizados em ensaios de IMS seguidos por PCR e cultivo celular, foram capazes de detectar e isolar o organismo. Os NCMp foram utilizados para a detecção de *Leptospira* spp. em fluidos biológicos de cães artificialmente contaminados com leptospiras e em amostras clínicas de cães positivos para a leptospirose. A introdução da IMS como passo anterior a PCR utilizando os NCMp foi capaz de detectar uma concentração 10 vezes menor de leptospiras quando comparada a PCR realizada diretamente a partir de amostras de urina e sangue artificialmente contaminados. Além disso, a IMS realizada com os NCMp aumentou a sensibilidade da PCR em amostras clínicas de cães positivos para leptospirose. Os dados obtidos em ambos os estudos sugerem que os NCMc e NCMp constituem importantes ferramentas para o desenvolvimento de novos testes de diagnóstico.

Palavras chave: Diagnóstico. Cobalto. *Leptospira.*

ABSTRACT

Monte, Leonardo Garcia **Magnetic particles: synthesis and applications of immunoseparation assays** 2011. 61f. Thesis (Doctoral Degree) – Biotechnology Post-Graduation Program. Universidade Federal de Pelotas.

The development of reagents for infectious disease diagnosis and prevention is strategic for the scientific and technological advance of the country. Promising results have been obtained with the use of magnetic nanocomposites (MNC) in health for several purposes as separation, purification and detection of cells or biomolecules. In this work, an immunomagnetic separation (IMS) method using carbon and cobalt-based (MNCc), synthesized, and other polystyrene and iron-based (MNCp) commercially available was applied, for the isolation and detection of pathogenic leptospires. The MNCc were synthesized through polymeric precursor method and characterized by scanning electron microscopy/transmission (SEM/TEM), X-ray diffractometry (XRD), infrared spectrometry (FTIR), and immunofluorescence (IF). The MNCc surface was functionalized with carboxylic groups through the incorporation of acrylic acid. MNCc, adsorbed with monoclonal antibodies (MAbs) against pathogenic leptospires, were used in the IMS assays followed by PCR and cell culture was able to detect and isolate the organism. MNCp were used for detecting *Leptospira* spp. in biological fluids from dogs artificially contaminated with leptospires, and clinical samples from dogs positive for leptospirosis. The introduction of IMS, as a step prior to PCR using MNCp, detected a concentration of leptospires ten times smaller when compared to PCR performed directly on artificially contaminated urine and blood samples. Moreover, the IMS performed with MNCp increased PCR sensitivity in clinical samples from dogs positive for leptospirosis. Data from both studies suggest that MNCc and MNCp are important tools for the development of new diagnosis tests.

Keywords: Diagnosis. Cobalt. *Leptospira.*

SUMÁRIO

1. INTRODUÇÃO GERAL

O desenvolvimento de nanocompósitos magnéticos (NCM) vem se destacando na última década visando inúmeras aplicações biotecnológicas (JAIN, 2003; KIM et al., 2009). Entretanto, esta tecnologia tem sido relatada desde os anos 70 e cerca de 35 anos após as primeiras descrições, diversos ensaios usando NCM foram elaborados (HORISBERGER, 1976; NAKAMURA; HASHIMOTO; MATSUNAGA, 1991; SCHRODER et al., 1986; NAGARAJAN et al., 2008; PARVEEN; MISRA; SAHOO, 2011). O núcleo magnético e a possibilidade de modificação da superfície proporcionam grande funcionalidade aos NCM e têm atraído a atenção dos grandes centros de pesquisas, já que o alvo magneticamente marcado pode ser detectado diretamente através de um magneto (OLSVIK et al., 1994; JAIN, 2008; LIU et al., 2011). NCMs podem ser sintetizados através de várias metodologias e com diversos materiais como metais (ferro, níquel e cobalto), silicatos, polímeros orgânicos ou biológicos, materiais a base de carbono e exibir grande diversidade morfológica tais como formas esféricas e cilíndricas. Além disso, a superfície dos NCM é facilmente manipulada por métodos químicos possibilitando o ancoramento de grupos funcionais incluindo carboxil, amino, hydroxil, hidrazida, clorometil e silanol (NAGARAJAN, 2008). Por esse motivo, uma grande variedade de moléculas pode ser imobilizada com o propósito de capturar o alvo específico (OLSVIK et al., 1994). Devido ao seu pequeno tamanho e alta área superficial, NCM possuem características superiores quando comparado com materiais de dimensões micrométricas.

Técnicas convencionais para a preparação de NCM incluem co-precipitação, processamento sol-gel, atomização, liofilização, spray a quente, evaporação-condensação, isolamento de matriz, reações de fase vapor induzidas por laser e aerossóis. Geralmente, a maioria desses métodos, envolve a utilização de equipamentos adequados para cada tipo de material, sofisticados, caros e, além disso, não é possível controlar com precisão a distribuição de poros superficiais e o tamanho dos NCM (PILLAI AND SHAH, 1996). Entretanto, metodologias alternativas vêm sendo desenvolvidas para a síntese de NCM, especialmente formulações que utilizem o elemento cobalto (CARREÑO et al., 2007; PARVEEN; MISRA; SAHOO, 2011). O alto desempenho magnético de compósitos a base desse elemento possibilita a elaboração de ferramentas biotecnológicas como, por exemplo, materiais para ensaios de imunoseparação magnética (BHATT, 2011; PARVEEN; MISRA; SAHOO, 2011).

Os avanços e as pesquisas realizadas no desenvolvimento de NCM, especialmente na área da saúde, estão se tornando cada vez mais comuns, (PARVEEN; MISRA; SAHOO,

2011) como a elaboração de ensaios para a prevenção de doenças, entrega de drogas, tratamento do câncer, transfecção celular e separação de células ou biomoléculas (MOHANRAJ E CHEN, 2006; PARVEEN; MISRA; SAHOO, 2011). Pesquisas recentes destacam a utilização de NCM para a melhoria na entrega de agentes quimioterápicos com significantes vantagens sobre as atuais metodologias (KIEVIT AND ZHANG 2011). Durante a entrega, os NCM carreadores são injetados na corrente sanguínea e são estrategicamente capturados na região tumoral via aplicação de um campo magnético local. Dessa forma, a droga pode ser liberada no momento adequado através de mudanças fisiológicas (PARVEEN; MISRA; SAHOO, 2011). Além disso, o tratamento de cânceres superficiais por hipertermia também vêm sendo melhorado com o auxílio de NCM, uma vez que é possível concentrar esse material via aplicação de um campo magnético externo no sítio tumoral aumentando a sensibilidade pelas células malignas à radiação e quimioterápicos (SOARES et al., 2010).

Como alternativa aos métodos de transfecção celular como a eletroporação, lipofecção e vetores virais, os quais demonstram lenta acumulação e conseqüentemente baixa concentração do analito na célula alvo, a utilização de NCM demonstra resultados promissores devido à maior eficiência de sedimentação do complexo partículas-tratamento sobre a célula alvo. O aumento obtido nos níveis de transfecção utilizando os NCM pode chegar a até 15 % (YIU et al., 2011).

Doenças infecciosas são causadas por micro-organismos patogênicos que podem ser disseminados diretamente ou indiretamente, de uma pessoa para outra ou para animais e vice-versa. Mais de 95% das mortes são causadas devido à falta de medidas eficazes de diagnóstico e tratamento (YAGER et al., 2006). A fim de melhorar a detecção destes patógenos em amostras clínicas e alimentares, os laboratórios necessitam de testes mais rápidos e baratos, como uma alternativa para as atuais metodologias.

Nos últimos anos, a imunoseparação magnética (IMS) tem sido citada como uma ferramenta versátil e poderosa para muitas aplicações clínicas (OLSVIK et al., 1994; FERNANDES et al., 2008; MOREIRA et al., 2009). Para a IMS, anticorpos e proteínas específicas podem ser ancorados a superfície dos NCM, os quais são capazes de capturar o micro-organismo ou biomolécula a partir das amostras em estudo (FERNANDES et al., 2008; MOREIRA et al., 2008; GORANSSON et al., 2010; ALBERT et al., 2011). Inúmeras vantagens são atribuídas a IMS como ferramenta laboratorial, tais como a capacidade de reduzir o tempo de isolamento do micro-organismo alvo e as possibilidades de eliminação de contaminantes, já que é possível realizar diversas lavagens com o complexo imunoseparado. Após a IMS, testes laboratoriais podem ser realizados para a detecção do alvo capturado,

dentre eles a reação em cadeia da polimerase (PCR) apresenta maior facilidade de execução, rapidez na obtenção de resultados e boa sensibilidade e especificidade quando comparados às metodologias convencionais de cultivo e detecção de micro-organismos (FDA, 1992; TORTORA et al., 2005). Além da aplicação no diagnóstico direto, a IMS pode ser utilizada na otimização do isolamento de agentes patogênicos fastidiosos a partir de fluídos ou tecidos de animais. Esta aplicação pode auxiliar na obtenção de novas cepas, possibilitando análises epidemiológicas, antigênicas e genéticas que contribuiriam para a formulação de novas formas de controle e prevenção de doenças causadas por micro-organismos patogênicos.

Devido à importância que a leptospirose representa para a saúde pública mundial, principalmente em países em desenvolvimento (BHARTI et al., 2003; ADLER E MOCTEZUMA, 2010), os ensaios de IMS deste estudo foram realizados com cultivos de leptospiras patogênicas e amostras clínicas de animais positivos para leptospirose.

A leptospirose é uma doença infecto-contagiosa causada por mais de 230 sorovares patogênicos pertencentes ao gênero *Leptospira* (ADLER E MOCTEZUMA, 2010). Dentre os ensaios laboratoriais de diagnóstico, a soroaglutinação microscópica (MAT) é considerada o teste referência no Brasil e no mundo (WHO, 2003). No entanto, algumas dificuldades são encontradas para a realização da MAT, tais como a necessidade de uma bateria viva de sorovares de *Leptospira* para a triagem dos soros de humanos e animais suspeitos e um alto grau de reações cruzadas entre diferentes sorovares, onde muitas vezes o resultado revela títulos elevados de anticorpos para sorovares que não são os causadores da enfermidade ou surto (LEVETT, 2003).

O isolamento do agente também é realizado para o diagnóstico, porém o crescimento das leptospiras em meios de cultura específicos é lento e pode durar até meses para a caracterização do organismo (WHO, 2003; FAINE et al., 1999). Contudo, o desenvolvimento de metodologias alternativas, como a IMS associada a PCR e ao isolamento, poderia contribuir para o entendimento da doença e a elaboração de testes de diagnóstico mais simples e eficientes, constituindo uma importante estratégia para a prevenção e tratamento da leptospirose. Embora NCM sejam comercialmente disponíveis, ainda existe a necessidade de desenvolver materiais de alta tecnologia em solo brasileiro que apresentem vantagens em relação aos produtos importados tais como NCM com alta estabilidade química e física, fáceis de sintetizar, capacidade de biofuncionalização, propriedades superparamagnéticas e sob custos reduzidos de produção.

O objetivo desse trabalho foi produzir NCM de carbono e cobalto usando um método simples de síntese química e avaliar NCM de poliestireno e ferro para o uso em ensaios de IMS visando à detecção e o isolamento de leptospiras patogênicas.

A hipótese desse trabalho foi que os NCM sintetizados à base de carbono e cobalto são capazes de ligar covalentemente proteínas e anticorpos; e a IMS realizada com os NCM de poliestireno como passo anterior a PCR aumenta a sensibilidade do teste quando realizada a partir de fluídos biológicos artificialmente contaminados e amostras clínicas de animais com leptospirose.

A tese é apresentada na forma de artigos científicos. O artigo 1 relata uma *mini-review* com aplicações e inovações biotecnológicas de partículas magnéticas, o qual será submetido ao periódico *Journal of Biosciences*. O artigo 2 relata o desenvolvimento e a caracterização de NCM a base de cobalto e carbono para a aplicação em ensaios de IMS. Esse trabalho será submetido ao periódico *Journal of Nanoparticle Research*. O Artigo 3 relata a aplicação da técnica de IMS utilizando NCM de poliestireno a partir de fluídos biológicos para a detecção de leptospiras patogênicas visando o controle da leptospirose canina. Esse trabalho foi aceito para publicação no periódico *Brazilian Journal of Microbiology*. Todos os artigos estão formatados conforme as exigências dos periódicos científicos em que serão submetidos ou publicados.

2. ARTIGO 1

MAGNETIC PARTICLES: A POWERFUL TOOL IN BIOTECHNOLOGY

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ABSTRACT

Magnetic particles (MP) varying nanometer to micrometer size can be easily manipulated by magnetic forces and have attracted much attention especially in biological and health area. This article focuses on the potential of MP in biotechnology and current methodologies, describing their advantages, limitations and challenges.

Keywords: Immunoseparation. Diagnosis. Diseases.

Magnetic particles (MP) varying nanometer to micrometer size have attracted attention as a tool in biotechnology. Several assays based on MP have been developed in which the magnetic field generated by the magnetically labeled targets is captured directly by a sensitive magnetometer, which minimizes the use of expensive laboratory equipments (Jain 2008).

MP can be artificially (AMP) or biologically (BacMP or magnetossomes) synthesized (Balkwill *et al.* 1980; Matsunaga *et al.* 2006). AMP can be made by several methodologies using diverse materials such as metals (iron, nickel and cobalt), metal oxides, silicates, non-oxide ceramics, polymers organics, carbon materials, organics or biological exhibiting great morphological diversity with shapes such as spheres, cylinders, disks, platelets, hollow spheres and tubes (Nagarajan 2008). The particle's surface is easily manipulated by chemical methods enabling several functional groups to be anchored onto the particle, including carboxyl, amino, hydroxyl, hydrazide, chloromethyl and silanol groups (Gossuin *et al.* 2008; Sun *et al.* 2009; Sun *et al.* 2010; Yuan *et al.* 2011). Therefore, a great variety of molecules can be immobilized on the surface particle with the purpose of capturing the specific target.

On the other hand, magnetotactic bacteria can produce intracellular BacMP or magnetossomes of iron oxide, iron sulphides or both that are individually enveloped by a lipid bilayer membrane (Bazylinski *et al.* 1994). Through genetic engineering, functional proteins such as enzymes, antibodies and receptors have been successfully displayed on BacMPs (Arakaki *et al.* 2008). Recently, the biotechnological potential of these platforms has been demonstrated in several assays such as high-performance DNA/RNA recovery, microorganism's detection, magnetic markers and the separation of mononuclear cells from peripheral blood (Yoza *et al.* 2003; Arakaki *et al.* 2008; Fernandes *et al.* 2008; Tanaka *et al.* 2008; Maeda *et al.* 2009; Yoshino *et al.* 2010). This article focuses the current methodologies

for biotechnological applications of MP, describing their advantages, limitations and challenges.

Over 95% of deaths caused by infectious diseases are due to lack of proper diagnosis and treatment (Yager *et al.* 2006). Conventional isolation methods are time-consuming and are not sufficiently sensitive for detection of low concentrations of cells from food and clinical samples (Olsvik *et al.* 1994). Immunomagnetic separation (IMS) has been cited as a versatile diagnostic tool (Fernandes *et al.* 2008; Moreira *et al.* 2009; Deng *et al.* 2011; Fedio *et al.* 2011). For IMS assay, specific proteins can be immobilized on the particles surface, which are able to capture target microorganism or molecule from samples (Gray and Bhunia 2005; Urdea *et al.* 2006; Yager *et al.* 2006; Fernandes *et al.* 2008; Moreira *et al.* 2009; Liebana *et al.* 2009; Goransson *et al.* 2010). So, the immune-captured complex can be characterized by routine laboratory tests including plating, biochemical, molecular, immunological, fluorescent or flow cytometry methods (Kim and Ligler 2010; Koktysh *et al.* 2011; Leung *et al.* 2011; Zhang and Qi 2011). In IMS procedure, the target is separated from the environment and is concentrated into amounts suitable for identification. Furthermore, growth or PCR inhibitory reagents and contaminants present in the samples are removed (Olsvik *et al.* 1994). Therefore, the association of the IMS step with current methodologies would enhance the degree of sensitivity, automation and reproducibility, which are parameters important to clinical applications.

MP have been applied in healthcare to improve delivery of chemotherapeutic agents in cancer treatment with the goal of minimizing toxic effects on healthy tissues with a significant advantage over standard chemotherapies by increasing the drug delivery specifically to the tumor site (Yezhelyev *et al.* 2006; Cherry *et al.* 2010; Kievit and Zhang 2011). In magnetic drug targeting, magnetic carrier particles with surface-bound drugs are injected into the vascular systems that are then captured at the tumor via a locally applied

magnetic field. The surface-bound drugs can be released from the drug carriers by changing the physiological conditions, and are then taken up by the affected cells (McBain *et al.* 2008; Parveen *et al.* 2011). Particles must remain in circulation long enough for tumor accumulation. However, they are prone to clearance by the mononuclear phagocyte system. The most common strategy has been grafting PEG or other macromolecules such as polysaccharides onto the particle surface (Wang *et al.* 2011), preventing protein adsorption, interactions among particles and interactions with immune cells (Peracchia *et al.* 1998).

Hyperthermia is an old concept and it has long been established as an option in the treatment of cancer, mainly in superficial cancers. More recently the concept of intracellular hyperthermia emerged wherein magnetic particles are concentrated at the tumor site and heated remotely using an applied magnetic field to achieve hyperthermic temperatures. It is also well established that by exposing cancer tissue to elevated temperatures, the viability of the cells is reduced and an enhancement of their sensitivity to radiation and chemotherapy is observed (Soares *et al.* 2010).

Screening for circulating tumor cells in blood has been an object of interest for evidence of progressive disease, status of disease activity and for early diagnosis of cancer (Hoshino *et al.* 2011). It is known that several types of cancers over express specific proteins on the tumor cell surface which enter the blood stream and serve as clinical biomarkers for neoplastic lesions (Ferrari 2005). For highly specific separation of tumor cells, the introduction of immune separation-based detection is desirable, in which antibodies for tumor-specific markers are utilized to label target cells (Hoshino *et al.* 2011; Kievit and Zhang 2011).

The transfection of genes into eukaryotic cells has become an important technique for analysis of gene function, production of recombinant gene products and gene therapy. MP are being increasingly used in cell sorting and transfection (Yang *et al.* 2008) as an alternative

method over use of pure plasmids, electroporation, receptor-mediated gene transfer, particle guns, viral vectors and lipofection (Hoelker *et al*. 2007; Yang *et al.* 2008). However, each methodology has benefits and limitations, and to date there is still no ideal system for gene transfer (Hoelker *et al.* 2007). Among these limitations, the slow accumulation and consequently low concentration of analyte in the target cell have been identified as simple but strong barriers to effective gene transfection (Luo and Saltzman 2000). Magnetofection is a new method for gene transfer that has shown promising results. This assay involves the use of magnetic force and plasmid DNA/magnetic bead complexes, and it has been developed for enhancing delivery of gene vectors to target cells (Gersting *et al.* 2004; Kadota *et al.* 2005). In the magnetofection procedure, the efficient sedimentation of the target-magnetic particles complex generated by an external magnetic field provides the largest fraction t of target (cells) within a few minutes of incubation and may increase levels of transfection up to 15% (Plank *et al.* 2003; Yiu *et al.* 2011). In addition, cationic magnetic particles mediate cell binding which is followed by internalization (Scherer *et al.* 2002).

Scaffolds play a pivotal role in tissue regeneration, which provides specific macro and micro structure to modify the biological and biomechanical reaction of the cells during the healing process (Sangsanoh *et al.* 2007; Schnell *et al.* 2007). Cell adhesion onto scaffolds is the first fundamental step in bone regeneration, which will greatly influence the morphology and capacity of cell proliferation and differentiation (Ma *et al* 2005). The electrospinning, aided by MP, is a technique that has been used for the synthesizing of nanofibrous scaffolds and is proving to be a promising material for tissue regeneration. The key factor for use of this technique lies in the possibility of generating a surface tension by using MP and external voltage field during the biomaterial polymerization that facilitates the scaffolds formation. Electrospun nanofibrous membranes are considered to be of great potential in the field of tissue regeneration, since they can closely mimic the extracellular

matrix architecture. The electrospinning process also provides operational flexibility for incorporating multiple components into nanofiber, such as metal nanoparticles (Pt, Ag, Au) and inorganic compound nanoparticles (TiO_2, SiO_2). In addition, this process has helped in osteoinduction promoting significantly higher proliferation rate and faster differentiation of osteoblast cells (Meng et al. 2010).

Although the potential benefits of MP are considerable, there is a definite need to identify any potential negative responses in ecosystems and humans (He *et al.* 2011). Furthermore, drawbacks can be found during the synthesis process causing adverse effects for their use (Kwon *et al.* 2008; Morimoto *et al.* 2010). A recent study with *Escherichia coli* organism demonstrates that iron oxide magnetic particles induced genomic mutations leading to morphological changes (He *et al.* 2011). In addition, the effect of static magnetic fields that can lead to aggregation and cytotoxicity in biological system. So, different approaches to synthesis of MP are essentials to increase the biocompatibility these materials (Bae *et al.* 2011).

MP without any surface coatings can generate hydrophobic surfaces with a large surface area to volume ratio, which leads to agglomeration and formation of large clusters, resulting in increased particle size. This inherent aggregation behaviour of particles is a crucial limiting factor that reduces the quality of the material (Gupta and Gupta 2005). Aiming to avoid the particles potential toxic effects, the toxicological and chemical characterization of new composites is necessary (Oberdorster *et al.* 2005). In addition, immune system defense mechanisms studies against MP could elucidate and minimize adverse effects in animal and human health.

In conclusion, MP has been cited as a powerful tool in biological and health areas. Despite problems related to their synthesis and toxicity, this advance in technology is a new approach in the detection of microorganisms and molecules in food and clinical samples.

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3. ARTIGO 2

SYNTHESIS AND BIOFUNCTIONAL VALIDATION OF COBALT MAGNETIC NANOPARTICLES EMBEDDED IN CARBON MATRIX

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ABSTRACT

Carbon nanostructures and nanocomposites display versatile allotropic morphologies, physico-chemical properties and have a wide range of applications in mechanics, electronics, biotechnology, structural material, chemical processing and energy management. In this work we report the synthesis, characterization and biotechnological application of cobalt magnetic nanoparticles embedded in carbon structure (Co/C-MN). A single-step chemical process was used in the synthesis of the Co/C-MN. The Co/C-MN has presented superparamagnetic behavior at room temperature an essential property for immunoseparation assays carried out here. To stimulate covalent interactions between proteins and Co/C-MN, this nanocomposite was functionalized with acrylic acid (AA). We have showed the bonding of different proteins onto Co/C-AA surface by using immunofluorescence assay. A Co/C-AA coated with monoclonal antibody anti-pathogenic *Leptospira* spp. was able to capture leptospires, suggesting that it could be useful in immunoseparation assays.

***Keywords*:** Immunoseparation assays. Pechini method. *Leptospira*.

1. Introduction

Carbon nanostructures and nanocomposites (NC) display versatile allotropic morphologies as also physico-chemical properties and arewidely used in mechanics, biotechnology, electronics, structural materials, chemical processing and energy management (Chang et al. 2003; Carreño et al. 2007). Among these properties, much attention has been paid to nanostructured magnetic systems due to their magnetic behavior, which is related to particle size and the type of support matrix used. NC may provide an appropriate morphology derived from of type porous (microporous or mesoporous) surface, that allow host inorganic particles and organic substance , potential factor necessary to advance applications (McHenry et al. 1994).

Superparamagnetism has been demonstrated in Fe, Ni and Co ferromagnetic nanoparticles embedded in an amorphous matrix (Childress and Chien 1991; McHenry et al. 1994). Magnetic nanoparticles (MN) have been extensively studied in biomedical applications including magnetic resonance imaging (MRI) contrast agents, magnetic targeting of drug delivering vehicles and magnetic separation of DNA, proteins and cells (Çakmak et al. 2009).

Selection and assembly of materials are the main issues in the development of diagnostic assays, vaccines and epidemiological studies to control and prevention of diseases caused by pathogenic micro-organisms. (Gray and Bhunia 2005; Kim et al. 2010). Immunomagnetic separation (IMS) aided by proteins and antibodies is a technique that has been used for detection and isolation of specific organisms. After IMS, it is possible to cultivate and identify the target organism by traditional biochemical, immunological or molecular methods (Olsvik et al. 1994; Fernandes et al. 2008). Although magnetic particles are commercially available, there is still need to produce materials with improved properties, e.g. easy preparation, easy surface modification, low cost and superparamagnetic property. Conventional techniques for the preparation NC include bulk precipitation, sol-gel processing,

spray-drying, freeze-drying, hot-spraying, evaporation-condensation, matrix isolation, laser-induced vapor phase reactions and aerosols. Generally, in most types of NC prepared by these methods, it is usually not possible to control precisely the size and size distribution of the NC (Palli and Sohah, 1996). In this context, the aim of this study was to synthesize cobalt/carbon nanocomposite (Co/C-NC) by a single-step chemical process, anchor specific proteins onto Co/CNC surface and evaluate them in IMS assay for the capture of pathogenic leptospires.

2. Materials and methods

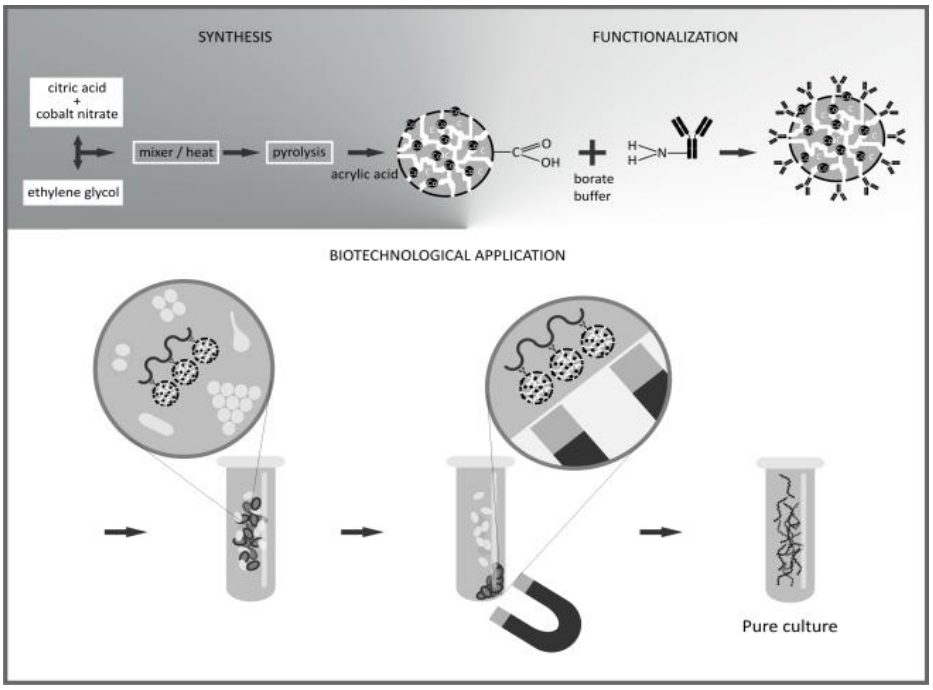

Figure 1. General schematic diagram of nanocomposite synthesis by polymeric precursor method and immunomagnetic separation of pathogenic leptospires.

2.1. Synthesis of the Co/C-*nanocomposite*

A single-step chemical process was used in the synthesis of the cobalt/carbon nanocomposite (Co/C-NC), figure 1. The synthesis procedure consists in dissolution of citric acid (CA; Synth, USA) and the cobalt salt, Co $(NO_3)_2$•$6H_2O$, using a CA:Co molar ratio of

3:1 in water (Pechini, 1967). This mixture was homogenized for 15 min at room temperature and polymerization was initiated by adding ethylene glycol at a mass ratio of 40:60 in relation to the CA, at approximately 80 °C. The final composites were obtained by a two step pyrolysis of the polymeric intermediate. The first step (2 h, 250 °C) promoted the breakage of the organic parcel of the polymeric intermediate. The powder was submitted to a second heat treatment, performed in a N_2 atmosphere ranging between 600 ° C and 800 °C, to produce the cobalt magnetic nanoparticles (Co/C-MN). The CO/CO_2 atmosphere resulting from the pyrolysis of the organic material promoted the reduction of the Co salt without using H_2 as a reducing agent (Leite et al. 2002).

2.2. Characterization of the Co/C-MN

Using SiO_2 as an external standard the crystalline phases and the cell volume were determined by examining X-ray diffractometry (XRD) patterns which were obtained with a Shimadzu XRD-6000 diffractometer with Cu Kλ radiation (λ=1.5406 Å). Morphological characterizations were performed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) using a Philips, model CM200 equipment.

Samples were characterized by N_2 adsorption/desorption isotherms obtained at the temperature of liquid nitrogen in an automated physisorption instrument (Autosorb-1C, Quantachrome Instruments). Prior to the analysis, the samples were degassed in a vacuum at 300^{o}C for 2-3 h. Specific surface areas were calculated according to the Brunauer–Emmett–Teller (BET) (Brunauer et al. 1938) method, and the pore size distributions were obtained according to the Barret-Joyner-Halenda (BJH) method from the adsorption branch data.

2.3. Co/C-MN surface modification

The Co/C-MN was functionalized using a physical coating method. The particle surface was coated with acrylic acid through a polymerization reaction. The Co/C-MN particles were dispersed in acrylic acid, 2.9 $mol.L^{-1}$ at approximately 100 °C. To improve the

coating, particle dispersion was increased by incubation in an ultrasonic bath for 2 h followed by drying at 50 °C. These coated MN were defined as Co/C-AA. For a comparative study, other concentrations (1 mol.L^{-1} and 0.5 mol.L^{-1}) of acrylic acid were also evaluated.

2.4. Characterization of the Co/C-AA

The samples functionalized and not functionalized were analyzed by Fourier Transform Infrared Spectroscopy (FT-IR, Varian, model 640-IR) to detect the presence of the carboxylic groups responsible for the covalent bonds between the Co/C-AA surface and proteins. In addition, magnetic measurements (Quantum Design, model PPMS) were carried out to verify the behavior of the NC in an external magnetic field. The zeta potential of the Co/C-AA was determined by a Zetasizer Nano ZS (Malvern Instruments, Malvern, U.K.) instrument at ambient temperature. The concentration of Co/C-AA suspension was 5 mg.mL^{-1} using the samples dispersed on phosphate buffer saline with pH 7.4.

2.5. Preparation of biofunctional Co/C-AA

Heating at 80 °C for 15 min was performed to reduce microbial contaminants prior to starting the preparation of the biofunctional Co/C-AA. Immobilization of proteins was carried out as previously described (Çakmak et al. 2009). Briefly, recombinant LipL32, the most abundant surface lipoprotein on the outer membrane of pathogenic *Leptospira* spp. (Haake et al. 2000), anti-LipL32 monoclonal antibody (MAb) (Fernandes et al. 2007) and protein A were attached to the Co/C-AA surface in 0.05 M borate buffer (pH 9.5). All proteins were used at high concentrations (1.2 mg.mL^{-1}) for nanoparticle surface saturation. To detect the bound LipL32, anti-LipL32 MAb or protein A onto Co/C-AA surface, anti-LipL32 MAb and/or goat-anti mouse fluorescent isothiocyanate (FITC) conjugate (Sigma) were used. Protein-Co/C-AA complexes were observed by immunofluorescence microscopy (Olympus BX 51) at an excitation wavelength of 450 nm. Control reactions were performed using an

anti-internalin A MAb specific for *Listeria monocytogenes* and phosphate buffer saline with 0.05% of Tween-20.

2.6. Biofunctional assay of the Co/C-AA

In order to assess the biotechnological application of these surface-modified MN, an IMS assay using *L. interrogans* serovar Canicola Hond Utrecht IV strain was carried out as previously described (Fernandes et al. 2008). Briefly, Co/C-AA attached to the anti-LipL32 MAb were added to a liquid culture of the *L. interrogans* strain. The biotechnological application of IMS is represented in figure 1. To perform the experiments an immunomagnetic separator MPC-S (Invitrogen, CA, USA) was used according to the manufacturer's instructions. The presence of DNA from the immunomagnetic-captured leptospires was confirmed by PCR using primers specific for *lipL32* gene as previously described (Haake et al. 2000). In addition, captured leptospires were inoculated into Ellinghausen-McCullough-Johnson-Harris liquid medium (EMJH, Difco-USA). To evaluate the nonspecific interaction between the Co/C-AA and the leptospires, a control reaction was performed using the Co/C-AA without the anti-LipL32 MAb. The cultures were examined daily for contamination and evidence of growth.

3. Results and discussion

Figure 2 shows the N2 adsorption/desorption isotherms obtained from the carbon nanocomposite. Figure 2a, according to the nomenclature of International Union of Pure and Applied Chemistry (IUPAC), shows isotherms with a loop similar to the type H4, that is often associated with narrow slit-like pores, but in this case the type I isotherm character is indicative of microporosity. Figures 2b illustrate an isotherm as a type IV isotherm with a type H2 hysteresis, usually associated with capillary condensation in mesopore structures.

Figures 2c demonstrate the results obtained with BJH method which indicated the distribution homogeneous of pore onto Co/C-AA surface.

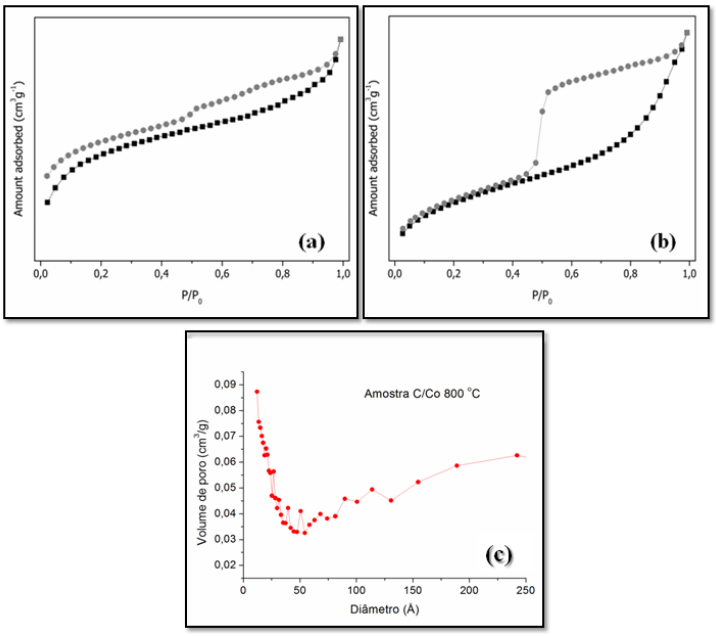

Figure 2. (a) and (c) nitrogen adsorption/desorption isotherms obtained by BET method for Co/C-AA samples pyrolyzed at different 600 °C and 800 ^{o}C, respectively. The dark symbols refer to the adsorption branch and gray symbols to the desorption branch. (c) pore size distribution obtained by BJH method for Co/C-AA calcined at 800 ° C.

Table 1 summarizes the surface specific area and average pore diameter. The results show a significant increase in the surface area and average pore diameter of samples with the rise in pyrolysis temperature from 600 °C to 800 °C. In addition, the results for the Co/C-MN coated with acrylic acid show a significant decrease in the sample's surface specific area with the rise in pyrolysis temperature from 600 °C to 800 °C and acrylic coated process, changing from microporous to mesoporous was also observed, respectively, suggesting that residual carbon is an important factor in the material's morphological properties and during the process of fixing of acrylic acid on the surface of Co/C-MN.

Table 1. Surface specific area and average pore diameter of cobalt magnetic nanoparticles embedded in carbon structure.

Samples	Surface specific area ($m^2.g^{-1}$)	Average pore diameter (Å)
Co600	134	29
Co800	116	37
Co800AA	2	167

Figure 3a shows a HRTEM image of the Co/C-AA with a diameter between 10 and 20 nm, embedded in a shell of the carbonaceous matrix. In Figure 3b it is possible to observe the atomic planes from cfc-Co (JCPDS 89-4307), with interplanar spacing $d_{(111)}$ = 0.2087 nm (Pola et al. 2010). Sharp peaks showed in XRD pattern confirmed the presence of cfc-Co, figure 3c. In addition, a region characteristic of amorphous carbon preserved from chemical synthesis was observed. Furthermore to the cfc-Co peaks, small intense peaks due to the presence of cobalt oxide from the first pyrolysis step were also observed. However, this did not affect the final application negatively due to the small amount of material present. Cobalt content in Co/C-AA sample was measured by inductively coupled plasma optical emission spectrometry (ICP OES), which showed about 42.3% cobalt.

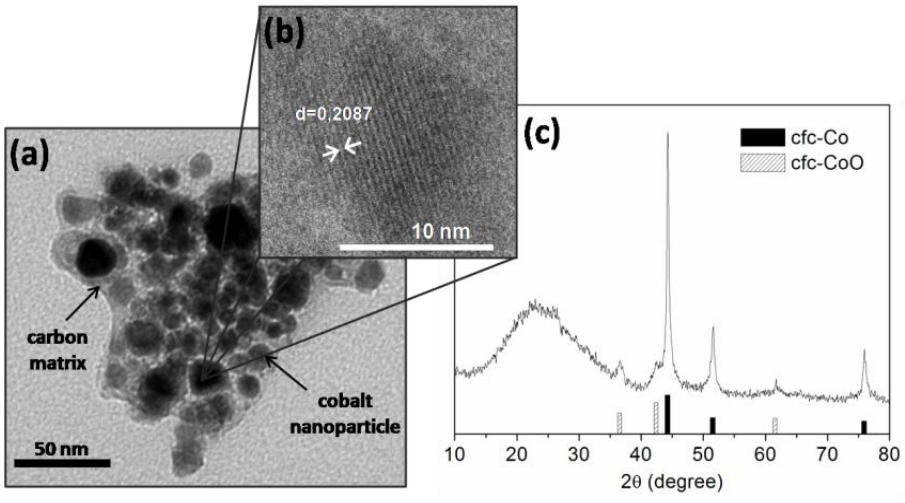

Figure 3. (a) and (b) show HRTEM images and (c) XRD pattern of cobalt/carbon calcined at 800 °C, in N_2 atmosphere.

As expected, there were no significant peaks in the FT-IR spectrum of the Co/C-AA (figure 4a). However, analysis of the Co/C-AA identified three peaks, located at 1732 cm^{-1}, 1411 cm^{-1} and 3493 cm^{-1} (figure 4b). These peaks were attributed to stretching of the C=O, C-O and O-H bonds, respectively. There was another peak due to C=C stretching at 1574 cm^{-1} and other bands of stretching out-of-plane bending and secondary absorptions (Liu et al. 2009; Zheng et al. 2005). These results suggested that the Co/C-AA surface was successfully coated with a polymeric layer that contained carboxyl groups. Furthermore, on comparing the MEV images, there were differences in the composite before and after functionalization (figure 4). This observation suggested that the acrylic acid was polymerized and that the granules of the Co/C-AA were maintained inside its polymer structure. The presence of carboxyl groups, as shown by infrared analysis, enabled interaction with biomolecules, mainly through the amino groups.

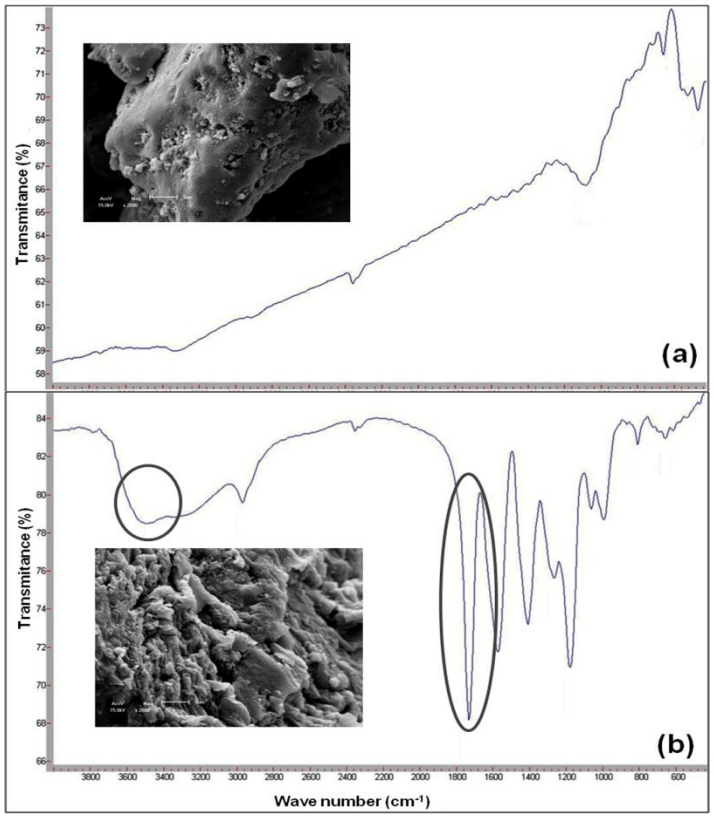

Figure 4. FT-IR spectrum and MEV image (2000 x) of cobalt/carbon nanoparticles (a) not functionalized and (b) functionalized with acrylic acid.

The results of the magnetic measurements of the Co/C-AA sample are showed in figure 5. The magnetic behavior at 300 K is indicated by the saturation magnetization (M_s), remanent magnetization (M_r) and coercive field (H_c). Magnetization ratio (M_r/M_s) and Hc values different from zero at room temperature indicate that there is a residual magnetic moment of the nanoparticles (Cheng et al. 2005). The value of the magnetization ratio presented for Co/C-AA was low (0.104), suggesting superparamagnetic behavior at room temperature (Sajitha et al. 2004). In addition, the Co/C-AA developed in this work presented M_r/M_s ratio of H_C = 125 Oe, consistent with values reported by other superparamagnetic nanoparticles. These nanoparticles have potential in several biotechnological applications, such as immunoassays, magnetic resonance imaging, magnetic cell separation, magnetic oligonucleotide and nucleic acid separation (Cheng et al. 2005; Yu and Qiu 2008).

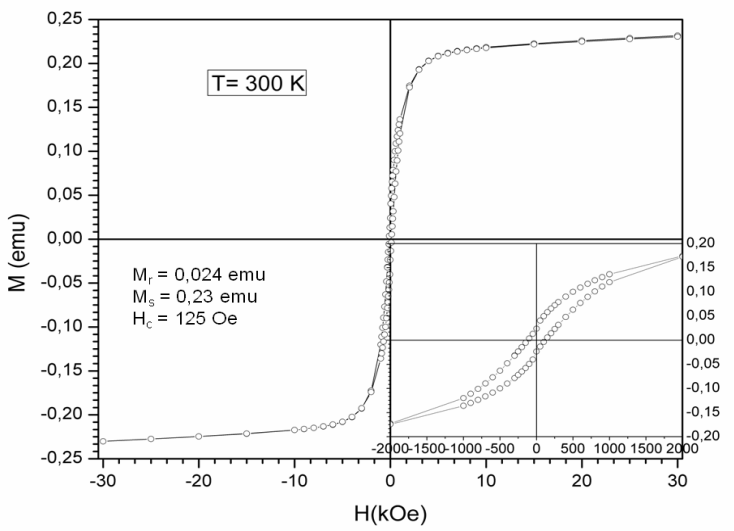

Figure 5. Magnetization curve for Co/C-AA sample at 300 K.

For a comparative study, other concentrations of acrylic acid to functionalize nanocomposites were also formulated, but these samples did not show good applicability and stability. Illustrated by zeta potential (table 2), which expresses the colloidal stability by surface charge of particles, it can be said that stability increased with the concentration of acrylic acid in the modification of the particles, since the zeta potential values became more negative when we increased the concentration of acid. Demir-Cakan et al. (2009) explain that this can be due to the fact that zeta potential is a surface technique and although most of the acidic functionalities are located onthe surface for carbonaceous materials and suggest that presumably some of the carboxylic groups for the acrylic acid coated samples are also located within the carbon matrix.

Additional information found in literature presented by Jans and coauthors (Jans et al. 2010) synthesized nanoparticles stabilized by poly (acrylic acid) with zeta potential near -50 mV, while at pH 7. This potential is very negative because there are many deprotonated

carboxylic groups. The same is presented in our composites coated with acrylic acid, but the carboxyl groups aremore protonated, maintaining the zeta potential close to -20 mV.

Table 2. Zeta potential values for Co/C-MN recovered with different acrylic acid concentrations.

Acrylic acid ($mol.L^{-1}$)	Zeta potential (mV)
Control (No acrylic acid)	-24.9
0.5	-8.31
1	-3,5
2.9	-19,5

Mohapatra and Pramanik (Mohapatra and Pramanik 2011) studied the behavior of phosphoric acid coupling agents on Co/C-AA in an aqueous medium and showed that functional nanoparticles were highly stable and presented potential for use in biomedical applications. Çakmak and co-workers (Çakmak et al. 2009) produced monodisperse poly (glycidyl methacrylate) microspheres by dispersion polymerization that covalently immobilized protein A. Similar results have been obtained comparing the method of functionalization presented in this article with those described in the literature (Çakmak et al. 2009; Mohapatra and Pramanik 2011), however, our process is simpler, with less steps in the synthesis, since it is possible to disperse directly the Co/C-MN into solution of acrylic acid. In addition, we avoid the steps of synthesis from organic precursor for carboxyl groups or activation of already existing groups and also a route using the functionalization system of high control, such as temperature, pH, among others.

We also used a simple method for the immobilization of the proteins onto the Co/C-AA surface, based on peptide ligation promoted by borate buffer (Çakmak et al. 2009). The maximum immobilization capacity from LipL32, anti-LipL32 MAb and protein A was found to be 0.9 $mg.mL^{-1}$, 0.7 $mg.mL^{-1}$ e 0.8 $mg.mL^{-1}$, respectively. Immunofluorescence assays

were used to confirm the immobilization effects. Mixes of Co/C-AA and proteins incubated with borate buffer produced fluorescent complexes, unlike those incubated with phosphate buffer saline (figure 6). These results suggest that proteins were efficiently ligated to Co/C-AA surface.

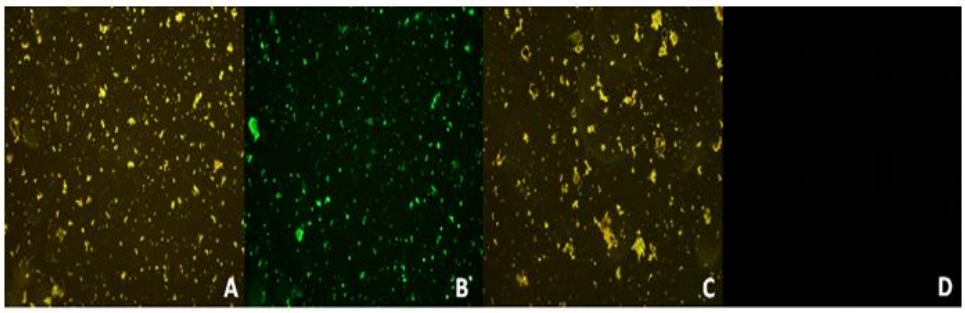

Figure 6. Immunofluorescence assay demonstrating the MAb anti-LipL32 attached to Co/C-AA. Panel "A" and "C", nanoparticles in dark field; Panel "B", anti-LipL32 MAb-Co/C-AA complex detected with goat anti-mouse fluorescent isothiocyanate (FITC) conjugate (Sigma); Panel "D", negative control using phosphate buffer saline with 0.05% of Tween-20 as ligation buffer. Labeling was visualized by fluorescence microscopy (Olympus BX 51) with excitation wavelength of 450 nm with a 100× objective.

Biofunctional assay demonstrated the capacity of the Co/C-AA coated with anti-LipL32 MAb to capture leptospires. Conversely, the Co/C-AA without the anti-LipL32 MAb was not able to capture leptospires (figure 7). These results were similar using commercial magnetic particles of iron and polystyrene for detection of pathogenic leptospires and *Salmonella* Typhimurium organisms (Fernandes et al. 2008; Moreira et al. 2009). In addition, the growth of leptospires after immunoseparation followed by subculture was observed, suggesting that Co/C-AA could help in obtaining new leptospira isolates and others pathogenic microorganisms.

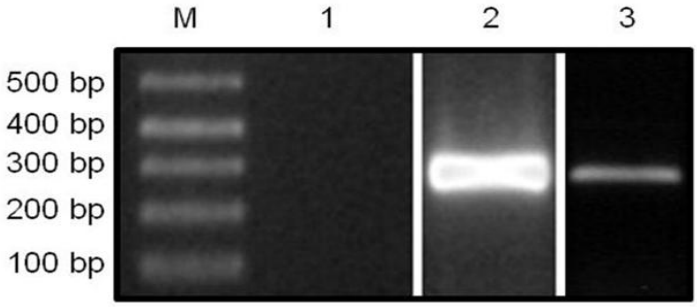

Figure 7. Agarose gel electrophoresis of IMS-PCR products from leptospiral strain culture showing the detection of *lipL32* gene (264 bp). M, 1kb Plus DNA Ladder; 1, Co/C-AA without anti-LipL32 MAb; 2, Positive control reaction (genomic DNA of *Leptospira* sp.); 3, Co/C-AA with anti-LipL32 MAb on the surface

4. Conclusions

The cobalt magnetic nanoparticles embedded in carbon matrix (Co/C-AA) produced in this article were prepared successfully using a single chemical one step method which varying 20 nm to 50 nm size. The effective incorporation of acrylic acid surface on carbon mesoporous material was a decisive factor in bonding specific proteins and developing a biofunctional material for the isolation of microorganisms and detection approaches.

The Co/C-AA synthesized here suggests an important material in biotechnological applications.

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4. ARTIGO 3

DIAGNOSIS OF CANINE LEPTOSPIROSIS USING AN IMMUNOMAGNETIC SEPARATION-PCR METHOD

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ABSTRACT

Diagnosis of leptospirosis by PCR is hampered due to the presence of substances on biological fluids. Here, we report an immunomagnetic separation step prior to PCR which improved the detection of *Leptospira* spp. in blood and urine samples from dogs. It resulted in a significant improvement on sensitivity for diagnosis of canine leptospirosis.

Keywords: Canine leptospirosis, IMS-PCR, LipL32.

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* spp., which occurs in many animal species and humans (1). Dogs may be exposed to leptospires in the environment by contact with urine of an infected host, contaminated water or moist soil, where the bacteria may survive for several months (5, 12). In the last years, several assays have been proposed for confirmation of canine leptospirosis, including serology, polymerase chain reaction (PCR), fluorescent antibody testing of urine or tissue samples, or organism isolation (8).

The recommended laboratory diagnostic test, microscopic agglutination test (MAT), is based on detection of antibodies against *Leptospira* spp. in dog sera; however, pathogen-specific antibodies may remain in the blood stream for a long period, even after recovering from disease. Besides serology, demonstration of leptospires by culture of blood, tissues or urine is definitive; it also identifies the infecting leptospire (7).

PCR assay is highly sensitive, but the presence of PCR inhibitory substances on biological fluids can prevent amplification, resulting in false negative (10). Recent studies report the immunomagnetic separation (IMS) technique prior to PCR assay as an approach to reduce the effect of inhibitory substances present in biological fluids and food samples (2, 3, 4, 6, 7, 9, 14, 15). For diagnosis of leptospirosis, the IMS-PCR approach was reported to

detect *Leptospira* spp. in bovine urine (15) and in human biological fluids (6). In order to improve PCR sensitivity and specificity, we developed a novel IMS-PCR approach by using both magnetic beads in house coated with a monoclonal antibody (mAb) and specific PCR primers for pathogenic *Leptospira* spp. (6, 9). Blood and urine samples were obtained from dogs suspected of having leptospirosis (n=5) at the Veterinary Hospital/ Universidade Federal de Pelotas, Brazil. Blood samples were centrifuged at 2,000 x *g* for 5 min, the supernatant was collected, and one drop was observed under microscope to investigate the presence of spirochetes by darkfield microscopy (DFM) on an Olympus BX 51 microscope. Control samples were obtained from healthy dogs (n=5) negative by MAT. The MAT was performed according to Faine *et al*. (5), using reference strains of 19 different leptospiral serovars. Reciprocal agglutination titres of greater than or equal to 1:100 were considered positive reactions.

For PCR assay, blood sera and urine samples were centrifuged at 15,000 x *g* for 10 min. The cells were washed with sterile 0.01 M phosphate-buffered saline (PBS, pH 7.2) and the DNA was extracted by heating at 95 ^{o}C for 15 min in 50 µL of lysis solution (1:1 of 0.125% SDS and 0.05 M NaOH). In experiments in which lysis followed IMS, 20 µL of the lysis solution were directly added to immune separated products in microtubes and then heated at 95 ^{o}C for 15 min. Primers *lipL32* F: 5' CGCTTGTGGTGCTTTCGGTGGT 3' and *lipL32* R: 5' CTCACCGATTTCGCCTGTTGGG 3' were used, resulting in a 264 bp amplicon of the *lipl32* coding region (9). Amplification was carried out in a Peltier Thermal Cycler PTC-100® (Bio-Rad) with 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by an extension for 7 min at 72 °C at the end of the final cycle. Aliquots were analyzed by electrophoresis in 1% agarose gel with ethidium bromide and visualized under UV transillumination. A specific PCR internal amplification control (IAC) was used in all assays, which consists of a DNA fragment containing 501 bp

not related to *Leptospira* spp., flanked by target sequences of *lipL32* primers (6). The IAC concentration was estimated spectrophotometrically at 260 nm and the optimal concentration for use in the *lipL32* PCR was determined by titration, and the lowest reproducible concentration was determined using decimal dilutions of IAC (50 to 0.05 pg) as template DNA in a PCR with *lipL32* primers. PCR and IAC-PCR were performed using a single pair of *lipL32* specific primers.

Determination of the minimal concentration of DNA and number of leptospiral cells required to result in amplification of the *lipL32* gene sequence by PCR was carried out according to Fernandes *et al.* (6). Briefly, genomic DNA from *L. interrogans* was diluted with sterile 10 mM Tris-HCl, 1 mM EDTA (TE, pH 8.0) to concentrations ranging from 20 to 1 pg/µL, and pellets from control samples artificially contaminated with *Leptospira interrogans* serovar Canicola strain Hond Utrecht IV at concentrations ranging from 10^8 to 10^0 cells per mL. Then, the pellets from each DNA concentration and bacterial dilution were washed with PBS and suspended in 50 µL of lysis buffer for DNA extraction. DNA extracted from saprophytic *L. biflexa* serovar patoc Patoc I or *E. coli* were used as negative controls. These experiments were repeated three times.

The IMS-PCR was performed with protein A-magnetic beads (Bangs Laboratories Inc, Fishers, IN, USA) adsorbed with a mAb against leptospira LipL32 protein according to the manufacturer's instructions. The mAb used in this work is specific to pathogenic leptospires (6). Briefly, 10 µL of mAb-coated beads were added to clinical and control samples. The immunocapture complex was washed three times, suspended in DNA extraction buffer, boiled and used on PCR assay. To perform the experiments, an immunomagnetic separator MPC-S (Invitrogen, CA, USA) was used.

All 5 dog serum samples were MAT positive with titers that varied from 100 to 3200. The antibodies most frequently found in the MAT recognized serovars Canicola

CCZ463 (5/5), Canicola Hond Utrecht IV (4/5), Icterohaemorrhagiae (4/5), Copenhageni (4/5), Ballum (3/5) and Grippotyphosa (3/5). The highest agglutination titers corresponded to serovars Canicola CCZ463 and Canicola Hond Utrecht IV. No agglutination was observed in sera from healthy dogs. Conventional PCR assay was able to detect 2 pg per 25 µL of pure genomic *Leptospira* DNA and 10^3 cells per mL^{-1} either in artificially contaminated canine serum or urine samples (data not shown). When IMS was applied on artificially contaminated samples prior to PCR the detection limit decreased to 10^2 cells mL^{-1} (Fig. 1A). No amplification was observed after IMS-PCR performed with saprophytic strains or *E. coli*, or when non-sensitized beads were used for IMS with pathogenic strains (data not shown). PCR performed without previous IMS treatment amplified *lipL32* gene sequence from four urine and one serum samples (data not shown). The IMS-PCR performed with canine leptospirosis clinical samples was able to amplify *lipL32* sequence gene in all urine and in two out of five serum samples tested. These results are demonstrated in Fig. 1B. The IMS-PCR approach enhanced the PCR method since the conventional PCR failed to detect one positive sample.

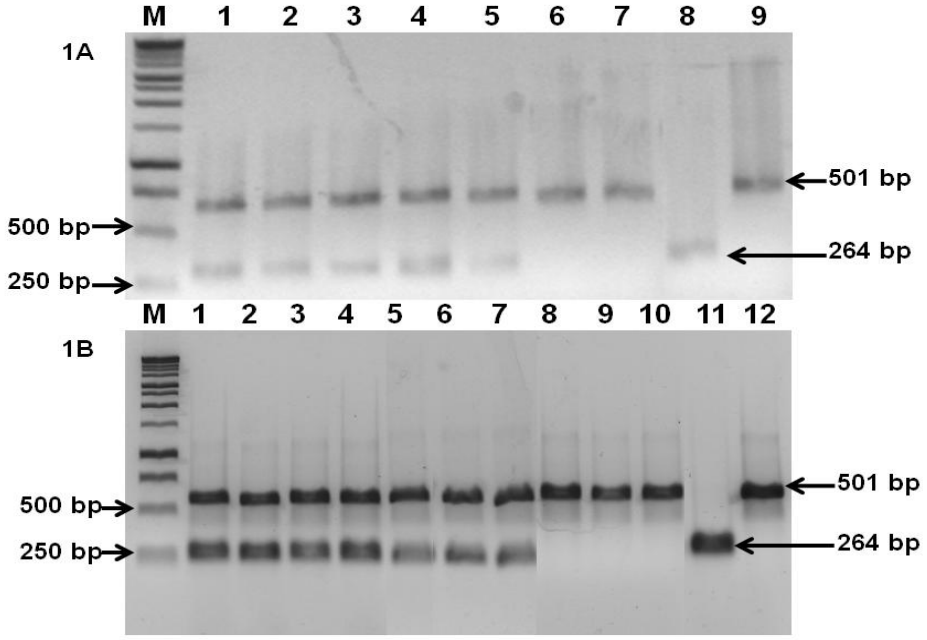

Figure 1. Agarose electrophoresis of IMS-PCR from artificially contaminated and clinical fluid samples in presence of 0.5 pg of IAC. Panel A, detection limit of *L. interrogans* serovar

Canicola strain Hond Utrecht IV in artificially contaminated dog urine samples: M, 1 kb DNA Ladder; Lanes 1-7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 leptospires per mL, respectively; lane 8, DNA only, lane 9, IAC only. Panel B, detection of *Leptospira* spp. amplified from dog clinical samples. M, 1kb DNA Ladder; lane 1-5 (urine) and 6-10 (blood), respectively. Lane 11 DNA only; Lane 12 IAC only.

Canine leptopirosis has a variable clinical presentation but, as a consensus, it results in leptospira renal scarring (5). The recommended diagnostic test has to be done by testing paired acute and convalescent sera to confirm the diagnosis, which is helpful in unvaccinated dogs, but hard to interpret in vaccinated ones (6). The antibodies detection methods are not useful before seven days after leptospira infection and the standard method, MAT; require paired samples to detect seroconversion (5). For those reasons, antigen detection tests offer potential advantage over tests based on antibody detection both for early diagnosis and identification of renal carrier status (5). Therefore, research focused on highly sensitive and specific routine tests for leptospira detection in blood and urine samples led to development of several molecular methods for diagnosis of leptospirosis (4, 6, 9, 14). However, the sensitivity and kinetics of PCR diagnostic tests may be dramatically reduced when applied directly to biological samples, such as urine and serum samples (11). The presence of inhibitor molecules and heterogeneous bacteria in the samples can affect PCR performance (13).

Here, we demonstrated that the IMS step can be useful for concentrating leptospires in clinical samples to allow detection by PCR and for reducing inhibitory substances which led to increase in sensitivity. Although the IMS-PCR was able to detect leptospires in only two out of five serum samples tested, high agglutination antibodies titers were found in those sera and no spirochetes were visualized under DFM suggesting absence of leptospiremia in

those animals. The use of a sensitive leptospira detection method such as the IMS-PCR, may constitute an important tool for identification of leptospira renal scarring.

In conclusion, this study demonstrated that IMS using an extensively characterized mAb against LipL32, a surface exposed outer membrane protein present in all pathogenic leptospires, is efficient in capturing pathogenic leptospiral cells. In addition, the IMS coupled to PCR has the potential to improve sensitivity and specificity of a diagnostic test for leptospirosis. The same approach may be useful for detection of other pathogens.

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5. CONCLUSÕES

- Os nanocompósitos magnéticos sintetizados a base de carbono e cobalto foram capazes de ancorar diferentes proteínas e auxiliar a captura e detecção de leptospiras patogênicas;

- A introdução da técnica de IMS utilizando os nanocompósitos magnéticos de poliestireno e ferro, como passo anterior a PCR, aumentou o limite de detecção da PCR a partir de amostras artificialmente contaminadas e a sensibilidade a partir de fluídos biológicos de cães positivos para a leptospirose.

6. PERSPECTIVAS

- Produção de anticorpos policlonais contra *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis* e *Mycoplasma flocculare* visando à detecção e isolamento de *M. hyopneumoniae*;

- Avaliar a técnica de IMS/PCR utilizando os anticorpos produzidos para detectar *M. hyopneumoniae* a partir de cultivos puros, artificialmente contaminados com outros micro-organismos e amostras clínicas;

- Avaliar a técnica de IMS/isolamento utilizando os anticorpos produzidos para isolar *M. hyopneumoniae* a partir de cultivos puros, artificialmente contaminados com outros micro-organismos e amostras clínicas;

- Avaliação dos anticorpos anti-*M. hyorhinis* e anti-*M. flocculare* em ensaios de inibição do crescimento, visando otimizar o isolamento de *M. hyopneumoniae*;

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