Molecular typing: what is the role in pediatric infection control?

MOREL, Anne-Sophie

Abstract
Les différentes méthodes de typisation ont considérablement enrichi la réalisation d'investigations des infections nosocomiales, particulièrement aux soins intensifs de pédiatrie. La phénotypisation fournit les premiers indices suspects d'une épidémie et complète la typisation moléculaire. En l'absence de "gold standard", l'électrophorèse à champs pulsés et l'amplification au hasard de segments d'ADN sont les techniques de génotypisation les plus fréquemment utilisées au laboratoire de microbiologie clinique. La typisation binaire, encore réservée aux laboratoires de recherche, semble très prometteuse. Sans se substituer aux données cliniques et épidémiologiques, la typisation moléculaire joue un rôle complémentaire. Elle implique le concept "d'épidémiologie moléculaire". Ce travail présente une revue des différentes méthodes de typisation, illustrée d'exemples de leurs rôles dans le contrôle des infections nosocomiales en pédiatrie. Leurs forces et faiblesses y sont analysées comparativement. Le compte-rendu d'un cas de transmission "nosocomiale" de "Staphylococcus aureus" résistant [...]
“Molecular Typing: What is The Role in Pediatric Infection Control?”

Thèse préparée sous la direction du Professeur Susanne SUTER

par

Anne-Sophie MOREL

de

Lancy (GE)

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INTRODUCTION

Les différentes méthodes de typisation ont considérablement enrichi la réalisation d’investigations des infections nosocomiales, en général et en particulier aux soins intensifs de pédiatrie. La phénotypisation fournit les premiers indices suspects d’une épidémie et complète la typisation moléculaire (génotypisation).

En l’absence de «gold standard», l’électrophorèse à champs pulsés et l’amplification au hasard de segments d’ADN sont les techniques de génotypisation les plus fréquemment utilisées au laboratoire de microbiologie clinique. La typisation binaire, encore réservée aux laboratoires de recherche, semble très prometteuse. Sans se substituer aux données cliniques et épidémiologiques, la typisation moléculaire joue un rôle complémentaire. Elle implique le concept «d’épidémiologie moléculaire».

Ce travail présente une revue des différentes méthodes de typisation, illustrée d’exemples de leurs rôles dans le contrôle des infections nosocomiales en pédiatrie. Leurs forces et faiblesses y sont analysées comparativement.

Dans une deuxième partie est présenté le compte rendu d’un cas de transmission «nosocomiale» de *Staphylococcus aureus* résistant à la méticilline d’une mère à trois de ses quadruplés en milieu hospitalier.
RÉSUMÉ EN FRANÇAIS

LA TYPISATION MOLÉCULAIRE: QUEL RÔLE EN PÉDIATRIE?

Les infections nosocomiales, principales complications d’une hospitalisation, sont responsables d’un important taux de morbidité et mortalité en pédiatrie. Leur fréquence est inversément proportionnelle à l’âge de l’enfant et concerne des populations de patients dits «à risque» (nouveaux-nés, enfants atteints de maladies chroniques, ou hospitalisés aux soins intensifs). Associée aux études épidémiologiques classiques, la typisation est utile pour la prévention, le diagnostic et le traitement des infections nosocomiales. Elle compare rigoureusement les isolats provenant de patients colonisés et/ ou infectés ainsi que de leur environnement afin de déterminer s’il s’agit de la même souche (souche épidémique). La typisation moléculaire est devenue un outil indispensable pour améliorer notre compréhension de la transmission des nombreux pathogènes nosocomiaux impliqués en pédiatrie.

Typiser des micro-organismes signifie analyser de multiples isolats cliniques ou environnementaux au sein d’une espèce donnée (bactéries, champignons, plus rarement parasites et virus) afin de préciser si ces isolats sont issus d’un même clone ou dérivés de différents clones. La typisation est basée sur la présomption que les isolats clonalement apparentés partagent des caractéristiques. Celles-ci doivent être différenciées de celles des isolats non-apparentés. L’utilité d’une caractéristique dépend de sa stabilité dans la souche ainsi que de sa diversité au sein de l’espèce. La diversité reflète l’accumulation d’événements génétiques. Pour être largement utile, un système de typisation devrait être applicable à un grand nombre d’organismes, reproductible, relativement bon marché, simple à réaliser et à interpréter. Les résultats devraient être disponibles rapidement afin d’assurer la prise en charge thérapeutique efficace du patient et l’instauration de mesures de surveillance de l’infection. Actuellement, il n’existe aucune méthode de typisation idéale remplissant tous ces critères. La phénotypisation examine les caractéristiques exprimées par l’organisme (expression de gènes: taille, composition...). Le test de susceptibilité aux antibiotiques (antibiogramme), la biotypisation et la sérotypisation sont les plus utilisés. Plusieurs techniques ont été incorporées dans des kits.
commerciaux et/ou automatisées. Généralement bon marché, largement disponible, rapide, simple à réaliser et à interpréter, la phénotypisation souffre d’une reproductibilité faible à moyenne, ainsi que d’un modeste pouvoir discriminatif; de nombreuses espèces ne sont pas typisables par ces méthodes. Malgré ses limitations, elle révèle souvent les prémices d’une épidémie et complète la génotypisation ou typisation moléculaire. Cette dernière examine la structure génétique de l’organisme (phénomènes liés aux acides nucléiques) en analysant directement l’ADN. Doté d’un pouvoir discriminatif puissant, d’une reproductibilité très élevée, et de la typisabilité théorique de toutes les espèces (exception: l’analyse de plasmides), la génotypisation a largement supplanté la phénotypisation. La typisation moléculaire est surtout représentée au laboratoire de microbiologie clinique par l’électrophorèse à champs pulsés (Pulsed-field gel electrophoresis: PFGE) et l’amplification au hasard de segments d’ADN (Ramdom amplified polymorphic DNA: RAPD), techniques cependant coûteuses et exigentes en temps.

De chaque investigation d’épidémie nosocomiale émane un enseignement («leçon») convertible en nouvelles stratégies de prévention et de traitement. Les rôles de la typisation moléculaire dans le contrôle des infections nosocomiales en pédiatrie sont multiples.

Premièrement, la typisation a identifié de nouvelles sources d’épidémies incluant par exemple, Enterobacter cloacae multirésistant détecté dans l’eau distillée des supports ventilatoires mécaniques par l’analyse du profil plasmidique, Candida parapsilosis identifié par caryotypisation comme contaminant de la glycérine utilisée pour l’administration de suppositoires, ou Pseudomonas aeruginosa multirésistant identifié par PFGE dans des jouets de bain. Le traitement de l’infection associé à l’élimination de la source et des mesures d’hygiène simples (isolement, port de blouse, masque et gants, hygiène des mains à bon escient: friction hydro-alcoolique) limitent la dissémination du pathogène et jugulent l’épidémie. Par ailleurs, la typisation permet de comprendre la transmission entre patient: la ribotypisation et le RAPD ont confirmé l’hypothèse d’une transmission entre patients de Burkholderia cepacia chez les patients mucoviscidosiques (CF). La transmission hospitalière, la transmission ambulatoire ainsi que certains contextes sociaux tels que les camps CF ont été reconnus par ces techniques.
Les recommandations de séparer les patients CF infectés avec ce germe ont réduit avec succès de nouvelles acquisitions de *B. cepacia* parmi ces enfants.

La typisation permet également de démontrer le rôle du personnel soignant et médical dans les infections croisées. L’emploi du PFGE a prouvé que le port d’ongles artificiels parmis le personnel soignant et médical est lié à la transmission de *P. aeruginosa* à des nouveau-nés hospitalisés aux soins intensifs. En toute logique, ce matériel cosmétique devrait être proscrit chez tout personnel soignant et médical.

De plus, l’épidémiologie moléculaire peut être utilisée pour déterminer précisément des réservoirs potentiels. Exemple: deux nouveau-nés de très bas poids de naissance hospitalisés aux soins intensifs présentent un syndrome d’exfoliation staphylococcique. La rareté de cette entité chez cette population de patients à haut risque d’infection nosocomiale rend indispensable l’identification de tous les patients ou personnels porteurs asymptomatiques afin d’éviter la dissémination du pathogène. Le PFGE a distingué dans ce cas la souche épidémique de *Staphylococcus aureus* sensible à la méticilline, et l’éradication du portage asymptomatique d’un autre nouveau-né et de trois employés de l’unité a limité le risque d’épidémie secondaire.

Enfin, la typisation a clarifié le rôle de la flore endogène dans le développement de maladies systémiques. Le tractus gastrointestinal des patients oncologiques peut être rapidement colonisé par des entérocoques résistants à la vancomycine. Le PFGE a confirmé que le même clone colonisait leur tractus digestif et «transloquait», provoquant une maladie invasive. De nombreux experts recommandent dorénavant le dépistage de ces pathogènes multirésistants chez ces patients à haut risque. Des observations similaires ont été faites pour *C. albicans* et *E. cloacae* dont la colonisation digestive a été suivie d’infection sanguine avec le même clone.

En conclusion, la typisation moléculaire est devenue un outil indispensable pour le contrôle des infections nosocomiales en général et en pédiatrie en particulier. Elle permet de contribuer à l’identification de l’origine d’une épidémie, de confirmer sa clonalité et de prouver sa réalité en distinguant les infections concomitantes mais indépendantes (souches endémiques/ cas sporadiques) des épidémies causées par un clone (souche épidémique) et des pseudo-épidémies. La typisation n’est donc pas le substitut d’une investigation épidémiologique classique et ne devrait être utilisée qu’en complément, intégrée dans le contexte clinique, en associant une équipe multidisciplinaire composée de cliniciens, d’épidémiologistes et de collaborateurs du laboratoire de microbiologie clinique.

N.B. Cette revue ne traite volontairement pas du rôle de la génotypisation des souches de staphylocoques à coagulase négative. Ces principaux germes pathogènes en réanimation pédiatrique restent un sujet extrêmement difficile que peu de groupes dans la littérature médico-scientifique ont abordé avec systématique.
LIST OF ABREVIATIONS

BT: Binary typing
CF: cystic fibrosis
CMV: Cytomegalovirus
DNA: desoxyribonucleic acid
ERSA: erythromycin-resistant *Staphylococcus aureus*
ESBL *Klebsiella pneumoniae*: Extended-spectrum beta-lactamase producing *K. pneumoniae*
GI tract: gastrointestinal tract
HCW: healthcare workers (including physicians)
HSV: Herpes simplex virus
IC: infection control
IS: insertion sequence
MRSA: methicillin-resistant *S. aureus*
MSSA: methicillin-sensitive *S. aureus*
NI: nosocomial infection
NICU: Neonatal Intensive Care Unit
PCR: Polymerase chain reaction
PFGE: Pulsed-field gel electrophoresis
PICU: Pediatric Intensive Care Unit
RAPD: Random amplified polymorphic DNA
REA DNA: restriction enzyme analysis of chromosomal DNA
REAP: restriction enzyme analysis of plasmid
RFLPs: Restriction fragment length polymorphism’s
SSSS: staphylococcal scalded skin syndrome
VRE: vancomycin-resistant *Enterococcus* spp.
A nosocomial infection (NI) is defined as an infection that is not clinically apparent or incubating on admission to hospital and believed to be acquired during the hospital stay. Usually the nosocomial pathogen can be eradicated by appropriate treatment.

Colonization is the presence of a microorganism in or on a host with growth and multiplication that is not associated with clinical symptoms. Colonization is sometimes very difficult to eradicate and represents reservoirs for potential pathogens.

An isolate refers to a pure culture of pathogen obtained by subculture of a single colony from a primary isolation plate, presumed to arise from a single organism.

A strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic or genotypic characteristics or both.

Clones, or genetically related isolates, are isolates that are indistinguishable from each other by a variety of genetic tests or that are so similar that they are presumed to be derived from a common parent.

Epidemiologically related isolates are isolates cultured from specimens collected from patients, or the environment during a certain time or in a well-defined area as part of an epidemiologic investigation that suggests that the isolates may be derived from a common source.

An outbreak is the increased incidence of an infectious condition in a specific place during a given period that is above the baseline rate for that place and time. Thus outbreak strains are isolates of the same species that are both epidemiologically and genetically related.
**Pseudo-outbreak** occurs when the existence of an epidemic is postulated and none is actually present. It may be due to laboratory contamination of clinical specimens.

**Endemic strains** are isolates that are recovered frequently from infected patients in a particular healthcare setting or community and that are indistinguishable or closely related to each other by typing methods but for which no direct or epidemiologic linkage can be demonstrated.

**A reservoir** of an infecting agent is the place where the organism maintains its presence in the hospital setting. Example: sinks for *Pseudomonas aeruginosa*.

**The source** is the place from which the infectious agent directly contacts its victims. Reservoirs and sources may be identical or may differ. Example: hands of healthcare workers for *Staphylococcus aureus*.

**The incidence** is the number of new cases of a disease in a particular population during a specific period of time.
PART I

Long version of the article entitled:

A. ABSTRACT

Typing methodologies have greatly improved our ability to perform epidemiologic investigations of nosocomial infections, in all setting including the Neonatal and Pediatric Intensive Care Unit (NICU/ PICU). Phenotyping techniques may provide the first clue that an outbreak is occurring and be used to complement molecular typing. Pulsed-field gel electrophoresis and Random amplified polymorphic DNA are the most widely used techniques to investigate nosocomial infections by clinical microbiology laboratories. Binary typing is an emerging molecular technique with much promise, but is currently limited to research laboratories. Molecular typing should be used in conjunction with clinical and epidemiologic data and should supplement and not substitute for a carefully conducted epidemiologic investigation.
B. INTRODUCTION

Nosocomial infections (NI) are the major complications of hospital stay and represent an important cause of morbidity and mortality. Pediatric patient populations such as newborns (especially premature babies), children admitted to the PICU or the NICU, children suffering from chronic diseases such as Cystic Fibrosis (CF), as well as oncologic patients or children in chronic care facilities are at high risk of NI. These patients should be detected as early as possible, assessed for colonization or infection, and appropriate infection control measures should be put in place to minimize transmission between patients.

Hospital epidemiologists are frequently asked to investigate increased rates of NI in pediatric population. Bacteria are mostly involved followed by fungi, which are of increasing concern. Nosocomial viral infections are of particular concern in pediatric compared with adult patients. The pediatric rate of NI as well as the site of infection vary with the units in which the child is admitted. Rates vary from 1% in general pediatric units to 23.6% in PICU with an overall incidence of 2.5%. Bloodstream infections occur mostly in the NICU, lower respiratory tract infections in the PICU, and viral gastrointestinal diseases in general pediatric units. Numerous pathogens may be involved: S. aureus, including coagulase negative staphylococci, and methicillin-resistant S. aureus (MRSA), P. aeruginosa, Burkholderia cepacia (former name for Pseudomonas cepacia), Enterococcus faecium, Enterococcus faecalis, including vancomycin-resistant strains (VRE), multiply antibiotic resistant Gram negative bacilli such as extended-spectrum beta-lactamase producing Klebsiella pneumoniae (ESBL K. pneumoniae), Candida spp., rotavirus and respiratory syncytial virus.

For decades, hospital epidemiologists have utilized typing strategies to assist their investigations. Typing microorganisms is the analysis of multiple isolates from clinical or environmental sites within a given species of organisms (mostly bacteria or fungi, rarely parasites or virus) to determine if these isolates are clonally related, i.e. they represent strains derived from the same parent versus multiple parents. Typing is useful in Pediatrics for prevention, diagnosis and appropriate treatment
of nosocomial infections (NI). It rigorously compares isolates from colonized/infected patients with isolates from their environment in order to determine the origin of the strain. Typing can be used to identify the source of an outbreak or to distinguish unrelated strains from endemic or epidemic strains and pseudo-outbreak(s). 

For the past three decades numerous new molecular techniques have been developed and supplanted old phenotypic systems in order to track the epidemiology of outbreaks and expand our study of infectious diseases. Molecular typing has been an important tool in Pediatrics for epidemiologists to evaluate the transmission of nosocomial pathogens, for clinicians to study the pathogenesis of infection (virulence factors) and for researchers to develop new insights in both epidemiology and pathogenesis of infection. The concept of «molecular epidemiology» has been born.

Molecular typing has become an invaluable tool in improving our understanding of transmission of pathogens. Typing has identified new sources of outbreaks including environment, patient-to-patient transmission and the role of healthcare workers (HCW) in cross-infection. During an investigation, molecular epidemiology can be used to accurately detect additional patient reservoirs, i.e., patients colonized with the epidemic strain. Finally, typing has clarified the role of endogenous flora in the development of invasive disease.

However, typing methods are not a surrogate for classical epidemiological methods and should be used in complement and considered within the overall clinical context. Such investigations require a multidisciplinary team consisting of epidemiologists, clinicians and microbiology laboratory personnel.

The first part of this work presents a descriptive and chronological review of typing methodologies illustrated with examples of their role in pediatric infection control. A focused assessment of the most currently used typing techniques in clinical microbiology laboratories is described by comparing their strengths and weaknesses (Table 1, p. 53).
C. STEPS IN INVESTIGATING AN OUTBREAK 31-32

The practice of modern epidemiology requires a multidisciplinary team consisting of epidemiologists, clinicians and microbiology laboratory personnel. Their respective missions are to perform an active surveillance of high-risk patients for NI, investigate potential outbreaks and their source, implement a strategy for controlling the outbreak and prevent its spread. The laboratory’s challenge is to identify the responsible pathogen and use the best typing method to perform a reliable analysis that differentiates epidemiologically related isolates from unrelated isolates of the same species (concomitant infections; do not belong to the outbreak). Each collaborator has to work conjointly through the 4 following steps:

Step 1. The recognition of the problem
- Early warning from the laboratory or increased infection noted by clinicians or infection control (IC) surveillance.
- Make a case definition for example; a patient in a particular unit with a positive culture for a particular pathogen isolated from any body site during the past year (can be colonized and/or infected).
- Calculation of the incidence rate and comparison with previous rates.
- Identification and storage of isolates.

Step 2. The characterization of the outbreak
- Where? Type of unit: NICU/ PICU, ward, delivery room, well baby nursery, entire unit, single room, proximity of other cases, type of environment?
- When? New outbreak, previous outbreak (year, month ago), last surveillance.
- What? What is the responsible pathogen, is it unique? Type of infection? Typing isolates*, how many strains involved? Increased recovery of other pathogens?
- How? Type of transmission, repeat cultures.
Step 3. Form and test an hypothesis

- Literature review.
- Mode of spread.
- Potential reservoirs and vectors: culture environment, perform surveillance cultures in patients and staff (*typing*).
- Test the hypothesis.

Step 4. Implementation of control and surveillance measures

- Prevention of further cases: isolation, cohorting, assignment of designated HCW, eradicate colonization.
- Evaluation of the efficiency of control measures, ongoing surveillance (*typing*).
- Storage of isolates.

(* : molecular typing would play an important role)

Pseudo-outbreaks

However, although these steps should be followed in each outbreak investigation, misdiagnosis may occur leading to «pseudo-outbreak». This phenomenon includes clusters of «false» infections or false clusters of true infections. Pseudo-outbreaks can increase length of stay and exposes the patient to potential undesirable side-effects of unnecessary treatment and raises health costs by all the implementation needed.

Pseudo-outbreaks of NI can be due to the multidisciplinary team (incomplete/ unbalanced) or to the failure/ mistake or skipping of one of the steps in the investigation process described above. The clinician can make an erroneous diagnosis or fail to distinguish community-acquired infection from NI. A positive culture may represent colonization rather than infection. Moreover, contamination may occur during specimen collection, or contamination of the specimen may occur during transportation or processing in the laboratory through media/ solutions or equipment issue. The use of an inadequate typing method can also be responsible for wrong conclusions as will be described below. Finally, increased surveillance efficiency and improved laboratory techniques for
identification may be sources of pseudo-outbreaks as well. The problem is usually recognized when a disparity appears between the laboratory results and the patient’s clinical status. The majority of reported pseudo-outbreaks have been pseudobacteremia involving a single species. Fortunately, few cases have been reported in the pediatric setting and molecular typing has been successfully used in their investigations: for example, ribotyping (see p. 38) proved a pseudo-infection associated with intrinsic contamination of a povidone-iodine solution with \textit{B. cepacia} in a NICU. A significant increased rate of recovery of this pathogen from cultures of peritoneal fluid and blood was reported in this unit. The isolates from the children and those from the povidone-iodine solution had the same molecular type.

Beside misdiagnosis, misinterpretation of results occurs when the multidisciplinary team is not complete. The following example illustrates the phenomenon very well: two outbreaks of erythromycin-resistant \textit{S. aureus} (ERSA) were reported in a well baby nursery. Since the source was incorrectly identified on the first episode (a nurse was assumed to be the carrier on the basis of the epidemiologic report and the antibiogram only), recurrence occurred (after the nurse retired). Molecular typing was able to prove that the nurse was a carrier of an unrelated strain of ERSA and that two other employees (present on the first outbreak) were actually infected with the epidemic strain.

Molecular epidemiology combines classical epidemiology study with molecular typing. One without the other leads to either misdiagnosis and/or misinterpretation.
D. BASIC THEORETICAL/ TECHNICAL BACKGROUND IN MOLECULAR BIOLOGY

Typing methodologies are generally divided into phenotypic and genotypic techniques. The first examines the observed characteristics of the organism (gene expression). The second examines its genetic structure (chromosomal or plasmid DNA).

The central hypothesis involving typing investigations is that isolates obtained from an epidemiologic cluster or during an infection in a single patient are clonally related (have a common precursor). As epidemiologically unrelated isolates of the same species differ in several characteristics, clonally related isolates should share characteristics by which they can be differentiated from unrelated isolates. 1 The usefulness of a characteristic for typing depends on its stability within a strain as well as its diversity within the species. Diversity reflects the accumulation of genetic events such as random, non-lethal mutations, or acquisition of DNA. 1 However, since the genome is less subject to natural variations than the phenotype of microorganisms, genotyping has largely replaced phenotyping to evaluate the relatedness of isolates obtained from an epidemiologic cluster.

Molecular typing strategies involve two basic technical approaches. The first one is an electrophoresis-based approach, which allows the separation of elements (plasmid, or chromosomal DNA, which are the molecules most often studied for genotyping) according to their molecular size. The procedure includes cell lysis to extract the molecule(s) to study from the isolate, followed by separation of fragments with conventional agarose gel electrophoresis. The separation involves a unidirectional constant voltage-electrophoresis «pulling» negatively charged DNA molecules smaller than 40-50 Kb in size through an agarose matrix toward a fixed positive charge (the DNA migrates in a sizeand conformation dependant way). 34 The result is a banding pattern visualized after staining the gel with edithium bromide, which binds to DNA molecule and fluoresces when illuminated with ultra-violet light. However, chromosomal DNA is too large to be analysed in its entirety and must be cut into smaller pieces with restriction endonucleases for its study. These enzymes cleave DNA at specific nucleotide recognition sequences. The generated product consists
of hundreds of fragments ranging from 0.5 to 50 Kb in length. They are then separated by size by conventional electrophoresis and visualized with UV light as above, producing a profile or «fingerprint» of the DNA (REAP Fig. 3 p. 31 and REA DNA Fig. 7 p. 37). Fragment length variation can be detected directly by electrophoretic separation of the restricted chromosome in agarose gel-based systems with or without additional use specific DNA probe (Southern blotting p. 35). Conventional agarose gel electrophoresis is used for many typing methods such as protein cell electrophoresis (p. 26), plasmid analysis (p. 29), restriction enzyme analysis of plasmid (REAP p. 32) and restriction enzyme analysis of chromosomal DNA (REA DNA p. 33), restriction length fragment polymorphism’s (RLFPs p. 35), ribotyping (p. 38), polymerase chain reaction-ribotyping (PCR-ribotyping p. 49), PCR-RFLPs (p. 49), random amplified polymorphic DNA (RAPD p. 49), and binary typing (p. 51).

The second approach uses nucleic acid amplification procedures whose prototype is the polymerase chain reaction (PCR). Technically, PCR uses two primers (18 to 20 base pairs in length corresponding to a known DNA sequence) to define the initiation site of replication. Replication starts with denaturing the double-stranded DNA template. The primers bind to each strand of the template and the thermostable DNA polymerase synthesizes the complementary strand. The newly replicated templates are then denatured again allowing a new round of replication. Within 3 to 4 hours, 20 to 30 cycles are completed yielding millions of copies of a particular DNA segment with high fidelity. The product (amplicon) is visualized directly on a gel. PCR-RFLPs, PCR-ribotyping, RAPD and rep-PCR (pp. 49-50) are multiple variants of this principle which are also combined with electrophoretic-based methods.
E. CRITERIA TO EVALUATE TYPING SYSTEMS

To be widely useful a typing method should be applicable to a broad range of microorganisms, reproducible, relatively inexpensive, easy to perform and interpret. Results should be available quickly (i.e. within days) to be relevant to patient care and IC.

**Typeability**: ability to obtain an unambiguous result for each isolate analysed. Non-typeable isolates give either a null or an uninterpretable result. Therefore, a useful typing method has a low rate of «non-typeable» isolates within a given species.

**Reproducibility**: ability to obtain the same result when the same strain is tested repeatedly. Reproducibility is influenced by technical factors such as day to day variation (in vitro reproducibility) and biological factors such as the variation in the stability of the typing characteristic (in vivo reproducibility).

**Discriminatory power** differentiates between unrelated strains but varies from species to species, which may vary in their genetic stability under the influence of different selective pressures.

**Ease of interpretation** implies that multiple users of the typing system obtain the same results and analyze the results in the same way in order to achieve the same conclusions. Ideally a method has guidelines for its interpretation.

**Ease of performance** relates to the rapidity, convenience, cost of equipment and personnel needed to perform a technique.
Some important points to remember

1) Organisms cannot be tested unless they are available. It is easy to discard saved isolates but impossible to recover them from the trash.

2) Use techniques that are readily available and inexpensive: progress to more sophisticated tests only if the initial results and the epidemiologic data indicate that further investigation is necessary.

3) Typing results usually require interpretation about sameness or relatedness, not a positive versus negative result.

4) Results obtained with different typing systems do not always correlate completely because different typing methods evaluate different traits.

5) Be suspicious if the typing system shows that all organisms are the same even though not epidemiologically related, but also if it shows that they are different.

6) Isolates that are different by one or more typing systems are unlikely to represent the same strain even though they appear to be epidemiologically related.

7) Organisms may have unstable genetic properties (e.g., expression of different genes, plasmid content). When evaluating typing systems, consider whether the trait assessed is stable.

8) Some organisms (e.g., MRSA) have limited genetic diversity. Thus, epidemiologically unrelated organisms may appear the same regardless of the typing system and may be falsely interpreted to represent an outbreak.

9) In general, if a method has worked for a particular organism (e.g., *Escherichia coli*), it is likely to work for a related organism (e.g., other *Enterobacteriaceae*).

10) Any single method is not always the best method. Which method is best will differ by the organism tested, the epidemiologic problem under study, or the research project. Often the best methods is the one that works.

11) Some laboratories will do molecular typing for a fee. In addition, some researchers are willing to evaluate organisms if they can be a coauthor on a manuscript.
F. PHENOTYPIC SYSTEMS

Phenotypic systems were the first methods used for epidemic purposes. They take advantage of biochemical, physiological and biological phenomena. Many of them have been incorporated into commercial kits or automated devices. Usually cheap, widely available, fast, easy to perform and to interpret, phenotyping suffers from a lack of reproducibility and discriminatory power leading to a limited usefulness for epidemiologic studies. The major disadvantage is that stability of most phenotypes varies under the influence of ubiquitous environmental selective pressures. Moreover, non-typeable isolates are common (Table 1 p. 53). Despite their limitations, phenotypic characteristics are usually the first clue that an outbreak may be occurring. Furthermore, many of these traits are standardly reported by clinical microbiology laboratories (antibiogram, serotyping).

1. Biotyping

Biotyping looks at a panel of metabolic activities expressed by an isolate such as morphology, environmental tolerance (specific growth media, pH, temperature) and biochemical reactions. This typing system allows species identification (mainly used for taxonomy) and helps to distinguish among strains of organisms. All strains are typeable. However, the method is limited by the poor ability to differentiate among strains within a species (poor discriminatory power) and a poor reproducibility due mainly to variations in the composition of the reagents (in vitro variations) and biological variations (in vivo variations).

_S. aureus_ is a well known nosocomial pathogen in Pediatrics. It is part of the normal skin flora, and up to 30% of children and 50% of adults are colonized in their anteriors nares. Postnatal skin colonization in babies occurs within days to weeks after birth. _S. aureus_ represents the second most common cause of NI in the NICU, whose main transmission is from patient-to-patient through HCW’ transient hands carriage. _S. aureus_ is responsible for a broad spectrum of clinical syndromes ranging from benign skin and soft tissue infections (impetigo, furuncle, pustulosis, wound infections), to osteomyelitis, arthritis and...
life-threatening diseases such as septicemia, endocarditis, toxic shock syndrome/ staphylococcal scalded skin syndrome (SSSS). Risk factors for colonization/ infection with this pathogen include prolonged hospitalization, underlying condition, needle and antibiotics use, understaffing and overcrowding. As an example, the biotype of *S. aureus* is presented in Table 2.

**Table 2: Phenotypic traits of *S. aureus***

<table>
<thead>
<tr>
<th>Phenotypic characteristics</th>
<th><em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Coccus</td>
</tr>
<tr>
<td>Gram</td>
<td>+</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
</tr>
<tr>
<td>Coagulase</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol fermentation</td>
<td>+</td>
</tr>
<tr>
<td>Hemolysis on blood agar plate</td>
<td>+</td>
</tr>
<tr>
<td>Salt tolerance</td>
<td>+</td>
</tr>
<tr>
<td>Anaerobic acid production from glucose</td>
<td>+</td>
</tr>
<tr>
<td>Acid production from glycerol + erythromycin</td>
<td>+</td>
</tr>
<tr>
<td>Susceptibility to lysostaphine</td>
<td>+</td>
</tr>
<tr>
<td>DNase production</td>
<td>+</td>
</tr>
</tbody>
</table>
2. Antimicrobial susceptibility testing (the antibiogram)

The antibiogram tests bacterial isolates for susceptibility to a panel of antimicrobial agents. The procedure exists in two forms (disk diffusion, which correlates the growth inhibition diameter with susceptibility or resistance to antimicrobial agents, and antimicrobial dilution, which looks at the turbidity of the growth of isolates by densitometry). Antimicrobial susceptibility testing is performed to aid in the selection of appropriate therapy. The usefulness of the antibiogram for epidemiologic studies is limited. Antibiotic resistance is common and can result from many genetic mechanisms, e.g. spontaneous mutations, acquisition or loss of resistance genes via plasmids and acquisition of transposons from other strains or species. Antibiotic resistance is a consequence of selective pressures within the hospital generated by change in medical practice and antibiotic use. Antimicrobial resistance can be unstable which leads to poor discriminatory power and lack of reproducibility (Table 1 p. 53).

However, the antibiogram remains an important method to detect multiresistant nosocomial pathogens such as MRSA, VRE and ESBL \textit{K. pneumoniae}.

Plasmid-mediated extended-spectrum beta-lactamases (ESBLs) have been described since the mid 1980’s in many gram-negative bacteria (mainly enterobacteria), but are particularly prevalent among hospital-acquired \textit{K. pneumoniae} isolates. ESBL \textit{K. pneumoniae}-producing strains can cause life-threatening infection in children. These enzymes, which confer high-grade resistance to third generation cephalosporins and extended-spectrum resistance to aminoglycosides (amikacin; also plasmid encoded), arose following the introduction of third generation cephalosporins. Most strains harboring ESBLs are inhibited by a beta-lactamase-inhibitor combination such as amoxycillin plus clavulanate, tazobactam or sulbactam. \cite{41} Risk factors for colonization/ infection with ESBL \textit{K. pneumoniae} include admission to the PICU or the NICU, recent surgery, instrumentation, prolonged hospitalization, antibiotic exposure to extended-spectrum beta-lactam agents. The main reservoir for ESBLs-producing pathogens is the GI tract of the patients, especially for patients in the NICU and immunocompromized children in whom bacteria may translocate and produce invasive disease. \cite{42} The double-disk synergy test is a reference method used to detect these strains. \cite{43} There is synergy
between a disk of amoxicillin plus clavulanate and disks of third generation cephalosporin (ceftriaxone, cefotaxim, ceftazidime) and monobactam (aztreonam) \(^43\) (Fig. 1). Most outbreaks caused by ESBL \textit{K. pneumoniae} have first been detected in the pediatric setting by the antibiogram and then proven by molecular typing techniques. \(^42,43\)

**Figure 1: Double-disk synergy test**

Fig. 1: The figure shows an ESBL producing \textit{K. pneumoniae} isolate tested by the double-disk synergy test: there is a synergy between a central disk of amoxycillin plus clavulanate (X) and disks of third generation cephalosporins (A= cefotaxime, B= ceftazidime, D= ceftriaxone) and a disk of monobactam (C= aztreonam). A clear-cut extension of the edge of the peripheral disk inhibition zone toward the clavulanate containing disk is interpreted as synergy (in French, this characteristic is referred to “image en bouchon de Champagne” as described by Vincent Jarlier, microbiologist). \(^43\)
3. Serotyping

Serotyping uses antibodies to detect unique antigenic determinants expressed on the cell surface of microorganisms.

This established typing method remains a useful tool for *Salmonella* and *Streptococcus pneumoniae* but is not generally applicable to other species with the exception of enterotoxigenic *Escherichia coli* species (ETEC) including *E. coli* 0157:H7 responsible for outbreaks of hemolytic colitis with hemolytic uremic syndrome in children (Table 1 p. 53).

4. Phage typing

Phage typing is of great historic interest. The concept was first developed in the 1940’s for typing *S. aureus* after noting that some *S. aureus* contained temperate phages, which lysed other bacteria of the same species. This technique has been the method of choice for investigating *S. aureus* epidemiology.

Phage typing is unique in that the International Subcommittee on Phage Typing Staphylococci has standardized this typing method. A set of 23 approved phages was recommended to characterize the strains by their pattern of resistance or susceptibility to the standard set of phages. Areas of lysis caused by each phage are noted as either a strong or a weak reaction. Phage typing has been used for epidemiological purposes to type other pathogens such as *Mycobacterium tuberculosis*, *Acinetobacter* species, *Clostridium perfringens*, *C. difficile*, *E. coli*, *Enterobacter* spp., *Enterococci* spp., *Helicobacter pylori*, *Proteus*, *Stenotrophomonas maltophilia*, *Salmonella* spp., and *Streptococci*. For a phenotypic assay, phage typing has good discriminatory power, but phages are not widely available and maintenance of phage stocks is costly. Moreover, some species are non-typeable with this technique (Table 1 p. 53).

5. Susceptibility to bacteriocins

Bacteriocins are proteins produced by bacteria and encoded by plasmid. They can inhibit the growth of closely related species. The producer strain is usually resistant. An isolate is assessed for susceptibility to a panel of bactericidal peptides produced by selected strains. This typing method is limited of course to bacteria and rarely used because of limitations similar to those of the phage typing technique.
6. Protein electrophoresis

This typing method allows the study of bacterial populations with limited application for epidemiologic analysis of clinical isolates. Protein electrophoresis can be used to delineate the origin of a microbial strain and to establish a relationship between isolates. The technique is very demanding and moderately discriminating. However, it has been successfully used to study a Candida albicans outbreak in a NICU. Different techniques are derived from this method.

- Material from the whole cell or cell surface is isolated and separated by sodium dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-Page) which separates proteins according to their respective molecular weight by conventional electrophoretic technique and stains the proteins to visualize them. The result is a complex pattern of about 50 major bands leading to difficult and subjective interpretation.

- Immunoblotting or Western-blotting is a variation of the above method, performed by transferring the separated cellular product from the SDS-Page to a nitrocellulose membrane (blotting) and exposing the separated proteins to antisera which contains reactive antibodies (an enzyme-labeled IgG) and a substrate to produce a color change. All strains are typeable. Results need some experience to be interpreted but lead to fewer bands than the whole cell electrophoresis.

- Multilocus enzyme electrophoresis (MLEE) differentiates organisms by studying the differences in the electrophoretic mobilities of numerous soluble metabolic enzyme(s) in a starch gel. The location of each enzyme is detected by exposing the gel to a substrate that produces a color reaction with that specific enzyme. Enzyme mobilities reflect variations in several genetic loci and yields an electrophoretic type (ET). Each distinct ET represents a multilocus genotype. Although individual enzymes may be absent (null) the evaluation of multiple enzymes ensures that all isolates are typeable.

- Zymotyping: is a variation of MLEE based on the differences in the electrophoretic properties of the bacterial esterase enzyme used for S. aureus.
7. Resistotyping and Yeast killer typing

These two phenotypic techniques are useful to type fungi/ yeasts but not exclusively. Nosocomial fungal infection especially caused by *Candida* sp. in the pediatric setting is of increasing concern. Not only the genus, but species is important to identify correctly these pathogens. *Candida* spp. are recovered from soil, food, and hospital environment. They are normal flora of the female genital tract and gastrointestinal (GI) tract as well. *Candida* are important opportunistic pathogens in Pediatrics. Clinical syndromes include late onset sepsis, central venous catheter bloodstream infection, meningitis, renal, splenic, or liver abscesses, endophthalmitis, osteomyelitis, arthritis, pneumonia or invasive dermatitis. The mortality rate caused by fungal infection is as high as 15 to 50%. Risk factors have been well documented for *C. albicans* colonization/ infection and include prematurity, immunocompromized hospitalized children treated with broad spectrum antibiotics, use of glucocorticoids, chemotherapy or H2 blockers, patients with invasive devices as well as preexisting fungal colonization.

Resistotyping is used to test the ability of fungal isolates to grow on solid media containing known concentration of various chemical inhibitors such as malachite green, boric acid, sodium arsenate, copper sulfate or selenite. Different dilutions enable a titration of the effect of each inhibitor on control strains. Resistotypes are based on the presence or absence of growth on each medium. Reproducibility is good but dependent on careful attention to media and strains preparation. Resistotyping is not an appropriate typing method for laboratories that do not routinely type fungi.

Killer yeast typing use a panel of killer yeasts to selectively inhibit the growth of fungal or bacterial isolates. The yeast killer phenomenon is represented by the production by yeasts, such as *Candida*, of exotoxins with antimicrobial activity mediated by specific cell wall receptors on susceptible microorganisms. The producer strain is resistant (immune to the activity of their own killer toxins). These exotoxins can kill susceptible cells from the same or congenic species. The typing is based on the presence or absence of growth inhibition of the isolates in the presence of a set of killer toxin-producing yeast (technique equivalent in yeasts to bacteriocines in bacteria).
However, «phenotypic switching» is a phenomenon involving changes in colony morphology as well as changes in cellular physiology of fungi that may occur and significantly affect the reproducibility of biotyping methods for these organisms.
G. GENOTYPING

Several DNA-based (genetic) methods have been developed with improved discriminatory power and reproducibility compared with phenotypic systems. Genotyping is useful for a broad range of organisms and can type strains that are non-typeable by phenotypic strategies. First confined to research laboratories, these molecular technologies are nowadays increasingly performed in clinical laboratories. They are aimed at detecting polymorphisms at the level of nucleic acids, as the genome of each individual strain is unique. Genetic variation can be documented by different molecular techniques. Although molecular typing methods represent the highest resolution techniques, they remain time consuming and require costly equipment (Table 1 p. 53).

1. Plasmid analysis

Plasmids are extrachromosomal double-stranded circular DNA mobile elements that replicate independently of the bacterial chromosome in the cytoplasm. Plasmid profile analysis was one of the first molecular techniques used for epidemiologic studies. It differentiates isolates by the number and size of plasmids (Fig. 2). This method is easy to perform and interpret, and relatively inexpensive. However, there are several disadvantages to plasmid typing. Strains lacking plasmids such as *P. aeruginosa* and Streptococci are non-typeable. Plasmids are mobile extrachromosomal elements and can be spontaneously lost or gained (by conjugation or transduction). Under the influence of selective pressures such as occurs in the presence of antibiotics or for virulence factors (toxins, adhesins), plasmids can be exchanged between strains. These genetic events can lead to lack of intrastrain stability (poor discriminatory power) as epidemiologically related isolates can have different plasmid profiles.
Fig. 2: The figure shows 2 bacteria A and B. A contains 2 plasmids (plasmid 1 and plasmid 2) and B possesses 3 plasmids (plasmid 3, plasmid 4 and plasmid 5). The plasmids are extracted from a lysed bacterial cell and detected by conventional agarose gel electrophoresis. The plasmid profile analysis of bacteria A and B shows 2 different banding patterns according to the number and size of plasmids.

Plasmid profile analysis also lacks reproducibility as plasmids can exist as different forms (monomers, supercoiled, nicked as well as linear molecules), which may migrate differently through the gel; bands of seemingly different sizes may in fact represent the same plasmid (Fig. 3).

The discriminatory power and the reproducibility of plasmid profile analysis can be improved by using restriction endonucleases, which digest (cut) specific nucleotide sequences within the
plasmid. This procedure, Restriction Enzyme Analysis of Plasmid (REAP) or plasmid fingerprinting, is simple, quickly performed, relatively inexpensive and correlates well with phenotypic procedures (Fig. 4). While REAP increases the discriminatory power of plasmid analysis, it is not useful for organisms with multiple large plasmids as many restriction fragments are produced or with low copy plasmids, which can make interpretation difficult. REAP is considered the method of choice for plasmid analysis 1, but at present the use of plasmid analysis is limited (Table 1 p. 53). The technique can be improved by southern hybridisation (p.35).

**Figure 3: Plasmid Analysis**

![Plasmid Analysis](image)

Fig.3: The figure shows the plasmid profile analysis from paired (A, B) *E. coli* strains isolated from 4 patients with recurrent bacteriuria: patient #22, #24, #25, and #37. The paired isolates from patients #22, #24 and #37 have different plasmid profiles. Only patient #25 shows an identical banding pattern consistent with relapse. However, the banding patterns for patients 22 and 24 only differ by 1 band confirming the lack of discriminatory power for this technique. Reprinted with permission. 4
Figure 4: Restriction enzyme analysis of Plasmid (REAP)

Fig. 4: The figure shows the restriction enzyme analysis of 2 different plasmids: plasmid 1 and plasmid 2 which possess different restriction sites leading to respectively 4 restriction fragments for plasmid 1 (fragments A, B, C, and D) and 2 restriction fragments for plasmid 2 (fragment E and F). REAP creates one or more bands of different sizes according to the number of restriction site on the plasmid. The number and sizes of the resulting restriction fragments are then compared on a conventional agarose gel electrophoresis. 3
However, plasmid analysis has been used to prove that an outbreak of sepsis due to *E. cloacae* in a PICU was due to contamination of the distilled water used for ventilatory support. Furthermore, in combination with other molecular techniques, REAP proved the genetic relatedness of strains causing an outbreak of neonatal meningitis due to *E. sakazakii* and an increased incidence of infection/colonization with ESBL *K. pneumoniae* in a NICU. However, REAP itself was clearly not sufficient alone to type the isolates of ESBL *K. pneumoniae*.

Plasmid profile analysis was also the typing method previously used to type *M. tuberculosis* before the high resolution genotyping techniques were available (see p. 40). A humorous example is the multistate outbreak of gastroenteritis caused by *Salmonella muenchen*. A link between the disease and smoking marijuana has been established by plasmid analysis. The isolates from patients and marijuana samples from different states carried the same plasmid.

### 2. Restriction enzyme analysis of chromosomal DNA (REA DNA)

or chromosomal fingerprint

The same molecular technique used to analyze plasmid DNA by conventional electrophoresis has been applied to chromosomal DNA. Compared with plasmid DNA, chromosomal DNA is more stable because it is less subject to loss, gain or exchange of genetic information (Fig. 5).

All isolates are typeable by this technique, but the results are difficult to interpret due to the complex patterns created by a large number of overlapping bands (Fig. 7A). Moreover, isolates with genetically identical chromosomal DNA may have different banding patterns as plasmid DNA may contaminate the genomic DNA.

In order to circumvent these inconveniences the technique has been modified or supplanted by other molecular methods (Table 1 p. 53).
Figure 5: RESTRICTION ENZYME ANALYSIS OF DNA (DNA FINGERPRINTING)

Patients

Isolates of a given nosocomial pathogen from different sites

1, 2, 3, 4, 5, 6, 7, 8

Restriction enzyme (frequent cutter)

9, 10, 11, 12

Bacterial chromosome (with frequent restriction sites)

Isolate 1

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
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</table>

Isolate 9

<table>
<thead>
<tr>
<th>A'</th>
<th>A''</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E'</th>
<th>G</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Conventional electrophoresis chamber

Migration of restriction fragments (0.5 to 25 KB)

HUNDREDS OF FRAGMENTS

Direction of DNA migration

Bandung pattern
3. Restriction Fragment Length Polymorphism’s (RFLPs)

A good alternative to plasmid analysis and REA DNA exploits naturally occurring chromosomal DNA polymorphism’s, called Restriction Fragment Length Polymorphism’s (RFLPs), using a probe for a known sequence of DNA within the chromosome. The first technique to exploit RFLPs was Southern blotting wherein probes for known DNA sequences are used to detect homologous DNA sequences (loci) within the chromosome. All strains with loci homologous to the probe are typeable. Microorganisms vary in the number of complementary DNA sequences, the location and number of restriction sites within those loci, and the number and sizes of subsequent DNA fragments. Probes that detect a region found several times within the chromosome produce more bands and therefore have better discriminatory power than probes detecting a region that occurs infrequently. Since the number of bands is limited (Fig. 6), interpretation is simplified (Fig. 7B). RFLPs are widely available, easy to perform and highly reproducible. However, there are limitations to RFLP; typing requires a cloned DNA sequence and often uses a radiolabeled probe (Table 1 p. 53).

Examples of the use of RFLPs for typing in Pediatrics include proving mother-to-infant transmission of *S. pyogenes* and the unrelatedness of strains associated with an outbreak of *VRE faecium* in four different wards of a children’s hospital. 12
Fig. 6: The figure shows the RFLPs banding pattern of 3 isolates (A, B, and C) using Southern blotting technique.

In figure A, the DNA is digested and separated electrophoretically as previously described. In figure B, the restriction fragments are then transferred (blotted) onto a nitrocellulose membrane following the method of Southern. Selected DNA probes are used to bind (hybridize) with complementary base-paired targets, matching only to the fragments containing homologous sequences. The restriction fragments containing specific sequences (loci) are then detected by radio- or chemical labeling.\(^1\)
Fig. 7: The figure shows isolates of *P. aeruginosa* from two CF patients analyzed by REA DNA (Fig 7A) whose banding patterns are unreadable due to the large number of overlapping bands. The corresponding Southern blot analysis (Fig 7B) using a radiolabeled DNA probe encoding a portion of the *Pseudomonas* exotoxin A gene shows a decreased number of bands thus simplifies the interpretation of the banding pattern. The figure shows 3 different types of banding patterns corresponding to 3 different strains of *P. aeruginosa* (lanes 1, 2, 5; lanes 3, 4, 6, 7, 8; and lanes 9, 10, 11, 12).
3a. Ribotyping

Ribotyping also exploits RFLPs. This method assesses DNA polymorphism’s by using the genes encoding the ribosomal RNA (ribosomal operon) as probes. These sequences are highly conserved in bacteria (Mark Wease, taxonomy phylogenic tree) and thus, different species can be typed using the same probe. All strains are typeable and results are reproducible (Fig. 8). Discriminatory power is fair and depends mainly on the number of ribosomal operons (e.g. *E. coli* possesses 7, *S. aureus* 5, and *M. tuberculosis* 1). Ribotyping has also been simplified by automatization, which uses a chemoluminescent rather than a radiolabeled probe, and a computerized imaging system to interpret banding patterns. Not surprisingly, these innovations are very costly (Table 1 p. 53).

Ribotyping has held an important role in pediatric infection control in CF patients. CF clinicians noted the emergence of *B. cepacia* in the early 1980’s. This pathogen is associated with increased mortality and morbidity in CF patients. Although patient-to-patient transmission was suspected, ribotyping and RAPD (p. 49) confirmed this hypothesis. Molecular epidemiology elaborated the sites of transmission including not only hospital transmission, but also transmission in outpatient clinics as well as social settings such as CF camps. These findings have lead to infection control recommendations to segregate infected CF patients from non-infected CF patients that have successfully reduced new acquisition of this pathogen among CF patients. Ribotyping has been involved in proving many outbreaks of nosocomial pathogens in the pediatric setting such as ESBL *K. pneumoniae*, and VRE. This typing method also proved the role of endogenous flora and NI for *E. cloacae* in NICU patients.
Fig. 8: The figure shows the hybridization of *E. coli* rRNA with EcoRI-digested DNA from 8 isolates of *Burkholderia cepacia* from different patients with cystic fibrosis (A-H). Lanes C and D have the same banding pattern; these strains are derived from two children in the same CF center providing evidence of cross-infection. Reprinted with permission.58
3b. Insertion sequences

Insertion sequences (IS) are mobile, transposable genetic elements, composed of unique nucleotide sequences usually present in numerous copies within a bacterial genome. Their number and location vary. Probes derived from bacterial insertion sequences (homologous sequences) 3-4 have been successfully used to type organisms by hybridization protocols (RFLPs). Each strain has a unique banding pattern. Although it seems paradoxical, the use of transposable elements (known to be unstable and potentially leading to many genetic rearrangements) has been proposed as the reference method for typing *M. tuberculosis*. 64 It has been demonstrated that these elements are very stable in this organism and fewer in number. 65

4. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) was first described in 1984 by Schwartz. 66 This technique is a variation of conventional agarose gel electrophoresis, but generates simpler banding patterns because it can resolve much larger DNA fragments (Fig. 9). The typical banding pattern of PFGE is composed of 5 to 20 fragments ranging from 10 to 800 Kb (megabase-sizes DNA which represent DNA molecules > 50 Kb in size and up to 1000 Kb in length) compared with conventional electrophoresis which generates many more fragments ranging from 0.5 to 25 Kb (Fig. 10). The results obtained by PFGE are highly reproducible, but the procedure is technically demanding. All bacterial species are theoretically typeable. However, isolation of intact chromosomal DNA can be difficult and DNA degradation by endogenous nuclease may occur prior to cleavage. 67 In addition, random genetic events can lead to loss of cleavage sites, which can alter the PFGE pattern (Fig. 11). PFGE requires expensive equipment and takes 5 to 6 days to perform. However, despite its limitations, PFGE is currently the most widely used typing technique (Table 1 p. 53). 26,34,68-70
Fig. 9: PULSED-FIELD GEL ELECTROPHORESIS

Fig. 9: Technically, PFGE starts with the embeding of an intact strain within an agarose plug, followed by the lysis of the microorganism and the digestion in-situ of the chromosomal DNA with a restriction endonuclease that cleaves infrequently (rare cutter). The agarose gel is placed in a chamber with 3 sets of electrodes that form a hexagon.

The current is first applied in one direction from one set of electrodes, then shifts to the second set for a short period of time (a pulse) and then shifts to the third set of electrodes. By varying periodically both the direction and the duration of the electric field, PFGE allows the separation of very large fragments of DNA (results are discussed in Fig. 12).
Fig. 10: Interpretation of PGFE banding pattern of an outbreak of *P. aeruginosa* in a NICU. Lane 1: ladder, lane 2: reference strain, lane 3: infant 1, lane 4: infant 2, lane 5: infant 3, lane 6: infant 4, lane 7: infant 5, lane 8: nurse 1, lane 9: nurse 1, lane 10: infant 6, lane 11: infant 7, lane 12: nurse 2, lane 13: nurse 2, lane 14: infant 8, lane 15: control. Lanes 3 through 9 show an identical banding pattern compatible with a unique strain (clone A). Lanes 10 and 11 show an other identical banding pattern compatible with an additional strain (clone B). Infection control measures would focus on infants 1 to 5 and nurse 1 (clone A). 73
Figure 11: Changes of PFGE patterns as a result of genetic events

Fig. 11: Regarding the number of genetic events, PFGE banding patterns can be read and classified as types and subtypes. Tenover et al. have proposed standardized guidelines for the interpretation of genetic relatedness of 30 or fewer isolates. The criteria are reliable if PFGE demonstrates at least 10 distinct fragments as less fragments reduce the discriminatory power. These criteria are presented below since PFGE is currently one of the most used tool for typing in clinical microbiology laboratories.

These recommendations represent the first attempt to standardize the interpretation of results obtained by a molecular typing method (Table 3 p. 44).
**Guidelines for PFGE interpretation (Table 3 and Fig. 12)**

- The isolate is **indistinguishable** (not identical!) if the tested strain shows no genetic difference and there is no fragment difference compared with outbreak strain. The isolate is part of the outbreak. Its restriction patterns have the same number of bands and the corresponding bands are the same size named (A).

- The strain tested is **closely related** to the outbreak strain when there is one genetic difference (consistent with one single genetic event, i.e. point mutation or insertion or deletion of DNA) visible by 2 or 3 fragment differences on the gel compared with the outbreak pattern. The isolate is said to be probably part of the outbreak named (A1).

- Up to 2 independent genetic events visible by 4 or 6 fragments pattern differences compared with the outbreak strain, the isolate is **possibly related** to the outbreak (A2).

- When there are more than 3 genetic differences visible by ≥ 7 fragment pattern differences compared with the outbreak strain, the isolate is considered **unrelated** to the outbreak (designated B) = different.

**Table 3: Criteria for interpreting PFGE patterns**

<table>
<thead>
<tr>
<th>Category</th>
<th>No. of genetic differences compared with outbreak strain</th>
<th>Typical no. of fragment differences compared with outbreak pattern</th>
<th>Epidemiologic interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indistinguishable</td>
<td>0</td>
<td>0</td>
<td>Isolate is part of the outbreak</td>
</tr>
<tr>
<td>Closely related</td>
<td>1</td>
<td>2-3</td>
<td>Isolate is probably part of the outbreak</td>
</tr>
<tr>
<td>Possibly related</td>
<td>2</td>
<td>4-6</td>
<td>Isolate is possibly part of the outbreak</td>
</tr>
<tr>
<td>Different</td>
<td>≥3</td>
<td>≥7</td>
<td>Isolate is not part of the outbreak</td>
</tr>
</tbody>
</table>
Fig. 12: PFGE Analysis interpretation: According to Table 3, isolates A and B from patient 1 are indistinguishable and thus represent the same strain (α). Isolates E and F from patient 3 are totally different from each other and from those of the other patients. They represent 2 additional strains of the pathogen (β and γ). Isolates C and D differ from strain α by one fragment (band 7 or 8 respectively) and differ from each other by 2 fragments (7 and 8). C and D could be derived from strain α by a simple chromosomal deletion (C7) or insertion event (D8). Isolates A through D are highly related and belong to the same strain group renoming them subtype α-1, α-2 and α-3 for A, B and C, D. Infection control measures would concentrate on patient 1 and 2.

**CHEF** (contour-clamped homogeneous Electric Field) and **FIGE** (Field-inversion Gel Electrophoresis) are two variations of PFGE. The migration speed of CHEF is slow (20-24 h) and uses an axis of 120° and the migration speed of FIGE is fast (4h) and the electric field’s orientation positive charge is periodically inverted by 180°. FIGE has been used in Pediatrics to...
prove an outbreak with *Legionella pneumophila*, and CHEF has evaluated the genetic relatedness of *Candida* in the NICU (electrophoresis karyotyping). The importance of PFGE in improving our understanding of transmission of NI in Pediatrics is tremendous:

1) **The role of hands of HCW in transmission of nosocomial pathogens.** It has been well demonstrated that hands with artificial nails have an increased colonization rate with Gram-negative bacilli compared with natural nails. Artificial nails have been linked by PFGE to a nosocomial outbreak of *P. aeruginosa* in NICU patients. Thus, cosmetic nail treatments including false nails, nailwraps and nail tips should not be permitted in HCW. Additional outbreaks of *P. aeruginosa* have been linked to HCWs with cosmetic nail treatment, with otitis externa and with *Candida* onychomycosis super-infected with *P. aeruginosa* (Fig. 10).

2) **The identification of potential carriage/ reservoir among HCW and other patients.** Two very low birth weight infants in the NICU presented with staphylococcal scalded skin syndrome (SSSS). To prevent further cases of this disease, detection of potential carriage among asymptomatic staff and infants is vital. In this case, the SSSS clone was methicillin-susceptible and PFGE was used to distinguish endemic strains from the outbreak strain. Eradication of carriage by three HCWs and another asymptomatic infant prevented a subsequent outbreak.

3) **The role of endogenous flora.** Vancomycin-resistant enterococcal (VRE) bacteremia is increasing among oncologic patients. The gastrointestinal (GI) tract of these patients may be rapidly colonized with VRE. Risk factors include long and repeated hospitalizations and antibiotic use. PFGE has confirmed that the same clone colonizes the GI tract and translocates into the blood causing invasive disease. Similar observations have been made for *C. albicans* and *E. cloacae* whereby GI colonization has been followed by blood stream infection with the same clone. In addition, rectal electronic thermometers may serve as a vector of nosocomial infection due to *E. faecium*. 
Many experts emphasize the usefulness of screening high-risk patients for potential pathogens, including patients transferred from other hospitals.

4) **Identification of new sources of NI.** As examples, outbreaks of *P. aeruginosa* caused by tap water and bath toy contamination, an outbreak of *E. cloacae* contaminating intravenous therapy in the NICU, and an outbreak of *C. parapsilosis* in NICU patients caused by contaminated glycerin suppositories. 15-18,69

In addition, PFGE solved many other outbreaks in the pediatric setting involving other nosocomial pathogens such as MRSA 56, MSSA 56, ESBL *K. pneumoniae*, 56 *C. difficile*, 70 VRE, 13,18 and *M. bovis*. 69

5. **Polymerase chain reaction (PCR)**

PCR rapidly and exponentially replicates (amplifies) a selected DNA sequence (template). PCR can also detect small amounts of DNA from organisms that cannot be grown such as *Pneumocystis carinii* or viruses or organisms which grow slowly such as fungi or mycobacteria. PCR is easy to perform and widely available. Contamination can readily occur and lead to false positive results. Thus, even with a single copy of target DNA, only moderate reproducibility is achieved. 1

Several methods that utilize PCR-based strategies for typing have been developed. These include PCR-RFLPs, PCR-ribotyping, random amplified polymorphic DNA (RAPD), also called arbitrarily primed PCR (AP-PCR) and Rep-PCR. For most of these methods the chromosomal sequences must be known to prepare the primers. These PCR-based methods can be further modified for typing by adding endonuclease digestion (Fig. 13B p. 48).
Fig. 13: PCR-based typing methods: The figure shows the analysis of *B. cepacia* strains isolated from patients at different CF centers. The strain pairs were analyzed by (RAPD) in Fig 13A, PCR-ribotyping in Fig 13B, and by restriction endonuclease digestion (*Hae* III) of the product of PCR-ribotyping in Fig 13C. The non-specific primer(s) used in RAPD (13A) hybridizes at random sites within the chromosome as one or more loci are amplified. The number and location of these random sites vary among strains and the number and size of generated fragments size are the basis for typing the isolates. Each CF center appeared to have a dominant RAPD type shared by the patients studied. However, PCR-ribotyping (13B) did not prove as discriminatory as RAPD. While paired isolates had the same banding pattern as one other, many centers shared the limited number of banding patterns. *Hae* III digestion of the PCR-ribotyping products improved the discriminatory power somewhat, but did not approach that of RAPD. Reprinted with permission. 4
5a. PCR-RFLP: consists of the amplification of the DNA to be tested followed by REA and conventional electrophoretic analysis. There is no need for Southern blotting. This procedure is limited by the requirement for species- or gene-specific oligonucleotide primers (Fig. 13C). It has been used to subtype *Bartonella henselae* and *Neisseria meningitidis* and to type Cytomegalovirus (CMV) and herpes virus (HSV).

5b. PCR-ribotyping: (Fig. 13B) uses the highly conserved 16S and 23S ribosomal sequences as templates. The discriminatory power of PCR-ribotyping reflects the heterogeneity of the length of the DNA between the ribosomal operon (intragenic spacer regions). Most importantly, a single primer pair can be used for any bacterial species. Like the PRC-RFLP there is no need for blotting or hybridization, but the advantage is that only a single primer pair is needed for all bacterial species (Table 1 p. 53).

PCR-ribotyping has been used to speciate the *B. cepacia* complex into multiple subspecies (genomovars), which has lead to a better understanding of the epidemiology of this pathogen among CF patients.

5c. AP-PRC or RAPD: (Fig. 14 p. 50) RAPD is useful for rapid screening of large numbers of isolates, is applicable to nearly all bacterial species, and results are available within a day at a low cost for a high-resolution technique. RAPD is more discriminatory than PCR-ribotyping and does not require the sequence of the target. It is fast and easy to perform. RAPD is currently one of the two methods of choice for genotyping in molecular epidemiology (Table 1 p. 53). However, the method suffers from assay to assay variability (Fig. 13A).
RAPD was used in combination with ribotyping to confirm patient-to-patient transmission of *B. cepacia* among CF patients (Fig. 13 and p. 38). RAPD has proved many outbreaks in the pediatric setting involving nosocomial pathogens such as ESBL *K. pneumoniae*, *E. cloacae*, *P. mirabilis*, and MRSA. RAPD was also useful in proving mother-to-infant transmission of a *S. aureus* strain expressing the staphylococcal scalded skin toxin.

**5d. Rep-PRC:** is another variation of the technique using short extragenic repetitive sequence primers present at many sites around the bacterial chromosome and complementary to specific repeated DNA sequences. When two sequences are located near enough to each other the DNA fragments between those sites is amplified. The number and location of the repeated sequences are variable. The number and size of the inter-repeated fragments generated can also vary from strain to strain. This technique has been used to study fungi and ameba. Rep-PCR was used in Pediatrics to prove a double outbreak of *S. marescens* and MRSA, which highlighted the role of overcrowding and understaffing as an important risk factor for NI.
H. FUTURE DIRECTION: Binary typing

PFGE and RAPD appear to be the methods of choice for typing many nosocomial pathogens in pediatric infection control. However, the lack of standardized interpretative criteria, the time expenditure and the high cost of these techniques make the development of an alternative method highly desirable.

Binary typing \(^{84,85}\) (Fig. 15 p. 52) appears promising and has been used to study the geographic distribution of different MRSA clones as well as the genomic evolution of this species. \(^{86}\) Binary typing has also been validated for methicillin-susceptible \textit{S. aureus} strains and MRSA strains. \(^{84}\)

This genotyping method uses a selected set of probes generated by RAPD to create a binary code (yes = 1 or no = 0). The resulting hybridization is then interpreted as a 15 digit binary code (e. g. Figure 15 cluster VIIIa: 100111110111101).

In a research setting BT generally had improved discriminatory power and reproducibility when compared with RFLPs, PFGE and RAPD (Table 1 p. 53). The simple binary output generated allows a straightforward interpretation; the technique is simple and potentially can be automated. \(^{87}\) Finally, BT can be extrapolated to many species. \(^{85}\) More widespread use in clinical settings will help to further delineate its potential limitations.
Figure 15: Binary Typing

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Fig.15: Binary typing: the figure shows 15 DNA-probes (AW-1 – AW-15) generated directly from the genome of 15 different MRSA strains. Three MRSA clusters VIII, IX, X, each derived from outbreaks and comprised of five strains (a, b, c, d, and e) are shown. Each cluster has a unique binary code that is shared by the strains within each cluster.

Because of the great diversity of possible binary codes this technique has great discriminatory power.

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I. TABLE 1: Comparison of currently used Typing Systems*, **

<table>
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<tr>
<th>Typing Methods</th>
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<th>Reproducibility</th>
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<th>Ease of interpretation</th>
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* Abbreviations used in table: REA DNA = restriction enzyme analysis of chromosomal DNA, RFLPs = restriction fragment length polymorphism’s, PFGE = pulsed-field gel electrophoresis, PCR-ribotyping = polymerase chain reaction-ribotyping, RAPD = random amplified polymorphic DNA

** Grading: 0 = unknown; + = poor; ++ = fair; +++ = good; ++++ = excellent
J. CONCLUSION

New Typing methodologies have improved both the rapidity of diagnosing infectious diseases, and the ability to perform accurate epidemiologic studies to identify the suspected organism and determine its association with the disease or the outbreak. Phenotyping techniques still remain useful in complement with molecular systems based on genetic characterization, which are highly discriminative and reproducible. PFGE and RAPD seem to be the most widely applicable techniques for most nosocomial pathogens.

It has to be emphasized that investigators of nosocomial infections should use typing in conjunction with clinical and epidemiologic data. Typing systems should supplement and not substitute for a carefully conducted epidemiologic investigation.

Choosing an appropriate typing system should take into account the five required criteria.

Molecular typing became an invaluable tool for infection control. It allows identification of the origin of an outbreak, the confirmation of clonality and can distinguish independent infections occurring during the same period (endemic strains/sporadic cases) from outbreaks caused by one clone (epidemic strain), and pseudo-outbreaks. Finally, molecular typing can facilitate the identification of appropriate infection control measures to lower the rate of mortality and morbidity of nosocomial infections among children.
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PART II

Presented as a poster at the 11th annual scientific meeting of

«Nosocomial» transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) from a mother to her quadruplet infants

**Abstract**

**Background**

Patient-to-patient transmission of methicillin-resistant *Staphylococcus aureus* (MRSA) in Neonatal Intensive Care Units (NICU) has been well described. We report the first documented outbreak of postnatal MRSA transmission from a mother to 3 of her preterm quadruplet infants.

**Methods**

Routine surveillance of clinical microbiology laboratory reports revealed an increased incidence of MRSA infections in our NICU, including 3 of 4 preterm quadruplets. Surveillance cultures of the anterior nares of all patients and the quadruplets’ parents were performed to detect MRSA carriage. The isolates were typed by Pulsed-field gel electrophoresis (PFGE) using *Sma*I. Infection control strategies included mupirocin treatment and contact isolation for infected/colonized infants.

**Results**

Clinical cultures from Quads «A», «C», and «D», and surveillance cultures of the quadruplets’ mother and 2 additional unrelated infants grew the same clone of MRSA. The mother’s only identified risk factors for MRSA acquisition were 2 prepartum hospitalizations related to the multiple gestation and previous treatment with antibiotics. All anterior nares cultures were negative after mupirocin treatment.

**Conclusions**

Empiric use of gowns and gloves by the family members of multiple gestations should be recommended to prevent transmission of potential pathogens in the NICU.
Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a well-known nosocomial pathogen of NICU patients. 1-5 Outbreaks have been attributed to intravenous catheters 3, use of topical gentamicin 2, and overcrowding and understaffing. 3 The most common sites of staphylococcal colonization among NICU patients include the anterior nares (88%), umbilicus (56%), groin (50%) and axilla (31%). 4 Skin colonization can occur within the first 24 to 48 hours of birth due to transmission from the hands of colonized healthcare workers. 5 Maternal-to-infant transmission of methicillin-susceptible *S. aureus* (MSSA) with subsequent newborn infection has been previously described via colonized genital secretions 6, placental transmission 7, and mastitis. 8 We present the first published report of an MRSA outbreak caused by postnatal transmission from a mother to 3 of 4 of her preterm quadruplet infants in the NICU.

Methods

In August 2000, routine surveillance of clinical microbiology laboratory reports revealed an increased incidence of MRSA infection among patients in our 45-bed level III-IV NICU including 3 quadruplet siblings (Quad «A», «C» and «D»). Surveillance cultures of the anterior nares of the quadruplets’ parents and the remaining 40 infants in the NICU were performed to detect MRSA carriage. Cotton-tipped swabs that had been inserted into both nares were smeared onto Mueller Hinton Agar plates containing 6 µg/ml of oxacillin (BD Microbiology Systems, Sparks, MD.) and incubated at 37° C for 24 hours. Colonies were identified as *S. aureus* (Staphaurex, Alexon-Trend, Inc. Ramsey, MN.), and antibiotic susceptibility testing was performed by MicroScan (Dade Behring Inc., Deerfield IL.). Mupirocin susceptibility testing was performed by disk diffusion (BD). 9 DNA from MRSA isolates was digested with *Sma*I endonuclease and the fragments were separated by Pulsed-field gel electrophoresis (PFGE; BioRad GenePath Strain Typing System, Hercule, CA.). Banding patterns were interpreted according to previously accepted guidelines. 10

To prevent further cases of MRSA infection/colonization, infection control strategies were implemented. These included contact isolation precautions, and topical mupirocin treatment of
the anterior nares twice daily for 7 days for colonized/infected infants and the mother of the quadruplets, phisohex baths for infants ≥ 1500 g, and daily phisohex showers for 5 days for the mother. For the remainder of the hospital stay, the family members wore gowns and gloves prior to contact with the quadruplets, which were changed between each baby. Repeat anterior nares cultures after mupirocin treatment assessed the efficacy of these strategies.

Results

The number of incident-cases of MRSA in our NICU is shown (Figure 1). This cluster of infections began in late July 2000. Quad «A», «B», «C», and «D» were 25-week preterm infants born by Caesarian-section. Quad «A» developed conjunctivitis due to MRSA on day of life (DOL) 25. Nine days later, on DOL 34, Quad «C» developed sepsis with MRSA followed by pustulosis on DOL 39. On DOL 37, Quad «D» presented with conjunctivitis due to MRSA. Each infant was placed on contact isolation; i.e. gown and gloves were worn by healthcare workers as well as the family of the quadruplets prior to contact with the infants.

The MRSA strains from the clinical cultures of Quad «A», Quad «C», and Quad «D», as well as the surveillance cultures of the anterior nares of the quadruplets’ mother, Quad «A», Quad «D» and two additional unrelated infants (infants 4 and infant 5) were susceptible to vancomycin, trimethoprim-sulfamethoxazole, rifampin, gentamicin, clindamycin, ciprofloxacin, and mupirocin. The anterior nares of Quad «B» grew MSSA, and the quadruplets’ father was negative for Staphylococcus spp. PFGE showed an identical banding pattern for the MRSA isolates from the mother, Quad «A», «C», «D», and the two additional unrelated infants (Figure 2). Repeat anterior nares cultures of the quadruplets’ mother and the 5 infants were negative for MRSA after mupirocin treatment.

The only identified risk factors for MRSA acquisition in the mother were a course of ampicillin and erythromycin a week prior delivery for ruptured membranes, and 2 prepartum hospitalizations related to the multiple gestation. No cases of MRSA infection/colonization were reported on either prepartum ward.
Discussion

Patient-to-patient transmission of MRSA among infants in the NICU is well documented, but previously linked to asymptomatic carriage by healthcare workers. In this report, we describe an outbreak caused by transmission from a mother to her infants that was most likely identified because of the multiple gestation involved. Maternal-to-infant transmission of MRSA was supported by molecular typing. However, it is also possible that the mother may have acquired MRSA from one infant and subsequently transmitted MRSA to her other infants. Further transmission to 2 unrelated infants is most likely due to carriage of MRSA by healthcare workers. There has been a previous report by Wenzel and colleagues demonstrating probable MRSA transmission from an infant to a mother to a subsequent preterm infant, but no additional unrelated infants were infected with this clone.

Infection control strategies implemented in our NICU included traditional methods: cohorting infected/colonized infants, use of contact isolation, reinforcing hand hygiene, and eradicating MRSA carriage with topical mupirocin applied to the anterior nares of the infants. However, expanded efforts to halt the outbreak included culturing the parents of the quadruplets and treating their mother as well. Because of the unique clustering of MRSA cases among quadruplet siblings and because no further transmission occurred after the cohort was established, healthcare workers were not cultured.

As no other cases of colonization/infection with MRSA were reported on either prepartum ward, it is unknown if the quadruplets’ mother had nosocomial or community acquisition of MRSA. Community-acquired MRSA (CAMRSA) strains have greater clonal diversity and are generally more susceptible to antimicrobial agents including clindamycin, gentamicin, and trimethoprim-sulfamethoxazole than hospital-acquired strains. The antibiotic susceptibility pattern of the MRSA strain from the mother suggests community acquisition. Thus, the increasing prevalence of CAMRSA could lead to reservoirs of this pathogen among the family members of hospitalized patients.
In conclusion, the epidemiology of the MRSA outbreak described in this report was unique as it clustered among quadruplet siblings due to maternal-to-infant transmission. We recommend that family members of multiple gestations empirically observe contact precautions in the NICU to prevent possible transmission of potential pathogens, particularly during an outbreak.
Fig. 1: The incident-cases of MRSA infection/ colonization in our NICU from June 1999 to December 2000 are shown.

Five infants, including Quad «A», «C», and «D» harbored the same clone of MRSA (Clone «A») in July and August 2000 only. In December 1999 and September 2000, two different MRSA clones were noted in two other infants.
Fig. 2: This figure shows the PFGE banding pattern of MRSA isolates. Lane 1: molecular weight markers, lane 2: control *S. aureus* strain, lane 3: isolate from the anterior nares of the quadruplets’ mother, lane 4: isolate from the anterior nares of Quad «A», lane 5: blood isolate from Quad «C», lane 6: isolate from the anterior nares of Quad «D», lane 7: isolate from the anterior nares of Infant 4, and lane 8: isolate from the anterior nares of Infant 5. Lanes 3, 4, 5, 6, 7, and 8 demonstrate identical banding patterns shared by MRSA strains from the quadruplets’ mother, Quad «A», «C», «D», and two additional unrelated infants.
REFERENCES


