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Detekce a genotypizace kmenů *Helicobacter pylori* ve Waldeyerově lymfatickém okruhu a jeho vztah ke vzniku patologií v této oblasti

Detection and genotypisation of *Helicobacter pylori* strains in Waldeyerś lymphatic tissue and its relationship to the formation of pathologies in this area

DIZERTAČNÍ PRÁCE

ŠKOLITEL: Prof. MUDr. Jaromír Astl, CSc.

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Abstrakt

Práce se zabývá studiem přítomnosti *Helicobacter pylori* (HP) v lymfatické tkáni Waldeyerova okruhu u skupiny dospělých a dětských pacientů a možnou souvislostí se vznikem benigních onemocnění v této oblasti (chronická tonzilitida, adenoidní vegetace a syndrom obstrukční spánkové apnoe-OSAS) a se vznikem karcinomu tonzily.

V naší práci jsme potvrdili hypotézu, že HP se nachází v lymfatické tkání Waldeyerova okruhu stejně tak jako v žaludku a lymfatická tkáň orofaryngu a epifaryngu tak plní roli extragastrického zdroje HP. Předpokládá se, že v lymfatické tkání Waldeyerova okruhu působí infekce HP podobnými imunitními a zánětlivými procesy jako v žaludku, neboť se jedná o lymfatickou tkáň vázanou na sliznici.

V dizertační práci jsme pomocí metody real-time PCR zjistili vysoký výskyt DNA HP v adenoidní a tonzilární tkáni. Ve skupině benigních onemocnění byly nejčastěji detekovány genotypy Cag-VacAs1bm1 a CagA-VacAs1bm2. Ve skupině pacientů s karcinomem tonzily byl nejčastěji detekován genotyp CagA-VacAs1bm1. Genotypizací zjištěné kmeny HP vykazovaly rozdíly v porovnání s převládajícími kmeny, které lze nejčastěji nalézt v žaludku. Prokázané kmeny se liší především menší expresí CagA genu. Byly tak detekovány méně virulentní kmeny. Předpokládá se, že dlouhodobá kolonizace lymfatické tkáně orofaryngu a epifaryngu méně virulentními kmeny může vést k ovlivnění imunitních mechanismů a teoreticky k nástupu procesu zánětlivého procesu a ke kancerogenezi. Korelace mezi výskytem určitých typů genotypů HP a chronickým zánětem či maligním onemocnění prokázána nebyla.

Otázka eradikace HP u pacientů s PCR pozitivitou a současnou sérologickou pozitivitou infekce v naší práci potvrzena nebyla a je předmětem dalších studií. Sérologická metoda nemá dobrou výpovědní hodnotu, zda se jedná o recentní či již proběhlou infekci HP a nelze rovněž zjistit, v jaké lokalizaci infekce proběhla/probíhá. HP protilátky se nevytváří v organismu hned po vzniku infekce a zároveň přetrvávají v organismu poměrně dlouho.

Výsledky dosažené v této práci přispívají k poznání významu HP infekce u dospělých a dětských pacientů a pokládají rovněž otázky pro další výzkum.

Klíčová slova: *Helicobacter pylori*, tonzily, adenoidní vegetace, PCR, genotyp

Abstract

The aim of this study was to reveal the presence of *Helicobacter pylori* (HP) in the Waldeyerś lymphatic tissue in the group of children and adults and its possible role in the etiology of benign diseases (chronic tonsillitis, OSAS, adenoids) and in the etiology of the tonsillar carcinoma.

In our study we have confirmed the hypothesis that HP is presented in the Waldeyerś lymphatic tissue as well as in the stomach and that the oropharynx and epipharynx are the an extragastric reservoir of HP. Mucosa associated lymphatic tissue in the stomach is similar to lymphatic tissue of Waldeyer ring. These conditions can be very favourable for the survival of HP and thus can promote inflammation changes and immune changes as well as in the stomach.

In our study, we have detected using real-time PCR method high incidence of HP DNA in adenoids and tonsillar tissue. In the group of benign diseases were the most frequent genotypes CagA-VacAs1bm1 and CagA-VacAs1bm2. In the group of the patients with tonsillar carcinoma was the most frequent genotype CagA-VacAs1bm1. Genotyping identified strains of HP showed differences in comparison with the predominant strains which are most frequently found in the stomach. Genotypic analysis of HP strains showed that prevailed less virulent strains of HP, so-called cagA negative and vacA positive. It is supposed, that the long colonization of less virulent strains in the oropharyngeal and epipharyngeal area leads to influence of immune mechanisms and can start the process of cancerogenesis. The correlation between particulary types of HP genotypes and chronic tonsillitis/tonsillar carcinoma was not proved.

The question of the eradication in the group of patients with PCR positivity and serology positivity was not confirmed in our study and it is the subject of the feature studies. The serology does not have a high sensitivity for the detection of acute infection and it is not possible to detect in which location the infection was/is taking place. The HP antibodies can persistent in the organism for a long time.

Our results contribute to the HP knowledge in children and adults.

Key words: *Helicobacter pylori*, tonsills, adenoids, PCR, genotype

Doktorská disertační práce byla vypracována v rámci prezenčního a kombinovaného studia postgraduálního studia biomedicíny na Klinice otorinolaryngologie a chirurgie hlavy a krku 1.lékařské fakulty Univerzity Karlovy v Praze a Klinice otorinolaryngologie 3.lékařské fakulty Univerzity Karlovy v Praze.

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1 Literární úvod a přehled dané problematiky

1.1 Mikrobiologická charakteristika a patogenetické působení *Helicobacter pylori*

Helicobacter pylori (HP) je spirální, pohyblivá, mikroaerofilní bakterie. Její přítomnost je spjata zejména s lidskou populací, avšak některé studie dokazují její přítomnost i u zvířecích druhů (Terio et al., 2005) . Infekce HP je považována za hlavní příčinu chronické gastritidy a hraje velmi důležitou roli v patogenezi vředové choroby, žaludečního lymfomu a karcinomu žaludku (Israel a Peek, 2001) Příčinná souvislost mezi působením HP a chronickou gastritidou se ukázala v roce 1983, kdy Marshall a Warren vykultivovali tuto bakterii z materiálu žaludeční sliznice.

Práci, postavenou na studii bioptických vzorků žaludeční sliznice u dětských pacientů s chronickou gastritidou, získaných sací metodou, provedla již v letech 1963 – 1966 Doc. MUDr. Miloše Sedláčková, CSc. v Praze. Její práce byla postavena na 224 případech dětí, na nichž popsala všechny charakteristiky gastritidy u dětí, etiologie zůstala však ve stavu hypotézy.

Výskyt infekce HP je odhadován mezi 40 – 80% a je spjat se zeměpisnou oblastí, věkem, rasou a socioekonomickým stavem dané země (Bureš et al., 2006). Zvýšený výskyt infekce HP je zaznamenán v rozvojových zemích. Ve státech střední a východní Evropy se promořenost udává mezi 60 – 95% (Bureš et al., 2006). Klinické projevy onemocnění se objevují pouze u 10 – 15% nakažených jedinců, což je pravděpodobně dáno rozdílnou imunitní odpovědí hostitele a odlišným stupněm virulence kmenů (Stromberg et al., 2003). Infekce může být získána již během dětství a probíhat latentně po celý život. Bakterie vyžaduje k růstu mikroaerofilní prostředí (5% O_2, 10% CO_2) a pro její kultivaci se používají půdy se sérem, koňskou krví a heminem (Bednář, 1996). Bakterie vyrůstá za 5 – 7 dní a vytváří okrouhlé kolonie 2 – 3 mm v průměru. Výrazným rysem je tvorba ureázy, katalázy a oxidázy. Ureáza pak štěpí ve tkáni přítomnou močovinu na NH_3 a CO_2 a amoniak pak neutralizuje volnou HCl v okolí bakterie v žaludku (Bednář, 1996). Přenosovou formou bakterie je forma kokoidní, která je více rezistentní k zevním vlivům. Cesty přenosu uvedeného patogenu nejsou doposud zcela objasněny, předpokládá se cesta oro – orální či oro – fekální (Brown, 2000).

Patologické působení HP je podmíněno interakcemi mezi hostitelem a mikrobem a je ovlivněno stupněm virulence jednotlivých kmenů (Stromberg et al., 2003). Progrese nemoci pak závisí na bakteriálním genotypu, vlivu prostředí, životním stylu i genetických predispozicích hostitele (Tegtmeyer et al., 2011). HP se vyznačuje produkcí celé řady faktorů virulence. Nejdůležitější jsou dva sekreční proteiny: vakuolizační cytotoxin A (VacA) a cytotoxin – asociovaný gen A (CagA). CagA je produkován asi 60% kmenů HP a je kódován genem *cagA*, který se nachází v tzv. ostrůvku patogenity – PAI (Olivares a Gisbert, 2006). Tento ostrůvek kóduje sekreční systém typu IV (T4SS), jehož

prostřednictvím se CagA protein dostává do hostitelské buňky (Höcker a Hohenberger, 2003). Kmeny s přítomností *cagA* jsou označovány jako CagA+. U pacientů s těmito kmeny byl zaznamenán vyšší výskyt peptických vředů, atrofických gastritid a karcinomu žaludku. T4SS je řízen řadou genů, mimo jiné *cagI, cagL, cagY* a *cagA*. Proteiny kódované těmito geny jsou integrovány prostřednictvím vazby s integrinem B1 do hostitelské buňky, kde dochází k jejich fosforylaci za účasti onkogenních tyrosinkináz. Tento proces pak vede k produkci cytokinů a růstových faktorů.

Dalším důležitým faktorem virulence je VacA protein. Ten interaguje s receptory hostitelské buňky, což má za následek modifikaci lysosomálních funkcí, buněčnou vakuolizaci, apoptózu a inhibici některých imunitních mechanismů (Backert a Selbach, 2008),(Hatakeyama, 2008). Nositeli vacA genu jsou všechny kmeny HP. VacA protein je však produkován jen zhruba u 50% kmenů. Je to dáno především tím, že jednotlivé kmeny vykazují rozdílnou strukturu vacA genu. Jsou známy alely s1 a s2 signální sekvence vacA a m1 a m2 střední sekvence vacA. Za vysoce toxické jsou považovány kmeny genotypu s1m1, které jsou spojovány s vysokým výskytem intestinálních metaplasií a gastritid (Höcker a Hohenberger, 2003). K dalším faktorům virulence patří lipopolysacharidy, adheziny (babA1, babA2) a enzym ureáza kódovaný ureázovým genem C.

Po proniknutí HP do lamina propria sliznice hrají hlavní roli v imunitní odpovědi hostitele dendritické buňky a monocyty. Tyto mají na svém povrchu receptory TLRs (Toll –like- receptors) (Portal-Celhay a Perez-Perez, 2006). Vazbou receptorů na dané buňky dochází k aktivaci NK buněk (natural killers) a Th1 imunitní odpovědi, což je dominantní imunitní reakce v patogenezi HP. Indukuje expresi IFN – gamma. Po kontaktu s bakterií dochází k vyplavování prozánětlivých cytokinů TNF – alfa, IL -1beta, IL-8,12,18 (Portal-Celhay a Perez-Perez, 2006).

V souvislosti s poškozením žaludeční sliznice, zvýšenou proliferací a nádorovou transformací žaludečního epitelu byly popsány také změny v expresi EGF, EGFR (Schiemann et al., 2002), TGF – beta (Stromberg et al., 2003) a iNOS (Sakaguchi et al., 1999). Rovněž bylo zjištěno, že zvýšenou stimulaci makrofágů k produkci i NOS a NO způsobuje HP produkovaná ureáza (Gobert et al., 2002)

1.2 Diagnostika *Helicobacter pylori*

Běžně používané detekční metody k průkazu infekce HP lze rozdělit na metody invazivní a neinvazivní. Při detekci infekce v oblasti orofaryngu se zpravidla užívají testy invazivní.

1.2.1 Invazivní testy

Kultivační průkaz – jedná se o metodu se 100% specificitou, sensitivita velmi závisí na řadě různých faktorů a na zkušenostech dané laboratoře (Velapatino et al., 2006). Kultivačně byl HP detekován řadou autorů (Bitar et al., 2006), (Pitkäranta et al., 2005). Kultivačním průkazem HP v

dutině ústní se jako první zabýval Krajden (Krajden et al., 1989). Jednou z podmínek úspěšné kultivace je dostatečně dlouhá doba bez užívání antibiotické terapie. Samotná kultivace pak probíhá na agarových půdách s přidanými obohacovadly, vhodnými pro růst této bakterie (Coumbia agar, Wilkins Chalgren agar atd.) (Mégraud, 2007). Kultivace vyžaduje mikroaerofilní prostředí. Kolonie se pak hodnotí po 3 – 4 dnech a mají většinou charakter drobných okrouhlých kolonií, 2 – 3 mm v průměru. Uvedená metoda je velmi časově náročná a při detekci v orofaryngu může přinášet falešně negativní výsledky, neboť v orofaryngu se vyskytují i jiné bakteriální kmeny, které mohou růst HP inhibovat (Ishihara et al., 1997). Dalším problémem této metody je předpoklad, že HP přežívá v oblasti orofaryngu v tzv. kokoidních formách, které jsou velmi špatně kultivovatelné (Bode et al., 1993). Kultivační průkaz v orofaryngu je tedy značně limitován.

Histopatologický průkaz HP se v současné době příliš neužívá. Jedná se však o metodu, která může jako jediná detekovat léze související s HP infekcí (atrofie a buněčná metaplasie). Vzorky k histopatologickému průkazu jsou fixovány v 10% formaldehydu. Tkáň je barvena nejčastěji metodou hematoxylin – eosin, Giemsa, Warthin Starry či Genta (Rotimi et al., 2000). V rámci histopatologického průkazu se rovněž využívá imunohistochemická detekce či elektronová mikroskopie. Imunohistochemický průkaz je pak schopný zachytit i kokoidní formy HP. Elektronová mikroskopie je časově velmi náročná a není doposud součástí rutinních diagnostických postupů. Hodnocení histologických řezů závisí především na správně odebraném vzorku tkáně a na zkušenostech dané laboratoře rozpoznat morfologické rysy daného mikroba (Mégraud, 2007) Histopatologickým průkazem HP v orofaryngeální tkání se zabývala řada studií (Bitar et al., 2005),(Jabbari Moghaddam et al., 2009). Na rozdíl od vzorků žaludeční sliznice jsou metody histopatologické detekce v orofaryngu limitovány přítomností velkého množství konkurenčních bakteriálních kmenů (Dowsett a Kowolik, 2003).

Detekce ureázy patří mezi rychlé, levné a jednoduché testovací metody, které byly užity v řadě studií (Khademi et al., 2007),(Unver et al., 2001). Tyto testy jsou založeny na průkazu ureázové aktivity HP (Qureshi et al., 1992) která spočívá v hydrolýze urey na oxid uhličitý a amonný iont. Koncentrace amonného iontu pak určuje pH, a tím vyvolává změnu barevného indikátoru (Vaira et al., 2000). Mezi nejvíce užívané ureázové testy patří CLO test (Campylobacter - like– organism) a RUT test (Rapid Urease Test). Některé práce ukazují významnou souvislost mezi pozitivitou RUT a histologickým průkazem HP (Madani et al., 2000) V orofaryngu je užití těchto metod značně omezeno, neboť v dutině ústní existují i jiné bakterie produkující ureázu – např. Streptococcus spp., Haemophilus spp., Actinomyces spp. atd. Zvyšuje se tak pravděpodobnost falešně pozitivních výsledků (Dowsett a Kowolik, 2003).

Molekulárně diagnostické postupy v detekci HP v oblasti orofaryngu a epifaryngu zahrnují v současné době především metodu PCR (polymerázová řetězová reakce). Tato technika byla koncipována v roce 1983 Kary Mullisem v rámci řešení exponenciální amplifikace oligonukleotidů v beta – globinovém genu. Základním principem PCR je opakovaná řízená denaturace dvouřetězcové DNA a následná renaturace osamocených řetězců se specifickými oligonukleotidy, které jsou v reakční směsi v nadbytku. Amplifikace DNA probíhá v opakujících se cyklech, které mají tři kroky: denaturace, hybridizace a polymerace. Denaturace probíhá při teplotě 95°C a spočívá v rozpadu vodíkových můstků spojujících vlákna DNA. Vzniká tak jednořetězcová DNA, neboli templát. Hybridizace (annealing) se uskutečňuje při teplotě 50 – 60°C, spočívá v dosednutí primerů a rovněž vede k obnově dimerů DNA. Elongace je fází, kdy dochází polymerací k syntéze nového řetězce DNA komplementárního s templátem. Pro PCR se používají termostabilní DNA polymerázy, především Taq polymeráza. Průkaz HP infekce v orofaryngu pomocí metody PCR je popsán v řadě prací (Abdel-Monem et al., 2011), (Bitar et al., 2005), (Bitar et al., 2006), (Cirak et al., 2003). Pro detekci HP je často používána detekce ureC genu pomocí 16S rRNA primeru. Identifikace tohoto genu však nevypovídá nic o virulenci mikroba. Ve většině publikovaných prací byly proto nejčastěji detekovány geny *cagA* a další geny ostrůvku patogenity, zejména *vacA* (a jeho polymorfismy). Pomocí PCR lze také detekovat geny kódující adhezní proteiny – Bab A2, Sab A, či další geny důležité pro patogenitu – *oipA, dupA, iceA* (Mégraud, 2007). Všechny tyto geny určují virulenci HP. Nejčastěji využívanými systémy jsou klasická end-point PCR a PCR v reálném čase (real – time – PCR). Real-time PCR je založena na měření množství produktu v průběhu amplifikace a v multiplexní aplikaci umožňuje provádět genotypizaci jednotlivých kmenů HP pomocí různě značených hybridizačních sond. End point PCR je klasickou PCR metodou a užívá se zejména k detekci *cagA* a *vacA* genů. V posledních letech byla publikována řada prací zabývajících se detekcí HP v žaludku prostřednictvím PCR metody. Byl například detekován glmM gen pomocí Novel-primeru (Espinoza et al., 2011). Rovněž byla provedena detekce babA2 a iceA genů u pacientů s benigní žaludeční diagnózou (Montealegre O´ et al., 2010). Senzitivita PCR metody je především závislá na typu transportního média a rovněž na způsobu uchovávání vzorku do doby izolace nukleové kyseliny.

1.2.2 Neinvazivní testy

Ureázový dechový test je v současné době považován za zlatý standart metod k detekci infekce HP v žaludku (Kocna, 2006). Vzorky jsou analyzovány metodou hmotnostní spektrometrie nebo metodou NDIRS (nondispersive infrared spektrometry). Tato metoda je založená na změnách oscilačního spektra molekul izotopů CO2 v infračerveném světle. Použití této metody spočívá v

měření absolutních koncentrací plynu a naměřené hodnoty více kolísají. Důležité je kritérium změny poměru vydechovaného CO2 v čase 30 oproti T0 –DOB větší než 5 promile (Kocna, 2006). Dechový test byl použit i v modifikovaném provedení jako endoskopický UBT, vykazující přesnost v detekci. Dle Maastrichtského konsenzu z roku 2000 se jedná o vyšetření vhodné k průkazu infekce HP a rovněž je pokládáno za vyšetření určené ke kontrole eradikace. Největším problémem dechových testů je kvantitativní interpretace. Objem vzduchu při dýchání se liší v závislosti na fyzické aktivitě, psychickém stavu a dalších faktorech. Výsledkem je pak jejich vysoká biologická aktivita.

Detekce HP antigenů ve stolici (Stool Antigen Test) většinou se jedná o sadu komerčně vyráběných testů založených na imunochromatografických či imunoenzymových metodách (Dzierżanowska-Fangrat et al., 2006). U dětí je pak považován za ekvivalent dechového testu (Doorn, 2001). Principem testu je vazba antigenů HP na polyklonální králičí anti-HP protilátku a stanovení probíhá in vitro metodou ELISA. Výsledná reakce se hodnotí vizuálně na základě změny barvy či fotometricky při 450 nm.

Sérologická detekce protilátek proti HP stojí na hranici mezi invazivními a neinvazivními metodami. Běžně jsou stanovovány protilátky IgG, IgA, IgM. Rovněž lze ze séra detekovat přítomnost protilátek proti CagA. K detekci protilátek se rutinně využívá metoda ELISA, která má sensitivitu 92% a specificitu 96% (Mégraud, 1995).

1.3 *Helicobacter pylori* ve Waldeyerově lymfatickém okruhu a dutině ústní

Waldeyerův lymfatický okruh je definován jako místa enormního nakupení lymfatické tkáně lamina propria mucosae hltanové úžiny (isthmus faucium). Waldeyerův okruh zahrnuje: patrové mandle (tonsillae palatinae), jazykovou mandli (tonsilla lingualis), nosohltanovou mandli (tonsilla pharyngea) a tonsillae tubariae. V rámci orálně - orálního či fekálně – orálního přenosu HP infekce lze očekávat přítomnost HP rovněž v dutině ústní a v orofaryngu. Do nedávna byl za jediný rezervoár infekce HP v lidském organismu považován žaludek. V posledních letech je diskutována otázka kolonizace orofaryngeální a epifaryngeální lymfatické tkáně tímto mikrobem a jejich možná funkce jako extragastrického zdroje infekce HP. Předpokládaný výskyt HP v lymfatické tkáni Waldeyerova okruhu je založen na podobnosti žaludeční a orofaryngeální/epifaryngeální lymfatické tkáně. Možný příčinný vztah HP a extra-gastrointestinálních chorob je zkoumán od roku 1993 (Konturek et al., 1999). Byla publikována řada prací, zabývajících se výskytem HP v dutině ústní a v orofaryngu/epifaryngu. Předpokládá se, že by HP v lymfatické orofaryngeální tkáni Waldeyerova okruhu mohl aktivovat imunitní systém (např. aktivací dendritických buněk) a zasahovat do exprese různých cytokinů a tím podporovat vznik chronického zánětu a eventuálně působit jako přímý karcinogen této oblasti. Oblast nosohltanu a patrových tonzil může být přímo exponována působením HP u nemocných s refluxní chorobou jícnu, zahrnující onemocnění vznikající na podkladě gastroezofageálního a extraezofageálního refluxu, který je častý především u dětí. Problematikou vztahu refluxního onemocnění a výskytu HP v oblasti orofaryngeální a epifaryngeální lymfatické tkáně se ve své práci podrobně zabýval Katra (Katra et al., 2014). Práce Pavlíka z roku 2007 podporuje možnost orofaryngu jako extragastrického zdroje HP nálezem odlišných kmenů HP v žaludku a v orofaryngu (Pavlík et al., 2007)

Bakterie byla detekována v dentálním plaku a ve slinách (Karczewska et al., 2002), (Czesnikiewicz-Guzik et al., 2005). Dentální plak a sliny jsou tedy považovány za extragastrický zdroj infekce HP. Sohrnným shrnutím literatury o existenci HP v dutině ústní se pak zabýval v roce 2014 Al Sayed (Al Sayed et al., 2014).

1.4 *Helicobacter pylor*i v oblasti orofaryngeální a epifaryngeální lymfatické tkáně

Význam tonzilární tkáně pro kolonizaci HP poprvé ve své práci zdůraznil Minocha (Minocha et al., 1997). V roce 2003 Cirak prokázal v tonzilární tkáni CagA pozitivní kmeny HP metodou PCR s použitím 16S ribozomálního primeru (Cirak et al., 2003). Stejný poznatek učinil o tři roky později Bulut (Bulut et al., 2006). Yilmaz M. a spol. detekovali pomocí 23S ribozomálního primeru HP v adenoidní tkáni (Yilmaz et al., 2004), Yilmaz T.a spol. pak detekovali v roce 2006 HP v tonsilární i adenoidní tkáni prostřednictvím ribozomálního primeru (Yilmaz et al., 2006). Khademi prokázal

přítomnost HP v tonsilární tkáni metodou CLO testu (Khademi et al., 2005). Tato metoda byla užita rovněž v roce 2001 k úspěšné detekci HP v adenoidní tkáni (Unver et al., 2001).

Ve studii Bitara a spol. byl zjištěn HP v adenoidní tkáni metodou RUT (rapid urease test) a histologickým průkazem (Bitar et al., 2005, 2006). Rovněž byl srovnáván výskyt HP na povrchu a uvnitř tonzil (Khademi et al., 2007). Práce Jelavice a spol. dokládá, že tonzilární tkáň není důležitým rezervoárem infekce HP u dětí podstoupivších tonzilektomii (Jelavic et al., 2007). Vayisoglu a spol. v roce 2008 zjistili pozitivitu HP ve dvou adenoidních vegetacích z celkového počtu 91 dětských pacientů pomocí RUT a ve dvou případech byl pozitivní rovněž imunohistochemický průkaz. Ve své práci zpochybňuje funkci tonzilární tkáně jako extragastrického zdroje infekce HP a rovněž vyvrací možnou souvislost chronické tonzilitidy s infekcí HP (Vayisoglu et al., 2008). Eyigor a spol. se v roce 2009 pokoušeli detekovat *glm* gen v tonzilární tkáni pomocí GJB2 primeru metodou PCR. V žádném vzorku tkáně tento gen nalezen nebyl, což vedlo k závěru, že tonzilární tkáň není extragastrickým zdrojem infekce HP (Eyigor et al., 2009). Jabbari Moghaddam a spol. ve své práci dokládají výskyt HP infekce v tonzilární tkáni pomocí metody RUT, kterou staví do pozice málo senzitivní metody pro detekci této infekce (Jabbari Moghaddam et al., 2009). V roce 2010 se zabývali detekcí HP infekce v tonzilární tkáni Vilarinho a spol. Pomocí RUT a imunohistochemického průkazu detekovali daný patogen v tonzilární tkáni pěti dětských pacientů. Metody PCR a FISH (Fluorescence in situ hybridization) v dané práci pozitivní výsledky nepřinesly, což dle autorů rovněž vyvrací teorii tonzilární tkáně jakožto extragastrického zdroje infekce HP (Vilarinho et al., 2010). Souvislostí kokoidních forem HP v tonzilární tkáni a IgA nefropatií se ve své práci zabývali Kussano a spol. Výstupy této práce ukazují na možnou souvislost onemocnění ledvin ve výskytu kokoidních forem HP (Kusano et al., 2010). V roce 2011 se studiem přítomnosti HP v tonzilární tkáni zabývaly dvě práce: Toros a spol. pomocí metody RUT a histologického průkazu přítomnost HP v tonzilární tkáni neprokázali (Toros et al., 2011), Abdel Monem a spol. pak publikovali prospektivní studii, ve které zachytili pozitivitu HP v tonzilární tkáni metodou RUT a rovněž metodou PCR, pomocí níž detekovali *ureC* gen (Abdel-Monem et al., 2011). V roce 2012 analyzoval Farivar et al. celkem 103 vzorků tonzilární tkáně pomocí metody Real-time PCR se sondami Scorpion s 21,53% pozitivních výsledků (Farivar et al., 2012). Vysokou pozitivitu HP ve vzorcích adenoidní tkáně prokázal v roce 2014 Kraus (Kraus et al., 2014). V roce 2015 detekoval Bayindir a spol. V celkem 57 vzorcích adenoidní a tonsilární tkáně cagA gen a gen pro fosfoglukosaminmutasu), úspěšnost metody PCR byla 10,9%, protilátky proti HP pak byly detekovány v 89% (Bayindir et al., 2015). V roce 2016 se zabýval detekcí HP v adenoidní tkáni Cedeno (Cedeño et al., 2016). Incidencí HP kolonizace se ve své práci zabýval také Güclü v roce 2013, který prokázal nízkou incidenci kolonizace HP v adenoidní tkáni a tonsilární tkáni, resp. Ze 49 tonsilárních vzorků bylo PCR pozitivních 6,1% (3 vzorky), RUT pozitivních 2 (4,1%), z 52 vzorků adenoidní tkáně byly 3 vzorky (5,8%) PCR pozitivní (Guclu et al.,

2013) . Kaymakci potvrdil pozitivitu HP v adenoidní tkáni pomocí PCR u 2 pacientů z 23 (Kaymakçı, 2014) .Kolonizaci adenoidní tkáně HP připustil na základě svých výsledků i Aydin v roce 2014. kdy detekoval 2 pozitivní vzorky pomocí PCR metody (Aydın et al., 2014). Narozdíl od něho Aliakbari v roce 2011 detekoval 0 HP pozitivních vzorků v adenotonsilární tkáni pomocí metody PCR (Aliakbari et al., 2011)

Následující tabulka (Tab.1) ukazuje přehledně práce, ve kterých se autoři zabývali detekcí HP ve tkáních Waldeyerova lymfatického okruhu za použití různých detekčních metod .

Autor	Rok	Počet případů	Typ odebrané tkáně	Diagnostická metoda	Počet pozitivních případů na H. pylori
Di Bonaventura	2000	36	tonzilární stěr	kultivace, imunohistochemický průkaz	0 (0 %)
Di Bonaventura	2001	75	tonzilární stěr a biopsie	PCR	0 (0 %)
Unver et al.	2001	19	adenoidní tkáň	CLO test	11 (58 %)
Skinner et al.	2001	50	tonzilární tkáň	CLO test, imunohistochemický průkaz	0 (0 %) CLO test a imunocytochemie
Uygur-Bayramicli	2002	27	tonzilární tkáň	histologie, imunohistochemický průkaz	0 (0 %) histologie, imunohistochemický průkaz
Cirak	2003	23	tonzilární a adenoidní tkáň	PCR (16S ribosomální RNA, CagA)	7 (30 %) pozitivních na H. pylori, 5 z nich (71 %) pozitivních na CagA gen
Yilmaz et. al.	2004	50	tonzilární a adenoidní tkáň	CLO test	0 (0 %)
Yilmaz et. al.	2005	38	adenoidní tkáň, středoušní tekutina	PCR (23S ribosomální RNA)	12 (67 %) ve středoušní tekutině, 1 (5 %) v adenoidní tkáni
Pitkaranta	2005	20	adenoidní tkáň, středoušní tekutina	Kultivace	0 (0 %)
Khademi et. al.	2005	56	tonzilární a adenoidní tkáň	CLO test	27 (48%)
Bitar	2005	25	adenoidní tkáň	RUT, histologie a nested PCR (UreA)	21 (84 %) pozitivních v RUT, 4 (16 %) pozitivních v histologii, 0 (0 %) pozitivních v nested PCR
Bulut	2006	71	tonzilární a adenoidní tkáň	PCR (CagA - glmM gen)	29 (24,6 %) pozitivních na H. pylori, 17 z nich (58,6 %) CagA pozitivních
Bitar	2006	28	adenoidní tkáň, středoušní tekutina	kultivace, RUT, PCR (ureC-gen)	0 (0 %) ve středoušní tekutině, 10 (77 %) v adenoidní tkáni podle RUT, 0 (0 %) podle PCR
Yilmaz et. al.	2006	22	adenoidní a tonzilární tkáň, středoušní tekutina, sliznice promotoria	kultivace, PCR (16S RNA)	středoušní tekutina: 2 pozitivní v kultivaci, 7 v PCR; sliznice promotoria: 1 v kultivaci, 7 v PCR; adenoidní tkáň: 11 (50 %) v kultivaci, 14 (64 %) v PCR; tonsilární tkáň: 12 (55 %) v kultivaci, 14 (64 %) v PCR

Jelarie et al.	2007	139	tonzilární a adenoidní tkáň	kultivace, RUT	17 (12 %) pozitivních v RUT, 0 (0 %) pozitivních v kultivaci
Kusano et. al.	2007	173	patrové tonzily	imunohistochemický průkaz, elektronová mikroskopie, hybridizace in-situ (16S RNA gen)	126 (72,9 %) pozitivních
Vayisoglu et. al.	2008	91	tonzilární a adenoidní tkáň	RUT, imunohistochemický průkaz	2 (2,2 %) v adenoidní tkáni, 0 (0 %) v tonsilární tkáni s RUT, 0 (0 %) v imunohistochemickém průkazu
Eyigor et. al.	2009	55	35 adenoidních tkání, 20 tonzil	RUT, PCR (glmM gen)	RUT 5,5 % pozitivních, 0 % PCR pozitivních
Ozcan	2009	25	adenoidní tkáň, středoušní tekutina	CLO, imunohistochemický průkaz	0 (0 %) CLO pozitivních, 0 (0 %) imunohistochemicky pozitivních
Jabbari Moghaddam	2009	285	tonzilární tkáň	RUT, histopatologie	113 (39,6 %) pozitivních s histopatologií, 40 (14 %) pozitivních s RUT
Vilarinho et. al.	2010	62	adenoidní a tonzilární tkáň	RUT, imunohistochemický průkaz, FISH, PCR-DNA (vacA gen)	3 pozitivní s RUT, 2 pozitivní s imunohistochemickým průkazem, 0 pozitivních s FISH, 0 pozitivních s PCR
Toros et. al.	2011	84	tonzilární a adenoidní tkáň	RUT, histopatologie	0 (0 %) pozitivních s RUT, 0 (0 %) poitivních s histopatologií
Abdel-Monem	2011	20	adenoidní a tonzilární tkáň	RUT, PCR (ureC-gen)	16 (53,3 %) pozitivních s RUT, 5 (16,6 %) pozitivních s PCR
Hussey et al.	2011	93	adenoidní tkáň	Nested RT-PCR (RNA)	1 positive (Wolinella africanus)
Farivar et al.	2012	103	tonzilární tkáň	Scorpion RT - PCR	21,35% positive
Kraus et al.	2014	49	adenoidní a tonzilární tkáň	RT-PCR	98% positive, 2% negat.
Bayindir et al.	2015	57	adenoidní a tonzilární tkáň	HP protilátky, PCR (cagA, fosfoglukosaminmutasa)	protilátky 89%, PCR 10,9%
Kaymaki	2014	23	adenoidní tkáň	PCR	8,7% pozitivních
Nártová	2014	89	tonzilární tkáň	PCR	80%PCR pozitivity u chronických tonsilitid, z toho cagA gen u 25%. Ve skupině SAS 82,76% pozitivitym cagA u 20,83%
Lukeš	2014		tonzilární tkáň	PCR	73,91% pozitivity ve skupině tonzilárních tumorů, 70,0% u chronických tonsilitid, 69,23% OSAS vzorků
Katra	2014	30	adenoidní tkáň	PCR	90% pozitivita, cagA gen 33,33%

Tabulka 1: Přehled relevantních vědeckých prací, zabývajících se detekcí Helicobacter pylori v lymfatické tkáni Waldeyerova lymfatického okruhu

1.5 Podíl *Helicobacter pylori* na karcinogenezi

Helicobacter pylori je řazen mezi karcinogeny I. typu dle IARC (Logan, 1994). Existují tři základní možné cesty karcinogeneze (Lukeš et al., 2008a).

1. působení HP jako přímého mutagenu (Hatakeyama, 2006)
2. imunitní inhibice T- buněk prostřednictvím vakuolizačního cytotoxinu (Boncristiano et al., 2003).
3. Karcinogenní působení HP zvýšením hladin řady cytokinů a regulačních molekul, zejména pak vzestup hladin TGF, EGF a NOS, kdy existuje předpoklad, že zvýšená exprese těchto molekul se podílí na orofaryngeální karcinogenezi (Lukeš et al., 2008a)

Práce Lukeše et al. z roku 2007 pak ukazuje na základě imunohistochemického průkazu zvýšené hladiny eNOS, iNOS a kaspázy-3 u chronických zánětů tonzil a u tonzilárních karcinomů (Lukeš et al., 2008b). Práce Pavlíka et al.z roku 2015 podporuje teorii dlouhodobé kolonizace HP v oblasti tonzil a orofaryngu celkově, rovněž tak připouští možnost ovlivnění HP imunitního systému a následný zlom v proces kancerogeneze (Pavlik et al., 2015).

Byl zkoumán především vztah mezi laryngeálními, popř. laryngopharyngeálními spinocelulárními tumory a HP. Existuje tak řada prací, které tuto teorii buď podporují, či vyvrací. Metaanalýza Zhuo et al. z roku 2008 připouští infekci HP jako potenciální rizikový faktor laryngeálního karcinomu (Zhuo et al., 2008). Rovněž Gong et al. svou prací v roce 2010 tuto teorii podporuje (Gong et al., 2010). Dayama et al. se zabýval ve své studii z Indie výskytem laryngeálních karcinomů a možnou souvislostí jejich vzniku s pozitivitou HP. Přiklání se tak ke vztahu HP a rakoviny v orální oblasti (Dayama et al., 2011). Pozitivní vztah HP a laryngopharyngeálních spinocelulárních tumorů potvrzuje i práce Guilemany et al. z roku 2014 (Guilemany et al., 2014). Naopak práce Amizadeh et al. z roku 2015 se k roli HP jako možného patogenu při vzniku spinocelulárních karcinomů laryngu nepřiklání (Amizadeh et al., 2015). Stejně tak práce Zhona et al. z roku 2016 (Zhou et al., 2016)

2 Cíle a hypotézy dizertační práce

Dizertační práce je zaměřena na studium kolonizace tkáně Waldeyerova lymfatického okruhu *Helicobacter pylori.* Zabývá se detekcí této bakterie v dané oblasti a následnou genotypizací. Zabývá se otázkou, zda HP může být etiologickým faktorem při vzniku benigních a maligních onemocnění v této oblasti.

2.1 Cíle dizertační práce

1. Průkaz přítomnosti *Helicobacter pylori* ve tkání Waldeyerova lymfatického okruhu pomocí metody real-time PCR a zhodnocení výskytu její DNA v této oblasti.
2. Popis genotypových rozdílů *Helicobacter pylori* a porovnání jednotlivých genotypů v tkáňových vzorcích patrových tonzil a adenoidních vegetací v rámci benigních a maligních onemocnění této oblasti.
3. Porovnání zastoupení *cagA* genů a *vacA* genů v dané oblasti.
4. Zhodnocení argumentu indikace eradikace *Helicobacter pylori* v rámci výskytu *cagA* genu ve Waldeyerově lymfatickém okruhu.

2.2 Hypotézy dizertační práce

Hypotéza 1: Ve Waldeyerově lymfatickém okruhu se vyskytuje infekce *Helicobacter pylori* stejně tak jako v žaludku.

Hypotéza 2: Nález kmenů *Helicobacter pylori* v oblasti Waldeyerova lymfatického okruhu a současný sérologický průkaz infekce je argumentem k provedení eradikace u daných pacientů.

Hypotéza 3: Existuje korelace mezi výskytem konkrétních genotypů a chronickým zánětem nebo maligním onemocněním ve Waldeyerově lymfatickém okruhu.

3 Materiál a metody

3.1 Sběr biologického materiálu

Všechny vzorky byly odebírány s řádným informovaným souhlasem pacientů na Klinice ORL a chirurgie hlavy a krku 1.LF UK a FN v Motole v letech 2009 – 2014 a dále pak na ORL oddělení nemocnice Rudolfa a Stefanie Benešov. Pacienti byli operováni pro chronickou tonzilitidu, pro hypertrofii tonzil při syndromu obstrukční spánkové apnoe, pro zvětšenou adenoidní vegetaci a pro orofaryngeální tumory. Z výzkumu byly vyřazeni pacienti, kteří v minulosti podstoupili eradikaci HP. Ve skupině dospělých pacientů bylo 145 vzorků tonzilární tkáně, resp. 80 vzorků tonzil s diagnózou chronické tonzilitidy (55,71%), 42 vzorků tonzil s diagnózou OSAS (28,97%) a 23 vzorků tonzil s diagnózou tonzilárního karcinomu (15,86%). Celkem bylo odebráno 79 dětských tkáňových vzorků, 62 z adenoidních vegetací (78,48%), 17 pak z tonzilární tkáně při OSAS (21,52%). U 12 dětských pacientů byla odebrána tkáň adenoidní i tonzilární. U skupiny pacientů s OSAS byla předpokládána přítomnost zdravé tonzilární tkáně. V úvodu chirurgického výkonu po zavedení endotracheální intubace byly odebrány tkáňové vzorky tonzilárních karcinomů a tonzil za sterilních podmínek, před aplikací anestetik a dezinfekčních prostředků do dutiny ústní. Vzorky byly následně vloženy do transportního media (Microtest^{R} M4RT, Remel Inc., USA) pro PCR zpracování a transportovány do laboratoře.

3.2 Vyšetření vzorků

3.2.1 Izolace nukleové kyseliny z tkáňových vzorků a její uskladnění

DNA *Helicobacter pylori* z tkáňových vzorků byla izolována s využitím MagNA Pure Compact Systému (Tegimenta AG, Rotkreuz, Switzerland) a soupravou MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics), s užitím protokolu Total_NA 400_100 a s předmytím v MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics). Izolované vzorky DNA *Helicobacter pylori* byly uskladněny při teplotě -80 °C.

3.2.2 Detekce nukleové kyseliny *Helicobacter pylori* a její genotypizace

Genetická analýza byla pro tuto práci založena na užití primerů a hybridizačních sond podle van Doorna (van Doorn et al., 1998). Pro cagA gen, střední region vacA genu a pro signální úsek vacA genu byly vyvinuty ve spolupráci s TIB – Molbiol GmbH v Berlíně tři real – time PCR assaye. Byly užity následující primery: cagA F (+), cagA R (-), HPMGF (+), HPMGR- (-), VAF1F(+) a VAXR (-) dle Van Doorna a spol. (van Doorn et al., 1998). Hybridizační sondy TaqMan byly vytvořeny pro detekci specifických sekvencí cagA, vacA m1 a vacA m2. Pro cagA esej byly vytvořeny FAM-BBQ

značené sondy (detekce 530nm), pro střední sekvenci vacA FAM-BBQ značené TM sondy pro M1 (530nm) a HEX-BBQ značené TM sondy pro M2 (560nm). TaqMan^{R} real-time PCR eseje byly prováděny na zařízení LightCycler^{R} , verze 2.0 s šestikanálovou detekcí: 530,570,610,640,670 a 705nm. Byl použit komerčně vyráběný LightCycler^{R} TaqMan^{R} MasterMix (Roche Applied Science, Basel.), Schwietzerland) – 15ul MasterMixu včetně primerů a sond a 5ul vzorku izolátu DNA na 20ul kapiláru.

Pro real-time PCR esej k detekci signální sekvence vacA genu byly použity hybridizační sondy S1aLC(LC610), S1bLC(LC640) a S2 LC (LC705) společně s komerčním LightCycler^{R} FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostics, Basel, Switzerland) – 15ul MasterMixu včetně primerů a sond a 5 ul vzorku izolátu DNA na 20ul kapiláru.

Amplifikace o 45 cyklech využívala protikontaminačního systému AmpErase – počáteční denaturace při teplotě 95stC po dobu 10 minut sloužila rovněž k denaturaci enzymu Uracil-N-glykosylázy. Následných 44 cyklů mělo amplifikační profil upravený dle pokynů výrobce primerů a sond. V každém cyklu probíhala detekce signálů v rámci multiplexních rtPCR fluorescenčních vlnových délek. Analýza dat byla provedena pomocí Roche LightCycler software version 3.5.3. Následující dvě tabulky ukazují přehled užitých primerů a sond metodou PCR. Přehled užitých sond a primerů ukazují souhrnně tabulky 2 a 3.

Gen	Typ	Počet nukleotidů	Sekvence	Detekce/nm
cagA	cag_TM	28	6FAM-ATA ACG CTG TCG CTT CAT ACG ATC CTG A–BBQ	530
vacA S	S1a_LC	21	LC Red610-GCR TTR GTC AGC ATC ACA CCG–PH	610
	S1b_LC	21	LC Red640-GCG TTG ATT AGY KCC ATA CCG–PH	640
	S2_LC	21	LC Red705-GCT AAY ACG CCA AAY GAT CCC–PH	705
vacA M	M1_TM	30	6FAM-ACC ACC ATT ACC CGT ATC AAT ACC TTT AAA–BBQ	530
	M2_TM	26	HEX-CTA GTG TTT AGC CCG TTA TCG CTC TT–BBQ	560

Tabulka 2: Přehled užitých sond

Gen	Primer	Počet nukleotidů	Sekvence
cagA	cagA F	24	5-TTG ACC AAC AAC CAC AAA CCG AAG-3
	cagA R	22	5-CTT CCC TTA ATT GCG AGA TTC C-3
VacA S	VA1F	21	5-ATG GAA ATA CAA CAA ACA CAC-3
	VA1R	19	5-CTG CTT GAA TGC GCC AAA C-3
vacA M	HPMGF	21	5-CAG AGC CAC TTT CAA TAA CGA-3
	HPMGR	21	5-CGT CCA AAT AAT TCC AAG GG-3

Tabulka 3: Přehled užitých primerů

3.2.3 Ověření přítomnosti *Helicobacter pylori* – specifické DNA v tkáňových vzorcích

V červnu 2012 se na trhu objevila nová komerční real-time PCR esej pro detekci flagelárního genu (fla genu) Helicobacter pylori – Bioron Real – Line Fla Format (Bioron, Germany). Archivované vzorky pacientských tkání byly rozmraženy a následně testovány výše uvedeným komerčním testem pro srovnání a potvrzení přítomnosti HP – specifické DNA ve tkáňových vzorcích.

3.2.4 Sérologická analýza sérových vzorků

K potvrzení infekce *Helicobacter pylori* u pacientů byla použita sérologická detekce. Sérové vzorky byly testovány kvantitativním komerčním ELISA testem (EIA H. pylori, Test-Line, Czech Republic) na IgG, IgA a IgM protilátky. Stanovení bylo vyjádřeno indexem pozitivity (IP= poměr průměrných absorbancí testovaného séra a cut off) a hodnoceno jako pozitivní pro IP.>1,2. detekci protilátek specifického anti-CagA proteinu byl použit komerční test Helicobacter p120 (CagA) ELISA Test-Line. Testy byly provedeny na Ústavu imunologie a mikrobiologie 1.LF UK a VFN.

4 Výsledky práce

V následujících kapitolách výsledků dizertační práce jsou přehledně shrnuty výsledky všech přiložených publikací.

4.1 Průkaz přítomnosti *Helicobacter pylori* ve tkání Waldeyerova lymfatického okruhu pomocí metody PCR a zhodnocení výskytu její DNA v této oblasti.

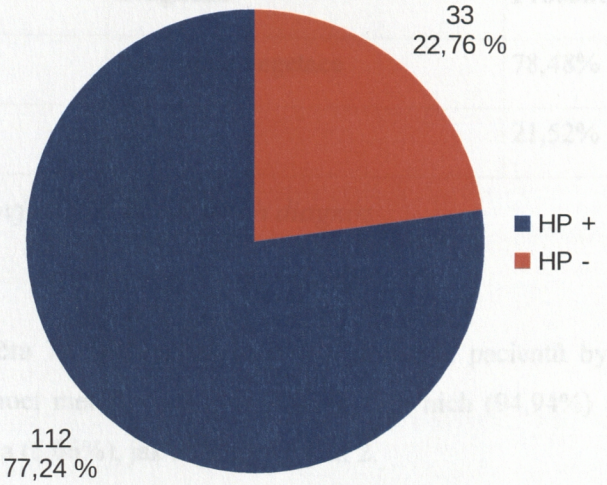
Soubor čtyř přiložených publikací se zabývá detekcí *Helicobacter pylori* ve tkáni Waldeyerova lymfatického okruhu prostřednictvím metody real-time PCR. Práce se zabývají jak skupinou dospělých pacientů (Nártová et al., 2014; Lukeš et al., 2014) tak skupinou pacientů dětských (Kraus et al., 2014; Katra et al., 2014).

Do skupiny dospělých pacientů bylo celkem zařazeno 193 pacientů. Diagnózami v této skupině byly chronická tonzilitida, OSAS (syndrom obstrukční spánkové apnoe) a tonzilární spinocelulární karcinom. Celkem bylo užito k detekci *Helicobacter pylori* metodou real-time PCR 145 vzorků tonzilární tkáně, resp. 80 vzorků tonzil s diagnózou chronické tonzilitidy (55,71%), 42 vzorků tonsil s diagnózou OSAS (28,97%) a 23 vzorků tonzil s diagnózou karcinomu (15,86%). Přehled ukazuje tabulka 4.

Počet vzorků	**Diagnóza**	**Procentuální zastoupení**
80	Chronická tonzilitida	55,17%
42	OSAS	28,97%
23	Karcinom tonzily	15,86%

Tabulka 4:Přehled procentuálního zastoupení diagnóz.

Přítomnost *Helicobacter pylori* byla z celkového počtu vzorků (n=145) prokázána pomocí metody real-time PCR u 112 pacientů (77,24% vzorků), u 33 pacientů (22,76% vzorků) přítomnost *Helicobacter pylori* v tonzilární tkáni prokázána nebyla. Následující graf ukazuje přehledně výskyt *Helicobacter pylori* v tonzilární tkáni u dospělých pacientů.



Graf 1: Výskyt HP v tonzilární tkáni u dospělých pacientů

Následující tabulka 5 pak počet HP pozitivity/negativity v rámci jednotlivých diagnóz.

Typ diagnózy	**HP +**	**HP -**
Chronická tonzilitida	62	18
OSAS	33	9
Karcinom tonzily	17	6

Tabulka 5: Srovnání HP pozitivity/negativity v rámci jednotlivých diagnóz

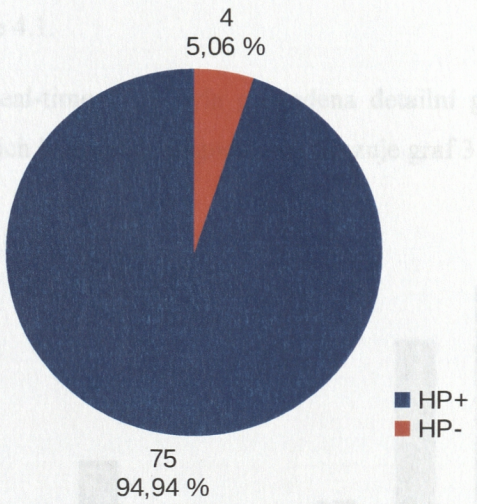
Bylo prokázáno, že DNA *Helicobacter pylori* byla v tonzilární tkáni u dospělých pacientů detekována, *Helicobacter* zde tedy přítomný je, stejně tak, jako v lidském žaludku, pro který je hlavním patogenem.

Do skupiny dětských pacientů (Kraus et al., 2014; Katra et al., 2014) bylo celkem zařazeno 67 dětí. Diagnózami v této skupině byly hypetrofická adenoidní vegetace a OSAS, byly tedy odebírány vzorky adenoidní tkáně a tonzilární tkáně. Celkem bylo odebráno 79 tkáňových vzorků, 62 z adenoidních vegetací (78,48%), 17 pak z tonzilární tkáně při OSAS (21,52%). U 12 dětských pacientů byla odebrána tkáň adenoidní i tonzilární. Přehled ukazuje tabulka 6.

Počet vzorků	Diagnóza	Procentuální zastoupení
62	Adenoidní vegetace	78,48%
17	OSAS	21,52%

Tabulka 6: Počet tkáňových vzorků dětských diagnóz

Z celkového počtu 79 odebraných vzorků u dětských pacientů byla prokázána přítomnost *Helicobacter pylori* pomocí metody real-time PCR u 75 z nich (94,94%) a u 4 vzorků přítomnost bakterie prokázána nebyla (5,06%), jak znázorňuje graf 2.



Graf 2: HP pozitivita / negativita u vzorků dětských pacientů.

Ve skupině vzorků adenoidních vegetací byla pozitivní PCR detekce HP v 59 vzorcích tkáně (n=62), což představuje 95,16%. Ve vzorcích tonzilární tkáně při OSAS byla PCR HP detekce pozitivní v 16 případech, což představuje 94,12%. Přehled ukazuje tabulka 7.

Typ diagnózy	HP +	HP -
Adenoidní vegetace	59	3
OSAS	16	1

Tabulka 7: HP pozitivita/HP negativita u vzorků dětských pacientů

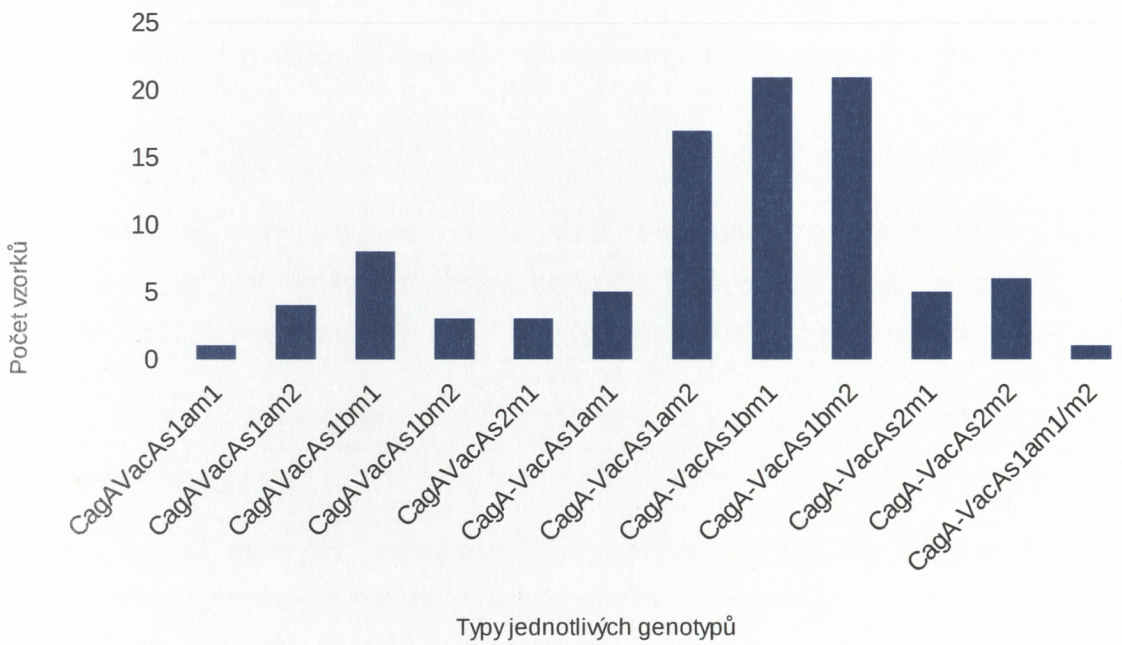
Tyto výsledky prokazují, že stejně tak jako u skupiny dospělých, tak i u skupiny dětských pacientů se ve tkáni Waldeyerova lymfatického okruhu DNA *Helicobacter pylori* nachází.

4.2 Popis genotypových rozdílů *Helicobacter pylori* a porovnání jednotlivých genotypů v tkáňových vzorcích patrových tonzil a adenoidních vegetací v rámci benigních a maligních onemocnění této oblasti.

4.2.1 Benigní diagnózy

Ve skupině dospělých pacientů byly zkoumány typy benigních diagnóz: chronická tonzilitida a OSAS. Ve skupině dětských pacientů byly pak benigními diagnózami adenoidní vegetace a OSAS. Výskyt HP pozitivity/negativity u jednotlivých diagnóz dospělých a dětských pacientů ukazují výše uvedené tabulky v kapitole 4.1.

Pomocí metody real-time PCR byla provedena detailní genotypizace kmenů *Helicobacter pylori* ve tkáňových vzorcích benigních diagnóz, což ukazuje graf 3.



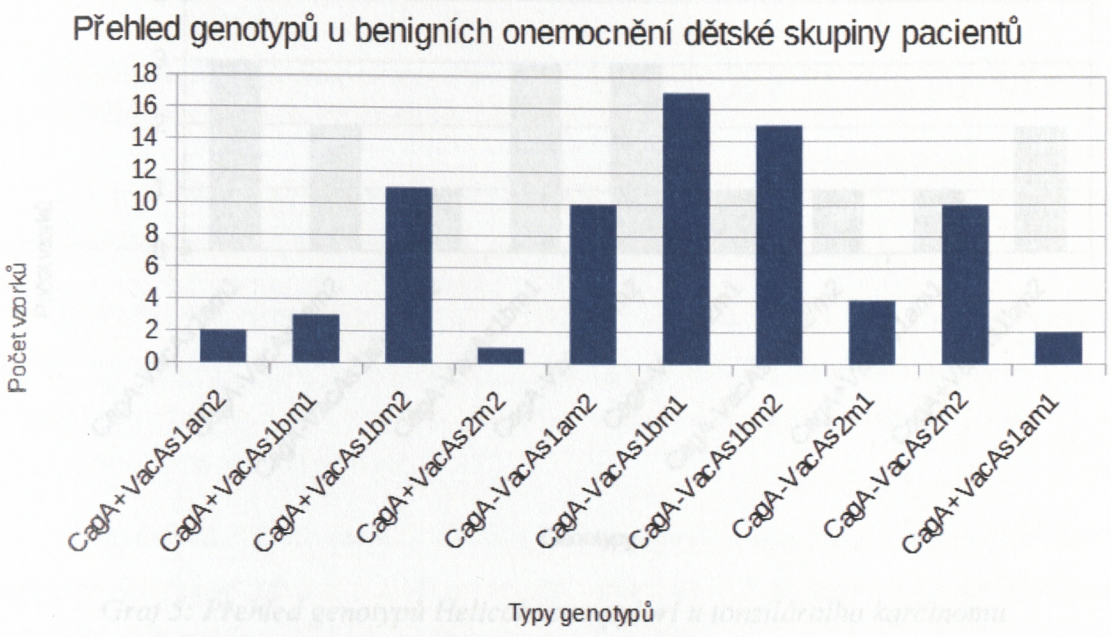
Graf 3: Přehled genotypů u benigních onemocnění dospělé skupiny pacientů

Z daného grafu vyplývá, že nejčastějším genotypem u benigního typu onemocnění (chronická tonsilitida a OSAS) u dospělých pacientů je genotyp **CagA-VacAs1bm1** a genotyp **CagA-**

VacAs1bm2, dalším nejčastějším genotypem u skupiny benigních onemocnění je genotyp **CagA-VacAs1am2.**

Tato analýza zjistila, že u benigních onemocnění dospělé skupiny pacientů převažuje VacA pozitivita nad CagA pozitivitou.

Přehled genotypů u benigních onemocněních dětské skupiny pacientů ukazuje graf 4.



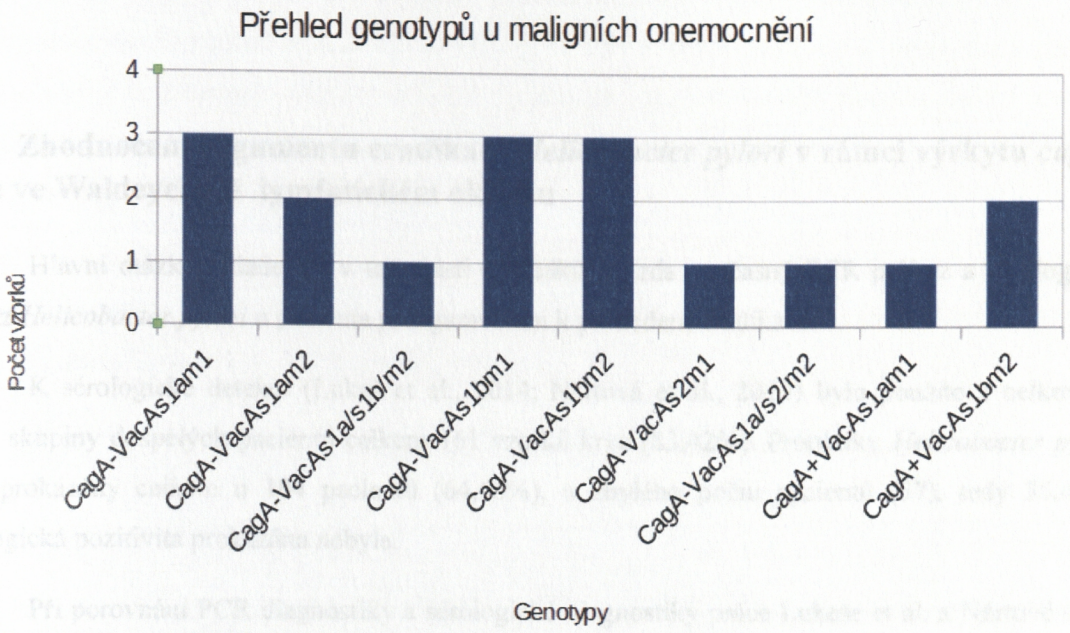
Graf 4: Přehled genotypů u benigních onemocnění dětské skupiny pacientů

Nejčastějším typem genotypu u dětské skupiny benigních onemocnění (adenoidní vegetace, OSAS) je genotyp **CagA-VacAs1bm1**, druhým nejčastějším pak genotyp **CagA-VacAs1bm2**. Lze tak říci, že u benigních onemocnění dětské skupiny opět převažuje pozitivita VacA genu nad CagA genem.

4.2.2 Maligní diagnózy

Do skupiny maligních onemocnění byly zařazeny tonzilární karcinomy (Lukeš et al., 2014).Počet vzorků tonzilárních karcinomů ukazuje tabulka v kapitole 4.1.

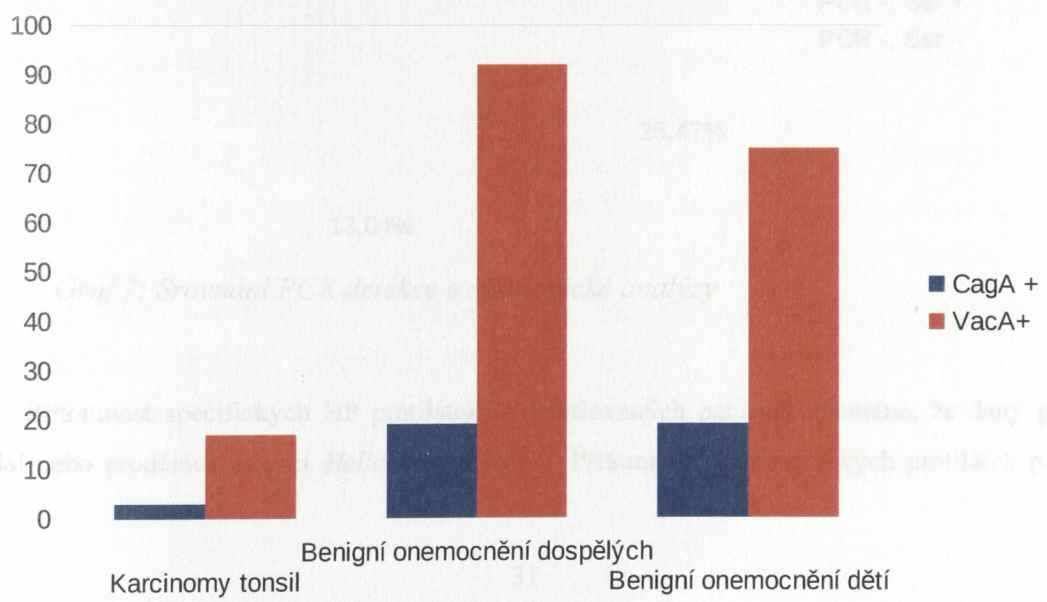
Nejčastějším typem genotypu u tonzilárního karcinomu byl genotyp **CagA-VacAs1bm1**, druhým nejčastějším pak genotyp **CagA-VacAs1bm2**, což znázorňuje graf 5. Stejně tak jako u skupiny benigních onemocnění, převažuje i zde pozitivita VacA genu.



Graf 5: Přehled genotypů Helicobacter pylori u tonzilárního karcinomu

4.3 Porovnání zastoupení *cagA* genů a *vacA* genů ve tkáni Waldeyerova lymfatického okruhu

Níže uvedený graf 6 dokumentuje, že celkově převyšuje zastoupení VacA genotypů v PCR pozitivních vzorcích. Nejvíce pak u skupiny benigních onemocnění dospělých (90%), nejméně u skupiny karcinomu tonzily (15%).



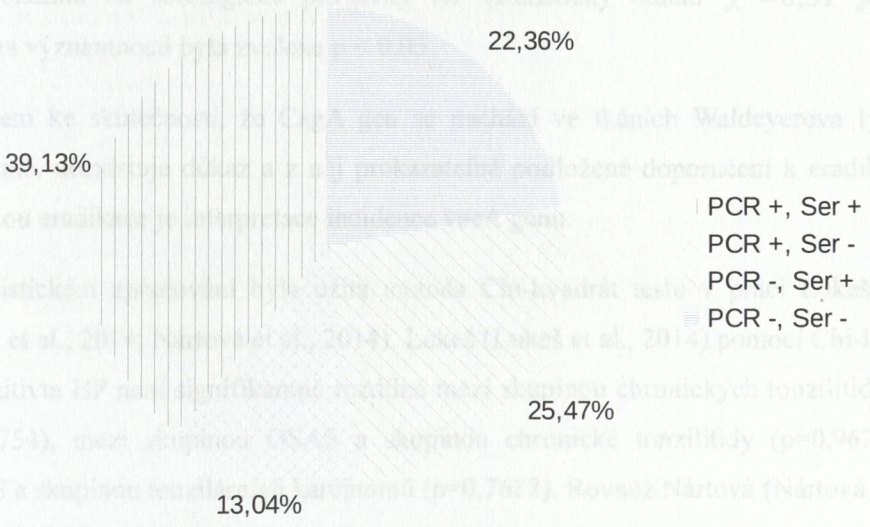
Graf 6: Porovnání zastoupení CagA pozitivity/VacA pozitivity ve tkáni Waldeyerova lymfatického okruhu

4.4 Zhodnocení argumentu eradikace *Helicobacter pylori* v rámci výskytu *cagA* genu ve Waldeyerově lymfatickém okruhu

Hlavní otázkou kladenou v teto části výsledků je, zda současný PCR průkaz a sérologický průkaz *Helicobacter pylori* u pacienta je argumentem k provedení eradikace.

K sérologické detekci (Lukeš et al., 2014; Nártová et al., 2013) bylo použito z celkového počtu skupiny dospělých pacientů celkem 161 vzorků krve (83,42%). Protilátky *Helicobacter pylori* byly prokázány celkem u 104 pacientů (64,60%), u zbylého počtu pacientů (57), tedy 35,40%, sérologická pozitivita prokázána nebyla.

Při porovnání PCR diagnostiky a sérologické diagnostiky práce Lukeše et al. a Nártové et al. (Lukeš et al., 2014; Nártová et al., 2014) popsaly, že celkem 63 PCR pozitivních vzorků bylo zároveň sérologicky pozitivních, 21 PCR pozitivních vzorků bylo sérologicky negativních, 41 PCR negativních vzorků bylo pozitivních sérologicky a 36 PCR negativních vzorků bylo rovněž sérologicky negativních. Uvedené údaje shrnuje graf 7.



Graf 7: Srovnání PCR detekce a sérologické analýzy

Přítomnost specifických HP protilátek u vyšetřovaných pacientů znamená, že daný pacient prodělal nebo prodělává infekci *Helicobacter pylori*. Přítomnost anamnestických protilátek pak pro

aktivní infekci HP nesvědčí. V případě přítomnosti IgA, IgM imunoglobulinů je vysoká pravděpodobnost, že pacient infekci HP prodělal, ale také v jiné oblasti než v orofaryngu, např. v žaludku a oblast patrových tonzil tak kolonizována nebyla. Lze rovněž říci, že nulová detekce protilátek neznamená, že *Helicobacter pylori* není v tonzilách přítomen. Tento fakt zdůrazňuje vysokou sensitivitu PCR metody v detekci *Helicobacter pylori.*

Detekce přítomnosti *Helicobacter pylori* oběma metodami ukázalo přehledově konkordanci u 99 vzorků (61,49%) a diskordanci u 62 vyšetřovaných vzorků (38,51%). 41 diskordantních výsledků bylo sérologicky pozitivních a PCR negativních.

K porovnání metod PCR a sérologie byl užit Chi-kvadrát test:

	Ser +	Ser -	Suma
PCR +	63	21	84
PCR -	41	36	77
Suma	104	57	161

Tabulka 8: Porovnání PCR a serologie

Na základě χ^2 testu lze konstatovat, že PCR pozitivita HP ve vyšetřovaných vzorcích je signifikantně rozdílná od sérologické pozitivity HP (statistický odhad $\hat{\chi}^2 = 8{,}31$ je větší než kvantil). Hladina významnosti byla zvolena $p < 0{,}05$.

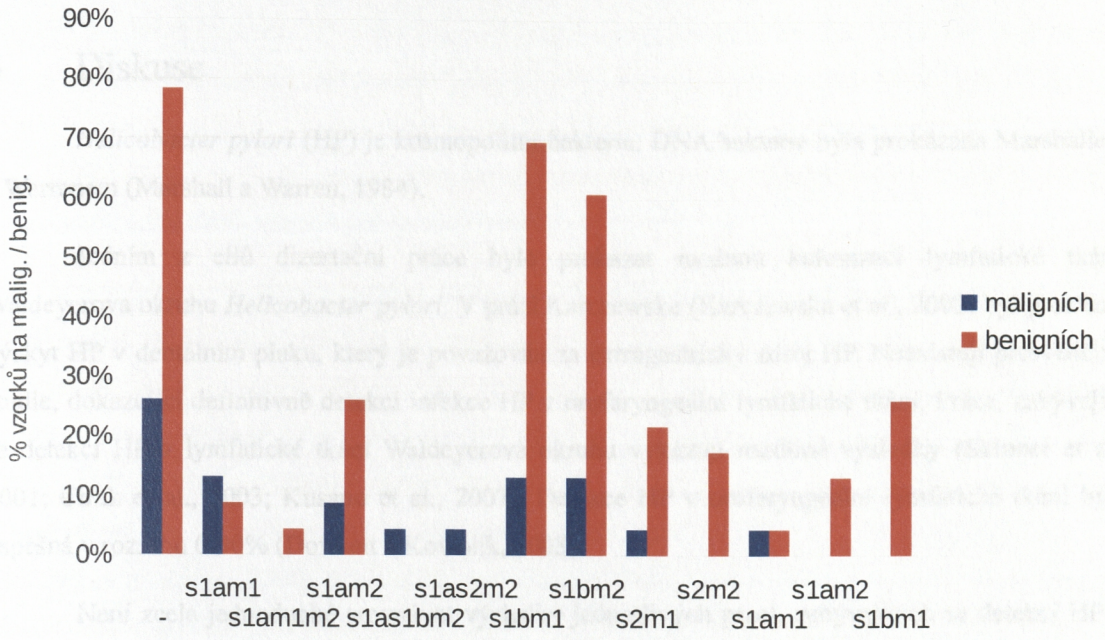
Vzhledem ke skutečnosti, že CagA gen se nachází ve tkáních Waldeyerova lymfatického okruhu v menšině, neexistuje důkaz a z něj prokazatelně podložené doporučení k eradikaci daných pacientů. Otázkou eradikace je interpretace incidence vacA genu.

Ve statistickém zpracování byla užita metoda Chi-kvadrát testu v práci Lukeše a v práci Nártové (Lukeš et al., 2014; Nártová et al., 2014). Lukeš (Lukeš et al., 2014) pomocí Chi-kvadrát testu dokázal, že pozitivta HP není signifikantně rozdílná mezi skupinou chronických tonzilitid a skupinou tumorů (p=0,7754), mezi skupinou OSAS a skupinou chronické tonzilitidy (p=0,9625) či mezi skupinou OSAS a skupinou tonzilárních karcinomů (p=0,7632). Rovněž Nártová (Nártová et al., 2014) prokázala pomocí Chi-kvadrát testu, že prevalence HP není signifikantně rozdílná u skupiny pacientů s chronickou tonsilitidou a OSAS (p=0,76), stejně tak, že není signifikantní rozdíl ve výskytu cagA genu mezi těmito dvěma skupinami pacientů (p=0,69) při p<0,05.

Pro jednotlivé typy genotypů byl spočítán počet vzorků tkáně chronické tonzilitidy a karcinomu tonzily. Na základě těchto hodnot byl vypočten **Pearsonův korelační koeficient**, který má hodnotu **0,59**. Na základě této hodnoty lze říci, že nebyla zjištěna signifikantní korelace mezi konkrétním typem genotypu a chronickým zánětem/karcinomem tonzily. (R =0,59, úplna korelace =1).

			maligni	benigni
cagA-	VacA-	-	6	18
	VacA+	s1am1	3	2
		s1am1m2	0	1
		s1am2	2	6
		s1as1bm2	1	0
		s1as2m2	1	0
		s1bm1	3	16
		s1bm2	3	14
		s2m1	1	5
		s2m2	0	4
cagA+	VacA+	s1am1	1	1
		s1am2	0	3
		s1bm1	0	6
		s1bm2	2	3
		s2m1	0	1
Celkem:			23	80

Tabulka 9: Počty jednotlivých genotypů u chronické tonzilitidy a tonzilárního karcinomu



Graf 8: Procentuální zastoupení jednotlivých genotypů u chronické tonsilitidy a tonzilárního karcinomu

Kultivace byla odebrána u 45 dospělých pacientů (11 pacientů s karcinomem tonzily, 22 s chronickou tonzilitidou a 12 pacientů se SAS), z čehož byl *Helicobacter pylori* prokázán pouze u 3 (6,67%).

5 Diskuse

Helicobacter pylori (HP) je kosmopolitní bakterie. DNA bakterie byla prokázána Marshallem a Warrenem (Marshall a Warren, 1984).

Jedním z cílů dizertační práce bylo prokázat možnou kolonizaci lymfatické tkáně Waldeyerova okruhu *Helicobacter pylori.* V práci Karczewske (Karczewska et al., 2002) byl prokázán výskyt HP v dentálním plaku, který je považován za extragastrický zdroj HP. Neexistují přesvědčivé studie, dokazující definitivně detekci infekce HP v orofaryngeální lymfatické tkání. Práce, zabývající se detekcí HP v lymfatické tkání Waldeyerova okruhu vykazují rozdílné výsledky (Skinner et al., 2001; Cirak et al., 2003; Kusano et al., 2007). Detekce HP v orofaryngeální lymfatické tkáni byla úspěšná v rozsahu 0-90% (Dowsett a Kowolik, 2003).

Není zcela jednoduché srovnávat výsledky jednotlivých prací, zabývajících se detekcí HP v lymfatické tkání Waldeyerova okruhu, neboť autoři každé práce užili k průkazu přítomnosti bakterie odlišné diagnostické metody, jak ukazuje tabulka v literárním úvodu práce. Obecně lze říci, že autoři využívají k průkazu jak metod invazivních, tak metod neinvazivních. Často užívanou invazivní metodou je RUT (rychlý ureázový test) a CLO test (campylobacter – like test). Tyto testy byly užity např. V práci Skinnera z roku 2001 (Skinner et al., 2001), který pomocí CLO testu detekoval z 50 vzorků tonzilární tkáně HP v 0% případů. Pozitivitu HP v tonzilární tkání pomocí CLO testu neprokázal ani Yilmaz v roce 2004 (Yilmaz et al., 2004). Naopak Bitar prokázal v roce 2005 pozitivitu v adenodní tkáni v 84% případů (Bitar et al., 2005). Vayisoglu pak v roce 2008 prokázal pozitivitu HP v lymfatické tkáni Waldeyerova okruhu pomocí RUT ve 2 vzorcích (Vayisoglu et al., 2008). Větší úspěch při použití RUT při detekci HP v této oblasti měl Abdel – Monem v roce 2011, který prokázal celkem 16 pozitivních vzorků (Abdel-Monem et al., 2011).

V našich pracích metoda RUT využita nebyla. Důvodem byl poměrně nízký záchyt pozitivity HP touto metodou v předchozích studiích a také předpoklad, že oblast lymfatické tkáně orofaryngu je osídlena také jinými bakteriálními kmeny, které produkují ureázu a mohou tak HP inhibovat. Testy pak vykazují falešně pozitivní výsledky (Dowsett a Kowolik, 2003). Metoda je proto vhodnější pro detekci žaludečních vzorků. Midolo zdůrazňuje v roce 2000 sensitivitu této metody 93% a specificitu 98% (Midolo a Marshall, 2000).

Metodou kultivační se ve své práci zabývali Lukeš et al. (Lukeš et al., 2014). Ve své práci prokázali kultivační metodou celkem 3 pozitivní vzorky ze 45. Hlavním argumentem je možné ovlivnění kultivační detekce externími vlivy (Dowsett a Kowolik, 2003) či přerůstání HP jinými kmeny. Přímou inhibici orálními kmeny in vitro popisuje ve své práci Ishihara v roce 1997 (Ishihara et

al., 1997). Možná je rovněž transformace v kokoidní formy, které nejsou kultivovatelné (Shahamat et al., 2004). Rovněž Yilmaz užil v roce 2006 ve své práci kultivační průkaz HP v lymfatické tkáni Waldeyerova okruhu, kdy celkem 11 adenoidních a 12 tonzilárních vzorků bylo pozitivních (Yilmaz et al., 2006).Obecně je kultivace považována za zlatý standard v detekci žaludeční HP s 80-90% sensitivitou a 90-100% specificitou (Makristathis et al., 2004).

Z metod neinvazivních jsme v našich pracích užili sérologický průkaz specifických protilátek HP (Lukeš et al., 2014; Nártová et al., 2014). K sérologické detekci bylo použito z celkového počtu skupiny dospělých pacientů celkem 161 vzorků krve (83,42%). Protilátky proti *Helicobacter pylori* byly prokázány celkem u 104 pacientů (64,60%), u zbylého počtu pacientů (57), tedy 35,40%, sérologická pozitivita prokázána nebyla. Obecně lze říci, že sérologická pozitivita nevypovídá o současné probíhající infekci HP a není určujícím faktorem pro zahájení antibiotické terapie HP (Testerman a Morris, 2014). Sérologický průkaz protilátek HP má klinický význam především pro dlouhodobé sledování po léčbě a pro monitorování úspěchu eradikace HP. Protilátky IgG v séru často přetrvávají až půl roku po prodělané infekci HP. Lukeš detekoval v lymfatické orofaryngeální tkáni 12 PCR pozitivních vzorků bez současného průkazu protilátek u daných pacientů. Možným vysvětlením je časná detekce HP, následující brzy po primární infekci, kdy imunitní systém ještě neprodukuje protilátky v nadbytku. Pro toto svědčí i fakt, že 9 z 12 lidí v Lukešově práci bez průkazu protilátek bylo PCR pozitivní pro méně virulentní genotypy HP s1/m2 v lymfatické tkáni orofaryngu. Důležitým faktem může být ale také skutečnost, že HP se v lymfatické tkáni orofaryngu mohl nacházet v kokoidních formách, dlouhodobě přežívajících. Podobně jako Lukeš se sérologií HP v oblasti orofaryngu zabýval Bürgers v roce 2008, který detekoval HP ve slinách bez průkazu protilátek (Bürgers et al., 2008).

V našich pracích jsme jako hlavní detekční metodu užili metodu invazivní molekulární – PCR, resp, real-time PCR. Jedná se o metodu s nejvyšší sensitivitou a specificitou 100% (Testerman a Morris, 2014). V experimentech bylo užito mnoho variací PCR s detekcí 0-90% (Dowsett a Kowolik, 2003). Různorodost spočívala v užití odlišných typů primerů a sond k detekci různých segmentů DNA HP, např primery po ureázový gen, primery pro 16s ribosomální RNA gen, 23S ribosomální RNA gen (Yilmaz et al., 2006; Abdel-Monem et al., 2011).Specificita a sensitivita primerů je velmi odlišná (Song et al., 1999). Genotypizace umožňuje rozlišit přítomné geny HP, kódující virulentní faktory a rozlišit tak vysoce virulentní a méně virulentní kmeny (Pavlík et al., 2007). Diskrepance mezi publikovanými PCR výsledky pak ukazuje na důležitost výběru vhodné PCR assaye.

V souboru přiložených prací jsme prokázali přítomnost DNA v lymfatické tkání Waldeyerova okruhu, resp. tonzilární a adenoidní tkáni. Prokázali jsme rovněž přítomnost vysoce virulentního cagA genu a méně virulentního vacA genu. Z celkového počtu vzorků lymfatické tkáně u dospělých

pacientů jsme prostřednictvím PCR metody prokázali přítomnost u 112 z nich (77,24%), v lymfatické tkáni Waldeyerova okruhu u dětí pak z celkového počti 79 vzorků u 75 z nich (94,94%). Jednoznačně lze tedy říci, že lymfatická tkáň Waldeyerova okruhu je extragastrickým rezervoárem HP. Toto tvrzení podporuje i práce Pavlíka z roku 2015(Pavlik et al., 2015) , oproti tomu práce Aliakbari z roku 2011 toto tvrzení zpochybňuje (Aliakbari et al., 2011).

Metodu PCR k detekci HP v lymfatické tkání Waldeyerova okruhu užila ve svých pracích řada autorů, jejichž výsledky jsou v souladu či v rozporu s našimi daty. Bonaventura v roce 2000 zpochybnil tonzilární tkáň jako extragastrický rezervoár HP a ve své práci detekoval 0 pozitivních vzorků metodou PCR (Bonaventura et al., 2001). Studie Ciraka z roku 2003 naopak zachycuje z 23 adenotonsilárních vzorků PCR pozitivitu v 7 (30%), z toho cagA pozitivitu u 5 vzorků. Stejně tak Bukut v roce 2005 prokázal z celkem 71 vzorků PCR pozitivitu u 29 z nich, v 17 z těchto pak cagA pozitivitu, což podporuje naše výsledky. Podpořil rovněž stejně jako naše práce možnou roli HP v rekurentních adenotonzilitidách a adenotonzilární hypertrofii (Bulut et al., 2006). Oproti tomu Bitar v roce 2005 detekoval 0 pozitivních vzorků metodou nested PCR (Bitar et al., 2005). V souladu s našemi pracemi je studie Yilmaze z roku 2006 (Yilmaz et al., 2006) a studie Kusana z roku 2007 (Kusano et al., 2007). Potenciální vliv HP na vznik chronické adenotonzilitidy u dětí byl zpochybněn prací Vayisogla z roku 2008 (Vayisoglu et al., 2008). Naše výsledky naopak podporuje práce Mogghadama z roku 2009 (Jabbari Moghaddam et al., 2009). Abdel- Monem pak detekoval ve své práci ureázový gen u 5 tkáňových vzorků (Abdel-Monem et al., 2011). Farivan a Najafipour detekovali užitím scorpion PCR vysoký výskyt HP v lymfatické tkáni u pacientů s chronickou tonzilitidou (Farivar et al., 2012; Najafipour et al., 2012). Práce Cedeno z roku 2016 nepotvrdila pozitivitu HP v adenoidní tkáni pomocí metody PCR. Hwang ve své metaanalýze z roku 2016 zcela popírá roli HP při vzniku chronické tonsilitidy (Hwang et al., 2015). Kaymakçı v roce 2015 vyhodnotil celkem z 23 vzorků adenotonzilární tkáně u dětí pomocí metody PCR 2 pozitivní vzorky. Připustil však fakt, že role HP ve vzniku hypertrofie adenoidní tkáně vyžaduje ještě další studie (Kaymakçı, 2014). Bayindir pak v roce 2015 detekoval pozitivní vzorky adenotonzilární tkáně pomocí metody PCR a připustil možnou roli HP při vzniku chronické adenotonzillitidy v endemických oblastech (Bayindir et al., 2015). Oproti většině prací detekovali ve svých studiích Kraus i Katra vysoké množství PCR pozitivních tkání Waldeyerova lymfatického okruhu na přítomnost HP (Kraus et al., 2014; Katra et al., 2014).

Dizertační práce ukazuje na zvýšený výskyt méně virulentních genotypů HP v lymfatické tkání Waldeyerova okruhu, tedy genotypů cagA negativních, vacA pozitivních.

Analýza genotypů HP v tonzilární tkáni u dospělých jedinců v dizertační práci ukazuje na zvýšený výskyt s1/m2 alel vacA genu a s1/m1 alel vacA genu. CagA gen byl nalezen pouze v malém

počtu případů, v práci Lukeše v 5 tkáňových vzorcích, v práci Nártové v 17 tkáňových vzorcích. Přehledný výskyt vacA a cagA genotypů ukazuje v kapitole Výsledky graf 5.

Tento nález vypovídá o tom, že méně virulentní kmeny HP mají v lymfatické tonzilární tkáni u dospělých jedinců v našich studiích dominanci. Nález genotypu s2/m1 byl v naší studii minimální, v literatuře byl publikován pouze v ojedinělých studiích (Letley et al., 1999). Na základě srovnání našich výsledků a výsledků jiných studií můžeme říci, že lymfatická tkáň Waldeyerova okruhu je extragastrickým rezervoárem HP. Naše data podporují vysokou sensitivitu a specificitu zvolené modifikace PCR metody. Rovněž podporují hypotézu, že HP je potenciálním etiologickým faktorem při patogenezi chronické tonzilitidy, neboť ve vzorcích detekovaných pacientů s touto diagnózou se ukázala vysoká přítomnost genotypů vacA s1m1.

Zajímavým faktem je přebytek cagA negativních genotypů v lymfatické tkáni Waldeyerova okruhu, což vede k úvaze, že CagA protein, jakožto hlavní virulentní faktor v žaludku, nehraje v oblasti lymfatické tkáně orofaryngu a epifaryngu důležitou infekční roli. Naopak dlouhodobá kolonizace cagA negativními kmeny HP v této oblasti a tedy potenciální pomalá alterací imunitního systému může vést ke vzniku chronického zánětu a v pozdější fází k iniciaci kancerogeneze. Tuto teorii popírá práce Aliakbari (Aliakbari et al., 2011), rovněž tak metaanalýza Hwanga z roku 2015 (Hwang et al., 2015).

Práce Katry a Krause rovněž prokázaly přítomnost HP v dětské adenoidní tkáni a v dětské tonzilární tkáni (Katra et al., 2014; Kraus et al., 2014). Hlavními genotypy u dětí byly stejně tak jako u dospělé skupiny pacientů s1m1 a s1 m2. Dle našich výsledků lze tedy i adenotonzilární tkáň u dětí považovat za extragastrický rezervoár HP.

V rámci tonzilárního karcinomu byl nejčastějším genotypem také s1m1/ s1m2, stejně jako u skupiny chronických tonsilitid. Tento fakt přispívá k úvaze vzájemného vztahu chronické tonsilitidy jako prekancerózy k malignímu onemocnění.

Naše práce si rovněž pokládá otázku významu eradikace u pacientů s nálezem HP v lymfatické tkání Waldeyerova okruhu, zvláště pak u pacientů s chronickou tonzilitidou. I přes významně vyšší výskyt cagA negativních kmenů by eradikace mohla bránit rozvoji dalších patologií. Teorii, že HP je rezidentem lymfatické tkáně Waldeyerova okruhu podporuje práce Pavlíka (Pavlik et al., 2015), která rovněž připouští možnou transformaci v maligní onemocnění a tedy nepřímo podporuje prospěšnost eradikace. Skutečnost, že HP je primárním kolonizátorem lymfatické tkáně Waldeyerova okruhu podporuje práce Lukeše z roku 2010, kdy byly nalezeny odlišné genotypy HP v orofaryngeální lymfatické tkáni a v žaludku u 6 jedinců (Katra et al., 2014). Tento fakt opět přispívá k provádění eradikace u pacientů s pozitivitou HP v lymfatické tkání Waldeyerova okruhu.

Otázka prospěšnosti eradikace však plně doposud zodpovězená není a je třeba, aby byla předmětem dalších studií. Z ekonomického hlediska by antibiotická terapie byla mnohonásobně výhodnější, než následná ORL - chirurgická a onkologická léčba pacientů. Je však nutno získat silnější data a podpořit je statisticky.

6 Závěr

Uložené cíle dizertační práce byly splněny.

V lymfatické tkání Waldeyerova okruhu, resp. v tonzilární a adenoidní tkáni dospělých a dětských pacientů, jsme pomocí užší modifikace metody PCR (real-time PCR) prokázali přítomnost DNA HP. Provedli jsme následnou genotypizaci tkáňových vzorků a vyhodnotili jsme tak zastoupení jednotlivých genotypů HP v avízované oblasti. Byl zkoumán jejich etiologický vliv na vznik benigních i maligních onemocnění ve Waldeyerově okruhu.

Oblast Waldeyerova okruhu lze dle našich dat považovat za extragastrický rezervoár HP u dospělých i dětí. Přítomnost HP není závislá na typu oro/epifaryngeálního onemocnění a je pozitivní jak u skupiny benigních, tak u skupiny maligních onemocnění.

Genotypizací byly zjištěny rozdíly mezi oro/epifaryngeálními kmeny a kmeny žaludečními, respektive byla zjištěna nízká přítomnost cagA pozitivních kmenů. U pacientů s karcinomem tonzily byl rovněž zjištěn vysoký výskyt vacA pozitivních, tedy méně virulentních kmenů.

Kultivační průkaz byl prokázán pouze ve třech případech a sérologická pozitivita rovněž nebyla prokázana u všech pacientů. Tento fakt vyzdvihuje vysokou sensitivitu a specificitu použité PCR metody.

Hypotéza č. 1 byla na základě výsledků potvrzena. DNA HP se nachází v lymfatické tkáni Waldeyerova okruhu u dospělé skupiny pacientů v 77,24% vzorků a u skupiny dětí v 94,94% vzorků. Výskyt tak lze považovat za vysoký.

Hypotéza č.2, eradikace u pacientů s PCR pozitivitou a zároveň sérologickou pozitivitou je indikována, **nebyla v našich pracích potvrzena, je předmětem dalších studií a nelze ji tedy přijmout.** PCR pozitivita a zároveň pozitivita protilátek se ukázala v 39,13% případů, PCR pozivita a sérologická negativita pak u 13,04%, 25,47% bylo PCR negativních a sérologicky pozitivních, zbytek (22,36%) bylo negativních sérologicky i PCR. Metoda sérologická není jednoznačně vypovídající o tom, zda se jedná o recentní či již proběhlou infekci, neboť HP protilátky se nevytváří ihned po vzniku primární infekce a zároveň přetrvávají v organismu poměrně dlouho. V některých případech může být sérologie negativní v důsledku přítomnosti málo virulentních kmenů, které neiniciují tvorbu protilátek či v důsledku výskytu kokoidních forem, což by i potvrzovalo nízkou kultivační výtěžnost v naší práci.

Hypotéza č. 3, tedy otázka existence korelace mezi konkrétními typy genotypů a chronickým zánětem či maligním onemocněním lymfatické tkáně Waldeyerova okruhu, **potvrzena nebyla.**

Pro jednotlivé typy genotypů byl spočítán počet vzorků tkáně chronické tonzilitidy a karcinomu tonzily. Na základě těchto hodnot byl vypočten **Pearsonův korelační koeficient**, který má hodnotu **0,59**. Na základě této hodnoty lze říci, že nebyla zjištěna signifikantní korelace mezi konkrétním typem genotypu a chronickým zánětem/karcinomem tonzily. (R =0,59, úplna korelace =1).

7 Příloha

7.1 Publikace *in extenso*, které jsou podkladem dizertační práce

7.1.1 Publikace s IF, které jsou podkladem dizertační práce

1. **Nártová, E.**, Kraus J., Pavlík E., Lukeš P., Katra, R., Plzák, J., Kolářová L., Šterzl I., Betka J., Astl J.: presence of different genotypes of Helicobacter pylori in patients with chronic tonsillitis and sleep apnoea syndrome, Eur Arch Otorhinolaryngol, 2014, 271:607–613 **(IF 1,458)**
2. Lukeš P., Pavlík E., Potužníková B., **Nártová E.** et al.: Detection of Helicobacter pylori in oropharyngeal lymphatic tissue with real – time PCR and asessment of its carcinogenic potencial, Eur Arch Otorhinolaryngol, 2014, 271:399–405 **(IF 1,458)**
3. Kraus, J., **Nártová E.,** Pavlík E., Katra, R. et al.: Prevalence of Helicobacter pylori in adenotonsillar hypertrophy in children, Acta Otolaryngol, 2014, 134: 88-92 **(IF 0,99)**
4. Katra, R., Kabelka, Z., Jurovčík M., Hradský, O., Kraus, J., Pavlík, E., **Nártová, E.** et al.: Helicobacter pylori in adenoid hyperplasia and reflux episodes detected by multiple intralunimanl impedance in children, Int J Pediatr Otorhinolaryngol, 2014, 78: 1243–1249 **(IF 1,447)**

7.1.2 Publikace bez IF

1. **Nártová E.**, Lukeš P., Pavlík E, Šterzl I., Astl J., Betka J., The presence of Helicobacter pylori in oropharynx and its influence to the gastric infection, Otorinolaryng. a Foniatr. (Prague), 58, 2009, č. 2, s. 97-101.
2. Lukeš P., Pavlík E., Potužníková B, Plzák J, **Nártová E**, Doseděl J, Katra R, Sterzl I, Betka J, Astl J.: Comparison of Helicobacter pylori genotypes obtained from the oropharynx and stomach of the same individuals - a pilot study, Prague Med Rep. 2012;113(3):231-9.
3. **Nártová, E.**, Astl, J., Lukeš,P., Katra,P., Pavlík,E., Betka,J.: Helicobacter pylori a jeho úloha v patogenezi a patologii orofaryngu a epifaryngu ve vztahu k ORL onemocněním, Otorinolaryng a Foniatr. (Prague), 61, 2012, č. 2, s. 120-129.
4. Pavlík, E., **Nártová, E.,** Astl, J. et al.: Detection of Helicobacter pylori and Human Papillomavirus in Peroperative Tissue Biopsies Collected from Malignancies in Oropharyngeal Area, AJCEM (American Journal of Clinical and Experimenatl Medicine), 2015; 3(6): 364 – 367.

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9 Obsah přílohy

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Příloha 1

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MISCELLANEOUS

Presence of different genotypes of *Helicobacter pylori* in patients with chronic tonsillitis and sleep apnoea syndrome

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Abstract *Helicobacter pylori*, a well-known gastric pathogen, has been detected in the oral cavity and oropharynx in tonsillar tissue. In our study, the presence of *H. pylori* in the tonsillar tissue of patients with chronic tonsillitis and sleep apnoea syndrome (SAS) was investigated. The aim was to detect and genotype *H. pylori* for a collection of data supporting the possible role of *H. pylori* in the aetiology of chronic tonsillitis and SAS. *Helicobacter pylori* was detected by real-time polymerase chain reaction (rt-PCR). 89 patients, 60 with a diagnosis of chronic tonsillitis and 29 with SAS, were tested. In the chronic tonsillitis group, *Helicobacter* was detected in 48 (80 %) specimens, cagA gene was detected in 12 samples (25 %) and 12 samples were negative. In SAS group, *Helicobacter* was found in 24 samples (82.76 %), cagA gene was detected in 5 (20.83 %) and 5 samples (17.24 %) were negative. *Helicobacter pylori*-specific immunoglobulins were tested by ELISA in the serum of 57 patients only with 41 (71.93 %) showing positive. Our results on *H. pylori* DNA detection and *H. pylori* seropositivity show 26.32 % discrepancy, slightly in favour of rt-PCR (15.79 % compared to 10.53 %). The *H. pylori* presence in tonsillar tissue does not depend on the type of oropharyngeal disease ($p = 0.756$). This study shows that oropharynx constitutes an extragastric reservoir of *H. pylori* infection which could serve as an aetiopathogenetic factor for chronic tonsillitis and tonsillar hyperplasia by SAS. No conclusion has yet been drawn about the mechanism of the process.

Keywords *Helicobacter pylori* · Tonsillar tissue · Sleep apnoea syndrome · Real-time PCR · Hybridisation probes

Introduction

Helicobacter pylori is gram-negative, microaerophilic, spiral, and cosmopolitan bacterium. It has been associated

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with common gastric diseases, especially with chronic and active gastritis and peptic ulcer disease and, in some cases, also with gastric cancer [1]. The prevalence in Central and Eastern Europe is estimated from 60 to 95 % [2]. Clinical manifestation appears in only 10–15 % of infected individuals. Although much information on its epidemiology is known, the mechanism of the transmission remains unclear. There are three routes of transmission: oral–oral, faecal–oral and gastric–oral [3]. The stomach was supposed to be the only reservoir of infection in humans.

The presence of *H. pylori* infection in oropharyngeal lymphatic tissue has been extensively studied in recent years. This bacterium was found in dental plaque and saliva [8–11]. As a result, the oral cavity has been suggested to be a reservoir for *H. pylori* infection. Mucosa associated lymphoid tissue in the stomach is similar to lymphoid tissue of the Waldeyer Ring [12]. Several studies have explored the presence of *H. pylori* in tonsillar and adenoid tissue using different types of detection [3, 12–18].

The most important virulence factors associated with gastric pathogenesis are CagA protein (cytotoxin) and VacA (vacuolating cytotoxin A). Genes encoding virulence factors of *H. pylori* are grouped in the cagPAI (pathogenicity island) region and some of them encode a type IV bacterial secretion apparatus. This apparatus translocates CagA into host cells in gastric mucosa [4]. The CagA protein stimulates cell signalling through the interaction with several host proteins. This interaction leads to an increased release of cytokines and regulatory molecule production [5].

VacA is also an important virulence factor as it causes cytoplasmic vacuolation [6]. There are differences among *H. pylori* strains in the structure of VacA [7]. Two types of signal regions are known—s1 and s2. There are also two types of mid-regions known—m1 and m2. Strains bearing the vacA s1m1 gene showed higher degrees of gastric colonisation [4].

The aim of this study was to detect genomic DNA of *H. pylori* in tonsillar tissue samples of patients with chronic tonsillitis and sleep apnoea syndrome (SAS) by a sensitive polymerase chain reaction (PCR) technique and type *cagA* and *vacA* gene regions using specific hybridisation probes and serology analysis. The reaction of the host to *H. pylori* infection was confirmed by detection of specific immunoglobulins of IgG, IgA and IgM classes in serum samples using ELISA and immunoblot testing.

Materials and methods

Patients

The study, conducted over 2 years, included 89 adult patients with a diagnosis of chronic tonsillitis or SAS. All these patients underwent tonsillectomy at the Department of Othorinolaryngology and Head and Neck Surgery. The patients were between 35 and 70 years of age.

The study includes 60 patients with a diagnosis of chronic tonsillitis and 29 patients with diagnosis of SAS. Excluding criterion was the eradication of *H. pylori* infection at the time prior to the surgery.

Specimen collection and transport

The samples from tonsils were collected using sterile instruments at the beginning of surgery. All of them were immersed into Microtest R M4RT transport media (Remel Inc., USA) and transported to the laboratory for analysis.

Nucleic acid isolation and storage

Genomic DNA of *H. pylori* was isolated using a MagNA Pure Compact System (Tegimenta AG, Rotkreuz, Switzerland) and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics), protocol Total_NA 400_100″, with pretreatment in MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics). Isolated nucleic acid specimens were stored at −80 °C.

Helicobacter pylori nucleic acid detection and genotyping

Helicobacter pylori genetic analysis was based on primers and hybridisation probes designed by van Doorn. Three real-time PCR assays had been developed in cooperation with TIB–Molbiol GmbH, Berlin, Germany for the cagA gene, middle region of the vacA gene and the last for the vacA gene signal region. The following primers and probes were used: cagA F (sense), cagA R (antisense), HPMGF+ (sense), HPMGR− (antisense), VAF1F+ (sense) and VAXR− (antisense). Taq Man hybridisation probes were prepared for cagA, vacA m1 and vacA m2-specific sequence detection. For the cagA assay, a FAM–BBQ-labelled probe (530 nm) was used, for vacA middle region assay FAM–BBQ labelling was used for M1 TM probe (530 nm) and HEX–BBQ labelling for M2 TM probe (560 nm). Taq Man real-time PCR assays were run on a LightCycler instrument, version 2.0 (six channels detection 530, 570, 610, 640, 670 and 705 nm). Commercial Light Cycler Taq Man Master (Roche Applied Science) was used: 15 µl of working Master Mix and 5 µl of sample DNA isolate per 20 µl capillary. For real-time PCR for vacA gene signal region, hybridisation probes S1a LC (LC 610), S1b LC (LC 640) and S2 LC (LC 705) were used together with commercial Light Cycler Fast Start DNA Master PLUS HybProbe (Roche Diagnostics)—15 µl of working MasterMix and 5 µl of sample DNA isolate per 20 µl capillary.

Confirmation of *H. pylori*-specific DNA presence in samples

In June 2012, a new commercial real-time PCR assay for detection of *H. pylori* flagellar gene Bioron Real-Line Fla Format (Bioron, Germany) was launched on the market. Archived patient samples were defrozen and new DNA isolates were prepared and tested by this new commercial test for comparison and confirmation of *Helicobacter* presence in the tonsillar tissue.

Serological analysis of serum specimens

To confirm *H. pylori* infection in patients, serological analysis was performed. Serum samples were tested by quantitative commercial ELISA tests (EIA *H. pylori*, Test-Line, Czech Republic) on IgG, IgA and IgM antibodies. For specific anti-CagA protein antibodies detection, the commercial test *Helicobacter* p120 (CagA) ELISA Test-Line was used.

For detection of specific IgG and IgM antibodies against separated *H. pylori* antigens (120, 87, 33, 30, 29, 26, 19, 17 kDa) commercial tests such as BLOT *H. pylori* IgG and IgA, Test-Line were used. The result of either 120 kDa (CagA) or 87 kDa (VacA), or 33 kDa was confirmed as positive. Alternatively, result of more than 2 of 30, 29, 26, 19, 17 kDa antigens was also confirmed as positive.

Results

All 89 patients in the study underwent tonsillectomy (100 %). 60 patients (67.42 %) for the diagnosis of chronic tonsillitis and 29 (32.58 %) for the diagnosis of SAS. The age of the patients in the group of chronic tonsillitis varied from 20 to 60 years (31.76 ± 9.11), median 30. The SAS group varied from 27 to 63 years (44.73 ± 11.45), median 45.

In all perioperative biopsy tissue samples, the PCR procedure for *H. pylori* genotypes detection was performed. PCR results were positive in 72 patients (80.90 %), and in 17 patients (19.10 %), *H. pylori* was not detected.

Out of 72 *H. pylori* DNA-positive patient samples, cagA gene was detected in 17 (23.61 %). 55 patient samples (76.39 %) were cagA negative. In the chronic tonsillitis group, 12 patients were cagA positive (20 %). 36 patients (60 %) cagA negative and *H. pylori* was not detected in 12 patients of this group (20 %).

In the SAS group, 5 patients (17.24 %) were cagA positive, 19 (65.52 %) cagA negative and *H. pylori* was not found in 5 patients (17.24 %).

The distribution of detailed genotypes in both diagnoses is shown in Tables 1 and 2 and Figs. 1 and 2.

Table 1 PCR detection of *Helicobacter pylori* in patients with chronic tonsillitis

Patient no.	Sex	PCR detection of HP	Genotyping		
		PCR	cagA	vacAs	vacAm
Patients with chronic tonsillitis					
1	Female	Pos	Pos	s1a	m1
1	Male	Pos	Pos	s1a	m2
2	Female	Pos	Pos	s1a	m2
3	Male	Pos	Pos	s1b	m1
1	Female	Pos	Pos	s1b	m1
1	Male	Pos	Pos	s1b	m2
2	Female	Pos	Pos	s1b	m2
1	Female	Pos	Pos	s2	m1
1	Female	Pos	Neg	s1a	m1
2	Male	Pos	Neg	s1a	m2
2	Female	Pos	Neg	s1a	m2
4	Male	Pos	Neg	s1b	m1
10	Female	Pos	Neg	s1b	m1
4	Male	Pos	Neg	s1b	m2
5	Female	Pos	Neg	s1b	m2
1	Male	Pos	Neg	s2	m1
4	Female	Pos	Neg	s2	m1
3	Male	Pos	Neg	s2	m2
10	Female	Neg	Neg	Neg	Neg
2	Male	Neg	Neg	Neg	Neg

Table 2 PCR detection of *Helicobacter pylori* in patients with sleep apnoea syndrome

Patient no.	Sex	PCR detection of HP	Genotyping		
		PCR	cagA	vacAs	vacAm
Patients with sleep apnoea syndrome					
1	Male	Pos	Pos	s1a	m2
1	Female	Pos	Pos	s1b	m1
1	Male	Pos	Pos	s1b	m1
2	Male	Pos	Pos	s2	m1
2	Male	Pos	Neg	s1a	m1
6	Male	Pos	Neg	s1a	m2
1	Female	Pos	Neg	s1a	m2
4	Male	Pos	Neg	s1b	m1
4	Male	Pos	Neg	s1b	m2
2	Male	Pos	Neg	s2	m2
1	Female	Neg	Neg	Neg	Neg
4	Male	Neg	Neg	Neg	Neg

Comparison of results of our real-time PCR assays and commercial Bioron Real-line *H. pylori* FLA test showed 100 % concordance.

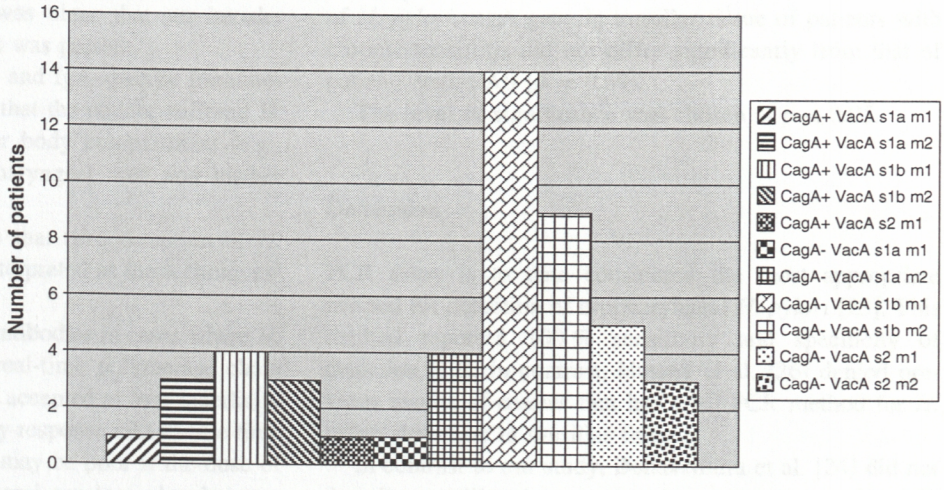


Fig. 1 Overview of detailed genotypes of *Helicobacter pylori* in patients with chronic tonsillitis

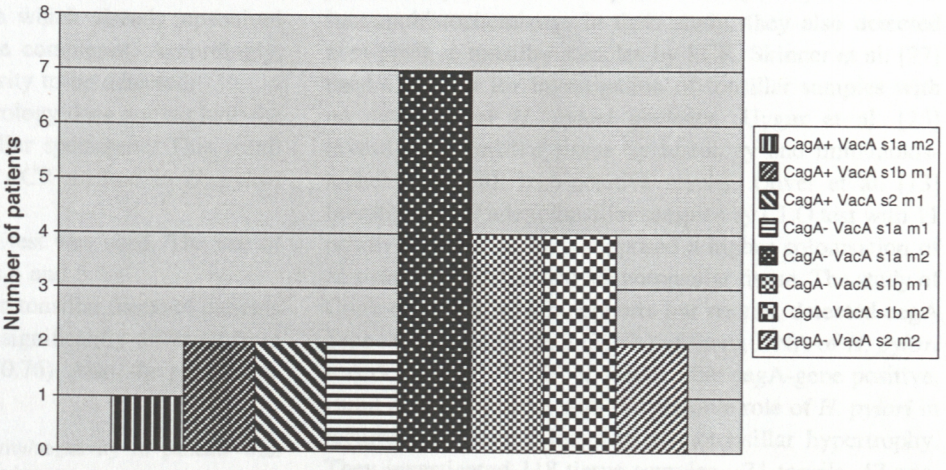


Fig. 2 Overview of detailed genotypes of *Helicobacter pylori* in patients with sleep apnea syndrome

Serological investigation (ELISA and immunoblot detection of specific *H. pylori* immunoglobulins) was performed in 57 patients.

Helicobacter pylori-specific immunoglobulins were detected in 41 patients (71.93 %); in the serum of 16 patients, no antibodies were detected (28.07 %).

The comparison of PCR positivity/negativity and seropositivity/seronegativity of *H. pylori* infection is shown in Table 3 and Fig. 3.

Investigation by both direct and indirect diagnostic techniques provided 42 (73.68 %) concordant and 15 (26.32 %) discordant results. Six discordant results were based on positive serology and negative PCR. In comparison of these two techniques, this value should not play an important role. From a diagnostic point of view, the presence of specific antibodies means that the host organism undergoes or underwent infection. In case anamnestic

Table 3 Comparison of PCR positivity/negativity and seropositivity/seronegativity of *Helicobacter pylori* infection

	Positive serology	Negative serology
Positive PCR	35 (61.40 %)	9 (15.79 %)
Negative PCR	6 (10.53 %)	7 (12.28 %)

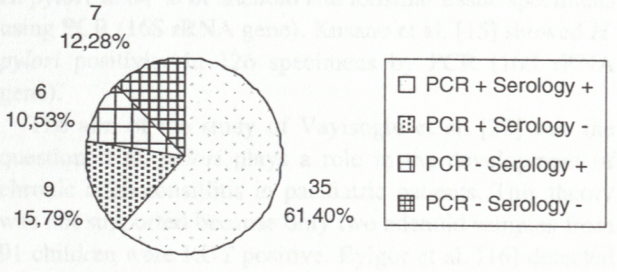


Fig. 3 Comparison of PCR detection and serology analysis (no. 57 patients)

antibodies were detected, it was clear that no invader (infectious agent or its antigen) was present.

In case of presence of IgM- and IgA-specific immunoglobulins, it is highly possible that the patient suffered *H. pylori* infection, but in another body compartment (e.g., stomach) other than the oropharyngeal area and his/her tonsils were not colonised.

Therefore, we can conclude that zero detection of *H. pylori* by PCR should not be interpreted as the techniques' poor sensitivity.

Conversely, no presence of antibodies in cases where *H. pylori* DNA was detected by real-time polymerase chain reaction (rt-PCR) should not be accepted as low specificity of this technique as the antibody response takes some time to develop and seroconversion may be poor if the dose of infectious agent was low. In general, serological techniques detect immunoglobulins that are free in blood and tissues being not able to detect those which already opsonised bacteria and/or formed immune complexes. Accordingly, antibodies must be in the majority to be detected.

This means that negative serology does not exclude the presence of *H. pylori* in tonsillar specimens. This result emphasises high sensitivity of PCR method in *H. pylori* detection.

In the study, the Chi-square test was used. The use of this method is shown in Tables 4 and 5.

The prevalence of *H. pylori* in tonsillar tissue of patients with chronic tonsillitis was not significantly different from that of patients with SAS ($p = 0.76$). Also, the prevalence of *H. pylori* cagA gene in tonsillar tissue of patients with chronic tonsillitis did not differ significantly from that of patients with SAS ($p = 0.69$).

The level of significance was chosen at $p < 0.05$.

Table 4 *Helicobacter pylori* positivity/negativity in patients with chronic tonsillitis or sleep apnoea syndrome

	Helicobacter pylori positive	*Helicobacter pylori* negative	Total
Chronic tonsillitis positive	48 (80 %)	12 (20 %)	60
Sleep apnoea syndrome positive	24 (82.76 %)	5 (17.24 %)	29
Total	72	17	89

Table 5 *Helicobacter pylori* CagA positivity/negativity in patients with chronic tonsillitis or sleep apnoea syndrome

	Helicobacter pylori CagA positive	*Helicobacter pylori* CagA negative	Total
Chronic tonsillitis positive	12 (25 %)	36 (75 %)	48
Sleep apnoea syndrome positive	5 (20.83 %)	19 (79.17 %)	24
Total	17	55	72

Discussion

PCR assay is to date considered the most appropriate method for detection of oropharyngeal *H. pylori* [23]. This method reported 100 % sensitivity and specificity of detection [12]. In contrast, Hussey et al. [26] denied previous results of studies that had used PCR method for *H. pylori* detection in tonsillar tissue.

In contrast to our study, Bonaventura et al. [24] did not describe tonsillar tissue as an extragastric reservoir of *H. pylori*. They detected zero-positive samples by culture and immunohistochemistry. In their study, they also detected zero-positive tonsillar samples by PCR. Skinner et al. [27] used CLO test for investigation of tonsillar samples with no evidence of *H. pylori* presence. Uygur et al. [25] investigated tonsillar tissue by histology and immunohistochemistry with zero-positive results. Unver et al. [13] investigated 19 adenotonsillar samples by CLO test with 11 positive results, so they supposed a higher colonisation of *H. pylori* in tonsillar and adenotonsillar tissue. The study of Cirak et al. [3], which supports our results, detected cagA gene in 23 patients. In 7 (30 %) of them, DNA of *H. pylori* was detected, 5 (71 %) of whom were cagA-gene positive. Bulut et al. [20] came with the possible role of *H. pylori* in recurrent adenotonsillitis and adenotonsillar hypertrophy. They investigated 118 tissue samples—71 tonsils, 47 adenoid tissues. CagA gene was positive in 12 samples of hypertrophic tonsils and in 5 samples of patients with recurrent adenotonsillitis. So they did not contradict the possible role of *H. pylori* in recurrent adenotonsillitis. On the other hand, Bitar et al. [19] found *H. pylori* positivity by RUT and histology, but no positivity by nested PCR. In their next study, they investigated middle ear fluids and adenoid tissue specimens using culture, RUT and PCR. No positivity by PCR was found [28].

In accordance with our study, Yilmaz et al. [21] detected *H. pylori* in 64 % of adenoid and tonsillar tissue specimens using PCR (16S rRNA gene). Kusano et al. [15] showed *H. pylori* positivity in 126 specimens by PCR (16S rRNA gene).

The aim of the study of Vayisoglu et al. [22] was the question if *H. pylori* plays a role in the development of chronic adenotonsillitis in paediatric patients. This theory was not supported because only two adenoid samples from 91 children were RUT positive. Eyigor et al. [16] detected zero-positive adenotonsillar specimens from 55 (PCR detection of glmM gene) and only 3 specimens were RUT

positive. They discussed the question of the role of *H. pylori* in upper respiratory tract diseases, especially in adenotonsillitis. Kurtaran et al. [29] permitted the possible role of *H. pylori* in pathogenesis of upper respiratory system diseases, especially pharyngitis by gastroesophageal reflux disease transmission. Our theory is supported by the study of Moghaddam et al. [17]. They admitted the possible role of tonsillar tissue as a reservoir of *H. pylori* infection [17]. Vilarinho et al. [30] found 3 adenoid and tonsillar specimens positive by RUT, 2 positive by immunohistochemistry, but none positive by fluorescence in situ hybridisation or PCR (vacA gene). Abdel-Monem et al. [12] detected 5 positive specimens by PCR (ureC gene). Toros et al. [31] investigated adenotonsillar tissue in 84 children by RUT and histopathology. They found no *H. pylori* colonisation in any specimens. Farivan and Najafipour [18, 32] wrote about high success of scorpion PCR method in detection of *H. pylori* in tonsillar tissue in patients with chronic tonsillitis.

Based on comparison of our results and the results of other studies, we can conclude that oropharyngeal lymphatic tissue is an extragastric reservoir of *H. pylori*. Our results support the high sensitivity and specificity of the real-time PCR technique. This fact is shown in Table 3. The seronegativity in some patients does not exclude the presence of *H. pylori* in tonsillar tissue where it is detectable by the real-time PCR method. The significance of IgG immunoglobulins detection in *H. pylori* infection is emphasised in Urita et al. [33]. Our results support the hypothesis that *H. pylori* plays a role in the pathogenesis of chronic tonsillitis because of the high presence of genotype vacA s1m1 (37.5 %). Another interesting fact is that the majority of cagA-negative genotypes isolated from tonsillar tissue (55 of 72–76.39 %) leads to idea that the main virulence factor in gastric infections CagA may not be of such importance in infections of the tonsils. Long-term colonisation of tonsillar tissue by *H. pylori* lacking CagA protein may lead to development of chronic inflammation and alteration of immune system mechanisms.

There are few studies investigating the presence of *H. pylori* in patients with SAS. Unal et al. [34] investigated the seroprevalence of *H. pylori* in patients with SAS. As they detected IgG immunoglobulins in 17 of 19 patients, they emphasised a high association between *H. pylori* infection and SAS. Ye et al. [35] found that the seroprevalence of *H. pylori* in patients with SAS was 75.5 %, and therefore concluded that *H. pylori* may be associated with SAS. The new study in 2012 by Stergiopoulos et al. [36] supported these former results. Contrarily to our study, all these authors used serology detection only.

In contrast to Unal and Ye, we used the DNA detection and genotype analysis for the presence of *H. pylori* in oropharynx. In our study, we verified the presence of *H. pylori* directly in tonsillar tissue in patients with SAS. Our detection of *H. pylori* DNA in 24 of total 29 samples (82.76 %), out of which 79.17 % were cagA negative, supports the hypothesis that *H. pylori* plays a role in pathogenesis of SAS.

Our results on *H. pylori* DNA detection and *Helicobacter* seropositivity show 26.32 % discrepancy, slightly in favour of real-time PCR (15.79 % compared to 10.53 %). This shows that absence of seropositivity does not mean absence of *H. pylori* infection, while presence of IgG may be anamnestic.

In conclusion, our study supports the possible role of *H. pylori* in chronic tonsillitis and SAS. Therefore, eradication of *H. pylori* infection, even if it concerns cagA-negative genotypes, may prevent future oropharyngeal pathology.

Statistical analysis on comparison of *H. pylori* infection in the groups of chronic tonsillitis and SAS using Chi-square test showed that the *H. pylori* presence in tonsillar tissue does not depend on the type of oropharyngeal disease ($p = 0.756$). Oropharyngeal tonsillar tissue is an extragastric reservoir of *H. pylori* infection. The mechanism of aetiological effect is still unclear.

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Příloha 2

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MISCELLANEOUS

Detection of *Helicobacter pylori* in oropharyngeal lymphatic tissue with real-time PCR and assessment of its carcinogenic potential

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Abstract *Helicobacter pylori* (HP) is considered a major gastric pathogen with oncogenic potential. The aim of this study was to determine whether HP is present in oropharyngeal lymphoid tissue and whether oropharyngeal HP strains carry virulence factor genes known to be involved in gastric carcinogenesis. The study included 104 subjects (41 patients with tonsillar carcinoma, 38 with chronic tonsillitis and 25 with obstructive sleep apnoea syndrome—OSAS). Detection of specific serum anti-HP antibodies was performed with an ELISA. The presence of HP in tissue was determined by culture and real-time PCR. Detection of virulence factors genes was also performed. Specific antibodies were found in 78.05 % of tumour cases, 34.21 % of chronic tonsillitis cases, and 72.0 % of OSAS cases. The presence of HP in the tissue was detected in 73.91 % of tonsillar tumours, 70.0 % of tonsillitis cases, and 69.23 % of OSAS specimens. The results of the virulence factor gene analysis showed the majority of the s1b (52.4 %) and m2 (59.5 %) alleles of *vacA* gene and limited abundance of *cagA* gene (12.5 %). Results confirm that HP may colonise oropharyngeal lymphoid tissue. Oropharyngeal HP colonisation was frequently found in the oropharyngeal cancer group and in patients with benign oropharyngeal diseases. A virulence factor gene analysis showed differences from the predominant strains most commonly found in the stomach. The strains obtained from the oropharynx differed primarily by the lower abundance of the *cagA* gene and carried the less virulent *vacA* gene allele combination.

Keywords *Helicobacter pylori* · Real-time PCR · Oropharynx · Oropharyngeal cancer · Sleep apnoea syndrome · Chronic tonsillitis

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Introduction

Helicobacter pylori (HP) is a gram-negative bacterium. It is a major cause of chronic gastritis and plays an important role in the pathogenesis of other diseases, such as gastric lymphoma and carcinoma [1]. HP infection is considered to be one of the most common chronic bacterial infections in humans [2]. Clinical manifestations of HP (e.g. ulcer disease), however, appear only in 10–15 % of infected persons [3]. The most significant virulence factors associated with gastric pathologies are cytotoxic associated gene A (*cagA*) and vacuolating cytotoxin A (*vacA*) [4]. Bacterial genome analysis has led to the identification of genes

encoding virulence factors, which are grouped in the so-called pathogenicityisland (PAI) [5]. The most important is the *cagA* gene, which encodes the high molecular weight protein CagA, which is highly immunoreactive. The *cagA* gene is present only in approximately 60 % of all HP strains [6]. Compared with *cagA*-negative strains, the strains producing CagA are associated with an increased risk of severe gastric pathologies (e.g. gastric cancer) [7]. Another virulence factor, *vacA* causes host cell vacuolation. It is encoded by the *vacA* gene, which is carried by all HP strains, but only approximately 50 % of strains produce the VacA protein [7]. This is due to variability of the primary sequence of the *vacA* gene. Different alleles can be found in the signal sequence (s1a, s1b, s2) and in the middle region (m1, m2) of the gene. Strains with type s1 signal sequence alleles produce a functional VacA toxin, whereas strains with type s2 alleles have little cytotoxic activity. s1/m1 strains are considered highly toxic [8, 9].

Oral–oral or faecal–oral transmission is considered to be the primary route of HP transmission among people [10]. Assuming an oral–oral or faecal–oral route of transmission, the presence of HP in the oral cavity and oropharynx can be expected. The first positive detections were in dental plaques and saliva [11, 12]. Recent research has focused on detecting HP in tonsillar and adenoid tissues. Attempts to detect HP in these locations, however, have so far yielded inconsistent results [13–15]. Whether oropharyngeal lymphoid tissues are colonised by HP and whether HP influences the host immune system response in this location have not yet been satisfactorily determined. It is expected that HP in the oropharyngeal lymphoid tissue of Waldeyer's circuit could activate the immune system and interfere with the expression of various cytokines, thereby promoting chronic inflammation and possibly acting as a direct carcinogen.

The aim of this study was to determine whether HP is present in oropharyngeal lymphoid tissue and whether oropharyngeal HP strains carry virulence factor genes involved in gastric carcinogenesis that may also play a role in oropharyngeal pathogenesis.

Methods

Collection of biological material

All specimens were collected at the Department of Otorhinolaryngology and Head and Neck Surgery, First Faculty of Medicine, Faculty Hospital Motol, from patients who underwent surgery for tonsillar squamous cell carcinoma, chronic tonsillitis or obstructive sleep apnoea syndrome (OSAS). The patients were enrolled after signing an appropriate informed consent form. The inclusion criterion for participation was at least a 2-month period without antibiotic use; patients who previously underwent HP eradication were excluded.

Overall, the study included 104 subjects, including 41 patients with carcinoma of the tonsil, 38 with chronic tonsillitis and 25 with OSAS. In the latter group, we assumed healthy tonsillar tissue. Indication for surgery in these subjects was to perform uvulo-palato-pharyngoplasty (UPPP), part of which was tonsillectomy in all cases.

In all patients, preoperative blood specimens were collected by standard venepuncture into Vacuette tubes (GBO, Kremsmünster, Austria) for serum anti-HP antibodies detection.

Tissue specimens from tonsillar carcinomas and tonsils were collected under strictly sterile conditions at the beginning of surgery, after endotracheal intubation and before application of anaesthetics or disinfectants into the oral cavity. Tissue specimens were loaded into a transport medium for culture and into MicrotestR M4RT transport media (Remel Inc., Lenexa, KS, USA) for PCR processing.

Examination of specimens

A. Detection of specific serum anti-HP antibodies in IgG, IgA, and IgM isotypes by an ELISA. Quantitative evaluation of specific anti-HP antibodies in sera was carried out in IgG, IgA and IgM isotypes by ELISA using commercial tests (EIA - *H. pylori*, Test Line, Brno, Czech Republic). The results were expressed by an index of positivity (IP = ratio of average absorbance of the test serum and the cut-off) and considered to be positive when IP > 1.2. Tests were performed at the Institute for Immunology, 2nd Faculty of Medicine, Charles University in Prague
B. Tissue detection of HP: culture, real-time PCR, virulence factor genes analysis

Culture

Culture specimens were placed in transport media (thioglycolate bouillon) immediately after collection. Culture examination was carried out in a micro-aerobic environment (Anaerocult C, Merck, Whitehouse Station, NJ, USA) at 37 °C for 4–7 days. Culture media were prepared from Columbia agar enriched with 4–10 % horse blood, 7 % horse serum and supplements A and B (Dulab, Dubné, Czech Republic). Bacteria identification was based on microscopic morphology, colony morphology and detection of urease, catalase and oxidase enzyme activity. Strains were subcultured on solid culture soil for every 3–4 days and incubated at 37 °C in a micro-aerobic environment.

Isolation of DNA

Isolation was performed on a Magna Pure Compact System (Tegimenta AG, Rotkreuz, Switzerland) using protocol "Total_NA 400_100" with a magna pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Basel, Switzerland) and Magna Pure Bacteria Lysis Buffer (Roche Diagnostics, Basel, Switzerland). DNA specimens were stored at −80 °C until real-time PCR was performed.

PCR amplification and gene analysis

For gene analysis, three real-time PCR TaqMan assays were developed in cooperation with TIB-Molbiol Berlin, Germany: one for the *cagA* gene, the second for the middle region of the *vacA* gene and the last assay for the *vacA* gene signal region. Primers used for the PCR detection of HP were: *cagA* F (sense), *cagA* R (antisense), HPMGF+ (sense), HPMGR− (antisense), VA1F (sense) and VA1R− (antisense), according to Atherton et al. and van Doorn et al. [6, 16, 17] (Table 1).

TaqMan hybridisation probes were developed for specific *cagA, vacA m1* and *vacA m2* sequence detection (Table 2). A FAM-BBQ labelled probe (detection 530 nm) was used for the *cagA* assay, and for the *vacA* middle region assay, FAM-BBQ labelling and a M1 TM-probe (530 nm) and HEX-BBQ labelling and a M2 TM-probe (560 nm) were used.

TaqMan® real-time PCR assays were run on a LightCycler® system, version 2.0 (six-channel detection) (Roche Applied Science, Basel, Switzerland). The commercial LightCycler® TaqMan® Master (Roche Applied Science, Basel, Switzerland) consisted of 15 µl of master mix including primers and probes, and 5 µl of sample DNA isolate per 20 µl capillary.

For real-time PCR for the *vacA* gene signal region, hybridisation probes S1a LC (LC610), S1b LC (LC640), and S2 LC (LC705) were used together with the commercial LightCycler® FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostics, Basel, Switzerland), consisting of 15 µl of Master Mix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

The real-time PCR assay program consisted of 45 cycles with an initial denaturation step at 95 °C for 10 min during the first cycle and 10 s in the remaining cycles. Annealing temperatures were 58 and 68 °C for 10, 15 or 20 s depending on the probes used. An extension step at 72 °C for 45 s was followed by real-time detection at the appropriate wavelength for each probe. Data analysis was performed using Roche LightCycler® software version 3.5.3.

Statistical analysis

Statistical analysis was performed using Statistica software (StatSoft, Inc., Tulsa, OK, USA). Fisher's exact test and Chi-square test were used.

Results

Serology

Serology analysis was performed in a total of 104 patients (41 in the tumour group, 38 in the chronic tonsillitis group and 25 in the OSAS group). Of these patients, 32 (78.05 %) in the tumour group, 13 (34.21 %) in the chronic tonsillitis group, and 18 (72 %) in the OSAS group were positive for anti-HP antibodies at least in one isotype. Statistically significant differences were found between the tumour and chronic tonsillitis groups (Chi-square test: $p = 0.0001$) and between the OSAS and chronic tonsillitis groups (Chi-square test: $p = 0.0049$) (Fig. 1).

Culture

Material for culture was collected from a total of 45 patients (11 tonsillar cancers, 22 chronic tonsillitis and 12 OSAS). Positive proof of HP by culture was only found in three specimens (one cancer and two tonsillitis).

PCR

Material for PCR was obtained from a total of 56 people (23 with cancer, 20 with chronic tonsillitis and 13 with

Table 1 PCR primers for *cagA* and *vacA* sequence amplification

Gene	Primer	No. of nucleotides	Sequence
cagA	*cagA* F	24	5-TTG ACC AAC AAC CAC AAA CCG AAG-3
	cagA R	22	5-CTT CCC TTA ATT GCG AGA TTC C-3
vacA S	VA1F	21	5-ATG GAA ATA CAA CAA ACA CAC-3
	VA1R	19	5-CTG CTT GAA TGC GCC AAA C-3
vacA M	HPMGF	21	5-CAG AGC CAC TTT CAA TAA CGA-3
	HPMGR	21	5-CGT CCA AAT AAT TCC AAG GG-3

Table 2 PCR probes for detection of *cagA* and *vacA* sequences

Gene	Type	No. of nucleotides	Sequence	Detection/nm
cagA	cag_TM	28	6FAM-ATA ACG CTG TCG CTT CAT ACG ATC CTG A–BBQ	530
vacA S	S1a_LC	21	LC Red610-GCR TTR GTC AGC ATC ACA CCG–PH	610
	S1b_LC	21	LC Red640-GCG TTG ATT AGY KCC ATA CCG–PH	640
	S2_LC	21	LC Red705-GCT AAY ACG CCA AAY GAT CCC–PH	705
vacA M	M1_TM	30	6FAM-ACC ACC ATT ACC CGT ATC AAT ACC TTT AAA–BBQ	530
	M2_TM	26	HEX-CTA GTG TTT AGC CCG TTA TCG CTC TT–BBQ	560

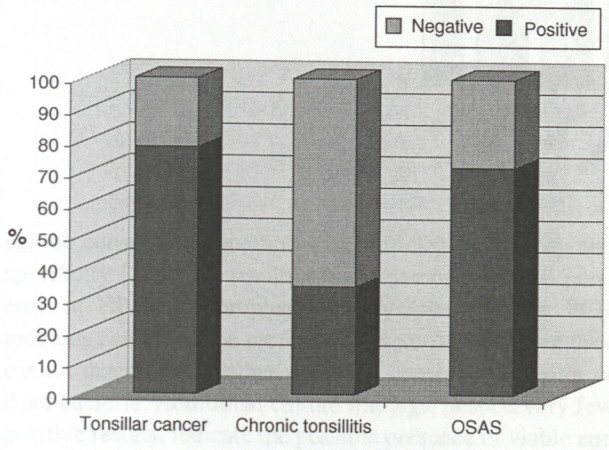


Fig. 1 Results of specific anti-HP antibody detection

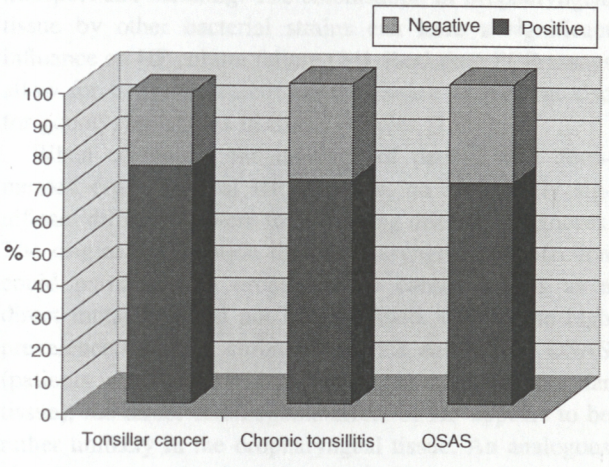


Fig. 2 Results of real-time PCR detection of HP in tissue specimens

OSAS). HP was detected by real-time PCR in 17 (73.91 %) tumours, 14 (70.0 %) tonsillitis samples, and 9 (69.23 %) OSAS samples (Fig. 2).

There were no significant differences between the tumour and chronic tonsillitis groups (Chi-square test: $p = 0.7754$), the OSAS and chronic tonsillitis groups (Chi-square test: $p = 0.9625$) or the tumour and OSAS groups (Chi-square test: $p = 0.7632$).

Virulence factors gene analysis of oropharyngeal HP strains

The distribution of virulence factor gene alleles found in each group is shown in Fig. 3. The results showed that the majority of the *vacA* gene alleles were s1b (52.4 %) and m2 (59.5 %), and there was a low abundance of the *cagA* gene (12.5 %). A very rare s2/m1 strain was found in one case. Mixed s1a/s1b, s1a/s2, and m1/m2 genotypes were detected in three specimens.

Comparison of PCR and serology results

In most patients (14 cancers, 7 tonsilitis, and 7 OSAS patients), PCR positivity was associated with the finding of antibodies in serum. Interestingly, we identified 12 individuals (3 cancer, 7 tonsillitis, and 2 OSAS patients) who were PCR positive for HP in oropharyngeal tissue without detectable serum antibodies. In two cases of chronic tonsillitis, we also obtained positive culture results from oropharyngeal tissues without finding serum antibodies.

Discussion

One of the aims of the study was to assess the possible colonisation of oropharyngeal lymphatic tissue by HP. Unlike dental plaque, which is considered a reservoir of extra gastric HP [12, 18], there are no convincing publications on the definitive detection of oropharyngeal HP infection; the results obtained to date vary considerably [19–21]. The detection of oropharyngeal HP is indicated in the range of 0–90 % [22]. Because various authors have used different methods of detection, it is not possible to compare study conclusions. Frequently used rapid urease tests (RUT) and campylobacter-like organism (CLO) tests seem to be inappropriate detection methods for diagnosing oropharyngeal HP. The presence of other urease-producing bacterial strains in the oropharynx can lead to false-positive results. Culture of HP was proven very difficult because it is not resistant to external influences, which may completely prevent successful detection. Real-time PCR assay

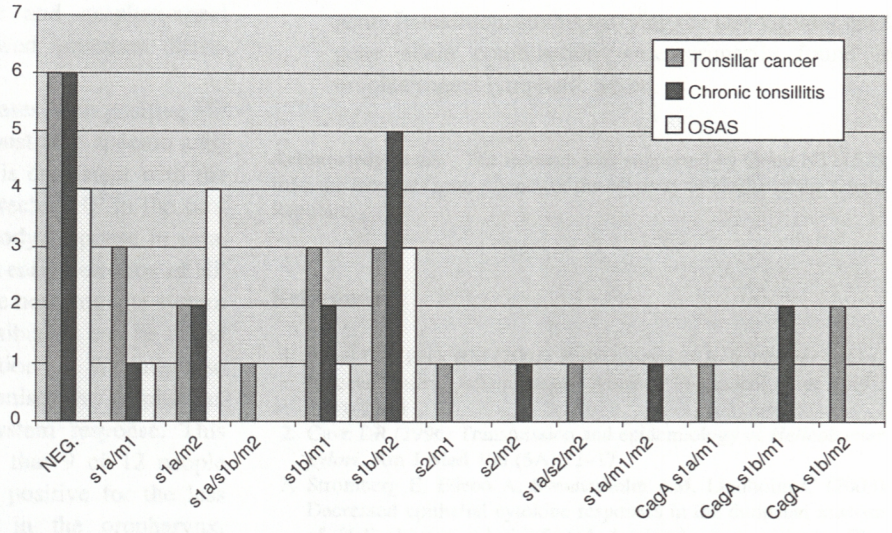


Fig. 3 *cagA* and *vacA* gene analysis results

can be considered a method with sufficient sensitivity and specificity [23]. Our results clearly demonstrate the presence of HP in the oropharyngeal lymphatic tissue. PCR methods can detect the presence of bacterial DNA, but they cannot determine whether the DNA comes from living or dead bacteria. Additional culture findings, despite very few positive results, indicate the possible presence of viable and replication capable bacteria. Low numbers of positive cultures can be explained by the high sensitivity of HP to transport and handling. The colonisation of oropharyngeal tissue by other bacterial strains can have a significant influence on HP culture failure [24]. Real-time PCR assays allow for not only detecting the presence of HP, but also for genotyping strains in tissue samples [15].

When comparing the numbers of people with documented oropharyngeal HP presence, no statistically significant differences were found among different diagnoses. The original assumption that oropharyngeal HP infection could participate in oropharyngeal carcinogenesis as a direct mutagen could not be confirmed. Due to the high prevalence of HP in chronic tonsillitis and also in OSAS (patients who would be expected to have healthy tonsillar tissue), the direct carcinogenic effect of HP appears to be rather unlikely in the oropharyngeal tissue. An analogous situation occurs in the stomach: although the HP seroprevalence is reported between 40 and 80 % [25], serious stomach problems such as gastroduodenal ulcer disease or gastric cancer only develop in 10–15 % of infected people [26]. Uemura et al. [27] performed a prospective study and found thatgastric cancer developed in 36 of 1,236 persons with gastric HP infection.

HP strain virulence varies depending on the production of several toxins, which is due to the presence of virulence factor genes, most importantly the *cagA* and *vacA* genes. The main carcinogenic effect of HP is in the presence of the *cagA* gene and the s1/m1 allele combination of the *vacA* gene [28]. A meta-analysis conducted by Huang et al. [29] showed that the risk of gastric cancer is twice as high in people who were positive for antibodies against CagA in sera. The analysis of oropharyngeal HP genotypes performed here showed increased abundance of the s1/m2 allele combination of alleles of the *vacA* gene. The *cagA* gene was found only in five (12.5 %) cases. This finding means that lessvirulent HP strains predominated in the oropharynx. In comparison with the geographical epidemiological studies of gastric HP genotypes performed by Van Doorn et al. [6], the allele distribution of the *vacA* gene and the presence of the *cagA* gene differ considerably. The dominantly expressed signal sequence allele of the *vacA* gene in Europe is s1a (88.8 %), and the dominant middle sequence allele is m1 (54.5 %). The *cagA* gene was found in 72.1 % of specimens. The results of our oropharyngeal genotype analysis, however, showed a majority of s1b (56.7 %) and m2 (59.5 %) alleles of the *vacA* gene and only the rare abundance of the *cagA* (12.5 %) gene in oropharyngeal genotypes. The finding of an s2/m1 genotype can be considered to be veryunique. The finding of this genotype was published in the literature only in isolated cases [30]. The finding of mixed genotypes of HP strains (found in three cases) is rare; however, it has been previously described [31].

Some authors [32] consider findings of HP in the oropharynx to be a sign of temporary contamination by the gastroesophageal reflux. Data that we have already published exclude this presumption [33]. Twenty patients with proven HP in the oropharynx were selected for routine urea breath testing (UBT) for the presence of HP in the stomach. Only eight of them showed concurrent presence of gastric infection. In gastric biopsies, the presence of HP was proven by real-time PCR in all six patients who underwent

endoscopy. Comparison of gastric and oropharyngeal genotypes in same individuals showed important differences in four of them.

Burgers et al. [34] reported ten cases with positive HP detection in the saliva without demonstrable specific anti-HP antibodies in sera. This finding is consistent with the results obtained in our work; we detected HP in the oropharynx in 12 people with no antibody response in sera. One possible explanation could be an early detection of HP following primary infection, when the host immune system has not yet produced antibodies. Antibodies can be found in sera up to a few months after infection [7]. We must also consider the possibility that HP colonises the oropharynx without causing a host immune system response. This hypothesis is supported by the fact that 9 of 12 people without an antibody response were positive for the less virulent genotype of HP (s1/m2) in the oropharynx. Another explanation is the possibility of presence of coccoid forms of HP. This viable form of bacteria cannotbe cultured by conventional microbiological techniques and is characterised by reduced virulence. HP in this form occurs in unfavourable environments [35].

Epidemiological data on the prevalence of HP infection published in the literature are often based on the serologic detection of specific anti-HP antibodies. The prevalence of infection in the Czech Republic is reported to be 45–83 % [36]. The presence of anti-HP antibodies has been considered in relation to gastric infection. Data obtained in our work, however, suggest the possibility of the presence of HP in other locations. This possibility should be considered in future epidemiological studies, which should determine the exact localisation of the bacterium in addition to evaluating the presence of antibodies.

In the future, it would be appropriate to focus attention on the local effect of HP in oropharynx lymphoid tissue. If oropharyngeal tissue can host highly virulent HP strains, then the translocation of toxins into oropharyngeal cells and a subsequent cytokine response identical to that observed in gastric mucosal cells can be expected. To date, this hypothesis has not been confirmed or refuted.

The most important results of this work can be summarised as follows.

A. We confirmed that HP is capable of colonising oropharyngeal lymphoid tissue. Frequent oropharyngeal HP infection was detected in the oropharyngeal cancer group and in benign oropharyngeal diseases. The possible effect of HP on the development of oropharyngeal carcinogenesis was not proven
B. Virulence factor gene analysis of oropharyngeal HP strains showed differences compared to the predominant strains most commonly found in the stomach. Strains differed primarily by the lower presence of the *cagA* gene. In addition, strains carrying the less virulent *vacA* gene allele combination were primarily found in oropharyngeal lymphatic tissue

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Pilot study: Association between *Helicobacter pylori* in adenoid hyperplasia and reflux episodes detected by multiple intraluminal impedance in children

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ABSTRACT

Objectives: The aim of this pilot study was to investigate an association between laryngopharyngeal reflux detected by combined multiple intraluminal impedance and pH monitoring and *Helicobacter pylori* in adenoid hyperplasia detected with real time polymerase chain reaction (PCR).
Methods: The study group consisted of 30 children (median age 5.34 years) with extraesophageal symptoms of gastroesophageal reflux disease with adenoid hyperplasia. All children underwent adenoidectomy with subsequent PCR detection of *H. pylori* DNA in the tissue and multiple intraluminal impedance and pH monitoring. The most proximal impedance sensor was located 1 cm caudal to the entrance of the oesophagus.
Results: We found significant differences in the number of reflux episodes among patients with PCR positivity (median 35) and negativity (median 0) of *H. pylori* (p-value of Mann–Whitney U-test 0.0056). Patients with PCR positivity of *H. pylori* had significantly more reflux episodes reaching the upper oesophageal sphincter (p-value of Mann–Whitney U-test 0.023). The absence of reflux episode was the only independent factor for PCR negativity of *H. pylori* in the multiple logistic regression model.
Conclusions: These results support the hypothesis that reflux episodes reaching the upper oesophageal sphincter may play an important role in the transmission of *H. pylori* into lymphoid tissue of the nasopharynx and thus may contribute to adenoid hyperplasia in children.
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1. Introduction

Helicobacter pylori (HP) is a gram-negative, spiral, microaerophilic and flagellated bacterium that colonises the gastric mucosa. Its prevalence has been estimated to range from 40 to 80% and it varies widely by geographic area, age and socioeconomic status [1]. Immune changes caused by HP infection and its consequences have been thoroughly investigated in the past in the stomach area [2,3]. It is well known that HP infection is acquired in childhood, often before the age of 5 years [4]. The mechanism of transmission remains unclear. There are three possible routes of transmission: the faecal–oral, the oral–oral and the gastric–oral routes. Cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) are the most important virulence factors associated with gastric pathogenesis. The CagA protein stimulates cell signalling through the interaction with several host proteins and this leads to an increased release of cytokines group production [5]. VacA protein causes cytoplasmic vacuolation [6]. Two types of signal regions (s1 and s2) and two types of mid-regions (m1 and m2) are known. Strains bearing the vacA s1m1 gene showed higher degrees of gastric colonisation [7]. Possible causal relationships between HP and extra- gastrointestinal diseases have been studied since 1993.

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Table 1
Demographic and clinical characteristics of patients.

	All patients ($n=30$)	Patients with PCR positivity of *Helicobacter pylori* ($n=27$)	Patients with PCR negativity of *Helicobacter pylori* ($n=3$)
Sex, M/F	20/10	18/9	2/1
Age in months, median (interquartile range)	64 (45–78)	64 (46–77)	76 (64–78)
Symptoms (%)			
Dysphonia or hoarseness	12 (40%)	10 (56%)	2 (67%)
Chronic cough	11 (37%)	10 (22%)	1 (33%)
Heartburn	8 (27%)	8 (44%)	0
Other symptoms[a]	3 (10%)	3 (17%)	0
Laryngoscopic findings (%)			
Laryngitis posterior	12 (40%)	11 (61%)	1 (33%)

[a] Dysphagia, stridor and globus sensations.

Gastroesophageal reflux (GER) could also be a cause for the enlargement and multiplication of lymphoid follicles [8]. Recent studies have shown that the exposure of upper aerodigestive tract to gastric secretion may cause inflammatory changes that could eventuate into adenoid hyperplasia [9] and the possible role of HP in the pathogenesis of adenoid hyperplasia by gastroesophageal and laryngopharyngeal reflux disease transmission [10]. The nasopharynx area may be directly exposed to the action of HP particularly in patients with gastroesophageal reflux disease (GERD), including diseases arising on the basis of gastroesophageal reflux (GER), as well as laryngopharyngeal reflux (LPR). There are many reports of HP detection in hyperplastic adenoids of children [11–14]; however, the detection rate is highly variable and the assumption that HP can colonise adenoids tissue has not yet been clearly established. In the literature, studies focused on the relationship between the presence of HP in adenoid hyperplasia and LPR in children are limited [15]. LPR is a condition in which the penetration of (duodeno) gastric refluxed fluid above the upper oesophageal sphincter (UES) occurs. We distinguish between physiological LPR, which does not cause any mucosal changes, and pathological LPR, which causes mucosal changes and clinical symptoms. Laryngopharyngeal reflux disease (LPRD) is a condition which causes reflux symptoms and/or complications in the pharynx, oral cavity, larynx and respiratory tract. Prevalence and incidence of LPRD in childhood and adolescence is not exactly known. Mechanisms of reflux in patients with GER and LPR are different. Pathological LPR is regarded as one of the factors which participates in inflammatory processes in the pharynx, larynx and upper respiratory tract. The most important factor in patients with LPR is a dysfunction of the upper oesophageal sphincter (UES) [16]. LPR is most commonly associated with the reflux/posterior laryngitis, vocal cord granulomas, globus pharyngeus, chronic cough and so-called postnasal drip syndrome in childhood [17] . Mucosa of the upper respiratory and swallowing tract in different patients is variously sensitive to aggressive components of refluxed fluid (hydrochloric acid, pepsin and bile acids) and the subjective perception of clinical symptoms is also very individual. We distinguish acid reflux (pH < 4), weakly acid (pH 4.01–6.9) and non-acid/alkaline (pH ≥ 7). The question of LPR is full of controversial opinions. There are two opinions still discussed in the pH-metric diagnosis of LPR. One group of authors believes that each episode of LPR is pathological [18,19], while the second group evaluates the LPR pathological if 3–7 reflux episodes occur within a period of 24 h [20,21]. Studies focused on defining the "cut-off" value for the reflux index (RI) are inconsistent, particularly with regards to the age range of the paediatric population. At present, there are no normative value dates that would determine the numbers of LPR as physiological. The aim of this study was to investigate an association between LPR detected by combined multiple intraluminal impedance and pH monitoring (MII-pH) and the presence of HP in adenoid hyperplasia detected with real time polymerase chain reaction PCR.

2. Material and methods

2.1. Patients

The study included 32 children with the diagnosis of adenoids and symptoms clinically suggestive of LPR for 2009–2012. We suggested enrolment in the study to the parents or legal guardians of children scheduled to undergo adenoidectomy and examination of LPR. The study was approved by the Ethics Committees of the authors' institutions, and a written informed consent was obtained from all participants or their guardians. Clinical characteristics of patients are listed in Table 1.

All patients underwent adenoidectomy at the Department of Ear, Nose and Throat, 2nd Faculty of Medicine, University Hospital Motol, Charles University, Prague, Czech Republic. All 32 patients had confirmed adenoid hyperplasia preoperatively using a flexible endoscopic examination of nasopharynx area. They have also well-known typical symptoms of enlarged adenoids. We did not include patients having tonsillar hypertrophy or any history of chronic tonsillitis in the study. All patients were asked about symptoms of GERD with oesophageal and extraoesophageal symptoms such as dysphonia, chronic cough, hoarseness or regurgitation and *pyrosis*. We evaluated the signs of posterios laryngitis (oedema and erythema of posterior commissure) or other possible LPR findings - pseudosulcus vocalis, ventricular obliteration, diffuse laryngeal oedema and posterior commissure hypertrophy. None of these patients was using anti-reflux therapy or had ever been diagnosed with GERD. However, 2 of these patients (2/32) were unable to be scheduled for MII-pH probe monitoring and were excluded from this study. Therefore, finally 30 patients underwent evaluation for LPR by MII-pH. The introduction of the measuring probe was performed 2 weeks after adenoidectomy, not later than one month.

2.2. Detection of HP

2.2.1. Specimen collection and transport

The samples from adenoids were collected using sterile instruments immediately after endoscopically assisted adenoidectomy. All of them were immersed into Microtest R M4RT transport media (Remel Inc., USA) and transported to the laboratory for analysis.

2.2.2. Nucleic acid isolation and storage

Genomic DNA of *H. pylori* was isolated using MagNA Pure Compact System (Tegimenta AG, Rotkreuz, Switzerland), and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics) protocol, "Total_NA 400_100", with pre-treatment in MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics). Isolated nucleic acid specimens were stored in −80 °C.

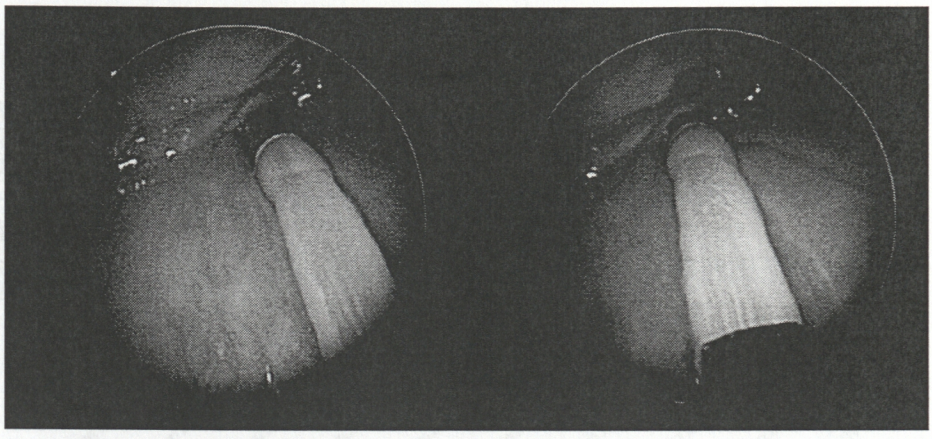


Fig. 1. Insertion of the proximal impedance ring (Z1) under visual control.

2.2.3. H. pylori nucleic acid detection and genotyping

H. pylori genetic analysis was based on primers and hybridisation probes designed by van Doorn. Three real-time PCR assays had been developed in cooperation with TIB Molbiol GmbH, Berlin, Germany: for the cagA gene, middle region of the vacA gene and the last for the vacA gene signal region. The following primers and probes were used: cagA F (sense), cagA R (antisense), HPMGF+ (sense), HPMGR– (antisense), VAF1F+ (sense) and VAXR– (antisense). TaqMan hybridisation probes were prepared for cagA, vacA m1 and vacA m2-specific sequence detection. For the cagA assay, a FAM BBQ-labelled probe (530 nm) was used, for vacA middle region assay FAM BBQ labelled probe (530 nm) was used, and for vacA middle region assay FAM BBQ labelling was used for M1 TM probe (530 nm) and HEX BQ labelling for M2 TM probe (560 nm). TaqMan real-time PCR assays were run on a Light Cycler instrument, version 2.0 (six channels detection 530, 570, 610, 640, 670 and 705 nm). Commercial Light Cycler TaqMan Master (Roche Applied Science) was used: 15 μl of working Master Mix and 5 μl of sample DNA isolate per 20 μl capillary. For real-time PCR for vacA gene signal region, hybridisation probes S1a LC (LC 610), S1b LC (LC 640) and S2 LC (LC 705) were used together with commercial Light Cycler Fast Start DNA Master PLUS HybProbe (Roche Diagnostics) – 15 μl of working MasterMix and 5 μl of sample DNA isolate per 20 μl capillary.

2.3. Multiple intraluminal impedance

2.3.1. Catheters and position of the probe

We used Omega-MMS (Medical Measurement Systems) with the paediatric catheter with 7 impedance sensors and one pH-sensitive antimony electrodes (ZNIS+7R2.0; Versaflex® Z, Given Imaging (Los Angeles), LLC). The segment between each pair of sensors (15 mm), known as the impedance sensor spacing, corresponds to 1 recording impedance channel. The pH electrode is positioned in the centre of either most distal impedance sensor spacing. Calibration was performed before the introduction according to manufacturer's instructions. The MII-pH catheter was passed transnasally, the most proximal impedance ring (Z1) was located within a distance of 1 cm caudal to the entrance of the oesophagus (Fig. 1).

For proper positioning of the most proximal impedance ring Z1, we used the flexible laryngoscopy technique introduced through second nasal passage. The catheter was fixed to the nose and face. During the measurement, a daily regime was recorded. The examination was terminated after a 24 h recording when the measuring catheter was removed and the data were processed by automatic analysis in MMS software – database software, version 8.19i. All impedance recordings were visually analysed for the typical MII pattern of reflux events. This was defined as any retrograde-passing bolus detected by channel one with duration of more than 5 s. A priori variables selected for comparison were as follows: bolus height (the highest channel reached by the reflux); proximal reflux was defined as reflux that reached the most proximal impedance channel Z1; total reflux events; all types of reflux events which reached the most proximal impedance channel Z1; acid reflux events (pH < 4), weakly acid reflux events (pH 4.01–6.9), non-acid/alkaline reflux events (pH ≥ 7).

2.3.2. Statistical analysis

The whole data analysis was performed using the R-project (version 3.0.1; [22]). Comparison of number of the reflux episodes among patients with and without PCR positivity of HP was tested by Mann–Whitney *U*-test. Association of absence of reflux episodes with PCR positivity of HP was evaluated using odds ratios (OR) with 95% confidence intervals (CI95%). Multiple logistic regression was used to assess dependency of tested factors.

3. Results

All 30 patients included into the study underwent adenoidectomy for the clinical symptoms of adenoids. The median age was 64.1 months; range 25–104 months. In all cases, tissue samples were collected and the PCR procedure for HP genotypes detection was performed. PCR result were positive in 27 patients (90%); in 3 patients (10%) HP was not detected. Out of 27 HP DNA positive patient samples, cagA gene was detected in 9 (33.33%) and 18 patient samples (66.67%) were cagA negative.

The distribution of detailed genotypes is shown in Table 2 and Fig. 2. All patients in study group underwent examination of larynx, 50% of them using flexible endoscope under local anaesthesia and 50% using rigid optical system during the induction of general

Table 2
PCR detection of *Helicobacter pylori* in study ($n = 30$).

Patient		PCR detection of HP		Genotyping		
No.	Sex	Pos	Neg	cagA	vacAs	vacAm
2/1	M/F	+		–	s1a	m2
4/3	M/F	+		–	s1b	m1
6	M	+		–	s1b	m2
1	M	+		–	s2	m1
1	M	+		–	s2	m2
1/1	M/F	+		+	s1b	m1
2/1	M/F	+		+	s1b	m2
2	M	+		+	s1a	m1
2	F	+		+	s1a	m2
2/1	M/F	–	–	–	–	–

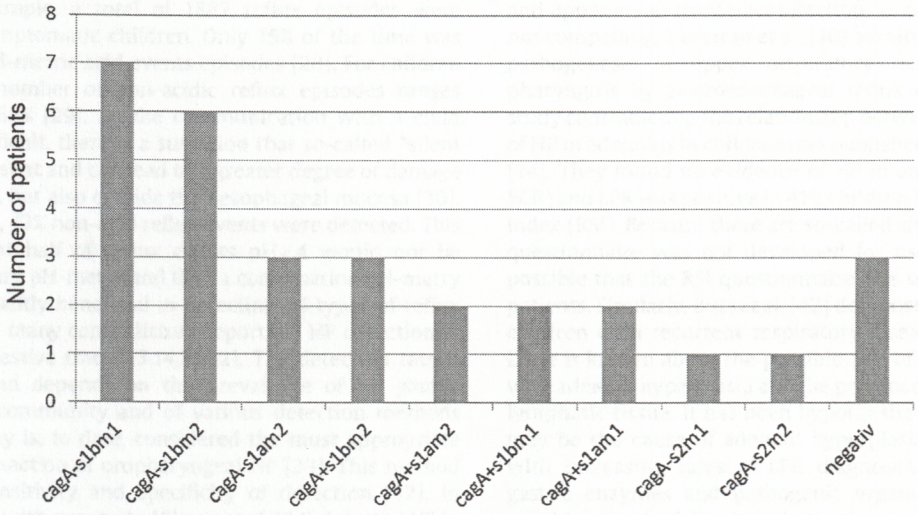


Fig. 2. Overview of detailed genotypes of *Helicobacter pylori* in study group.

Table 3
Reflux episodes detected using MII in patients with and without positivity of PCR for Hp.

	Patients with PCR positivity of *Helicobacter pylori* ($n=27$)	Patients with PCR negativity of *Helicobacter pylori* ($n=3$)	Test, *p*-value
Total number of reflux episodes, median (interquartile range)	35 (20–63)	0 (0–0.5)	Mann–Whitney *U*, 0.0056
Acid, median (interquartile range)	5.6 (3.1–29.4)	0 (0–0.005)	Mann–Whitney *U*, 0.0057
Weakly acid, median (interquartile range)	3.5 (0–9.4)	0 (0–0)	Mann–Whitney *U*, 0.014
Alkaline, median (interquartile range)	0 (0–0.32)	0 (0–0)	Mann–Whitney *U*, 0.31
Absence of reflux episodes (%)	1 (4%)	2 (67%)	Fisher Exact, 0.0069
Number of proximal reflux episodes, median (interquartile range)	2 (1–7)	0 (0–0)	Mann–Whitney *U*, 0.023
Acid	2 (0–5)	0 (0–0)	Mann–Whitney *U*, 0.046
Weakly acid	0 (0–2.5)	0 (0–0)	Mann–Whitney *U*, 0.17
Alkaline	0 (0–0)	0 (0–0)	Mann–Whitney *U*, 0.59
Absence of proximal reflux episodes (%)	5 (19%)	3 (3%)	Fisher Exact, 0.014

anaesthesia prior to adenoidectomy. The main symptoms and clinical findings of LPR in this group are shown in Table 1.

All 30 children included in the study underwent MII-pH detection of LPR. There were a total of 1363 reflux episodes, of which 777 (57.01%) were acid (pH ≤ 4) and 586 (42.99%) were non-acid (pH > 4.01). Out of 586 non-acid reflux only 33 (5.63%) reflux episodes were alkaline (pH ≥ 7), the rest 553 (94.37%) was weakly acid (pH 4.01–6.9).

The median of reflux episodes per hour was 1.89 (range 0–8.25 per hour). The median of the lowest pH was 0.99 pH (range 0.2–2.9 pH). Number of reflux episodes detected in upper position was 1203 (88.26%) and in supine position was 160 (11.74%). The number of reflux episodes reached the upper oesophagus (the highest impedance channel Z1) was 161 (11.81%), of which 118 (73.29%) were acid, 40 (24.85%) weakly acid and 3 (1.86%) alkaline. The results of reflux episodes in patients with and without PCR positivity are summarised in Table 3.

We found significant differences in the number of reflux episodes between patients with PCR positivity (median 35) and negativity (median 0) of HP (*p*-value of Mann–Whitney *U*-test 0.0056). Patients with PCR positivity of HP had significantly more reflux episodes reaching UES (*p*-value of Mann–Whitney *U*-test 0.023). The absence of reflux episodes was the only independent factor for PCR negativity of HP in the multiple logistic regression model.

4. Discussion

HP is one of the most common bacterial infections worldwide. The colonisation of gastric mucosa by HP is a well-established risk factor for atrophic or metaplastic gastritis, chronic gastritis and peptic ulcer diseases and constitutes a major risk factor for gastric adenocarcinoma and gastric mucosa associated lymphoid tissue (MALT) lymphoma [23]. The oral–oral route seems the most usual transmission mechanism among children. Paediatric LPR has gained increasing recognition over the past few years and it has been identified as a common disorder in infants and young children. The exposure of the upper aerodigestive tract to gastric contents in childhood may cause a variety of otolaryngologic manifestations [24]. GER is common in infants and young children and is usually physiological, although GER symptoms persists at 7.2% [25]. The prevalence of LPR/LPRD in children is not precisely known. MII-pH is a relatively new method for detecting GER/reflux episodes based on changes in electrical resistance to the flow of an electrical current between 2 electrodes placed on the MII probe, when a liquid, semisolid, or gas bolus moves between them. If impedance changes of a bolus appear first in the distal channels and proceed sequentially to the proximal channels, they indicate retrograde bolus movement, which is GER [26]. MII-pH can monitor not only acid reflux (pH < 4), but also weakly acid (pH 4.01–6.9), non-acid reflux (pH ≥ 7) and refluxed fluid composition (liquid, air, bolus). MII-pH is undoubtedly beneficial in the detection of pH-non-acidic reflux episodes even in the youngest children. Normal values of MII-pH monitoring have not yet been established and are the subject of many studies [27]. In our study, the most proximal impedance ring Z1 was located at a distance of 1 cm caudal to the entrance of the oesophagus and the flexible laryngoscopy technique was used for proper positioning. Through this process, X-ray control of the probe position was not performed. All types of reflux episodes can be measured. The prevalence of non-acidic reflux episodes was much higher than

expected. For example, a total of 1887 reflux episodes were detected in 50 symptomatic children. Only 15% of the time was correlated with pH-metric acid events episodes [28]. For children with GERD, the number of non-acidic reflux episodes ranges between 50 and 85% [29]. As the communication with a child patient is very difficult, there is a suspicion that so-called "silent reflux" may be present and can lead to a greater degree of damage of the oesophagus, but also outside the oesophageal mucosa [30]. In our study group, 43% non-acid reflux events were detected. This means that almost half of reflux events pH > 4 would not be detected by standard pH-metry and thus a combination pH-metry and MII is significantly beneficial in detecting all types of reflux episodes. They are many contradictory reports of HP detection in the upper aerodigestive tract [13,14,31,32]. The detection rate is highly variable and depends on the prevalence of HP gastric infections in the community and of various detection methods involved. PCR assay is, to date, considered the most appropriate method for the detection of oropharyngeal HP [33]. This method reported 100% sensitivity and specificity of detection [32]. In partial accordance with our study, Yilmaz et al. [34] detected HP in 64% of adenoid and tonsillar tissue specimens using PCR (16 S rRNA gene). Unver et al. [35] supposed a higher colonisation of HP in tonsillar and adenotonsillar tissue. They investigated 19 adenotonsillar samples by CLO test with 11 positive results. Nartova et al. [36] showed PCR positive detection of HP DNA in 80.90% patients who underwent tonsillectomy and they concluded that oropharyngeal lymphatic tissue is an extragastric reservoir of HP. In contrast, Toros et al. [37] investigated the adenotonsillar tissue in 84 children by RUT and histopathology. They found no HP colonisation in any specimens. Bonaventura et al. [38] did not describe tonsillar tissue as an extragastric reservoir of HP in dyspeptic patients. They detected zero tonsillar samples positive by PCR. Bitar et al. [31] found HP positivity by RUT and histology, but no positivity by nested PCR. In their study, no patient had a history of symptoms suggestive of LPR. Also, Eyigor et al. [39] detected zero positivity in 35 adenoid specimens (PCR detection of glmM gene). In our study, HP was detected in adenoids in 90% patients and in 30% genotype vacA s1m1 was identified. A high positivity of HP detection could be caused in a selection of patients because in this study, patients with LPR symptoms were chosen, particularly if we admit the possibility that HP may reach the nasopharynx by refluxed fluid movements. By this mechanism, HP could also be autoinoculated in nasopharyngeal lymphoid tissue. This is possibly why HP rises and settles in the lymphoid tissue of the nasopharynx. It is still not known how many reflux episodes are required to transfer HP to nasopharynx. The results of our study suggest (Table 3) that what is more important is the total number of reflux episodes than the number of episodes reaching the upper oesophagus (absence of reflux episodes, $p = 0.0069$, Fisher Exact test). Mucosa associated lymphoid tissue in the stomach is similar to lymphoid tissue of Waldeyer ring [32]. These conditions can be very favourable for the survival of HP and thus can promote inflammation and also contribute to the reactive hyperplasia of the lymphatic tissue in nasopharyngx. The exact mechanism of the induction of nasopharyngeal lymphatic tissue hyperplasia is unknown. The cause of hyperplasia of lymphoid tissue (adenoids) in the nasopharynx of children seems to be multifactorial. We speculate that a possible factor may be the host genotype interactions with the presence of HP. Another interesting fact is that the majority of cagA- negative genotypes (60%) lead to the idea that the main virulence factor in gastric infections cagA may not be of such importance in chronic inflammation and an alteration of immune system mechanisms which may contribute to adenoid hyperplasia in children. According to evidence-based guidelines from ESPGHAN and NASPGHAN for HP infection in children [40], current evidence for a causal relation between an infection of HP and upper respiratory tract infections or otitis media in children is not compelling. Kurtaran et al. [10] admitted possible role of HP in pathogenesis of upper respiratory tract diseases, especially pharyngitis by gastroesophageal reflux disease transmission. A study contradicting the relationship between LPR and the presence of HP in adenoids in children was published in 2011 by Hussey et al. [14]. They found no evidence of HP in any adenoid sample (RNA PCR) and LPR was positive in 41% children by using reflux symptom index (RSI). Because there are so-called silent refluxes and the RSI questionnaire was not developed for use in children [41], it is possible that the RSI questionnaire was underestimated by some patients. Similarly, Buts et al. [42] demonstrated that 18 of 30 (61%) children with recurrent respiratory disease have the silent GER. Little is known about the possible role of LPR or LPRD in children with adenoid hyperplasia and the presence of HP in this hypertrofic lymphatic tissue. It has been hypothesised [14,43] that the reflux may be the cause of adenoid hyperplasia in children, especially with increasing rates of LPR diagnoses. Refluxate can contain gastric enzymes and pathogenic organisms (HP) that may be capable of colonising lymphatic tissue of the nasopharynx and causing inflammation. This hypothesis is supported by the study of Keles et al. [15]. They reported that children with adenoid hyperplasia had a higher incidence of pharyngeal reflux than healthy children and the risk of development of adenoid hyperplasia was established 9.6 more times in children having pathologic pharyngeal reflux. Another study reported a correlation between the need for early adenoidectomy and the history of prolonged regurgitation in 153 children was published by Contencin et al. [44]. Meta-analysis reports (a total of 793 patients were studied in 16 reports) state that 24 h pH monitoring is a valuable method for LPR at 2 cm above the upper oesophageal sphincter. Up to 20% of asymptomatic normal individuals have small amounts of reflux to the pharynx when the upper probe is placed 2 cm above the UES. This confirms that the reflux episodes may be present in asymptomatic persons. The first paediatric study published by Skopnik et al. [45] used MII to describe and characterise impedance patterns of GER detected by pH monitoring in infants with symptoms of reflux disease. The catheter was placed transnasally with a total MII measuring length reaching from the hypopharynx to the cardia. In relation to the overall reflux events detected by MII, the pH monitoring demonstrated a sensitivity of 18.9% whereas the sensitivity of MII was 98.7% and more than 75% of all GER detected by MII reaching the pharyngeal space. Normal values of MII-pH have been reported in premature healthy infants [27]. In this study, oesophageal impedance was recorded for 24 h in 21 asymptomatic preterm neonates by a specially designed feeding tube that included 9 impedance electrodes. They reported a median of 70 reflux events, 25% of which were acid, 73% were weakly acidic and 2% were weakly alkaline. There are no universally accepted criteria for the diagnosis of LPR in various paediatric age groups, which is compounded by a lack of normative data regarding this. Up to now, there are no normative values that would regard the frequency of "physiologic" LPR events in children [26]. With increasing clinical use of MII-pH in children, normative data will soon be available. Our study did not include a control group of paediatric patients with/without evidence of the presence of HP in the adenoids without the typical symptoms of LPR. The main reason for not creating a control group was the difficulty in obtaining parental informed consent for the introduction of impedance catheters for 24 h in healthy children. The second reason is our view that the investigation of LPR by using MII-pH in asymptomatic patients is not considered justified. The mere presence of adenoids is not a sufficient indication for invasive and expensive tests. Our present study is only a pilot project. A certain limitation is the low numbers of enrolled patients; therefore, future studies will require a larger

patient population to confirm these results. Further studies will also tell us whether the location of the most proximal sensor is sensitive enough to detect all extraesophageal reflux episodes or whether it is necessary to place the most proximal sensor above the UES, where there is a risk that it will not capture the movement of fluid for excess space.

5. Conclusions

Our results may support the hypothesis that reflux episodes in childhood reaching UES may play an important role in transmission of HP into lymphoid tissue of the nasopharynx. Further studies investigating the relationship between LPR, HP and adenoid hyperplasia in children are essential before we can start concluding any treatment recommendations. MII-pH monitoring when proximal ring is placed one centimetre caudal to the entrance of the oesophagus could be a useful tool for evaluating proximal reflux episodes. However, larger studies, including control groups, are needed to find normal range values of LPR in children.

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Příloha 4

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ORIGINAL ARTICLE

Prevalence of Helicobacter pylori in adenotonsillar hypertrophy in children

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Abstract

Conclusion: Our results encourage the notion that the pharynx could be an extragastric reservoir of Helicobacter pylori (HP). The study confirmed the presence of HP in adenotonsillar tissue in children. It could have importance in the pathophysiology of upper respiratory diseases. However, its precise role in these processes remains unclear and requires further studies. *Objective:* A prospective study was carried out to evaluate the presence of HP in tonsillar and adenoid tissue in children. The study focused on real-time PCR analyzing CagA and VacA genotypes of HP strains. *Methods:* A total of 37 consecutive pediatric patients with adenotonsillar hypertrophy indicated for surgery were observed in a prospective study. Adenoidectomy and/or tonsillectomy was performed in each patient; 49 specimens were taken, 32 from adenoids and 17 from tonsils. The presence of HP and its genotype were tested in all samples by real-time PCR analysis. *Results:* Of 49 samples analyzed, 48 were positive for the presence of HP (98%), so only 1 sample was negative. While the genotype VacAs1bm2 was definitely dominant in adenoid tissue, wider distribution was observed in tonsillar tissue. Cag^+ strains represented one-fifth of all samples (21%).

Keywords: *Adenoid, tonsils, PCR, pharynx*

Introduction

Helicobacter pylori (HP) is a spiral, microaerophillic, Gram-negative bacterium that has been proven as a common gastric pathogen, causing gastric ulceration, gastritis, lymphoma, and even gastric cancer [1]. The prevalence of human HP infection has been estimated to range from 40% to 80% [2]. It has been declared a type I carcinogen by IARC WHO (1994, http://monographs.iarc.fr). Since its detection in the oral cavity and oropharynx, the potential pathogenic effect of HP in this area has been the subject of a number of studies [2–7].

The production of virulence factors determines the virulence of HP strains. The most important among these factors are CagA (cytotoxic associated gene A) and VacA (vacuolization cytotoxin A).

It has been shown that 60–70% of strains present the immunodominant CagA protein encoded by the group of genes called the CagA-PAI (pathogenicity island) [2]. The CagA-positive strains are associated with a significant higher risk for severe gastritis, ulcer disease, and gastric cancer compared with CagA-negative strains [8]. The other important gene is called the VacA gene, showing a marked polymorphism that is also carried by all strains of HP. Various combinations of VacA alleles generate different pathogenetic potentials [8]. The signal region, s1 or s2, and two mid-regions, m1 or m2, exist in all possible combinations. The finding of the presence of HP in

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the oropharyngeal tissue independently from the gastric infection confirms that HP can permanently colonize oropharyngeal tissue and the oropharyngeal presence is not caused merely by contamination by gastric content. This is further confirmed by the finding of different strains in the stomach and oropharynx in the same individuals [5,9]. The role of HP in the pathology of the pharynx such as chronic inflammation, adenotonsillar hypertrophy, and tumorogenesis still remains unclear. These contradictory findings correspond to the various findings of the mere presence of HP in the pharynx. While the adenoid and tonsillar tissue could present an extragastric reservoir of HP, some studies have refuted this possibility. Our research has been focused on real-time PCR analysis of CagA and VacA genotypes of HP strains in pediatric adenoid and tonsillar tissue.

Material and methods

The design of the study was prospective: 37 consecutive pediatric patients with adenotonsillar hypertrophy indicated for surgery were observed in a prospective study in the period from October 2011 to July 2012. They underwent different procedures according to clinical findings of hypertrophic tissue in upper airways, almost always in a combination of surgical procedures in one step. Adenoidectomy and/or tonsillectomy (tonsillotomy) was always performed in each patient. The ages of patients varied from 3 to 11 years at the time of surgery (average age 5.22 years, median 5 years). There were 28 males (aged 3–11 years, average 5.18 years, median 5 years) and 9 females (aged 3–10 years, average 5.22 years, median 5 years). A total of 49 specimens were taken, 32 from adenoid tissue and 17 from tonsillar tissue. We obtained samples from both areas in 12 patients. The presence of HP and its genotype was tested in all samples.

Specimen collection and transport

The samples from adenoids and/or tonsils were collected using sterile instruments at the beginning of surgery. All of them were immersed into Microtest R M4RT transport medium (Remel Inc., Lenexa, KS, USA) and transported to the laboratory for analysis.

Nucleic acid isolation and storage

Genomic DNA of HP was isolated using the MagNA Pure Compact System (Tegimenta AG, Rotkreuz, Switzerland) and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Basel, Switzerland), protocol, Total_NA 400_100", with pretreatment in MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics). Isolated nucleic acid specimens were stored at –80°C.

HP nucleic acid detection and genotyping

Genetic analysis of HP was based on specific primers and hybridization probes. Three real-time PCR assays had been developed in cooperation with TIB – Molbiol GmbH (Berlin, Germany): for the *cagA* gene, middle region of the *vacA* gene, and the *vacA* gene signal region. The following primers and probes were used: cag A F (sense), cag A R (antisense), HPMGF+ (sense), HPMGR– (antisense), VAF1F+ (sense), and VAXR– (antisense). Taq Man hybridization probes were prepared for *cagA*, *vacA* m1, and *vacA* m2 specific sequence detection. A FAM–BBQ-labelled probe (530 nm) was used for the *cagA* assay, FAM–BBQ labelling was used for the M1 TM – probe (530 nm) for the *vacA* middle region assay, and HEX–BBQ labelling for the M2 TM – probe (560 nm). Taq Man real-time PCR assays were run on a LightCycler instrument, version 2.0 (six channels detection: 530, 570, 610, 640, 670, and 705 nm). Commercial Light Cycler Taq Man Master (Roche Applied Science) was used: 15 µl of working Master Mix and 5 µl of sample DNA isolate per 20 µl capillary. For real-time PCR for *vacA* gene signal region hybridization probes S1a LC (LC 610), S1b LC (LC 640), and S2 LC (LC 705) were used together with commercial Light Cycler Fast Start DNA Master PLUS HybProbe (Roche Diagnostics) – 15 µl of working MasterMix and 5 µl of sample DNA isolate per 20 µl capillary.

Results

Forty-nine samples were taken from 37 patients. Of the 49 samples analyzed, 48 were positive for the presence of HP (98%), so only 1 sample was negative. We found six different genotypes of HP in various combinations (Table I). In all, 32 samples from adenoids and 17 from tonsils were analysed (Table II). While the genotype VacAs1bm2 was definitely dominant in adenoid tissue, in half with a Cag combination, a wider distribution of HP genotypes was observed in the tonsillar tissue (Figure 1). Twelve patients with samples from both areas – tonsils and adenoid – had the same genotype in eight cases in both localizations, while there were four patients with a different genotype in the tonsils and adenoid tissue. Cag^+ strains represented one-fifth of all samples (21%) and the findings in adenoids and tonsils were similar (22% and 19%, respectively). No correlation was found between subjective complaints, objective examination, and laboratory findings of certain types of HP genotypes.

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Table I. Single Helicobacter pylori (HP) genotypes and their combinations in positive samples.

	CagA			VacAs1am2	VacAs1bm1	VacAs1bm2	VacAs2m1	VacAs2m2
HP genotype	10			7	11	17	3	10
Genotype distribution	CagA + VacAs1bm1	CagA + VacAs1bm2	CagA + VacAs2m2	VacAs1am2	VacAs1bm1	VacAs1bm2	VacAs2m1	VacAs2m2
Total (48 samples)	1	8	1	7	10	9	3	9

Discussion

There have been several published studies concerning the presence of HP in oropharyngeal lymphatic tissue [2,4,6,10–19]. Despite this, no conclusive results regarding the role of HP in this region have been ascertained. Different invasive and non-invasive methods have been used to detect HP worldwide [20]. The most sensitive method seems to be the PCR technique, which has provided the best results in oropharyngeal lymphatic tissue [5,7,9]. Most methods commonly used in the gastric area are not fully suitable for extragastric sites. The rapid urease test (RUT) and immunohistochemistry can frequently give false-positive results [2]. The choice of method is consequently the most important element for detection of HP presence and can be crucial for study results. Whether the adenotonsillar tissue is the extragastric reservoir of the HP still remains unclear. Many authors have dealt with detection of HP in adenoid or tonsillar tissue and the results vary from 0 to 84% of positive samples. Culture, RUT, immunohistochemistry, Campylobacter-like organism (CLO) tests, histopathology, and PCR have been used as detection methods [2]. We suppose that the low capture of HP presence in the pharyngeal region reported in some papers is due to the use of less suitable methods. Histology with different staining methods demonstrates high sensitivity and specificity rates (up to 96%) in gastric mucosa specimens. However, there is low specificity in the oral cavity and pharynx, where other bacterial strains are often detected with the possibility to inhibit the growth of HP, and this makes detection of HP difficult [2]. Other common oropharyngeal bacteria (urease-producing species) somewhat devalue RUT or CLO tests based on the detection of urease production. This is again a very useful method in gastric mucosa specimens, but has a high false-positive rate in the oropharynx. Culture achieves 80–90% sensitivity and 90–100% specificity rates and is currently accepted as the gold standard for the diagnosis of gastric HP. However, culture from the oral cavity and oropharynx has been proven to be very difficult as there is often overgrowth by other bacterial species and special techniques may be necessary [2]. Immunohistochemistry to detect extragastric HP is used mostly experimentally and other authors have described false-positive results using this method [17]. Molecular methods show the best potential for detection of HP and its strains in oropharyngeal lymphatic tissue. For this reason, the PCR technique was selected for the present study and we compared our results with those of other authors using similar techniques. Di Bonaventura et al. used PCR for the first time to detect HP in tonsillar tissue, but no positive results were obtained [10]. Other authors concluded the same finding in the years following and no presence in the adenoid and tonsillar tissue has been detected by their studies. In contrast, other papers have proved the positivity of HP in the oropharynx in up to 64% of the tested samples. An overview of papers using the PCR method to detect HP in the adenoid and tonsillar tissue is summarized in Table III. In a recent paper Hussey et al. contradicted previous studies with positive

Table II. Genotypes and their combinations in adenoids and tonsils.

Genotype in adenoids	CagA + VacAs1bm1	CagA + VacAs1bm2	CagA + VacAs2m2	VacAs1am2	VacAs1bm1	VacAs1bm2	VacAs2m1	VacAs2m2
32 samples	0	7	0	5	4	7	2	7
Genotype in tonsils	CagA + VacAs1bm1	CagA + VacAs1bm2	CagA + VacAs2m2	VacAs1am2	VacAs1bm1	VacAs1bm2	VacAs2m1	VacAs2m2
17 samples (1 negative)	1	1	1	2	6	2	1	2

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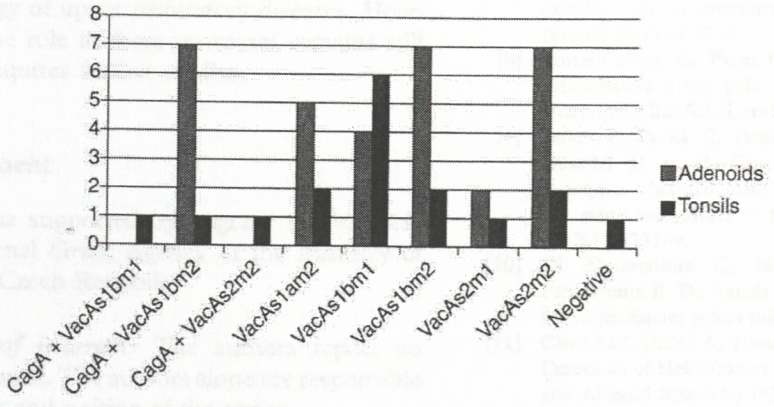


Figure 1. Distribution of Helicobacter pylori (HP) genotypes in adenoids and tonsils.

findings of HP in the adenoids because of negative results of their samples examined by nested RT-PCR [18]. However, the authors tried to detect the RNA of HP in adenoid tissue. We confirmed this method to be highly sensitive and specific, but it requires extremely precise techniques and correct implementation. In our opinion, the fragility of sample elaboration in this method could be the reason for false-negative results; moreover, this opinion a minority position worldwide. The latest study published in 2012 deals with the relationship of HP to chronic tonsillitis; the PCR method was again confirmed to be the best choice and the presence of HP in the tonsils was proved [19].

Conclusion

Our results encourage the notion that the pharynx could be an extragastric reservoir of HP. The study confirmed the presence of HP in adenotonsillar tissue in children. This could have importance in the

Table III. Overview of studies aiming to detect the presence of Helicobacter pylori (HP) using PCR and their results.

Author	Year	No. of subjects	Specimen	Diagnostic method	No. of specimens positive for HP
Di Bonaventura et al. [10]	2001	75	Tonsillar swab and biopsy	PCR	0 (0%)
Cirak et al. [11]	2003	23	Tonsillar and adenoid tissue	PCR (16S ribosomal RNA, CagA)	7 (30%) positive, 5 of them (71%) positive for CagA gene
Yilmaz et. al. [12].	2005	38	Adenoid tissue, middle ear effusion	PCR (23S ribosomal RNA)	12 (67%) in middle ear effusion, 1 (5%) in adenoid tissue
Bitar et al. [13]	2005	25	Adenoid tissue	Nested PCR (UreA)	0 (0%) positive
Bulut et al. [4]	2006	71	Tonsillar and adenoid tissue	PCR (CagA - glmM gen)	29 (24.6%) positive, 17 of them (58.6%) positive for CagA gene
Bitar et al. [14]	2006	28	Adenoid tissue, middle ear effusion	PCR (ureC-gen)	0 (0%) positive
Yilmaz et. al. [15].	2006	22	Adenoid and tonsillar tissue, middle ear effusion, promontorium mucosa	PCR (16S RNA)	Middle ear 7 (32%) positive, adenoid and tonsillar tissue 14 (64%) positive
Eyigor et. al. [16].	2009	55	35 adenoid tissue, 20 tonsillar tissue	PCR (glmM gen)	0 (0%) positive
Vilarinho et. al. [17].	2010	62	Adenoid tissue and tonsillar tissue	PCR - DNA (vacA gen)	0 (0%) positive
Abdel-Monem et al. [6]	2011	20	Adenoid and tonsillar tissue	PCR (ureC-gen)	5 (16.6%) positive
Hussey et al. [18]	2011	93	Adenoid tissue	Nested RT-PCR (RNA)	1 positive (Candidatus Wolinella africanus)
Farivar et al. [19]	2012	103	Tonsillar tissue	Scorpion RT-PCR	21.35% positive

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pathophysiology of upper respiratory diseases. However, its precise role in these processes remains still unclear and requires further studies.

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Příloha 5

Comparison of *Helicobacter Pylori* Genotypes Obtained from the Oropharynx and Stomach of the Same Individuals – A Pilot Study

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Key words: *Helicobacter pylori* – Real-time PCR – Genotyping – Oropharynx – Stomach – Comparison

Abstract: *Helicobacter pylori* has been recently detected in the oral cavity and oropharynx. However, the role it plays in oral and oropharyngeal pathogenesis remains unclear. The virulence of *H. pylori* strains can be distinguished according to the virulence factors genes carried. Our research has been focused on real-time PCR analysis of *cagA* and *vacA* genes of *H. pylori* strains in tonsils and tonsillar squamous cell cancer and their comparison with *H. pylori* strains obtained from

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the gastric mucosa of the same patients. Urea breath test (UBT) test was used to detect a gastric *H. pylori* infection in 20 patients with previously proven *H. pylori* in the oropharynx. Genotyping of *H. pylori* in gastric biopsies was performed in patients with positive gastric infection. Out of 20 patients positive for oropharyngeal *H. pylori*, 8 were positive for concurrent gastric *H. pylori* infection. In 6 of them gastric biopsies were obtained. Comparison of oropharyngeal and stomach *H. pylori* genotypes showed important differences. Four of 6 patients had different *H. pylori* strains in the oropharynx and stomach. The differences were found in *cagA* gene as well as in *vacA* gene. The finding of oral presence of *H. pylori* without concurrent stomach infection was confirmed using UBT. The results show that more than one *H. pylori* strain can be present in oropharynx and stomach in the same patient. The oropharyngeal infection seems to be independent to the gastric infection.

Introduction

Helicobacter pylori is a well known gastric pathogen (Peterson, 1991; Israel and Peek, 2001). *H. pylori* is a declared type I carcinogen by IARC (1994). Since it was detected in the oral cavity (Nguyen et al., 1993; Riggio and Lennon, 1999; Karczewska et al., 2002) and oropharynx (Bulut et al., 2006; Pavlik et al., 2007; Abdel-Monem et al., 2011) a hypothesis about its possible influence in oropharyngeal pathogenesis is being considered (Rubin et al. 2003; Lukes et al., 2008). *H. pylori* is spread from person to person by oral-oral or faecal-oral route (Brown, 2000). Assuming the oral cavity and oropharynx as a gateway of infection, we can expect that in the oral cavity and oropharynx of the same individual we should find *H. pylori* strains of the same genotype. The virulence of *H. pylori* strains differs according to the production of several virulence factors. The most important virulence factors, which were described as associated with gastric pathogenesis, are CagA (cytotoxic associated gene A) and VacA (vacuolizating cytotoxin A). Genome sequence analysis of *H. pylori* DNA led to the identification of genes encoding these virulence factors. The immunodominant CagA protein is encoded by the group of genes called the cagA-PAI (pathogenicity island), which is present in 60–70% of strains (Mobley, 1996). *H. pylori cagA+* strains are associated with a significantly increased risk for severe gastritis, atrophic gastritis, peptic ulcer disease and distal gastric cancer compared with *cagA–* strains (Portal-Celhay and Perez-Perez, 2006). All *H. pylori* strains carry the *vacA* gene. Significant polymorphism of this gene exists. *VacA* alleles possess one of two types of signal region, s1 or s2, and one of two mid-regions, m1 or m2, occurring in all possible combinations. *H. pylori* strains with different forms of *vacA* have different pathogenetic potential (Portal-Celhay and Perez-Perez, 2006). The question whether the finding of oropharyngeal *H. pylori* indicates permanent colonization or it is only a sign of contamination of this area by gastric juices still remains unclear.

The presence of different genotypes of *H. pylori* in the oral cavity and stomach is now being considered (Wang et al., 2002; Loster et al., 2006). The comparison of oropharyngeal and stomach genotypes has not previously been studied. The aim of this study was to find out whether one person could host different *H. pylori* strains in different locations. The other aim was to determine whether the oropharyngeal presence could be caused by contamination by gastric contents or it is a permanent colonization which could be independent from gastric infection. Previous research in Department of Otorhinolaryngology, Head and Neck Surgery, First Faculty of Medicine, Charles University in Prague, has been focused on real-time PCR analyzing *cagA* and *vacA* genotypes of *H. pylori* strains in tonsils and tonsillar squamous cell cancer (Lukes, 2010). In 6 cases we have compared the DNA sequences of *H. pylori* strains obtained from oropharyngeal area with the strains obtained from gastric mucosa of the same patients.

Material and Methods

Collection of clinical specimens

The study was approved by the appropriate ethics committee, and informed written consent was obtained from every participant.

Twenty patients with previously proven *H. pylori* in the oropharynx were selected for routine urea breath testing (UBT) for the presence of *H. pylori* in the stomach. The patients were part of a study on the detection of oropharyngeal *H. pylori* (Lukes, 2010). They underwent surgery and subsequently the tissue samples were analysed for the presence of *H. pylori*. Indications for surgery were tonsillar spinocellular cancer (SCC) (9 patients), obstructive sleep apnea syndrome (OSAS) (5 patients) and chronic tonsillitis (6 patients).

The tonsillar specimens were collected using sterile instruments at the beginning of surgery, immediately after insertion of endotracheal tube, prior to application of local anaesthetics or disinfection substances into the oral cavity. Biopsies were immersed into Microtest^{R} M4RT transport media (Remel Inc., USA) and transported to the laboratory for real-time PCR analysis. After positive results for *H. pylori* presence in tonsillar tissue, the presence of gastric infection was confirmed using UBT.

Eight patients were found positive for *H. pylori* by UBT. Six of them (3 with cancer, 2 with OSAS and 1 with tonsillitis) gave their consent for gastroscopy (remnant two patients declined any further examination). The gastroscopy was performed subsequently at the Department of Gastroenterology, Hospital of Merciful Sisters of St. Charles Borromeo, Prague. Gastric mucosa samples were taken from the antrum and body of the stomach and immersed in Microtest^{R} M4RT transport media for real-time PCR analysis. The genotyping of gastric strains was performed. The gastric genotypes were compared to oropharyngeal genotypes in the same individuals.

Preparation of genomic DNA

H. pylori DNA isolation was performed on MagNA Pure Compact System (Tegimenta AG, Rotkreuz, Switzerland) using Protocol "Total_NA 400_100" and MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Basel, Switzerland) with pre-treatment in MagNA Pure Bacteria Lysis Buffer (Roche Diagnostics, Basel, Switzerland). Isolated nucleic acid specimens were stored in –80 °C until genotyping assays were performed.

PCR amplification and genotyping

For genotyping, 3 real-time PCR TaqMan assays had been developed in cooperation with TIB-Molbiol Berlin, Germany; one for the *cagA* gene, the second for the *vacA* gene middle region and the last one for the *vacA* gene signal region. Primers used for PCR detection of *H. pylori* were: cagA F (sense), cagA R (antisense), HPMGF+ (sense), HPMGR– (antisense), VA1F (sense) and VA1R– (antisense) according to Atherton et al. (1995) and van Doorn et al. (1998, 1999) (Table 1).

TaqMan hybridisation probes were developed for *cagA, vacA m1* and *vacA m2* specific sequence detection (Table 2). For the *cagA* assay FAM-BBQ labelled probe was used (detection 530 nm), for *vacA* middle region assay FAM-BBQ labelling was used for M1 TM-probe (530 nm) and HEX-BBQ labelling for M2 TM-probe (560 nm).

TaqMan^{R} real-time PCR assays were run on a LightCycler^{R} instrument, version 2.0 (six channel detection: 530, 570, 610, 640, 670 and 705 nm). Commercial LightCycler^{R} TaqMan^{R} Master (Roche Applied Science, Basel, Switzerland) was used – 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

For real-time PCR for *vacA* gene signal region hybridisation probes S1a LC (LC610), S1b LC (LC640) and S2 LC (LC705) were used together with commercial LightCycler^{R} FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostics, Basel, Switzerland) – 15 µl of MasterMix including primers and probes and 5 µl of sample DNA isolate per 20 µl capillary.

The real-time PCR assay program consisted of 45 cycles with an initial denaturation step at 95 °C for 10 minutes in first cycle and than for 10 s in remnant cycles. Annealing temperatures were selected 58 and 68 °C for 10, 15 or 20 s according to used probes. An extension step at 72 °C for 45 s was followed by real-time detection at proper wavelength according to used probe.

Results

Only 8 of 20 patients with proven oropharyngeal presence of *H. pylori* showed concurrent presence of gastric infection by UBT.

In gastric biopsies the presence of *H. pylori* was proven by real-time PCR in all 6 patients who underwent endoscopy. Endoscopic examination showed mucosal changes typical for gastric inflammation in four patients, the inflammation was

clinically asymptomatic. None of the patients admitted any gastroduodenal complaints.

Comparison of gastric and oropharyngeal genotypes showed important differences. Results are shown in Table 3. Three patients (nos. 1, 2 and 5) had the *cagA* positive *H. pylori* strain in the stomach but negative in the oropharynx. Two of them also showed differences in the *vacA* gene S region (nos. 1 and 5). Patient no. 6 showed a difference in S region alone. In two patients (nos. 3 and 4) the *H. pylori* strains from the oropharynx and stomach were identical.

Table 1 – PCR primers for amplification of *cagA* and *vacA* sequences

Gene	Primer	No. of nucleotides	Sequence
cagA	cagA F	24	5'-TTG ACC AAC AAC CAC AAA CCG AAG-3'
	cagA R	22	5'-CTT CCC TTA ATT GCG AGA TTC C-3'
vacA s	VA1F	21	5'-ATG GAA ATA CAA CAA ACA CAC-3'
	VA1R	19	5'-CTG CTT GAA TGC GCC AAA C-3'
vacA m	HPMGF	21	5'-CAG AGC CAC TTT CAA TAA CGA-3'
	HPMGR	21	5'-CGT CCA AAT AAT TCC AAG GG-3'

Table 2 – PCR probes for detection of *cagA* and *vacA* sequences

Gene	Type	No. of nucleotides	Sequence	Detection (nm)
cagA	cag_TM	28	6FAM-ATA ACG CTG TCG CTT CAT ACG ATC CTG A-BBQ	530
vacA s	S1a_LC	21	LC Red610-GCR TTR GTC AGC ATC ACA CCG-PH	610
	S1b_LC	21	LC Red640-GCG TTG ATT AGY KCC ATA CCG-PH	640
	S2_LC	21	LC Red705-GCT AAY ACG CCA AAY GAT CCC-PH	705
vacA m	M1_TM	30	6FAM-ACC ACC ATT ACC CGT ATC AAT ACC TTT AAA-BBQ	530
	M2_TM	26	HEX-CTA GTG TTT AGC CCG TTA TCG CTC TT-BBQ	560

Table 3 – Results of *Helicobacter pylori* investigation

No.	Diagnosis	Oropharyngeal genotype	Gastric genotype	Gastritis
1.	tonsillar cancer	cagA– S1aM1	cagA+ S1a/bM1	–
2.	tonsillar cancer	cagA– S1bM2	cagA+ S1bM2	–
3.	tonsillar cancer	cagA+ S1bM2	cagA+ S1bM2	+
4.	OSAS	cagA– S1aM2	cagA– S1aM2	+
5.	OSAS	cagA– S1aM2	cagA+ S1a/bM2	+
6.	tonsillitis	cagA+ S1bM1	cagA+ S1aM1	+

OSAS – obstructive sleep apnea syndrome

Discussion

The results of the present study showed that oropharyngeal lymphatic tissue can harbour *H. pylori* strains independently to the stomach infection. Real-time PCR genotyping of oropharyngeal and gastric strains in patients with present infection in both areas showed that different *H. pylori* strains can be found in oropharynx and stomach of the same individual. Four of 6 investigated patients had different *H. pylori* strains in oropharynx and stomach. The differences were found in *cagA* gene as well as in *vacA* gene.

These results are in concordance with the first study that compared cytotoxin genotypes of *H. pylori* between the stomach and oral cavity published by Wang et al. (2002). Authors used saliva for genotyping of oral *H. pylori*. They showed that more than one *H. pylori* strain may exist in the saliva and the stomach of the same individual. According to our knowledge, our study is the first to compare *H. pylori* genotypes between the stomach and tonsillar tissue biopsies. In this study the patients were selected according to the oropharyngeal *H. pylori* infection. None of them admitted any gastrointestinal complaints. Gastroscopy showed the presence of gastritis in the majority of patients although all were clinically asymptomatic. This differs from previous studies, which were primarily focused on patients with gastric disease. Only a few other studies focused on comparison of *H. pylori* strains in the oral cavity and stomach. Shames et al. (1989), Khandaker et al. (1993), Zhang and Lu (1997) and Hu et al. (2002) showed evidence, that both sites are infected with the same *H. pylori* strain. These authors used a DNA fingerprinting method, PCR adopted single strand conformation polymorphism (SSCP) or restriction endonuclease analysis respectively. None of these studies compared the different gene sequences of VacA gene. The variance of our results could be explained by different diagnostic methods used. Nevertheless real-time PCR genotyping is considered to be highly specific with high degree of reliability (Burgers et al., 2008).

Another study that used real-time PCR to detect *H. pylori* in the oral cavity (Martinez-Gomis et al., 2006) did not find any individual positive for oral *H. pylori*.

The authors did not mention in their paper the use of any transport medium for preserving *H. pylori* DNA, which seems to be very important for successful PCR detection (Pavlik et al., 2007).

The presence of oropharyngeal *H. pylori* without concurrent stomach infection was confirmed using UBT and is in accordance with previously published data by Burgers et al. (2008), who showed that *H. pylori* can be present in the oral cavity independently of stomach colonization. UBT has proved to be one of the most accurate methods for assessing gastric *H. pylori* status (Gisbert and Pajares, 2004). Loster et al. (2006) used a modified UBT for detecting oral *H. pylori*. The solution containing of ^{13}C-urea was kept in the oral cavity for 5 min to detect oral urease producing bacterium. When using a standard UBT procedure, the solution of ^{13}C-urea is swallowed immediately, so the time of contact with oropharyngeal mucosa is too short detect oropharyngeal *H. pylori*.

The finding of presence of *H. pylori* in the oropharyngeal tissue independently from the gastric infection confirms that *H. pylori* can permanently colonize oropharyngeal tissue and the oropharyngeal presence is not caused only by contamination by gastric contents. This is further confirmed by the finding of different strains in the stomach and oropharynx in the same individuals.

On the other hand the results re-open the question of *H. pylori* transmission. When we consider the oral cavity and oropharynx as a gateway for infection of stomach, we would expect to find identical strains in both areas. This inconsistency must be resolved by further analysis.

Although *H. pylori* is proved to be highly pathogenic bacterium with carcinogenic potential, the data about oropharyngeal pathogenesis are insufficient. Culture of *H. pylori* from oral and oropharyngeal samples is extremely difficult (reviewed by Lukes et al., 2008). PCR techniques are not able to solve the question of viability of bacterial cells. The lower number of *cagA* positive oropharyngeal strains compared to gastric strains could shed some light on the question of oropharyngeal pathogenesis, nevertheless the number of investigated patients is very small. Further improvement of investigation methods such as culture is needed for obtaining more information about possible involvement of *H. pylori* in oropharyngeal diseases.

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